PROJECT 1

THE CONTROL OF *PHYTOPHTHORA* IN NATIVE PLANT COMMUNITIES

PART A

APPLICATION TECHNOLOGIES AND PHOSPHONATE MOVEMENT IN THE HOST

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1 SUMMARY

Phytophthora cinnamomi is a vigorous pathogen that kills a wide range of plant species by attacking their root system. The fungus has a widespread though discontinuous distribution in the south-west of Western Australia and it is estimated that up to a quarter of the 8000 (or more) species of vascular plants in the south-west may be susceptible to infection (Shea, 1991). Many of these endemic species have been brought to the brink of extinction. Apart from impacting on the structure and genetic diversity of heath and shrublands, animal communities are seriously affected due to severe alteration of their habitat. Dieback disease currently poses one of the greatest threats to conservation of Western Australia's flora. Although various hygiene strategies have been employed to minimise the spread of infections into healthy areas these are regarded as an interim solution pending the development of better methods.

Recently, research by the Department of Conservation and Land Management (CALM) has demonstrated that phosphonate is an effective chemical tool for the protection of native plant

species from mortality or infection caused by *P. cinnamomi*. Aerial spraying of phosphonate is a promising method of application which permits cost-effective treatment of the entire plant canopy, even in remote areas.

A new experimental system involving miniplots and the use of an ultra-low volume sprayer has been established. This will allow economical testing of a range of options for foliar application of phosphonate to protect susceptible species regenerating in disease centres infested by *P. cinnamomi*. Ultra-low volume application of 10% phosphonate to the foliage protects plants for 12-18 months but this concentration is generally too low to confer long term protection. Higher concentrations (20% and 40%) ensure protection for at least three years due to attainment of much higher initial levels of active ingredient (phosphite ion) in plant tissue.

Phosphonate is distributed in the shoots and roots of treated plants and an experiment involving *Banksia telmetia* has demonstrated that the relative distribution of phosphite ion between shoots and roots changes progressively. In all our trials we demonstrated that although the overall concentration of phosphite decreased with time, it always became significantly higher in roots than shoots.

In some cases treated plants showed signs of slight chemical burning of leaf margins but in our field trials these effects were only present on non-target species mainly eucalypts. Chemical burning did not occur in any of the rare and endangered species including *Banksia brownii* and *Andersonia* which were treated with two applications of 40% phosphonate at 60l ha^{-1}.

Although phosphonate is commercially available at a maximum concentration of 40%, the undiluted chemical has not been used to treat plants before. Application of 40% phosphonate at 60l ha^{-1} is the greatest concentration and delivery rate that can be applied to native species in multi storey situations characterised by an extensive range in plant size. In situations where only young or small plants are present, the application rate should be decreased to about 15l ha^{-1}. The required frequency of treatment with phosphonate decreases with increasing plant age. The relatively high growth rates of young plants result in a more rapid, internal dilution of phosphite than that occurring in slower growing, older individuals. Excessive application of phosphonate can cause chemical burning of the foliage or plant mortality and the rate of 90l

ha^{-1} used in one trial at Eneabba should not be applied to native species. Thus, phosphonate application rates should take into account the identity and age of target species as well as canopy structure and density.

The fate and distribution of the phosphite ion in native plant species is unknown and must be studied in detail if phosphonate applications are to be managed effectively. There are reports (Groussol *et al.*, 1986) that phosphonate is subject to source-sink relationships in plants and that the effectiveness of the chemical application depends on its timing (Whiley *et al*, 1986). Therefore, studies of fungicide distribution within the host are essential for optimising disease control. We have commenced experiments to determine the factors that control phosphonate translocation in native plants. Knowledge of this will allow timing of fungicide applications to coincide with particular growth stages and climatic conditions thus maximising the effects of treatment on native flora.

An *in vitro* experiment was carried out to determine the sensitivity of Western Australian isolates of *P. cinnamoni* to phosphonate. The toxicity of phosphonate was enhanced on media containing low concentrations of phosphate and decreased with increasing concentration of phosphate in the medium. The ED_{50} values (expected dosage for 50% inhibition) recorded in this report were lower than those published in the literature for the same species and showed that local isolates of P. *cinnamomi* are extremely sensitive to phosphonate. This may partially explain why phosphonate is so effective for control of the pathogen in native species growing in predominantly phosphorous-deficient soils.

Phosphonate oxidises to phosphate at temperatures above 65°C thus the chemical must be added to culture media after autoclaving. Our work demonstrated that growth rates of two *P. cinnamomi* isolates were significantly higher on media autoclaved before addition of phosphonate than on media supplemented with phosphonate after sterilisation. This result was obtained on media comprising both high and low, initial concentrations of phosphate. It is suggested that some published data on similar work may be invalid since the effect of autoclaving on phosphonate was not taken into account.

The results of nutritional analysis show that application of phosphonate to three species of *Banksia* slightly affected the uptake of certain cations, but the significance of this for plant

health and development is unknown and warrant further investigation. There was no effect of phosphonate application on flowering and seed germination.

The extensive experimental field trials conducted thus far have demonstrated that phosphonate has the capacity to protect several *Banksia* spp. as well as a number of other susceptible plant taxa. The fungicide is a safe and effective agent and earlier doubts that phosphonate could not be used on native vegetation (mainly Proteaceae) due to sensitivity to phosphorous compounds have proved to be unjustifiable.

This research has documented safe prescriptions for the use of fungicide phosphonate to protect native flora. The prescriptions will be incorporated in the management of *Phytophthora* infections in Western Australia and the protection of rare flora.

2 INTRODUCTION

2.1 *PHYTOPHTHORA CINNAMOMI* - THE ORGANISM

Phytophthora cinnamomi Rands is a virulent soil-borne pathogen belonging in the Oomycetes or "water moulds" (Shearer and Tippett, 1989). The fungus requires warm, moist conditions for sporulation, dispersal and infection. In the presence of susceptible tissue, it produces sporangia that release motile zoospores. The zoospores are transported in free water often in sub-surface flow or are spread in infected soil. *P. cinnamomi* is also dispersed through direct root-to-root contact from one susceptible host to another. Thick walled oospores may also be a source of infection but since only one mating type (A_2) is usually present in the south-west of Western Australia, sexual reproduction appears to be infrequent.

The pathogen was probably introduced to Western Australia early this century. It was first discovered by Rands in Sumatra in 1922 and was later identified as the cause of jarrah dieback in *Eucalyptus marginata* in south-western Australia (Podger *et al.*, 1965).

2.2 IMPACT OF *PHYTOPHTHORA CINNAMOMI* ON NATIVE PLANT COMMUNITIES

P. cinnamomi is the most common and destructive of the *Phytophthora* species found in native plant communities in the south-west of the State. It occurs in the area bounded by Eneabba 250 km north of Perth to Cape Arid and east of Dryandra near Popanyinning (Shearer, 1994). The experimental field trials established during this project were located in areas of natural infections (Figure 1).

P. cinnamomi kills its' hosts by destroying the roots and girdling the stem base, thus depriving the plant of access to nutrients and water (Shearer, 1994). As the amount of root and shoot necrosis increases, infected plants exhibit a range of symptoms that include leaf chlorosis followed by gradual crown decline often referred to as "dieback". Groups of apparently healthy plants can die suddenly and infection fronts or dead patches may be present. The impact of the disease varies in different areas depending on many factors including vegetation type, geographic location, soil type and climatic conditions.

P. cinnamomi is widespread in the south-west of Western Australia where it causes great destruction to a broad spectrum of flora in state forests, national parks and reserves (Shearer and Fairman, 1991). Many popular Western Australian *Banksia* spp. including *B. coccinea* (Figure 2), *B.baxteri* and *B. hookeriana* are increasingly affected by the fungus (McCredie *et al.*, 1985).

A significant proportion of the estimated 8000 species of native vascular plants may be susceptible to infection (Shea, 1991). It has also been established that over 3000 species are endemic to the south-west of Western Australia and many of these are both geographically restricted and susceptible to *Phytophthora*. The pathogen poses a great threat to a number of Declared Rare Flora (Table 1) with some being considered at risk of extinction due to the disease (Keighery, 1992).

Figure 1 Location of experimental field sites in Western Australia

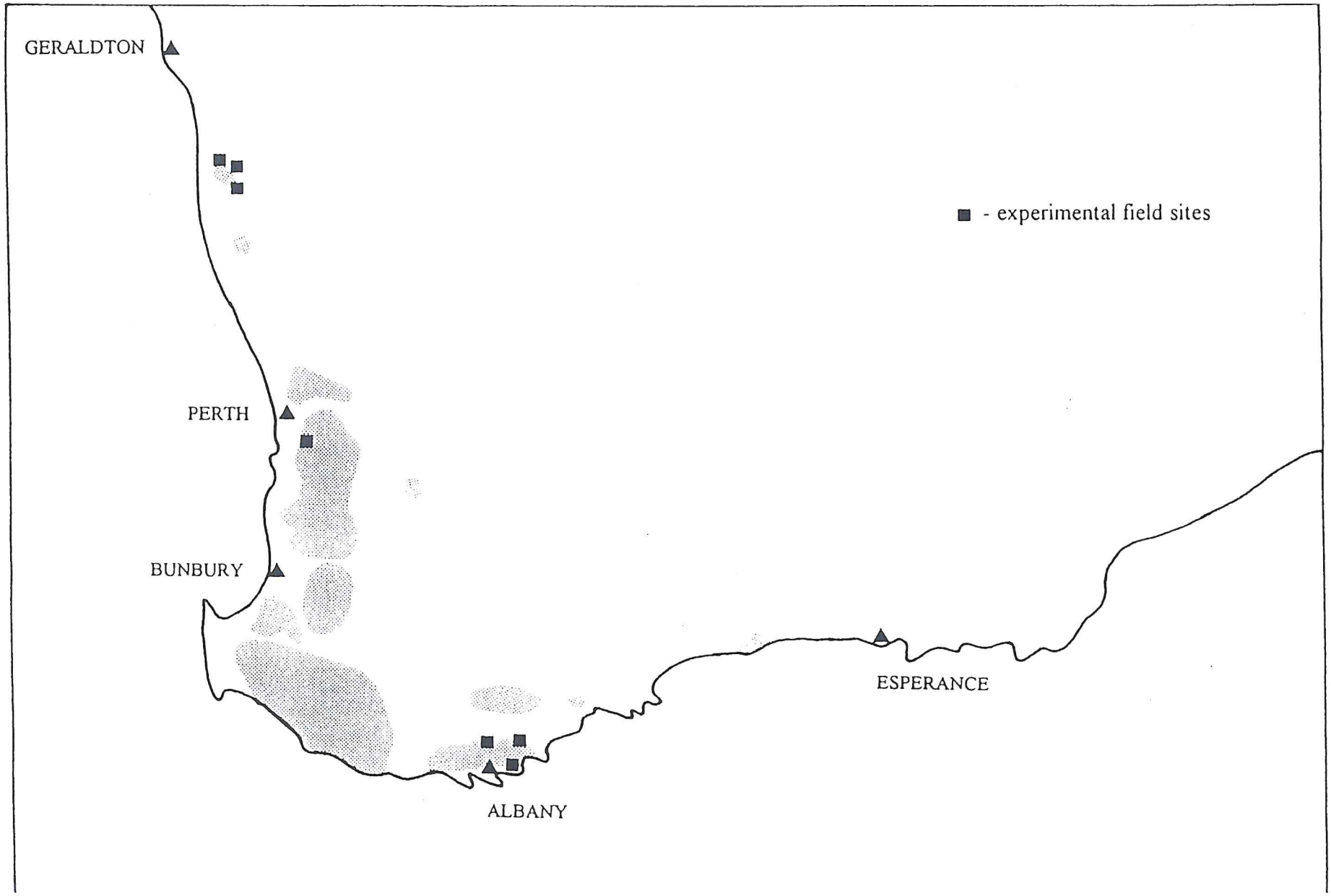


Figure 2. *Banksia coccinea* is frequently harvested for its beautiful scarlet flower.

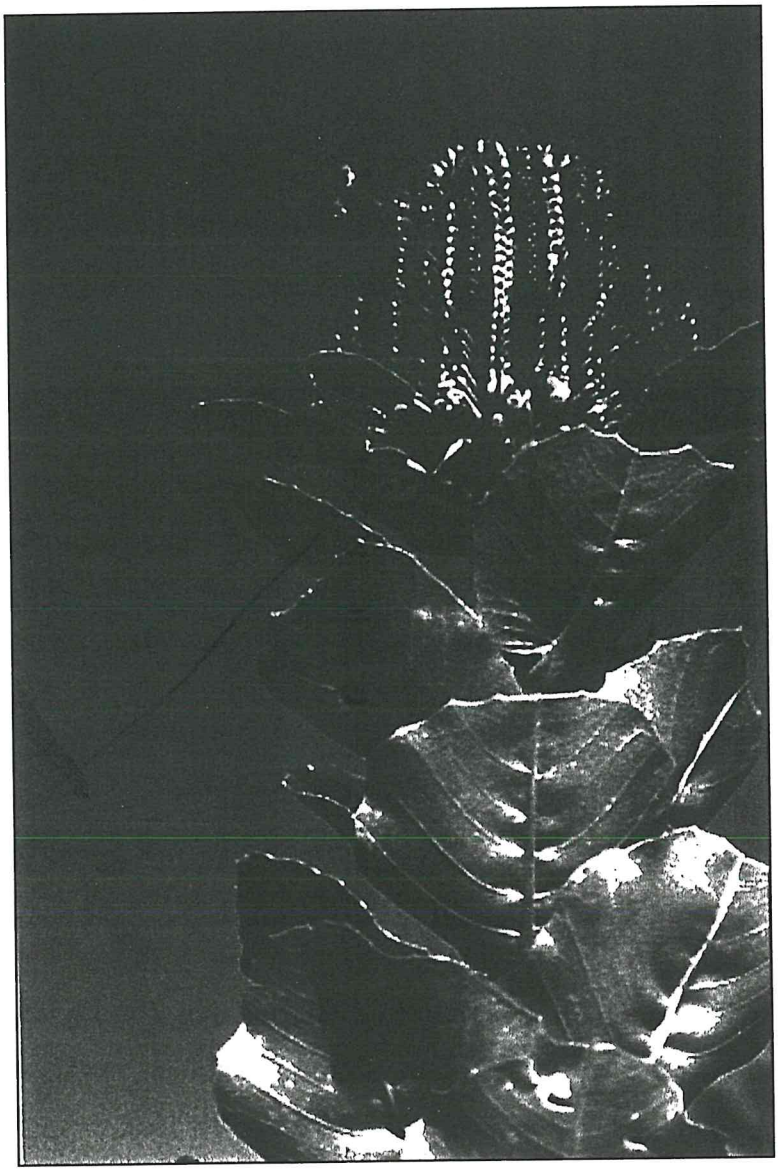


Table 1. Susceptibility of Declared Rare Flora to *Phythphora cinnamomi* (from Keighery, 1992).

Highly susceptible	Susceptible
Andersonia sp. (GK 8229)	*Banksia goodii*
Adenanthos cunninghamii	*B. tricuspis*
A. dobagii	*Chamelaucium erythrochlora*
A. ellipticus	*C. griffinii*
A. ileticos	*C. roycei*
A. pungens ssp. Pungens	*Darwinia collina*
A. velutinus	*D. macrostegia*
Banksia brownii	*D. meeboldii*
B. verticillata	*D. oxylepis*
Dryandra sp. (Kamballup)	*D.squarrosa*
D. *sp. (Lullfitz 3379)*	*D. wittwerorum*
Isopogon uncinatus	*D. sp. (Keighery 5732)*
Lambertia echinata ssp. echinata	*Dryandra serratuloides*
L. fairallii	*Grevillea calliantha*
L. orbifolia	*G. saccata*
	Leucopogon obtectus

The fungus also has a devastating effect on many plant species that are not necessarily rare, but are a major structural element of some plant communities. When these species are eradicated by the activity of *P. cinnamomi*, affected areas decrease in structural complexity and change from diverse heaths, shrublands or woodlands into species-poor, bare areas bearing grasses and sedges. These changes result not only in further losses of many plant species but also impact on animal communities that are unable to survive in altered habitats. Although hosts of *P. cinnamomi* occur mainly amongst members of the Proteaceae, Myrtaceae, Papilionaceae, Epacridaceae and Dilleniaceae, species belonging in other families are also affected.

2.3 DISEASE MANAGEMENT OF *P. CINNAMOMI* IN NATIVE PLANT COMMUNITIES

Current strategies to control *P. cinnamomi* in plant communities in Western Australia aim to minimise the vectored spread of the pathogen and to restrict intensification of disease to protect various conservation and economic values (Shearer and Tippett, 1989). Strategies which have been researched include recognition of vulnerable sites, risk assessment of introduction and spread of the pathogen in a particular area, application of quarantine, and manipulation of conditions disfavouring the pathogen or enhancing host resistance. In addition, hygienic procedures have been developed for the public and for industry to help protect large areas of healthy bush from the disease. Although when integrated, the above methods minimise the introduction and spread of infection to healthy areas and susceptible hosts, they are only regarded as an interim solution until a better approach can be developed.

2.4 CHEMICAL CONTROL OF *PHYTOPHTHORA* DIEBACK

In the past, diseases caused by *Phytophthora* were usually controlled using specific cultural practices, disinfectants or soil fumigation (Coffey, 1991). However, these methods are unpracticable in natural vegetation. The chemicals were non-selective, toxic to plants or animals, and could not be used to control the pathogen in native vegetation.

Systemic fungicides such as the phenylamides and phosphonates developed in over the past two decades have provided new opportunities for chemical control of soil-borne *Phytophthora* species that cause diseases of many crops. The discovery of fungicides that were distributed systemically within plant tissues, opened new possibilities for disease control and prompted new research into the effectiveness of such chemicals in controlling some of the *Phytophthora* spp. present in forests, heath and shrublands.

2.4.1 Phosphonate for Control of *P. cinnamomi* in Parks and Reserves

When used as a part of integrated management strategy, phosphonate (mono di-potassium

phosphonate) promises to be a very useful agent for the control of *P. cinnamomi* in native plant species. Phosphonate is an aqueous solution of mono- and di-potassium phosphonate, held in equilibrium at pH 5.7 to 6.0. In its use as a fungicide it is formulated from neutralised phosphonic acid and sold as a potassium salt under various brand names and strengths of 20% or 40%. The active component of the fungicide is the phosphite ion (PO_3-).

In the post war period there were attempts to use phosphonate as a phosphorous fertiliser. However, it was found to be ineffective in that role due to its' very low rate of transformation from phosphonate to phosphate in field conditions (McIntire *et al.*, 1953). The discovery that phosphonate salts were effective fungicides was made in the 1970's and a derivative of phosphonic acid (fosetyl-Al) called Aliette, was first released in 1977. Later, the discovery that phosphonic acid had the same fungicidal activity as fosetyl-Al, led to a successful challenge to the patent protection obtained by the manufacturer of Aliette in Australia. As a result of that challenge, Australia is the only country where phosphonate is readily available and legally used as a fungicide.

Plants absorb and translocate the phosphite ion readily, but there is no evidence that either mammals or plants are able to metabolise it (Guest and Grant, 1991). Non-enzymic transformation can occur at extreme pH and temperatures not present in biological systems. The only oxidation that does occur, takes place in the soil where phosphonate is rapidly oxidised to phosphate by soil microbes (Adams and Conrad, 1953).

For several years the chemical has been used to control *P. cinnamomi* in economically significant crops including avocado (Pegg *et al.*, 1985), pineapples (Rohrbah and Schenck, 1985) and cocoa (Guest *et al.*, 1994) , but its use on native species is novel and has been pioneered by CALM in Western Australia (Shearer and Fairman, 1991). Phosphonate was found to be very effective against infection in jarrah and some species of *Banksia* (Shearer, *pers. comm.*). The chemical is cheap, biodegradable and non-toxic to people, animals or many important segments of the soil microflora. Foliar application of phosphonate did not affect microbial numbers in the rhizosphere of avocado seedlings (Wongwathanarat and Sivasithamparam, 1991). Also, growth of mycorrhizal fungi was unaffected by application of the fungicide to maize (Wellings *et al.*, 1990).

Phosphonate has been tested in field trials by CALM in various areas in the south-west on plant communities already infected with *P. cinnamomi*. The trials have shown that one application of phosphonate gives excellent control of the fungus over five to seven years in some *Banksia* spp. (Shearer, *pers. comm.*). The chemical can be injected into the trunk using a hydraulic injector. This technique is successful because the phosphite ion is transported in the conductive tissues of the tree to the leaves and from there to the roots. Phosphonate can also be sprayed on the foliage using a backpack sprayer. Both of these methods of application have been excellent for controlling infections occurring in small areas where the chemical can be applied manually.

P. cinnamomi has caused severe damage to a population of rare and endangered *Banksia brownii* at Millbrook Reserve, 30 km north of Albany. The results of a foliar application field trial (sprayed to runoff with a backpack) undertaken several years ago, has since demonstrated that phosphonate protected the plants for at least three years. The infection front moved three metres past the experimental plots leaving the sprayed plants healthy. The trial was subsequently discontinued as the whole *B. Brownii* population was included in an aerial application trial.

To control spread of infection effectively over moderately sized to large areas, the chemical must be applied by aircraft. Aerial application of phosphonate permits treatment of remote areas cost-effectively and without disturbance to the treated or neighbouring areas.

Although phosphonate does not eliminate disease, it is a powerful prophylactic fungicide and it offers the only available option for the prevention of further losses of rare and endangered plant species due to dieback. Phosphonate treatment provides effective protection of healthy plant communities in areas where the pathogen is present in the short to medium term. Also, it is the only chemical that can be safely applied to extensive areas of native flora without posing any significant danger to non-target plant or animal species.

3 OBJECTIVES

The objectives of this work were:

- To evaluate the efficacy of aerial application of phosphonate in the control of *P. cinnamomi* in native plant communities in Western Australia.

- To determine the long term effects of phosphonate on native plants.

- To determine the appropriate rates of application and the duration of plant protection conferred by aerial application of phosphonate.

- To provide a scientific basis for the management of phosphonate application in parks and reserves, where *P. cinnamomi* is active and causes rapid destruction of the native flora.

- To examine the effects of phosphonate on *in vitro* growth of Western Australian isolates of *P. cinnamomi* in relation to phosphate availability.

4 RESEARCH

This report provides an overview of research undertaken since the inception of this project in 1992. To simplify reporting, different components of the project are covered in five separate sections numbered 4.1 through to 4.5.

4.1 ASSESSMENT OF THE EFFICACY OF AERIAL APPLICATION OF PHOSPHONATE

4.1.1 Spray Application: General

The fungicide has been field tested during the past five years in several areas of the south-west on plant communities already infected with *P. 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 using aircraft. This method of application is the most suitable if long fronts of infection are to be treated. It also permits cost-effective treatment of most remote areas without disturbance to treated or neighbouring areas, thereby eliminating any possibility of accidental spread of disease.

In contrast to most manually applied sprays, aerially applied systemic fungicides are delivered in low volumes, usually 20-50l ha^{-1} (Jacobsen, 1986). Also, because of aircraft speed and other physical parameters, various factors must be considered. These 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 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.

All our field trials were treated by a local contractor (Giles Aviation) and both of the planes used in the experiments were equipped with a Micronair spraying system that ensures even droplet size.

In order to determine spray coverage within the plant canopy and droplet size, water-sensitive papers were attached to the upper leaves and also placed directly on the ground below trees. The use of water-sensitive papers is not directly quantitative but does give a quick visual estimate of droplet size and density at various points in the plant canopy. The relationship between the volume of liquid 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 100-500 μm with 70% of droplets being in the range of 100-300 μm while droplet density was 50-60 drops cm^{-2}. It is generally recommended that droplet VMD should be 300-500 μm as this 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. Droplet penetration

through the canopy was good, as the water-sensitive papers laid under the trees intercepted only 20% fewer drops than papers attached to upper leaves.

The field trials were treated with 8.5% and 10% phosphonate applied at 26-30l ha^{-1} in earlier experiments, and in the recent work, with 20% and 40% applied at 30-60l ha^{1}. Synertrol (canola oil) was used as a wetting agent. In order to boost the concentration of active ingredient in plant tissues, a second application was carried out, about one month after initial spraying in all the experiments. Shoot and/or root samples were taken periodically and analysed for phosphite. Plant health was assessed.

4.1.2 Phosphonate Analysis

Determination of the longevity of phosphonate residues in plant tissue was a significant part of this research. Chemical analysis was required to establish the time span over which the fungicide persisted in shoots and roots of various species. This information was essential in making decisions on appropriate treatment concentrations and application rates as well as timing of subsequent sprays.

Plant samples were collected from the field, washed, and dried in a low temperature oven (35°C) for 24 hours. There is some evidence in the literature (also supported by our *in vitro* study) that phosphite is oxidised to phosphate at temperatures above 65°C.

Dried leaf or root tissue was finely ground and samples of 500 ± 2mg were placed in centrifuge tubes to which 4.5ml of 0.1N H_2SO_4 was added. Samples were shaken for over two hours and centrifuged for approximately 10 minutes at 8000 rpm. One ml of methanol was pipetted into 5ml volumetric flasks and 0.25ml of the clear acid extract was added. The volumetric flasks containing sample extract and methanol were transported to the Western Australian Chemistry Centre where standards of varying concentrations (1 , 5, 10, 50, 100, 500, 1000 $\mu g\ g^{-1}$) were prepared. Diazomethane was added to the samples and standards in excess, until a persistent yellow colour developed. Excess diazomethane was neutralised using a few microlitres of isopropanol. Diazomethane was prepared using chemical Diazald and a Diazald kit to generate its' solution in ethyl ether.

using a few microlitres of isopropanol. Diazomethane was prepared using chemical Diazald and a Diazald kit to generate its' solution in ethyl ether.

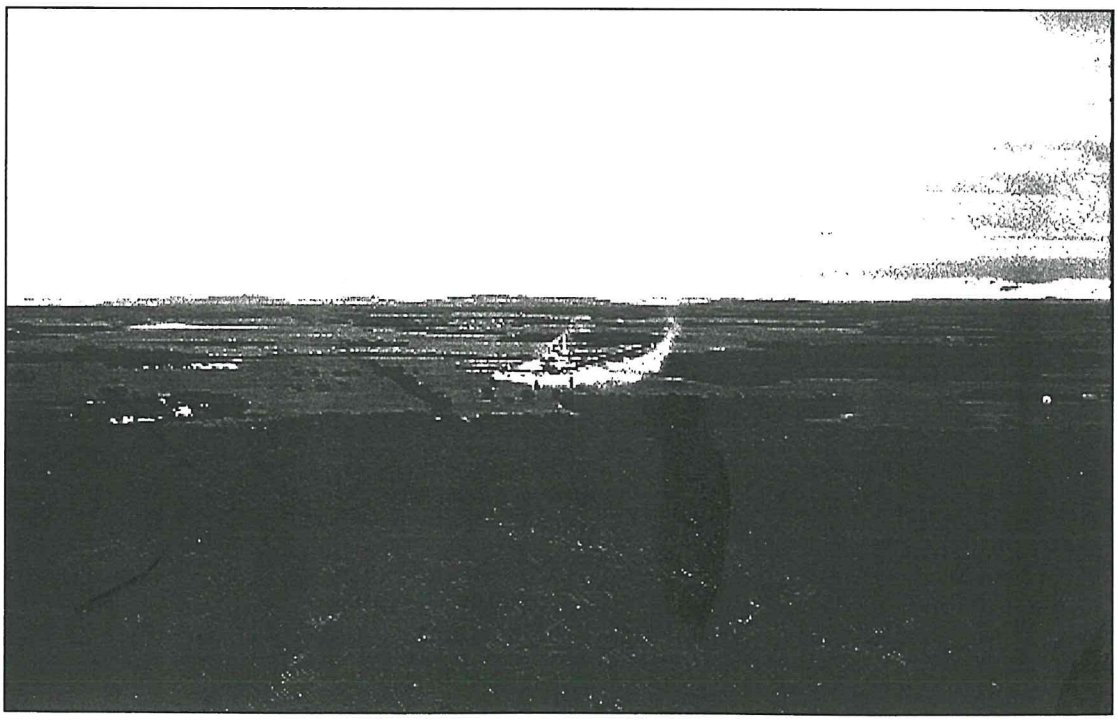
When diazomethane is added to the filtrate, phosphorous acid is converted to dimethyl phosphonate. The methylated compound was then determined by gas chromatography using a phosphorous-specific flame photometric detector.

4.1.3 Aerial Application Trial at South Sister Nature Reserve

4.1.3.1 Methods

In early May 1993 we commenced our first aerial application trial at South Sister Nature Reserve (30 Km E of Albany) where a population of rare and endangered *Banksia brownii* is located. This species, which is very susceptible to infection by *Phytophthora*, occurs only at a few locations, all of which are infested with the pathogen.

Figure 3. Spraying of the South Sister Nature Reserve.



The South Sister site (8.8 ha) was sprayed with 8.6% potassium phosphonate, using Synertol (0.5%) as a wetting agent. The chemical was applied by aircraft, in a low volume of 26l ha^{-1} (Figure 3). A follow-up spray was applied six weeks later.

Twenty *B. brownii* trees were randomly selected, marked, and their health was assessed. Leaf samples were taken for chemical analysis to determine the concentration of phosphite ion in the plant tissue. The marked trees were inspected for any visible signs of phytotoxicity, but none were observed. The site was monitored every six months to assess plant health and changes in phosphite concentration within plant tissues.

4.1.3.2 Results and Discussion

The results of chemical analyses of leaves are presented in Table 2. The concentration of phosphite after the first application of phosphonate was 1.3 $\mu g\ g^{-1}$ (mean for 20 trees) and this was boosted to 6.2 $\mu g\ g^{-1}$ by the second application. The concentration then declined to 0.6 $\mu g\ g^{-1}$ after six months but trees remained healthy for one year. Eighteen months after spraying, the concentration of phosphite decreased to 0.17 $\mu g\ g^{-1}$ and two marked trees died. Two years after spraying, phosphite could not be detected in the leaves.

The application rate and concentration of fungicide used at this site were probably too low to ensure long term protection of treated plants. Although plants were protected for up to eighteen months, phosphonate would have to be applied at a higher rate and concentration in order to prevent further deaths in the area.

The results from recent trials indicate that a much higher (than previously used) concentration of phosphonate can be applied to plants without causing phytotoxicity (section 4.1.6). After careful assessment of the latest work, the area was re-sprayed with 40% phosphonate at 60l ha^{-1} and later at 30l ha^{-1} in May 1996. The area is monitored every six months.

Table 2. Concentrations ($\mu g\ g^{-1}$) or phosphite ion (PO^{-}_{3}) in leaf tissue of *Banksia brownii* before and after ultra low volume spraying with 8.5% phosphonate.

Sampling time	$[PO^{-}_{3}]$
Before 1st spray	0
After 1st spray	1.3
Before 2nd spray	1.3
After 2nd spray	6.2
6 months after 2nd spray	0.6
18 months after 2nd spray	0.17
2 years after 2nd spray	0

4.1.4 Aerial Application Trial in Gull Rock National Park

4.1.4.1 Methods

A fully replicated field trial in the Gull Rock area near Albany was established in April 1993 to determine the effectiveness of phosphonate for the control of *P. cinnamomi* in *Banksia coccinea*. The fungus has had a high impact in the area and numerous infection fronts are causing widespread destruction (Figure 4).

The trial consists of eight plots (four pairs of sprayed and control) which were set up on infection fronts. The plots measure 40 m x 20 m. Within each sprayed plot a sub-plot was marked (20 m x 10 m) and 20 plants were tagged in each of sub-plots. Plant height and mortality were measured every six weeks for six months before spraying, and periodically thereafter. Reduction in plant growth rate is a good indicator of phytotoxicity thus pre- and post-spray measurements allowed assessment of the effect of phosphonate on plant health. Samples were taken for chemical analysis to determine the concentration of the phosphite ion in plant tissues.

Figure 4. Active infection front of *P. cinnamomi* killing *B. coccinea* at Gull Rock. Most *B. coccinea* plants are dead in the infected area (lower right).

The plots were treated in early November and a follow-up spray was applied in the first week of December 1993. The plots were sprayed twice with 10% phosphonate at 30l ha^{-1}. The second spraying in December was carried out in the same way. In order to monitor spray coverage and droplet size, water-sensitive papers were placed in the treated plots as described already.

4.1.4.2 Results and Discussion

After the first application of phosphonate, it was noted that coverage (only 20-30 droplets cm^{-2}) was unsatisfactory. This was caused by excessive drift despite only slight to moderate wind speed of 10-25 Km hr^{-1}. For the second spraying droplet size was increased from 100-300 μm to 300-600 μm and this significantly improved coverage to about 60 droplets cm^{-2}. Despite the increased droplet size, penetration of the plant canopy was excellent. Application of larger droplets can be beneficial in areas where windy conditions prevail and,

when only a small area is to be sprayed, increased droplet size is preferable as it improves precision of the application and minimises loss through drift.

The concentrations of the active ingredient (phosphite) in plant tissue ranged from 0.9-4.3 μg g^{-1} after the first spraying and 4.1-34.2 μg g^{-1} after the second application (Table 3). One year after treatment the concentration of phosphite had decreased to between 0.18 and 0.4 μg g^{-1} and after two years it could not be detected in plant tissue.

The concentration of phosphite in leaf tissue was significantly increased by the second application of phosphonate (Table 3). Improved coverage due to increased droplet size in the second spray resulted in significantly higher concentrations of phosphite in plant tissue. The substantial differences in phosphonate residues between plots resulted from uneven application of spray due to difficulty in targeting the small experimental plots from the air.

Table 3. Concentrations (μg g^{-1}) of phosphite ion in leaf tissue of *B. coccinea* at stated times after treatment with phosphonate.

Replicate	Before 1st spray	After 1st spray	Before 2nd spray	After 2nd spray	After 1 year	After 2 years
A	0	0.9	0.15	4.15	0.4	0
B	0	4.3	5.1	13.2	0.3	0
C	0	1.4	1.2	6.2	0.18	0
D	0	2.5	2.4	34.2	0.32	0

Plant mortality is the ultimate measure of the effectiveness of phosphonate treatment. Information on plant mortalities in treated and control plots is provided in Figure 5. In comparison with unsprayed, control areas, plant mortality was reduced in all plots treated with phosphonate and aerial application of the chemical consistently controlled the disease for 12-18 months. One year after treatment, the number of deaths in sprayed plots started to increase in two replicates (A and C) and after 16 months the mortality rate in sprayed plots and controls was similar.

There was a substantial increase in the number of dead plants in all control plots 90-200 days after spraying (Figure 5) and this coincided with a very dry period during summer and early autumn in 1993/94. That no such increase in mortality was recorded in the treated plots over the same period, strongly suggests that the sprayed plants were protected by phosphonate and were more able to survive drought conditions because their roots had sustained less damage due to *P. cinnamomi.* In replicates B and D there was excellent control of the disease in both treated plots with very few deaths recorded after eighteen months (Figure 5). The number of dead plants in the control plots in B and D increased substantially in the same period and at a similar rate to the controls in A and C.

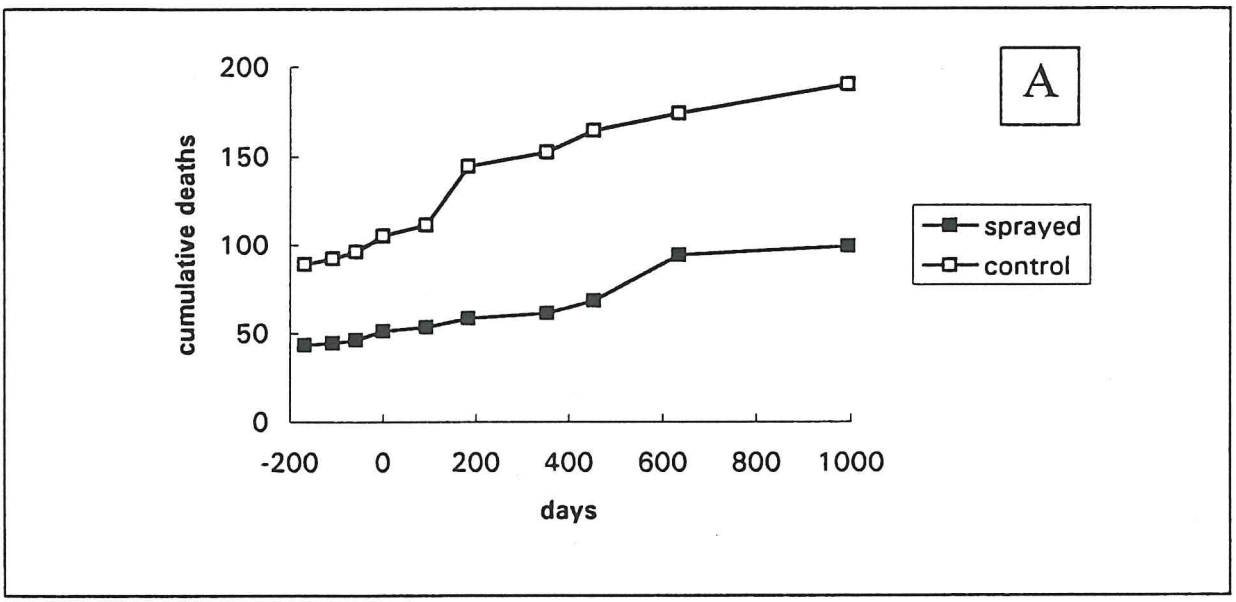
The *B. coccinea* plants in B and D had greater initial concentrations of phosphite ion in their leaf tissue than treated plants in the other replicate plots (Table 3). The populations in these plots were younger (3-8 years old) and possibly absorbed more phosphonate due to a higher leaf area to total plant mass ratio than that associated with older plants in the other treated areas.

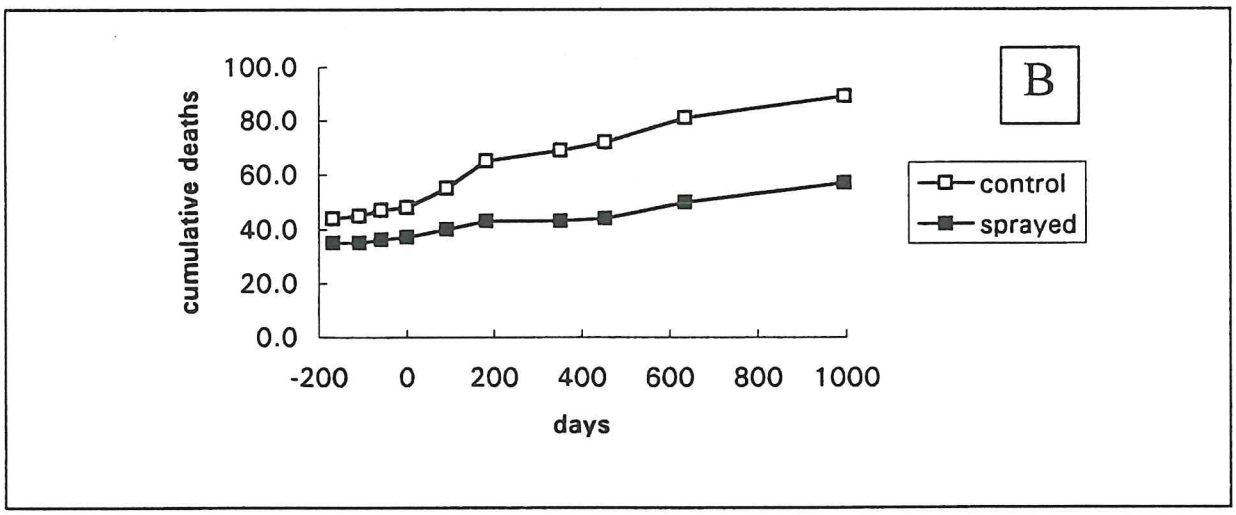
One year after spraying we collected root samples from the treated plots. The samples were analysed and the estimated concentrations of phosphite ion ranged between 0.2 and 0.7 $\mu g\ g^{-1}$. This result indicates that phosphite is distributed in shoots and roots.

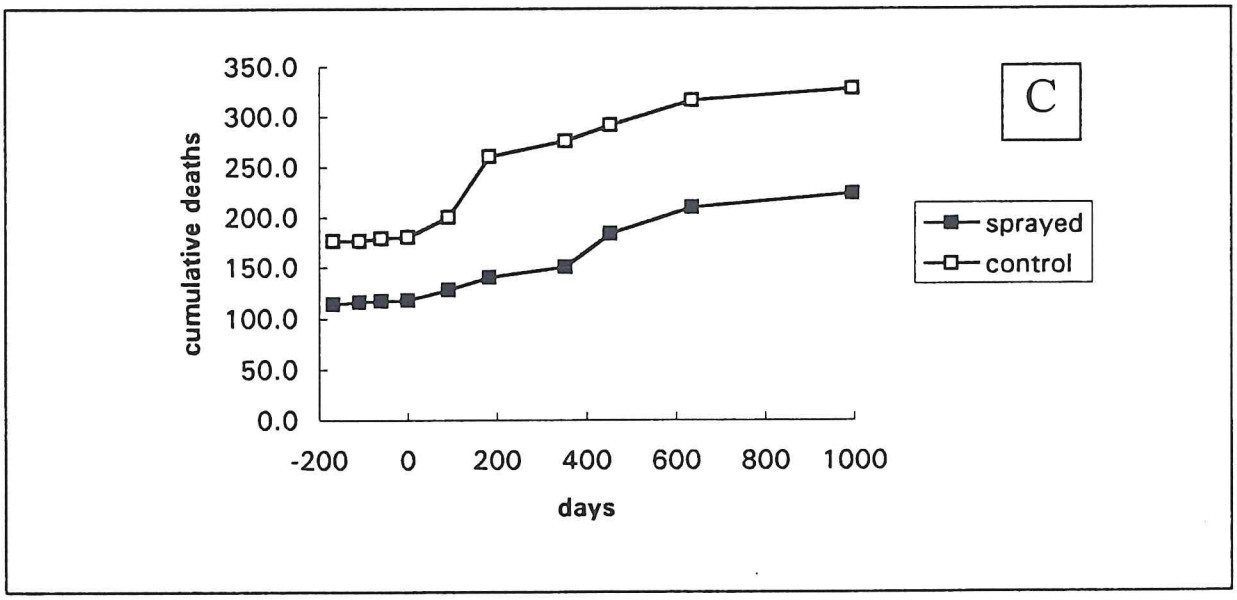
Plant growth was monitored before and after spraying and no significant differences in the rate of growth between treated and untreated plants was observed. Application of 10% phosphonate at 60l ha^{-1} did not affect plant growth.

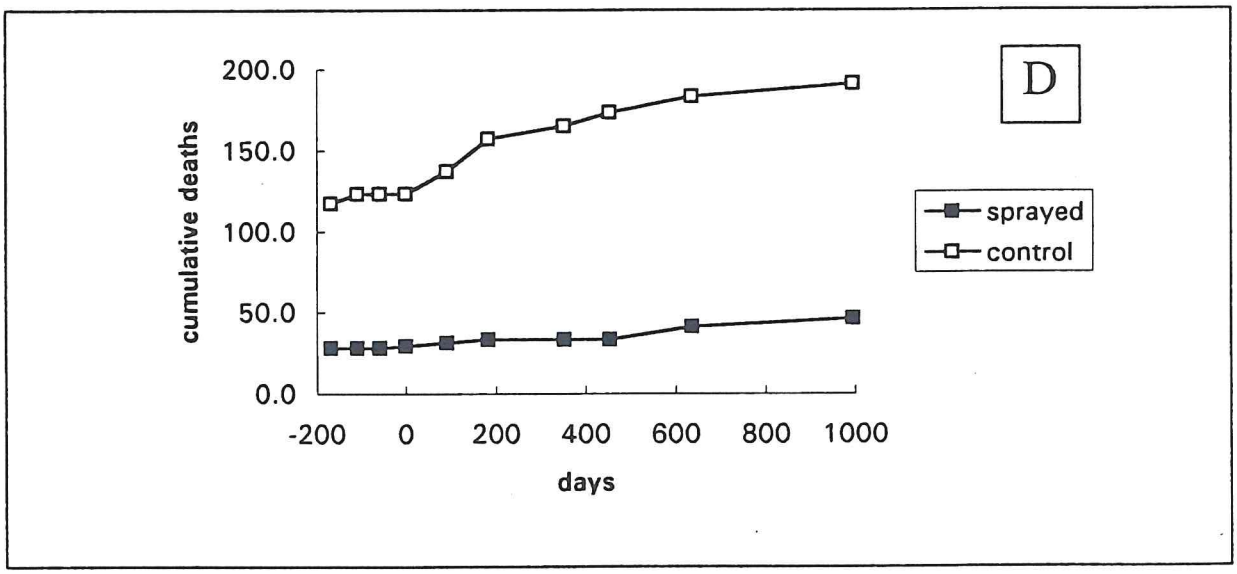
In May 1996 the plots were sprayed with 40% phosphonate at 60l ha^{-1}, as it had been established that the low concentration (applied initially) only protected plants for 12-18 months. Re-spraying at the higher concentration resulted in some minor leaf burning in a few non-target species (mainly eucalypts).

Figure 5. Gull Rock aerial application trial. The cumulative number of dead plants in replicate plots (A - D) sprayed with phosphonate (on day 0) or unsprayed (controls).



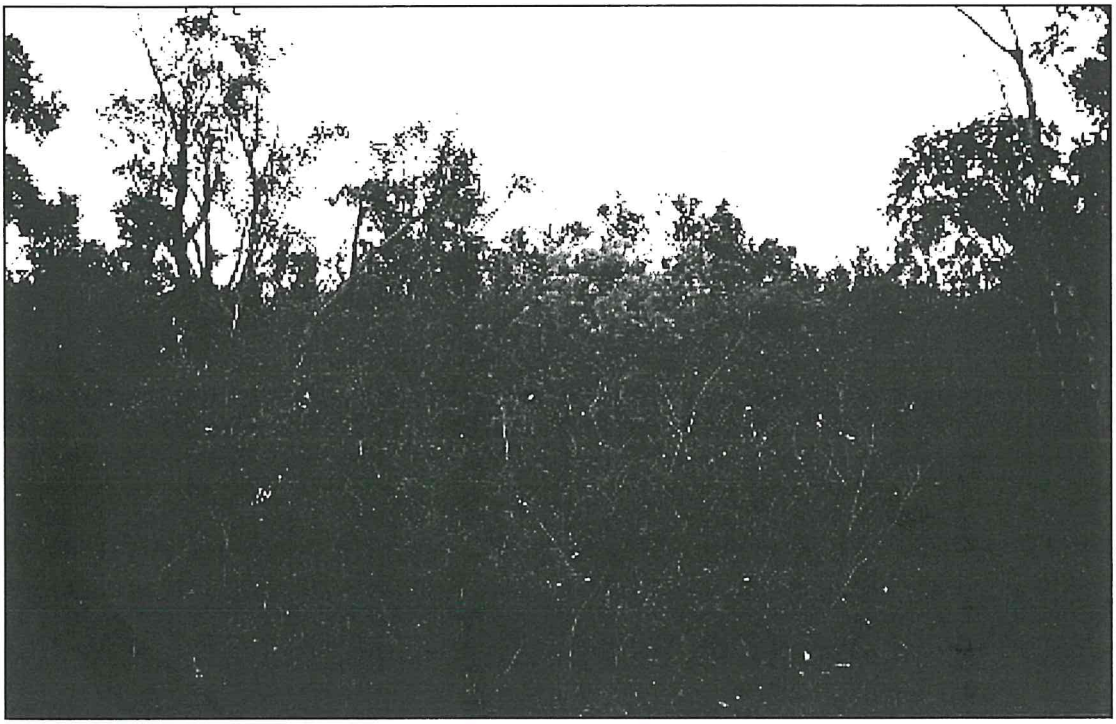



C
cumulative deaths
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
-200
0
200
400
600
800
1000
days
sprayed
control


D
cumulative deaths
200.0
150.0
100.0
50.0
0.0
-200
0
200
400
600
800
1000
days
sprayed
control

4.1.5 Aerial Spray Trial at Millbrook Reserve

Figure 6. Active infection front of *P. cinnamomi* in a stand of *B. brownii* at Millbrook Reserve. All *B. brownii* plants were killed in the infected area (bottom of the photograph).



4.1.5.1 Methods

P. cinnamomi has severely damaged a population of *Banksia brownii* at Millbrook Reserve 30 km north of Albany (Figure 6). A foliar application field trial (using backpack sprayer) conducted by CALM has demonstrated that phosphonate protected plants for up to three years (Shearer, *pers. comm.*). In that trial experimental plots were located on the edge of an active infection front and sprayed with phosphonate. After three years, the infection front had moved more than three metres past the plots in which the sprayed plants remained healthy.

The aerial spray trial described here is the second one involving *B. brownii* and it allowed comparison of the effectiveness of phosphonate application in controlling *Phytophthora* infection in a single plant species in two different areas. In addition application of the

fungicide at Millbrook Reserve will help protect another population of this endangered plant species from extinction.

Almost the whole population of *B. brownii* (6 ha) was sprayed aerially, using low volume application in November, 1993. Plants in the Millbrook Reserve and at Gull Rock were sprayed on the same days and the follow-up application at the former location also involved larger droplet size. The plots were sprayed twice with 10% phosphonate at 30l ha^{-1} (with 0.5% Synertrol) to give an effective application rate of 60l ha^{-1}. The second spraying in December was similarly conducted. Chemical analyses and plant health measurements were carried out for twenty selected trees and the duration of protection was established.

4.1.5.2 Results and Discussion

The concentration of phosphite in leaf tissue was significantly increased by the second application of phosphonate (Table 4). Improved coverage due to increased droplet size in the follow-up application resulted in significantly higher concentrations of phosphite in plant tissues.

The concentration of phosphite found in leaf tissues after the second spraying was almost four times higher than that noted at South Sister Reserve. This was due to the use of increased spray concentration, application rate and droplet size, the latter to minimise drift loss and improve coverage.

Root samples were also collected one year after the second spray. The samples were analysed and the concentration of phosphite ion ranged from 0.3 to 1.9 $\mu g\ g^{-1}$. This result showed that phosphite was distributed in the roots and shoots. No plant mortalities were observed up to November, 1996.

Table 4. Concentrations ($\mu g\ g^{-1}$) of phosphite (PO^{-}_{3}) in leaf tissue of *B. brownii* after ultra low volume spraying with 10% phosphonate.

Sampling time	$[PO^{-}_{3}]$
Before 1st spray	0
After 1st spray	4.5
Before 2nd spray	1
After 2nd spray	22
1 year after 2nd spray	0.41
2 years after 2nd spray	0

4.1.6 Aerial Application Trial at North Dandalup

4.1.6.1 Methods

In October,1994 a fully replicated aerial application trial was established on the Swan Coastal Plain near North Dandalup. Three species of *Banksia* were included in the trial: *B. attenuata, B. ilicifolia and B. menziesii.*

Plots (60 m x 30 m) were located on the edge of an active infection front in remnant bushland vegetation on private property. An assessment sub-plot (40 m x 20 m) was marked within each sprayed plot. Two plants of each species were marked in each assessment plot and plant samples for chemical analysis were taken from these at each harvest.

Three concentrations of phosphonate (10, 20 and 40 %) were applied twice at 60l ha^{-1} using aircraft (Giles Aviation). In this trial higher concentrations were tested than those used near Albany (see 4.1.3). Forty percent phosphonate is the most concentrated preparation available and has not previously been used to treat plants. The concentration of Synertrol was increased to 2% in this trial due to the prevalence of higher temperatures (30°C) than those experienced in other work. Spraying was conducted in early November, and again four weeks later.

Shoot samples were taken for chemical analysis immediately after the first and second applications, six months after treatment and then annually. The number of dead plants was estimated in all plots to establish the duration of fungicidal activity.

4.1.6.2 Results and Discussion

The average concentrations of phosphite found in leaf tissue were 5.3, 18.9 and 53.4 $\mu g\ g^{-1}$ for treatments with 10, 20 and 40% phosphonate respectively (Figure 7). These concentrations would be expected to provide adequate protection especially in the latter two treatments. Analysis of variance indicated no significant differences between replicates, plant species, trees or samples. The differences between treatments were significant (P=0.01). The standard errors presented in Figure 7 are asymmetric because the data for statistical analysis were transformed to logs.

Figure 7. Phosphite concentration ($\mu g\ g^{-1}$) in leaf tissue of *Banksia* plants after initial ultra low volume spraying of 10, 20 and 40 % phosphonate.

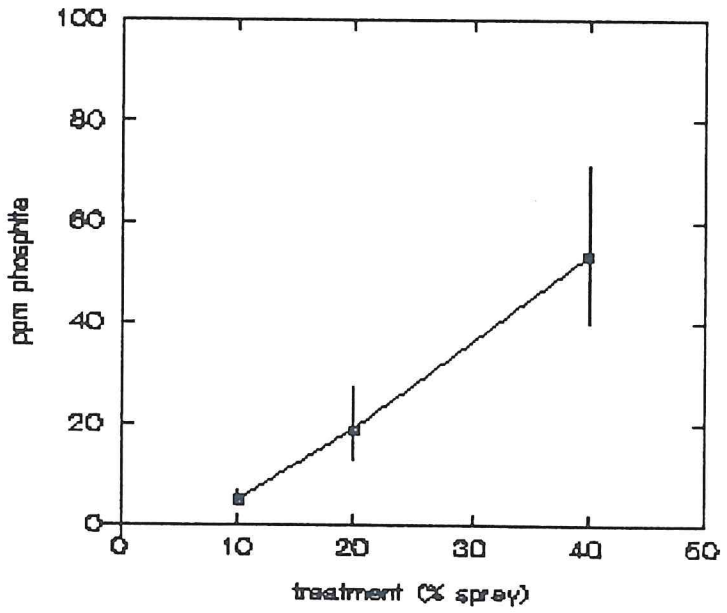


Figure 8 shows the concentration of phosphite in leaf tissue of individual species of *Banksia* after follow-up spraying. The concentration of fungicide residues varied between individual plant species but the differences were not significant and average values for all three species are presented in Figure 9.

Figure 8. Phosphite concentrations ($\mu g\ g^{-1}$) in leaf tissue of three *Banksia* spp. after follow-up ultra low volume spraying of 10, 20 and 40% phosphonate.

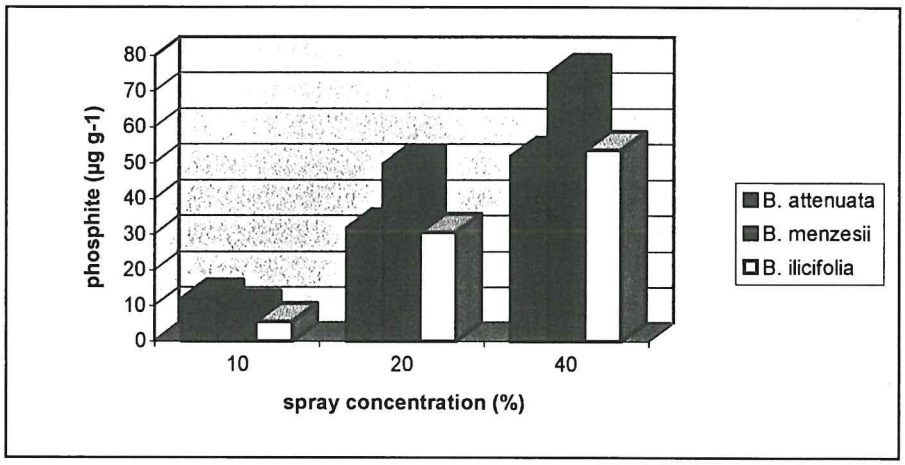
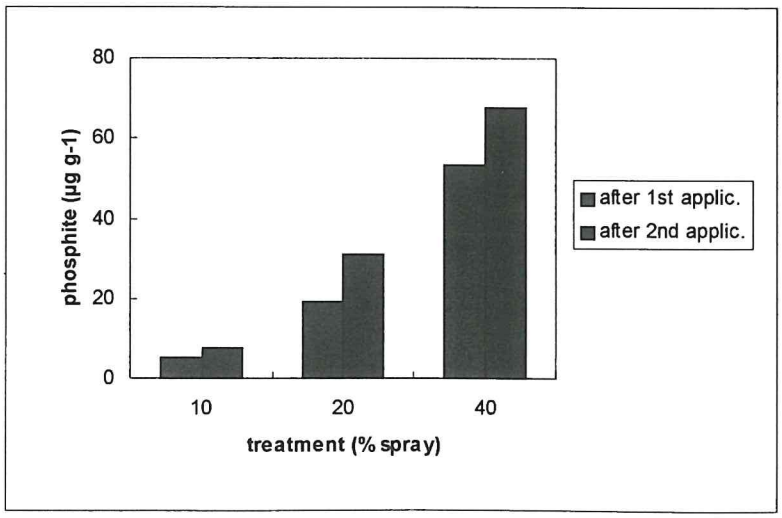


Figure 9. Phosphite concentration ($\mu g\ g^{-1}$) in leaf tissue of *Banksia* after first and follow-up ultra low volume spraying of 10, 20 and 40 % phosphonate.



The follow up treatment increased the concentration of the active ingredient by 20% to 25% (Figure 9). This increase was smaller than expected as a result of technical problems associated with non- availability of the Micronair spray system.

Application of 40% phosphonate was considered likely to ensure long term protection due to the high initial phosphite concentration attained in plant tissue (Figures 8 and 9). Although this treatment did not result in any burning of leaf margins it was decided to investigate whether the high concentration had any detrimental effect on flowering or seed setting.

Plants treated with 20% and 40% phosphonate remained healthy at least until November, 1996. However, there were six deaths in the 10% treatment which again proved too low for long term protection.

4.1.7 The Effect of Phosphonate on Seed Number and Germination in *Banksia* spp.

4.1.7.1 Seed Extraction

In order to determine the effect of high concentrations of phosphonate on flowering, seed setting and seed viability, cones from *B. menziesii, B. attenuata* and *B. ilicifolia* were collected from plots sprayed with 40% phosphonate. The cones had developed after spraying. Two plants of each species were selected at random and up to five cones were collected from each plant.

Collected cones were placed a few at a time, in oven trays and moistened with ethanol which was then ignited. After the fire was extinguished, the cones were placed in labelled foil trays and soaked in water overnight. The cones were then transferred to an oven at approximately 50°C to dry out, and to open the seed capsules thereby simplifying extraction of the seed. The seeds were counted and it was also noted that a significant number were damaged or eaten by insects, aborted, mouldy, undeveloped or trapped in the capsules.

4.1.7.2 Germination

The seeds were surface sterilised in chlorox (5%) for five minutes and rinsed in sterile, distilled water for 30-60 seconds. The seeds were then placed on top of wet filter paper overlying a layer of vermiculite in plastic petri dishes and sprayed with distilled water. The petri dishes were placed in an illuminated incubator at 15°C for up to six weeks or until germination had ceased. All components in the system had been sterilised by autoclaving (20 min at 15lb cm^{-2}) unless otherwise indicated. Numbers of germinated seed were recorded.

4.1.7.3 Results and Discussion

The quantity of seed per cone in three *Banksia* spp. was very variable but no significant differences in seed numbers were found between cones from sprayed and control treatments (Table 5). The number of seeds per cone was generally lower in *B. menziesii* than in the other two species. Cone production also varied considerably between species, with *B. ilicifolia* having only 0-3 harvest-suitable cones per tree with the other two species bearing 6-8 cones.

Table 5. Mean numbers of extracted seeds per cone for three species of *Banksia.* Standard errors are shown in parentheses.

Treatment	*B. attenuata*	*B. ilicifolia*	*B. menziesii*
40 % phosphonate	10.3 (1.1)	10 (2.3)	6 (1.74)
Control	14.8 (1.4)	8.5 (4.9)	5.6 (2.1)

The mean percentage of seed germination per sample differed between species, but no significant difference in seed germination was noted between samples from sprayed and control treatments (Table 6). *B. menziesii* had the highest germination percentage of 60-80%, whereas germination in *B. attenuata* was less than 5%. Application of 40% phosphonate at 60l ha^{-1} did not have any effect on flowering or seed germination in three *Banksia* spp. in the North Dandalup trial.

Table 6. Mean percentages of seeds germinating after extraction from cones of three species of *Banksia*. Standard errors are shown in parentheses.

Treatment	*B. attenuata*	*B. ilicifolia*	*B. menziesii*
40% phosphonate	2.5 (0.7)	54.2 (16.3)	60 (13.7)
Control	4.6 (1.2)	24.6 (11.2)	80 (11.2)

4.1.8 The Effect of Phosphonate on Plant Nutrition in Three *Banksia* spp.

4.1.8.1 Methods

Application of high concentrations of phosphonate can result in some chemical burning of foliage in a number of plant species. In plants that do not show any visible signs of chemical injury, the fungicide can still cause physiological changes or result in other phytotoxic responses.

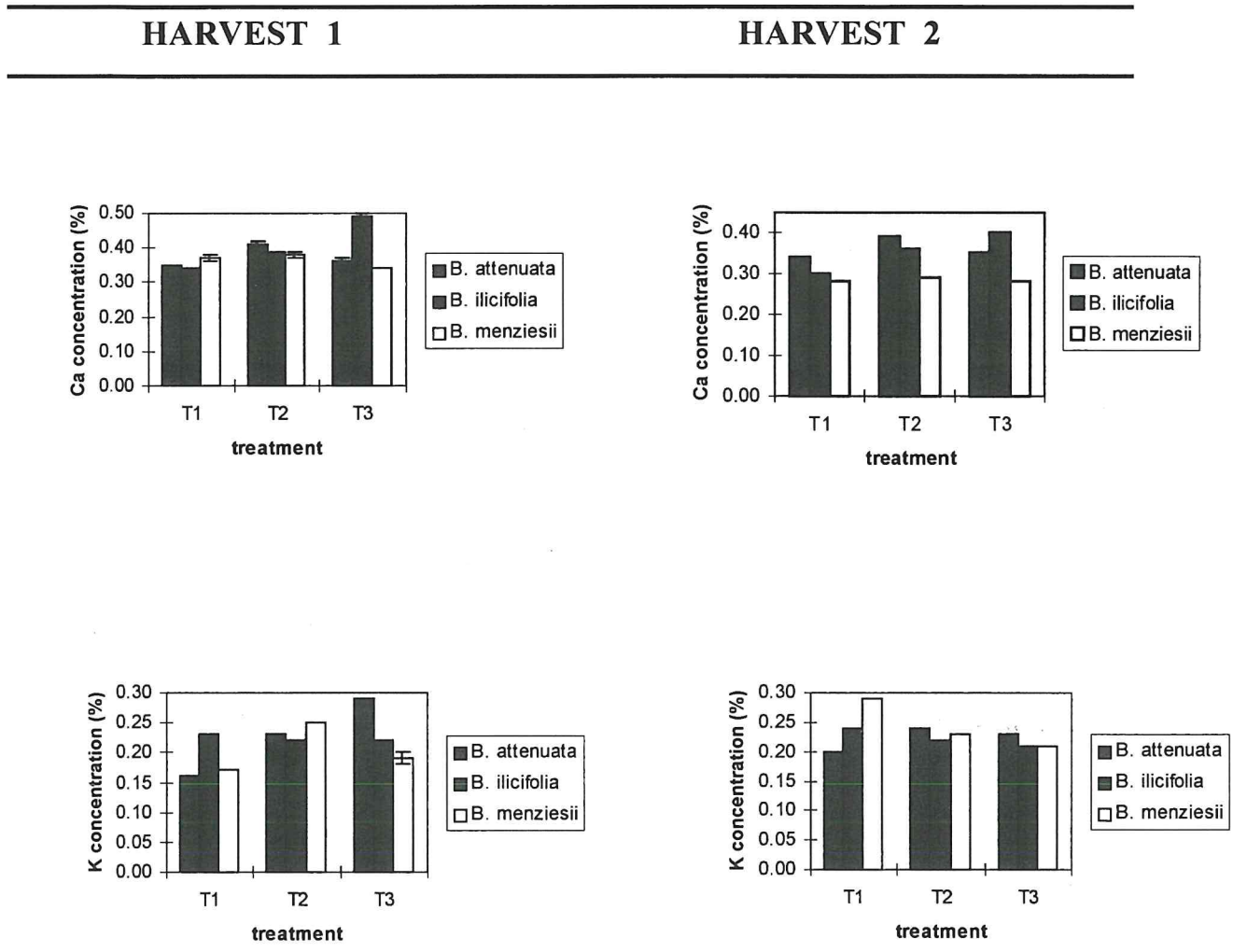
In this experiment we investigated the effects of 20% and 40% phosphonate on the concentrations of macro- and micro-nutrients in order to determine the influence of the fungicide on plant nutrition. Plant samples were collected from three *Banksia* spp. at North Dandalup and analysed for the following cations: Ca, Mg, K, Na, Mn, Zn and Fe. Samples (0.5g) were placed in a cylinder, 4.5 ml triacid (a mixture of HNO_3, H_2SO_4 and $HClO_4$) was added and the samples were digested, diluted and analysed. Concentrations of K and Na were determined using a flame photometer, while Ca, Mg, Mn, Fe and Zn were estimated, using a Perkin Elmer atomic absorption spectrophotometer.

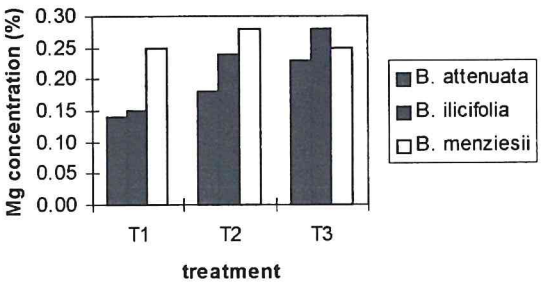
4.1.8.2 Results and Discussion

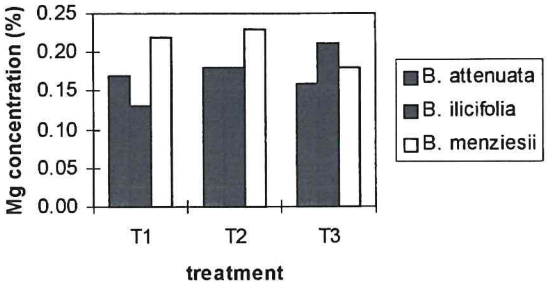
The results of chemical analyses, which are presented in Figure 10, show that application of concentrated phosphonate affected the uptake of certain cations to some degrees but the

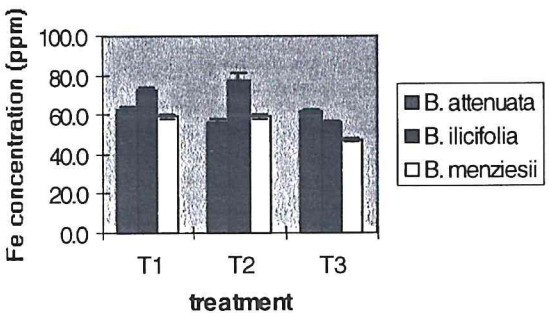
significance of these effects (positive or negative) for plant health and development are unknown and need further investigation.

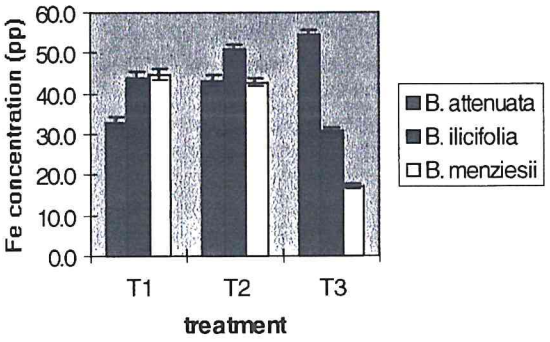
Figure 10. Concentration of cations in three species of *Banksia* sprayed with phosphonate at concentrations of 20% (T2), 40% (T3) or unsprayed (T1; control)

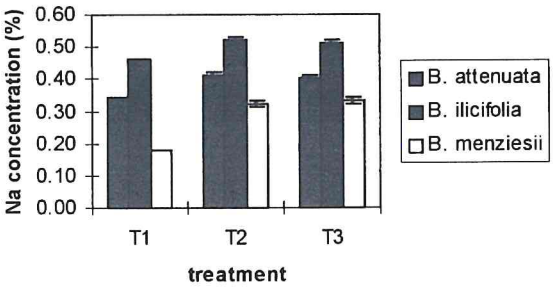


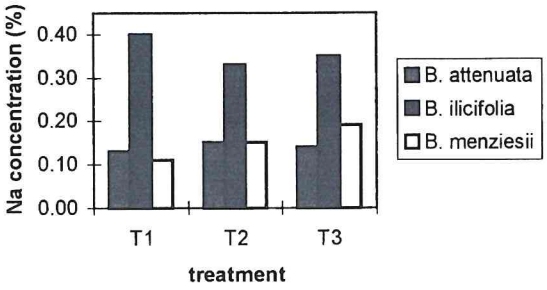

Mg concentration (%)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
T1
T2
T3
treatment
B. attenuata
B. ilicifolia
B. menziesii

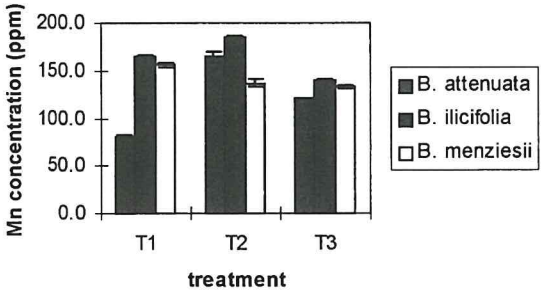

Mg concentration (%)
0.25
0.20
0.15
0.10
0.05
0.00
T1
T2
T3
treatment
B. attenuata
B. ilicifolia
B. menziesii

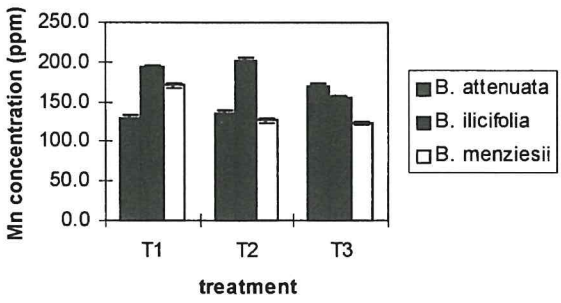

Fe concentration (ppm)
100.0
80.0
60.0
40.0
20.0
0.0
T1
T2
T3
treatment
B. attenuata
B. ilicifolia
B. menziesii

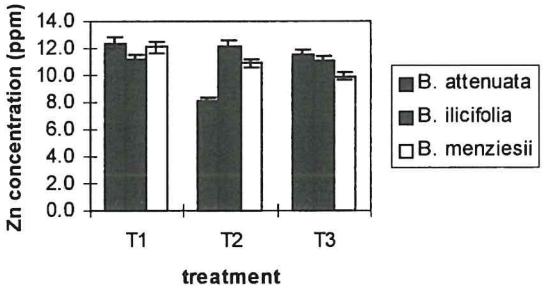

Fe concentration (pp)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
T1
T2
T3
treatment
B. attenuata
B. ilicifolia
B. menziesii

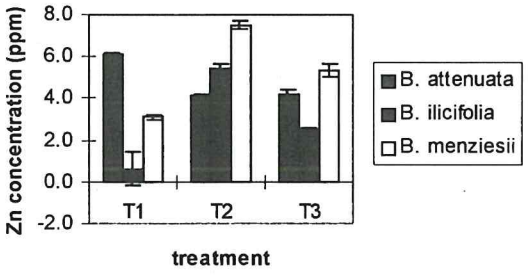

Na concentration (%)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
T1
T2
T3
treatment
B. attenuata
B. ilicifolia
B. menziesii


Na concentration (%)
0.40
0.30
0.20
0.10
0.00
T1
T2
T3
treatment
B. attenuata
B. ilicifolia
B. menziesii









4.1.9 Cost of Aerial Application of Phosphonate

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

At present the cost of treating one hectare with 40% phosphonate is $171 for a single spraying when applied at 60l ha^{-1}, and $342 if a follow-up spray is applied. The cost of aircraft hire is $300-$500/hour. The time required to spray any particular area can vary considerably depending mainly on the following factors:

- size, shape and location of the target area
- distance from airstrip as frequent chemical and fuel reloading is required
- maximum load of the aircraft
- type of aircraft spraying system

As an example, the time required to spray South Sister Nature Reserve (~9 ha) including loading was around 1.5 hours. The above cost of aircraft hire applies only to experimental applications and the cost of treating large areas is expected to be significantly lower.

4.2 ASSESSMENT OF THE EFFICACY OF PHOSPHONATE APPLICATION USING A NOVEL EXPERIMENTAL SYSTEM

4.2.1 Field Trial at Eneabba - West (Phosphonate Distribution Trial)

4.2.1.1 Methods

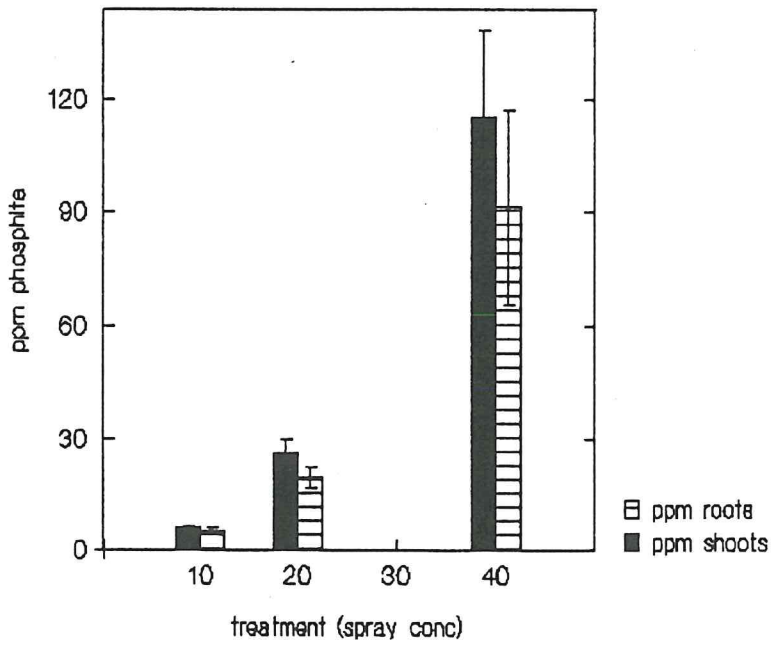
A new experimental system to test phosphonate application was established in November, 1994. Small plots (4 m x 1 m) containing regenerating seedlings of *Banksia telmetia* (post-fire) were sprayed twice with three different concentrations of phosphonate (10, 20 and 40%) using a hand-held, ultra-low volume sprayer. Each plot was sprayed at a rate equivalent to 15l ha-1 and the spray was mixed with 2% Synertrol. The number of droplets per unit area, and droplet size, were similar to that achieved by aerial application at North Dandalup (as determined by water sensitive papers).

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

4.2.1.2 Results and Discussion

Leaf and root samples from the first (spring) harvest were analysed and the data is presented in Figure 11. The average concentrations of phosphite in leaf tissue were 5.9, 26.3 and 115.4μg g^{-1}, for treatments with 10, 20 and 40% phosphonate respectively, while corresponding values for root tissue were 5.2, 19.6 and 91.3μg g^{-1} in that order. Analysis of variance indicated no significant differences (P=0.01) between replicates or samples but the differences between treatments were highly significant. The concentration of phosphite ion was slightly higher in shoots than in roots at all spray concentrations but the differences were not significant (P=0.01).

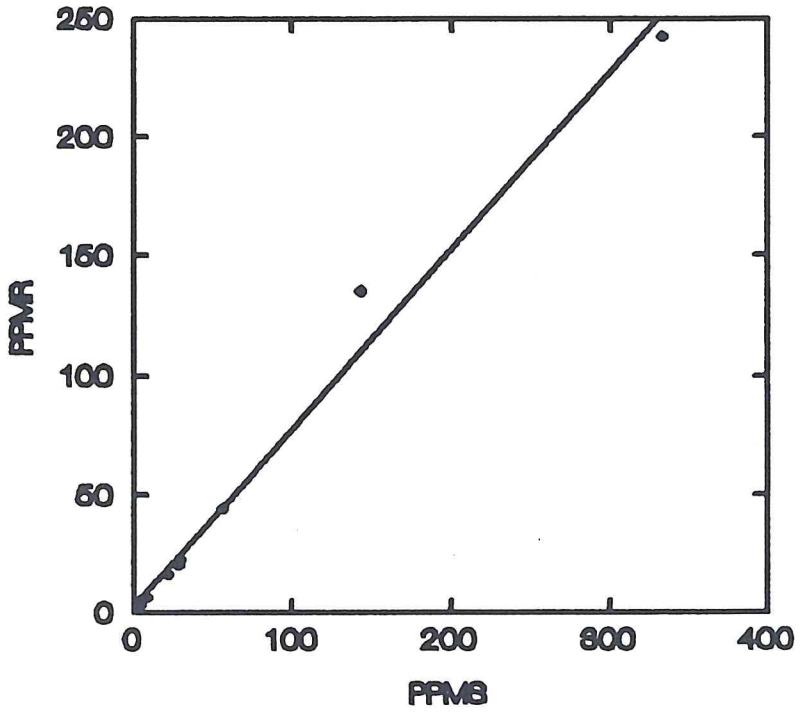
Figure 11. Concentrations of phosphite (μg g^{-1}) in leaf and root tissue of *B. telmetia* after spraying with 10, 20 and 40% phosphonate using a hand-held, ultra low volume sprayer.



There was a linear relationship between the concentrations of phosphite in shoots and roots (R^2 = 0.986) of *B. telmetia* (Figure 12). Phosphonate, as a systemic fungicide, is distributed through xylem and phloem (Groussol *et al.*, 1986). It has been demonstrated that the relative seasonal distribution of phosphite ion is influenced by source-sink relationships in

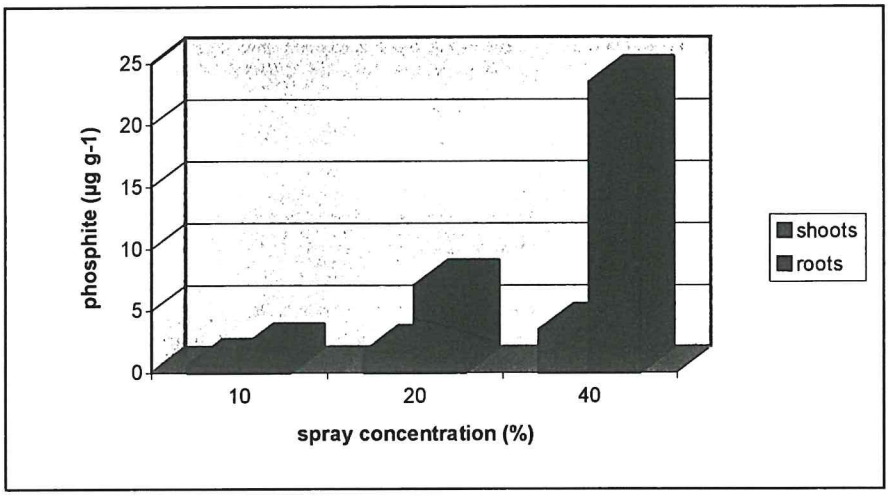
the plant. For example, in avocados the best time for phosphonate application is thought to be late summer when the roots are the main metabolic sink of the plant (Whiley *et al.*, 1986).

Figure 12. Regression line showing a linear relationship between concentrations of phosphite in roots and shoots of *B. telmetia* at harvest 1. (PPMS and PPMR: shoot and root concentrations of phosphite ion in $\mu g\ g^{-1}$).



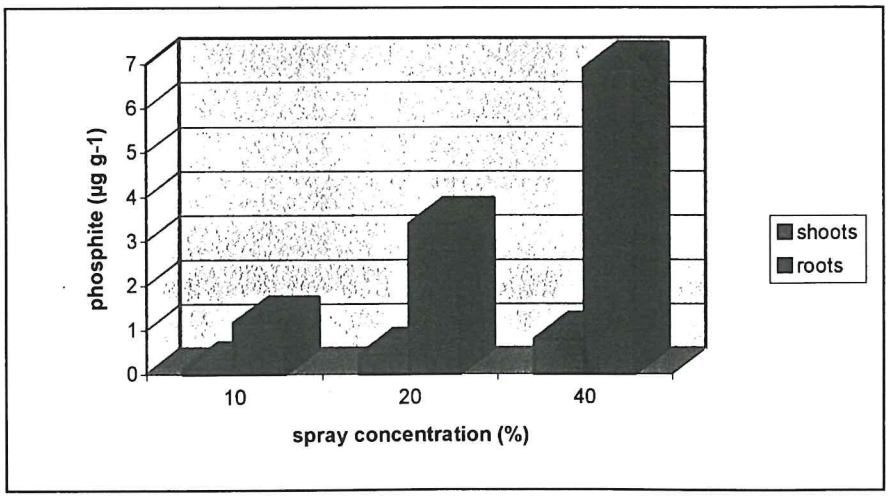
When samples were collected and analysed six months after spraying, the concentration of phosphite had decreased significantly in all treatments, but was now significantly higher in roots than in shoots (Figure 13). This reduction in overall plant tissue phosphite concentration coincided with a large increase in plant mass. Shoot dry weight of the two-year-old seedlings (post-fire regeneration) had increased by 400% in all treatments including controls and this would be expected to dilute the concentration of phosphite in plant tissue.

Figure 13. Concentration of phosphite (µg g^{-1}) in leaf and root tissue of *B. telmetia* six months after treatment with 10, 20 and 40% phosphonate sprayed using a hand-held ultra low volume sprayer.



The concentrations of fungicide residue had further decreased in all treatments when samples were collected and analysed one year after spray application but still remained significantly higher in the roots than in shoots (Figure 14). Two years after spraying, there was no detectable phosphite in shoot or root tissue.

Figure 14. Concentration of phosphite (µg g^{-1}) in leaf and root tissue of *B. telmetia* one year after treatment with 10, 20 and 40% phosphonate sprayed using a hand-held ultra low volume sprayer.



The results from this trial indicate that the concentration of phosphite in seedlings decreases substantially over a relatively short time due to a dilution factor associated with the high growth rate of young plants. The relative distribution of the active ingredient between shoots and roots changes, although it appears that in the long term phosphite is stored preferentially in the roots.

The ultimate fate of the phosphite ion is unknown and must be studied in detail if phosphonate applications are to be managed effectively and efficiently. Young plant populations have to be treated more frequently than older plants due to their high rate of growth and greater root turnover.

4.2.2 Field Trial at Eneabba - West (Active Infection Front)

4.2.2.1 Methods

An experiment involving *Banksia attenuata* and *B. menziesii* was established in October, 1994. Plots were set up on the edge of an active infection front of *P. cinnamomi* and ten plants of each species were marked within each plot. Individual plants were sprayed with three different concentrations of phosphonate (10, 20 and 40%) applied once or twice using a hand-held ultra-low volume sprayer. The sprayed plants ranged between one and seven years old. The first spray was applied in late October and the second about four weeks later

This experiment was designed predominantly to monitor plant mortality and to determine the appropriate application rates for the chemical. The benefits of a follow-up application were also assessed.

4.2.2.2 Results and Discussion

B. menziesii plants in the Eneabba trial showed much lower susceptibility to *P. cinnamomi* than *B. attenuata* in the same area and this difference was expressed in both treated and control plots (Figures 15 and 16). There was also a marked difference in mortality between plants sprayed with different concentrations of phosphonate (Figures 15 and 16). In both species, plants sprayed with phosphonate at 20% twice, or at 40% once or twice, stayed

healthy for at least two years. Application of lower concentrations (10% sprayed twice and 20% sprayed once) protected the plants only for a short period, and as early as one year after treatment mortalities were observed. The plants were not adversely affected by application of 40% phosphonate.

Two years after application, chemical analysis failed to detect phosphite in leaf tissue of the sprayed plants in any treatments, but plants which had received the three highest doses of phosphonate remained healthy.

The roots were not analysed in this trial as extraction of root material would have caused damage to plants that were required to remain undisturbed for future assessments. However it is possible that some residual phosphite was present in the roots despite lack of detectable residues in the shoots. The results of the other trials certainly indicate that the concentration of the chemical consistently attains higher levels in roots than in shoots.

The trial will be finally assessed and harvested in November, 1997. The degree and duration of plant protection associated with the various phosphonate application regimes will then be determined and destructive harvesting will allow analysis of roots for phosphite.

Figure 15. Eneabba field trial: Mortality of *B. menziesii* treated with 10, 20 and 40% phosphonate sprayed using a hand-held ultra low volume sprayer.

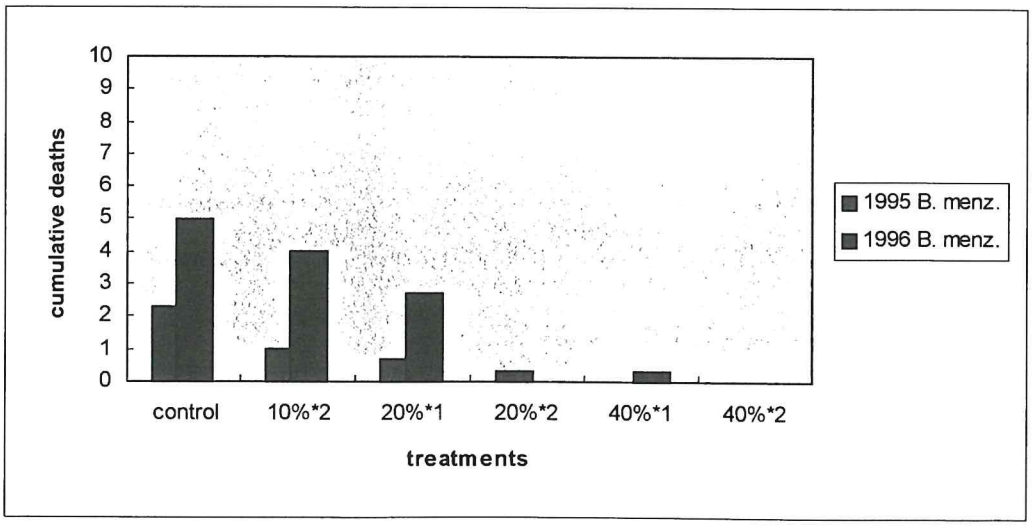
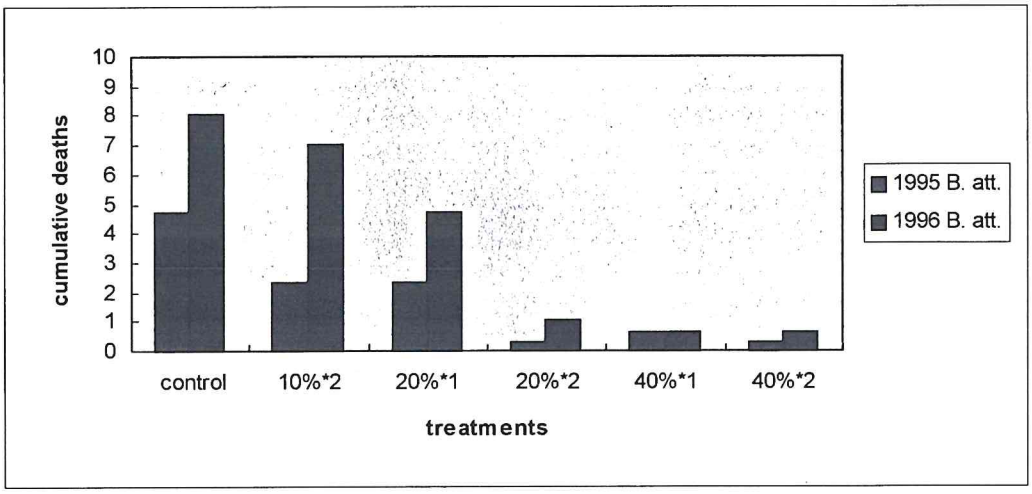


Figure 16. Eneabba field trial: Mortality of *B. attenuata* treated with 10, 20 and 40% phosphonate sprayed using a hand-held ultra low volume sprayer.



4.2.3 Field Trial at Eneabba - South (Phytotoxicity Trial)

4.2.3.1 Methods

An experiment involving *Lambertia multiflora* was established in November, 1994 to observe the ability of plants to recover from injury caused by high application rates. Single plants (located in an area affected by *P. cinnamomi*) were sprayed twice with three concentrations of phosphonate (10, 20 and 40 %) using a hand-held ultra low volume sprayer to apply the equivalent rate of 90l ha^{-1}.

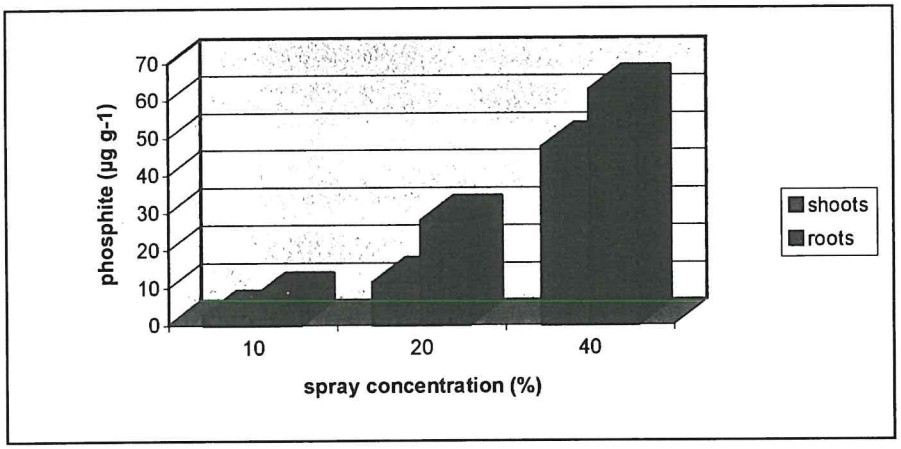
Spraying was carried out in late October and again about four weeks later. The experiment involved destructive harvests. Plant samples (roots and shoots) were taken for chemical analysis immediately after spraying and thereafter every six months. Plant mortality was recorded for all sprayed and control plots.

4.2.3.2 Results and Discussion

Chemical burning of foliage was evident on plants sprayed with phosphonate at concentrations of 20% or 40% and half of the plants sprayed with the latter concentration were killed.

The concentrations of phosphite in plant tissue several weeks after treatment were extremely high and ranged from a few hundred to several thousand μg g^{-1}. One year later, the concentration of the active ingredient had decreased significantly and as in other trials was higher in the roots than in the shoots (Figure 17).

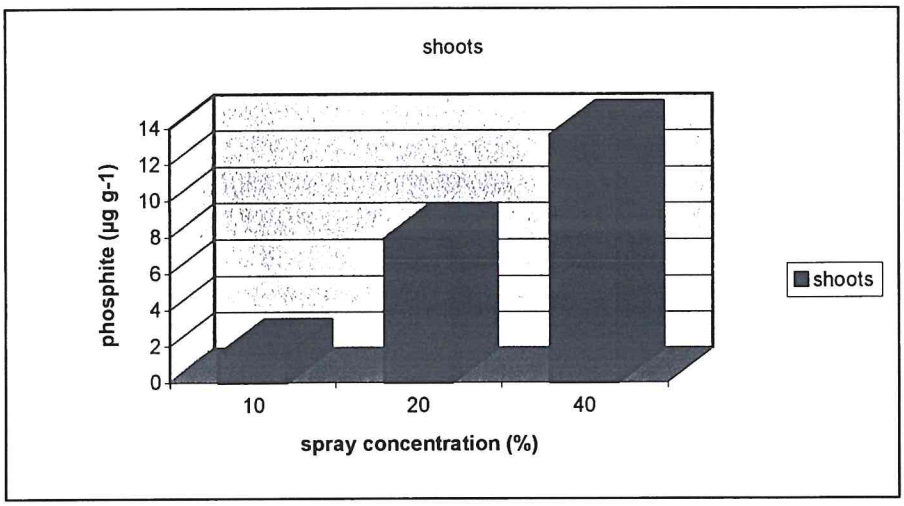
Figure 17. Concentrations of phosphite (μg g^{-1}) in leaf and root tissue of *L. multiflora* one year after treatment with 10, 20 and 40% phosphonate sprayed using a hand-held ultra low volume sprayer.



Two years after treatment the concentration of phosphite ion had decreased further in all treatments. Figure 18 shows the concentration of the fungicide residues in the leaf tissue. The ground was extremely dry and hard during the harvest and we were unable to extract the roots.

All surviving plants that were burned during spraying subsequently recovered, and produced new growth within six months. Phosphonate when applied in excessive doses causes chemical burning of the foliage and death of plants. The rate of 90l ha^{-1} applied in this trial was excessive and should not be used on native species.

Figure 18. Concentrations of phosphite ($\mu g\ g^{-1}$) in leaf tissue of *L. multiflora* two years after treatment with 10, 20 and 40% phosphonate sprayed using a hand-held ultra low volume sprayer.



4.3 EFFICIENCY OF ABSORPTION OF PHOSPHONATE

4.3.1 Methods

Phosphonate will often be used in adverse environmental conditions. Periods of rain are frequent in the south-west of Western Australia in autumn to spring, when the chemical will usually be applied. Therefore a glasshouse experiment was carried out to determine the effects of different concentrations of surfactant (Synertrol oil) and the subsequent incidence of rainfall on absorption of the fungicide by plants.

Two-year-old *Banksia brownii* plants were sprayed with 0.5% phosphonate supplemented with 0.3% or 0.6% Synertrol, and the foliage was exposed to simulated heavy rainfall (overhead sprinklers for 15 minutes) 1, 3, 5 or 7 hours after spraying. Control plants were unwatered. Two days later plants were harvested and samples taken for analysis. The results are presented in Table 7.

4.3.2 Results and Discussion

Table 7. Leaf phosphite concentrations ($\mu g\ g^{-1}$) in plants sprayed with 0.5% phosphonate and 0.3 or 0.6% Synertrol. Plants were exposed to simulated rainfall at stated times after fungicide application. Standard errors are shown in parentheses.

[Synertrol]	1 hour	3 hours	5 hours	7 hours	Control
(0.3%)	0	2.7 (0.3)	66.7 (5)	89.3 (7)	175.6 (18)
(0.6%)	0	3.0 (1)	69.3 (6)	99.7 (10)	183.7 (22)

Timing of rainfall had a major effect on the amount of phosphonate residues found in the plant tissue (Table 7) . When simulated rainfall was applied one hour after spraying no phosphite (sensitivity of analysis = 1 $\mu g\ g^{-1}$) was detectable. In the treatment watered three hours after fungicide application, low concentrations of phosphite were detected in leaf tissue and in the five or seven hour treatments absorption of phosphonate residues was significantly increased. Absorption of residues by unwatered control plants was much greater than that recorded for any other treatment. Doubling the concentration of Synertrol did not significantly improve absorption (Table 7).

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

4.4 SPECIES SENSITIVITY TO HIGH CONCENTRATIONS OF PHOSPHONATE

Three trials, initially sprayed with 10% phosphonate in 1993, were re-sprayed by aerial application of 40% phosphonate in May, 1996. During the course of this work it was established that 10% phosphonate protected plants in the experimental plots for only 12-18 months. Re-spraying with 40% phosphonate resulted in some leaf margin burning in a few

non-target species. All recently sprayed, experimental trials and some operational trials sprayed by Albany District in 1996 were surveyed for signs of chemical burning. Information derived from this included the applied concentration, the rate per hectare and severity of burning. This data provides important information on the sensitivity of plant species to different phosphonate concentrations and sets safe upper limits for prescriptions that will be used in planning future treatment trials. The species affected or unaffected by the applications of phosphonate are listed in Tables 8 and 9 respectively.

Table 8. Species affected by application of 40% phosphonate at 60l ha^{-1}. The plants had 10 to 15% of leaf area burned.

Species	Family
Agonis hypericifolia	Myrtaceae
Eucalyptus sterii	Myrtaceae
Isopogon cuneatus	Proteaceae
Petrophile diversifolia	Proteaceae
Xanthorrhoea preissii	Xanthorrhoeaceae

Table 9. Species not affected by application of 40% phosphonate at 60l ha^{-1}

Species	Family
Andersonia 2PB	Epacridaceae
Banksia attenuata	Proteaceae
Banksia brownii	Proteaceae
Banksia gardneri	Proteaceae
Banksia ilicifolia	Proteaceae
Casuarina	Casuarinaceae
Daviesia fluxuosa	Papilionaceae
Dryandra formosa	Proteaceae
Dryandra sessilis	Proteaceae
Hakea cucullata	Proteaceae
Hakea lasiantha	Proteaceae
Kingia australis	Xanthorrhoeaceae

4.5 EFFECTS OF PHOSPHONATE ON *IN VITRO* GROWTH OF WESTERN AUSTRALIAN *P. CINNAMOMI* AT DIFFERENT CONCENTRATIONS OF PHOSPHATE

4.5.1 Introduction

Phosphonic 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 infection in jarrah (*Eucalyptus marginata)* and some species of *Banksia* (Shearer and Fairman, 1991). However, the mechanism by which long term protection is afforded to native plants is unknown. Frequently, conclusions about the mode of action of phosphonate (direct or indirect) are drawn on the basis of comparison between concentrations of phosphite ion found to control the pathogen *in vivo* and those providing *in vitro* inhibition (Fenn and Coffey, 1984, Smillie *et al,* 1989).

For the purpose of this study phosphonate is the same as partially neutralised (with KOH at pH 5.7 - 6.0) phosphonic acid. Each of these terms are used to describe the commercial preparation of mono-di potassium phosphonate.

The aims of this study were to determine:

- The effect of autoclaving phosphonate amended growth media on radial growth *P. cinnamomi*

- The effect of phosphate concentration on the *in vitro* growth of isolates affecting Western Australian native plants

- The effect of phosphonate on the growth of isolates at different phosphate concentrations.

4.5.2 Materials and Methods

4.5.2.1 Design

In Experiment 1, the effect of autoclaving phosphonate-amended agar media on the growth of *P. cinnamomi* was investigated. A completely randomised block design was used with growth rate the dependent variable. Independent variables included the number of isolates tested (two), concentrations of phosphate (four), and concentrations of phosphonate (four) added to media either before or after autoclaving. There were five replicates for each combination of treatments. Controls for the experiment were media including the highest level of phosphate (10 mM) and no phosphonate. Phosphate at 10 mM was selected as the optimum concentration for growth of *P. cinnamomi*.

The third experiment compared growth rates of *P. cinnamomi* on media amended with combinations of phosphate and phosphonate at various concentrations. Phosphonate was added to the media after autoclaving in all treatments. A completely randomised block design was used with three Western Australian isolates of *P. cinnamomi*, five levels of phosphonate and five levels of phosphate. Controls for this experiment were media including the highest level of phosphate (20 mM) and no phosphonate.

4.5.2.2 Media Preparation

Media preparation was similar in Exps. 1 and 2. The basal medium was modified from Ribeiro *et al.*, (1975) with omission of β-sitosterol, and adjustment of potassium dihydrogen phosphate to levels determined for the experiments.

In Exp. 1 phosphate was added to media at the rates of 0.1 mM, 1 mM, 10 mM, or omitted. Media were then buffered to pH 6.2. A commercial preparation of neutralised phosphonic acid was added to half of the media (the heated treatments) at each concentration of phosphate to give phosphonic acid concentrations of 0, 5, 10, and 20 $\mu g\ ml^{-1}$. Agar was added and the media were autoclaved then cooled to 45°C. Prior to pouring, the phosphonate solution was added through a 0.45 μm millipore filter to each of the remaining media (the unheated

treatments) to give final concentrations of phosphonic acid of 5, 10, or 20 μg ml^{-1}. Thiamine was also added at this stage, and then media were poured, 20 ml per 15 cm petri dish. Exp. 2 did not involve phosphonate and all ingredients were added to media before autoclaving.

In Exp. 3, preparation of media was also similar to that in Exp. 1. Phosphate concentrations were 0.1 mM, 1 mM, 5 mM, 10 mM and 20 mM. However phosphonate was always added to media after autoclaving to give phosphonic acid concentrations of 0, 5, 10, 20 and 40 μg ml^{-1} for each of the five phosphate levels. The ED_{50} and ED_{90} values were calculated at each phosphate concentration for the three isolates of *P. cinnamomi* tested in the experiment.

4.5.2.3 Inoculations

Isolates of *P. cinnamomi* were stored on agar pieces in distilled water. Prior to experimentation, the fungi were subcultured to corn meal agar (CMA), and then to defined media (Ribeiro *et al.*, 1975) with β-sitosterol omitted. The experimental test dishes were centrally inoculated with 8 mm disks taken from the advancing edge of cultures growing on the defined media. Petri dishes were then incubated at 20°C in Exp. 1 and 25°C in Exp. 2.

4.5.2.4 Measurements

Measurements of radial growth were made by marking the edge of a colony at four places on each dish at various time intervals. At the end of the experiments vernier callipers were used to measure increases in colony radii and growth rates were estimated. The ED_{50} values were calculated for each phosphate concentration and were obtained from a regression line by plotting percent mycelial inhibition against log concentration of phosphonate. Extrapolating from this curve, ED_{90} values were also estimated.

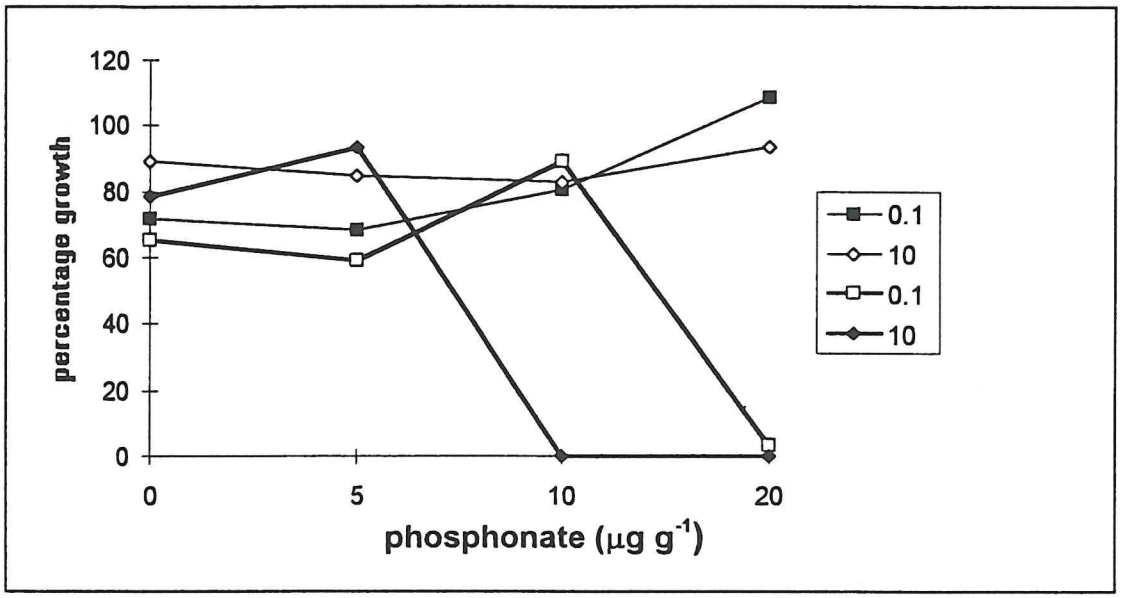
4.5.3 Results

4.5.3.1 The Effect of Autoclaving Phosphonate-amended Culture Media on Growth of *P. cinnamomi* (Experiment 1)

Growth rates of two isolates of *P. cinnamomi* were significantly higher in the heated treatment (phosphonate added before autoclaving) on media containing either low or high concentrations of phosphate (Figures 19 and 20). The growth of isolate 3478 (Figure 19) was stimulated at phosphonate concentrations above 10 $\mu g\ ml^{-1}$ in the heated treatment at both phosphate levels but particularly at the lowest phosphate concentration (0.1 mM) where it increased to around 110% of the control at 20 $\mu g\ ml^{-1}$ phosphonate. At the higher phosphate concentration (10 mM) growth of this isolate reached 90% of the control. The growth of isolate Sc72 (Figure 20) was slightly stimulated when the level of phosphonate increased to 10 $\mu g\ ml^{-1}$ in the heated treatment (at 10 mM phosphate) but dropped to less than 80% of the control when phosphonate was increased from 10 to 20 $\mu g\ ml^{-1}$.

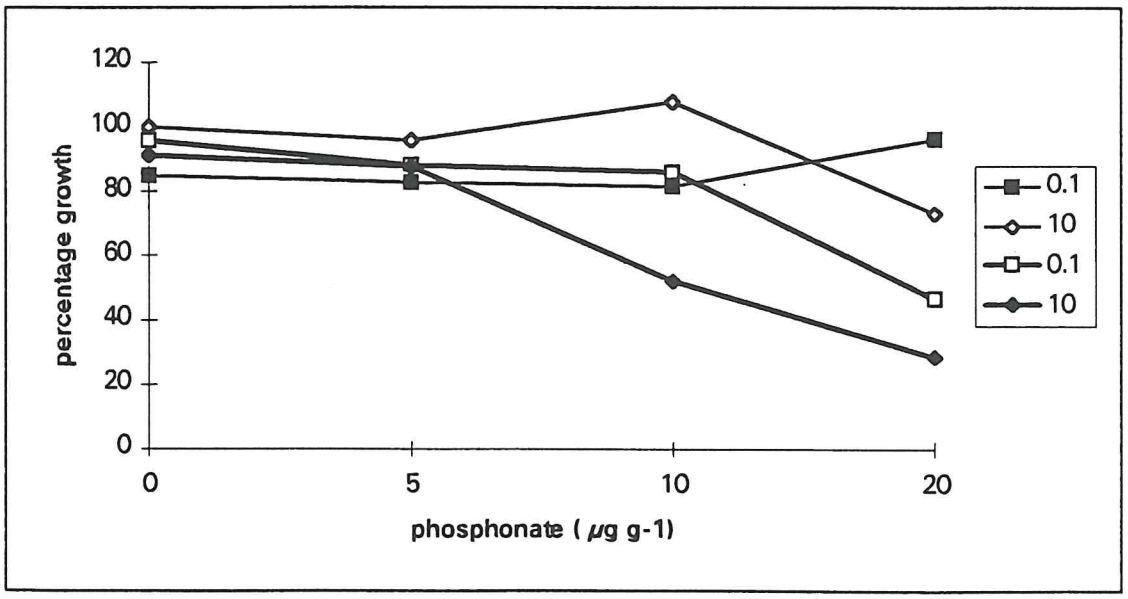
In the non-heated treatments there were differences between the two isolates in their response to increased levels of phosphonate in the medium. Isolate 3478 was much more sensitive to phosphonate and its growth was completely inhibited at 20 $\mu g\ ml^{-1}$ phosphonate, whereas the growth of isolate Sc72 was reduced to a lesser degree and ranged between 30% and 48% of the control at the same phosphonate level.

Figure 19. Growth of *P. cinnamomi* isolate 3478 (expressed as % of control growth) at various combinations of phosphate (0.1 or 10.0 mM) and phosphonate incorporated into media before or after autoclaving.



Thin lines indicate phosphonate added before autoclaving

Figure 20. Growth of *P. cinnamomi* isolate Sc72 (expressed as % of control growth) at various combinations of phosphate (0.1 or 10.0 mM) and phosphonate incorporated into media before or after autoclaving.

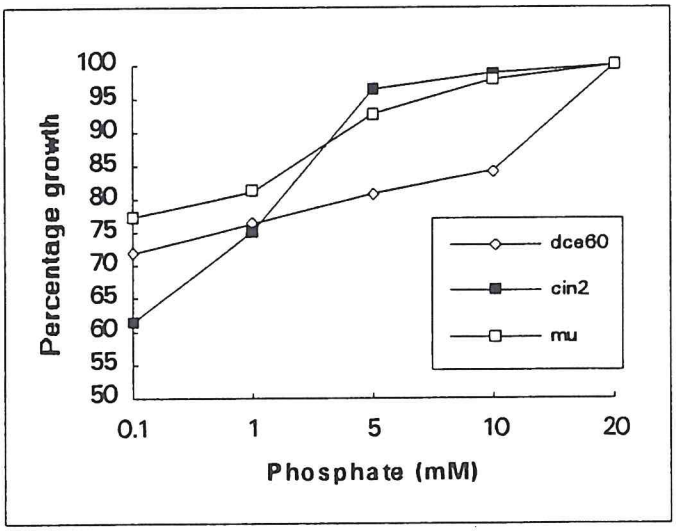


Thin lines indicate phosphonate added before autoclaving

4.5.3.2 The Effect of Phosphate on the Growth of *P. cinnamomi* (Experiment 2)

A comparison of growth between three isolates of *P. cinnamomi* at various levels of phosphate demonstrated that stimulation of growth occurred at concentrations up to 20 mM (Figure 21).

Figure 21. Growth of three isolates of *P. cinnamomi* at different concentrations of phosphate (expressed as % of control growth).



The three isolates differed in their response to phosphate. At 0.1 mM phosphate, growth of isolate cin2 was 61% of the control but this increased substantially at 5 mM or higher. Growth of isolate dce60 was 72% of the control at 0.1 mM phosphate and was gradually stimulated with increasing concentrations of phosphate. The optimum phosphate concentration for this isolate was higher than the 20 mM used in this study. The growth rate of isolate mu was 77% of the control at the lowest (0.1 mM) phosphate concentration tested and the optimum concentration for this isolate was 20 mM.

4.5.3.3 Effect of Phosphate on the Toxicity of Phosphonate in *P. cinnamomi* (Experiment 3)

The effect of phosphate on the toxicity of phosphonate to *P. cinnamomi* is illustrated in Figures 22 and 23. The three isolates varied markedly in their sensitivity to phosphonate. Isolate mu was the least sensitive and grew even at 40 μg ml^{-1} phosphonate although its rate of growth was reduced by more than 75% compared to that on a phosphonate-free medium at the same level of phosphate (0.1 mM). This isolate was still the least sensitive at the highest phosphate concentration used (20 mM) and its' rate of growth was reduced by 50% at 40 μg ml^{-1} phosphonate. Isolate dce60 was most sensitive at all phosphate concentrations and produced almost no growth at 40 μg ml^{-1} phosphonate. Growth of dce60 was reduced by around 80% at the highest phosphate level. The third isolate (cin2) exhibited intermediate sensitivity to phosphonate.

The toxicity of phosphonate was enhanced on low phosphate media for all isolates and decreased with increasing concentration of phosphate (Figures 22 and 23). At 0.1 mM phosphate, increased concentration of phosphonate resulted in marked reductions in growth of all isolates. At 20 mM phosphate (Figure 23) the reductions in growth rate at higher levels of phosphonate still occurred but were less pronounced than those noted at the lower concentration of phosphate.

ED_{50} and ED_{90} values were calculated at each phosphate concentration for the three isolates tested in Exp. 3 (Table 10). The ED_{50} values for inhibition of radial growth were very low and ranged from 1.7 μg ml^{-1} to 4.3 μg ml^{-1} phosphonate on low phosphate media (0.1 mM) and 10.5 μg ml^{-1} to 17.8 μg ml^{-1} on high phosphate media (20 mM). The extrapolated ED_{90} values ranged from 21.2 μg ml^{-1} to 81.8 μg ml^{-1} for media containing 0.1 mM phosphate and 64.6 μg ml^{-1} to 332.4 μg ml^{-1} for high phosphate media (20 mM).

Figure 22. Radial growth of three isolates of *P. cinnamomi* on media containing 0.1 mM phosphate and phosphonate at concentrations of O (A), 5 (B) or 40 μg ml^{-1}(C).

□ - isolate mu. ■ - isolate cin2. ÷ - isolate dce60.

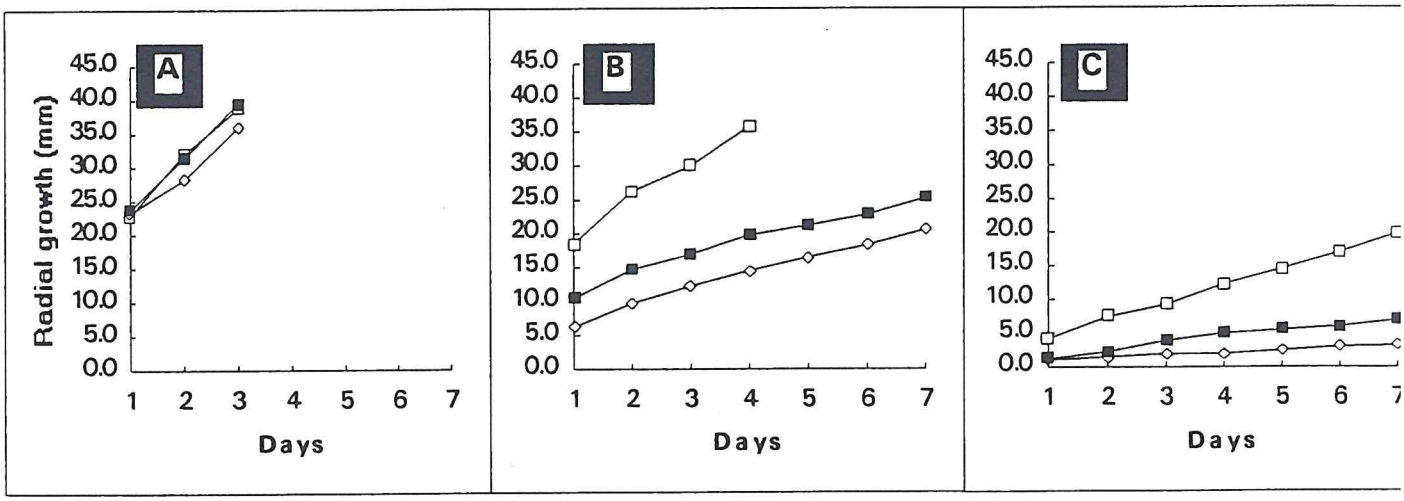


Figure 23. Radial growth of three isolates of *P. cinnamomi* on media containing 20 mM phosphate and phosphonate at concentrations of O (A), 5 (B) or 40 μg ml^{-1} (C). □ - isolate mu. ' - isolate cin2. ÷ - isolate dce60.

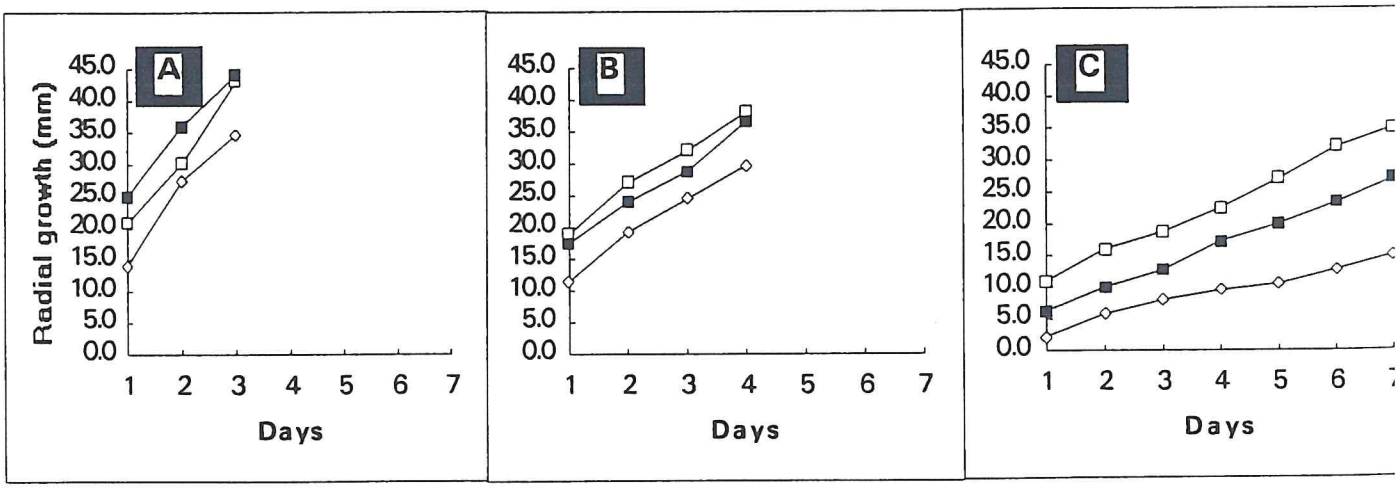


Table 10. Summary of ED_{50} and ED_{90} phosphonate concentrations for inhibition of radial growth of three isolates (mu, cin2 and dce60) of *P. cinnamomi.*

Isolate	ED_{50} ($\mu g\ ml^{-1}$)	ED_{90} ($\mu g\ ml^{-1}$)	Phosphate (mM)
mu	4.3	81.8	0.1
mu	6.4	160.9	1
mu	8.3	169.4	5
mu	13.2	203.8	10
mu	17.8	160.2	20
cin2	2.1	21.2	0.1
cin2	3.5	32	1
cin2	4.8	36.2	5
cin2	6.5	46.1	10
cin2	10.5	64.6	20
dce60	1.7	56.2	0.1
dce60	11.2	177.1	1
dce60	13	230.2	5
dce60	15.7	320.0	10
dce60	15.8	332.4	20

4.5.4 Discussion

Despite considerable research, uncertainty about the mechanism of action of phosphonate remains. Frequently conclusions are drawn on the basis of comparisons made between concentrations of phosphite ion found to control the pathogen *in vivo* and inhibitory levels recorded *in vitro* (Fenn and Coffey, 1984, Smillie *et al.*, 1989, Rochrbach and Schenck, 1985). Concentrations of phosphonate found to completely inhibit growth of the pathogen *in vitro* differ considerably in different experiments depending mainly on the level of phosphate

included in growth media. They also vary greatly between different *Phytophthora* species and between isolates of a single species (Coffey and Bower, 1984, Fenn and Coffey, 1984).

In some *in vitro* studies on the effectiveness of phosphonate in control of *Phytophthora,* the chemical was added to media before autoclaving (Coffey and Bower, 1984; Barchietto *et al.*, 1988). In our experiment it was concluded that autoclaving can cause the oxidation of phosphite ions to phosphate. Robertson and Boyer (1956) determined that the phosphite ion is stable at temperatures up to 60°C. Since autoclaving is carried out at 121°C the actual concentration of phosphite ion in the medium could be much lower than that added initially. When phosphite is oxidised, the growth of the pathogen is actually stimulated because the concentration of phosphate increases in the growth medium. This was observed in our experiment which demonstrated that growth rates of two *P. cinnamomi* isolates were significantly higher on media autoclaved after addition of phosphonate than on media supplemented with the chemical at 45°C. Controls for the experiment utilised media containing 10 mM phosphate and the observed increase in growth rate of isolate 3478 to 110% of that on control media reflected the presence of additional phosphate due to oxidation of phosphite during autoclaving. Also, isolate 3478 was much more sensitive to phosphonate than Sc72. The results of this experiment indicate that phosphonate may inhibit growth of *P. cinnamomi* at lower concentrations than previously reported. Thus, it is clear that phosphonate should be added to media after autoclaving and sterilised by microfiltration. Also, plant samples collected in the field for phosphonate analysis should be dried in low heat.

The effect of phosphate on the *in vitro* growth of P. *cinnamomi* was evaluated. The three Western Australian isolates varied in their responses to phosphate but growth stimulation was observed for all isolates at concentrations up to 20 mM in modified Ribeiro's medium. Fenn and Coffey (1984) demonstrated that increasing the concentration of phosphate in liquid culture to 8.4 mM resulted in a 11% decrease in growth of *P. cinnamomi*. Also Grant *et al.* (1992) demonstrated that for a range of *Phytophthora* species phosphate concentrations above 0.3-5 mM can be toxic and cause reduction in growth. Our study showed that the optimum phosphate concentration for growth of some isolates of *P. cinnamomi* can exceed 20 mM.

It is widely accepted in the scientific literature that the concentration of phosphate in the experimental medium affects the toxicity of phosphonate (Barchietto *et al.*, 1988; Griffith *et*

al., 1989). In the current study it was consistently demonstrated that toxicity of phosphonate is enhanced at low concentrations of phosphate and diminished by increasing the concentration of phosphate irrespective of phosphonate concentration (Figures 22 and 23). This confirms results from the literature (Fenn and Coffey, 1984) and suggests that phosphate metabolism may be one metabolic target of phosphonates. It also partially explains why phosphonate is effective in the control of the pathogen in native species (Shearer and Fairman, 1991) that are low in phosphate. In their natural state, most Australian soils are deficient in phosphorous and native plants have evolved over time to adapt to these infertile conditions (Costin and Williams, 1983). Some species developed a special mechanism, for example the proteoid roots of Banksia spp. which efficiently utilise a limited phosphorous supply. Many of the oligotrophic families including Proteaceae, have extremely low concentrations of P in their shoots (0.1 to 2.2 mg g^{-1} for Proteaceae) (Foulds 1993), whereas the phosphate content of agricultural plants can be as high as 20 mM (Bielski and Ferguson 1983).

The ED_{50} and ED_{90} values were calculated at each phosphate concentration for the three isolates tested in the experiment. The ED_{50} values for inhibition of radial fungal growth on media containing 20 mM phosphate were consistently much greater than the corresponding values for media low in phosphate (0.1 mM). There is considerable inter- and intra-specific variability in sensitivity to phosphonate within the genus *Phytophthora.* Reported concentrations for inhibition range between 5.2 and 224.4 μg ml^{-1} phosphonate (Coffey and Bower, 1984) with *P. cinnamomi*, *P. citriphthora* and *P. citricola* being the most sensitive and *P. infestans* the most resistant species. The values vary depending on the phosphate concentration in the experimental medium. However Fenn and Coffey (1984) demonstrated that concentrations of phosphate in the range of 0.084 to 8.4 mM did not affect the action of phosphonic acid on *P. cinnamomi*. The ED_{50} values estimated in our study differed at different phosphate concentrations and were lower than similar values reported in the literature for the same species. Our data show that local isolates of *P. cinnamomi* are very sensitive to phosphonate.

The *in vitro* study demonstrated that phosphate concentration affects the growth response of *P. cinnamomi* to phosphonate. However, to establish the importance of plant phosphate to the toxicity of phosphonate *in vivo* further research is required. In order to obtain a better

understanding of the mechanism of phosphonate action there is also a need to study microdistribution and translocation of phosphonate residues in plants as well as fungal phosphate metabolism.

5 OUTCOMES

- Aerial application of phosphonate is a practical tool for the control of *P. cinnamomi* in native vegetation in Western Australia. The chemical significantly reduced mortality of *B. coccinea* and *B. brownii* and can be applied to the entire plant canopy. Aerial application of phosphonate permits cost-effective treatment of medium to large areas without disturbance of the treated or neighbouring plant communities.

- Extensive experimental field trials demonstrated that phosphonate is a safe and effective agent in the control of *P. cinnamomi* in susceptible flora. The chemical does not have a phytotoxic effect when used at the prescribed concentrations and application rates.

- Safe and effective application rates have been formulated and the duration of protection achieved with the prescribed treatments has been established.

- The research has documented efficacious prescriptions for the use of fungicide phosphonate and it has provided a scientific basis for the management of chemical application in native plant communities affected by *P. cinnamomi*. The research findings are currently being implemented and a number of populations of rare species have been treated in several operational trials designed to protect them from infection by *P. cinnamomi* in the south-west of the State.

- The effect of phosphonate on *in vitro* growth of *P. cinnamomi* was established and Western Australian isolates of the pathogen were found to be extremely sensitive to the chemical.

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FINAL REPORT

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ENVIRONMENT AUSTRALIA

MAY 1997

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