FI

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

## ANNUAL REPORT TO THE ENDANGERED SPECIES UNIT, AUSTRALIAN NATURE CONSERVATION AGENCY

## March 1994



Department of Conservation and Land Management
PO Box 104, Como, WA 6152

# CONTENTS

4. Validate the test by field trials
   - 4.1 Method for extraction of DNA from soil
   - 4.2 Validation of the test by reconstruction experiments with soil amended with *P. cinnamomi*
   - 4.3 Validation of the test with natural soils and plant material
   - 4.4 Using the test to study colonization of the host plant

**PROJECT 3**
**DEVELOPMENT OF GIS-BASED DECISION-SUPPORT TOOLS AND THE DATABASING OF *PHYTOPHTHORA*-SENSITIVE TAXA**

Objective

1. Obtain data sets

2. Interrogate data sets

3. Develop a Predictive model

Appendix

References

**PROJECT 4**
**THE CONTROL AND MANAGEMENT OF *PHYTOPHTHORA MEGASPERMA* IN THE NATIVE PLANT COMMUNITIES OF WESTERN AUSTRALIA**

Summary

1. Control of *P. megasperma*
   - 1.1 Sensitivity of *P. megasperma* to phosphonate *in vitro*
   - 1.2 Efficacy of aerial applications of phosphonate to retard the damage and spread of the disease caused by *P. megasperma*

2. Management of *P. megasperma*
   - 2.1 *In vitro* influence of temperature on radial growth, sporangium and oospore formation
     - 2.1.1 *In vitro* influence of temperature on radial growth, sporangium and oospore formation
     - 2.1.2 The effect of season and site factors on oospore formation in the field
     - 2.1.3 The main environmental factors controlling oospore dormancy and germination
   - 2.2` Variability of *P. megasperma*

3. The occurrence of *P. megasperma* in the national parks of WA

4. Liaison

**PROJECT 5**
**IDENTIFICATION, GERMPLASM STORAGE AND IN VITRO PROPAGATION OF *PHYTOPHTHORA* AND CANKER THREATENED TAXA**

1. Identification of rare and threatened flora at risk from dieback disease

2. In vitro propagation

3. Cryostorage

4. Seed collection
   - 4.1 Sampling strategy
     - 4.1.1 Number of populations per taxa
     - 4.1.2 Number of source plants per population
     - 4.1.3 Number of seed per source plant
   - 4.2 Population reconnaissance
   - 4.3 TFSC accessions
   - 4.4 Future collections and proposed developments

5. Seed storage, viability testing and inventory system

   - 5.1 Registration
   - 5.2 Cleaning and fumigation
   - 5.3 Quantifying the seedlot
   - 5.4 Germination/viability testing
   - 5.5 Moisture content determination, reduction and drying of seed
   - 5.6 Packaging, storage and monitoring regime
   - 5.7 Collation of data

References

**PROJECT 6**
**CONTROL AND MANAGEMENT OF *DIPLODINA* CANKER THREATENING *BANKSIA COCCINEA***

Summary

Introduction

1. Control measures
   - 1.1 Burning
     - 1.1.1 Survey of disease and seed reserves in *B. coccinea* stands

# PROJECT 1

# THE CONTROL OF *PHYTOPHTHORA* IN NATIVE PLANT COMMUNITIES

**B. Komorek, B. Shearer, B. Smith and R. Fairman**
*Science and Information Division, Department and Conservation and Land Management, 50 Hayman Rd., Como WA 6152.*

**Annual report to the Australian Nature Conservation Agency (February 1994)**

## Summary

Phosphonate is a very useful chemical in the control of *Phytophthora* and can become an important element of integrated control strategy. It is the only available option in the prevention of further losses of rare and endangered plant species from dieback, in areas where the pathogen is present, in the short to medium term.
The results show that fungicide residues are retained in the plant tissue for some time and the concentration of phosphite can be increased significantly by follow-up spraying. Experiments are continuing to determine the longevity of the fungicide in plant tissues.
Aerial application of the chemical is a promising method of application. The fungicide can be applied to the whole plant canopy effectively. Appropriate climatic conditions are a prerequisite to successful treatment as the absorption of the chemical is affected by rain. The required dry period after spraying was found to be at least seven hours after heavy rainfall.
*In vitro* bioassay demonstrated that phosphite is oxidised to phosphate by heat (autoclaving) thus the concentrations inhibitory to *P. cinnamomi* may be lower than previously reported in the literature.
The toxicity of phosphonate is enhanced on low phosphate media. This may explain why phosphonate is so effective in the control of the pathogen in native species which are low in phosphate.

## Introduction and main objectives

*Phytophthora cinnamomi* is a vigorous pathogen that kills a wide range of plant species by attacking their root system. The fungus is widespread in the South-West of Western Australia causing great destruction to a large proportion of vegetation in state forests, national parks and reserves. Apart from impacting on the structure and genetic

diversity of heath and shrublands it also affects animal communities by altering their habitat.
It is estimated that 1500 to 2000 species of the estimated 8000 species of vascular plants in the South-West may be susceptible to infection (Shea, 1991). Many of them are highly endemic and have been brought to the brink of extinction. Therefore, dieback disease currently poses the greatest conservation threat facing Western Australia.
There are several measures that can be applied to control *Phytophthora* dieback. These include various quarantine and hygiene procedures which have been developed for public and industries to help protect large areas of healthy bush from the disease. However, these methods of disease management are regarded as a holding action until better methods are developed.

Chemotherapy is another important measure which can be used in the fight against *Phytophthora*. Phosphorous acid applied as the potassium salt (phosphonate), has been successfully used in horticulture to control *P. cinnamomi* and was also found to be very effective against the infection in jarrah and some species of banksia. It is cheap, biodegradable and non-toxic to people, animals and soil microflora. In terms of safety and environmental acceptability, there is much to recommend phosphonate. The active component of the fungicide is the phosphite ion ($PO_3^-$). It is readily translocated through plants including the roots and it persists within plant tissues. Although phosphonate does not eliminate disease, it is a powerful prophylactic fungicide and offers the best currently available option in the disease control. It will allow prevention of further losses from *Phytophthora* dieback disease through effective protection of healthy plant communities.
Phosphonate can be applied in already infected areas of special significance (eg. rare flora or beauty spots) where it will protect endangered plant species against extinction.

The main objectives of the project are as follows:
1. To evaluate the efficacy of aerial application of phosphonate in the control of *Phytophthora* in native plant communities of Western Australia..
2. To determine the effective concentration of phosphonate residues that control the pathogen
3. To investigate the nature of the resistance response induced in the plant by the application of the chemical.
4. To study the long term effects of the fungicide on native plants.

## 1. Assessment of the efficacy of aerial application of phosphonate

The fungicide has been field trialed in the past five years in several areas in the South-West on plant communities already infested with *Phytophthora cinnamomi*. Foliar application and trunk injection trials have shown that one application of phosphonate gives excellent control of the fungus over several years (Shearer, pers. comm.). However, in order to effectively control spread of infection, the chemical must be applied aerially using an aircraft. This method of application would be most suitable if long fronts of infection were to be treated. It would also permit treatment of most

remote areas cost-effectively, without disturbance to the treated and neighbouring areas, therefore eliminating any possibility of accidental spread of the disease. There are some fundamental differences between the two methods of application. Aerially applied systemic fungicides are applied in low volumes, usually 20-50 l/ha (Jacobsen, 1986). Also, because of the aircraft's speed and the dynamics of the physics involved, various factors must be considered. These should include the size of the target and the need for adequate coverage (number of droplets deposited per unit area at various levels of the plant canopy), droplet size, effective application swath width and uniformity of application. It is critical that before any of these factors can be considered, proper calibration of the aircraft is achieved.

The aim of the aerial trials is to provide information on the appropriate rates of application and the duration of protection of phosphonate achieved by this particular method of application. The 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 (30 km east of Albany)

In the beginning of May 1993 we commenced an initial 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 is very susceptible to infection from *Phytophthora*, occurs only in a few locations and all populations are infected with the pathogen.

The South Sister site (8.8 ha) was sprayed with 8.5% concentration of potassium phosphonate, using Synertrol (0.5%) as a wetting agent. The chemical was applied from an aeroplane, in a low volume of 26 l/ha. A local contractor was selected to do the job (Giles Aviation). A follow-up spray was carried out six weeks later.
Twenty *B. brownii* trees were randomly selected and marked. Their health was assessed. In order to determine the coverage of the spray within the plant canopy and droplet size, water-sensitive papers were attached to the upper leaves and also put directly onto the ground below the trees. The use of water-sensitive papers is not directly quantitative but does give a quick visual estimate of droplet size and density in various points of the plant canopy. The relationship between the volume of liquid carrier used and the density of spray deposition on the plant is important because the biological efficacy of a fungicide is determined not only by rate of application but also by spray droplet density on the plant.
In our trial, droplet size (VMD-volume mean diameter) ranged from 100 to 500 µm with 70% of droplets being in the range of 100 to 300 µm and droplet density was 50-60 drops/cm$^2$.
It is generally recommended that droplet VMD should be between 300 and 500 µm as it prevents excessive drift. However in our situation smaller droplets are preferred as they penetrate the dense canopy much better due to good horizontal movement of the spray. The wind speed was only 3 km/hr so spray drift was minimal. Droplet

penetration through the canopy was good as the water-sensitive papers laid under the trees had only 20% fewer drops than the papers attached to the upper leaves.
Leaf samples were taken for chemical analysis to determine the concentration of phosphite ion in the plant tissue. The marked trees were assessed for any visible signs of phytotoxicity, but none were observed.
Preliminary results from the first five harvests are presented in the following table. The concentration of the active ingredient was boosted by the second application of phosphonate but it decreased to a low level of 0.6 ppm six months after spraying. Despite the low concentration of the phosphite ion in plant tissues the plants remain healthy.
The site has been monitored every six months to assess plant health and changes in phosphite tissue concentration.

**Table 1** Phosphite concentration ($\mu g\ g^{-1}$) in leaf tissue of *B. brownii*
The vaues represent preliminary results as they are the means of only five samples

| Sampling time | $PO_3$ ($\mu g\ g^{-1}$) |
|---|---|
| before 1st spraying | 0 |
| after 1st spraying | 1.3 |
| before 2nd spraying | 1.3 |
| after 2nd spraying | 6.2 |
| six months after spraying | 0.6 |

## 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 and infection fronts are causing wide spread destruction.
*B. coccinea* is a keystone species as its flowers are an important food source for small marsupials and birds. It is also a commercially important species.

The trial consists of eight plots ( 4 pairs of sprayed and control) which have been set up on the infection front. The plots are 40 m long and 20 m wide. Within each sprayed plot an assessment sub-plot was marked (20 m long 10 m wide). Twenty plants were marked in each of the assessment sub-plot. Plant height was measured every six weeks before spraying and will be measured biannually after the treatment. Change in the rate of plant growth is a good indicator of phytotoxicity so the pre- and post-spray measurements will allow to determine whether the chemical has any deleterious effects on the plants.
The number of dead plants within each plot was also measured before spraying and will be recorded twice a year after spraying to establish the duration of the effectiveness of the fungicide. Plant samples are taken for chemical analysis to determine the concentration of the phosphite ion.

The plots were treated in early November and a follow-up spraying was carried out in the first week of December 1993.
The plots were sprayed twice with 10% phosphonate (with 0.5% Synertrol as a wetting agent) in a low volume of 30 l/ha which gave the effective rate of 60 l/ha. The second spraying in December was done at the same rate.
In order to monitor the coverage of the spray and droplet size, water-sensitive papers were placed in the treated plots. After the first spraying we noticed that the coverage had not been satisfactory which was shown by a low number of droplets per unit area (20-30 /$cm^2$). Poor coverage was caused by excessive drift that occurred despite only slight to moderate wind (10-25 km/hr).
In the second spraying we increased the droplet size from 100-300 µm to 300-600µm which improved coverage significantly to a desired number of around 60 droplets/$cm^2$. Despite the increased droplet size the penetration of the plant canopy was excellent. It appears that application of bigger droplets is more beneficial in areas where windy conditions prevail such as South-West. In addition, when only small area is to be sprayed, increased droplet size is preferred as it will contribute to improved precision of the application and minimise losses through drift.
The results from the first four harvests are presented in the following table.
The concentration of the active ingredient was significantly increased by the second application of phosphonate. Improved converge due to increased droplet size in the follow-up application resulted in significantly higher concentration of phosphite in plant tissue. The substantial differences in phsphite concentration between plots resulted from uneven application as the pilot had some difficulties in targeting the small experimental plots.

**Table 2** Phosphite concentration in leaf tissue of *B. coccinea*

| | before 1st spr. ($\mu g\ g^{-1}$) | after 1st spr. ($\mu g\ g^{-1}$) | before2nd spr. ($\mu g\ g^{-1}$) | after 2nd spr. ($\mu g\ g^{-1}$) |
|---|---|---|---|---|
| plot 1 | 0 | 0.9 | 0.15 | 4.15 |
| plot2 | 0 | 4.3 | 5.1 | 13.2 |
| plot3 | 0 | 1.4 | 1.2 | 6.2 |
| plot4 | 0 | 2.5 | 2.4 | 34.2 |

## 1.3 Aerial spray trial at Millbrook Reserve

*Phytophthora cinnamomi* has had a high impact on the population of rare *Banksia brownii* at Millbrook Reserve 30 km north of Albany. A foliar application field trial (using backpack sprayers) carried by CALM demonstrated that phosphonate protected the plants for up to three years. The infection front has moved three metres past the experimental plots leaving the sprayed plants healthy.
This aerial spray trial is a second one involving *Banksia brownii* and it will allow comparison of the effectiveness of phosphonate application in controlling *Phytophthora* infection in the same plant species but in two different areas. In addition

the application of the fungicide at Millbrook will save another population of this endangered plant species from extinction.
Almost the whole population of *B. brownii* (6 ha) has ben sprayed aerially, using low volume application. Plants in the Millbrook Reserve and Gull Rock were sprayed on the same days so the follow-up application at Millbrook also involved larger droplet size. The plots were sprayed twice with 10% phosphonate (with 0.5% Synertrol as a wetting agent) in a low volume of 30 l/ha which gave the effective rate of 60 l/ha. The second spraying in December was done at the same rate. Chemical analysis and plant health measurements have been carried out and the duration of protection will be established. The results from this trial have been compared with the ones obtained from South Sister. It will allow to determine whether phosphonate acts similarly in plants grown in different conditions.
The results from the first four harvests are presented in the following table.
The concentration of the active ingredient was significantly increased by the second application of phosphonate. Improved coverage due to increased droplet size in the follow-up application resulted in significantly higher concentration of phosphite in plant tissue.
The "after2nd spraying" concentration of phosphite at Millbrook was almost 4 times higher than at South Sister due to the following factors:
- spray concentration was increased from 8.5% to 10%
- application rate was increased from 30 l/ha to 60 l/ha
- droplet size was increased (lower losses through drift and improved coverage)

**Table 3** Phosphite concentration ($\mu g\ g^{-1}$) in leaf tissue of *B. brownii*

| Sampling time | $PO_3$ ($\mu g\ g^{-1}$) |
|---|---|
| before 1st spraying | 0 |
| after 1st spraying | 4.5 |
| before 2nd spraying | 1 |
| after 2nd spraying | 22 |

## 2. The efficacy of ground application of phosphonate on declared rare flora

CALM recently treated with phosphonate, spot infections of declared rare flora species. They included a population of *Andersonia grandiflora*, a small shrub that occurs only in a few localities in the South-West and three years old regeneration of rare *Banksia brownii*.
Samples of foliage have been taken for analysis to determine the longevity of the chemical within young plants. Health of the populations will be assessed periodically to determine whether phosphonate can protect young susceptible plants against infection for any length of time.
Plant samples are being processed for residue analysis which will be carried out in April 1994.

## 3. Determination of the effective concentration of phosphonate residues that controls the pathogen

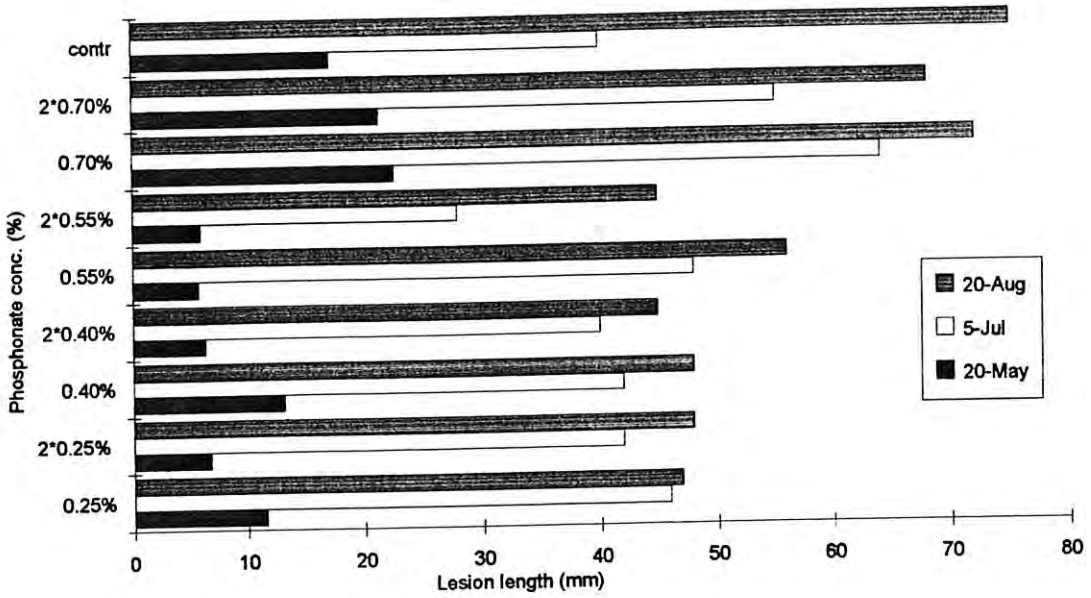
Currently we have no knowledge of the persistence of phosphite ions in native plant species. This information is essential in developing an effective management program of protecting native plant communities from further decline and imperative as a first step in understanding the mode of action of the chemical.

A glasshouse experiment was set up in April 1993 to measure the concentration of phosphite ion over time and correlation with plant performance as well as lesion development. The effect of repeated application of the fungicide on the duration of protection will also be evaluated. It is probable that two applications at a lower rate are more effective than a single application at a higher and less likely to cause phytotoxicity.

Two years old *Banksia grandis* plants were stem inoculated with *P. cinnamomi*. The fungicide treatment was applied 5 days after inoculation when the lesions were well established (about 10 mm diam.). Four concentration rates sprayed once and twice will be tested: 0.25%, 0.40%, 0.55% and 0.70%.
The experiment is planned to continue for two years which will allow five harvests to monitor phosphite concentration in leaf tissue. Lesion extension, crown health, plant height and dry weights are also measured.

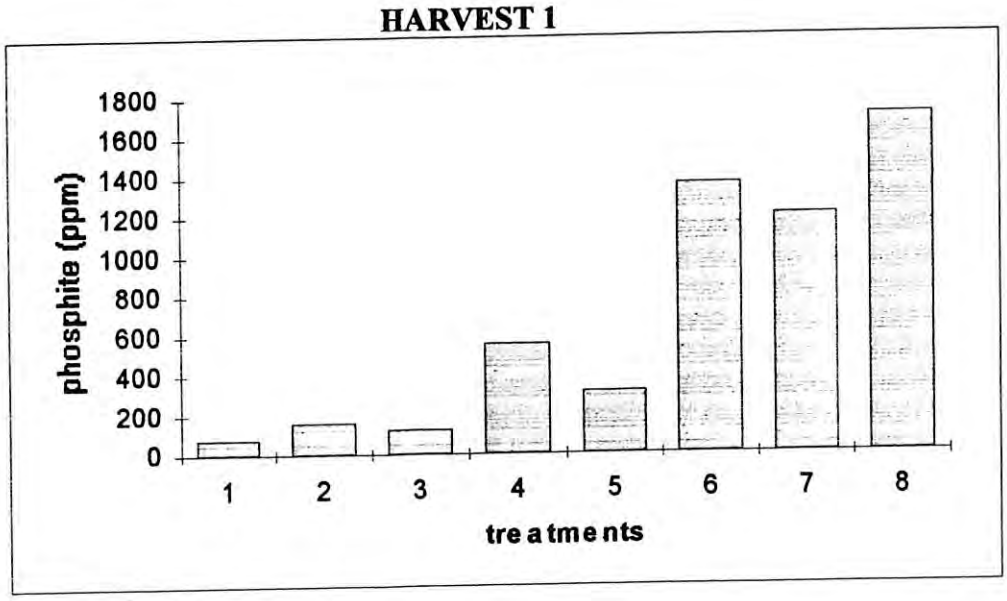
In the initial period, lesions were measured every 6 weeks. The results (Figure 1) show that there was no difference among the treatments in the lesion length in the first two months. In the third month the growth rates of the lesions in the plants sprayed with 0.25% to 0.55% phosphonate decreased, but increased substantially in the control. The application of the highest concentration of phosphonate caused some phytotoxicity that was visible as slight burning of young foliage. After about three weeks the plants recovered. Lesions in plants sprayed with 0.70% phosphonate were initially similar to the controls but later they stopped growing.
All the controls died by September 1993.

**Figure 1** Lesion length (mm) in the first four months after inoculation and spraying. 2* indicates plants sprayed twice.

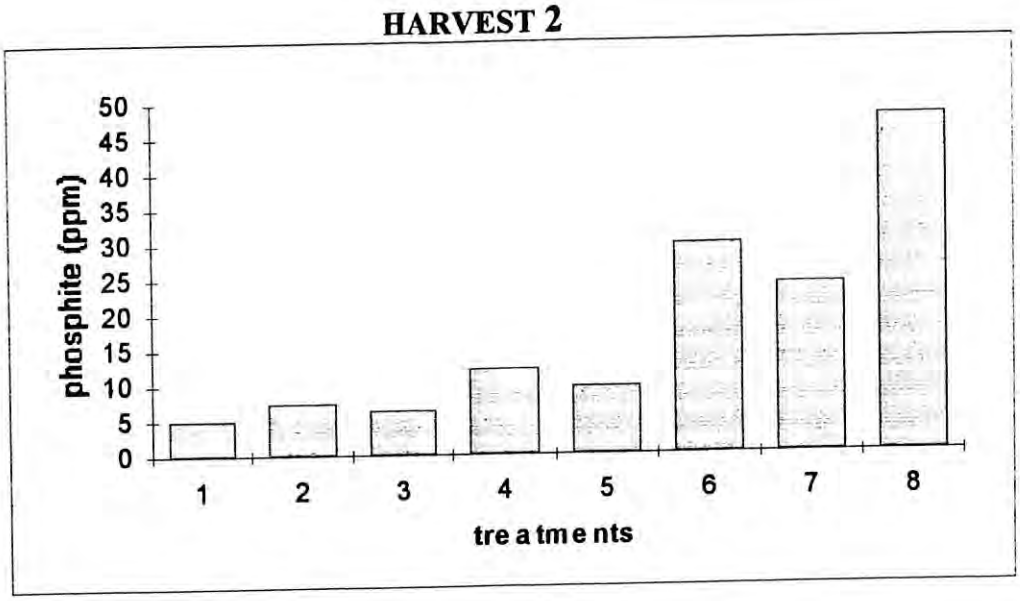



One month after the follow-up spraying, plant samples were taken for chemical analysis (first harvest). Second harvest was done six months later. The concentrations of the phosphite ion were measured and the results the first two harvests are presented in Figure 2

**Figure 2** Leaf tissue concentration of phosphite in plants treated with various concentrations of phosphonate.



**HARVEST 2**

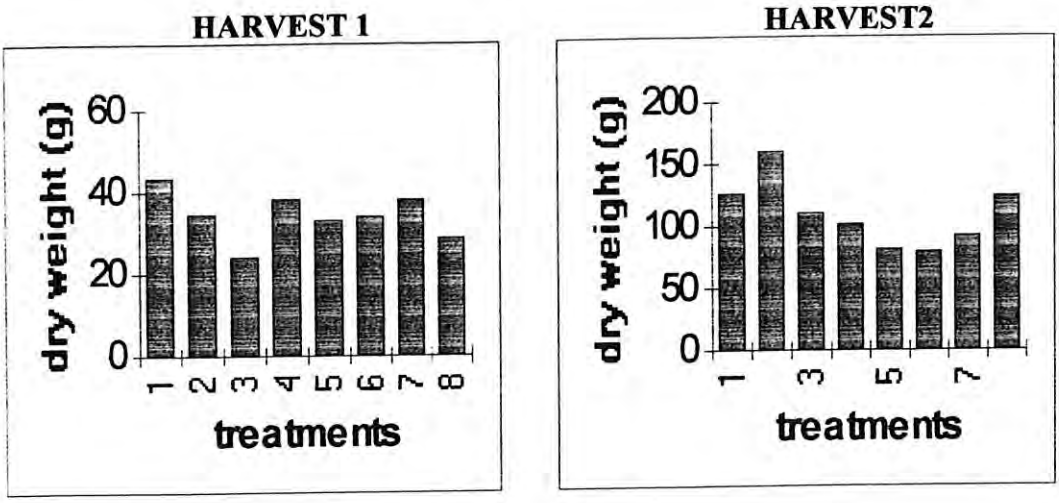



***Treatments:***

**1** - 0.25% applied once
**2** - 0.25% applied twice
**3** - 0.40% applied once
**4** - 0.40% applied twice
**5** - 0.55% applied once
**6** - 0.55% applied twice
**7** - 0.70% applied once
**8** - 0.70% applied twice

The second spraying increased the level of the active ingredient significantly at all spray concentrations and the effect persisted into the second harvest.
The concentration of phosphite decreased 20 to 30 times in the period of six months whereas the dry weights (Figure 3) increased about 3 times in the same time in all treatments. Therefore, this substantial decrease in tissue phosphite concentration cannot be explained by the increase in the dry weight alone.
The loss of phosphite from plant tissues was probably caused mainly by losses through root turnover and root exudation and also through some leaf senescence.
Further experiments are planned to determine the fate of phospite ion in plants and the extent of loss through the roots.
Although the phosphite concentration dropped significantly in six months it is still high enough to have a direct effect on the pathogen in all treatments. Further harvests will demonstrate how the phosphite tissue concentration changes over a longer time and whether the longevity as well as the efficacy of the fungicide can be improved by the second application of the chemical.
The differences in dry weights between the treatments are not significant in both harvests.

**Figure 3** Dry weight of above ground plant parts in plants treated with various concentration of phosphonate.




## 4. Efficiency of absorption of phosphonate

The fungicide phosphonate will often be used in adverse environmental conditions. Rain is a frequent occurrence in the South-West of Western Australia in autumn to spring - the period when the chemical will be either aerially or ground applied. Therefore a glasshouse experiment was carried out to determine the effect of different concentrations of surfactant (Synertrol oil) on the absorption of the fungicide and a requisite dry period after spraying for maximum absorption.

Two year old *Banksia brownii* plants were sprayed with 0.5% phosphonate that had either 0.3% or 0.6% of Synertrol added and the foliage was exposed to simulated heavy rainfall (overhead sprinklers for 15 minutes) 1, 3, 5 and 7 hours after spraying and compared with control that was not exposed to rain for 12 hours.
Two days later plants were harvested and samples taken for analysis. The results are presented in Table 4.

**Table 4** Leaf phosphite concentration ($\mu g\ g^{-1}$) in the treated plants after simulated heavy rainfall.

| | 1 hour | 3 hours | 5 hours | 7 hours | control |
|---|---|---|---|---|---|
| Syn. 0.3% | 0 | 2.7 (0.3) | 66.7 (5.0) | 89.3 (7.0) | 175.6 (18.0) |
| Syn. 0.6% | 0 | 3.0 (1.0) | 69.3 (6.0) | 99.7 (10.0) | 183.7 (22.0) |

Numbers in brackets show standard errors

The results show that the timing of rainfall has a major effect on the amount of phosphonate residues found in the plant tissue. When rain occurred 1 hour after spraying no phosphite (sensitivity of analysis was 1 ppm) was absorbed into the tissues. It also appeared that even after 7 hours, phosphonate was still being absorbed, although the high level of phosphite in the control could be attributed to some residual fungicide on the leaf surface.
Doubling Synertrol in the spray did not improved absorption.

The results from this experiment have important impact on the management of the fungicide application. It is obvious that phosphonate should not be applied when rainfall is expected within less than seven hours. It is also important to point out that light drizzle would probably not have as significant effect on absorption as heavy rain.

# 5. Mechanism of action of phosphonate

## 5.1 Bioassay of the activity of phosphonate

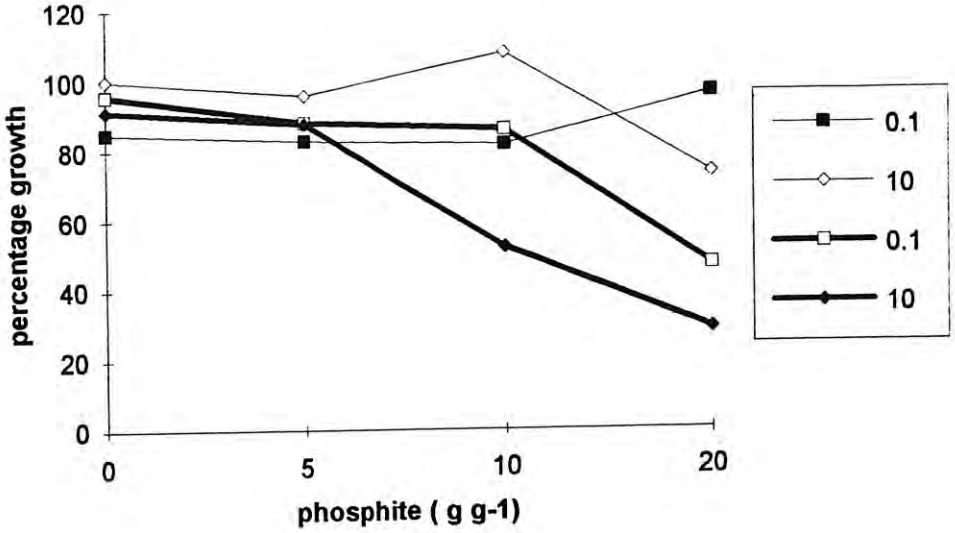
Despite considerable research uncertainty about the mode of action of phosphonate remains. Frequently the conclusion is drawn on the basis of comparing the concentrations of phosphite ion found *in vivo* that control the pathogen with inhibitory levels obtained *in vitro* (Fenn and Coffey 1984, Smillie *et al.*1989). Concentrations of phosphite ion that completely inhibit growth of the pathogen *in vitro* are very variable in different experiments depending mainly on the level of phosphate. They also vary greatly between different *Phytophthora* species and even isolates.
In most of the *in vitro* experiments phosphonate is added to the media before autoclaving (eg. Coffey and Bower 1984) which can cause the $PO_3$ ions to oxidise to $PO_4$. Robertson and Boyer (1956) determined that phosphite is stable only in the temperatures up to 60°C. The temperature of autoclaving is 121°C. Therefore the actual levels of the phosphite ion in the medium that affect the growth of the fungus could be much lower than the levels added. When phosphite is oxidised, the growth of the pathogen is actually stimulated because the concentration of phosphate increases in the growth medium.

An *in vitro* experiment was carried out to determine the effects of heat (autoclaving) on the growth of *Phytophthora* isolates in the modified Ribeiro's medium amended with phosphonate and three levels of phosphate. The results of this experiment will contribute to better understanding of the mechanism of action of phosphonate.

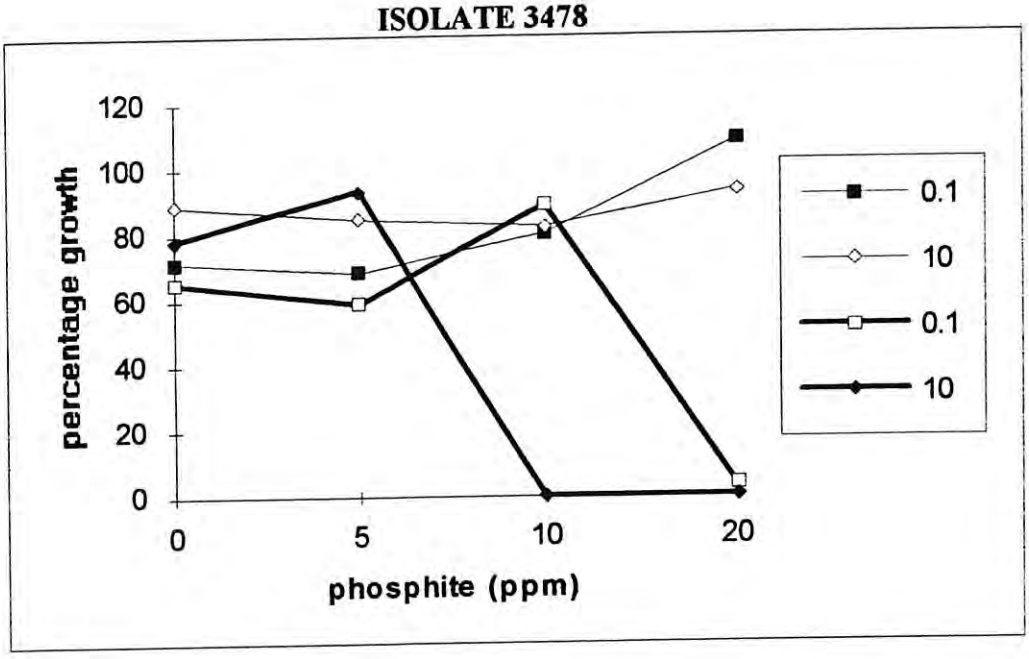
Initial trials show growth rates of *P. cinnamomi* were significantly higher in the heated treatment in cultures at both low and high in phosphate due to transformation of phosphite to phosphate.(Figure 4). The differences are significant at $PO_3$ levels above 10 $\mu g\ g^{-1}$ (in the high phosphate treatment) and above 15 ppm (in the low phosphate treatment). The results indicate that inhibitory levels of phosphonate may be lower than previously reported.
**Figure 4** Rate of growth of a *P. cinnamomi* isolate expressed as percentage of

control vs concentration of $PO_3$ at varying levels of $PO_4$ (0.1 and 10 Mm) Thin lines indicate heat treatment.

**ISOLATE Sc 72**




**ISOLATE 3478**




## 5.2 *In vitro* variability among four isolates of *Phytophthora* species

The previous experiment demonstrated that autoclaving growth media amended with phosphonate causes transformation of phosphite to phosphate. Therefore the levels of phosphonate that inhibit the growth of the pathogen *in vitro* may actually be lower than the ones reported in the literature.
In our experiment we measured growth rates of four isolates at five levels of phosphite and five levels of phosphate. The levels of $PO_3$ and $PO_4$ as well as growth medium were similar to ones commonly used by other researches. Phosphorous acid (adjusted to pH 6.2) was added to agar media **after** autoclaving.

### 5.2.1 The effect of phosphate on the toxicity of phosphonate in the growth medium

The toxicity of phosphonate was enhanced on low phosphate media. At 1 mM of phosphate, increased concentration of phosphite resulted in the large reduction of growth of all isolates. At 20 mM the reduction was much lower and also varied between the isolates. This confirms the results from the literature (eg. Fenn and Coffey 1985) and it suggests that phosphate metabolism may be one metabolic target of phosphonates. This result also explains why phosphonate is so effective in the control of the pathogen in native species which are low in phosphate.

**Figure 5** Radial growth of four different isolates of *Phytophthora cinnamomi* at 1 mM of $PO_4$

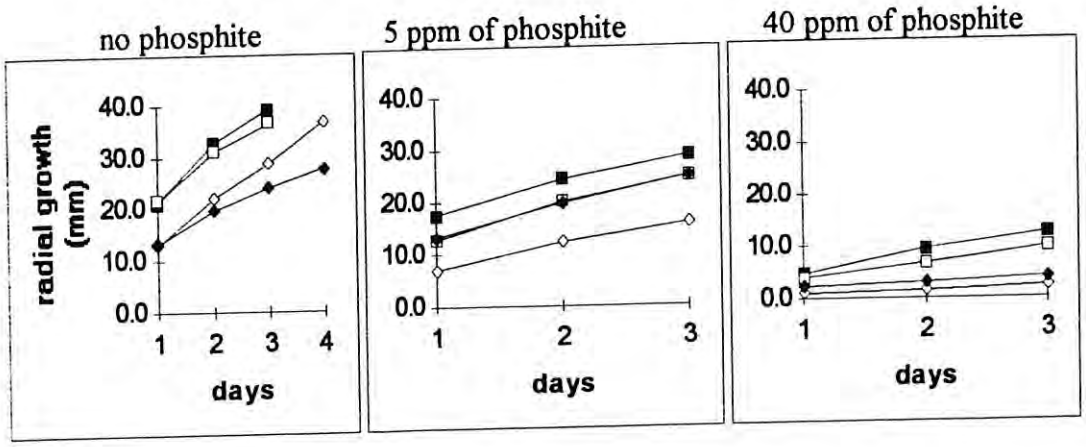
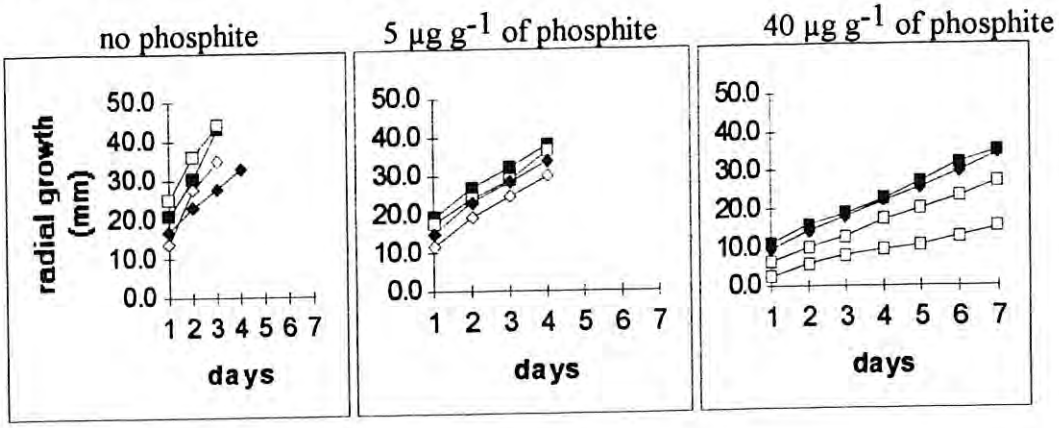


**Figure 6** Radial growth of four different isolates at 20 mM of $PO_4$




## 5.2.2 *In vitro* responses to phosphonate.

Inhibition percentages of radial growth by 5 µg $g^{-1}$ phosphorous acid at 1 mM of phosphate were calculated and compared with similar values quoted in the literature (Coffey and Bower 1984). Our values of 1% for *P. cinnamomi* isolate Rsc72, 18% for isolate Cin2, 40.5% for isolate Mu and 46% for isolate Dce60 are generally higher when compared with other A2 mating type isolates. It suggests that the levels of phosphonate that inhibit the growth of the pathogen *in vitro* may actually be lower than the ones reported.
Further data from this study will be presented in the next report.

## References

Coffey, M.D. and Bower, L.A. (1984). *In vitro* Variability Among Isolates of Eight *Phytophthora* Species in Response to Phosphorous Acid. Phytopathology 74:738-742. The American Phytopathological Society

Fenn, M. E. and Coffey, M. D. (1984). Studies on the *in vitro* and *in vivo* antifungal activity of fosetyl-Al and phosphorous acid. Phytopathology 74: 606-611.

Jacobsen, B.J. (1986). Calibration and Use of Aircraft for Applying Fungicides. In Hickey K.D. ed. Methods for evaluating Pesticides for Control of Plant Pathogens.

Robertson, H.E. and Boyer, P.D. 1956. Orthophosphite as a buffer for biological studies. Arch. Biochem. Biophys. 62:396-401.

Smillie, R.H., Grant, B.R. and Guest, D.I (1989). The mode of action of phosphite: Evidence for both direct and indirect modes of action on three species of *Phytophthora* in plants. Phytopathology 79: 921-926.

CALM (1991). Dieback disease in Western Australia. CALM Briefing Paper 5/91.

# PROJECT 2

# DEVELOPMENT OF A DNA DIAGNOSTIC TEST FOR *PHYTOPHTHORA CINNAMOMI*

**P. O'Brien**

## 1. ESTABLISH A COLLECTION OF *PHYTHOPHTHORA* ISOLATES

Complete.

## 2. IDENTIFICATION OF *PHYTOPHTHORA* TAXON SPECIFIC DNA SEQUENCES

We have reported previously the isolation of DNA sequences specific for *P. cinnamomi*. These can be used as the basis of a diagnostic test for detection of *P. cinnamomi*. Upon isolation of these sequences we decided to proceed with the development of the diagnostic test. The intention is to isolate sequences specific for other taxa at a later stage.

*Sequences specific for other taxa (P. megasperma and P. citricola) will be isolated during 1995/1996, and developed into PCR assays (see timetable). During the validation period these tests can be validated coincidentally with the test for P. cinnamomi. the development of PCR tests for these sequences will be greatly facilitated by the techniques developed for the P. cinnamomi test.*

## 3. DEVELOP A QUANTITATIVE PCR TEST FOR EACH SEQUENCE

### 3.1 Construction of pairs of oligonucleotide primers

Oligonucleotide primers have been synthesised based on the sequence of the cloned DNA fragment. A number of primers were synthesised from each end of the sequence and they can be used in pairwise combinations. The primer pairs were tested and it was found that each pair gave a number of bands rather than a single band. he pattern consisted of one or two intense bands and a number of weakly staining bands. Inclusion of DMSO in the PCR reaction to increase the specificity of primer binding was found to eliminate the weakly staining bands from the pattern. These bands are thought to be due to nonspecific annealing of the primers to the template. The best results were seen with 8%DMSO and 2mM$MgCl_2$. The products were analyzed by electrophoresis on acrylamide.

*We need to further optimise the PCR reaction by investigating parameters such as the concentration of NTP's, the amount and brand of Taq polymerase (there are substantial differences between brands), and the effect of hot start. We also need to test primers for the other P. cinnamomi sequences we have isolated.*

### 3.2 Test primer pairs against target *Phytophthora* taxon

The primers give an amplification product in reactions with *P. cinnamomi* DNA. No such products are observed in reactions with no added DNA. In addition to testing the detection of mycelium, we have also tested detection of zoospores and chlamydospores. Zoospores were produced and mixed with soil at a concentration of $2x10^2$/g soil. This concentration was easily detectable.

*We are still working on the production and detection of chlamydospores.*

### 3.3 Testing the specificity of the primer pairs against the target *Phytophthora* taxon

The primer pairs were tested in PCR reactions with template DNA from different *Phytophthora* species. Amplification products were obtained only in reactions containing *P. cinnamomi* DNA.

*We need to test an expanded range of isolates before undertaking field trials.*

### 3.4 Development of an internal standard

Use of the diagnostic test under field conditions will require development of an internal standard to preclude false negatives. The standard can be developed from the cloned fragment by insertion of a DNA sequence (150-300bp) into a suitable restriction site within the cloned fragment. This insertion will result in a larger amplification product than would be observed from the original fragment. Addition of the standard to a sample containing *P. cinnamomi* DNA will result in two amplification products observed by gel electrophoresis, the larger from the internal standard and the smaller from the *P. cinnamomi* DNA in the sample. Failure to observed the larger would indicate inhibition of the PCR reaction by components in the sample. the test would need a period of validation under field conditions during which results obtained with the test were compared with results obtained by current procedures.

## 4. VALIDATE THE TEST BY FIELD TRIALS

### 4.1 Method for extraction of DNA from soil

Soil contains a number of substances variously referred to as "humic acids". These are extracted along with DNA and they inhibit subsequent enzymatic reactions such as PCR and restriction digestion. A number of methods for extraction of bacterial DNA from soil have been developed, but these are too cumbersome and not applicable to fungi. Our purpose was to develop a method which is simple and effective and not too laborious.

We have used five different types of soils in the development of the extraction procedures. They are representative of the soil types in which *P. cinnamomi* is expected to be prevalent. We found that extraction of the soils with buffer caused the release of substances which are potent inhibitors of the PCR reaction. These have to be removed.

The method which we have developed involves boiling the soil in a buffer to lyse the mycelium and release the DNA. The DNA is then concentrated by binding to glass beads (Geneclean proprietary product). However we found that substances

released from the soil during the extraction were not removed by the gene clean step and further purification was required. this can be achieved by chromatography through a sieving gel such as Sephadex. Initially we used gravity flow but have also successfully used a centrifugal column which takes less time and maintains the DNA in a more concentrated form. A number of gel exclusion columns are available commercially, and we have tried these unsuccessfully. They are not effective at removing the inhibitors. This may relate to the size of the gel column. The commercial columns are no more than 1.5 cm in length whereas the columns we use are 4-5 cm in length. We are currently purchasing some columns from Pharmacia which are longer than the more commonly used ones and hopefully they will be successful.

*We have to (i) resolve the problems with the sieving columns, (ii) test all of the soil types*

**4.2 Validation of the test by reconstruction experiments with soil amended with *P. cinnamomi***

This experiment is to determine the sensitivity of the test under field conditions. The approach being taken is to mix dilutions of *P. cinnamomi* DNA with soil extract, and after purification amplify the DNA using the specific primers. The amended soil samples will be analyzed concurrently with the baiting procedure so that the tests can be accurately compared for sensitivity.

*This item has not yet started.*

**4.3 Validation of the test with natural soils and plant material**

In this activity samples of soil and plant material are analyzed for *P. cinnamomi, P. megasperma* and *P. citricola* by (a) baiting technique, (b) DNA probe by hybridization analysis, and (c) PCR test so that we can evaluate the performance of the test under field conditions, and compare the effectiveness of the test with the baiting method. We include the hybridization test as an independent confirmation of the results obtained with the PCR test. The field samples are likely to be more heterogeneous than the laboratory samples and contain material from a much wider range of microbial, animal and plant species, it is possible that the PCR test may interact nonspecifically with some of these species, but it is unlikely that the hybridization test would.

*This item has not yet started.*

**4.4 Using the test to study colonization of the host plant**

The presence of the fungal mycelium within tissues can be detected by in sity PCR of tissue sections. Sections can be taken at intervals after infection and analyzed. this will enable us to follow the progress of the fungus through the plant. By comparing resistant and sensitive plants we might get a clue as to the factors affecting the sensitivity of the host. We can also use the technique to determine which tissues and which cells within a tissue are colonized.

*This item has not yet started.*

Information on the time required for these items is given in the timetable.

## PROJECT 3

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

**R. Wills, A. Conacher, A. Chapman and G. Behn**

## OBJECTIVE

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

The project has three elements:

1. assemble, verify and automate appropriate data sets;
2. interrogate data sets to answer basic management questions;
3. develop a model with predictive capabilities and test validity of predictions.

The Two People's Bay-Mt Manypeaks area in the Albany District (Fig. 1) was chosen as the prototype target area because of its long history of *Phytophthora* impact, the presence of 22 priority plant taxa including 7 Declared Rare Flora (DRF) (eg *Banksia brownii*) and the large representation of susceptible species, combined with the availability of classified satellite imagery resulting from a joint project by CALM's Land Information Branch and CSIRO's Division of Mathematics and Statistics.

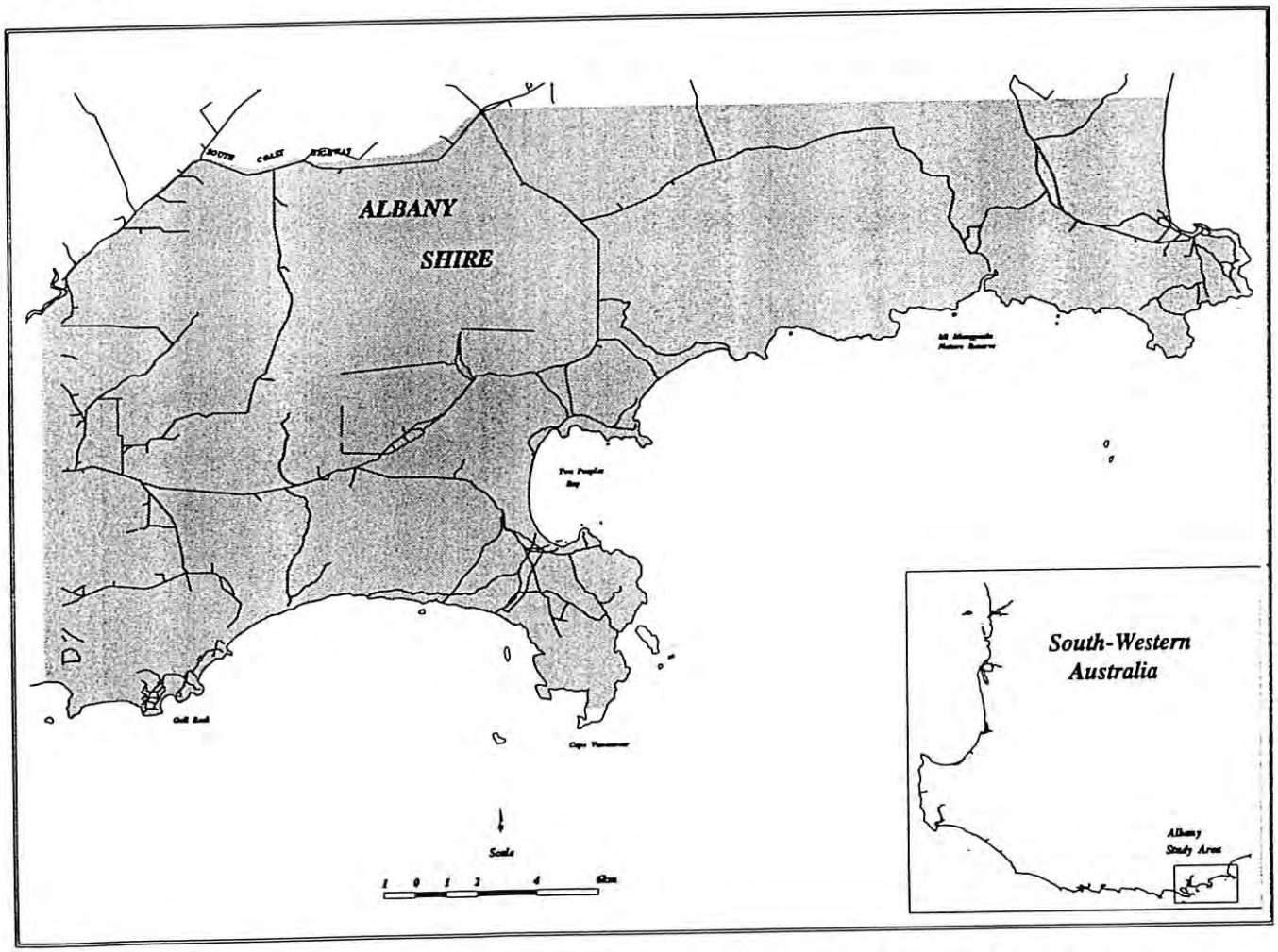



**Figure 1. The Two People's Bay-Mt Manypeaks study area in the Albany District.**

## 1. OBTAIN DATA SETS

Contour data (10 m) were digitised from 1:50 000 topographic maps obtained from the Department of Land Administration (DOLA) and used to generate a Digital Terrain Model using the Arc/Info module TIN. The DTM was then used to build an Elevation Lattice (30m resolution) using GRID. Additional GRIDs of aspect, slope, flow direction and flow accumulation were also built.

The distribution of dieback based on disease impact on vegetation (Fig. 2) was derived from the classification by one of us (G. Behn) of two co-registered Landsat images from February and April 1991. The classification data were imported into Arc/Info and used to generate polygon coverages of dieback distribution.

Landforms and soils for the study area (Appendix 1) are taken from Churchward *et al.* (1980).

Other data sets are still to be supplied by their custodians.

Integration with the Herbarium's existing specimen data will be achieved when a SQL-compliant RDBMS compatible with ARC/Info is obtained later this year.

## 2. INTERROGATE DATA SETS

To date, the mapping of dieback has relied on aerial photograph interpretation together with ground validation of mapping. Use of Landsat imagery for mapping combined with GIS to interrogate dieback maps has a number of immediate benefits. A range of questions relevant to both managers and researchers can be quickly answered that were previously impossible to attempt or extremely time consuming. For example:

**Which areas are infected?**

Estimates of the amount of area infected with dieback have been obtained for the first time. In this study area, 9.6% of the area is identified, using the Landsat imagery, as having dieback present (Table 1)[1]. Of the six landforms individually making up more than 5% of the study area, four have above average levels of dieback present. In examining landforms, it is apparent that Gardner type Gs is highly impacted, while Gardner type Gg appears much less affected by dieback (Table 1).

**What landforms are severely affected by dieback?**

While the overall level of apparent infection (<10%) is relatively small, some units of landform exhibit severe impact (Table 2), especially in the eastern end of the study area (Fig. 3). For example, one unit of the Dempster landform type Dc has 78% of its area classified as dieback infected (Table 2).

**Are there areas of high hazard landform that are not infected?**

**Are there any areas which appear to be of low risk?**

While less than 10% of the study area is classified as infected, only 93 polygons representing 5889 ha (14% of study area) have less than 1% of their total area infected (Fig 4.). Only 3 of the 30 Gardner type s landforms are uninfected, and while only a small proportion of Gardner type g landform is infected, 16 out of 20 polygons are infected.

---

[1]Note that the study area includes about 8750 ha of farmland (21%) which has been classified from the Landsat processing as "non-dieback". If this amount is taken into account, 12.1% of the remainder appears infected. Digital tenure information necessary to clip the cleared private land from the study area is still to be received from the data custodian.

**Figure 2. The distribution of dieback based on impact on vegetation derived from the classification of Landsat images for the Two People's Bay- Mt Manypeaks study area.**

**Figure 3. The distribution of landform polygons with greater than 20% infection in the study area.**

**Figure 4. The distribution of "No dieback" landforms with individual polygons having infections < 1% of total area.**

**Table 1. Area of infection by landform (Landforms making up >5% of the study area highlighted in bold)**

| Landform code | Landform description | Area of infected landform (ha) | Area of landform (ha) | % landform infected | % of infection in study area | % of landform in study area |
|---|---|---|---|---|---|---|
| BAf | Barrow | 28.1 | 1287.6 | 2.2 | 0.07 | 3.0 |
| BAg | Barrow | 8.0 | 233.0 | 3.4 | 0.02 | 0.6 |
| BO | Boulongup | 85.3 | 1288.0 | 6.6 | 0.20 | 3.1 |
| BWp | Blackwater | 13.8 | 197.2 | 7.0 | 0.03 | 0.5 |
| **Dc** | **Dempster** | **763.0** | **8679.0** | **8.8** | **1.80** | **20.5** |
| Ds | Dempster | 30.0 | 186.2 | 16.1 | 0.07 | 0.4 |
| **F** | **Fernley** | **283.0** | **2671.5** | **10.6** | **0.67** | **6.3** |
| **Gg** | **Gardner** | **155.4** | **4603.2** | **3.4** | **0.37** | **10.9** |
| **Gs** | **Gardner** | **543.9** | **3237.5** | **16.8** | **1.29** | **7.7** |
| Mc | Meerup | 80.7 | 623.6 | 12.9 | 0.19 | 1.5 |
| Mf | Meerup | 17.7 | 253.3 | 7.0 | 0.04 | 0.6 |
| Mp | Meerup | 120.3 | 1046.8 | 11.5 | 0.28 | 2.5 |
| Mr | Meerup | 7.9 | 71.6 | 11.1 | 0.02 | 0.2 |
| Ms | Meerup | 94.5 | 984.1 | 9.6 | 0.22 | 2.3 |
| Mu | Meerup | 1.1 | 437.9 | 0.2 | 0.002 | 1.0 |
| My | Meerup | 18.1 | 397.2 | 4.6 | 0.04 | 0.9 |
| OW | Owingup | 15.9 | 811.7 | 2.0 | 0.04 | 1.9 |
| PN | Pillenorup | 5.1 | 172.5 | 3.0 | 0.01 | 0.4 |
| S6 | Minor Valleys | 219.6 | 1960.9 | 11.2 | 0.52 | 4.6 |
| **S7** | **Minor Valleys** | **322.0** | **3020.4** | **10.7** | **0.76** | **7.1** |
| S9 | Minor Valleys | 257.9 | 1505.0 | 17.1 | 0.61 | 3.6 |
| **TK** | **Takalarup** | **980.7** | **8261.4** | **11.9** | **2.32** | **19.5** |
| V1 | Major Valleys | 1.3 | 42.4 | 3.0 | 0.003 | 0.1 |
| V7 | Major Valleys | 2.1 | 180.8 | 1.2 | 0.005 | 0.4 |
| WATER | Water | 0.1 | 135.4 | 0.1 | 0.00 | 0.3 |
| Total | | 4055.4 | 42287.7 | 9.6 | 9.59 | 100 |

**Table 2. Examples of landform polygons with greater than 20% infection (19 records with areas greater than 20 ha presented)**

| Landform | Infected area | Total area | % Infected |
|---|---|---|---|
| Dc | 287.5 | 812.2 | 35.4 |
| Dc | 30.1 | 57.2 | 52.6 |
| Dc | 51.1 | 87.9 | 58.1 |
| Dc | 38.1 | 48.8 | 78.0 |
| F | 108.8 | 517.7 | 21.0 |
| F | 31.9 | 86.0 | 37.1 |
| Gs | 94.6 | 432.3 | 21.9 |
| Gs | 34.6 | 150.7 | 23.0 |
| Gs | 128.8 | 550.4 | 23.4 |
| Gs | 27.7 | 99.0 | 28.0 |
| Gs | 21.9 | 38.5 | 56.8 |
| Gs | 21.5 | 30.6 | 70.2 |
| S6 | 35.2 | 95.3 | 36.9 |
| S6 | 26.8 | 72.4 | 37.1 |
| S6 | 63.1 | 94.6 | 66.6 |
| S7 | 56.7 | 188.1 | 30.1 |
| S7 | 95.4 | 212.4 | 44.9 |
| S9 | 81.7 | 250.5 | 32.6 |
| S9 | 79.1 | 153.4 | 51.6 |

**Table 3. "No dieback" landforms with individual polygons having infections < 1% of total area.**

| Landform code | Landform description | Total area | Area <1% infected | % "No dieback" |
|---|---|---|---|---|
| BAf | BARROW | 1287.6 | 1037.4 | 81% |
| BAg | BARROW | 233.0 | 72.1 | 31% |
| BO | BOULONGUP | 1288.0 | 789.5 | 61% |
| BWp | BLACKWATER | 197.2 | 0 | 0% |
| Dc | DEMPSTER | 8679.0 | 407.4 | 5% |
| Ds | DEMPSTER | 186.2 | 53.5 | 29% |
| F | FERNLEY | 2671.5 | 275.3 | 10% |
| Gg | GARDNER | 4603.2 | 35.0 | 1% |
| Gs | GARDNER | 3237.5 | 190.6 | 6% |
| Mc | MEERUP | 623.6 | 134.8 | 22% |
| Mf | MEERUP | 253.3 | 75.8 | 30% |
| Mp | MEERUP | 1046.8 | 137.2 | 13% |
| Mr | MEERUP | 71.6 | 0 | 0% |
| Ms | MEERUP | 984.1 | 79.0 | 8% |
| Mu | MEERUP | 437.9 | 434.1 | 99% |
| My | MEERUP | 397.2 | 11.6 | 3% |
| OW | OWINGUP | 811.7 | 214.5 | 26% |
| PN | PILLENORUP | 172.5 | 48.9 | 28% |
| S6 | MINOR VALLEYS | 1960.9 | 597.1 | 30% |
| S7 | MINOR VALLEYS | 3020.4 | 740.3 | 25% |
| S9 | MINOR VALLEYS | 1505.0 | 102.6 | 7% |
| TK | TAKALARUP | 8261.4 | 315.3 | 4% |
| V1 | MAJOR VALLEYS | 42.4 | 2.2 | 5% |
| V7 | MAJOR VALLEYS | 180.8 | 0 | 0% |
| WATER | WATER | 135.4 | 135.4 | 100% |
| Total | | 42287.7 | 5889.5 | 14% |

**Does infection vary with aspect?**

*Phytophthora cinnamomi* prefers warmer temperatures as growth of the fungus is limited below 15 °C and ceases below temperatures of 5 °C (Zentmyer 1980, Shearer *et al.* 1987b). While the fungus may be vegetatively active at temperatures of 10 °C (Weste & Marks 1987), sporulation is very limited below 15 °C (Shearer *et al.* 1987b). Optimum growth occurs at soil temperatures of 20 - 32 °C (Zentmyer 1980, Shearer *et al.* 1987b). Because of the effect of temperature on growth, that north facing slopes should carry a higher level of infection.

When rates of infection are examined by aspect, within a framework of 45° units, north facing slopes do indeed have a higher rate of infection (average 10.9%) than south facing slopes (average 8.0%) (Table 4, Fig. 5). This is also reflected in the ditribution of largest infections, with 56% of infections greater than 50 ha in extent found on north facing slopes.

However, ground truthing must be undertaken to validate these results, because other factors may alter reflectance values (*eg* possible higher reflectance values on north facing slopes and shadowing on south facing slopes). Logically, given the time of image capture (10am), false positives (higher reflectance) may result on north-eastern aspects, while false negatives (lower reflectance, especially from shadowing) might be expected on south-western aspects.

**Table 4. Area infected by aspect.**

| Aspect-code | Sector | Area infected (ha) | Total area (ha) | % area infected | Area of infected polygons > 5 ha | % of infected area |
|---|---|---|---|---|---|---|
| Flat | | 681.2 | 7180.0 | 9.5 | 351.9 | 51.7 |
| 1 | N-NE | 679.5 | 5006.3 | 13.6 | 452.4 | 66.6 |
| 2 | NE-E | 450.4 | 3850.3 | 11.7 | 274.9 | 61.0 |
| 3 | E-SE | 380.2 | 4330.1 | 8.8 | 186.6 | 49.1 |
| 4 | SE-S | 398.7 | 5971.4 | 6.7 | 154.3 | 38.7 |
| 5 | S-SW | 387.2 | 5042.1 | 7.7 | 160.4 | 41.4 |
| 6 | SW-W | 345.8 | 3514.6 | 9.8 | 170.2 | 49.2 |
| 7 | W-NW | 330.1 | 3560.8 | 9.3 | 144.1 | 43.7 |
| 8 | NW-N | 487.4 | 4586.2 | 10.6 | 245.2 | 50.3 |
| Total | | 4140.4 | 43041.8 | 9.6 | 2139.9 | 51.7 |

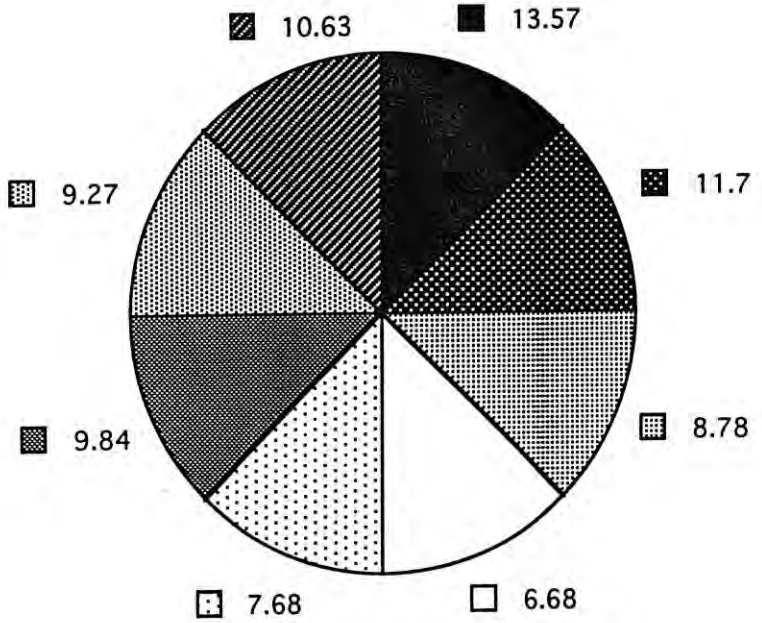



**Figure 5. Proportion (%) of area infected by aspect (North to top of page)**

Other questions which can be simply answered by interrogating the data sets using GIS are:

- How many DRF populations within an area are infested?
- What is the area within a particular watershed to be treated with phosphonate?
- What susceptible taxa, especially priority taxa, might be threatened?

The design of a user interface to provide a tool for people untrained in the operation of Arc/Info will be important in making these data accessible to managers, enabling the integrated management of *Phytophthora*, and, if successful, will contribute to the development of an expert system for the management of lands in the south-west of WA. We expect that the tool will be applied to management of existing areas, facilitate conservation effort across lands of different tenure and

between landscape units, and in the identification of areas in need of rehabilitation or restoration. It should also have application as a model for other land managers in Australia. The design of a user interface will proceed in concert with the ongoing development of a model with predictive capabilities.

## 3. DEVELOP A PREDICTIVE MODEL

### Where is *Phytophthora cinnamomi* likely to be twelve months from now?

A prototype model with predictive capabilities is now being tested . The prototype dieback model relies on GRIDs of aspect, slope, flow direction and flow accumulation. The model is being developed using a beta-release of ARC/Info version 7. The advantages provided by multi-layering capabilities of GRID 7 over the previous version will be fully exploited by this model.

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 vegetations, aspect, road networks will increase the in an area. The results of data interrogation are being used to derive relevant indices of susceptibility for the construction of the model.

If the first stage of model validation is successful - to be determined by testing patterns of spread with information obtained from historical data sets - additional levels of complexity will be added to the model, to reflect factors such as probablity of introduction by humans or animals, seasonal variation, *etc.*

## APPENDIX

## Legend from: Churchward (1988)

| | | |
|---|---|---|
| BAf | Barrow | Yellow duplex soils sands gravels J-M-Y forest. |
| BAg | Barrow | Granite outcrop. |
| BO | Boulongup | Yellow solonetzic soils in swamps: Ys-Mel thickets,reeds. Podzols in sands; J-B-Sh woodland. |
| BWp | Blackwater | Humus podzols on plains; Kg sedgelands. Tt heath. Peat in swamps; Wt thickets. POdzols on dunes; B woodland. |
| Dc | Dempster | Sands and laterite on elongate crests: J-Ab-M forest. |
| Ds | Dempster | Sands and gravels on smooth slopes: Ab-Sh low forest. |
| F | Fernley | Sandy or gravelly yellow duplex soils on rises: J-Bu woodland. Humus podzols in broad depressions: Kg sedgeland: Tt heath. |
| Gg | Gardner | Granite outcrop. |
| Gs | Gardner | Leached sands and podzols: Mallee-heath. |
| Mc | Meerup | Calcareous sand: Pp heath and woodland. |
| Mf | Meerup | Podzols on interdune plains: B-Bu-Y woodland. |
| Mp | Meerup | Podzols over calcareous sand: B-Bu-Y woodland. |
| Mr | Meerup | Beach ridges: Pb heath and B woodland. |
| Ms | Meerup | Podzols in siliceous sand: B-Bu-Y-Sh woodland. |
| Mu | Meerup | Unstable sand. |
| My | Meerup | Calcareous sand: Pp heath and woodland. |
| OW | Owingup | Yellow solonetzic soils organic loams and diatomaceous earths: Wt-Mel thickets. Tt heath and reeds. Podzols on dunes: B S |
| PN | Pillenorup | Gravelly yellow duplex soils sands and laterite: J-M-Y low forest. |
| S6 | Minor Valleys | Narrow V-shaped valleys in sedimentary rock: <10m relief. |
| S7 | Minor Valleys | Broad valleys in sedimentary rocks: 30m relief: smooth slopes: swampy floor (f). |
| S9 | Minor Valleys | Valleys in sedimentary rocks: 40m relief: steep slopes: much siltstone: swampy floor (f). |
| TK | Takalarup | Gravelly yellow duplex soils on plains: J-M woodland mallee heath. Yellow solonetizic soils in depressions: Ys-Mel thic |
| V1 | Major Valleys | Valleys in granitic areas: >40m relief: smooth steep slopes: narrow terrace: Red earths yellow duplex soils on slop |
| V7 | Major Valleys | Valleys in sedimentary rocks: 20-40m relief: short steep irregular slopes: much siltstone: occasional granite outc |
| WATER | Water | |

## REFERENCES


Churchward, H. M.; McArthur, W. M.; Sewell, P. L.; & Bartle, G. A. (1988) Landforms and soils of the South Coast and Hinterland, Western Australia. Northcliffe to Manypeaks. Division of Water Resources Divisional Report 88/1, CSIRO, Canberra

Shearer B. L. & Shea S. R. (1987) Variation in seasonal population fluctuations of *Phytophthora cinnamomi* within and between infected *Eucalyptus marginata* sites of southwestern Australia. *For. Ecol. & Manage.* **21**, 209-30.

Weste G. & Marks G. C. (1987) The biology of *Phytophthora cinnamomi* in Australasian forests. *Ann. Rev. Phytopath.* **25**, 207-29.

Zentmyer G. A. (1980) *Phytophthora cinnamomi* and the diseases it causes. *Am. Phytopath. Soc. Monogr.* **10**

# PROJECT 4

## ANNUAL REPORT

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

**S. Bellgard, B. Shearer, C. Crane and B. Smith**

This report covers the period February 27, 1993 to February 26, 1994.

## CONTENTS

## SUMMARY

*Phytophthora megasperma* is an active plant pathogen in National Parks directly to the north of Perth, extending up to Eneabba, and along the south coast in the Fitzgerald River National Park (FRNP). Where active, *P. megasperma* has high impact on species contributing to habitat structure, e.g. *Banksia attenuata* in the northern sandplain and *B. speciosa* and *B. attenuata* on the south coast. *In vitro* studies have demonstrated that phosphonate can inhibit mycelial growth and oospore production in *P. megasperma*. A field-trial to evaluate the efficacy of foliar applications of phosphonate in retarding the damage caused by *P. megasperma* was commenced in October 1993 in the post-fire re-growth at the foot of East Mount Barren in the FRNP. Three months have elapsed since a 0.3% foliar spray application was carried out, and at this stage, the efficacy of the phsophonate application is not fully apparent.

An understanding of how particular environmental conditions affect the life cycle of *P. megasperma* is fundamental to the development of an efficacious disease management strategy. *In vitro* temperature studies have shown that the optimum temperature for mycelial growth and oospore formation in *P. megasperma* is 25°C. Comparable numbers of sporangium were found to be produced at 15, 20 and 25°C for isolates retrieved from the FRNP. In order to get an insight into the major environmental factors controlling oospore formation, mycelial mats of *P. megasperma* were placed into surface field soil at the start of each season. To date, three seasons have been assayed, with Summer proving to be the season when oospore formation was at its minimum. Summer was also associated with the driest soil and highest soil temperature. Controlled experiments partitioning and testing the influence of individual environmental parameters on oospore function have been initiated. The first stage of the work has investigated the proportion of viable v. dormant v. non-viable oospores produced by *P. megasperma in vitro*. It was found that the majority of oospores produced by *P. megasperma* were viable, yet dormant. No activated/germinated oospores of *P. megasperma* were observed.

*P. megasperma* is a highly variable organism. There is currently great world-wide interest in the exact taxonomic affinity of fungal isolates within the *P. megasperma* species complex. We have commenced morphometric analysis on all locally held isolates of *P. megasperma*, and have obtained isolates available from within Australia, and representative isolates from overseas. To date, we have examined half of the isolates with respect to; radial growth rate, oogonium diameter, and sporangium dimensions. Radial growth rate of the isolates examined appears to adhere to a normal distribution. On the basis of oogonium diameter, we have identified 12 morphologically distinct groups. Six of the *morphotypes* have been described previously, while, the other six may prove to be endemic to WA. It is hoped that isozyme studues, to be carried out early in 1994, will facilitate the classification of the *P. megasperma* isolates recovered from the national parks of WA.

## INTRODUCTION AND AIM

*Phytophthora megasperma* is the most common *Phytophthora* species recovered from dieback affected vegetation of the northern sandplain (Hart *et al.* 1991) (Fig. 3.1). The infections extend over a range of approximately 160 km: commencing in Moore River National Park, and extending through areas adjacent to Badgingarra and Nambung National Park to just north of Eneabba (CALM 1990). *P. megasperma* has also been identified as an active pathogen in the area around East Mount Barren within the Fitzgerald River National Park (FRNP) and around the Hopetoun area to the east of the FRNP (CALM 1991) (Fig. 3.1). It has now become apparent that *P. megasperma* is active in the western side of the FRNP, e.g. the Point Ann Area, West Mount Barren and Quaalup. In addition, *P. megasperma* has also been recovered from the Esperance area and within Cape Arid National Park (Fig. 3.1). Because of its wide geographic distribution, and high impact where active, it is critical that work on this species continues to determine its occurrence, behaviour and avenues of control. This project aims to test a number of control options to retard the spread of the disease caused by *P. megasperma* and to provide a scientific basis for the management of native plant communities infested by *P. megasperma* to minimise future impacts.

## 1 CONTROL OF *P. MEGASPERMA*

### 1.1 Sensitivity of *P. megasperma* to phosphonate *in vitro*

To date, phosphonate has proven to be the most efficacious fungistatic agent in controlling the disease caused by *P. cinnamomi*. Thirty isolates of *P. megasperma* were screened for sensitivity to three levels of phosphonate *in vitro*. The impact of phosphonate (0, 20, and 50 $\mu$g/ml) on radial mycelial growth and oospore formation was assessed. Overall, radial growth rate and oospore formation was hindered by increasing dosage of phosphonate (Table 1.1.1). Earlier overseas works identified the $ED_{50}$ value for mycelial inhibition in *P. megasperma* to be between 22.3 and 91.2 $\mu$g/ml (Coffey and Bower 1984). From our research, we calculated the $ED_{50}$ value to be 482.5 $\mu$g/ml for the 30 isolates tested. It would appear that our WA isolates are less sensitive than the agricultural isolates examined by Coffey and Bower (1984). However, in general, lower concentrations of phosphonate than *in vitro*-defined levels are effective in the host (B. Shearer, unpubl.).

**Table 1.1.1 Overall mean radial growth rate and oospore formation response at each of 0, 20, and 50 $\mu$g/ml of amended phosphonate**

Data given are means (±s.e.) of 5 replicates of 30 tested fungal isolate at each concentration.

| [Phosphonate] ($\mu$g/ml) | Radial growth rate (mm/day) | Oospore formation |
|---|---|---|
| 0 | 4.50 (0.12)a | 27.30 (0.79)a |
| 20 | 4.21 (0.13)b | 26.77 (0.70)a |
| 50 | 3.97 (0.13)c | 22.97 (0.69)b |

N.B. Values followed by dissimilar letters are significantly different at p=0.05 according to Duncan's New Multiple Range test.

## 1.2 Efficacy of aerial applications of phosphonate to retard the damage and spread of the disease caused by *P. megasperma*

The effective dose for foliar application of phosphonate for the control of *P. cinnamomi* is between 0.2 and 0.5% phosphonate (B. Shearer, unpubl.). The vegetation at the foot of East Mount Barren (EMB) is comprised largely of four year old seedlings of the sites' Proteaceae-Myrtaceae mixed rock heath. Little or no information is available on the sensitivity of native seedlings to phosphonate foliar applications, or the use of the chemical to control *P. megasperma*. However, a 0.2% foliar application has been successfully used to confer resistance to *Phytophthora* in seedlings of protea (R. Fairman, *pers. comm.*). Consequently, two 0.3% doses of phosphonate (with a two-week interval between sprays) were applied to plots of infested regenerating vegetation on the southern side of EMB and a gravel pit along Hamersley Dve within the EMB area.

Three 10 m × 12 m plots were permanently established in each of the two sites chosen (i.e. EMB site and Gravel Pit site), and marked using short wooden stakes. Each of the three plots was comprised of two 5 m × 5 m squares separated by a 2 m buffer. One hundred and twenty (i.e. 60 plants in each 5 × 5 m square) of the perennial plant species within each of the plots were permanently tagged and their disease status recorded according to the schedule outlined in Table 1.2.1. At the time of scoring disease status, plant and soil samples were taken of diseased plants and submitted to the Vegetation Health Service to check for disease activity in the various plots.

**Table 1.2.1 Disease rating scale used to assess disease expression and hence plant performance in the phosphonate foliar spray trial (*sensu* Shearer 1993)**

| Disease rating | Visual symptoms of disease expression |
|---|---|
| 0 | plant healthy |
| 1 | 10% lower leaves necrotic |
| 2 | 10-20% lower leaves necrotic |
| 3 | 20-<50% lower leaves necrotic |
| 4 | 50-<75% lower leaves necrotic |
| 5 | 75-<90% lower leaves necrotic |
| 6 | 90-<100% |
| 7 | plant dead |

The experiment (carried out in each of two sites) was a randomised complete block design, replicated three times. A five litre 0.3% dose of phosphonate (as *Phospot 200*, neutralised form of phosphonic acid), using synertrol (0.3%) as a surfactant constituted the "treatment", while the "control" spray comprised of five litres of water with 0.3% synertrol added. Two weeks following the spraying, plants were re-assessed for phytotoxic reactions. No phytotoxic symptoms were noticed, and a follow-up spray of similar concentration was carried out on 27.10.93 (i.e. a total of 10 l/5 × 5 m plot). To date, assessments have been made one and three months after the second spray. The change in disease expression of the tagged plants in both the "treated" and "control" plots will be re-assessed one, three, six and 12 months after spraying in order to gauge the efficacy of the phosphonate application in

conferring host resistivity. At the time of the one, six, and 12-month assessments, root, shoot and leaf samples will be removed for B. Komorek to determine residual tissue phosphonate levels.

*P. megasperma* was retrieved from diseased plants situated within each plot chosen for remedial spray treatment (Table 1.2.2). This confirms that the plots chosen for spray treatment were indeed infected by *P. megasperma*.

**Table 1.2.2 Summary of disease activity in the sprayed study sites**

| Site | Plot | Plant spp. sampled | Pathogen recovered |
|---|---|---|---|
| EMB | 1 | *Dryandra falcata* | Pm |
| | | *D. quercifolia* | Pm |
| | 2 | *D. falcata* | Pm |
| | | *D. falcata* | Pm |
| | | *Banksia lemanniana* | Pm |
| | | *D. quercifolia* | Pm |
| | 3 | *B. lemanniana* | Pm |
| | | *D. quercifolia* | Pm |
| GP | 1 | *D. falcata* | Pm |
| | 2 | *D. plumosa* | Pm |
| | 3 | *Hakea ferruginea* | Pm |

N.B. EMB = East Mount Barren, GP = Gravel Pit, Pm = *P. megasperma*.

As only three months have elapsed since the second spray, and as such, there are insufficient data to warrant detailed statistical analysis at this stage. Provided is a figure summarising the changes in disease expression of individuals in "treated" v. "control" plots (Fig. 1.2.1). In this figure, the smaller the change in disease rating may be equated with effective control. At the EMB site, *Banksia lemmaniana* was the only diseased plant species in which the phosphonate spray treatment was associated with a greater change in disease expression than in the unsprayed "control" plots (Fig. 1.2.1). For all the other plant species showing disease, i.e. *B. speciosa*, *Dryandra cuneata*, *D. falcata*, and *D. quercifolia*, spray treatment with phosphonate resulted in a smaller change in disease expression than the unsprayed "control" (Fig. 1.2.1). For the Gravel Pit site, effective control was not so apparent, but disease expression was much less than that exhibited by the plants comprising the EMB site (Fig. 1.2.1). For *D. cuneata* and *D. plumosa*, the change in disease expression was generally greater in the phsphonate "treated" plots than in that of the unsprayed "control" plots (Fig. 1.2.1). For *D. falcata*, there was no difference between "treated" and "control" plots.

So it appears that there is some effective control, but only in some plant species, and possibly also depending upon site conditions. It is hoped that by the six month assessment, more definitive patterns with respect to treatment and plant species will become apparent, especially as the results of the tissue analysis will also be available.

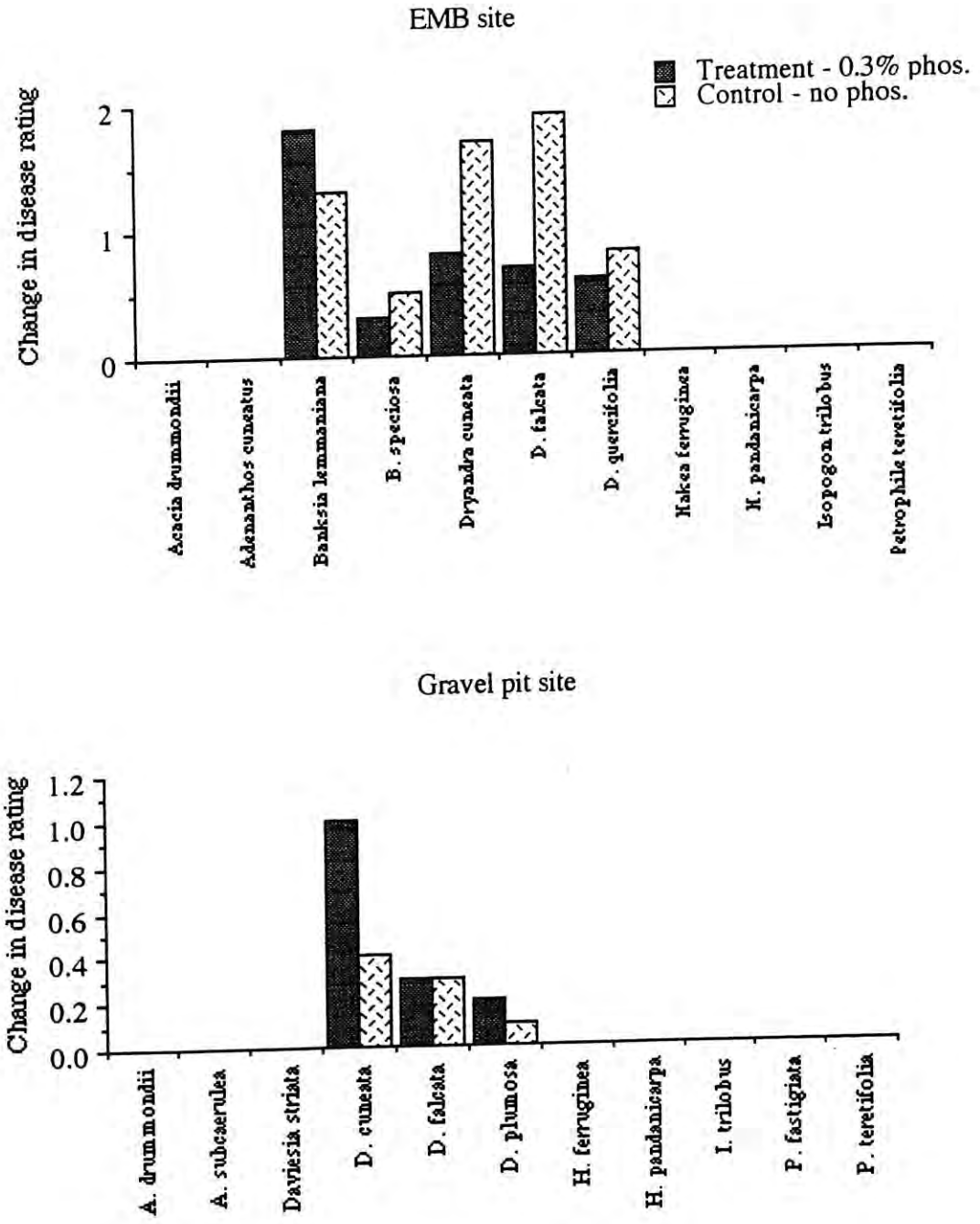



Fig. 1.2.1 Summary graph depicting change in rating of diseased plant species sprayed with phosphonate v. control. Data represent the change in disease rating over a three month period for five representatives of each plant species randomly chosen from within each site.

# 2 MANAGEMENT OF *P. MEGASPERMA*

## 2.1 Significance of oospores

### 2.1.1 *In vitro* influence of temperature on radial growth, sporangium and oospore formation

Pratt and Mitchell (1975) reported that temperature and soil moisture were the major determinants of the infective activity of *P. megasperma* in naturally infested soils. In a fully replicated, controlled experiment, we compared radial growth rate, sporangium and oospore formation at 5, 15, 25, and 35°C for 12 isolates of *P. megasperma* (Table 2.1.1.1).

**Table 2.1.1.1 List of *P. megasperma* isolates used in temperature experiment**

| Isolate (spp.) | Isolated from... | Location |
|---|---|---|
| DCE 439 (Pmm) | *Banksia hookeriana* | Eneabba |
| DCE 440 (Pms) | *Conospermum triplinervum* | Eneabba |
| TH2 (Pmm) | *B. ilicifolia* | Wongonderrah Nat. Reserve |
| SEB 108 (Pm?) | *Hypocalymma angustifolium* | Wongonderrah Nat. Reserve |
| DCE 441 (Pmm) | *B. attenuata* | Minyolo Brook |
| DCE 442 (Pmm) | *B. attenuata* | Mullering Brook |
| DCE 177 (Pms) | *Pinus radiata* | Jarrahwood |
| DCE 187 (Pms) | *Eucalyptus caesia* | Baldivis |
| SEB 109 (Pm?) | *B. nutans* | FRNP |
| SEB 110 (Pm?) | *B. speciosa* | FRNP |
| DP 20 (Pmm) | *B. speciosa* | Cape Arid |
| DP 21 (Pmm) | *Dryandra sessilis* | Cape Arid |

NB. Pmm = *P. megasperma* var. *megasperma*, Pms = *P. megasperma* var. *sojae*, Pm? = taxonomic affinity not yet determined, FRNP = Fitzgerald River National Park.

Very little growth was observed at 5°C (Fig. 2.1.1.1). The only isolates capable of any mycelial growth at 5°C (after 10 days), were isolates SEB109 and 110: both isolates were retrieved from the FRNP (Table 2.1.1.1). The temperature at which maximum growth rate was observed was consistently 25°C (Fig. 2.1.1.1). No mycelial growth was observed for any isolate at 35°C (Fig. 2.1.1.1). Over all temperatures, the fastest growing isolates were SEB108 and DCE441 [p=0.05] (Fig. 2.1.1.1).

No sporangium formed at 5 and 35°C. Sporangium numbers produced at 25°C were approximately fifity times greater than that produced at 15°C for all isolates after 10 days (Fig. 2.1.1.2). Isolates SEB109, 110, and DP20, 21 were the only isolates which produced sporangia at 15°C (Fig. 2.1.1.2). These four isolates were retrieved from FRNP and Cape Arid National Park respectively (Table 2.1.1.1): both localities on the south coast.

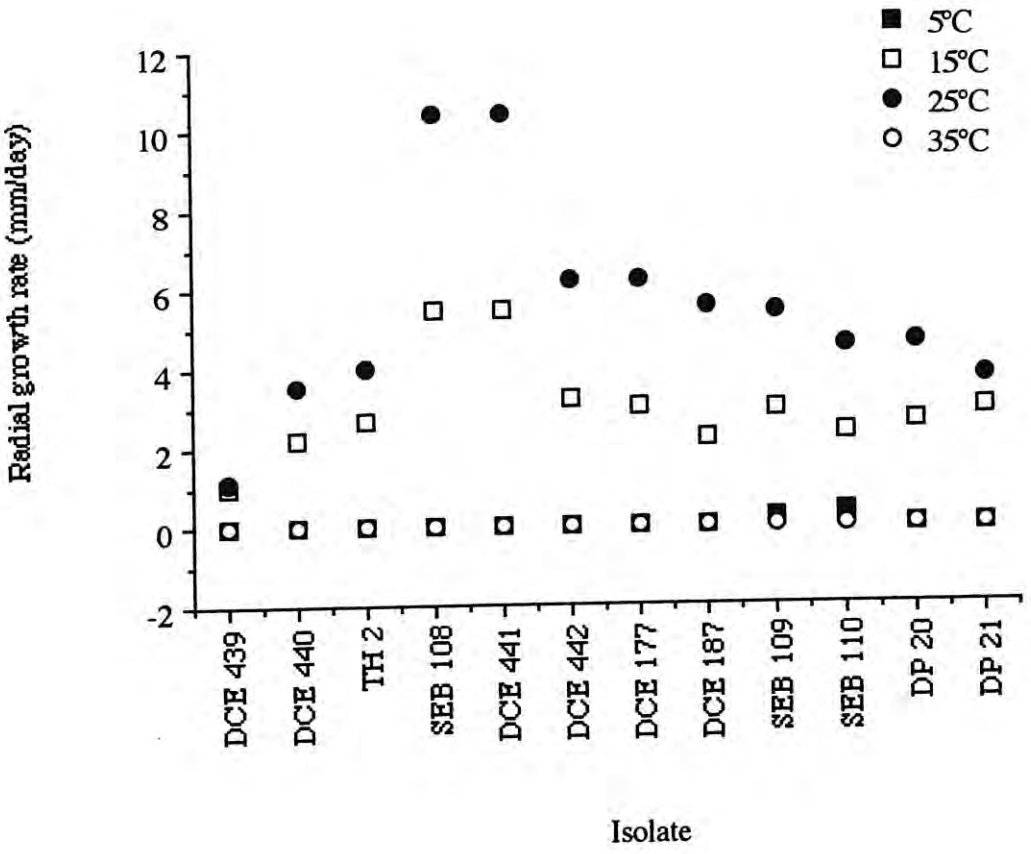



Fig. 2.1.1.1 Radial growth rate (mm/day) after 10 days for 12 isolates of *P. megasperma* grown at 5, 15, 25, and 35°C. Data given are the mean±s.e.m. of four replicates at each temperature for each isolate.

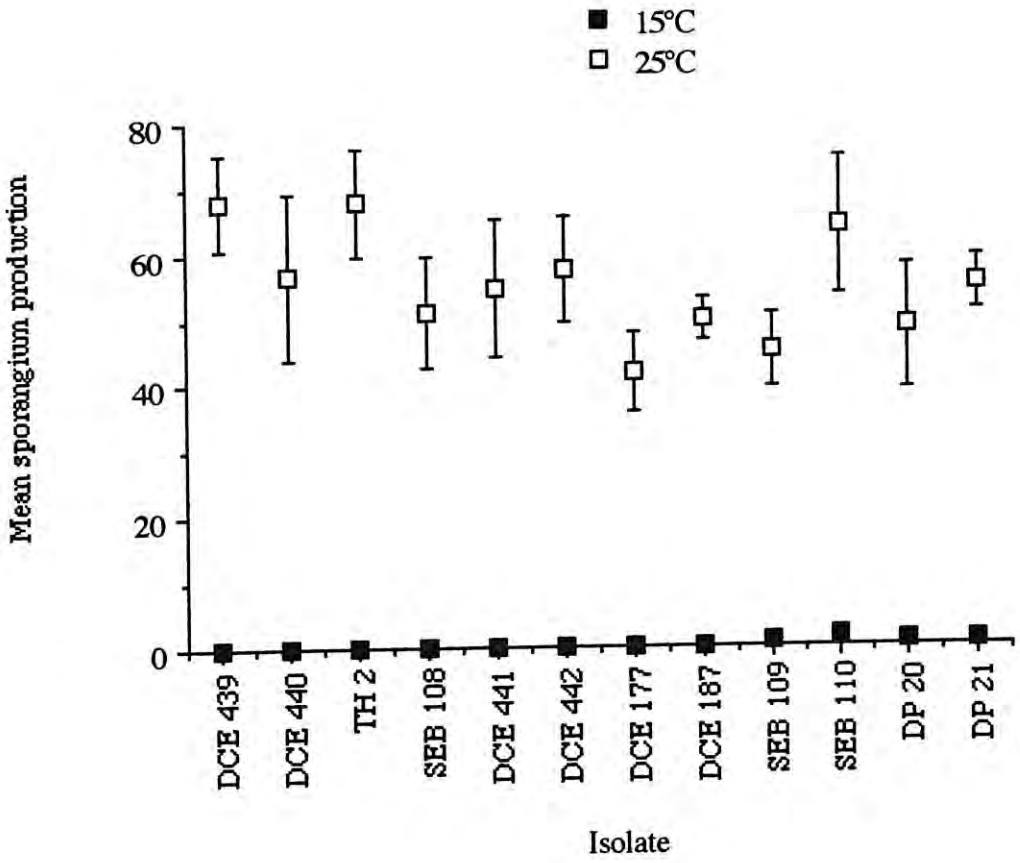



Fig. 2.1.1.2 Sporangium production of 12 *P. megasperma* isolates after 10 days at 15 and 25°C. Data given are the mean±s.e.m. of four replicates at each temperature for each isolate.

No oospores were produced at either 5, 15, or 35°C after 10 days. Oospores were only produced at 25°C. There was no significant difference between the numbers of oospores produced by each individual isolate at 25°C at any of the sampling periods. Oospore numbers ranged from 17 (isolate DP21) to 31 (isolate DCE187), but there was considerable variability within isolates.

The temperature study described above identified that sporangium were formed by *P. megasperma* isolates recovered from the south coast at 15 and 25°C. This result may have implications for the modification of the baiting procedure used to recover *P. megasperma* from soil and plant material. Baiting of soil samples from high impact sites in the FRNP consistently fails to retrieve *P. megasperma* (C. Wilkinson, *pers. comm.*). More recently, plating of surface sterilised roots onto selective media has successfully and consistently isolated *P. megasperma*, even when baiting of soil from around the roots of these diseased plants failed to return *P. megasperma*. It has been observed however, that *Pythium* spp. are consistently recovered from soils baited at 25°C. In a recent trial, soil samples from the FRNP were divided into two aliquots and baited separately at 15 and 25°C. Recoveries of *P. megasperma* were obtained from the soil baited at 15°C, but, not from that baited at 25°C. This suggests that there may be a lower temperature optimum for sporangium production in *P. megasperma*, and/or, that at 25°C, the zoospores of *Pythium* spp. out-compete the zoospores of *P. megasperma* for available bait tissue. Consequently, a further study examined the formation of sporangium of 20 *P. megasperma* isolates retrieved from diseased plants in the FRNP at; 15, 20, and 25°C in an attempt to determine a temperature optimum.

This experiment was a randomised complete block design, with temperature as a blocking factor. No significant difference in sporangium production was observed with respect to isolate or temperature (Table 2.1.1.2). The results of this experiment suggest that *P. megasperma* isolates recovered from the FRNP are able to produce comparable numbers of sporangium in a temperature range from 15 to 25°C. In an earlier study, Pfender *et al.* (1977) found that in flooded soil, isolates of *P. megasperma* produced sporangia at temperatures ranging between 8-24°C, but not at 28°C. In this earlier study, the optimum temperatures for sporangium production were found to be between 12 and 16°C. Taken together, these results tend to indicate that incubation of soil baits suspected of containing *P. megasperma* at temperatures less than 25°C may be more efficacious. Our temperature study provided evidence to suggest that *P. megasperma* has a fairly broad range for optimum sporangium production. It was mentioned previously that at 25°C, *Pythium* spp. were consistently recovered from colonised bait tissue. It may well be that 15 and/or 20°C are not optimum for *Pythium* spp. to sporulate, and thus, the reduction in competition for available bait tissue results in *P. megasperma* being recovered preferentially.

### 2.1.2 The effect of season and site factors on oospore formation in the field

*P. megasperma* is homothallic, and can form oospores quite readily in sterile culture on 10% V8 juice agar. It has been demonstrated that *P. megasperma* can form oospores in sterile soil culture, with or without a plant host, in dry or wet soil conditions. Oospores of *P. megasperma* have also been found in the roots of diseased host plants (G. Hardy, *pers. comm.*). To date, no quantification of the seasonal variation in oospore production of *P. megasperma* has been carried out. Such information is necessary to quantify the population dynamics of *P. megasperma* from year-to-year.

Seasonal variation in oospore formation was monitored using a deliberate inoculation of surface field soil with mycelial disks harbouring *P. megasperma* (as per Shea *et al.* 1979).

**Table 2.1.1.2 Mean number of sporangia formed at each of 15, 20, and 25°C for 20 isolates of *P. megasperma* retrieved from the FRNP**

| Isolate[1] | Isolated from... | Sporangium[2] | | |
|---|---|---|---|---|
| | | 15°C[3] | 20°C[3] | 25°C[3] |
| SEB 200 | *Dryandra quercifolia* | 8.7±0.8 | 10.0±3.2 | 2.7±0.5 |
| SEB 201 | *D. quercifolia* | 17.5±0.6 | 10.0±2.3 | 15.2±1.2 |
| SEB 202 | *D. falcata* | 5.0±2.0 | 3.7±0.8 | 3.2±0.5 |
| SEB 203 | *D. tenuifolia* | 7.0±2.0 | 18.0±2.2 | 13.5±0.3 |
| SEB 204 | *D. plumosa* | 8.2±1.1 | 3.7±0.8 | 9.0±0.4 |
| SEB 205 | *D. plumosa* | 22.7±2.1 | 8.7±2.1 | 13.7±1.5 |
| SEB 206 | *Banksia attenuata* | 15.2±0.8 | 6.0±1.1 | 10.0±3.7 |
| SEB 207 | *B. attenuata* | 20.5±2.4 | 8.2±3.4 | 7.0±2.7 |
| SEB 208 | *Conospermum distichum* | 13.7±1.9 | 7.0±0.7 | 9.0±3.0 |
| SEB 209 | *D. cuneata* | 5.2±0.8 | 13.7±0.8 | 21.7±3.3 |
| SEB 210 | *D. cuneata* | 18.7±3.5 | 12.5±3.6 | 14.2±3.2 |
| SEB 211 | *D. plumosa* | 12.0±2.4 | 11.5±2.1 | 8.5±3.7 |
| SEB 212 | *B. attenuata* | 11.0±1.3 | 11.0±1.3 | 10.2±1.1 |
| SEB 213 | *B. lemanniana* | 7.2±0.5 | 20.7±1.9 | 14.7±1.5 |
| SEB 214 | *D. falcata* | 6.7±1.0 | 20.0±1.7 | 20.0±3.7 |
| SEB 215 | *Hakea pandanicarpa* | 15.7±1.4 | 7.7±1.5 | 13.5±0.9 |
| SEB 216 | *D. falcata* | 18.0±3.8 | 13.0±0.4 | 9.2±5.1 |
| SEB 217 | *D. quercifolia* | 13.7±1.4 | 9.7±0.7 | 10.2±1.4 |
| SEB 218 | *Isopogon formosus* | 17.7±3.6 | 12.0±1.8 | 8.0±2.9 |
| SEB 219 | *B. lemanniana* | 25.5±5.1 | 14.5±1.8 | 13.5±1.5 |

N.B. [1] = No significant difference in sporangium formation between isolates ($F = 1.31$, $F_{19,38} = 2.09$, $p=0.05$).

[2] = Mean number of sporangia±s.e.m. calculated on four replicates of each isolate at each temperature regime.

[3] = No significant difference in sporangium formation between temperatures ($F = 1.43$, $F_{19,38} = 2.09$, $p=0.05$).

Colonised mycelial disks (enveloped in a 30 $\mu$m nylon mesh bag) of five locally retrieved fungal isolates were placed into the soil at a depth of approximately 10 cm, at the start of each season at the Wongonderrah site. One and two weeks after emplacement, the mycelial disks were retrieved from the field, stained, and the number of oospores produced assessed. At the time of each retrieval; soil moisture, soil temperature, air temperature, and rainfall were also recorded.

To date, three seasons have been assayed, viz. Winter, Spring, and Summer. The Autumn assessment will be commenced in April 1994. The results of the experiment to date are presented in Fig. 2.1.2.1. The graph tends to suggest that there is a peak in oospore production in Spring. However, according to the nested 2-factor ANOVA, there is no significant difference in oospore production between seasons (Fig. 2.1.2.1).

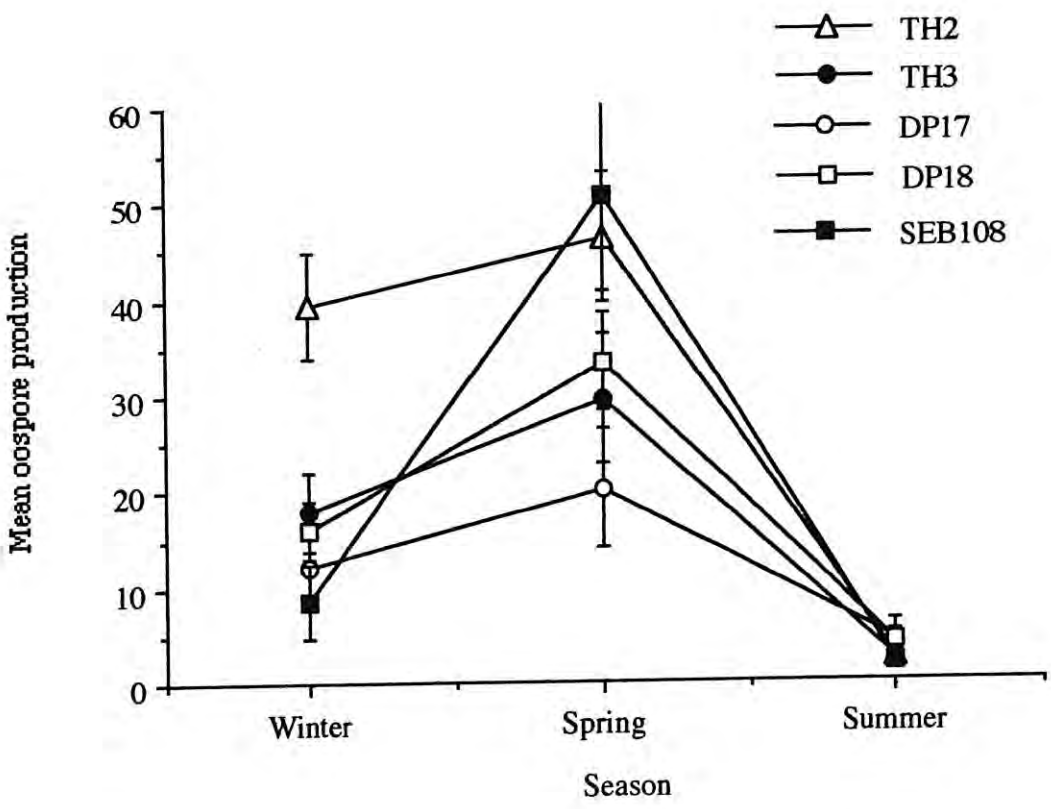



Fig. 2.1.2.1 Mean oospore production by five *P. megasperma* isolates retrieved from the Wongonderrah site deliberately emplaced in field soil at the start of each season. Each data point represents the mean±s.e.m. of ten replicates ($F_{season} = 6.68$, $F_{2,3} = 9.55$, p=0.05).

A contrast of individual seasonal means (Multiple comparisons test, Snedecor and Cochran 1980) identified that there was a significant difference in oospore production between Summer v. Spring and Winter (Table 2.1.2.1).

It would appear that Summer is the season when oospore production is at its minimum (Table 2.1.2.2). Summer was also associated with the lowest soil moisture and highest soil temperature (Table 2.1.2.2). So it would appear that dry soils associated with high soil temperatures is somewhat inhibitory to oospore formation in *P. megasperma*.

**Table 2.1.2.1 Contrast of individual seasonal means for oospore production of deliberately field-emplaced mycelial disks of *P. megasperma***

| | Winter | Spring | Summer |
|---|---|---|---|
| Winter | - | n.s | n.s. |
| Spring | n.s. | - | n.s. |
| Summer | sig. | sig. | - |

N.B. Winter v. Spring/Summer, $F = 0.30$, $F_{1,3} = 10.13$, $p=0.05$.
Spring v. Winter/Summer, $F = 8.23$, $F_{1,3} = 10.13$, $p=0.05$.
Summer v. Winter/Spring, $F = 11.67$, $F_{1,3} = 10.13$, $p=0.05$.

**Table 2.1.2.2 Environmental conditions and mean number of oospores produced in each of the three seasons assayed**

| Season | Soil moisture[1] (%) | Soil temp.[2] (°C) | Air temp.[2] (°C) | Rainfall[3] (total mm) | Oospores[4] |
|---|---|---|---|---|---|
| Winter '93 | 12.1±1.2 | 13.0±0.1 | 11.7±0.1 | 93.4 | 3.8a |
| Spring '93 | 3.1±0.8 | 19.2±0.2 | 14.6±0.3 | 9.2 | 5.6a |
| Summer '93 | 0.7±0.0 | 29.6±0.2 | 20.2±1.1 | 0.0 | 1.5b |

N.B. [1] = Data represents the mean±s.e.m. of two weekly measurements.

[2] = Data represents the mean±s.e.m. of 14 daily measurements.

[3] = Data represents the total rainfall recorded over a two week assay period.

[4] = Data represents the mean of five isolates assayed at each of five plots within the Wongonderrah site at the start of each season. Numbers followed by the same letter are not significantly different according to Multiple comparisons test, p=0.05 (refer to Table 2.1.2.1).

## 2.1.3 The main environmental factors controlling oospore dormancy and germination

Sexual reproduction in *P. megasperma*, resulting in the production of oospores, provides the means of long-term survival, as oospores are resistant structures, often difficult to germinate (Elliot 1983). Indeed, oospores formed in soybean tissue by *P. megasperma* var. *sojae* are thought to be the primary survival propagule in soil (Jimenez and Lockwood 1982). Dormancy of oospores of *P. megasperma* is considered to be constitutive (Erwin and McCormick 1971), i.e. involving endogenous constraints that are not overcome simply by

supplying conditions suitable for growth (Griffin 1981). It has been reported that oospores often require a period of pre-treatment at higher or lower temperatures than at ambient temperature before they will germinate. Additionally, soil extracts and/or soil matric potential can stimulate oospore germination. Commencing in April 1994, we will be assessing the impact of pre-treatments on oospore activity of *P. megasperma*. This information will identify the environmental conditions under which dormant oospores of *P. megasperma* are most likely to be activated. The first stage in this study was to determine the percentage of viable oospores produced *in vitro*, and the results of this work are reported below.

Oospores of 12 *P. megasperma* isolates (as per Table 2.1.1.1) were obtained, and oospore viability was examined using the tetrazolium bromide test (Jiang & Erwin 1990). Colours of oospores were determined microscopically, with 1) black-coloured oospores being considered nonviable, 2) rose-coloured oospores being considered dormant, and 3) blue-coloured oospores being considered activated (pre-germination phase) (El-Hamalawi & Erwin 1986). No activated oospores were found *in vitro* for the 12 fungal isolates assayed (Table 2.1.3.1).

**Table 2.1.3.1 *In vitro* assessment of nonviable, dormant and activated oospores of *P. megasperma***

Black (bl) = nonviable and rose (r) = dormant. Data represent the number of oospores out of 25 examined with each colour response

| Isolate | Rep. I | | Rep. II | | Rep. III | | Rep. IV | | Rep. V | |
|---|---|---|---|---|---|---|---|---|---|---|
| | bl | r | bl | r | bl | r | bl | r | bl | r |
| DCE 439 | 4 | 21 | 0 | 25 | 0 | 25 | 3 | 22 | 3 | 22 |
| DCE 440 | 1 | 24 | 4 | 21 | 6 | 19 | 5 | 20 | 3 | 22 |
| TH2 | 0 | 25 | 0 | 25 | 0 | 25 | 3 | 22 | 1 | 24 |
| SEB 108 | 0 | 25 | 0 | 25 | 1 | 24 | 3 | 22 | 4 | 21 |
| DCE 441 | 6 | 19 | 0 | 25 | 4 | 21 | 3 | 22 | 2 | 23 |
| DCE 442 | 7 | 18 | 9 | 16 | 0 | 25 | 3 | 22 | 6 | 19 |
| DCE 177 | 2 | 23 | 2 | 23 | 5 | 20 | 3 | 22 | 4 | 21 |
| DCE 187 | 2 | 23 | 2 | 23 | 4 | 21 | 5 | 20 | 6 | 19 |
| SEB 109 | 3 | 22 | 1 | 24 | 5 | 20 | 5 | 20 | 2 | 23 |
| SEB 111 | 3 | 22 | 2 | 23 | 4 | 21 | 5 | 20 | 4 | 21 |
| DP 20 | 3 | 22 | 2 | 23 | 4 | 21 | 6 | 19 | 8 | 17 |
| DP 21 | 4 | 21 | 5 | 20 | 5 | 20 | 2 | 23 | 2 | 23 |

N.B. 1) $\chi^2_{11} = 15.993$, p=0.8556, $\chi^2_{0.05,11} = 19.675$: reject $H_o$.

2) black- v. rose-coloured oospores, 191 v. 1309, i.e. 12.7% v. 87.3%. Now, the 95% confidence interval for Binomial distribution for 1500 observations is 705 v. 795, i.e. 47% v. 53% (Snedecor and Cochran 1980).

There was no significant difference between isolates with respect to the proportion of black- and rose-coloured oospores produced *in vitro* ($\chi^2_{11}$ = 15.993, p=0.8556, Table 2.1.3.1). The majority of oospores assessed were rose-coloured i.e. viable, yet dormant (Table 2.1.3.1).

## 2.2 Variability of *P. megasperma*

*P. megasperma* has been described as an extremely diverse and variable plant pathogen (Hansen and Hamm 1983). Several attempts have been made to define sub-groups on the basis of morphology, pathogenicity, and host plant species. More recently, cytological and biochemical techniques have been employed to augment traditional morphological studies. We have commenced morphometric analysis on all locally held isolates of *P. megasperma*, and isolates representative of the six sub-groupings of *P. megasperma* currently recognised on the basis of; morphology, electrophoretic pattern of total proteins, chromosome number, and nuclear DNA content (Hansen *et al.* 1986). Isozymic analysis will commence in March 1994, while analysis of pathogenic variability will be commenced mid-year.

To date, we have analysed; radial growth rate, oogonium diameter, and sporangium dimensions of 59 isolates of *P. megasperma.* Included in the isolates examined, were six isolates provided by Clive Brasier (Table 2.2.1). Radial growth rate of the isolates examined appears to adhere to a normal distribution, however there are three isolates with growth rates in excess of 6 mm/day (Fig. 2.2.1). For the *P. megasperma* isolates examined, radial growth rate varied between 1 and 10.3 mm/day. The majority of the isolates had a growth rate between 3 and 5 mm/day (Fig. 2.2.1).

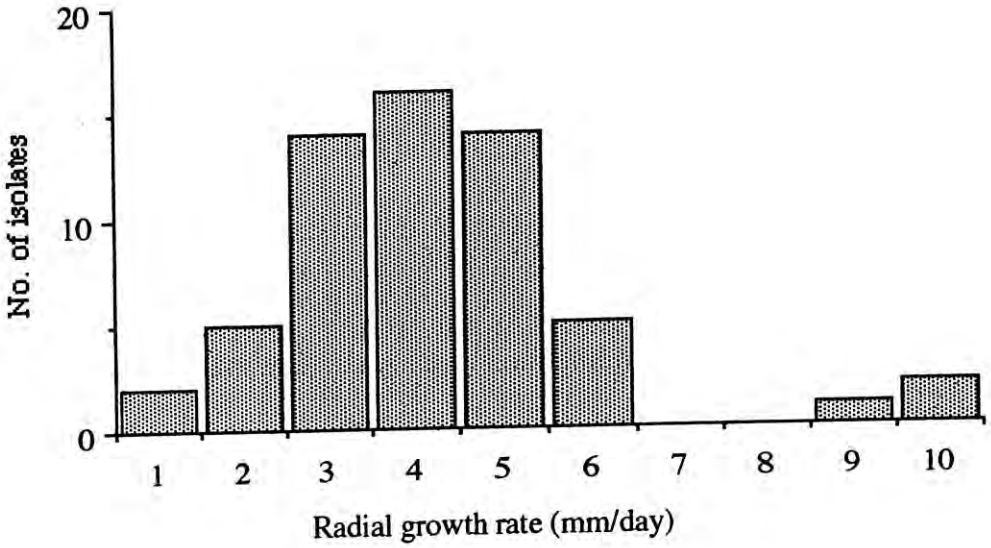



Fig. 2.2.1 Frequency histogram of radial growth rates (mm/day) of 59 isolates of *P. megasperma* grown on CMA (12ml/plate) in the dark for four days at 25°C.

Mean oogonium diameter ranged between 23 and 47 $\mu$m for the 59 *P. megasperma* isolates examined (Table 2.2.1). The six *isotypes* (as per Hansen *et al.* 1986) formed the foci of six morphologically distinct groups (Morphometric groups 'a' to 'f', Table 2.2.1), around which a number of our WA isolates clustered. In addition to the six groups formed around the six

**Table 2.2.1 Morphological characteristics diagnostic of the twelve identified *P. megasperma* isolate groupings.**

| Morphometric group* | No. in group | Growth rate (mm/day) | Oogonium diam. ($\mu$m) | Sporangium length ($\mu$m) | Sporangium width ($\mu$m) | Sporangium pore ($\mu$m) |
|---|---|---|---|---|---|---|
| a (e.g. P452)[1] | 7 | 1.9-6.0 | 44.7-47.5 | 39.4-71.5 | 25.4-46.7 | 8.7-11.7 |
| b (e.g. P450)[1,2] | 3 | 2.5-6.1 | 43.6-43.9 | 42.1-46.1 | 28.5-32.2 | 8.6-9.9 |
| c (e.g. P471)[1] | 8 | 3.0-6.5 | 40.7-42.5 | 38.7-53.5 | 25.2-43.3 | 8.7-14.9 |
| d (e.g. P439)[1,3] | 3 | 1.0-6.9 | 39.9-40.3 | 34.3-53.0 | 33.7-36.8 | 8.9-12.7 |
| e (e.g. P445)[1,4] | 6 | 2.5-5.1 | 38.1-39.1 | 33.9-60.8 | 24.5-41.7 | 7.7-14.4 |
| f (e.g. P484)[1] | 13 | 2.4-9.3 | 35.5-37.7 | 33.6-53.4 | 26.1-41.7 | 8.2-12.9 |
| g (e.g. SEB 110) | 4 | 3.6-6.0 | 32.9-34.9 | 47.0-55.1 | 37.8-38.9 | 10.1-12.4 |
| h (e.g. DP20) | 7 | 3.5-10.1 | 31.3-32.1 | 33.6-50.0 | 24.8-41.8 | 8.4-15.9 |
| i (e.g. R3-6 283) | 2 | 3.3-5.2 | 29.2-29.8 | 48.8-51.7 | 29.4-30.9 | 8.5-8.7 |
| j (DP18) | 1 | 2.8 | 28.6 | 42.9 | 35.9 | 10.9 |
| k (e.g. SEB 117) | 3 | 4.2-5.3 | 25.5-26.2 | 38.1-64.6 | 27.7-42.2 | 9.9-13.0 |
| l (e.g. DCE187) | 2 | 3.7-5.0 | 23.7-24.8 | 38.8-57.2 | 25.1-38.5 | 9.1-9.5 |

NB. * = Groupings based upon oogonium diameter determined using the Scott-Knott procedure ($\lambda$=500, p=0.002).

[1] = Six *isotypes* provided by Clive Brasier representative of the six sub-groups of *P. megasperma* currently recognised (Hansen *et al.* 1986).

[2] = *P. trifolii*, [3] = *P. sojae*, [4] = *P. medicaginis* (Hansen and Maxwell 1991)

*isotypes*, were six other morphologically distinct groups (Morphometric groups 'g' to 'l', Table 2.2.1). Isolates from groups 'g' to 'l' had mean oogonia diameters significantly smaller than those isolates belonging to groups 'a' to 'f' (Table 2.2.1). Sporangium length ranged in size between 33 and 71 $\mu$m and sporangium width ranged between 24 and 43 $\mu$m (Table 2.2.1). All sporangia examined were non-papillate, and pore size ranged in size between 8 and 15 $\mu$m (Table 2.2.1).

On the basis of oospore morphology alone, it would appear that here in WA we have *P. megasperma* isolates similar to the six *isotypes* identified and described by Hansen *et al.* (1986) (Table 2.2.1). Additionally, there appears to be another six morphologically distinct groups characterised by oogonium diameters less than 35 $\mu$m (Table 2.2.1). The isolates belonging to this group may prove to be endemic to WA, however, we have only analysed the morphometrics of half of the held isolates. It is hoped that isozymic analysis of all isolates will make apparent any genetical basis for the observed morphological variability, and hence, elucidate the taxonomic affinities of WA isolates of *P. megasperma*.

## 3 The occurrence of *P. megasperma* in the national parks of WA

To date, we have identified 33 plant species as being susceptible to infection by *P. megasperma* in the northern sandplain, with the majority of susceptible plants belonging to the Proteaceae family (Table 3.1). These diseased plants have been retrieved from within and adjacent to a number of national parks (N.P.) and/or nature reserves (N.R.) including; Moore River N.P., Nambung N.P., Badgingarra N.P., and Wongonderrah N.R. In total, we know of 27 plant species within the FRNP to be susceptible to infection by *P. megasperma* (Table 3.2). Again, the majority of susceptible species belonging to the family Proteaceae (Table 3.2). Consequently, it is becoming increasingly apparent that a number of plant species are indicative of infections caused by *P. megasperma*. The *Banksia* spp. are particularly sensitive in the northern sandplain, while symptoms of disease expression in *Banksi* and *Dryandra* spp. are indicative of *P. megasperma* activity in the FRNP.

## 4 Liaison

*P. megasperma* isolates to be examined in the morphometrics and isozyme study have been provided by; Dr Clive Brasier (The Forest Authority, Research Division, UK), Drs Barbara Hall and David Paton (The University of Adelaide), Roz Hart (Hart, Simpson & Associates, Environmental Consultants), and Carla Wilkinson and Janet Webster (Vegetation Health Service, S.I.D., CALM).

With permission of ANCA, copies of this annual report will be distributed to; Frank Batini (Manager, Protection Branch, CALM), Kelly Gillen (Regional Operations Manager, South Coast Region, CALM), Drew Griffiths (District Manager, Albany District, CALM), Nathan McQuoid (Head Ranger, FRNP, CALM), David Rose (District Manger, Moora District, CALM), Carla Wilkinson, Roz Hart, and Anthea Pate (Environmental Officer, TIWEST Joint Venture). In this way, land managers and other interested scientists will be brought up-to-date with the major findings relating to the control and management of *P. megasperma*.

**Table 3.1 Host plant species susceptible to infection by *P. megasperma* in the northern sandplain (as per 14.2.94).**

| Family | Plant species |
|---|---|
| Dilleniaceae | *Hibbertia pachyrrhiza* |
| | *H. subvaginata* |
| Epacridaceae | *Leucopogon conostephioides* |
| | *L. striatus* |
| Haemodoraceae | *Phlebocarya ciliata* |
| Iridaceae | *Patersonia occidentalis* |
| Myrtaceae | *Calytrix aurea* |
| | *C. flavescens* |
| | *Eremaea* sp. |
| | *Hypocalymma angustifolia* |
| | *Scholtzia involucrata* |
| | *Verticordia drummondii* |
| Papilionaceae | *Daviesia aff. incrassata* |
| | *Jacksonia ulicina* |
| | *J. spinosa* |
| Proteaceae | *Adenanthos cygnorum* |
| | *Banksia attenuata* |
| | *B. grandis* |
| | *B. hookeriana* |
| | *B. ilicifolia* |
| | *B. menziesii* |
| | *B. prionotes* |
| | *B. sphaerocarpa* |
| | *B. telmatiaea* |
| | *Conospermum stochaedis* |
| | *C. triplinervium* |
| | *Grevillea polybotryoides* |
| | *Hakea auriculata* |
| | *Isopogon buxifolius* |
| | *I. formosus* |
| | *Petrophile linearis* |
| | *Stirlingia latifolia* |
| Xanthorrhoeaceae | *Xanthorrhoea preissii* |

**Table 3.2 Host plant species susceptible to infection by *P. megasperma* along the south coastal region (as per 14.1.94).**

| Family | Plant species |
|---|---|
| Myrtaceae | *Actinobium cunninghamii* |
| Papilionanaceae | *Daviesia striata* |
| Proteaceae | *Adenanthos cuneatus* |
| | *Banksia attenuata* |
| | *B. baxteri* |
| | *B. gardneri* |
| | *B. lemanniana* |
| | *B. media* |
| | *B. nutans* |
| | *B. occidentalis* |
| | *B. repens* |
| | *B. speciosa* |
| | *Conospermum distichum* |
| | *Dryandra blackiana* |
| | *D. cuneata* |
| | *D. falcata* |
| | *D. nivea* |
| | *D. plumosa* |
| | *D. quercifolia* |
| | *D. sessilis* |
| | *D. tenuifolia* |
| | *Hakea pandanicarpa* |
| | *H. varia* |
| | *H. victoria* |
| | *Isopogon formosus* |
| | *I. buxifolius* |
| | *I. trilobus* |
| | *Petrophile teretifolia* |
| Rutaceae | *Boronia megastigma* |
| Xanthorrhoeaceae | *Xanthorrhoea platyphylla* |

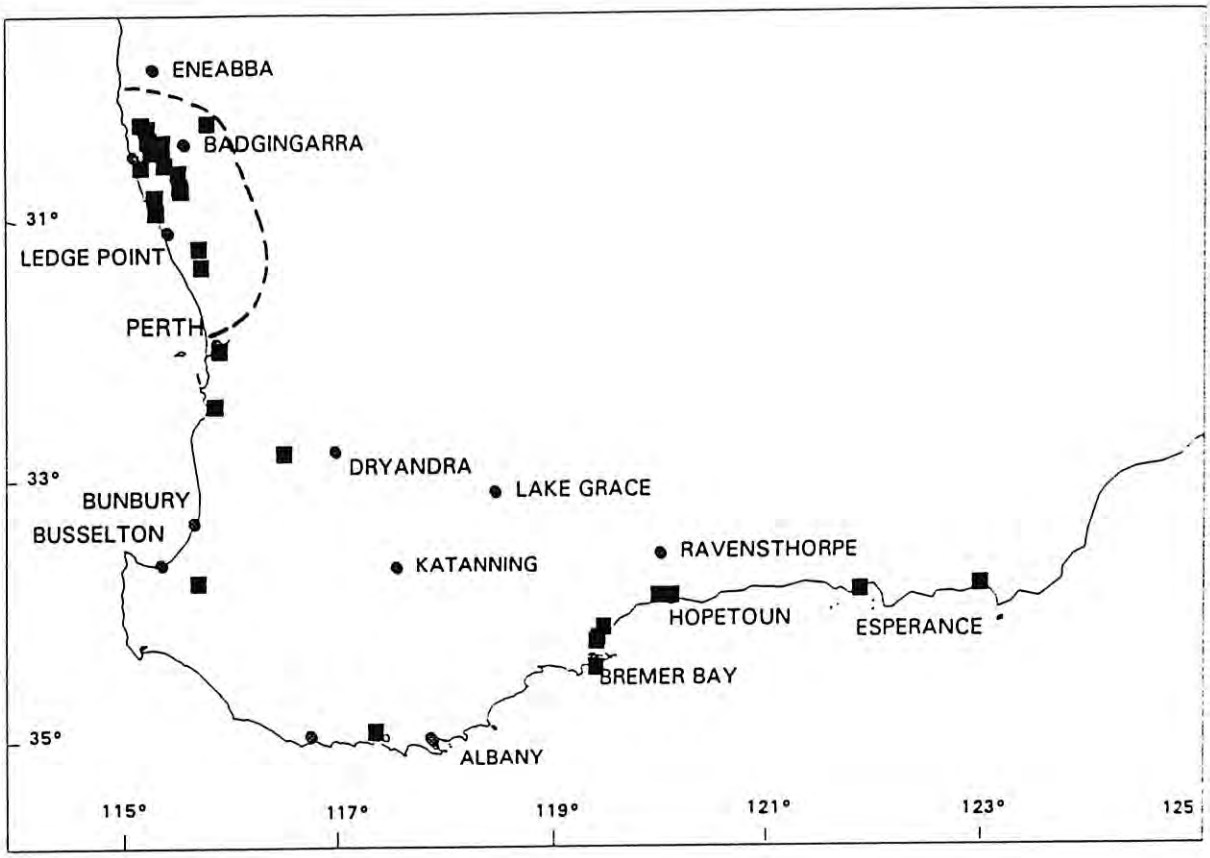



Fig. 3.1 The geographic distribution of recoveries of *P. megasperma* (■) within WA. The area delineated by hatched lines (---) represents the northern sandplain.

## REFERENCES


CALM. (1990). *Moora District Dieback Protection Plan 1990-1994*. Department of Conservation and Land Management, Perth, WA.

CALM. (1991). *Fitzgerald River National Park Management Plan 1991-2001*. Management Plan No. 15, Department of Conservation and Land Management, Perth, WA.

Coffey, M.D. and Bower, L.A (1984). *In vitro* variability among isolates of eight *Phytophthora* species in response to phosphorus acid. *Phytopathology* **74**, 738-742.

El-Hamalawi, Z.A. and Erwin, D.C. (1986). Plant, enzymatic, and chemical factors affecting viability and germination of oospores of *Phytophthora megasperma* f.sp. *medicaginis*. *Phytopathology* **76**, 503-504.

Elliot, C.G. (1983). Physiology of sexual reproduction in *Phytophthora*. *Phytophthora - its Biology, Taxonomy, Ecology, and Pathology* (Ed. by D.C. Erwin, S. Bartnicki-Garcia, and P.H. Tsao), pp. 71-80. The American Phytopathological Society, St. Paul, Minnesota.

Erwin, D.C. and McCormick, W.H. (1971). Germination of oospores produced by *Phytophthora megasperma* var. *sojae*. *Mycologia* **63**, 972-977.

Griffin, D.H. (1981). *Fungal Physiology*. John Wiley and Sons, New York.

Hansen, E.M. and Hamm, P.B. (1983). Morphological differentiation of host-specialised groups of *Phytophthora megasperma*. *Phytopathology* **73**, 129-134.

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

Hansen, E.M., Brasier, C.M., Shaw, D.S. and Hamm, P.B. (1986). The taxonomic structure of *Phytophthora megasperma*: evidence for emerging biological species groups. *Transactions of the British Mycological Society* **87**, 557-573.

Hart, Simpson and Associates. (1991). *Dieback Infections in the Northern Sandplain*. Unpublished consultancy to the Northern Sandplains Dieback Working Party. March 1991.

Jiang, J., and Erwin, D.C. (1990). Morphology, plasmolysis, and tetrazolium bromide stain as criteria for determining viability of *Phytophthora* oospores. *Mycologia* **82**, 107-113.

Jimenez, B. and Lockwood, J.L. (1982). Germination of *Phytophthora megasperma* f. sp. *glycinea* in the presence of soil. *Phytopathology* **72**, 662-666.

Pfender, W.F., Hine, R.B. and Stanghellini, M.E. (1977). Production of sporangia and release of zoospores by *Phytophthora megasperma*. *Phytopathology* **67**, 657-663.

Pratt, R.G. and Mitchell, J.E. (1975). The survival and activity of *Phytophthora megasperma* in naturally infested soils. *Phytopathology* **65**, 1267-1272.

Shea, S.R. Gillen, K.J. and Kitt, R.J. (1979). Variation in sporangial production of *Phytophthora cinnamomi* Rands on jarrah forest sites with different understorey compositions. *Australian Forest Research* **8**, 219-226.

Snedecor, G.W. and Cochran, W.G. (1980). *Statistical Methods 7 ed*. The Iowa State University Press, Ames, Iowa.

# PROJECT 5:

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

**A. Cochrane, D. Coates and M. McDonald**

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

Priority listings of taxa under immediate threat of dieback are derived from laboratory research and field observations by CALM staff and consultants and are constantly being revised. A currently updated list of some 198 taxa incorporates the South Coast, Wheatbelt, Central Forest, Metropolitan, Northern Forest and Greenough regions. This represents about 10% of flora listed on the Department of Conservation and Land Management's *Declared Rare and Priority Flora List* for Western Australia (Ken Atkins 02/02/94).

The list comprises species, status and regional distribution (Table 1). The family Proteaceae comprise 91 taxa in 6 genera, with 54 taxa in 9 genera of the family Epacridaceae, 14 taxa in 2 genera of the Papilionaceae, 5 taxa in 1 genus of the Casuarinaceae, 1 taxon in the Xanthorrhoeaceae and 1 genus in the Myrtaceae.

## 2. IN VITRO PROPAGATION

Discontinued (see Year 1 report)

## 3. CRYOSTORAGE

Discontinued (see Year 1 report)

## 4. SEED COLLECTION

The objective of the Threatened Flora Seed Centre (TFSC) is to ensure the maintenance of genetically representative seed collections of West Australian threatened flora under long term storage conditions as an interim solution for the prevention of genetic degradation or local extinction of critical populations. The TFSC will initially be a repository for genes from critically affected genepools that face extinction. It is envisaged that the stored seed will be used for regeneration programs should a control for dieback fungi be successfully implemented. The germplasm accessed will also be available for genetic studies on population structure to enhance the management and conservation of threatened taxa.

Seed collection attempts to systematically capture 90-95% of the common alleles from a threatened taxon on a representative range-wide basis (Brown and Briggs, 1991). However, many threatened taxa are not well known either taxonomically, geographically and/or phenologically. Poorly known seed biology and ecology also contribute to make effective systematic sampling difficult. This is often exacerbated by small population size or low and sporadic seed production or high seed predation levels. In addition, all sampling strategies are subject to the bias derived from fluctuations in the genetic contribution of individuals to the genepool due to disturbances, such as fire or drought, causing temporal genetic fluctuations in the genepool. Therefore, obtaining seed samples that have a wide genetic base (routine for common taxa) is usually difficult for rare taxa. Even so, the genetic quality of any seed sampled warrants some critical considerations to ensure that the genepool has optimal representation. The following TFSC seed collection protocols are primarily derived from work by Brown (1978) and Brown *et al* (1989a, 1989b, 1991) and based on guidelines provided by the Kew Seed Centre (Royal Botanic Gardens Kew, Wakehurst Place) and CSIRO's Australian Tree Seed Centre (ATSC). The design of genebanks and protocols recommended for use in genebanks have been formulated by the International Board for Plant Genetic Resources (IBPGR) (Cromarty *et al* 1985, Ellis *et al* 1985a and Ellis *et al* 1985b).

## 4.1 SAMPLING STRATEGY

### 4.1.1 Number of Populations per Taxa

The number of populations to be sampled will depend on the genetic variation between populations, but for most threatened taxa this information will be unknown. Sampling should be standardised but will require flexibility and adjustment according to the known distribution patterns and the resources available to carry out the collections. As a guideline, all populations should be sampled if the number of populations is less than five and a maximum of five populations if the number of populations exceeds five.

### 4.1.2 Number of Source Plants per Population

To capture 90-95% of the common alleles the optimum sample size for a given population is between 10 and 50 families (Brown and Briggs, 1991). Brown and Briggs point out that the capture of greater than 90-95% of the common alleles may become an exponential drain on resources requiring a sample of hundreds of families to achieve marginal genetic gains. Optimal sampling is dependent on factors affecting population genetic structure, particularly the breeding system. For the majority of target TFSC taxa population genetic structure is unknown.

Sampling unrelated families within a given population is a prerequisite for all family seedlots (seed from unrelated individual parent plants). This may be achieved by spacing source individuals according to the seed dispersal mechanisms involved; the root suckering ability or extent of lignotubers present; and/or the movement patterns or type of pollination vectors involved. Each family sampled from a population should contribute seed in equal quantities to avoid biasing the seedlot. The maintenance of family seedlots, must have the highest priority for the TFSC during all seed collections. This will allow the widest range of end uses that include attributing morphological variation to a specific family, genetic studies to assess breeding

systems, and/or bulking highly representative sub samples using viability figures. Field bulked seedlots (ie., seed from each plant collected in the population pooled to comprise a single seedlot in the field) are warranted when seeding events produce sparse and erratic seed crops that yield inadequate quantities of seed for the maintenance of family seedlots in long-term storage. In cases where the size of the population is such that collection from unrelated families is impossible, collection of seed from as widely spaced individual plants as possible is desirable.

#### 4.1.3 Number of Seed per Source Plant

A thousand seeds per population is the ideal minimum number of seeds required to adequately represent and conserve a genepool under long-term storage conditions (Brown and Briggs, 1991). If a 10 family seedlot is collected this amounts to 100 seeds per source plant. Fewer seeds are required from source plants if the number of families sampled increases. Ideally a healthy excess of 1000 viable seed per seedlot should be collected to enable the extraction of representative sub-samples for germination and viability testing, determination of moisture content and subsequent long-term monitoring of viability at intervals of 3-5 years (over a minimum of 50 years). Invertebrate predation can affect seed yields and must be taken into account during collections.

The above objectives will be difficult to achieve when sampling most rare flora populations. Quantities adequate for long-term storage may be accessed by implementing a repetitive (albeit benign) seasonal sampling regime until an adequate sample is obtained. Depletion of the population of the contribution of seed from any source individual should be avoided. Exceptions to this may be necessary at various sites that have critical levels of pathogen infestation.

### 4.2 POPULATION RECONNAISSANCE

To obtain a genetically representative seed collection population reconnaissance is necessary. The status of each plant needs to be carefully assessed with respect to

- the number of breeding plants contributing to the target seed crop;
- the seed production status of each plant;
- maturation status of seed crops;
- an estimation of the probable population spatial structure.

Population reconnaissance enables the optimum number of families from which adequate and uniform quantities of seed to be sampled as well as the systematic spacing of source plants to minimise the possibility of sampling closely related progeny.

### 4.3 TFSC ACCESSIONS

Accessions for 128 seedlots have been collected or incorporated into the Threatened Flora Seed Centre over the past 12 months with date, location, type and quantity of seed collected (Table 2). These collections cover *Phytophthora* and canker

threatened species present on the south coast, the southern wheatbelt and the Swan coastal plain.

Seed traps were devised and tested in spring and early summer 1993 for the first time on a number of species of the genus *Adenanthos* on the south coast. Differential fruiting of some taxa has warranted this method of collection. Some success has been forthcoming, although high levels of seed predation has been noted. Monitoring of the traps is required on a fortnightly, if not weekly, basis to prevent excessively high levels of predation and subsequent seed loss.

## 4.4 FUTURE COLLECTIONS AND PROPOSED DEVELOPMENTS

Threatened taxa on the northern sandplains will be targeted for collection in 1994, with continued sampling in the southern coastal regions. Seasonal resampling of populations with low viable seed production levels will commence this year. Existing seed traps will continue to function in the Stirling Range National Park and the Fitzgerald River National Park over the ensuing 12 months. Additional traps are likely to be erected at other sites following the success of the previous ones. A number of accessions will soon require monitoring, and subsamples will be unfrozen and tested to ascertain viability.

# 5. SEED STORAGE, VIABILITY TESTING AND INVENTORY SYSTEM

A fully operational germplasm storage facility based on medium and long term seed storage techniques is now in place (The Threatened Flora Seed Centre). This facility has already been referred to in a report to the ESU (Morse et al, 1993) as a model system for the seed-based germplasm storage of threatened species. Full details of the facility and protocols which have been developed are presented in this final report.

The Threatened Flora Seed Centre laboratory protocol involves the registration, cleaning, fumigation, testing and storage of all seed collected.

### 5.1 Registration

Documentation of all details of the collection, testing and storage of accessed seed is critical if resampling and regeneration programs are to be successfully implemented. Voucher specimens may require identification after drying and freezing prior to incorporation into the main collection. The registration of relevant collection details such as the location of species and the number of seed collected, as well as phenological data for the population and environmental data for the site is made on the WAHERB/TFSC database. The WAHERB number is linked to the unique, chronologically sequential TFSC seedlot number. The TFSC seedlot numbers are a five digit sequence with TD appended indicating Threatened by Dieback or P1 to P4 indicating Priority ranking if the collection was made opportunistically. The TFSC seedlot number is linked to the unique collectors field number (recorded on the Provenance Information datasheet). The TFSC numbers for each seedlot are appended to all relevant label data and seed bags.

### 5.2 Cleaning and fumigation

Most seed is brought into the TFSC uncleaned from the field. In most cases, seed needs to be extracted from cones/follicles or capsules prior to testing and storage. Seed is cleaned to attain the highest practical level of purity possible. For some small seeded species, however, the time taken to achieve 100% purity may use excessive resources and a lower level of purity may be justified. Subsamples extracted from the impure seedlots to be used for germination and viability tests must be pure seed to avoid fungal contaminants.

Fumigation will be required to eliminate invertebrate predation of seed, prior to its ultimate storage. Predation of collected seed may be arrested by fumigating the seedlot with $CO_2$ using heat-sealed laminated plastic bags. Carbon dioxide also has a sterilising effect on fungal growth and micro organisms. This technique is employed by the CSIRO's Australian Tree Seed Centre. Fumigation is complete after two weeks. Seedlots stored as woody cones can be fumigated directly into their ultimate storage skin (laminated foil replacing air with $CO_2$).

### 5.3 Quantifying the seedlot

The type of quantification used will vary according to fruit and seed type. Seed collected from small, easily extracted seed types are most efficiently recorded as the number of seed per gram. To do this a small subsample is taken, counted then weighed and the figure for the subsample extrapolated to the total weight of the seedlot. Other species warrant a careful seed count. Detailed quantification of seedlots is important and requires accurate assessment and databasing to ensure that monitoring procedures during storage can readily determine if adequate viable seed is being maintained.

### 5.4 Germination/viability testing

Once the seedlot has been cleaned and fumigated it must undergo germination/viability determination and moisture content analysis. Representative sub-samples need to be obtained from each seedlot sufficient for these tests. In most cases the optimum pre-treatment for germination of TFSC taxa will be unknown due to poorly understood seed biology and testing for dormancy breaking may be required. A range of dormancy-breaking techniques such as the use of differing concentrations of Gibberellic Acid and heat treatment (boiling water) have been trialed to aid germination. Temperature is a critical germination parameter for species in the South West winter rainfall zone, and all seedlots derived from this zone are initially tested at a constant 15°C with an 12 hour/12 hour light/dark regime. Consideration of optimal recruitment events in the species' natural habitat is recommended when dealing with difficult seedlots.

Once the optimal germination has been achieved (i.e., > 90% if possible) viability is determined using a representative subsample with at least three replicates. The number of seeds used for each replicate will clearly be determined by the amount of seed constituting the seedlot. In the case of low numbers of seed per seedlot (i.e. between 500 and 1000 seeds) the total quantity used for the replicates should be 5% of the total number. For critically low seedlots (i.e. less than 500 seeds) it may be necessary to use as little as 2.5% of the total number of seeds.

All germinants produced by TFSC laboratory that are not used in the genetics laboratory at the WA Herbarium are donated to the Kings Park and Botanic Gardens for incorporation into the Rare Flora display or for on-going experimentation on the biology of rare plants.

**5.5 Moisture content determination, reduction and drying of seed.**

Two important factors in the requirement of seed storage are seed moisture content and temperature. Seed is dried to between 4 - 7% moisture content, placed in sealed containers and stored to -18°C or less. Hermetically sealed containers such as laminated foil are used to keep relative humidity low. The Karl Fischer Auto-Titrator or the Oven Dry Method (103°C for 17hrs) are used to assess moisture content. Ideally all moisture content analyses should be determined using the former method as it specifically detects water molecules in the sample (i.e., the volatile components are not measured cf. the oven dry method). The precision obtained by this method enables as few as two or three seed to be used, however it is recommended that at least 50 seed per replicate are tested, selectively covering the range in seed size and maturation. The number of replicates to use will be dependant on the stability of results obtained during the analyses. An acceptable level of discrepancy between replicates is probably around 0.2% (cf. the oven-dry method). Desiccators with silica gel are used for drying the seed. Regularly regenerated silica gel at 15°C and relative humidity of 10-15° is optimal for the lowering of moisture content of seed to 4 - 7%.

**5.6 Packaging , storage and monitoring regime.**

Laminated aluminium foil is used as a packaging medium. This foil consists of 3 layers: polyethelene on the inside provides the sealing property, aluminium foil in the middle acts as a barrier to moisture and polyester on the outside protects the foil from damage and oxidation. Shrestha *et al* (1985) found carbon dioxide to be an effective storage medium, second to nitrogen but cheaper and easier to use. During extensive laboratory testing, it was found that germination success and seedling vigour were maintained at high levels over periods of time when oxygen was excluded from the storage atmosphere. With an increase in storage temperature, the rate of loss of viability accelerates when oxygen is the storage medium. Laminated foil bags are made up with the use of a heat sealer, the germplasm is placed in to the bags which are filled with $CO_2$ then sealed. This is a simple and inexpensive technique for storage of germplasm for the short, medium and long term.

The maintenance of seed germination vigour and capacity during storage is critical for any genebank (Hanson, 1985). Seed viability and seedling vigour are known to decline with increased length of storage, and seed stored at ambient temperatures and relative humidity loses viability quickly. Long term storage conditions (-18°C) are used for base collections when seed is fully extracted. Some seed is stored for the long term unextracted in cones/follicles at room temperature, also sealed in foil with $CO_2$ and stored in large black plastic storage drums for safety. Storage at room temperature when seed remains in cones as near as possible emulates storage on the plant. As germination and moisture content testing may take 6-8 weeks to conclude, interim storage during this period should be at 4°C if the seed is fully extracted or at room temperature (20-25°C) if seed is enclosed in cones, follicles or bracts. Low

temperatures are preferable for all seedlots during pre-storage handling. Prior to storage of base collections, subsamples for retesting are placed in separate packages, carefully labelled and sealed in foil with $CO_2$ and stored at -18°C.

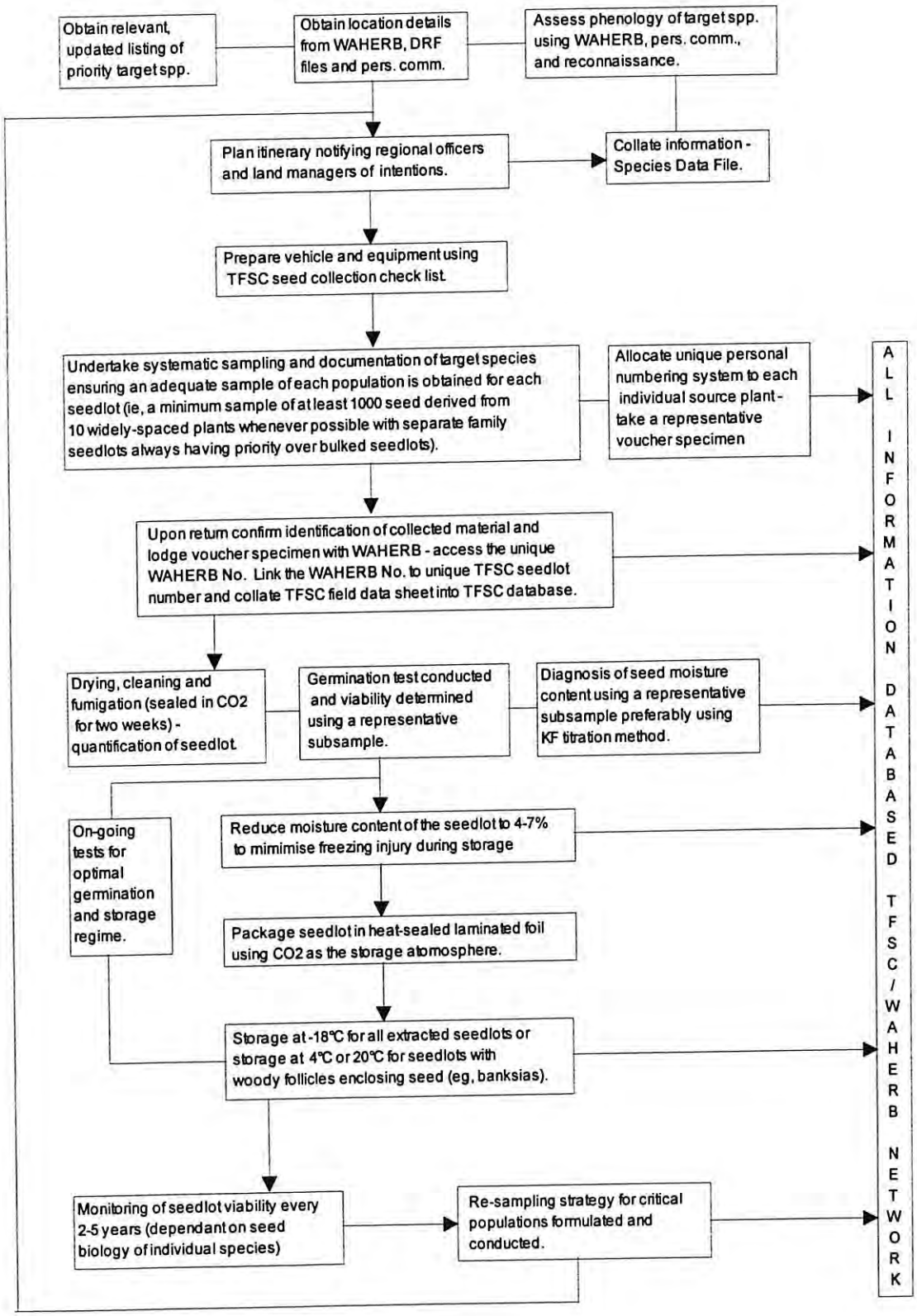
The determination of an appropriate on-going monitoring regime to assess seedlot response to storage at -18°C is dependent on the quantity of seeds collected and its original viability and moisture content. The monitoring interval is some fraction of the regeneration interval. The time taken for viability to reach a regeneration standard is the estimate that the monitoring interval is based on (Cromarty *et al* 1982). In a newly established genebank it is advised to begin with a short monitoring interval to assess the species' response to medium and long term storage. The IBPGR recommend monitoring at least every 5 years for active collections or those with low initial viability or storage life. Australian flora response to storage is virtually unknown therefore a conservative monitoring regime is warranted. It is prudent to initially check the accessions after one year in storage at -18°C, and thereafter at intervals of 3-5 years dependent upon the species and its response to below-zero storage temperatures. Any significant decline in seed quality must be countered by formulating a new storage strategy before resampling.

### 5.7 Collation of data

All of the above information is collated into the WAHERB/TFSC database as an ongoing process. Meticulous adherence to detail is imperative to maintain the integrity of the TFSC genebank in the long term.

A number of new databases have recently been designed to facilitate the transfer of data to the manager of the TFSC. A Seed-in-Store database has been set up to enable a quick check on the amount of seed in storage and the dates for monitoring of samples to be made. This will ensure correct and timely monitoring of accessions over the long term. Secondly, a Fruiting Time database has been initiated to allow future collections to be timed according to phenological information. To date, botanists have generally been interested in flowering times without reference to seed production. This database will focus the planning of seed collection trips and target optimal seed collection periods. This new database will be updated from information from herbarium specimens, field observations, Kings Park and Botanic Gardens collections records and from research from scientific papers and published literature. In addition, an on-site TFSC reference library has been established and will continue to be updated.

**Figure 1:** Seed collection and storage protocol for the Threatened Flora Seed Centre (TFSC), WA Herbarium, CALM.



## REFERENCES


Brown, A.H.D. and Briggs, J.D. 1991. Sampling strategies for genetic variation in ex-situ collections of endangered plant species. In , *Genetics and Conservation of Rare Plants*. Falk, D.A. and Holsinger, K.E., (eds.) Oxford University Press.

Brown, A.H.D., Clegg, M.T., Kahler, A.L. and Weir, B.S. (eds.) 1989a. *Plant Population Genetics, Breeding and Genetic Resources.* Sinauer, Sunderland, Mass.

Brown, A.H.D., Frankel, O.H., Marshall, D.R. and Williams, J.T. (eds.) 1989b. *The Use of Plant Genetic Resources.* Cambridge University Press.

Cromarty, A.S., Ellis, R.H. and Roberts, E.H. 1985. *The Design of Seed Storage Facilities. Handbooks for Genebanks No.1.* IBPGR. Rome.

Ellis, R.H., Hong, T.D. and Roberts, E.H. 1985a. *Handbook of Seed Technology for Genebanks, Vol.1: Principles and Methodology., Handbooks for Genebanks No.2.* IBPGR Secretariat, Rome.

Ellis, R.H., Hong, T.D. and Roberts, E.H. 1985b. *Handbook of Seed Technology for Genebanks, Vol.2: Compendium of Specific Germination Information and Test Recommendations. Handbooks for Genebanks No.3.* IBPGR Secretariat, Rome.

Hanson, J. 1985. *Procedures for Handling Seed in Genebanks. Practical Manuals for Genebanks. No.1* IBPGR, Rome.

Morse, G.J., Whitfeld, S. and Gunn, B.V. 1993. *Australian Seedbanks and Cryogenic Storage of Endangered Flora.* Consultancy Report to the Endangered Species Unit, Australian Nature Conservation Agency. CSIRO, Division of Forestry, Australian Tree Seed Centre. ANCA, Canberra.

Shrestha, K.B., Shepherd, K.R. and Turnbull, J.W., 1985. Controlled atmosphere storage for *Pinus radiata* seed. *Comm. For. Rev.* 64(2):141 - 150.

## TABLE 1: LIST OF 198 PRIORITY TAXA CONSIDERED OR KNOWN TO BE MODERATELY OR HIGHLY SUSCEPTIBLE TO DIEBACK OR CANKER

| SPECIES | STATUS | REGION | DISTRICT |
|---|---|---|---|
| Adenanthos pungens subsp. effusa | E | WB | Kt |
| Adenanthos pungens subsp. pungens | E | WB | Kt,Al |
| Adenanthos velutinus | E | WB | Kt |
| Andersonia sp. Two Peoples Bay (G Keighery 8229) | E | SC | Al |
| Banksia brownii | E | SC | Al |
| Darwinia apiculata | E | SW | |
| Darwinia carnea | E | WB,GRE | Na,Mo |
| Darwinia ferricola ms | E | CF | |
| Daviesia bursarioides ms | E | GRE | Mo |
| Daviesia oxylobium ms | E | WB | Na,Me |
| Dryandra mimica | E | CF,SW | Pe |
| Dryandra sp. Kamballup (M Pieroni 20.9.88) | E | SC | Al |
| Dryandra sp. Stirling Range (F Lullfitz 3379) | E | SC | Al |
| Isopogon uncinatus | E | SC | Al |
| Lambertia echinata subsp. echinata | E | SC | Es |
| Lambertia fairallii | E | SC | Al |
| Lambertia orbifolia | E | SF,CF | Wl,Bu |
| Leucopogon marginatus | E | WB | Me |
| Adenanthos cunninghamii | V | SC | Al |
| Adenanthos dobagii | V | SC | Al |
| Adenanthos ellipticus | V | SC | Al |
| Adenanthos eyrei | V | SC | Es |
| Adenanthos ileticos | V | SC | Es |
| Allocasuarina fibrosa | V | WB | Me,Na |
| Allocasuarina tortiramula | V | WB | Kt |
| Banksia cuneata | V | WB | Na |
| Banksia goodii | V | SC,SF | Al,Wl |
| Banksia oligantha | V | WB | Kt |
| Banksia sphaerocarpa var. dolichostyla | V | WB | Me,Kt,Na |
| Banksia tricuspis | V | GRE | Mo |
| Banksia verticillata | V | SC,SF | Al,Wl |
| Darwinia acerosa | V | GRE,SW | Mo |
| Darwinia collina | V | SC | Al |
| Darwinia macrostegia | V | SC | Al |
| Darwinia masonii | V | GRE | Ge |
| Darwinia meeboldii | V | SC | Al |
| Darwinia oxylepis | V | SC | Al |
| Darwinia sp. Stirling Range (GJ Keighery 5732) | V | SC | Al |
| Darwinia squarrosa | V | SC | Al |
| Darwinia wittwerorum | V | SC | Al |
| Daviesia euphorbioides | V | WB | Me |
| Daviesia speciosa ms | V | GRE | Mo |
| Daviesia spiralis | V | WB | Me,Na |
| Dryandra serratuloides | V | GRE | Mo |
| Leucopogon obtectus | V | GRE | Mo |
| Leucopogon cryptanthus | X | GRE | |
| Adenanthos cygnorum ssp. chamaephyton | 1 | SW | |
| Allocasuarina tessellata | 1 | GRE | Ge |
| Andersonia longifolia | 1 | GRE | Mo |
| Astroloma sp. Nannup (RD Royce 3978) | 1 | CF,SW | Mu,Bu,Nn |

| | | | |
|---|---|---|---|
| Banksia elegans (Fraser Rd form) | 1 | GRE | Ge |
| Coleanthera coelophylla | 1 | WB,SC | Kt,Al,Es |
| Darwinia calothamnoides ms | 1 | SC | Es |
| Darwinia chapmaniana ms | 1 | GRE | Mo |
| Darwinia repens | 1 | GRE | Ge |
| Darwinia sp. Carnamah | 1 | GRE | Mo |
| Darwinia sp. Gardner | 1 | ? | ? |
| Darwinia sp. Mt Baring (KR Newbey 9775) | 1 | SC | Es |
| Darwinia sp. Mt Ney | 1 | SC | Es |
| Darwinia sp. Williamson (GJ Keighery 12717) [aff. apiculata] | 1 | CF | Bu |
| Daviesia pteroclada ms | 1 | GRE | Mo |
| Dryandra sp. 15 (EA Griffin 3453) [aff.hewardiana] | 1 | GRE | Mo |
| Dryandra sp. 20 (AS George 16787) [aff. armata] | 1 | GRE | Mo |
| Dryandra sp. 23 (AS George 16788) [aff. fraseri] | 1 | GRE | Mo |
| Dryandra sp. 30 (AS George 11657) [aff. squarrosa] | 1 | CF | Bu |
| Dryandra sp. 31 (AS George 16754) [aff. conferta] | 1 | WB | Na |
| Dryandra sp. 36 (AS George 16721) [aff. ferruginea] | 1 | SC | Es |
| Dryandra sp. 38 (AS George 16695) [aff. drummondii] | 1 | WB | Kt |
| Dryandra sp. 41 (AS George 16879) [aff. calophylla] | 1 | WB | Kt |
| Dryandra sp. 42 (AS George 16789) [aff. hewardiana] | 1 | GRE | Mo |
| Dryandra sp. 46 (AS George 16866) [aff. sclerophylla] | 1 | GRE | Mo |
| Epacridaceae gen. nov. [aff. Melichrus] | 1 | GLD,WB | Kg,Me |
| Isopogon drummondii | 1 | GRE,SW | Mo,Pe |
| Isopogon sp. Dale [aff. scaber] | 1 | SW | Mu |
| Leucopogon blepharolepis | 1 | SC,WB | Es,Kt |
| Leucopogon florulentus | 1 | SC | Al,Es |
| Leucopogon plumuliflorus | 1 | GRE | Mo |
| Leucopogon pogonocalyx | 1 | SC | Al |
| Leucopogon sp. Bonnie Hill (KR Newbey 9831) | 1 | SC | Es |
| Leucopogon sp. Cascades (MA Burgman 3700) | 1 | SC | Es |
| Leucopogon sp. Clyde Hill(MA Burgman 1207) | 1 | SC | Es |
| Leucopogon sp. Condingup (MA Burgman 1377) | 1 | SC | Es |
| Leucopogon sp. Coujinup (MA Burgman 1085) | 1 | SC | Es |
| Leucopogon sp. Dundas (MA Burgman 1482) | 1 | SC | Es |
| Leucopogon sp. Kau Rock (MA Burgman 1126) [aff. allittii] | 1 | SC | Es |
| Leucopogon sp. Munglinup (KR Newbey 8123) | 1 | SC | Es |
| Leucopogon sp. Peak Charles (MA Burgman 1476) | 1 | SC | Es |
| Leucopogon sp. Roberts Swamp (KR Newbey 8173) | 1 | SC | Es |
| Leucopogon sp. South Coast (KR Newbey 8213) | 1 | SC | Es |
| Acrotriche patula | 2 | SC,WB | Me,Es,Na |
| Adenanthos cacomorphus | 2 | SC | Al |
| Adenanthos linearis | 2 | SC | Al |
| Andersonia auriculata | 2 | SF | Wl,Al |
| Andersonia axilliflora | 2 | SC | Al |
| Andersonia bifida | 2 | WB | Na |
| Andersonia carinata | 2 | GLD,WB | Kg,Me |
| Andersonia macranthera | 2 | SC | Es |
| Astroloma foliosum | 2 | SW | Pe,Mu |
| Astroloma sp. Eneabba (N Marchant s.n.) | 2 | GRE | Mo |
| Banksia epica | 2 | SC | Es |
| Banksia occidentalis subsp. formosa | 2 | SC,SF,CF | Al,Pm,Nn |
| Darwinia luehmannii | 2 | SC | Es |
| Darwinia sp. Peak Charles | 2 | SC | Es |
| Darwinia sp.humb Peak (KR Newbey 4847) [aff. diosmoides] | 2 | SC | Al |
| Daviesia chapmanii ms | 2 | GRE | Mo |

| | | | |
|---|---|---|---|
| Daviesia dielsii | 2 | GRE | Mo |
| Daviesia lineata ms | 2 | WB | Kt |
| Daviesia physodes | 2 | GRE,SW | Mo,Mu,Pe |
| Dryandra cynaroides | 2 | WB | Na,Kt |
| Dryandra erythrocephala var. 44 (AS George 16743) | 2 | WB | Kt,Na |
| Dryandra foliosissima | 2 | WB,SC | Kt,Al |
| Dryandra sclerophylla | 2 | GRE | Mo |
| Dryandra seneciifolia | 2 | SC | Al |
| Dryandra serra | 2 | SC | Al,Wl |
| Dryandra sp. 1 (AS George 16647) [aff. hewardiana] | 2 | WB | Kt |
| Dryandra sp. 12 (K Newbey 2226) [aff. ?plumosa] | 2 | SC | Al |
| Dryandra sp. 25 (AS George 16763) [aff. hewardiana] | 2 | WB | Me |
| Dryandra sp. 37 (AS George 16740) [aff. proteoides] | 2 | WB | Na |
| Dryandra sp. 45 (K Alcock 486) [aff. serratuloides] | 2 | WB | Kt,Na |
| Dryandra sp. 48 (AS George 16886) [aff. falcata] | 2 | SC | Al |
| Dryandra sp. 49 (K Alcock 472) [aff. ferruginea] | 2 | SC | Al |
| Dryandra sp. 50 (AS George 16713) | 2 | WB | Kt |
| Dryandra sp. 52 (T Aplin 6537) | 2 | CF | Bu |
| Dryandra sp. Fitzgerald (M Burgman s.n.) [aff. pteridifolia] | 2 | SC | Al |
| Dryandra sp. Watheroo (RD Royce 9625) | 2 | GRE | Mo |
| Isopogon alcicornis | 2 | SC | Es |
| Leucopogon bracteolaris | 2 | SC | Al |
| Leucopogon breviflorus | 2 | SC | Al,Es |
| Leucopogon denticulatus | 2 | SC | Al |
| Leucopogon glaucifolius | 2 | SC,GRE,(SW) | Al,Mo,(Pe) |
| Leucopogon interruptus | 2 | SC | Es |
| Leucopogon lasiophyllus | 2 | SC | Al |
| Leucopogon multiflorus | 2 | SC | Es |
| Leucopogon pleurandroides | 2 | SC | Es |
| Leucopogon polystachyus | 2 | SF | Ma,Wl,Pm |
| Leucopogon rotundifolius | 2 | SC | Al,Es |
| Leucopogon sp. Yanneymooning (F Mollemans 3797) | 2 | WB | Me |
| Leucopogon tamariscinus | 2 | SC | Al |
| Lysinema elegans | 2 | GRE,SW | Pe,Mo |
| Monotoca leucantha | 2 | GRE | Mo |
| Petrophile crispata | 2 | WB | Kt,Na |
| Petrophile incurvata | 2 | WB,GRE | Ge,Me |
| Adenanthos glabrescens ssp. exasperatus | 3 | SC | Al,Es |
| Adenanthos gracilipes | 3 | SC | Es |
| Allocasuarina grevilleoides | 3 | GRE | Mo |
| Allocasuarina ramosissima | 3 | GRE,SW | Mo,Mu |
| Andersonia echinocephala | 3 | SC | Al |
| Andersonia grandiflora | 3 | SC | Al |
| Andersonia setifolia | 3 | SC,SF | Al,Wl |
| Banksia lullfitzii | 3 | GLD,WB,SC | Kg,Me,Es |
| Banksia micrantha | 3 | GRE,SW | Mo,Pe |
| Banksia scabrella | 3 | GRE | Ge |
| Conostephium minus | 3 | GRE,SW | Mo,Pe,Mu |
| Darwinia pimelioides | 3 | SW | Mu |
| Darwinia sp. Morawa | 3 | GRE | Ge,Mo |
| Daviesia debilior subsp. sinuans | 3 | WB | Me |
| Daviesia epiphyllum | 3 | GRE | Mo |
| Dryandra horrida | 3 | WB | Me,Na |
| Dryandra polycephala | 3 | SW | Mu |
| Dryandra sp. 7 (AS George 11703) [aff. polycephala] | 3 | GRE,SW | Mo,Mu |

| | | | |
|---|---|---|---|
| Dryandra sp. 16 (AS George 9446) [aff. horrida] | 3 | SC,WB | Es,Na |
| Dryandra sp. 22 (AS George 16779) [aff. pteridifolia] | 3 | GRE | Mo |
| Dryandra speciosa | 3 | WB,GRE | Mo,Me |
| Dryandra subpinnatifida | 3 | WB | Na,Kt |
| Dryandra tortifolia | 3 | GRE | Mo |
| Isopogon tridens | 3 | GRE | Mo |
| Jacksonia carduacea | 3 | GRE | Mo |
| Jacksonia sericea | 3 | SW | Pe,Dw |
| Leucopogon amplectens | 3 | WB | Me |
| Leucopogon apiculatus | 3 | SC | Es |
| Leucopogon brevicuspis | 3 | SC | Al,Es |
| Leucopogon oliganthus | 3 | GRE,SW | Mo |
| Petrophile biternata | 3 | GRE | Mo |
| Petrophile plumosa | 3 | GRE,SW | Mo,Mu |
| Adenanthos detmoldii | 4 | CF | Bu,Nn |
| Adenanthos labillardierei | 4 | SC | Al |
| Astroloma sp. Cataby (EA Griffin 1022) | 4 | GRE,SW | Mo,Mu |
| Banksia benthamiana | 4 | GRE,WB | Ge,Me |
| Banksia chamaephyton | 4 | GRE | Mo |
| Banksia elegans | 4 | GRE | Mo |
| Banksia laevigata ssp. laevigata | 4 | SC | Al |
| Banksia meisneri var. ascendens | 4 | CF | Bu,Na |
| Banksia solandri | 4 | SC | Al |
| Darwinia hypericifolia | 4 | SC | Al |
| Darwinia lejostyla | 4 | SC | Al |
| Darwinia polycephala | 4 | SC | Es |
| Darwinia sanguinea | 4 | GRE | Mo |
| Darwinia sp.Chiddarcooping (SD Hopper 6944)[aff. purpurea] | 4 | WB | Me |
| Darwinia sp. Dryandra | 4 | WB | Na |
| Darwinia sp. Mt Burdett | 4 | SC | Es |
| Darwinia thymoides subsp. nov. (J Alford & GJ Keighery 64) | 4 | WB,SW | Na,Mu |
| Dryandra comosa | 4 | WB | Me |
| Dryandra preissii | 4 | SC,WB | Al,Kt |
| Dryandra pulchella | 4 | WB | Me |
| Dryandra shanklandiorum | 4 | WB | Me,Na |
| Dryandra sp. 3 (AS George 16629) [aff. blechnifolia] | 4 | SC,WB | Al,Kt |
| Xanthorrhoea brevistyla | 4 | WB,SC | Al,Na |
| Adenanthos detmoldii x obovata+ | 8 | CF | Bu |

[1]Scale used to rank threatened taxa (based on Hopper *et al.*, 1990)

**X** - presumed extinct
**E** - endangered
**V** - vulnerable

**1** - few poorly known pops on threatened lands
**2** - few poorly known pops on conservation lands
**3** - several poorly known pops, some on conservation lands

## TABLE 2: List of Accessions held in the TFSC as of 02/02/94

| Seedlot number | Colln. date | Species | Location | *Type |
|---|---|---|---|---|
| 00001 TD | 09/08/87 | *Banksia brownii* | Mt Hassell | I/4 |
| 00002 TD | 02/11/88 | " " | Bluff Knoll | I/17 |
| 00003 TD | 09/02/88 | *B. cuneata* | Lake Mears Road | I/6 |
| 00004 TD | 09/02/88 | " | Swamp Road | I/10 |
| 00005 TD | 09/02/88 | " | Badjaling NR | I/13 |
| 00006 TD | 1988 | *B. brownii* | Cheyne Beach | I/38 |
| 00007 TD | | " | Quaranup | I/29 |
| 00008 TD | | " | Cheyne Bch Road | I/49 |
| 00009 TD | 26/04/88 | *B. epica* | Toolina Cove | I/45 |
| 00010 TD | 09/02/88 | *B. cuneata* | Water Res. 12397 | I/30 |
| 00011 TD | 09/02/88 | " | Simpsons Farm | I/15 |
| 00012 TD | 08/02/88 | " | Bruce Rock Road | I/30 |
| 00013 TD | 23/05/88 | " | Quairading | I/23 |
| 00014 TD | 24/09/90 | " | Laze Away | I/29 |
| 00015 TD | 06/87 | *B. verticillata* | Mt Hopkins | I/24 |
| 00016 TD | 10/05/86 | " | The Gap, Torndirrup | I/11 |
| 00017 TD | | *B.?gardneri* x goodii | Millbrook Road | I/18 |
| 00018 TD | 08/03/88 | *B. oligantha* | Wongeling N | I/10 |
| 00019 TD | 08/03/88 | " | Wongeling W | I/20 |
| 00020 TD | 08/03/88 | " | Wongeling E | I/10 |
| 00021 TD | 26/11/86 | *B. verticillata* | Mermaid Point Tk | I/20 |
| 00022 TD | 1987 | " | Poison Hill | I/9 |
| 00023 TD | 10/05/86 | " | Stony Hill Creek | I/13 |
| 00024 TD | 28/11/90 | *Dryandra* sp.Kambellup | Kambellup S | I/19 |
| 00025 TD | 09/08/90 | *Banksia oligantha* | Marribank site 1 | I/14 |
| 00026 TD | 09/08/90 | " " | Marribank site 2 | I/11 |
| 00027 TD | 09/08/90 | " " | Marribank site 3 | I/10 |
| 00028 TD | 09/08/90 | " " | Marribank site 4 | I/30 |
| 00029 TD | 1985 | *Lambertia orbifolia* | Narrikup 1 | I/9 |
| 00030 TD | 13/12/92 | " " | Narrikup 2 | B/15 |
| 00031 TD | 12/12/92 | *Isopogon uncinatus* | Muttonbird | I/16 |
| 00032 TD | 12/12/92 | *Banksia verticillata* | Stony Hill Creek | I/10 |
| 00033 TD | 13/12/92 | *Andersonia "pinaster"* | Boulder Hill | B/50 |
| 00034 TD | 14/12/92 | *A. echinocephala* | Mondurup | B/30 |
| 00035 TD | 15/12/92 | *A. grandiflora* | Toolbrunup | I/12 |
| 00036 TD | 18/12/92 | *Adenanthos pungens ssp effusa* | Tambellup | B/10 |
| 00037 TD | 22/01/93 | *Dryandra seneciifolia* | Red Gum Pass | I/13 |
| 00038 TD | 22/01/93 | *Andersonia grandiflora* | Stirling Drive | I/10 |
| 00039 TD | 23/01/93 | *Dryandra* sp.Kambellup | Kambellup N | I/10 |
| 00040 TD | 25/01/93 | *Banksia brownii* | South Sister | I/22 |
| 00041 TD | 29/01/93 | *Lambertia echinata ssp. echinata* | Lucky Bay | I/3 |
| 00042 TD | 30/01/93 | *Isopogon alcicornis* | Mt Burdett | I/10 |
| 00043 TD | 31/01/93 | *Dryandra serra* | South Sister | B/20 |
| 00044 TD | 18/09/92 | *Banksia verticillata* | Jimmy Newhills Harbour, Torndirrup | B/6 |
| 00045 P3 | 24/01/93 | *Thomasia solanacea* | Limeburners Lane | I/5 |
| 00046 P3 | 24/01/93 | *Eucalyptus acies* | South Sister | B/5 |
| 00047 P1 | 30/01/93 | *Melaleuca coccinea ssp. eximia* | Mt Burdett | B/10 |
| 00048 E | 30/01/93 | *Eucalyptus merrickiae* | Kent Road | I/5 |
| 00049 TD | | *Banksia brownii* | Bells End | I/5 |
| 00050 TD | 14/05/86 | *B. brownii* | Millbrook Road | I/3 |
| 00051 E | 09/05/86 | *B. goodii* | Millbrook Nat. Res. | I/11 |
| 00052 E | 29/08/86 | *B. goodii* | Redmond S.F. | I/3 |

| | | | | |
|---|---|---|---|---|
| 00053 E | 29/08/86 | *B. goodii* | Chorkerup Road | I/11 |
| 00054 E | | *B. ?semi-nuda* | Mt. Hopkins | I/8 |
| 00055 TD | 04/04/93 | *B. verticillata* | Channel Point | I/25 |
| 00056 TD | 06/04/93 | *B. verticillata* | Stony Hill | I/15 |
| 00057 TD | 07/04/93 | *B. verticillata* | Woolbale Hills | I/25 |
| 00058 TD | 15/05/93 | *Lambertia orbifolia* | Dennis Road | B/15 |
| 00059 TD | 15/05/93 | *L. orbifolia* | Brennans Ford | B/21 |
| 00060 TD | 18/05/93 | *Banksia brownii* | Quaranup Road | I/16 |
| 00061 TD | 19/05/93 | *B. brownii* | Millbrook Nat. Res. | I/41 |
| 00062 TD | 20/07/93 | *Dryandra sp.30* | Ruabon/Tutunup | I/10 |
| 00063 TD | 21/07/93 | *Dryandra sp.30* | Smith Rd, Wicher R. | I/17 |
| 00064 TD | 22/07/93 | *Banksia ?verticillata* | Thompson Cove SW | I/17 |
| 00065 TD | 22/07/93 | *B. ?verticillata* | Aldridge Cove SW | I/14 |
| 00066 TD | 23/07/93 | *B. seminuda ssp remanens* | Poison Hill carpark | I/3 |
| 00067 TD | 23/07/93 | *B. seminuda ssp remanens* | Poison Hill SW face | I/18 |
| 00068 TD | 24/07/93 | *B. brownii* | Mt. Hassell | I/25 |
| 00069 TD | 06/08/93 | *Isopogon scaber* | 42mp Brookton Hwy | |
| 00070 TD | 18/08/93 | *Dryandra sp. 48 (aff. falcata)* | Mt. Talyuberlup | B/15 |
| 00071 TD | 19/08/93 | *Dryandra seneciifolia* | Lookout-Stirling Range Drive | I/16 |
| 00072 TD | 19/08/93 | *Dryandra sp. 49 (aff. ferruginea)* | Lookout-Stirling Range Drive | I/17 |
| 00073 TD | 20/08/93 | *Andersonia sp. nov (Mt. Lindesay)* | Mt. Lindesay | B/10 |
| 00074 TD | 21/08/93 | *Dryandra serra* | Hunwick Rd(East) | B/32 |
| 00075 TD | 21/08/93 | *Dryandra serra* | Hunwick Rd(West) | I/11 |
| 00076 TD | 21/08/93 | *Banksia brownii* | Hazzard Rd | I/4 |
| 00077 TD | 22/08/93 | *Banksia brownii* | Hassell Beach Rd | I/15 |
| 00078 TD | 23/08/93 | *Banksia verticillata* | Mermaid/Channel Pt | I/8 |
| 00079 TD | 23/08/93 | *Banksia verticillata* | Mermaid Pt Track | I/16 |
| 00080 TD | 24/08/93 | *Dryandra seneciifolia* | Hamilla Hills | I/10 |
| 00081 TD | 14/09/93 | *Dryandra sp. 1* | Carter Rd | I/12 |
| 00082 TD | 14/09/93 | *Dryandra sp. 41* | Woodanilling Orchard/Dinwoodie Rds, Woodanilling | B/20 |
| 00083 TD | 15/09/93 | *Dryandra sp. 45* | Katanning-Nyabing Rd | B/10 |
| 00084 TD | 15/09/93 | *Dryandra sp. 38* | Nyabing-Kukerin Rd | I/20 |
| 00085 TD | 16/09/93 | *Dryandra sp. 44* | Hopkins NR | I/21 |
| 00086 TD | 16/09/93 | *Dryandra sp. 37* | Hopkins NR | I/21 |
| 00087 TD | 17/09/93 | *Dryandra sp. 31* | Corrigin-Quarading Rd | I/17 |
| 00088 TD | 21/9/93 | *Lambertia fairallii* | Stirling Range Drive | B/100 |
| 00089 TD | 09/11/93 | *Dryandra mimica* | Old Kent Rd, Wicher Range | B/1 |
| 00090 TD | 09/11/93 | *Lambertia orbifolia* | Dennis Road | B/10 |
| 00091 TD | 10/11/93 | *Astroloma sp. Nannup* | Scott River Rd. Reserve | I/20 |
| 00092 TD | 11/11/93 | *Dryandra sp. Kambellup* | Kambellup N | I/16 |
| 00093 TD | 11/11/93 | *Dryandra sp. Kambellup* | Kambellup S | I/15 |
| 00094 TD | 11/11/93 | *Lambertia fairallii* | Stirling Range Drive | B/50 |
| 00095 R | 03/11/93 | *Daviesia microcarpa* | Norseman | B/12 |
| 00096 TD | 25/11/93 | *Daviesia pseudaphylla* | Site 1,East Pillenorup Track, SRNP | I/11 |
| 00097 TD | 25/11/93 | *Daviesia pseudaphylla* | Site 2,East Pillenorup Track, SRNP | B/25 |

| | | | | |
|---|---|---|---|---|
| 00098 TD | 25/11/93 | ***Daviesia pseudaphylla*** | Site 1/2,East Pillenorup Track, SRNP | B/20 |
| 00099 TD | 26/11/93 | ***Andersonia echinocephala*** | Stirling Range Dr., SRNP | I/22 |
| 00100 TD | 28/11/93 | ***Banksia laevigata ssp. laevigata*** | Dorrie Hill, Twertup,FRNP | I/20 |
| 00101 TD | 30/11/93 | ***Adenanthos dobagii*** | Quoin Head, FRNP | B/12 |
| 00102 TD | 30/11/93 | ***Adenanthos ellipticus*** | East Mt. Barren | B/8 |
| 00103 TD | 01/12/93 | ***Daviesia megacalyx*** | Elverdton Rd. | B/30 |
| 00104 TD | 01/12/93 | ***Dryandra sp. 36 (aff. ferruginea)*** | Elverdton Rd. | I/13 |
| 00105 TD | 01/12/93 | ***Dryandra folisissima*** | Mt. Desmond | I/10 |
| 00106 TD | 02/12/93 | ***Dryandra folisissima*** | Ravensthorpe 2 | I/10 |
| 00107 TD | 02/12/93 | ***Dryandra sp. 36 (aff. ferruginea)*** | Ravensthorpe 2 | I/10 |
| 00108 TD | 02/12/93 | ***Daviesia megacalyx*** | Ravensthorpe 2 | I/14 |
| 00109 TD | 02/12/93 | ***Daviesia megacalyx*** | Floater Rd. | I/5 |
| 00110 P3 | 03/12/93 | ***Dryandra sp. 16 (aff. horrida)*** | Hatter Hill | I/23 |
| 00111 R | 03/12/93 | ***Banksia sphaerocarpa var. dolichostyla*** | South Ironcap | I/20 |
| 00112 TD | 04/12/93 | ***Daviesia oxylobium*** | Wamenusking NR | I/8 |
| 00113 R | Nov 1990 | ***Eucalyptus crucis ssp. crucis*** | Sandford Rocks NR | B/? |
| 00114 TD | 08/09/93 | ***Dryandra serra*** | Mt. Hallowell | I/10 |
| 00115 R | Oct 1992 | ***Lepidium catapycnon*** | Garden Gorge, Hammersley Range | I/1 |
| 00116 TD | 06/01/94 | ***Adenanthos pungens ssp effusa*** | Tambellup | B/3 |
| 00117 TD | 06/01/94 | ***Adenanthos pungens ssp pungens*** | Hamilla Hill, SRNP | B/12 |
| 00118 TD | 06/01/94 | ***Adenanthos pungens ssp pungens*** | Hamilla Hill, SRNP | I/10 traps |
| 00119 TD | 07/01/94 | ***Adenanthos velutinos*** | Geekabee Hill | I/4 |
| 00120 TD | 09/01/94 | ***Banksia verticillata*** | Mermaid Point | I/13 |
| 00121 TD | 10/01/94 | ***Adenanthos ellipticus*** | East Mt Barrren | B/40 |
| 00122 TD | 11/01/94 | ***Adenanthos dobagii*** | Quoin Head Track | I/10 |
| 00123 TD | 11/01/94 | ***Adenanthos dobagii*** | Quoin Head T/o | I/10 traps |
| 00124 TD | 14/01/94 | ***Lambertia echinata ssp echinata*** | Lucky Bay | I/3 |
| 00125 R | 14/01/94 | ***Myoporum turbinatum*** | Heywood Rd | I/10 |
| 00126 TD | 15/01/94 | ***Astroloma sp. Grass Patch*** | Ridley Rd, Grass Patch | B/40 |
| 00127 TD | 16/01/94 | ***Daviesia campophylla*** | Griffith/Edwards Rd | I/21 |
| 00128 | 07/07/93 | ***Banksia cuneata*** | Johnston's property Pop10 | B/? |

***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.

**Figure 2:** Field data collection form.

**CONSERVATION AND LAND MANAGEMENT**
**THREATENED FLORA SEED CENTRE**
**SPECIES DATA FILE 1 : Provenance information**

**Seedlot no.:**

**Species:**

**Coll. no.:**
**WAHERB no.:**

**Coll. type:**
**Date:**

**Location:**

**Lat.:** ° ' "S **Long.:** ° ' "E **Alt.:** m

**Description:**

**Density/frequency:** **Sample spacing:**

**Vegn type:**

**Climate:**

**Landform:**

**Soil type:** **pH:**

**Parent material:**

**Associated spp.:**

**Phenology:** **bud:** **flower:** **fruit:**

**fruit type:** **no./plant:** **seed/fruit:**

| Coll. no. | Quantity | Coll. no | Quantity | Coll. no | Quantity | Coll. no | Quantity |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |

**Comments:**

**Figure 3:** Germination and viability test form.

**CONSERVATION AND LAND MANAGEMENT**
**THREATENED FLORA SEED CENTRE**
**SPECIES DATA FILE 2: Germination and viability information**

**Species:** **Seedlot No.:**
**Provenance:** **Collectors No.:**
**Collection type:** **WAHERB No.:**
**Collection date:**
**Test start date:** **Rep. seed weight:**

**Pretreatment Trials**

**Treatment** **substrate/temp(°C)/ light/replicates**
**1st:**
**2nd:**
**3rd:**
**4th:**

| | | Treatment 1 | | | | 2 | | | | 3 | | | | 4 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Replicate | -> | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| No. seeds | -> | | | | | | | | | | | | | | | | |
| Date | Days | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | |
| Abnormal | | | | | | | | | | | | | | | | | |
| Soft | | | | | | | | | | | | | | | | | |
| Mouldy | | | | | | | | | | | | | | | | | |
| Hard | | | | | | | | | | | | | | | | | |
| TOTAL | | | | | | | | | | | | | | | | | |
| No. germ. | | | | | | | | | | | | | | | | | |
| No. viable | | | | | | | | | | | | | | | | | |
| % germ.n | | | | | | | | | | | | | | | | | |

**Comments/recommendations:**

**Figure 4:** Moisture content analysis data form.

**CONSERVATION AND LAND MANAGEMENT**
**THREATENED FLORA SEED CENTRE**
**SPECIES DATA FILE 3: Moisture content test sheet**

**Seedlot number:**
**Species:**
**Collectors no.:**
**WAHERB no.:**
**Coll.n type:**

**Status of sample used for test:**

**Test date:**

**Method:**

**Drying time start:**
**Drying time finish:**

**Replicate No. 1**

| Vial No. | **M1** (vial + lid) | = |
|---|---|---|
| | **M2** (vial + lid + sample) | = |
| | **M3** (vial + lid + sample) | = |

**% MC f.w. = (M1 - M3)/(M2 - M1) x 100**

**% MC f.w. =** ( )/( )

=

=

**Replicate No. 2**

| Vial No. | **M1** (vial + lid) | = |
|---|---|---|
| | **M2** (vial + lid + sample) | = |
| | **M3** (vial + lid + sample) | = |

**% MC f.w. = (M1 - M3)/(M2 - M1) x 100**

**% MC f.w. =** ( )/( )

=

=

**Difference between rep.s:**

**Comments:**

**Figure 5:** Form used for storage and monitoring status.

**CONSERVATION AND LAND MANAGEMENT**
**THREATENED FLORA SEED CENTRE**
**SPECIES DATA FILE 4: Moisture content, storage and monitoring**

**Species:** **Seedlot No.:**
**Provenance:** **Collectors No.:**
**Collection type:** **WAHERB No.:**
**Collection date:** **Initial viability test date:**
**Initial viability:** **Est. or known number of seed:**

***Moisture content determination:**

| Date: | Method | Rep. seed wt. | % MC (f.w.) |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

**Moisture content attained prior to storage:**

**Final seed wt. = initial wt. x (100 - initial % MC)/(100 - final % MC)**

**=**

| Initial MC | Drying time | Est. MC in storage |
|---|---|---|
| | | |

***Storage regime:**

| | Method | Quantity | Date |
|---|---|---|---|
| **Long term** | | | |
| **Med. term** | | | |
| **Short term** | | | |

***Monitoring regime.No. of sub-samples packaged for monitoring:**
**No. or weight of seed per sub-sample:**
**Frequency of monitoring:**
**Initial retest date:**
**Thereafter:**
**Comments:**

## PROJECT 6

# CONTROL AND MANAGEMENT OF *DIPLODINA* CANKER THREATENING *BANKSIA COCCINEA*

**J. Bathgate, B. Shearer, L. Röhl & C. Crane**
*Science and Information Division, Department of Conservation and Land Management*
*50 Hayman Rd Como, W.A. 6152*

NB. This report covers the period February 27, 1993 to February 27, 1994

## SUMMARY

*Diplodina* canker is a serious threat to the survival of *Banksia coccinea*. Results are reported from experiments and field studies aimed at establishing the major factors linked to the spread of the disease and options for control. A survey of 49 *B. coccinea* stands was conducted to decide whether burning diseased stands can be used to improve the health status of stands. Disease incidence, severity and mortality were low in reproductively immature plants and increased with plant age. Stands over 10 years of age were more likely to be severely infected. Seed reserves were also larger in these stands giving them an increased potential for regeneration over younger stands. The results support the case for using control burning to control the disease. A range of fungicides were screened for their activity against *Diplodina* sp. Of the fungicides tested, Carbendazim, Benomyl and Propiconazole were the most effective at inhibiting germination, germ tube development and mycelial growth. Assessments of permanent transects in four *B. coccinea* populations showed mortality, and disease incidence and severity, increased over the study period. Rate of increase in disease was not constant, being higher between January and April and October and November at two of the sites. An assessment of the location of cankers was carried out in November, 1993 and showed new cankers to be common in inflorescences. Insect damage was also common in inflorescences, however there was no definable association between insect damage and the incidence of cankers. Inoculation trials in cultivated *B. coccinea* at Wanneroo, north of Perth, and in a wild stand in the Stirling Range, on the south coast, showed varying results. *Diplodina* sp. infected both wounded and unwounded stem tissue at Stirling Range and wounded tissue only at Wanneroo. Lesion development was greatest in April at Wanneroo. At Stirling Range lesion development was the same in June and September.

# CONTENTS

## INTRODUCTION

*Banksia coccinea* is a plant of both conservation and commercial importance. It occurs on deep white sands from Albany, north to the Stirling Range and east to Young River in south-western Western Australia. *Diplodina* canker was first identified as a serious disease of *B. coccinea* in 1989. The disease is responsible for the death of large numbers of *B. coccinea* and has been found throughout the geographic range of *B. coccinea*. The disease causes cankers on the aerial portions of the plant. Cankers expand rapidly, girdling branches and eventually killing the plant. Diseased plants are characterized by dead, defoliated limbs. Fruiting bodies are produced by the fungus in dead plant tissues. Dead plant material is thought to be a major source of infective propagules (inoculum) of the fungus. Research aims to identify the factors affecting spread of the disease and options for controlling the disease in natural populations.

## 1. CONTROL MEASURES

### 1.1 BURNING

The spread of plant diseases can be retarded by removing the source of inoculum. This can be achieved through burning infected plant residues. *Banksia coccinea* is killed by fire, so regeneration is entirely from seed that is stored in the canopy. Seed storage is mainly determined by plant age but may be reduced where disease has degraded a stand. The prospect for managing disease in *B. coccinea* stands by control burning depends upon (1) low levels of disease intensity and mortality being a characteristic of young stands and (2) sufficient viable seed production prior to burning for adequate seed storage and hence recruitment of seedlings after the burn. The study has been divided into two parts: (1) a survey of disease and seed reserves in *B. coccinea* stands aimed at determining the influence of plant age on a) the incidence and severity of disease and b) canopy seed storage; and c) the impact of disease on canopy seed storage; and (2) a burning study aimed at determining levels of seedling recruitment and the rate of disease development *B. coccinea* stands after burning.

#### 1.1.1. SURVEY OF DISEASE AND SEED RESERVES IN *B. COCCINEA* STANDS

Forty nine of the most significant *B. coccinea* stands within the geographic range of the species were surveyed in April, May and September, 1993. Disease intensity was estimated on 60 to 100 randomly selected individuals per site. Disease intensity was expressed as incidence (percentage of infected individuals per site) and severity (percent limb area with cankers). Cones with developed follicles that contained seeds were counted on each plant. Stand age was estimated from node counts of individual *B. coccinea* (Lamont, 1985). Cone numbers reflect the reproductive maturity and reproductive output of stands and are taken here as a measure of the seed stored in the stand. Most stands were uniform in age, five sites however had two age strata, the younger stratum was considered a separate stand and was rated separately.

The stands surveyed varied in age from 2 to 25 years. Aerial canker was widely distributed, being present in 71% of the sites surveyed. The disease was most

frequently observed in older stands. All stands over 16 years of age were infected. Only 22% of stands aged 5 years or younger were infected (Table 1.1.1.1).

**Table 1.1.1.1** Average age and percent of stands in a range of age group in which *Diplodina* canker was detected.

| | Age Group | | | | |
|---|---|---|---|---|---|
| | 1 (0-5yrs) | 2 (6-10yrs) | 3 (11-15yrs) | 4 (16+yrs) | Mean |
| Number of stands | 9 | 17 | 10 | 13 | |
| Average Age | 4 | 8.6 | 13.2 | 19.8 | 11.7 |
| Percent stands infected | 22 | 65 | 90 | 100 | 71 |

The effects of stand age on disease incidence and severity, mortality and cone numbers was examined by analysis of variance, differences between means were tested by the Tukey Test. Percentages were arcsine-transformed prior to analysis. The effect of age group was significant for all disease variables ($P$=0.05). Disease incidence, severity and mortality were low (averages less than 9%) in stands under 10 years old. Stands 11 years and over had significantly greater levels of infection than younger stands ($P$<0.05). Disease incidence, severity and mortality averaged 50, 34 and 27% respectively in these stands (Figure 1.1.1.1 A-C).

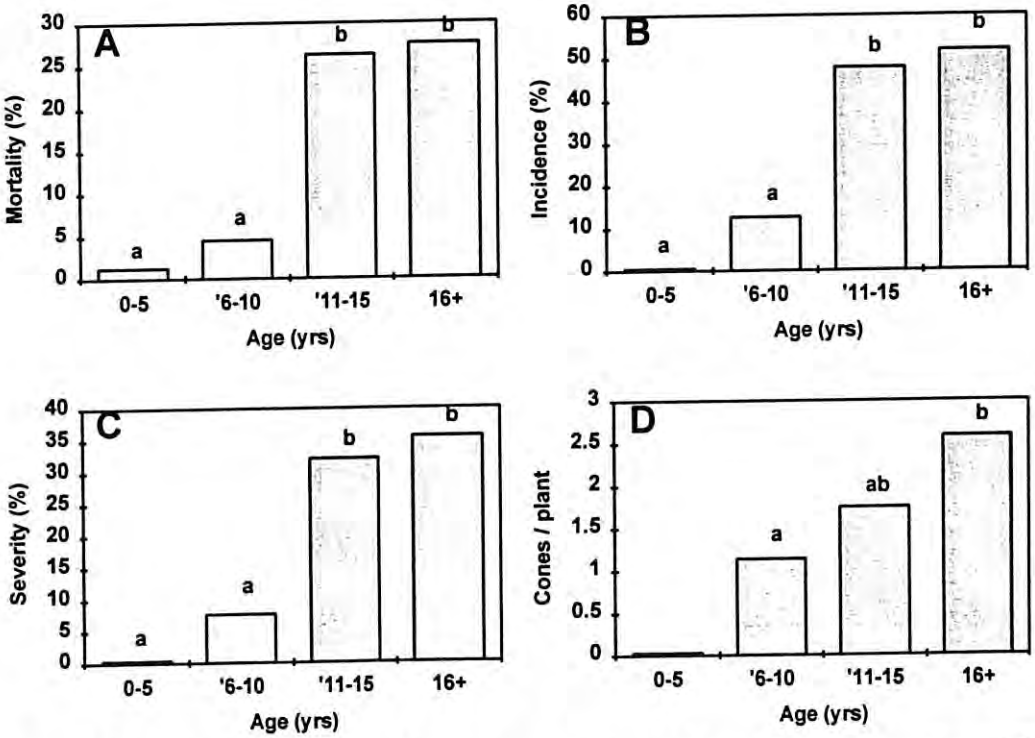



**Figure 1.1.1.1** Relationship between A. mortality; B. disease incidence; C. disease severity; and D. cones per plant and stand age group from a survey of 49 *Banksia coccinea* stands. Columns with different letters are significantly different by Tukey test ($P$=0.1). NB in D. most stands in the 0-5 yrs age group had not produced cones; this age group was therefore excluded from the analysis.

Cone numbers were low (average 0.03 cones/plt) in stands less than 5 years old. The effect of stand age ( in age group 2 to 4 only ) on cone number per plant was significant ($P$=0.1). Cone numbers in stands aged 6-10 years were slightly lower than stands aged 15 years or more, 1.2 compared with 2.6 cones per plant (Figure 1.1.1.1

D). Witkowski *et al.* (1991), showed seed production by *B. coccinea* peaked around 16 years of age. The low number of cones per plant recorded in stands aged 10 years or younger shows that these stands have not reached reproductive maturity and seed production is increasing. Variability in cone numbers between stands in the same age group was high. Some sites had been badly damaged by cockatoos or flowers had been harvested by wildflower pickers leaving them with low number of cones.

The results from the survey suggest that disease control by burning stands aged 10 years or less is not warranted because of the low levels of disease in these stands. Stands over 10 years of age are more likely to be severely infected. Seed reserves are approaching peak levels as stands reach this age and burning could be carried out without threatening the potential for post-fire recruitment. The stage of disease development at which seed reserves are impinged upon is being assessed separately and will be discussed in a forthcoming report. Future work will be aimed at identifying a threshold level of disease which can be used to gauge when to burn a particular stand of *B. coccinea.*

### 1.1.2. BURNING STUDY

A site in Stirling Range National Park was burnt in October 1993. Disease was assessed prior to burning and will be assessed in the regrowth at yearly intervals. Further *B. coccinea* stands in areas in which burns are scheduled by CALM and private landowners will be monitored. Seed reserves and recruitment will also be monitored at these sites.

## 1.2 FUNGICIDES

Fungicides are used extensively on agricultural and horticultural crops to control fungal diseases. The main aim of application is to decrease the inoculum potential of the pathogen. There are many fungicides registered for use in Australia, but before attempting disease control in the field it is important to screen fungicides on the basis of their inhibitory effect on spore germination and mycelial growth.

The objective of this study was to evaluate fungicides for the control of *Diplodina* canker through *in vitro* screening.

**Table 1.2.1** Fungicides evaluated *in vitro* for the control of *Diplodina* canker.

| Common name | Trade name |
|---|---|
| Benomyl | Benlate |
| Bordeaux Mixture | Bordeaux Mixture |
| Carbendazim | Bavistin |
| Iprodione | Rovral |
| Fenarimol | Rubigan |
| Phosphonate | Fosject |
| Propiconazole | Tilt |
| Thiabendazole | Tecto |

Eight fungicides have been evaluated to date (Table 1.2.1). Experiments were conducted to determine the effect of fungicides on (1) conidial germination and germ tube growth and (2) mycelial growth. Four concentrations of each fungicide (0.1, 1, 10 and 100 ppm) were incorporated into the media. There were five replications of each fungicide by concentration combination. Plates were incubated at 25°C. For conidial germination experiments, spore suspensions ($10^6$ conidia/ml)were placed on the surface of culture plates containing 1.5% water agar (WA) and the appropriate fungicide. Five additional plates containing WA only were inoculated and served as controls. After 24 hours incubation, germination was halted by adding a drop of formalin to the plate. One hundred spores in each replicate were examined to determine the number germinated. Germ tubes of 10 spores per replicate were measured. For mycelial growth experiments, a 3 mm plug of *Diplodina* sp. growing on 1/2 strength potato dextrose agar (1/2PDA) was placed in the centre of each culture plate containing 1/2PDA and incorporated fungicide. Five additional plates containing 1/2PDA only were inoculated and served as controls. The plates were incubated for five days and the colony diameter measured at this time.

The percent inhibition of germination, germ tube growth and mycelial growth in the fungicide amended treatments compared to the controls was calculated. Analysis of variance was performed on the angular-transformed values.

Significant differences in germination, germ tube growth and mycelial growth occurred between fungicides, concentrations and their interaction ($P<0.01$). Benomyl, Carbendazim and Thiabendazole reduced spore germination and germ tube growth at 1, 10 and 100 ppm (Table 1.2.2). Germ tube growth was completely inhibited at 100 ppm. At 1 and 10 ppm the fungicides effectively inhibited germ tube growth by 85% or more. Although a low percentage of spores germinated at 100 ppm, germ tube growth was limited and often distorted. Carbendazim, Benomyl and Thiabendazole were most effective at inhibiting mycelial growth, completely inhibiting growth at 1 ppm. Propiconazole and Fenarimol were also effective from 1 ppm. Phosphonate and Bordeaux mixture performed poorly in all tests.

*In vitro* screening separated the fungicides according to their efficacy against the pathogen. Carbendazim, Benomyl and Propiconazole were highly effective. Field evaluation of these fungicides is not justified at present due to the high frequency of application required with these groups of fungicides. Studies will be conducted to determine when inoculum is released and when plants are most susceptible to infection so that field applications can be targeted to the most infectious period of the pathogen.

**Table 1.2.2.** Evaluation of fungicides against *Diplodina* sp. using three screening techniques.

| Fungicide | Concentration (ppm active ingredient.) | Percent inhibition over control | | |
|---|---|---|---|---|
| | | Conidial Germination | Germ Tube Length | Colony Diameter |
| Carbendazim | 0.1 | 46.0 | 90.1 | 98.4 |
| | 1 | 63.2 | 93.8 | 100.0 |
| | 10 | 65.4 | 94.3 | 100.0 |
| | 100 | 99.8 | 100.0 | 100.0 |
| Benomyl | 0.1 | 2.0 | 2.5 | 45.3 |
| | 1 | 52.4 | 92.5 | 100.0 |
| | 10 | 89.0 | 98.1 | 100.0 |
| | 100 | 99.2 | 100.0 | 100.0 |
| Thiabendazole | 0.1 | 0.0 | 0.0 | 0.0 |
| | 1 | 39.2 | 86.4 | 100.0 |
| | 10 | 58.0 | 94.5 | 100.0 |
| | 100 | 99.8 | 100.0 | 100.0 |
| Fenarimol | 0.1 | 4.5 | 11.9 | 45.4 |
| | 1 | 7.8 | 13.5 | 88.9 |
| | 10 | 6.3 | 25.6 | 100.0 |
| | 100 | 90.7 | 98.9 | 100.0 |
| Propiconazole | 0.1 | 7.5 | 47.3 | 39.6 |
| | 1 | 0.5 | 11.3 | 95.0 |
| | 10 | 4.2 | 41.7 | 100.0 |
| | 100 | 47.2 | 91.0 | 100.0 |
| Iprodione | 0.1 | 0.2 | 2.1 | 6.1 |
| | 1 | 0.7 | 2.6 | 47.6 |
| | 10 | 14.9 | 49.9 | 73.4 |
| | 100 | 99.3 | 100.0 | 88.9 |
| Bordeaux Mixture | 0.1 | 0.7 | 0.0 | 0.0 |
| | 1 | 0.8 | 0.1 | 0.1 |
| | 10 | 5.0 | 29.2 | 6.0 |
| | 100 | 78.2 | 96.5 | 83.6 |
| Phosphonate | 0.1 | 6.2 | 28.5 | 3.6 |
| | 1 | 3.0 | 12.4 | 3.8 |
| | 10 | 11.5 | 76.9 | 0.3 |
| | 100 | 13.4 | 78.5 | 13.3 |
| LSD ($P$=0.05) | | 1.9 | 3.1 | 0.47 |

## 2. DISEASE MANAGEMENT

### 2.1 LIFE CYCLE OF *DIPLODINA* SP.

The causal organism of the aerial canker disease of *B. coccinea* was identified from cultures of the anamorph (asexual stage of the fungus) as the Coelomycete, *Diplodina* sp. (Shearer, unpublished). Cultures were derived from the margins of cankers from infected *B. coccinea*. The pathogenicity of the fungus was demonstrated through inoculation experiments (Shearer, unpublished data). The genus of the teleomorph (sexual stage) most frequently connected to *Diplodina* is *Cryptodiaporthe,* an Ascomycete in the family Diaporthaceae. At the commencement of the study, samples of cankered branches were collected and examined to find the sexual stage of the life cycle. The two stages in the life cycle of Ascomycetes generally play different roles in the epidemiology of the diseases they cause. Sexually produced ascospores are usually windborne and are responsible for long distance spread of the fungus whereas the asexually produced conidia are oozed out of the fruiting bodies and are splash dispersed.

Examination of field material collected in Stirling Range confirmed the presence of a Diaporthaceous fungus. Single ascospores from this fungus were cultured and produced cultures morphologically identical to *Diplodina* sp., thus establishing an anamorph-teleomorph connection. The fungus had many of the characteristics of the genus *Cryptodiaporthe*, however it differed from all previously described species and is currently being described as a new species of *Cryptodiaporthe*.

The general life cycle of the fungus is shown in Figure 2.1.1. Fruiting bodies are produced beneath the bark in cankers. Conidioma (asexual fruiting bodies) are produced soon after the death of the plant tissues whereas the development of perithecia (sexual fruiting bodies) is restricted to older cankers. Moisture plays a major role in stimulating spore release in Ascomycetes and Coelomycetes (Ingold, 1971). The conditions required for infection and production of symptoms on *B. coccinea* will be examined through artificial inoculations with the pathogen.

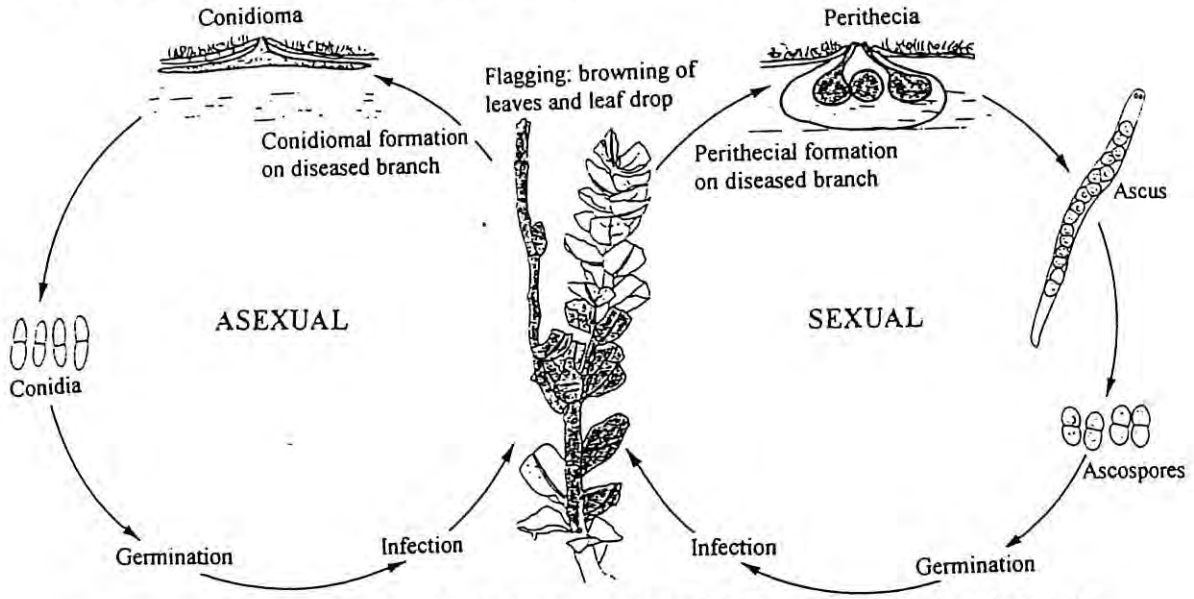



**Figure 2.1.1** The life cycle of *Diplodina* sp., including the *Cryptodiaporthe* sexual stage, on *Banksia coccinea.*

## 2.2 MEANS OF SPREAD

### 2.2.1 THE ROLE OF ASCOSPORES AND CONIDIA IN DISEASE SPREAD

A spore trapping study coupled with direct observation of diseased plant material has been commenced to determine the importance of ascospores and conidia in the epidemiology of the disease.

### 2.2.2 INFECTION OF *B. COCCINEA* BY *DIPLODINA* SP.

Field studies, isolations and inoculations have demonstrated the link between *Diplodina* sp. and the severe canker disease of *B. coccinea*. However it is not known whether the fungus is a primary or secondary incitant of the disease. Many plant pathogens, especially canker fungi, gain entry through wounds, although direct penetration or penetration through natural openings, such as lenticels and stomata, is possible. Host and environmental conditions can influence the ability of a pathogen to cause a disease.

This section reports results from inoculation experiments. The objective was to determine whether *Diplodina* sp. can infect uninjured shoots of *B. coccinea*.

Two inoculation experiments have been commenced using adult *B. coccinea* plants, one at Wanneroo, 40 km North of Perth, in an abandoned plantation, the other in a natural stand in Stirling Range National Park. The design is a factorial combination of date of inoculation (5 times at 3 month intervals) and method of inoculation (unwounded, and wounded by removing a leaf) with an added wounded control (uninoculated). Sampling will continue over the next 18 months, preliminary results are presented.

At Wanneroo, the fungus did not invade unwounded leaf nodes. Lesions developed on inoculated branches that were wounded. Lesions were significantly longer in the March than January, August and October inoculations (Table 2.2.2.1). *Diplodina* sp. was re-isolated from the inoculated and wounded treatments only.

At the Stirling Range stand, the fungus invaded both wounded and unwounded leaf nodes in the June inoculation. Unwounded stem tissue was not invaded in the September inoculation. The effects of inoculation date and wounding on lesion length and tangential spread were not significantly different ($P$=0.05). The frequency of lesion development in unwounded treatments was low in the June inoculation: only two stems developed lesions after 8 weeks compared with all 8 wounded stems. *Diplodina* sp. was isolated from 50% and 100% respectively of the unwounded and wounded inoculations.

Results from the two sites differ with respect to development of lesions on unwounded plants and the size of lesions at 8 weeks. Variations in climate and soil together with the past history of the site- plantation vs. wild population- may account for the differences. The infection of unwounded leaf nodes at the Stirling Range indicates fungus is not an obligate wound pathogen. Infections can occur either through wounds or via direct penetration of the plant given favourable conditions. The lack of

lesion development in unwounded tissue in September shows there may be a seasonal change in the susceptibility to invasion. Results presented below in 2.3 from a field survey of cankers initiated in shoot apices support these findings. Cankers developed with similar frequency on insect damaged and intact shoot apices. Further experiments will be conducted to partition the effects of host phenology and environmental conditions on lesion development.

**Table 2.2.2.1** Mean canker length and tangential spread in wounded and unwounded *Banksia coccinea* stems inoculated with *Diplodina* sp.

| Site | Date inoculated | Treatment * | Length | Tangential Spread (°) | *Diplodina* sp. re-isolated (%) |
|---|---|---|---|---|---|
| Wanneroo | 21/1/93 | Wounded | 1.8 | - | 83 |
| | 29/4/93 | Wounded | 29.2 | 121 | 88 |
| | 6/8/93 | Wounded | 4.1 | 19.8 | 50 |
| | 28/10/93 | Wounded | 10.6 | 29.4 | 100 |
| P | | | < 0.01 | < 0.01 | |
| LSD | | | 14 | 47.5 | |
| Stirling Range | 16/6/93 | Unwounded | 15.5 | 48.4 | 33 |
| | | Wounded | 72.2 | 97.6 | 100 |
| | 22/9/93 | Wounded | 20 | 36.9 | 50 |
| P | | | 0.1 | 0.6 | |
| LSD | | | 57 | 123 | |

* Means of treatments which developed lesions are shown. All uninoculated treatments and some unwounded treatments failed to develop lesions and have been excluded from the analysis.

## 2.2.3 FACTORS INFLUENCING DISEASE INTENSITY

Occurrence of plant disease is dependant upon a combination of a susceptible host, infective pathogen and favourable environmental conditions. Host susceptibility can change throughout a season as vegetative and reproductive growth occurs, and throughout the life of the plant as it ages. Levels of inoculum can change with seasonal patterns in the life cycle of the pathogen. Environmental conditions such as temperature, moisture, light, nutrition and soil pH can significantly affect the initiation and development of infectious plant diseases (Agrios, 1988). An understanding of these relationships is necessary to determine the importance of factors regulating an epidemic and for the development of management plans.

The aim of this study is to determine the major factors influencing disease intensity in *B. coccinea* populations.

Permanent transects were installed in four *B. coccinea* populations. Site details are shown in Table 2.2.3.1. Sites were visited at two to three month intervals and disease incidence and severity (see Section 1.1), numbers of cankered shoots, insect damage, foliar symptoms, sporulation and host phenology were assessed. Assessments will continue for the duration of the project. Disease progress at the four sites is shown in Figure 2.2.3.1

**Table 2.2.3.1** Details of sites in which permanent transects were installed.

| Location | Abbreviation | Size (m) | No Plants | Approx Age | Date 1st visit |
|---|---|---|---|---|---|
| Stirling Range National Park | SEBR | 3 x 50 | 141 | 16 | 26/8/92 |
| Hassell National Park | HASH | 2 x 50 | 374 | 8 | 1/9/92 |
| Waychinicup National Park 1 | CBR1 | 2 x 50 | 160 | 12 | 1/10/92 |
| Waychinicup National Park 2 | CBR2 | 2 x 50 | 309 | 4 | 11/11/92 |

Disease increased over the study period at all sites. The greatest increase in disease incidence and severity occurred at CBR1, where disease incidence increased by 40% and severity by 21%. Mortality increased most rapidly at SEBR. At CBR1 and SEBR, two periods of increased activity of the fungus were identified. These were from January to April and Oct to November 1993 at CBR1 and February to April 1993 at SEBR. Data will be collected over 2 more years to determine whether this pattern of disease development occurs seasonally or randomly.

The pattern of disease development in the stands may have implications for the burning study discussed in 1.1. Disease progress in the stands with low levels disease intensity at the commencement of the study was slow, indicating that in the short term the stands will remain with relatively low levels of disease. In comparison the disease progress curves at CBR1 show rapid development of disease and suggest disease development has entered an exponential phase. In the short term infection will increase rapidly in this stand. Further monitoring of disease development in stands with low to moderate levels of infection will show whether the rate of disease development increases once disease incidence passes a certain level. If this is the case, it may provide an indicator for determining when to burn a particular stand.

The location of new cankers at CBR1 in 1993 varied throughout the year. Apical cankers occurred at all observation times and were more frequent in December 1992 and October and November 1993. Internodal cankers were common from April to October, 1993. (Figure 2.2.3.2). Two additional sites were surveyed in November to determine the frequency of different canker types, Table 2.2.3.2 shows combined canker location data for the two sites. Apical cankers which developed in inflorescences were most common and accounted for 67% of new cankers. Insect damage was common in shoot apices, with 85% of the samples collected at the Stirling Range site being insect damaged. Analysis of the frequency of insect damage in cankered and healthy apices showed there to be a similar proportion of damaged apices in cankered and healthy samples ($\chi^2 = 3.48$, 1 d.f., $P$=0.06). It is interesting to compare these findings with those from 1.1, in which it was observed that the frequency of cankers in stands which had not reached reproductive maturity was very low. Proteoid plants, such as *B. coccinea*, are adept at concentrating major and minor nutrients in their seeds (Pate and Dell, 1982). Reproductive development may place the plant under a degree of nutritional stress, predisposing it to infection. Nutritional analysis of plants from different aged stands is being conducted to examine this possibility.

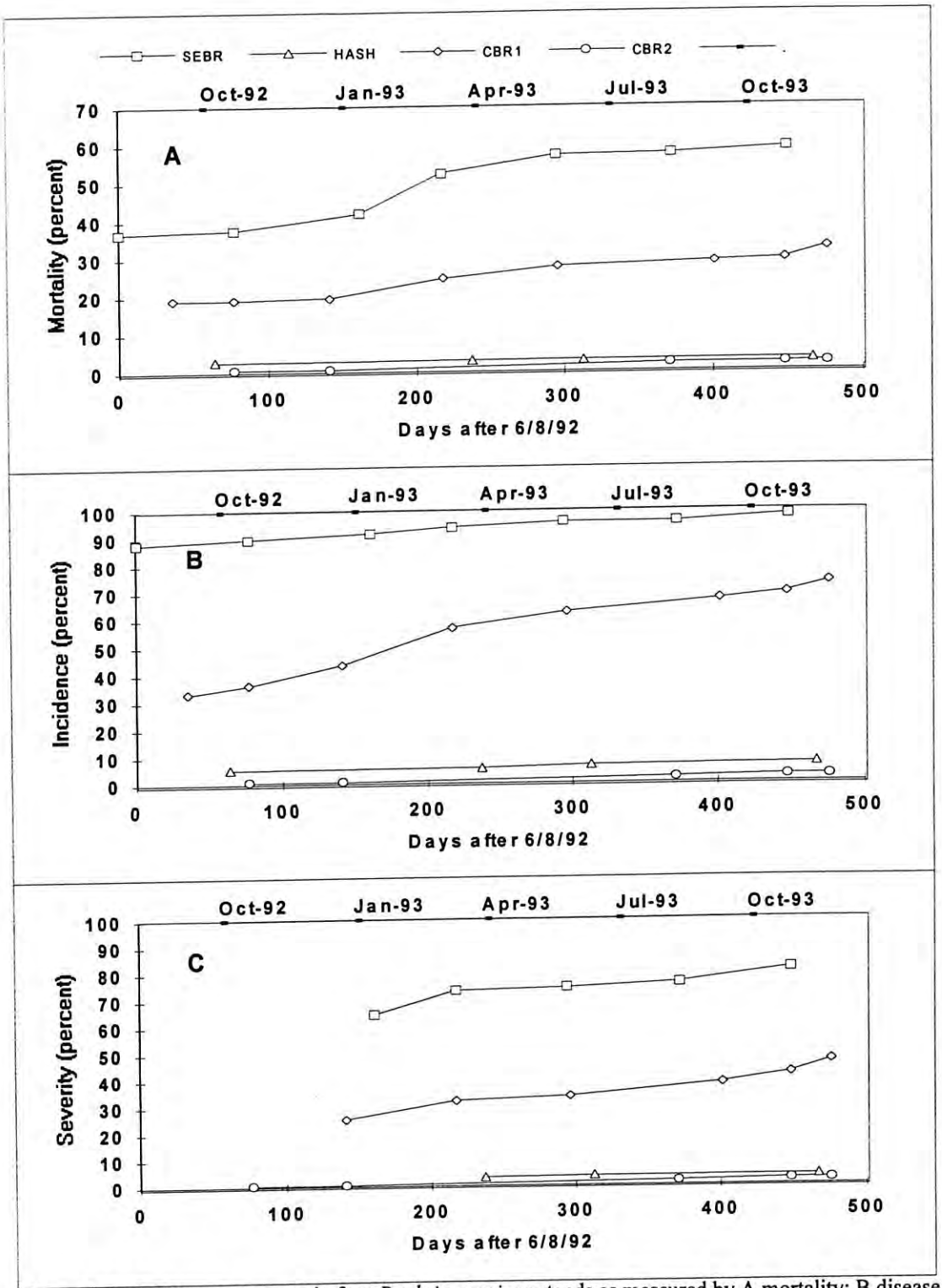



**Figure 2.2.3.1** Disease progress in four *Banksia coccinea* stands as measured by A mortality; B disease incidence and C disease severity.

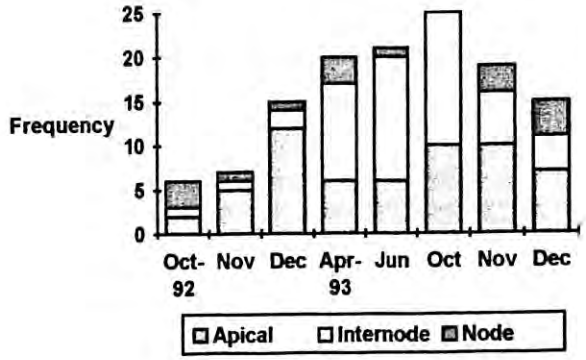



**Figure 2.2.3.2** Frequency of apical, nodal and internodal cankers on *Banksia coccinea* shoots at CBR1

**Table 2.2.3.2** . Frequency of canker types and dead plants in two 10-yr old *Banksia coccinea* stands in November 1993. Combined data from Stirling Range and Cheyne Beach.

| Canker Type | | Frequency * | |
|---|---|---|---|
| Apical | | | |
| | Inflorescence | 34 | (67) |
| | Infructescence | 4 | (8) |
| | Vegetative | 1 | (2) |
| | Total | 39 | (76) |
| Internodal | | 7 | (14) |
| Nodal | | 5 | (1) |

* Numbers in parentheses are percentages.

## REFERENCES

Agrios, G.N. (1988). "Plant Pathology." Academic Press, San Diego.

Ingold, C. T. (1971). "Fungal Spores - Their Liberation and Dispersal." Clarendon Press, Oxford.

Lamont, B. (1985). Fire responses of sclerophyll shrublands - a population ecology approach, with particular reference to the genus Banksia. In: J. Ford (Ed.) "Ecology and Management of Fire in Natural Ecosystems of Western Australia", pp 41-46, Environmental Studies Group, Western Australian Institute of Technology, Bentley.

Pate, J. S. and Dell, B. (1982) Economy of mineral nutrients in sandplain species. In: Pate, J. S. and Beard, J. S. (Ed.) "Kwongan, plant life of the sandplain", pp 227-252. University of Western Australia Press, Nedlands.

Witkowski, E. T. F., Lamont, B. B. and Connell, S. J. (1991). Seed bank dynamics of three co-occurring Banksias in south coastal Western Australia: the role of plant age, cockatoos, senescence and interfire establishment. Aust. J. Bot. 39:385-397