M 188: Biology and ecology of *Phytophthora citricola* in native plant communities affected by mining.

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Summary

The aim of this project was to develop an understanding of the disease dynamics caused by *Phytophthora citricola*, and to provide a scientific base for the management and control of the pathogen in native plant communities affected by mining. *Phytophthora* related diseases are a well-known threat to some plant communites of the southwest of Australia. *P. cinnamomi* in particular is a destructive pathogen, causing dieback in forests, woodlands and heathlands. *P. citricola* is geographically the most widespread of the *Phytophthora* species recovered from the southwest of Australia, and is tolerant of a more extreme range of environmental conditions. *P. citricola* is also unique in that it has been recovered from upland positions in the landscape.

P. citricola Sawada is the second most frequently isolated *Phytophthora* species from the northern jarrah (*Eucalyptus marginata* Donn. ex Smith) forest and the northern sandplains. Recovery from natural vegetation elsewhere in Australia however has not been widely reported. In 1983, the morphology of two isolates of *P. citricola* from New South Wales was described. Prior to this, the pathogen had only been reported twice from Australian forests. *P. citricola* was first recorded in Western Australia in 1970, in a description of a number of *Phytophthora* species pathogenic to citrus in Western Australia. Since then, *P. citricola* has been extensively recorded from plant communities north and south of Perth. Impact of disease caused by *P. citricola* in native plant communities is often low in comparison to *P. cinnamomi*. Where *P. citricola* is often a problem however, is in regeneration of jarrah on rehabilitated bauxite mined pits, where significant losses of regenerating seedlings occur in some years.

The objectives of the project were addressed by examining the biology, ecology and taxonomy of isolates of *P. citricola* local to the southwest. It was previously known that *P. citricola* could survive asymptomatically in soil, and was frequently recovered from soil baiting undertaken by Alcoa of Australia Limited in their soil surveys prior to mining. A survey was conducted in order to determine if *P. citricola* was associated with forest roads and had been introduced via the roads, or was evenly distributed throughout the forest and a natural component of the soil mycoflora. Results showed that *P. citricola* was positively associated with roads surveyed, with recovery of *P. citricola* declining away from the road into the adjoining forest. The most probable source of introduction is from infested soil on vehicles using the roads.

This project established the importance of oospores in the life-cycle of *P. citricola* in soil and plant tissue. *P. citricola* is a homothallic species, and readily produces a sexual spore type, known as oospores, in culture. The role of oospores in the lifecycle of *P. citricola*, and in plant tissue and soil had hitherto been unreported, with little understanding of the mechanisms employed by *Phytophthora* species to survive in the absence of host material. Oospores were shown to be produced in soil and plant material, and were able to survive extended dry and hot periods *in situ*. Oospores were still viable after six months at two field sites,

and 18 months in soil in the laboratory. Oospores were capable of further development into sporangia if environmental conditions were conducive for sporangial development. Examination of the relative persistence of the different propagule types of *P. citricola* established that only oospores were important in long-term survival in soil.

The intraspecific variation of *P. citricola* was addressed in a number of ways. Analysis of genetic variation at isozyme level resolved two major subgroups within local isolates of *P. citricola*. One subgroup was confined to the forested areas of the state, and was genetically invariable. The other subgroup was more widespread, and conformed more closely to published descriptions of *P. citricola*. The forest subgroup was also distinct in cultural and morphological characters. The presence of such variation identified between the two subgroups may warrant a redescription of the species; with the forest subgroup instated as a separate taxon.

Options for control available to managers of infested land include the use of fungicides. Phosphonate is currently the most promising chemical in use against *Phytophthora* related diseases, and has been used successfully against *P. cinnamomi* in native plant communities in the southwest. The efficacy of phosphonate against *P. citricola* was examined using a common northern sandplains overstorey species *Banksia prionotes*. Phosphonate successfully inhibited lesion development of all isolates tested.

Management recommendations.

Current methods of disease control and management are based on prevention of infestation of a healthy area, or the minimisation of impact of outbreaks on already infested land. The development of these management strategies were a result of improved understanding of the life-cycle of *P. cinnamomi*, and of factors affecting disease dynamics. *P. cinnamomi* readily produces water-borne zoospores in moist soil, with higher population densities in soil in winter during times of high soil moisture. Movement of *Phytophthora*-infested soil on vehicles travelling between tracts of land and watersheds is a primary means of spread of the fungus. Thus most management strategies are aimed primarily at preventing spread of soil infested with zoospore cysts.

The presence of heat and dessication-resistant oospores of *P. citricola* in soil means that spread of *P. citricola* in soil may not be restricted to winter. Additionally, infestations of *P. citricola* are often asymptomatic, increasing the possibility of inadvertent spread of *P. citricola* by movement of infested soil. Consequently, management practices developed for *P. cinnamomi* may not always be appropriate for *P. citricola*. However the above factors preclude the development of management practices feasible in their application to field based operations. In situations where the presence/absence of *P. citricola* is critical, soil baiting may be carried out. However as this is a time and resource-consuming exercise, it is not always a practical management option, particularly given the ubiquitous nature of the pathogen. It is therefore

recommended that standard dieback hygiene be carried out in the symptomatic presence of *P. citricola*. In the absence of symptoms, dieback hygiene at the discretion of the land manager is appropriate.

The application of phosphonate may be an effective method of controlling disease in minesite rehabilitation. Preliminary studies of phosphonate against *P. citricola* on a single host alleviated disease symptoms. In incidences of high impact, or where suppression of disease symptoms is critical, application of phosphonate may be a useful tool for land managers, however further work needs to be done to fully evaluate the effectiveness of phosphonate for large-scale application in disease control.

Section 1. Distribution of *Phytophthora citricola* on soil surface and at depth in the northern jarrah forest.

Abstract

A survey of three roads in the northern jarrah forest was conducted to determine the relationship between *P. citricola* and roads. Three roads were sampled from the surface, 10- 20 cm depth and from the caprock surface at 10 or 20 m intervals along, and up to 30 m into adjoining forest.. Greater recovery of *P. citricola* was recorded from the surface of two of the three roads, with recoveries declining with distance into adjacent forest. *P. citricola* was also recovered more often from the caprock surface than the road surface. Introduction was probably via infested soil deposited by vehicles using the roads.

Introduction

Phytophthora citricola Sawada root rot is well established in plant communities of southwestern Australia, in particular on the sandplains north and south of Perth and the jarrah *(Eucalyptus marginata* Donn ex Smith) forest. *P. citricola* has been frequently isolated from dead and dying jarrah seedlings on rehabilitated bauxite mined pits in the jarrah forest, and from a host of species from the northern sandplains. Unlike other *Phytophthora* species recovered from this region, *P. citricola* has been isolated from upland positions in the landscape. Additionally, the presence of *P. citricola* is not always apparent - *P. citricola* is frequently recovered from soil with no obvious symptoms of disease in plant overstorey. This may be due either to *P. citricola* being dormant in these instances, or *P. citricola* may be active in the soil but not affecting plant hosts.

Shea (1975) has shown that impact of *P. cinnamomi* Rands is greatest in low-lying water gaining areas of the northern jarrah forest. However, it can survive in upland sites with impeded drainage (Shearer and Shea 1987), and sporulation is favoured by subsurface lateral water flow (Kinal *et al* 1993). Shea *et al* (1983) detected *P. cinnamomi* up to eight times more frequently in soil taken from the surface of the lateritic layer (caprock) than from surface soil at a diseased site. In contrast to *P. cinnamomi*, the epidemiology of *P. citricola* has been poorly studied under local conditions.

In an attempt to exclude or confine the spread of *P. cinnamomi* in the northern jarrah forest, quarantine of designated areas with low incidence of *Phytophthora*-induced dieback disease was imposed in the mid-1970's (Shearer and Tippett 1989). Existing roads into these areas were closed by the use of physical barriers. However in some instances, these barriers were not been effective in controlling access, and the roads have been used by vehicles. These roads have been classified as not effectively quarantined (NEQ). Forest operations permitted in quarantined areas include logging of timber and bauxite mining. For purposes of dieback hygiene during logging and mining, and rehabilitation after mining, the *P. cinnamomi* status of an area is assessed before commencement of operations. The area is mapped by interpretation of the

understorey, with the presence of *P. cinnamomi* evident by the death of susceptible understorey and overstorey species. Sites are then demarcated as dieback or dieback-free, and mined or logged separately.

Current methods of interpretation and mapping of *P. cinnamomi* presence rely upon assessment of disease impact. These methods are unsuitable for the detection of *P. citricola* because of the asymptomatic or dormancy factor shown by *P. citricola*. Management options developed for minimisation of spread and impact of *P. cinnamomi* are therefore not suitable for *P. citricola* if the presence of the fungus cannot be mapped.

In this study, the following alternate hypotheses were proposed to explain the pattern of occurrence of *P. citricola* in the northern jarrah forest: the pathogen was associated with forest roads and had been introduced via the roads, or the pathogen was evenly distributed throughout the forest and was a natural component of the soil mycoflora. We also determined whether *P. citricola* was present at the surface of the caprock or impeding layer.

In addition, an assessment of the efficacy of established methodology for the detection of *P. citricola* in soil was undertaken. Other studies with homothallic (oospore-forming) *Phytophthora* species have shown that detection of these fungi has increased after manipulation of soil moisture prior to baiting. We therefore postulated that germination of any dormant propagules would be stimulated by manipulation of soil moisture. Methods for enhanced detection of *Phytophthora* varied from complete air-drying of soil before remoistening (*P. megasperma*, Stack & Miller 1985, *P. cactorum*, Jeffers & Aldwinkle 1987), to a period of incubation at soil moistures suitable for production of sporangia followed by flooding (*P. megasperma*, Canaday & Schmitthenner 1982 and Stack & Millar 1985).

Materials and Methods

Association of P. citricola with forest roads, and caprock surface beneath roads. Three roads, Taranna Rd, Leena Rd and a road off Banya Rd (Banya site) in the northern jarrah forest near Dwellingup were sampled between November 1992 and March 1993. Taranna Rd and Banya site were classified as NEQ, while Leena Rd was classified as dieback-free. All roads were poorly formed single roads with no constructed drainage, and little or no evidence of plant overgrowth. A high percentage of canopy cover in adjacent forest was present at all sites. Banya site and Taranna Rd were last logged under dry soil conditions in summer 1990/91, while Leena Rd was logged prior to 1980. The NEQ roads showed signs of recent use by forest visitors.

Roads were sampled twice; a preliminary survey to determine whether *P. citricola* was present on roads was conducted in spring 1992, and an intensive follow-up survey into the adjacent forest was conducted in summer 1992/93. In the preliminary survey, road surfaces were sampled every 10 or 20 m for up to 420 m. In the intensive survey, surface samples were taken from the road centre in the same positions as in the

preliminary survey. Samples into the adjacent forest were taken at each 10 or 20 m interval, at 3-5, 8-10, 18-20 and 28-30 m into the forest from both sides of the road (Fig. 1.1). Samples were taken at 0-5 cm depth, and from the caprock surface after removal of the overburden. Additionally, at Taranna Rd and Banya site, samples at 10-20 cm depth were taken from the centre of the road and 8-10 m into the forest. At each sample point, ten subsamples of 20-30 g were randomly taken over two m^2. Soil at 10-20 cm was sampled by driving a 5 cm diameter pipe into the soil to the appropriate depth (Fig. 1.2).

P. citricola in undisturbed forest. Two roads in relatively undisturbed jarrah forest at both Jarrahdale and Dwellingup were sampled in May 1993. Sites were chosen because no recent logging or other operation involving extensive soil movement had occurred in the recent past. The Dwellingup site was in an area designated as a Management Priority Area (MPA), with restricted access to forest visitors. No record of logging was available for the Jarrahdale sites. Dwellingup sites were probably last logged in the 1940's or 1950's (CALM logging records).

For each road, four transects perpendicular to the road were established 20m apart. Each transect was sampled at 20m intervals up to 300m into the forest. Surface soil only was sampled, with subsamples from each sample point taken as described above.

Baiting for presence of P. citricola. In the laboratory, all samples were thoroughly mixed and a subsample baited for presence of *Phytophthora* species using *Eucalyptus sieberii* seedlings, using the method of Marks and Kassaby (1974). An adaptation of the extended bioassay of Stack and Millar (1985) was also used; samples were allowed to dry out for 5-7 d before being re-flooded and baited a second time in an attempt to break dormancy of any propagules present.

Results

Association of P. citricola with forest roads. P. citricola was isolated from the surfaces of all three roads, and from adjoining forest at Taranna Rd and Leena Rd. No apparent symptoms of disease were evident at any site. At the Banya site, only three of 252 (1.2%) samples yielded *P. citricola,* all of which were taken from the road surface.

At Taranna Rd, *P. citricola* was positively associated with the road ($(\chi^2 = 16)$ p <0.001, Fig. 1.3, Table 1.1). The road centre (0 m) yielded the greatest percentage (92%) of isolations of *P. citricola* from samples taken from the soil surface. There was no apparent spatial relationship of *P. citricola* along the road, with recoveries evenly distributed the length of the road sampled. Recovery of *P. citricola* declined away from the road into the adjacent forest, with isolations from within 8-10 m of the road greater than from distances >8m from the road ($(\chi^2 \geq 3.6)$ $p< 0.10$). At this site, *P. citricola* was recovered more frequently from the

Fig. 1.1. Taranna Road, Dwellingup. Soil sampled for presence of *Phytophthora citricola* from around pegs. Samples taken from road centre, 3-5, 8-10 18-20 and 28-30 m into adjacent forest.

Fig. 1.2. Taranna Road, Dwellingup. Soil sampled for presence of *Phytophthora citricola* at 10-20 cm depth by the use of a soil corer.

surface of the caprock than either the soil surface or 10-20 cm depth (1.2 and 1.8 times respectively) (Table 1.1).

0m	*				* o	o	o	o	o
20m	*	*			* +	o	*	o	*
40m					* +	*	o		*
60m				*	* +		o	*	
80m					* + o	o	+		
100m					* o	o	+ o		
120m					* o	o	o		o
140m				o	* o	* o			*
160m	*			o	* o	* + o	o	*	
180m					* o	*			
200m				* o	*		*		
220m		*							
	28-30	18-20		8-10	3-5	0	3-5	8-10	18-20	28-30

Distance from road centre (m)

Fig. 1.3. Distribution of *Phytophthora citricola* along and up to 30 m into adjoining *Eucalyptus marginata* forest at Taranna Rd.

* samples positive for *P. citricola* at soil surface
+ samples positive for *P. citricola* at 10-20 cm depth
o samples positive for *P. citricola* at caprock surface

No association between proximity to the road and frequency of isolation of *P. citricola* was found at Leena Road (Table 1.2). Greater recovery of *P. citricola* from the caprock surface at this site was consistent with

that of Taranna Rd, with the fungus being isolated 2.3 times more frequently from the caprock surface than from the soil surface.

	Distance from road (m)									
Soil depth (cm)	28-30	18-20	8-10	3-5	0	3-5	8-10	18-20	28-30	%
0-5	3	2	2	4	11	3	3	1	3	29%
10-20	*	*	0	*	5	*	2	*	*	19%
Caprock	*	*	3	5	5	7	5	2	2	35%
%	25%	17%	14%	38%	58%	42%	28%	12%	21%	

Table 1.1. Total number of soil samples yielding *Phytophthora citricola* from road surface and up to 30 m into adjoining *Eucalyptus marginata* forest at Taranna Rd. Samples taken every 20m for 220m. * samples not taken from these positions, % percent samples positive for presence of *P. citricola*.

P. citricola in undisturbed forest. P. citricola was not detected at either of the two undisturbed Jarrahdale sites or at one of the two Dwellingup sites (total of 200 samples taken). However, from one site at Dwellingup, *P. citricola* was isolated from 4 of a total of 64 samples (6%). These four isolations were all clustered around an old disused log landing.

Suitability of standard baiting technique for detection P. citricola. Detection of *P. citricola* increased after the soils were dried and re-wet (Table 1.3). Of a total of 91 positive samples, taken from Taranna Rd and Leena Rd, 50 (55%) were positive for the presence of *P. citricola* after the first baiting bioassay. After the second baiting, *P. citricola* was recovered from 60 (66%) samples, 41 (45%) of which were new isolations. *P. citricola* was not able to be recovered after the second baiting from 19 (21%) of samples initially positive for the fungus.

	Distance from road centre (m)					
Soil depth (cm)	8-10	3-5	0	3-5	8-10	%
0-10	1	0	3	1	2	10%
Caprock	4	5	3	3	1	23%
%	18%	18%	21%	14%	11%	

Table 1.2. Total number of samples yielding *Phytophthora citricola* from road surface and into adjoining *Eucalyptus marginata* forest at Leena Rd. Samples were taken every 10m for 130m. % percent samples positive for presence of *P. citricola*.

	Surface			10-20 cm			Caprock		
Distance from road	1st baiting	2nd baiting	New isolations	1st baiting	2nd baiting	New isolations	1st baiting	2nd baiting	New isolations
Road centre	5	13	9	2	3	3	8	5	0
3-5 m	2	6	6	*	*	*	12	13	8
8-10 m	5	4	3	0	2	2	8	9	5
18-20 m	3	0	0	*	*	*	1	1	1
28-30 m	4	2	2	*	*	*	0	2	2
% samples +ve for *P. citricola*	11%	14%	11%	6%	14%	14%	19%	19%	10%

Table 1.3. Numbers of soil samples from Taranna and Leena Rds positive for presence of *Phytophthora citricola* after first and second baiting. Samples taken from road surface, 10-20 cm depth and caprock surface. * locations not sampled, New isolations - *P. citricola* only recovered after second baiting.

Discussion

A considerable spatial variation was shown by *P. citricola* within and between sites examined. Recoveries of *P. citricola* at Banya site and Taranna Rd were consistent with the initial source of introduction of the fungus into the area being via the roads. The most probable source of introduction is from infested soil on vehicles using the roads.

The distribution pattern of *P. citricola* into adjacent forest at Leena Rd was not consistent with that observed at Taranna Rd. This may be due to the 8-10 m from the road centre sampled at Leena Rd being too short a distance to avoid road effects. At Taranna Rd, *P. citricola* was isolated more frequently from distances up to 8-10 m from the road than from distances beyond 10 m. Secondly, Leena Rd was classified as dieback-free. Vehicular access had been more effectively restricted than either of the two other roads surveyed, with consequent reduced probability of infection occurring since quarantine measures were initiated in the 1970's. However, minor forest and wood products had recently been gathered from this site prior to mining, consequently *P. citricola* may have been introduced and dispersed from the road surface during these operations. This would have resulted in an irregular pattern of *P. citricola* dispersal, as these activities are not necessarily centred around the road. Quarantine measures prior to this possibly account for reduced recovery from the road surface itself.

Only three recoveries of *P. citricola* were made from the road surface at Banya site, with no recoveries from either the adjacent forest or the caprock surface. This pattern is consistent with the hypothesis that *P. citricola* has only recently been introduced to this site, and had not yet dispersed.

Recoveries of *P. citricola* at Taranna Rd from all depths sampled declined as distance from road increased. Higher recoveries of *P. citricola* at the surface of the caprock than at the soil surface or at 10-20 cm depth

agree with the observations of Shea et al. (1983) and Shearer and Shea (1987) for *P. cinnamomi*. The temperature and moisture buffered environment at depth favours the survival and sporulation of *P. cinnamomi*, and lateral transmission of the fungus was aided by subsurface water flow over a concreted duricrust horizon (Kinal *et al.* 1993). The pattern of spread of *P. citricola* from the road suggests that spread of this species may also be aided by lateral transmission in subsurface water flow across the caprock surface, and may have been transferred in water percolating down through the soil horizon.

In southwestern Australia, *P. citricola* is more tolerant of drier soil conditions than other *Phytophthora* species (Stukely, pers. comm.), and has been frequently isolated from upland sites in the northern sandplains (Hart *et al.* 1991). Soil matric potentials at the caprock surface at time of sampling for *P. citricola* were below -1500 kPa (after overburden removal and a number of days exposure of caprock surface to the summer sun). This tolerance of dry soil conditions is in strong contrast to *P. cinnamomi*. In a study on the seasonal population fluctuations of *P. cinnamomi* on infested jarrah sites, Shearer and Shea (1987), could not recover *P. cinnamomi* from surface soil of upland sites in the jarrah forest during the dry summer period. Recoveries decreased with soil matric potentials below -10kPa, with highest recoveries and inoculum densities occurring in mid-winter.

The reasons for the detection of *P. citricola* from an apparently undisturbed site at Dwellingup are unclear. Samples at this site were inadvertently taken from outside the area protected by the MPA, and may have been subjected to disturbance from bush-users. Only remnants of old-growth forest remain in the northern jarrah forest, and are consequently a strong attraction for recreationsts and researchers. It is possible that *P. citricola* was introduced to the area in infested soil by a forest visitor, or alternatively the fungus was introduced during logging operations and has persisted since that time. Logging history for this site was the same as for the MPA (probably 1940's or 50's), and a disused log landing was apparent near the site of the isolations. Further inspection of the site for signs of disturbance was impossible as the area was burnt a few weeks after sampling. However, the possibility that *P. citricola* is a natural component of the jarrah forest mycoflora occurring at low densities cannot be dismissed.

Detection of *P. citricola* from soil samples was considerably improved after manipulation of soil moisture. The inclusion of a second period of flooding further enhanced recovery of *P. citricola*, though not always from the same sample. The importance of the first flooding period is not lessened as a result.

The importance of oospores as a survival mechanism for *P. citricola* in local soils has not been shown, however the inclusion of a dry/wet cycle has enhanced detection of homothallic (oospore forming) species from soils in other studies. These include increased detection of *P. citricola* and *P. cactorum* from nursery soils (Drilias *et. al.* 1984), and *P. megasperma* f. sp. *glycinea* and *P. megasperma* f. sp. *medicaginis* from naturally infested soil (Canaday and Schmitthenner 1982, and Jeffers and Aldwinkle 1987). Oospores of *P. megasperma* f. sp. *medicaginis* when added to field soil were able to be detected both before and after drying

of the soil (Stack and Millar 1985). Oospores of other *Phytophthora* species have been shown to be important in long-term survival in the field. Bowers (1990) stated that *P. capsici* overwintered in the field in low numbers of viable oospores, while oospores of the heterothallic species *P. infestans* survived for up to 8 months when buried in non-sterile field soil (Pittis and Shattock 1994).

P. citricola has the ability to form oospores in soil (see section 4) and is probably surviving in the field in this form. It is widely accepted that zoospores, sporangia and hyphae of *Phytophthora* species do not persist under dry soil conditions (Duniway 1983). Recovery of *P. citricola* from the road centre and the caprock surface in the absence of vegetation at soil matric potentials well below -1500 kPa in summer suggests survival by means of a dormant or resilient propagule. In addition, the increased isolation of *P. citricola* from samples after manipulation of soil moisture would infer persistence by oospores. Samples positive for the presence of *P. citricola* after the first baiting often did not yield the fungus a second time. This would be expected if only a single, or dormant propagule/s was surviving in the sample, which was spent after the initial baiting. The second baiting after drying of the sample probably detected the presence of oospores of *P. citricola* after dormancy had been broken.

The recovery of *P. citricola* from soils that had initially tested negative prior to being allowed to dry out, suggests that the population levels of *P. citricola* have been underestimated in the past, when the standard baiting procedure has only included one baiting. Thus the extent of the fungus in southwestern Australia may also be in question.

Section 2. Differentiation of local isolates of *P. citricola* into subgroups based on isozyme analysis, morphology and cultural characteristics, and variability in pathogenicity.

Abstract

Three clearly defined subgroups were identified by isozyme analysis of 132 isolates of *P. citricola* recovered from plant communities of the southwest. One subgroup was confined solely to forested areas of the state. This subgroup was also distinct from other subgroups with slower growth on corn meal agar, the presence of sporangia with elongated necks and was insensitive to phosphonate in vitro. The presence of such variation may warrant a redescription of the species, with the forest subgroup instated as a separate taxa.

Introduction

Morphological features used in the taxonomy of *Phytophthora* were described by Waterhouse (1963) in her "world key", in which 43 species of *Phytophthora* were recognised. However, when developing this key, she noted that it was not intended for definitive use in identifying *Phytophthora* species, and was only to be regarded as an interim document. A more refined key has yet to be developed, and the systematics of *Phytophthora* taxonomy are continuously debated in the literature. The continuous rather than discrete nature of the small number of morphological characters used for species identification has often led to difficulties in correctly identifying and delimiting species.

The taxonomy of *P. citricola* has in the past been the subject of dispute. Tucker (1931) considered *P. citricola* to be synonymous with *P. cactorum* on the basis of growth and temperature characters, and similarities of oogonia between the two species. Chester (1932) reinstated *P. citricola* as a separate taxon to *P. cactorum*, when he described two distinct forms pathogenic to lilac. This second form he called *P. cactorum* var. *applanata*, without identifying its affinity with *P. citricola*. In 1957, Waterhouse recognised *P. cactorum* var. *applanata* as a separate taxon on the basis of semi-papillate sporangia, and *P. citricola* was resurrected as a valid species.

P. citricola is now thought to be one of the more morphologically well characterised *Phytophthora* spp. However, molecular and biochemical techniques are becoming increasingly important in detecting variation and resolving taxonomic boundaries between *Phytophthora* species.

Determination of intraspecific variability of *P. citricola* is also important when considering if a pathogen has been introduced or is indigenous to native plant communities.

In this section, cultural, morphological and pathogenic variation of isolates of *P. citricola* recovered from the known geographical range in southwest Australia was examined. Biochemical variation was determined by isozyme analysis.

2.1 Isozyme analysis

Using isozyme analysis Oudemans *et al* (1994) recently resolved 125 *P. citricola* isolates recovered world-wide into five distinct subgroups. They also observed a close relationship between a subgroup of avocado host-specific isolates of *P. citricola* with *P. capsici* and *P. citrophthora*; this subgroup was more closely related genetically to the morphologically divorced species than to the other *P. citricola* subgroups. Vallavieille and Erselius (1984) and Erselius and Vallavieille (1984) also noted genetic diversity within *P. citricola*, and a molecular affinity between *P. citricola* and *P. citrophthora*, in a comparison of total protein profiles of three and six *Phytophthora* species.

To determine the genetic relatedness of isolates of *P. citricola* recovered from plant communities in southwestern Australia, isozyme variation of 132 isolates was examined. Four isolates from non-native plant species and one from beneath forest canopy from New South Wales were included for comparative purposes.

Materials and methods

Isolates studied and culture maintenance. Information on isolates studied is listed in Table 2.1.1. All local isolates were recovered from soil or infected host material by baiting with either *E. sieberii* cotyledons, or lupins. Isolates were maintained on corn meal agar (CMA, Oxoid) at 23°C in the dark.

Isozyme analysis and scoring. Fungal material for analysis was prepared using the method described by Oudemans (1991). Twenty mL V8 broth was inoculated and allowed to grow for 6 d at 23°C in the dark. Mycelium was harvested by vacuum filtering off supernatant broth and thoroughly rinsing hyphal mat with distilled water. This material was then placed into Eppendorf vials and stored at -70°C. Enzymes were extracted by grinding frozen mycelium in 50-100 µL of extraction buffer (0.1 M Tris, pH 7.0 + 1 drop β mercaptoethanol). TEM running buffer was used for all enzyme systems, comprising Tris 19.4 g, EDTA 0.74 g, MgCl 0.8 g, glycine 8 g, histidine 2 g, and glutamic acid 1 g.

Cellulose acetate plates were used for separation of enzymes by electrophoresis according to the methods of Coates (1988). Details of enzyme systems examined are listed in Table 2.1.2.

Data analysis. One isolate was included in all gels for standardisation of scoring. Isolates were grouped into electrophoretic types of identical phenotypes, and data analysis was undertaken using the BIOSYS-1 computer package. A matrix of genetic similarity and genetic distance (Nei 1978) were calculated and a dendrogram generated from the distance matrix.

Table 2.1.1. Details of isolates of *P. citricola* used for isozyme analysis. E.T.; electrophoretic type. Cultures supplied by DDS; Dieback Detection Service (CALM), BS; Dr B. Shearer (CALM), Alcoa; Alcoa of

Australia Limited, DCE; Dr E. Davison (Dept of Conservation and Environment), ANU; Australian National University, HSA; Hart Simpson and Associates. * described by Gerrettson-Cornell (1983).

E.T.	Isolate	Isolated from	Location	Source	Alternative number
1	558	Soil	Ellis Creek	DDS	
1	1723	Soil	Gordon block	DDS	IMI 329676
1	2912	Soil		DDS	
1	2952	*Andersonia sp.*	Nannup	DDS	
1	0M 28R	Soil	Dwellingup	Alcoa	
1	0M 8RA	Soil	Dwellingup	Alcoa	
1	100M 3L	Soil	Dwellingup	Alcoa	
1	120 M 28R	Soil	Dwellingup	Alcoa	
1	120M 3L	Soil	Dwellingup	Alcoa	
1	120M 3L	Soil	Dwellingup	Alcoa	
1	140M 0	Soil	Dwellingup	Alcoa	
1	140M 8L	Soil	Dwellingup	Alcoa	
1	160M 3L	Soil	Dwellingup	Alcoa	
1	160M 8L	Soil	Dwellingup	Alcoa	
1	200M 3R	Soil	Dwellingup	Alcoa	
1	200M 8L	Soil	Dwellingup	Alcoa	
1	20M 3R	Soil	Dwellingup	Alcoa	
1	2A 200M	Soil	Dwellingup	Alcoa	
1	2A 80M	Soil	Dwellingup	Alcoa	
1	3A 80M	Soil	Dwellingup	Alcoa	
1	40M 8R	Soil	Dwellingup	Alcoa	
1	52K	Soil	Dwellingup	Alcoa	
1	53I	Soil	Dwellingup	Alcoa	
1	53K	Soil	Dwellingup	Alcoa	
1	60M 8R	Soil	Dwellingup	Alcoa	
1	8M 18R	Soil	Dwellingup	Alcoa	
1	9G	Soil	Dwellingup	Alcoa	
1	140M 3R	Soil	Dwellingup	Alcoa	
1	160M 0	Soil	Dwellingup	Alcoa	
1	160M 3R	Soil	Dwellingup	Alcoa	
1	180M 0	Soil	Dwellingup	Alcoa	
1	52P	Soil	Dwellingup	Alcoa	
1	52Q	Soil	Dwellingup	Alcoa	
1	53C	Soil	Dwellingup	Alcoa	
1	53H	Soil	Dwellingup	Alcoa	
1	53M	Soil	Dwellingup	Alcoa	
1	90D	Soil	Dwellingup	Alcoa	
1	HRD	Soil	Dwellingup	Alcoa	
1	MP49	Soil	Dwellingup	Alcoa	
1	MP50	Soil	Dwellingup	Alcoa	
1	MP53	Soil	Dwellingup	Alcoa	
1	DCE 236	*E. marginata*	Mundlinup block	DCE	
1	H 1046 *	Soil	Eden, NSW	ANU	DAR 35047
1	ARB12	Soil	Jarrahdale	Alcoa	
1	BBUTT14	Soil	Jarrahdale	Alcoa	
1	BBUTT27	Soil	Jarrahdale	Alcoa	
1	NX16	Soil	Jarrahdale	Alcoa	
1	NX18	Soil	Jarrahdale	Alcoa	

1	NX22	Soil	Jarrahdale	Alcoa	
1	NX29	Soil	Jarrahdale	Alcoa	
1	OM 3R	Soil	Jarrahdale	Alcoa	
1	DP11	Soil	Dwellingup	BS	
2	1177	Soil	Cape Leeuwin	DDS	
2	3480		Kings Park	DDS	
2	HSA 1210	*Gastrolobium spinosum*	Coorow	HSA	
2	HSA 1211	*Acacia sp.*	Coorow	HSA	
2	HSA 1215	*Gastrolobium spinosum*	Eridoon Road	HSA	
2	HSA 1216	*Gastrolobium spinosum*	Eridoon Road	HSA	
2	HSA 1228	*Daviesia sp.*	Tootbardi Road	HSA	
2	HSA 1237	*Banksia attenuata*	Mullering Raod	HSA	
2	HSA 1238	*Gastrolobium spinosum*	Waddi Road	HSA	
2	HSA 1239	*Gastrolobium spinosum*	Waddi road	HSA	
2	HSA 1657	Water	Cataby	HSA	
2	HSA 1680	Water	Cataby	HSA	
2	HSA 1799	*Banksia prionotes*	Cataby	HSA	
2	MOOR S12		Mooralup	DDS	
2	WANG		Wangara	DDS	
2	DP33	*Gastrolobium spinosum*	Coorow	BS	
3	3445	*Patersonia sp.*	Rocky Gully	DDS	
3	1160	Soil		DDS	
3	3460	*Grevillea prostrata*	Kings Park	DDS	
3	3465	*Patersonia sp.*	Rocky Gully	DDS	
3	3466	Soil	Eneabba	DDS	
3	3472	*Patersonia sp.*	Rocky Gully	DDS	
3	3485	*Hakea victoriae*	Fitzgerald River	DDS	
3	3486	*Dryandra quercifolia*	Fitzgerald River	DDS	
3	3487	*Jacksonia sp.*	Mt Lesueur	DDS	
3	3533	*Acacia sp*	Fitzgerald River	DDS	
3	3567	*Patersonia sp.*	Rocky Gully	DDS	
3	3576	*Banksia media*	Fitzgerald River	DDS	
3	756 A	Soil	Woodada Road	DDS	
3	756 B	Soil	Woodada Road	DDS	
3	ARB17	Soil	Jarrahdale	Alcoa	
3	BBUTT19	Soil	Jarrahdale	Alcoa	
3	C176			DDS	
3	CADDA 1	*Banksia prionotes*	Cadda	DDS	
3	DCE 10	*Actinidia chinensis*		DCE	IMI 133316
3	DP38	Soil	Beaton block	BS	
3	DP45	Soil		BS	
3	DP46	Soil	Williams Bay	BS	
3	DP48	Soil	Challar block	BS	
3	FRNP 10	*Allocasuarina sp.*	Fitzgerald River N.P.	DDS	
3	HSA 1148	*Conospermum triplinervum*	Eneabba	HSA	
3	HSA 1151	*Conospermum triplinervum*	Eneabba	HSA	
3	KAR 1	Soil	Gidgegannup	CALM	
3	KAR 2	Soil	Gidgegannup	CALM	
3	1148			DDS	
3	3286	Soil	Eneabba	DDS	
3	170M 8L	Soil	Dwellingup	Alcoa	
3	DCE 239	Soil	Taree Block	DCE	
3	DJ 785	*Banksia baxteri*	Mt Pleasant	DDS	

3	DP12	Soil		DDS	
3	DP43	*Hakea amplexicaulis*	Beaton block	DDS	
3	DP34	*Gastrolobium spinosum*	Coorow	DDS	
3	3387	Soil	Black Point	DDS	
3	3571			DDS	
3	BBUTT9	Soil	Jarrahdale	Alcoa	
3	3573			DDS	
3	CSHG	*Banksia prionotes*	Jurien	DDS	
3	170M 8R	Soil	Dwellingup	Alcoa	
4	DCE 454	*Gastrolobium spinosum*	Northern Sandplains	DCE	
4	LES B	*Macrozamia sp*	Lesueur Nat. Park	CALM	
5	3398	*Eucalyptus marginata*	Crawley	DDS	
5	571	Soil	Hamel Nursery	DDS	
5	BBUTT23	Soil	Jarrahdale	Alcoa	
5	H 1157	Soil	Geraldton	ANU	
5	OOG 234				
6	3253	*Banksia attenuata*	Yanchep	DDS	
6	3457	*Billardiera sp.*	Kings Park	DDS	
6	3458	*Conospermum sp.*	Kings Park	DDS	
6	120M 8RA	Soil	Dwellingup	Alcoa	
6	DP23	Soil	Yanchep	BS	
7	DCE 11		Dept Agriculture	DCE	IMI 134765
7	DCE 9	*Citrus aurantium*		DCE	IMI 129904
7	H 1056			ANU	
7	H 1017			ANU	
8	1221	Soil	Kalbarri	DDS	
8	3237	*Banksia prionotes*	Jurien	DDS	

Table 2.1.2. Details of enzyme systems.

Enzyme system	Locus	No. loci
Phosphoglucose isomerase	PGI	1
Fructose-1,6-phosphate	F16DP	2
Triose phosphate isomerase	TPI	1
Malate dehydrogenase	MDH	2
Glucose-6-phosphate hydrogenase	G6PDH	1
Phosphogluconate dehydrogenase	6PGD	1
Isocitric dehydrogenase	IDH	1
Malic enzyme	ME	1

Results

Eight enzyme systems were consistently active for all isolates (Table 2.1.3). Ten putative loci were resolved from the eight successful enzyme systems. F16DP and MDH stained for two loci. A further five enzyme systems frequently showed activity, however resolution of these enzymes was too inconsistent for accurate interpretation of results.

Table 2.1.3. Allelic frequencies of eight enzyme systems for three subgroups of *P. citricola*. ET; electrophoretic type, n; number isolates examined.

Sub-group	ET	n	PGI	FDP -1	FDP -2	TPI	MDH-1	MDH-2	G6PDH	6PGD	IDH	ME
1	1	54	BB	AA	CC	DD	CC	BB	CC	AA	AA	AA
2	2	15	BB	AA	AA	AA	CD	CC	BB	AA	AA	CC
	3	44	BB	AA	AA	AA	DD	CC	BB	AA	AA	CC
	4	2	BB	AA	AA	AA	BB	CC	BB	AA	AA	CC
	5	5	BB	AA	AA	CC	DD	CC	BB	AA	AA	CC
	6	6	BB	AA	AA	DD	DD	CC	BB	AA	AA	CC
	7	4	BB	AA	AA	CC	DD	BB	BB	AA	AA	BB
3	8	2	AA	AA	BB	BB	AA	AA	AA	AA	BB	BB

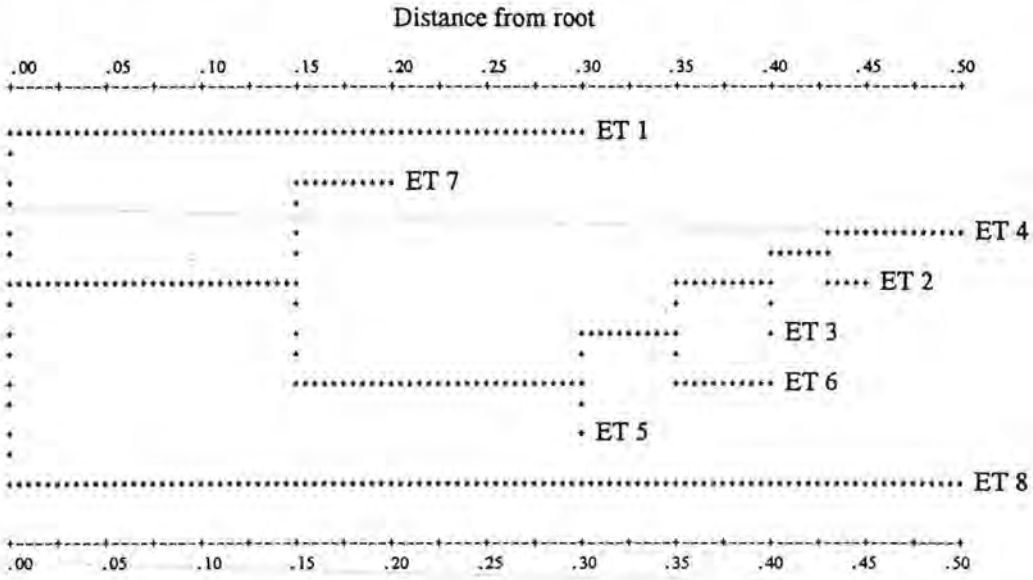
Eight electrophoretic types (ETs) were determined from the 132 isolates examined. A genetic distance matrix (Table 2.1.4) was generated (Nei 1978) from which three subgroups were resolved, based on a genetic distance between ETs of more than 0.5. Two subgroups (SG 1 and SG 3) contained only a single ET, while SG 2 contained five ETs (Table 2.1.3, 2.1.4). The evolutionary relationship between SGs based on Wagners phylogenetic tree is presented in Fig. 2.1.1.

Table 2.1.4 Nei (1978) unbiased genetic distance between subgroups.

SG	ET	1	2	3	4	5	6	7	8
1	1		**						
2	2	.772	**						
	3	.916	.025	**					
	4	.916	.079	.105	**				
	5	.916	.136	.105	.223	**			
	6	.693	.136	.105	.223	.105	**		
	7	.693	.404	.357	.511	.223	.357	**	
3	8	1.609	1.583	1.609	1.609	1.609	1.609	1.204	**

SG 1 was composed entirely of isolates recovered from the range of the jarrah forest of the southwest, with the exception of one isolate. This isolate was recovered from beneath a mixed forest canopy of *E. sieberi* L. Johnson, *E. obliqua* L'Herit and *E. cypellocarpa* L. Johnson in New South Wales. The majority of isolates were recovered directly from soil from Dwellingup and Jarrahdale while undertaking the survey detailed in Section 1. All 54 isolates of this SG were morphologically distinct from isolates of other SGs, but identical to each other (see Section 2.2).

Fig. 2.1.1 Wagner phylogenetic tree. Tree produced by rooting at midpoint of longest path.



Total length of tree = 1.418

SG 2 contained six electrophoretic types, three (ET 2, 3 and 4) of which differed only at one allele of one loci, MDH-1. MDH contained two putative alleles, with MDH-1 both homozygous and heterozygous. Isolates in SG 2 were mainly recovered from sandplains north and south of Perth (Table 2.1.1), predominantly from dying host plants. ETs 3, 5 and 6 also included isolates recovered from soil from Dwellingup and Jarrahdale (Section 1). ET 7 was the most distantly related of the ETs contained within SG 2, and was represented by four isolates - all of which were supplied by external sources. Information on hosts is only available for DCE 9, however all isolates were originally recovered by non-forestry institutions, probably from non-native vegetation.

SG 3 contained only two isolates. Isolate 1221 was recovered from Kalbarri, the northern-most extreme of the known range of *P. citricola*. This subgroup was genetically distinct from both SG 1 and SG 2, though more closely aligned to SG 1. Additionally, this subgroup was morphologically and culturally more similar to SG 1 than SG 2 (see section 2.2 and 2.3).

Discussion

Three distinct genetic subgroups of W.A. isolates of *P. citricola* were resolved by the use of isozyme analysis. This work confirms results of recent studies Erselius & Vallavielle (1984), Forster *et al* (1990) and Oudemans *et al* (1994) that show significant genetic diversity exists between isolates morphologically defined as *P. citricola*.

SG 3 was genetically distant from both SG 2 and SG 1, and comprised only two isolates. The identity of these two isolates has been confirmed as *P. citricola* by the Vegetation Health Service (formerly Dieback Detection Service) of CALM, although neither isolate of this subgroup has been identified by the International Mycological Institute (IMI).

Isolates of SG 1 have not been recovered from non-forested areas. This SG was homogenous, and was not closely related to either of the other two SGs. No variation was observed at any isozyme loci within any of the isolates of this subgroup, including an isolate from a forested area in N.S.W. This lack of genetic diversity is particularly interesting given that this subgroup contained the largest number of isolates of any subgroup. The number of isolates tested, and the range from which they were recovered would pobably have been sufficient to determine if genetic diversity at isozyme level existed at the enzymes tested within this SG. Further testing of a greater range of isozymes would reveal if this was so.

SG 2 contained isolates from a wide diversity of hosts. Isolates in ETs 2, 3, 4 5 and 6 were mainly recovered from native plants on the northern and southern coastal sandplains. These five ETs were closely related.

In a recent study on the genetic diversity of a worldwide collection of isolates of *P. citricola*, Oudemans *et al* (1994) identified five distinct subgroups. This current study included two isolates used by Oudemans *et al* (1994), DCE 10 and DCE 9. DCE 10 is contained in SG 2, ET 3 and equates to Oudemans *et al* (1994) subgroup CIT3. Isolates in CIT3 were recovered mainly from California, Australia and South Africa.

ET 7 contained only four isolates, all from sources external to the Dept of CALM; three isolates were originally isolated by personnel from the Dept of Agriculture. DCE 9 was recovered from *Citrus aurantium*. ET 7 of SG 2 equates to CIT1 of Oudemans *et al* (1994). CIT1 contained the majority of Oudemans *et al* (1994) isolates, including all isolates recovered from *Citrus* spp worldwide. It is interesting to note that ET 7 was not recovered from Australian native vegetation in either study. Perhaps the sampling intensity of agricultural/horticultural situations compared to native plant communities is reflected in this result - historically, native plant communities have been comparatively poorly studied.

Oudemans *et al* (1994) noted that the most commonly represented ETs of their study were geographically widespread, and one particular ET (CIT1) contained the majority of isolates sampled. They suggested that *Phytophthora* may exist with a clonal population structure in many situations. The total lack of genetic diversity at isozyme level exhibited by SG 1 complies with this suggestion, particularly in light of this SG including a geographically separate isolate.

2.2 Cultural variation between isolates of *P. citricola*

Introduction

Cultural patterns on media have in the past been considered a variable taxonomic character (Waterhouse, 1970), however certain species do have persistent cultural patterns that under standard conditions have been used in taxonomy. *P. citricola* is one of these, exhibiting a 'chrysanthemum' pattern. Cardinal temperatures for growth, and growth rates have also been used to differentiate *Phytophthora* to species level (Waterhouse *et al* 1983).

Cultural variation and growth rate on two media were examined to determine the extent of variation within the electrophoretic types of *P. citricola*.

Materials and methods

Isolates. A number of isolates from each ET representing the geographical range of that type were chosen.

Media. Two media were used; cornmeal agar (CMA) and Ribero's synthetic medium Ribeiro, 1978. Both media were designed specifically for growth of *Phytophthora*. CMA is a natural media, while Ribero's is chemically defined, and thus able to be reproduced identically. CMA was used for maintenance of cultures.

Isolates were inoculated onto petri dishes containing 17 mL of either media. A 3 mm diam. inoculum plug was taken from the margin of a culture actively growing on the media to be inoculated. Plates were sealed with a strip of Clingwrap, to minimise evapouration. Three replicate plates of each isolate were used for each temperature.

Incubation conditions. Plates were incubated at 15, 20 and 25 ^{o}C in the dark. Measurements were taken daily for up to 8 days. Colony patterns were determined after 5 days, using pattern descriptions compiled by (Waterhouse, 1983).

Experimental design and statistical analysis. The experiment was a randomised complete block design. A post hoc comparison of means of media and temperatures was determined by Tukey HSD test.

Results

Growth rates. Isolates with ET 1 were particularly consistent in their response to increases in temperature and changes in media. Very little variablility in any character was observed between isolates of this ET.

ET 1 was the only group to be significantly affected by media; at all temperatures, the growth rate of this group was slower on CMA than on Ribero's media (Table 2.2.1, $P<0.01$ at 15°C, $P<0.00$ at 20 and 25°C). ET2 was significantly affected by media only at 25°C, with the growth rate on Ribero's 40% faster than the growth rate on CMA at this temperature ($P<0.00$). At 20°C, the difference in growth rate between the two media was only 13%, with no difference at 15°C (Table 2.2.1). No differences in growth rates between media at any temperature was observed in the other ETs.

ET 8 was the slowest growing ET at all temperatures and both media. This ET comprised only two isolates (section 3.1), one from the northern most extreme of the range of *P. citricola* in this state. The group was particularly favoured by increasing temperatures, with growth rates increasing 3.3 times between 15 and 25°C, compared with ET 2 (the next most favoured ET) 2.7 times. In contrast, a decline in rate of growth between 20°C and 25°C, compared with 15°C and 20°C was most apparent in ET 1 on both media. The rate of growth on Ribero's at 25°C was only marginally greater (1.02 times) than at 20°C (12% on CMA), compared with an increase of 1.4 times from 15 to 20°C (42% on CMA).

Table 2.2.1. Growth rates of electrophoretic types (E.T.) of *Phytophthora citricola* at 25, 20 and 15°C on cornmeal agar (CMA) and Ribero's synthetic media. n (R) and n(C); number of isolates tested Ribero's and CMA respectively..

			25°C		20°C		15°C	
E.T.	n (R)	n (C)	Ribero's	CMA	Ribero's	CMA	Ribero's	CMA
1	13	30	4.8 ± 0.1	3.6 ± 0.03	4.7 ± 0.1	3.2 ± 0.03	3.3 ± 0.1	2.3 ± 0.03
2	8	8	10.1 ± 0.7	7.2 ± 0.2	6.2 ± 0.6	5.5 ± 0.1	3.7 ± 0.5	3.7 ± 0.1
3	17	22	7.3 ± 0.3	6.9 ± 0.2	5.6 ± 0.2	5.9 ± 0.1	4.3 ± 0.2	4.0 ± 0.1
5	2	2	7.1 ± 0.1	6.6 ± 0.2	5.4 ± 0.1	5.3 ± 0.1	2.9 ± 0.2	3.7 ± 0.1
6	2	2	5.0 ± 1.2	6.2 ± 0.2	4.2 ± 0.9	5.3 ± 0.2	3.5 ± 0.5	3.4 ± 0.2
7	3	2	6.1 ± 1.4	6.5 ± 0.2	5.3 ± 1.1	6.1 ± 0.3	4.3 ± 0.9	4.4 ± 0.2
8	2	2	3.0 ± 0.1	3.7 ± 0.04	2.0 ± 0.3	2.8 ± 0.1	0.9 ± 0.3	1.2 ± 0.04

Culture and hyphal pattern. ET 1 was invariable in culture patterns displayed by individual isolates on CMA. This pattern was "chrysanthemum", with narrow petaloid sectors. Other ETs were variable within and between groups. Patterns were predominantly uniform (without any obvious pattern) or radiate (uniformly radiate hyphae), with some isolates having a stellate and rose pattern.

Morphology of hyphae of ET 1 was also invariable. This ET had multi-branching corolloid hyphae compared with the striated hyphae with limited branching of other ETs.

Discussion

ET 1 was the only ET to be affected by media, with significantly greater growth rates on Ribero's synthetic medium than on CMA at all temperatures tested. Ribero's synthetic medium has glucose as the sole energy

source, a simple sugar readily available for fungal growth. In contrast, CMA is made from an infusion of corn, and consequently has more complex starches as the source of energy. It is possible that ET 1 is biochemically less able to metabolise this energy source than the other ETs, resulting in the slower growth rate of this ET on CMA than on Ribero's.

The smaller increase in growth rate of ET 1 between 20 and 25°C, compared with the increase between 15 and 20°C implies that the optimum growth rate for this ET is lower than for the other ETs. All other ETs showed a near linear relationship between growth rate and temperature on both media at the range of temperatures tested. Further temperatures need to be examined to determine optimum, minimum and maximum temperatures for growth for all ETs.

The uniformity of all isolates of ET 1 in growth rates, culture pattern and ability to utilise simple sugars is in contrast to other ETs, in which variability within groups in these characters was noted. Individual cultural patterns and growth rates varied markedly within other ETs, even though genetically identical at isozyme level. Interestingly, an isolate recovered from beneath an *E. sieberii* forest in Eden, N.S.W. was also identical in the above characters.

To summarise, ET 1 was the only ET to be affected by media, with significant increases in growth observed on a media containing simple sugars as the energy source. ET 1 probably had a lower optimum temperature for growth than any other ET. ET 1 also had a culture pattern different from other ETs on both media.

2.3 Variability in sporangia and oospore dimensions between electrophoretic types of *P. citricola.*

Introduction

Morphological characters used for differentiating *Phytophthora* species include various dimensions of both the sexual and asexual organs, presence/absence of chlamydospores, heterothallic/homothallic, amphigynous or paragynous antheridia and oospore abundance. Each character is assessed in conjunction with others, although not all characters of a particular species need be present for an individual isolate. The relative importance and stability of each character determines the reliability of that character for use in identification.

P. citricola is contained within Group III of the tabular *Phytophthora* key (Waterhouse, 1963) (Newhook *et al* 1978). Features consistent with this group are homothallism, paragynous antheridia and a semi-papillate apical thickening of the sporangia. Additionally, *P. citricola* has ovoid to obpyriform sporangia with an average length:breadth ratio of <1.6. Elongated sporangial necks in water are not a character of *P. citricola*. Oospores are ellipsoid or ovoid.

The purpose of this section was to compare two important morphological characters, oospore size and sporangia length:breadth ratio, of the two major electrophoretic subgroups discerned by the isozyme analysis. Oospore and ooogonia size are commonly used to differentiate local isolates of *Phytophthora* to species level, as is length:breadth ratio of sporangia. This character however may vary depending on substrate and environmental conditions.

Materials and methods

All isolates chosen were identified as *P. citricola*, being homothallic with paragynous antheridia and semi-papillate sporangia.

Oospore dimensions. Isolates were grown on Ribero's media containing β-sitosterol (Ribeiro, 1975) for oospore production. Oospore and oogonia dimensions of 30 oospores were measured after maturity, usually 10-14 days after subculturing.

Sporangia length:breadth ratio. Sporangia were produced as follows; one cm^2 sections were taken from a culture actively growing on V8 agar and transferred to a petri dish containing 15 mL of sterile soil extract. Plates were incubated in the light at between 20 and 25°C for 24 h. Sporangia were produced around the edge of agar sections. The length and breadth of 20 randomly selected mature sporangia was measured. Statistical analyisis was undertaken using Tukeys HSD test.

Results

Oospore dimensions. All oogonia and oospores measured complied with the diameters given by (Newhook, 1978) for *P. citricola* of between 21-44 and 18-38 μm respectively (Table 2.3.1 and 2.3.2). There was no significant difference in oogonia or oospore diameters between SGs or ETs ($P< 0.423$). Oospores of ET 1 ranged from 23.9 - 31.0 μm, while ET 3 oospores ranged from 24.2 - 26.1 μm.

Table 2.3.1. Oospore and oogonia diameters (μ) of isolates of three electrophoretic types of *P. citricola*.

Isolate	ET	Oogonia	s.e.	Oospores	s.e.
52Q	1	23.9	0.5	19.8	0.6
DCE236	1	25.2	0.7	21.5	0.5
53K	1	25.6	0.8	22.3	0.6
2952	1	25.6	0.5	22.0	0.5
MP50	1	27.6	0.7	22.5	0.9
NX22	1	31.0	0.5	27.0	0.6
HSA 1238	2	23.5	0.6	19.0	0.5
HSA1215	2	26.5	0.5	23.2	0.5
JW20	3	24.2	0.8	18.8	0.7
DP38	3	24.2	0.6	20.0	0.4
DCE10	3	25.6	0.5	19.7	1.6
DP48	3	26.1	0.7	22.1	0.6
3485	3	26.3	0.6	21.0	0.4

Table 2.3.2. Means of oospore and oogonia diameters of three electrophoretic types of *P. citricola*.

E.T.	n	Oogonia	s.e.	Oospores	s.e.
1	6	26	0.6	22.5	0.7
2	2	25.0	0.6	21.1	0.5
3	5	25.3	0.6	20.3	0.7

Sporangia length:breadth ratio. Sporangia were mostly semi-papillate, and attached either laterally or terminally to the sporangiophore. Shapes ranged from ovoid to obpyriform; sporangia with elongated necks were common in ET 1 (Fig. 2.3.1, 2.3.2). This character was not noted in the sporangia of any isolate of any other ET. These sporangia had a small length:breadth (L:B) ratio compared to sporangia with normal necks. Numbers of sporangia with elongated necks frequently comprised around 30% of sporangia for most isolates of this ET. When included in a random selection of sporangia for measurements, the L:B ratio for that isolate was consequently affected. Sporangial dimensions of 20 sporangia per isolate of seven ETs is presented in Table 2.3.3.

Table 2.3.3. Sporangia dimensions (μm) of isolates of seven electrophoretic types. Values are means of 20 sporangia ± standard error. Values with the same letter are not statistically different.

E.T.	n	L	B	L:B
1	5	42	23	1.83 a
2	4	43	30	1.42 b
3	12	41	28	1.50 b
4	2	42	29	1.46 b
5	5	37	26	1.40 b
6	4	38	28	1.38 b
7	3	34	28	1.21 b

Discussion

All ETs tested had similar oospore and oogonial dimensions and were morphologically indistinct in this character. Only three ETs were examined - ETs 2 and 3 were chosen to be representative of isozyme subgroup 2.

Elongated necks of sporangia were a distinguishing feature of ET 1. In the tabular key for *Phytophthora*, elongated necks are not a characteristic of *P. citricola*. The morphological characters examined in this study comply with those listed by Waterhouse (1957) in her description of *P. citricola*. Isolates within SGs 1 and 2 have been identified as *P. citricola* by the International Mycological Institute (IMI)

Fig. 2.3.1 Sporangia of subgroup 1 with elongated necks.

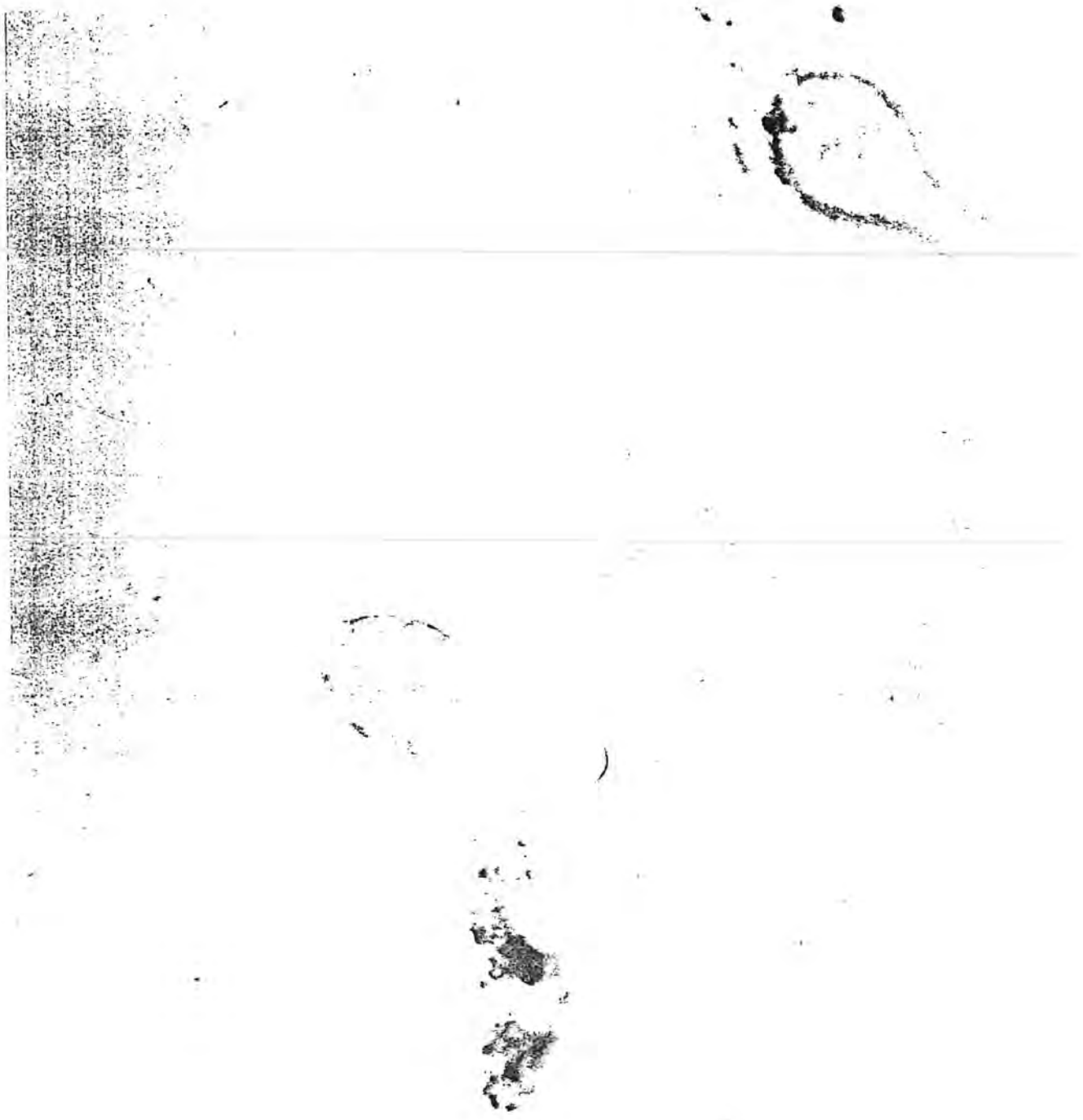
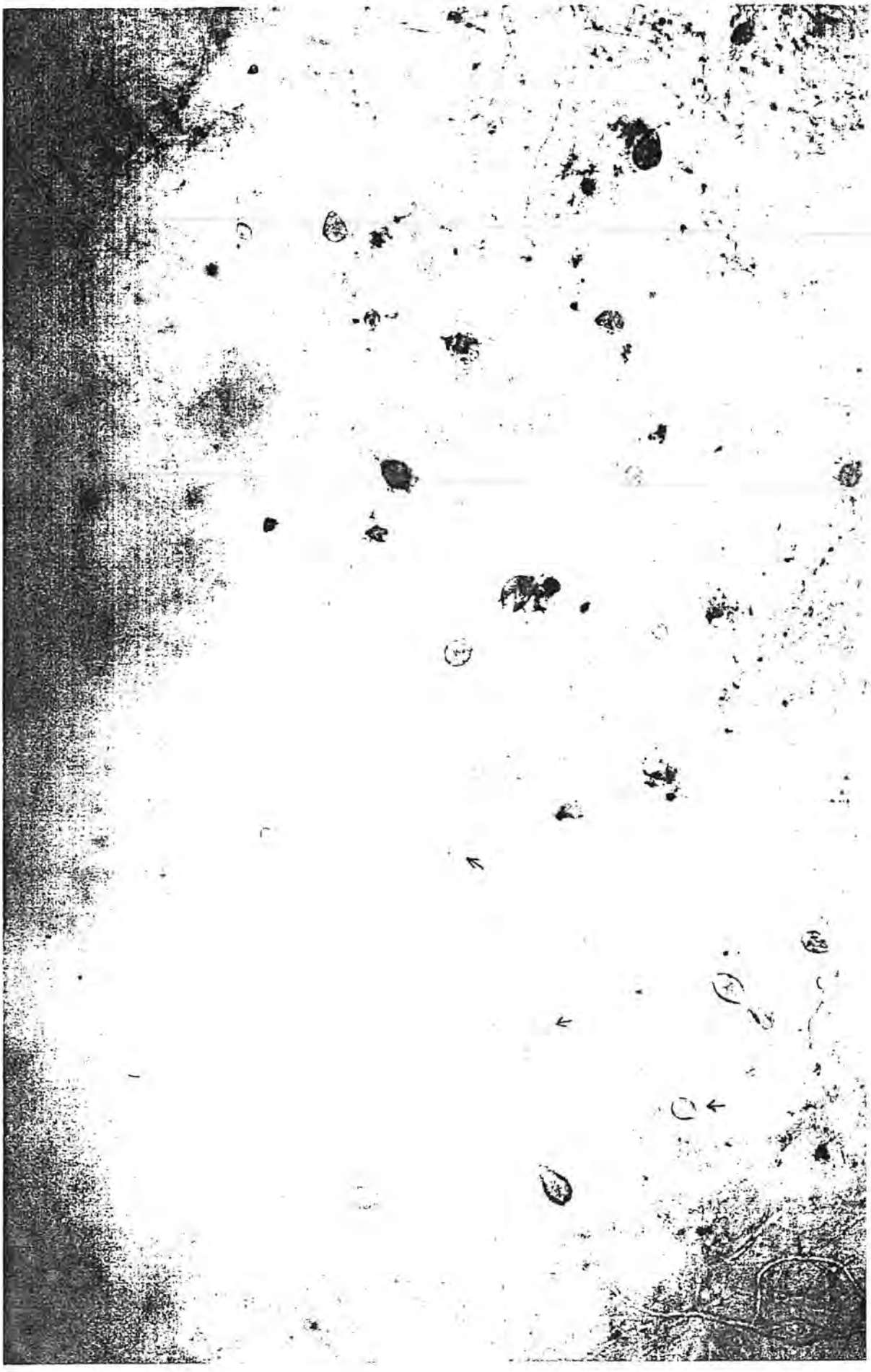


Fig. 2.3.2. Sporangia of subgroup 1 with elongated necks. Note normal shaped sporangia (arrows).



on the basis of morphological characters. SG 1 (ET 1) however was distinct from the other two SGs in a number of characters. The presence within ET 1 of sporangia with elongated necks, coupled with the other distinguishing features such as variation in culture pattern, growth rate and genetic variation at isozyme level may warrant a redescription of the species.

2.4 Variability in pathogenicity of isolates of *Phytophthora citricola* on jarrah (*Eucalyptus marginata*) clones.

Introduction

Determination of pathogenic variability is important for a number of reasons when studying the epidemiology of a pathogen. Environmental conditions may be a factor in disease expression, with disease impact exacerbated or ameliorated by favourable or unfavourable environmental conditions. It is important to establish whether this variability is due to environmental conditions alone, or if species can vary in their pathogenicity independent of ennvironmental conditions. Areas infested with highly aggressive isolates may be chosen for more intensive management than isolates less aggressive.

Isolates of proven pathogenic aggressiveness should be chosen when selecting individual isolates for resistance studies. Pathogenic variability between isolates of *P. cinnamomi* has established by Dudzinski *et al* (1993). For improved rehabilitation of bauxite mined pits infested with *P. cinnamomi*, individual jarrah (*Eucalyptus marginata* Donn ex Sm.) trees selected for genetically based resistance to *P. cinnamomi* have been micropropagated (Cahill *et al*, 1992). Several clonal lines with a range of resistance and susceptibility have been developed and screened for their ability to survive on field sites infested with *P. cinnamomi*. The relative susceptibility of jarrah clones bred for resistance to *P. cinnamomi* to other species of *Phytophthora* likely to be present in bauxite mined pits has yet to be determined.

The primary objective of the work presented here was to establish the variability in pathogenicity of geographically and genetically variable isolates of *P. citricola*. An isolate of *P. cinnamomi* of known pathogenicity to jarrah was included for comparative purposes. A second objective was to determine the relative susceptibility to *P. citricola* of jarrah clones resistant and susceptible to *P. cinnamomi*.

Materials and methods

Eight geographically widespread isolates of *P. citricola* and one isolate of *P. cinnamomi* (Table 2.4.1) were obtained either from soil or infected native vegetation. Two isozyme-typed subgroups of *P. citricola* were chosen, one confined to the jarrah forest, and one extensively isolated from plant communities of the southwest.

Jarrah clones used for inoculation included one bred for resistance to *P. cinnamomi* (1JN30 (RR)), and two susceptible clones (11JN402 (SS)and11JN379 (SS)) (Colquhoun pers. comm). Plants around 40 cm high (diameter between 4-7 mm) were stem inoculated with a five mm diam circles of Miracloth (CalBiochem) colonised with either *P. citricola* or *P. cinnamomi*. Circles were prepared by inoculating Pea Agar plates with the appropriate isolate and allowing the fungus to colonise Miracloth circles for 5 days. A colonised Miracloth circle was inserted into a small incision into the phloem tissue of the jarrah clone, covered with petroleum jelly to prevent dessication, and wrapped with nylon tape. Plants were grown in a glasshouse at ambient temperature in April 1993. Ten replicate plants per treatment were used. Experimental design was a randomised complete block design.

Lesion extension was measured above and below the inoculation point after 11 days. To determine total lesion length, stem material taken from 3 cm below to 3 cm above the visible lesion front was cut into 1 cm lengths and plated onto media selective for *Phytophthora* isolation (Tsao 1977).

Results

Variability in pathogenicity of P. citricola isolates. Lesions were visible as darker tissue extending from the point of inoculation. The fungus was often isolated ahead of the visible lesion by as much as 2 cm. Lesion length was calculated as the total length of stem tissue from which *P. citricola* was recovered on selective media.

Table 2.4.1. Geographical origin of isolates tested for variability in pathogenicity in jarrah clones.

Isolate	Host	Location	Isozyme SG
JW 20	*Banksia grandis*,	Gidgegannup	1
2952	Soil,	Nannup	2
NX 22	Soil	Jarrahdale	2
Arb 17	Soil	Jarrahdale	1
HSA 1211	*Acacia* spp	Eneabba	1
3286	Soil	Eneabba	1
1450	Soil	Walpole	1
P. cinnamomi	*Hibbertia subvaginata*		

Isolates displayed significant differences ($P< 0.01$) in their ability to invade phloem of all three jarrah clones (Table 2.4.2). In general, the two isolates recovered from the jarrah forest (SG 1) were more aggressive, resulting in consistently, if not significantly longer lesions in all clones (Table 2.4.2). All isolates of *P. citricola* produced longer lesions in the phloem of the susceptible clones than the isolate of *P. cinnamomi*.

Relative susceptibility of jarrah clones. The jarrah clone bred for resistance to *P. cinnamomi* (1JN30) was significantly less ($P<0.0002$) susceptible to invasion by all isolates of *P. citricola* than either of the two susceptible clones (11JN402 and 11JN379, Table 2.4.2). However length of *P. cinnamomi* induced lesions in the resistant clone were not significantly different from those in either of the two susceptible clones.

Table 2.4.2. Lesion length of *Phytophthora citricola* and *P. cinnamomi* after inoculation of stems of three jarrah *(Eucalyptus marginata)* clones after 11 days. Values are means of 10 seedlings, ± standard error. Means followed by the same letter are not significantly different (Fischers Least Significant Difference $P< 0.05$).

1JN30 (RR)	Lesion length (mm)	11JN402 (SS)	Lesion length (mm)	11JN379 (SS)	Lesion length (mm)
Arb 17	31.3 ± 2.9 ^{a}	*P. cinnamomi*	41.8 ± 2.9 ^{a}	*P. cinnamomi*	49.8 ± 4.2 ^{a}
JW20	35.9 ± 3.2 ^{abc}	Arb 17	48.5 ± 4.4 ^{ab}	JW20	60.4 ± 3.9 ^{b}
HSA 1211	44.8 ± 5.2 ^{bc}	JW20	49.7 ± 6.2 ^{ab}	Arb 17	63.0 ± 2.8 ^{b}
P. cinnamomi	45.3 ± 5.7 ^{bcd}	1450	57.4 ± 3.9 ^{abc}	HSA 1211	63.6 ± 5.0 ^{b}
3286	46.8 ± 4.7 ^{bcd}	HSA 1211	62.9 ± 2.6 ^{bcd}	2952	68.7 ± 2.8 ^{bc}
NX 22	47.5 ± 4.0 ^{cd}	2952	68.8 ± 2.9 ^{cd}	1450	69.2 ± 4.4 ^{bc}
1450	51.7 ± 5.7 ^{de}	3286	70.6 ± 5.7 ^{cd}	3286	74.1 ± 3.1 ^{c}
2952	60.7 ± 3.2 ^{e}	NX 22	73.5 ± 3.2 ^{d}	NX 22	77.0 ± 2.9 ^{c}

Discussion

The work reported here provides evidence for variability in pathogenicity between isolates of *P. citricola* recovered from the southwest of Australia. The use of cloned jarrah seedlings ensured minimisation of host genetic variability, enhancing detection of variation in the pathogen. Such variation in pathogenicity should be considered in biological and ecological studies of the pathogen, in particular if host resistance is an objective.

Greater pathogenic aggressiveness (the negative effect of infection on plant fitness) of *P. citricola* than *P. cinnamomi* in stem phloem of *E. marginata* is in agreement with previous work by Shearer *et al* (1988). *P. citricola* produced consistently longer lesions than *P. cinnamomi* in trees in the field. *P. cinnamomi* is however, a far more virulent pathogen than *P. citricola* in the northern jarrah forest (a virulent pathogen can infect many different plant genotypes (Jarosz, 1995), often affecting community structure. *P. citricola* is comparatively avirulent, and needs specific environmental conditions in order to cause disease symptoms in the jarrah forest (Bunny, unpublished). Community structure is rarely affected. *P. cinnamomi* however is extremely aggressive in other natural systems of the southwest, often killing all infected individuals (Shearer *et al* 1991).

Based on the tentative attempts to determine evolutionary trends in *Phytophthora* in the literature (Cooke, 1986; Brasier 1983, Brasier 1991, Brasier 1992, Hansen 1987) it can be argued that *P. cinnamomi* is more "advanced" than *P. citricola*. Saprophytism is considered to be more primitive than necrotrophy or biotrophy (Cooke 1986), and neither *P. cinnamomi* or *P. citricola* can be considered specialised. However the frequency with which *P. citricola* is recovered from soil, and the ephemeral nature of *P. cinnamomi* in soil after infection (Shea *et al* 1980), coupled with the possession of "primitive" characters by *P. citricola*

and "advanced" characters by *P. cinnamomi* (Brasier 1983) supports this argument. A wide host range (of unspecialised soil-borne pathogens) is one of the characters listed by Brasier (1983) as belonging to "advanced" species of *Phytophthora*.

Successful inhibition of *P. citricola* lesions in the *P. cinnamomi* resistant clone suggests similar mechanisms of host resistance are involved in interactions with both pathogens. The lack of variation between clones in resistance to the *P. cinnamomi* isolate is unexplained. In a similar experiment using the same clones inoculated with four isolates of *P. cinnamomi* however, the resistant clone was significantly more able to resist invasion by all four isolates than either susceptible clone.

2.5 Effect of phosphonate amendment of media on *in vitro* growth of *P. citricola*.

Introduction

Control of *Phytophthora* related diseases in natural ecosystems has not been feasible in the past. Limited control of Oomycetes in horticultural and agricultural situations has been gained by application of systemic fungicides of the classes cymoxanil, carbamate and acylalanine. The most promising class for broad scale application has proven to be the ethyl phospites (e.g. phosphonate).

In recent times Shearer *et al* (1991) has shown phosphonate to be highly effective in controlling *Phytophthora*-related dieback in native plant communities of southwestern Australia. Control of *P. cinnamomi* has been achieved for up to five years in *Banksia brownii* communities on the southcoast, and is effective in controlling *P. cinnamomi* induced lesions in jarrah (*Eucalyptus marginata*) and *B. grandis* (Shearer pers. comm.)

The mode of action of phosphonates is uncertain. Initially, it was considered that phosphonates acted by directly inhibiting growth of the fungal pathogen within the host plant (Dolan and Coffey 1988, Fenn and Coffey 1984). Phosphonates have been shown to be highly inhibitory to mycelial growth of several *Phytophthora* species *in vitro*, with a parallel observation for *in vivo* sensitivity (Dolan and Coffey 1988). *P. citricola* has been shown to be extremely sensitive to amendment of media with phosphonate (Coffey and Joseph 1985). More recently however, it has been shown that phosphonates show a complex mode of action, (Guest and Bompeix 1990 and Grant *et al* 1990) and act to stimulate a resistance response in the host, thereby inhibiting or restricting growth of the pathogen (Smith 1994).

The objectives of this section were to assess the relative *in vitro* sensitivity of the two major subgroups of *P. citricola* identified in Section 2.1.1. This was done by firstly assessing a large number of representatives from each SG to a single low concentration of phosphonate, and secondly by subjecting a fewer number of isolates to three higher concentrations of phosphonate.

2.5.1 Effect of amendment of media with 10 ppm phosphonate on isolates of SG 1 and SG 2.

Materials and methods

Forty one isolates representative of SG 1 and SG 2 were selected for study. Ribeiro's (Ribeiro, 1978) chemically defined medium was amended with 10 ppm neutralised phosphonate after autoclaving. Seventeen mL was dispensed into 9 cm Petri plates. Control plates were not amended with phosphonate. Plates were inoculated near the edge (to allow for further growth than a centre inoculation) with a 5 mm diam. disk taken from the growing edge of an actively growing colony. Plates were incubated in the dark at 20^{o}C. The colony edge was marked daily at two points, and the average growth rate per day calculated. Three replicate plates per isolate were used.

Results

A clear difference in sensitivity to phosphonate between the two SGs was observed (Fig. 2.5.1). Mycelial growth of isolates of SG 1 were unaffected by the presence of phosphonate in media at 10 ppm. Isolates within this SG were relatively invariable in their *in vitro* response to phosphonate, with percent growth of the unamended control ranging from 116% to 96%.

In contrast, isolates of SG 2 were strongly inhibited by 10 ppm phosphonate, and had a wider range of inhibition than SG 1 (Fig 2.5.1). The mycelial growth rate of the least sensitive isolate of this SG was 62% of the unamended control, falling to 8% for the most sensitive isolate.

Discussion

This study established a clear differential response to phosphonate amended media compared to unamended media of the two electrophoretic SGs identified in section 3.1. SG 1 was insensitive to phosphonate at the concentration used, in fact mycelial growth rate was slightly enhanced for some isolates. SG 2 was comparatively intolerant, with almost complete inhibition of radial growth of mycelium occuring in some isolates.

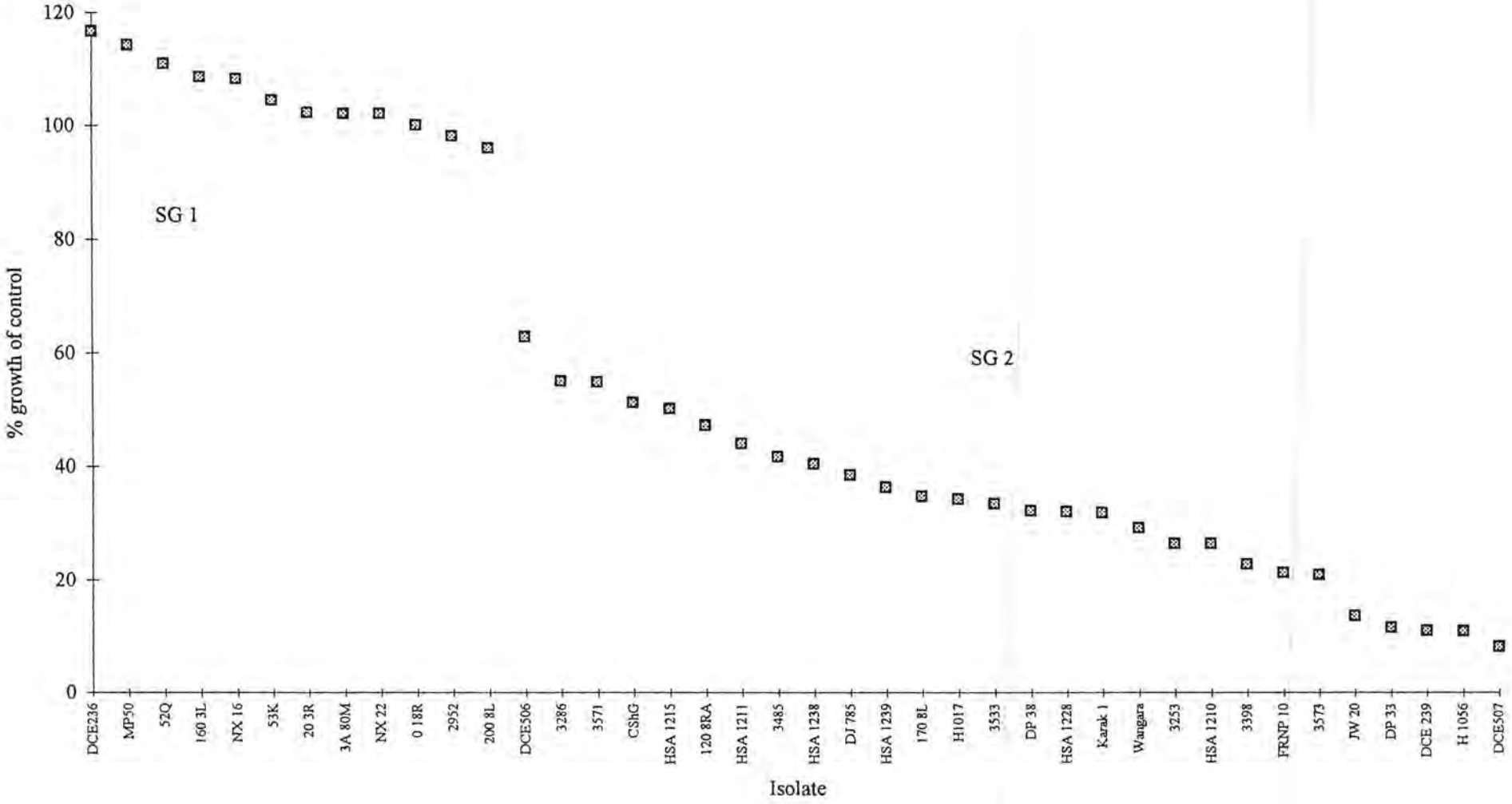


Fig. 2.5.1 Response of 41 isolates of *Phytophthora citricola* to amendment of media with 10 ppm phosphonate.

2.5.2 In vitro effect of 15, 30 and 50 ppm phosphonate on isolates of *P. citricola* of SG 1 and SG 2.

Isolates exhibiting a range of responses to phosphonate in vitro in section 2.5.1 were selected for further, more intensive exposure to phosphonate.

Materials and methods

Five isolates of SG 1 and nine isolates of SG 2 were used. Media was prepared, inoculated and measured as above. Phosphonate was added to media after autoclaving to final concentrations of 15, 30 and 50 ppm.

Results

As found in section 2.5.1, a differential susceptibility to amendment of media with phosphonate between the two SGs was found. Both SGs displayed increasing sensitivity to increasing levels of phosphonate, from 15 ppm to 50 ppm (Table 2.5.1, Fig. 2.5.1). This was more evident for SG 2 - the more sensitive SG identified in section 2.5.1.

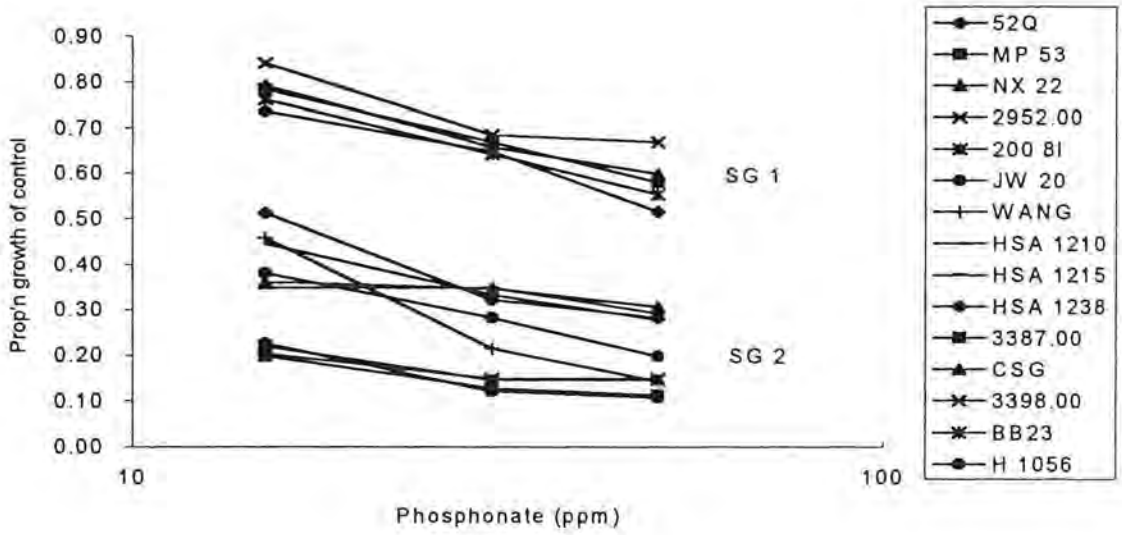


Fig. 2.5.2. Inhibition of *Phtyophthora citricola* isolates in response to 15, 30 and 50 ppm phosphonate.

Subgroup	n	Proportion inhibition of unamended media		
		15 ppm	30 ppm	50 ppm
SG 1	5	0.78 ± 0.017	0.66 ± 0.007	0.58 ± 0.027
SG 2	10	0.33 ± 0.037	0.24 ± 0.031	0.20 ± 0.025

Table 2.5.1. Proportion inhibition of unamended media of isolates of SG 1 and SG 2 in response to 15, 30 and 50 ppm phosphonate. Isolates for each SG have been combined. Values are means ± standard error.

The greatest reduction in growth rate of the most sensitive isolate of SG 1 at 50 ppm (maximum phosphonate concentration) (51%) was identical to the smallest inhibition of the most tolerant isolate at 15 ppm (lowest phosphonate concentration) of SG 2 (Fig 2.5.1).

Discussion

Radial growth of mycelium of both SGs was inhibited by the lowest concentration of phosphonate. Little variability in response to phosphonate between isolates in SG 1 was shown both in this study and in the preceding section. This finding supports the lack of variability shown by this SG in other characters examined (sections 2.1, 2.2 and 2.3). In contrast, SG 2 displayed a greater variability between isolates, possibly as a result of the slight genetic differences between ETs of this SG. This SG also showed differences in other characters tested in sections 2.1, 2.2 and 2.3.

In a study comparing variability between eight *Phytophthora* species, Coffey and Bower (1984) showed *P. citricola* to be one of the more sensitive isolates, being inhibited by up to 68% in the presence of 5 ppm phosphonate. Coffey and Joseph (1985) also showed extreme sensitivity of *P. citricola* to phosphonate, with 50% inhibition of radial growth occuring between 1.3 - 1.7 ppm. Oospore formation was also inhibited. These concentrations of phosphonate are much lower than those used in this current study. However isolates of *P. citricola* used by Coffey and Bower (1984) and Coffey and Joseph (1985) were all recovered from avocado. Oudemans *et al* (1994) showed avocado isolates to be a separate electrophoretic subgroup from other isolates collected worldwide. The results of this present study, showing a differential in vitro response to phosphonate by individual SGs, coupled with those of Coffey and Bower (1984) and Coffey and Joseph (1985) would suggest that in vitro responses to phosphonate are characteristic for a SG within a species, rather than between species as stated by Coffey and Bower (1984).

3. Seasonal effect of soil temperature and moisture on survival of different propagules of *Phytophthora citricola* at two field sites.

Abstract

The relative ability of different propagule types of *Phytophthora citricola* to survive was established at two field sites. Nylon mesh pouches containing zoospore cysts, oospores and colonised wood plugs of *P. citricola* were buried in the field at 10 cm depth each season for one year, and harvested at given intervals. Zoospore cyst decline was greater in sandy soil than gravelly loamy sand for autumn, winter and summer. In spring, amplification of numbers of zoospore cysts occurred at both sites. Oospore viability was site dependent, with greater numbers surviving after six months in sandy soil. Oospore viability decreased to at least 40% after six months. In soil under controlled conditions, oospore viability was unaffected after six months. Recovery of *P. citricola* from colonised wood plugs did not decline over six months. Only oospores, free in soil or in infected plant material are capable of long-term survival in soil.

Introduction

The role of oospores in the lifecycle of *P. citricola* in plant tissue and soil has hitherto been unreported, with little understanding of the mechanisms employed by *Phytophthora* species to survive in the absence of host material. Oospores are known to be important propagules for long-term survival of other pythiaceous fungi. Dormant thick walled oospores of *Pythium ultimum* are resistant to heat, dessication and fungicides (Stasz and Martin 1988), and Hoppe (1966) found other *Pythium* species still viable after 12 years in stored muck soil. Oospores of the alfalfa host-specific *P. megasperma* Drechsler f. sp. *medicaginis* Kuan & Erwin have been shown to be important in longterm survival in soil by Stack and Millar (1985), and Bowers (1990) concluded oospores were important propagules of *P. capsici* Leonian to overwinter in sub-zero soil temperatures.

P. citricola is a homothallic species, able to produce abundant numbers of oospores in culture. The persistence of some species of *Phytophthora* in the absence of host material has previously led to the implication, though not establishment of the presence of oospores in soil. *P. citricola* has been recovered from the road surfaces in the northern jarrah forest (section 1) (Woodman and Colquhoun 1992). Such surfaces were often devoid of vegetation, and exposed to extremes in heat and moisture, suggesting that the survival of *P. citricola* in these situations was dependant upon a spore type resistant to such environmental extremes.

Different propagule types are known to survive for significantly different periods in soil. The environmental conditions are different for each propagule type to be initiated, and the strategies employed by the fungus may be designed to optimise spread and survival of the fungus. Producton of large numbers of zoospores with short generation times (*r* strategy) may enable wide dispersal and colonisation of *Phytophthora* species,

while production of smaller numbers of oospores with a long life expectancy (*K* strategy) may allow the pathogen to remain established in a community.

The ability of *P. citricola* to produce oospores in soil was addressed by this project. A trial using four isolates of *P. citricola* established that oospores were indeed produced in soil and in infected host tissue (Bunny unpublished). This study was inititated to determine the seasonal effect of soil temperature and moisture on the ability of *P. citricola* zoospores, oospores and colonised wood plugs to survive under field conditions at two edaphically distinct sites. A *P. cinnamomi* isolate was included for purposes of comparison in the colonised wood plugs trial. Survival of oospores and plant material colonised by *P. citricola* were also assessed under controlled conditions.

Materials and methods

Experimental design and treatments. The experiment was a randomised complete block design. Two field sites with five replicate holes per season per site. Treatments consisted of zoospores and oospores of *P. citricola*, and plant material colonised by *P. citricola* and *P. cinnamomi* (colonised wood plugs). Samples were buried at 20 cm depth.

Isolates. Isolates used were recovered either from soil or infected plant material from a wide geographical range within the southwest (Table 2.1). The isolates comprised the two major SGs resolved in section 2.1. Isolates were cultured and maintained on corn meal agar (CMA) media.

Table 3.1. Geographic origin of isolates of *P. citricola* and *P. cinnamomi*. * indicates isolate included in this treatment.

Isolate	SG	Zoospores	Oospores	Colonised wood plugs	Host	Origin
JW20	1	*	*	*	*Banksia grandis*	Gidgegannup
2952	2		*	*	Soil, jarrah forest	Nannup
NX22	2		*	*	Soil	Jarrahdale
Arb17	1	*	*	*	Soil	Jarrahdale
HSA 1210	1	*	*	*	*Gastrolobium spinosum*	Eneabba
HSA 1211	1	*	*	*	*Acacia* sp	Eneabba
3286	1	*	*	*	Soil	Eneabba
1450	1	*	*	*	Soil	Walpole
P. cinnamomi				*		IMI
P. cinnamomi				*		

Field sites. Two sites were chosen. The Dwellingup site was situated in the northern jarrah (*E. marginata*) forest south of Perth. Soil type was a gravelly loamy sand. The second site was at Eneabba on the northern sandplains where kwongan heathland was the dominant plant community and soil type was a grey sand

(Fig. 3.1). Soil physical properties were analysed by Soil Science Service of CALM. Average annual rainfall for the Dwellingup site is 1100 mm yr^{-1} and 520 mm yr^{-1} at Eneabba.

Sites naturally infested with *P. cinnamomi* were selected in preference to *P. citricola* infested sites. *P. cinnamomi* is considered a more severe pathogen of jarrah and some kwongan plant species, and was to be introduced to the sites.

Propagule production and preparation. Zoospores. Zoospores were produced using a modification of the Byrt and Grant (1979) technique as follows: sterile 8 cm diameter miracloth (Calbiochem Ltd) mats were placed onto the surface of uncleared V8 juice agar (Ribeiro 1978) plates and inoculated with the appropriate isolate. After 4-5 days incubation at 22°C in the dark, mats were peeled off the agar and placed in approximately 15 mL of 2% V8 broth and incubated for 24 hours under light. Mats were then removed from the V8 broth, washed twice in minerals salts solution and returned to the incubator in 10 ml of the same solution. Sporangia were formed within 24 hours. Zoospores were induced to release by rinsing mats with sporangia in sterile distilled water and incubating the mats at 5-10°C for 5 min, then allowing mats to return to room temperature. To encyst zoospores, zoospore suspension was shaken vigourously for 2 min.

Encysted zoospores were placed into the field using the method of Morgan (1990). Cores made of 2 cm diameter PVC piping were cut into 2 cm lengths. Cheesecloth was secured over the bottom of the core, to allow close contact with soil in the field. About 10 g of soil from the respective field site was placed into the cores and wet up to field capacity. Cores were equilibrated to field capacity for 24 hours, after which a suspension of approximately 1000 zoospores of the appropriate isolate was dispensed into a hole in the centre of each core. Zoospores were covered over with soil. Cores were tied together for easy recovery at harvest, and buried in the field at both sites on the same day.

Oospores. Oospores were produced by inoculating a 9 cm diam. miracloth piece placed on V8 juice agar and allowed to grow for 30 days at 25°C in the dark. Oospores embedded into the Miracloth, which was then peeled cleanly off the agar and cut into approx. 3 cm^2 squares. Squares of all isolates were then placed in nylon mesh pouches and mixed with soil prior to being buried in the field.

Pieces of miracloth prepared as above were also placed in soil collected from both sites and kept in plastic cups in the dark at room temperature. Five cups containing 300 g of soil were used for each isolate. The same cups were sampled at each harvest. Soil moisture of soil in cups was maintained at summer soil moisture contents for both sites. To simulate summer rainfall, half of the cups containing oospores were wet up to 8% (approx. -500kPa) and 4% (approx. -50kPa) for Dwellingup soil and Eneabba soil respectively, six months into the trial. Soils were maintained at this moisture content for 7 days, allowed to naturally dry for a further 28 days then re-wet to the same soil moisture.

Fig. 3.1. Site of propagule survival trial at Eneabba, with kwongan heathland the dominant vegetation.

Colonised wood plugs. Plant material (colonised wood plugs) for inoculation was prepared by cutting 1-2 cm diameter *Banksia attenuata* R. Br. stems into 2 cm lengths. Plugs were placed into 2 litre Erlenmyer flasks and autoclaved twice, 24 hours apart. Flasks were inoculated with the appropriate isolate of *P. citricola* or *P. cinnamomi* grown on CMA for 5-7 days. Flasks were incubated for four weeks in the dark at 22°C. Fifteen colonised wood plugs per isolate were then placed into separate appropriately labelled nylon mesh pouches, mixed with soil and buried in the field.

Harvests and assessment of survival. Five replicates (one from each hole) of each propagule type per isolate from each site were sampled at each harvest.

Zoospores. Zoospores were placed in the field mid-season in autumn, winter and spring of 1993, and summer of 1993/94. Harvests were conducted at 0, 2, 7, 14, and 28 days for each inoculation period. Soil from retrieved cores was flushed with 4 ml deionised water onto a 9cm petri dish containing 15 mL P_{10}VARP selective media (Tsao 1977). The soil suspension was gently swirled to ensure even spread of soil slurry on the agar surface. Plates were incubated at 20°C. After 24-36 hours soil was rinsed off and colonies of *P. citricola* counted. To confirm identity, randomly selected colonies were transferred to CMA and allowed to grow a further 7 days.

Oospores and colonised wood plugs. Oospores and colonised wood plugs were placed into the field at the same times as zoospores in autumn, winter and spring. Oospores were harvested 3 and 6 months after inoculation, and colonised wood plugs harvested 3, 6, 9, 12 and 18 months after inoculation.

Viability of oospores was measured using the vital stain Tetrazolium Bromide (MTT) (Sutherland & Cohen, 1983). Miracloth pieces recovered from the field were flooded with 0.1% MTT and incubated for 48 hours at 35°C. After 2 rinses in deionised water, samples were examined microscopically. Viable (respiring) oospores stained pink, inviable oospores did not stain, and dead oospores stained black. At each harvest, thirty oospores per sample (replicate) were recorded, giving a total of 150 oospores (5 replicates) per isolate per site.

Assessment of oospore viability was conducted on a proportional basis, with scoring of viable, inviable, germinated and dead oospores. Percentage viability of a predetermined number of oospores has been used by other workers (Pittis & Shattock 1994 Jiminez & Lockwood 1982 and Bowers 1990). This method assumes that the total number of oospores retrieved from the field at harvest is similar to numbers initially placed into the field.

Survival of the fungus within colonised wood plugs recovered from the field was assessed by a baiting bioassay of individual colonised wood plugs using *E. sieberii* L. Johnson seedling cotlyledons. Colonised

wood plugs were immersed in approx. 50 mL glass distilled water in plastic cups and baited with 3 seedlings for up to 7 days. Cotyledons were then plated onto selective media (Tsao and Guy 1977). Initially, colonised wood plugs were split and plated directly onto selective media and observed for growth of *P. citricola*. Apparently negative colonised wood plugs were then baited. However when baited, recovery of the fungus was much greater than by plating, and further harvests were assessed by baiting alone.

Colonised wood plugs stored under controlled conditions was also assessed using the above method. As recoveries declined, colonised wood plugs were re-baited, using an adaptation of the extended alfalfa seedling bioassay of Stack and Millar (1985) as follows; after initial baiting with *E. sieberii*, all cups containing colonised wood plugs were allowed to dry out completely. After 4-7 days, the bioassay procedure was repeated.

Environmental parameters. Soil temperature. Soil temperature at two depths for each season was measured at both sites for the 28 day period zoospores were in the field. Thermistor probes connected to dataloggers were placed on the soil surface and covered with 2 mm sand to reduce radiation, and buried at 20 cm depth. Temperature was logged hourly.

Soil moisture. Soil moisture release curves for both soil types were determined using Buchner funnels and ceramic pressure plates. Seasonal soil moisture was determined from soil collected at each zoospore harvest period. Percent moisture content of field soil was determined, and used to derive soil water matric potentials from the soil moisture release curve.

Statistical analysis. Zoospores. LD_{90} of zoospores was assessed for each isolate for each season. Data were log transformed to minimise differences between observed and predicted outcomes. The regression equation for time was solved using the proportional hazards model by the equation

$$LD_{90} = (LN(10/9)) / \exp \text{intercept}^{1/\text{time}}$$

For determination of significant differences in survival between isolates within and between seasons and sites, an ANOVA on the proportion of zoospore cysts surviving at each harvest with those at day 0 was conducted. To satisfy ANOVA assumption of normal distribution, data were transformed by using the arcsin of the squareroot. As the initial zoospore population (day 0) varied between isolates, a comparison of means of numbers of zoospores recovered at each harvest was inappropriate.

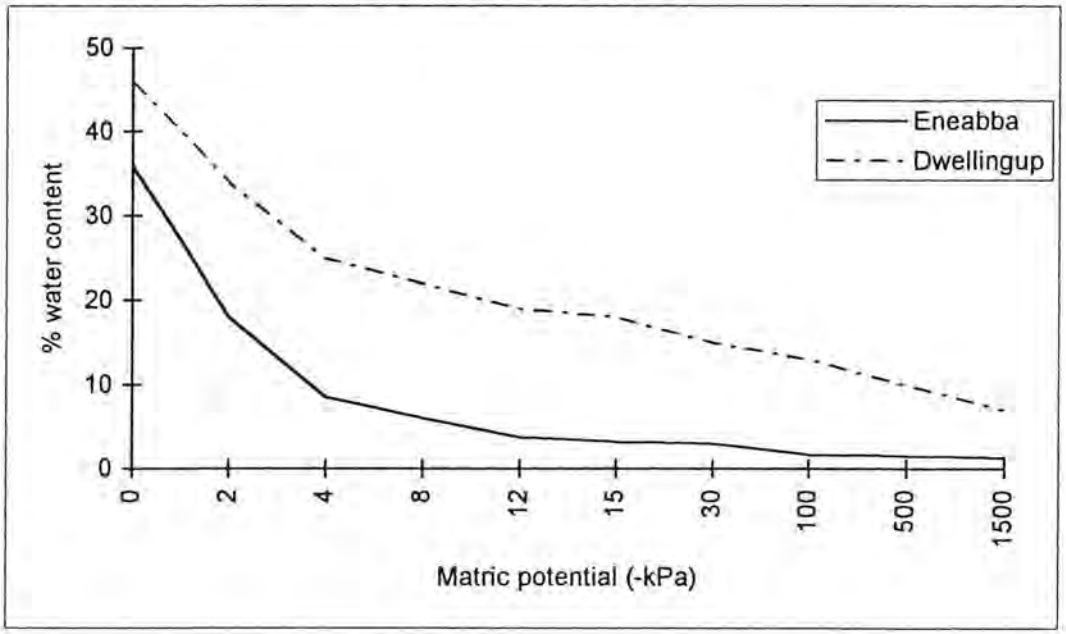
Oospores. Differences in oospore viability was determined by an analysis of variance for a randomised complete block design. An ANOVA on the proportion of viable and germinated oospores for each harvest was conducted. As with zoospore cyst recovery, data were transformed using the arcsin of the squareroot to normalise data.

Colonised wood plugs. No statistical test of differences in survival between seasons or *P. citricola* isolates was necessary, as survival of *P. citricola* in colonised wood plugs was unaffected for the duration of the experiment. A χ^2 test of significance for differences in survival between *P. cinnamomi* and *P. citricola* was conducted for each harvest.

Results

Soil properties. Dwellingup gravelly loamy sand had a greater water holding capacity than Eneabba sand at all matric potentials tested (Fig. 3.2). Water drained quickly from the sand, to below 5% at matric potentials below -2 kPa. In contrast, the Dwellingup soil at -1500 kPa had a water content of around 20% of saturation.

Fig 3.2 Water retention curves for Eneabba and Dwellingup soil.



Soil physical properties are presented in Table 3.2. Dwellingup soil had a higher proportion of silt and clay, resulting in the greater water holding capacity observed in Fig. 3.2.

Seasonal soil temperatures. Seasonal temperatures experienced at 20 cm depth at both sites for the period of the experiment show a range of temperatures within the cardinal extremes tolerated by *P. citricola* (Fig 3.3 a, b, c and d) (Smith & Davison 1992). The highest average temperature at 20 cm of all seasons recorded was 29.3^{o}C at Eneabba in summer. This was 8.6^{o}C higher than the average of 20.7^{o}C at Dwellingup for the same period. Surface temperatures over 60^{o}C were not uncommon at Eneabba for this period, with 69.8^{o}C being the highest surface temperature recorded. Winter temperatures at the two sites were similar at 20 cm,

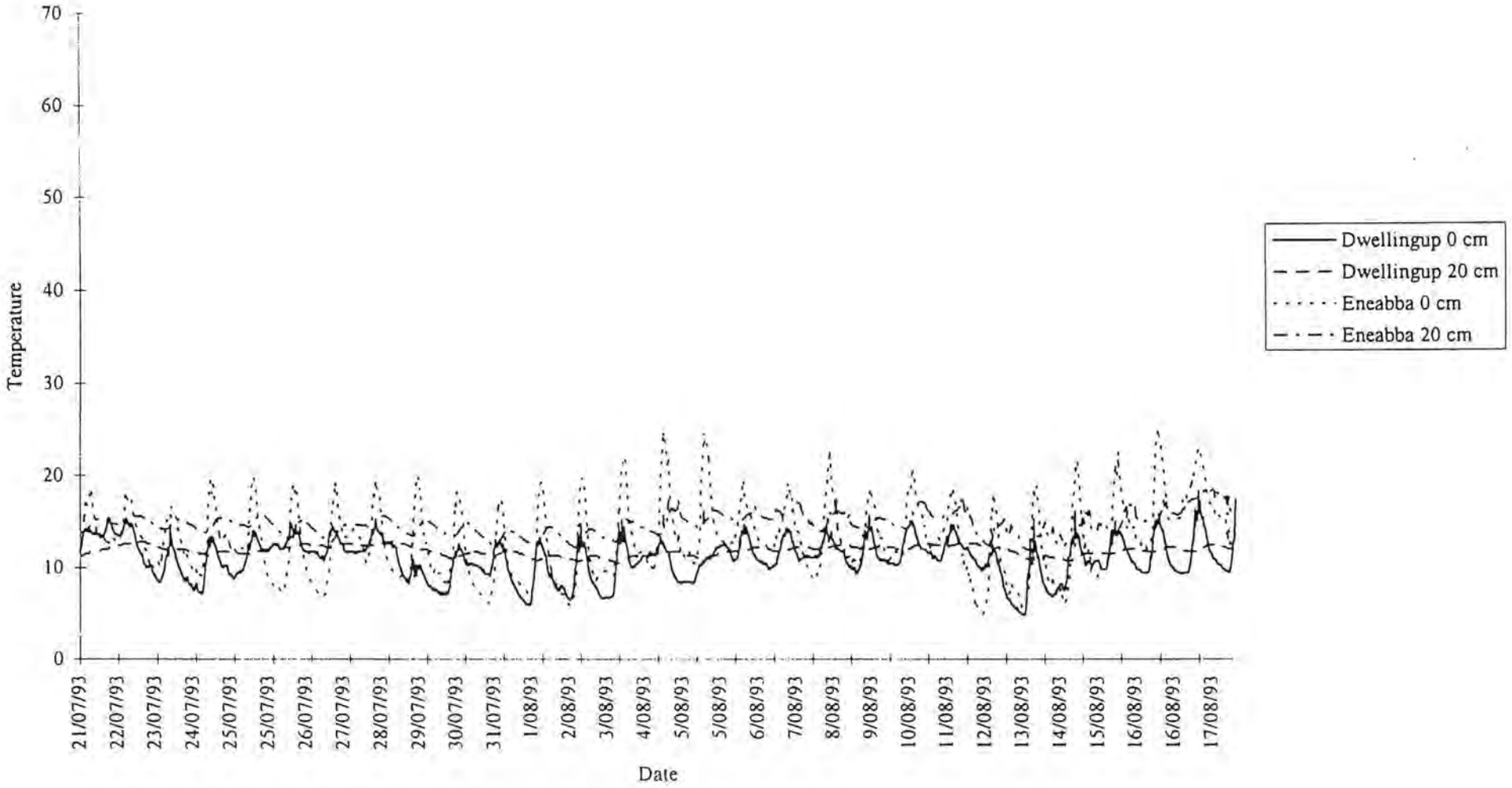


Fig. 3.3 b. Winter soil temperatures at Dwellingup and Eneabba during propagule survival trial.

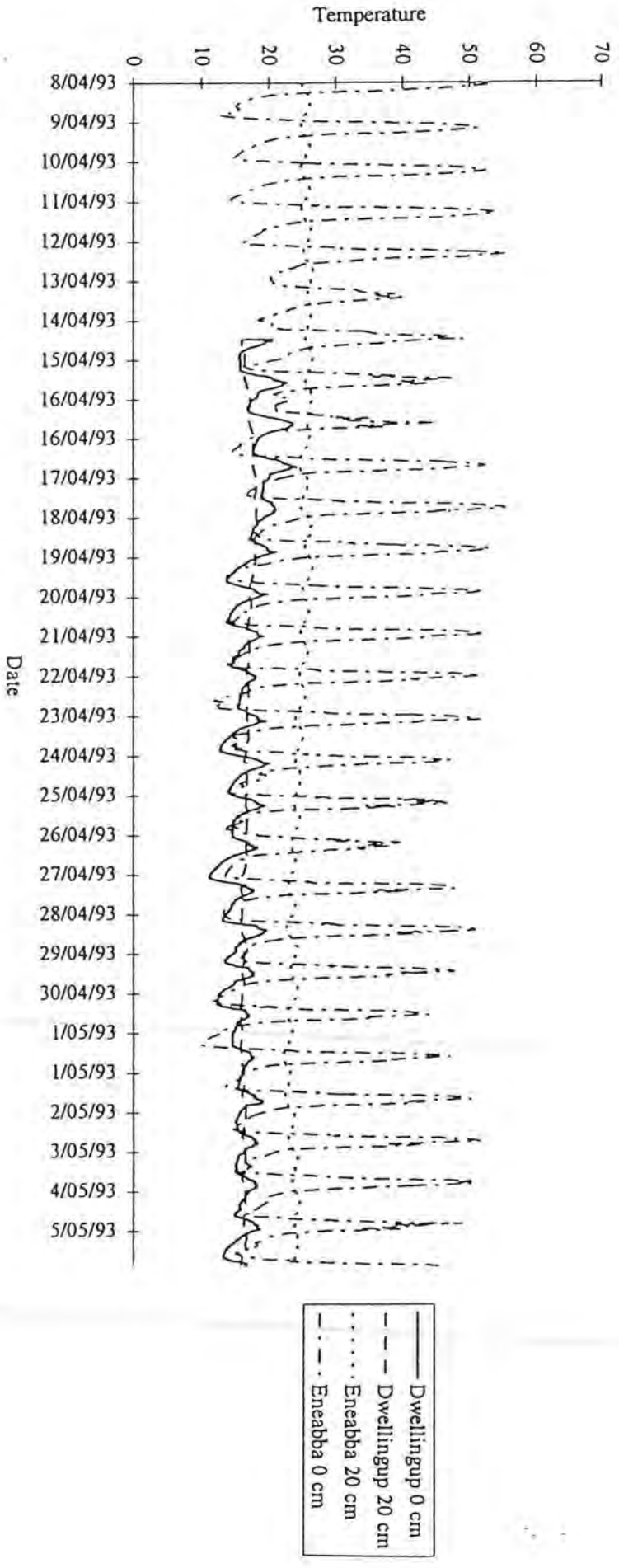


Fig. 3.3 a Autumn soil temperatures at Dwellingup and Eneabba during propagule survival trial.

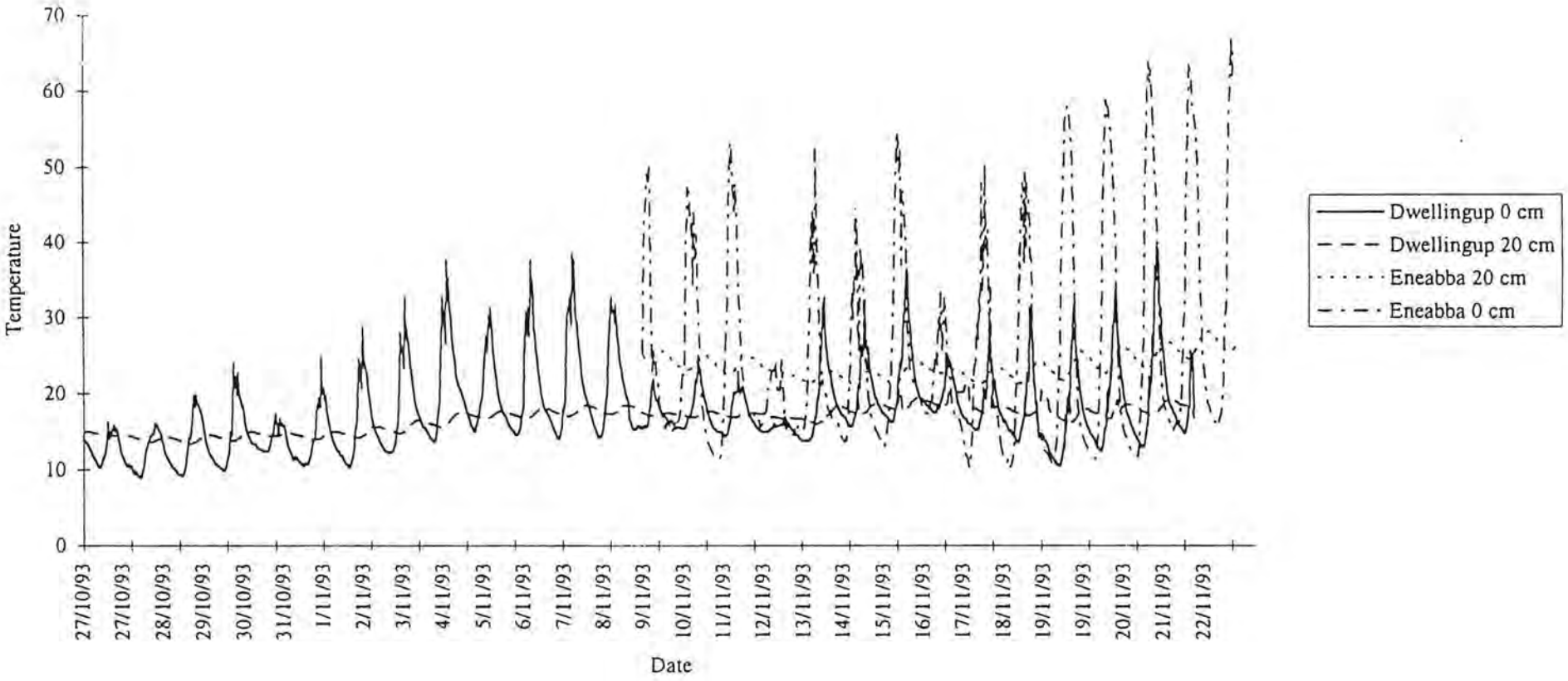


Fig. 3.3 c. Spring soil temperatures at Dwellingup and Eneabba during propagule survival trial.

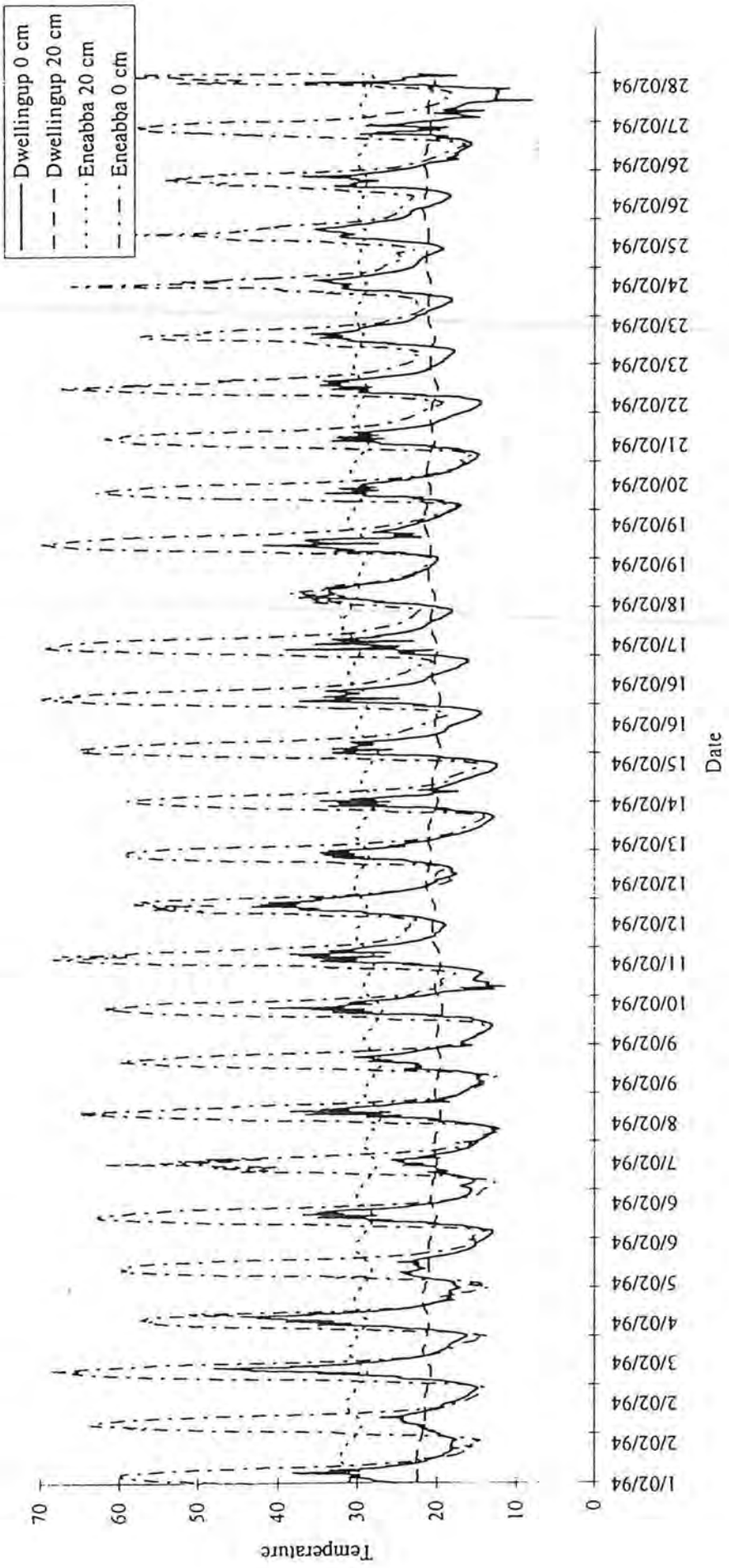


Fig. 3.3 d. Summer soil temperatures at Dwellingup and Eneabba during propagule survival trial.

averaging of 11.8°C at Eneabba and 14.7°C at Dwellingup. The lowest surface minimum temperatures for each site received during the experiment were 5°C at Dwellingup and 4.8°C at Eneabba, both in winter.

Table 3.2. Soil physical properties of Eneabba and Dwellingup soils.

Site	Particle size analysis (%)		Organic carbon	pH (%) (Water)	Electrical conductivity	Total soluble salts
Dwellingup	Coarse sand	46	1.6	5.3	18.3	0.4
	Fine sand	45				
	Silt	4				
	Clay	5				
Eneabba	Coarse sand	85	0.3	3.2	54.1	1.31
	Fine sand	14				
	Silt	0				
	Clay	1				

Zoospore cyst survival. LD_{90} values of individual isolates at Eneabba were smaller than for Dwellingup in autumn, winter and summer (Fig 3.4). Only in spring did zoospores of any isolate at Eneabba survive up to 28 days, whereas small numbers of zoospores of most isolates at Dwellingup survived longer than the 28 days tested in autumn, winter and summer (Fig. 3.4).

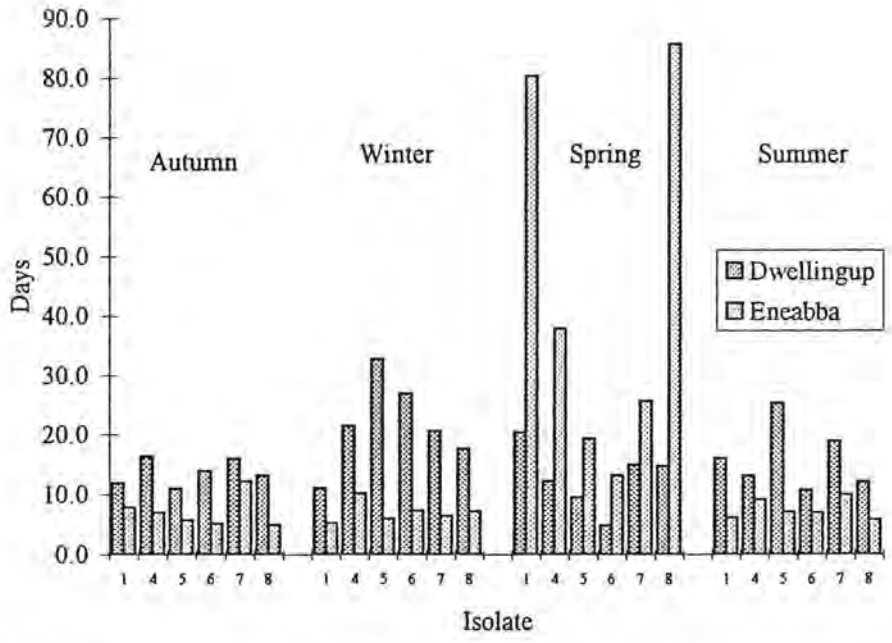


Fig. 3.4. LD_{90} of survival of zoospore cysts of six isolates of *Phytophthora citricola* at two field sites in four seasons.

An increase in the number of zoospore cyst recoveries was observed at Eneabba in spring at day 7, and Dwellingup in winter after day 14. These increases coincided with rainfall and consequent increases in soil moisture a few days prior to these times (Fig. 3.5). The soil matric potential at Dwellingup on day 14 of winter climbed to around -100 kPa, from -200 kPa at day 7 and -600 kPa at day 2, while at Eneabba on day 2 of the spring inoculation, soil matric potential rose to -6kPa from below -1500 kPa (Fig 3.5).

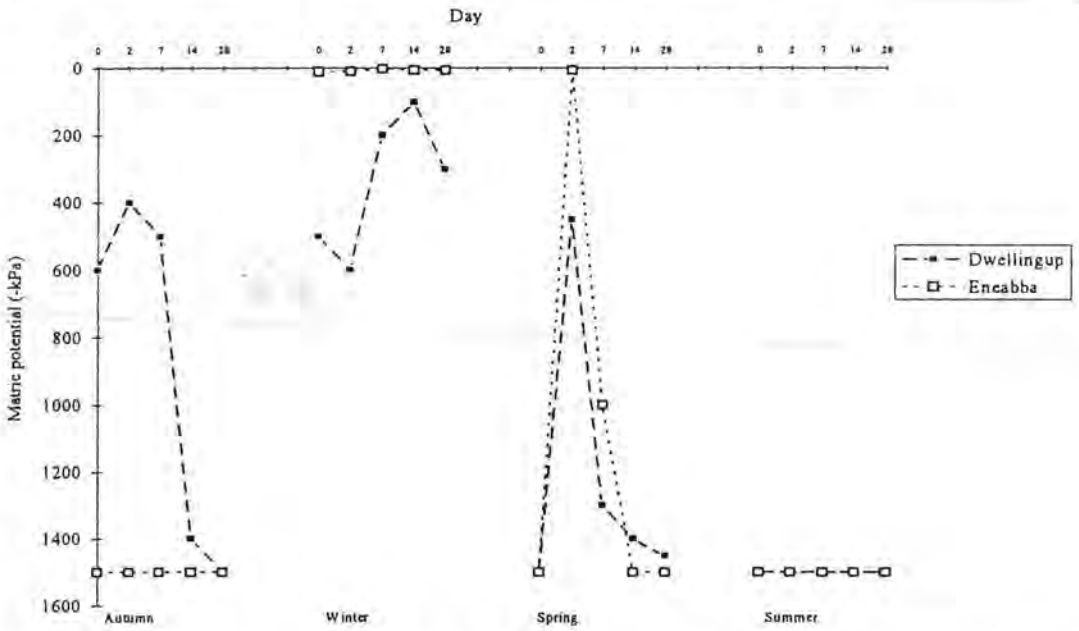


Fig. 3.5 Soil matric potential at two field sites at time of harvest of *Phytophthora citricola* zoospore cysts.

A significant difference ($P<0.05$) in zoospore survival between isolates for some harvests was determined by an ANOVA of the proportion of zoospores surviving at each harvest and the number of zoospores of each isolate placed into the field at day 0. Differences were particularly evident by day 28 at Dwellingup and day 14 at Eneabba (Table 3.3). However, no consistent differences or pattern between isolates were apparent, and it is probable that these differences were not real. For example, at Eneabba in autumn, numbers of zoospore cysts of isolate 3286 recovered at day 2 were significantly greater than any other isolate for this time. By day 7 and thereafter however, no difference in zoospore cyst survival between isolate 3286 and other isolates studied was observed. At day 28 when a significant difference was most often observed, no single isolate consistently survived in greater numbers than any other.

For all isolates combined, survival of zoospore cysts were consistently greater at Dwellingup than Eneabba for autumn and winter for all harvest periods. In summer, the two sites were not significantly different at day 2 and day 7, after which time there was no recovery of zoospore cysts of any isolate from Eneabba. In spring, site differences were minimal for the first two harvests. However, the increase in the number of zoospore cysts recovered from Eneabba after the rain prior to day 7 meant that a significantly greater number of zoospore cysts were retrieved from this site than from Dwellingup for the day 14 harvest. By day 28, site differences were again not significant.

Table 3.3. Differences in zoospore cyst survival between six isolates of *Phytophora citricola* placed in the field in autumn, winter, spring and summer, and recovered after 2, 7, 14 and 28 days. * indicates survival between isolates for that season was significantly different ($P<0.05$).

Site	Season	Day 2		Day 7		Day14		Day 28	
		F	*P*	F	*P*	F	*P*	F	*P*
Dwellingup	Autumn	2.3	0.08	0.97	0.46	0.34	0.89	8.11	0.00*
	Winter	0.73	0.61	1.26	0.32	0.57	0.72	5.76	0.00*
	Spring	1.76	0.17	1.0	0.14	2.67	0.053	2.26	0.09
	Summer	0.6	0.71	0.74	0.6	0.24	0.94	1.07	0.41
Eneabba	Autumn	10.1	0.00*	1.08	0.4	4.0	0.01*	-	-
	Winter	1.88	0.14	1.28	0.31	0.31	0.9	-	-
	Spring	1.63	0.19	1.76	0.17	2.86	0.04*	2.13	0.10
	Summer	0.42	0.83	0.26	0.93	-	-	-	-

Oospore viability. Oospores were placed into the field seasonally in autumn, winter and spring, and retrieved at 3 and 6 months for each season. Percentages of viable, dead and germinated oospores were calculated for each site for each season and harvest. A separate ANOVA (Table 3.4) for viable and germinated oospores was necessitated because of germination of oospores after placement in the field (Fig. 3.6). Oospores were capable of further development into sporangia (Fig. 3.7).

Initial oospore viability did not vary between SGs or isolates, with average viability of all isolates at time of inoculation consistently exceeding 90%. After placement in the field, neither decline in oospore viability nor numbers of germinated oospores varied between isolates ($P=0.52$ and 0.13 respectively (for seasons and harvests combined)).

Site was not a significant factor in the decline of oospore viability in soil for the first three months after inoculation ($P=0.62$), but by six months significant site differences were apparent. Greater numbers ($P>0.0001$) of oospores were viable after six months in the relatively infertile Eneabba sand than the organic-rich Dwellingup soil for the six month harvest of all inoculation dates. Conversely, a significantly greater number ($P>0.0001$) of black or dead oospores were observed at Dwellingup than Eneabba for the six month harvest, while germinated oospore numbers were not significantly different between sites ($P=0.37$).

Season was the most significant factor affecting oospore viability and germination. Decline in oospore viability for the first three months of the autumn inoculation was less than for the other two inoculation periods (Table 3.4). At time 0, 95% of oospores were viable, after three months at Dwellingup and Eneabba in autumn, oospore viability had declined to 59% and 56% respectively, compared to 50% and 43% in winter and 37% and 50% in spring. Decline in oospore viability during the second three months in the field

Fig. 3.6. Germinating oospore recovered from samples placed in field soil. Note remnants of previously germinated oospores.

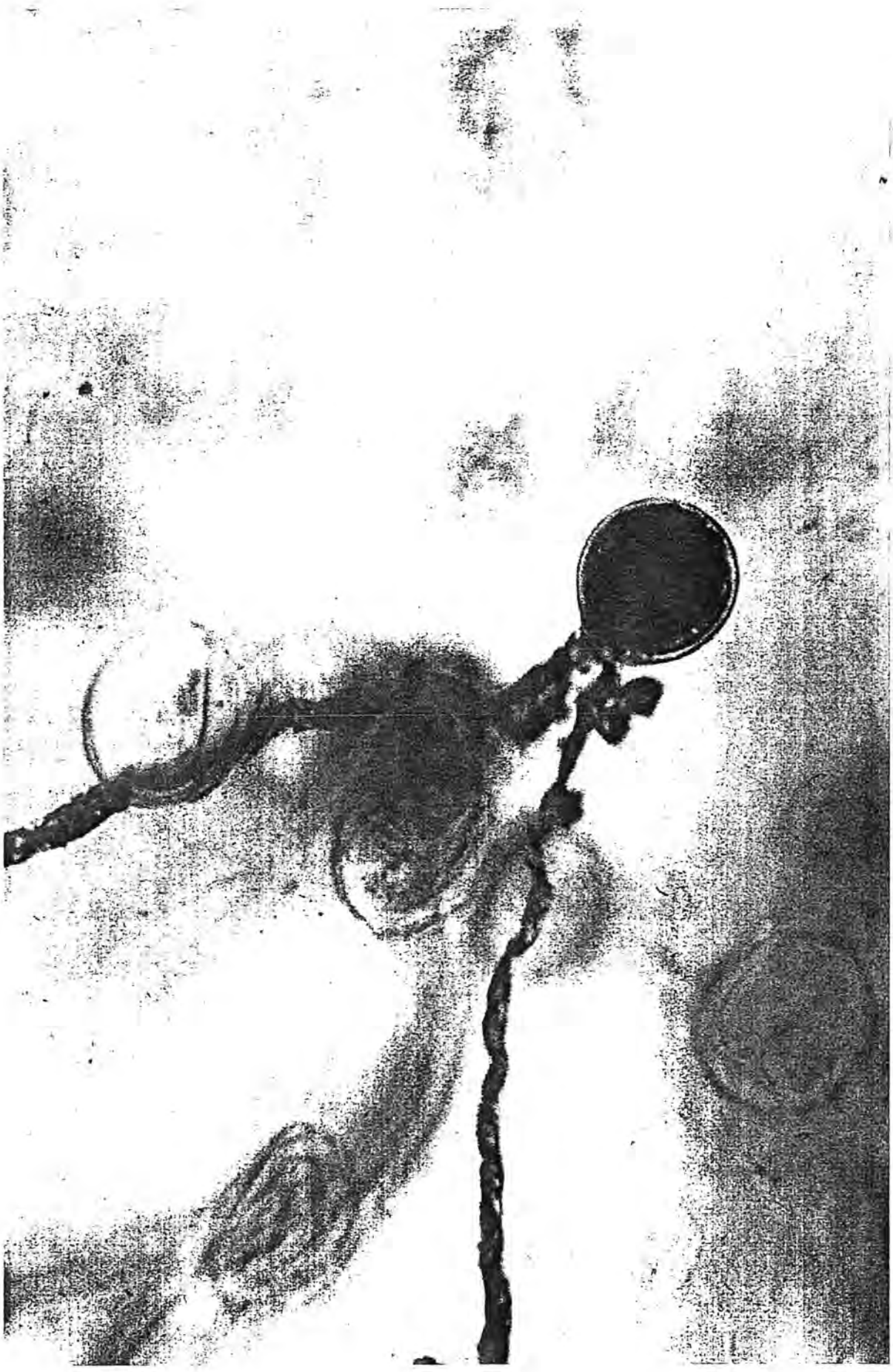


Fig. 3.7 Viable (pink), dead (black) and germinated (transparent) oospores recovered from soil. Oospores were capable of further development into sporangia (arrow).

was minimal compared with the first three months. Viability of autumn inoculated oospores harvested in spring at Eneabba remained at 56%, while at Dwellingup oospore viability had declined to 48%.

Table 3.4. ANOVA of survival of viable and germinated oospores of eight isolates of *Phytophthora citricola* in soil at Eneabba and Dwellingup. Oospores placed in field in autumn, winter and spring, and harvested at three and six month intervals.

Three month harvest, viable oospores.

Source	d.f.	m.s.	F value	pr > F
Treatment (site x season)	5	0.27	9.00	0.0001
sites	1	0.004	0.25	0.62
autumn vs winter	1	0.51	31.75	0.0001
autumn vs spring	1	0.79	49.79	0.0001
winter vs spring	1	0.03	2.02	0.16
Rep (treatment)	24	0.030	1.88	0.01
Isolate	7	0.011	0.71	0.66
Treatment*isolate	35	0.011	0.75	0.84

Three month harvest, germinated oospores

Source	d.f.	m.s.	F value	pr > F
Treatment (site x season)	5	0.16	4.47	0.005
sites	1	0.023	0.81	0.37
autumn vs winter	1	0.345	11.99	0.0007
autumn vs spring	1	0.091	3.18	0.08
winter vs spring	1	0.081	2.82	0.10
Rep (treatment)	24	0.036	1.24	0.21
Isolate	7	0.017	0.59	0.76
Treatment*isolate	35	0.029	1.03	0.43

Six month harvest, viable oospores

Source	d.f.	m.s.	F value	pr > F
Treatment (site x season)	5	0.228	8.18	0.0001
sites	1	0.635	30.59	0.0001
autumn vs winter	1	0.059	2.86	0.09
autumn vs spring	1	0.146	7.04	0.01
winter vs spring	1	0.392	18.89	0.0001
Rep (treatment)	24	0.036	1.34	0.14
Isolate	7	0.017	0.56	0.78
Treatment*isolate	35	0.029	1.22	0.21

Six month harvest, germinated oospores

Source	d.f.	m.s.	F value	pr > F
Treatment (site x season)	5	0.99	31.44	0.0001
sites	1	0.011	0.36	0.55
autumn vs winter	1	0.011	0.39	0.53
autumn vs spring	1	2.980	100.54	0.0001
winter vs spring	1	3.361	113.4	0.0001
Rep (treatment)	24	0.031	1.06	0.39
Isolate	7	0.065	2.19	0.04
Treatment*isolate	35	0.044	1.49	0.052

Germination of oospores for the six month period from spring to autumn was significantly less than for either of the two other inoculation periods (Fig 3.8). This period encompassed the dry summer months of December to March.

Colonised wood plugs. Recovery of *P. citricola* from colonised wood plugs retrieved from the field remained at 100% at both sites for all isolates of *P. citricola* for all inoculation and harvest periods. However, after the first harvest at 3 months, wood plugs initially colonised with *P. cinnamomi* yielded *P. citricola* from both sites. *P. cinnamomi* was not recovered from colonised wood plugs from any hole at either site for any inoculation period. To determine if *P. citricola* had infested soil around the holes, soil from the base of holes at both sites was sampled and baited with *E. sieberii* 12 months into the trial. *P. citricola* was recovered from all samples.

The *P. citricola* isolates comprised the two major SGs, SG 1 and SG 2 (section 2.1). SG 1 was successful in invading the plant material initially colonised by *P. cinnamomi*, as determined by the colony morphology of the isolate recovered from this material. The cultures recovered from each of the samples initially colonised by SG 2 however were true to the original, indicating that SG 1 was not successful in competitively colonising these wood plugs.

In the laboratory, plant material colonised by *P. citricola* and *P. cinnamomi* was stored in both soils under two soil moisture regimes for 18 months. The first was at a soil moisture likely to be present at both sites during summer, and the second was in similar conditions, with the inclusion of two simulated rainfall events six months into the trial. Under the dry soil conditions, survival of all of the *P. citricola* isolates did not decline for the first 12 months. After 18 months, *P. citricola* was unable to be recovered after the first baiting from two of the eight isolates stored in Dwellingup soil, and four of the eight stored in Eneabba soil (Table 3.5).

Table 3.5. Survival of 10 wood plugs colonised by isolates of *P. citricola* and *P. cinnamomi* maintained in the laboratory after 18 months. Plugs re-baited after 4-7 days dessication. new isolations; *P. citricola* only recovered after second baiting.

Dwellingup	1st baiting	2nd baiting	new isolations	Eneabba	1st baiting	2nd baiting	new isolations
JW20	7	9	2	JW20	1	8	7
2952	6	6	1	2952	6	8	3
NX22	7	6	2	NX22	2	1	1
Arb17	0	1	1	Arb17	0	6	6
HSA 1210	3	9	6	HSA 1210	2	7	7
HSA 1211	1	10	9	HSA 1211	0	9	9
3286	0	7	7	3286	0	8	8
1450	2	8	8	1450	0	10	10
P. cinnamomi	0	1	1	9	0	0	0
P. cinnamomi	0	0	0	10	0	0	0

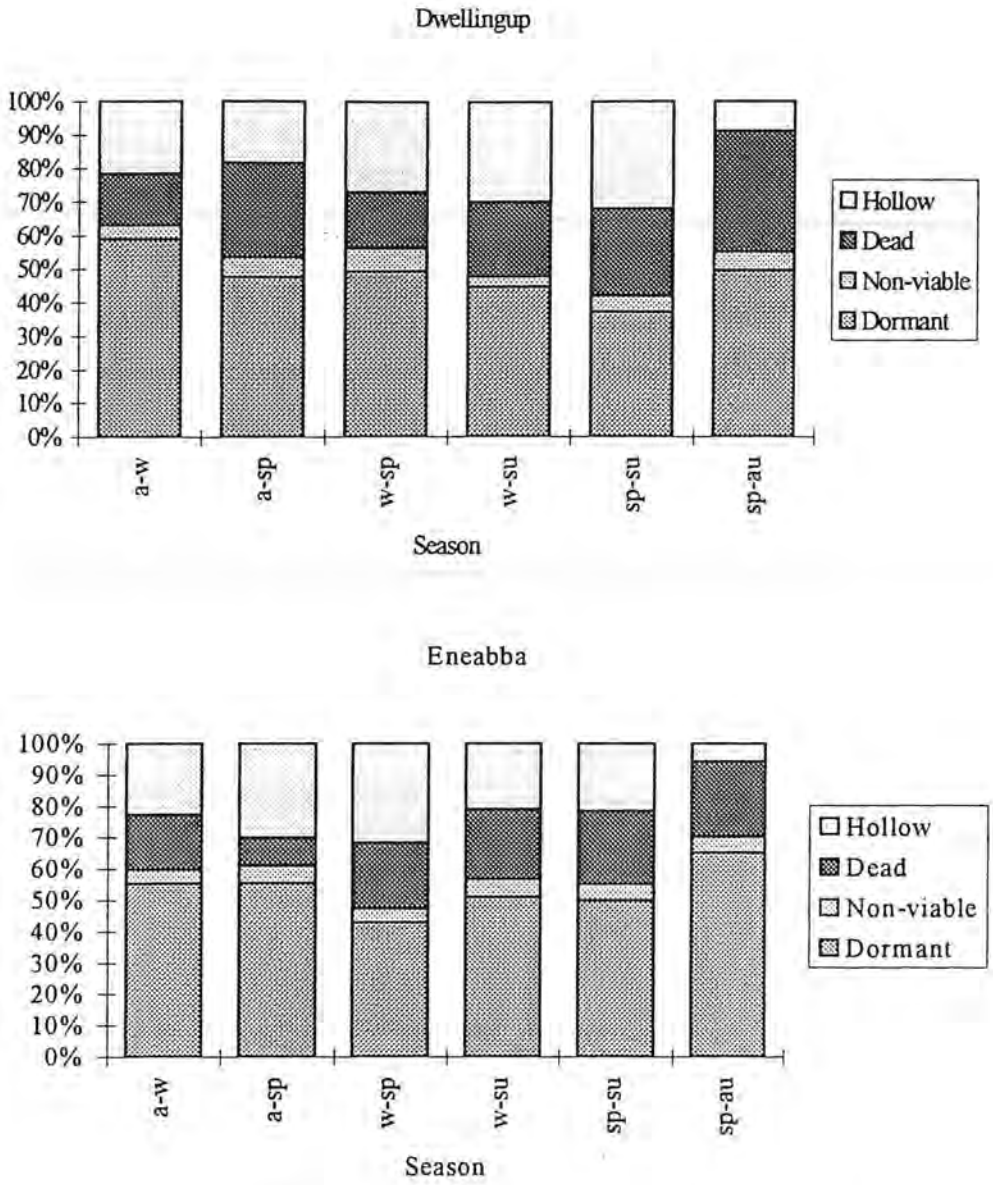


Fig. 3.8. Proportion of viable, dead, non-viable and dormant oospores of *Phytophthora citricola* after seasonal harvests at three and six months at two field sites. Oospores harvested at three months = autumn to winter (a-w), winter to spring (w-sp) and spring to summer (sp-su). Oospores harvested at six months = autumnn to spring (a-sp), winter to summer (w-su) and spring to autumn (sp-au).

In soil in the laboratory, oospore viability remained unaffected over six months in both Eneabba and Dwellingup soils at matric potentials below -1500kPa. Oospores did not germinate in either soil under these conditions.

Overall, the first baiting was successful in recovering *P. citricola* from 33% and 14% of all samples stored in Dwellingup and Eneabba soil respectively (Table 3.5). After being allowed to naturally dessicate for 4-7 days, all colonised wood plugs were re-baited. Recovery of *P. citricola* after this second baiting increased to 70% and 72% for Dwellingup and Eneabba respectively, 45% and 64% from colonised wood plugs initially negative. *P. citricola* was also not always recoverable from colonised wood plugs initially positive for *P. citricola*, with 8% (Dwellingup) and 6% (Eneabba) of these samples not yielding *P. citricola* after the second baiting. For the two bioassays combined, the total numbers of colonised wood plugs from which *P. citricola* could be successfully recovered after 18 months was 77% for both Dwellingup and Eneabba.

As with *P. citricola*, *P. cinnamomi* was unaffected by storage under dry conditions for the first 12 months. At 18 months, *P. cinnamomi* could only be successfully recovered from one of twenty (5%) samples baited. This recovery was made from Dwellingup soil after the second bioassay.

Simulated rain favoured survival of *P. citricola*, with the fungus being successfully recovered from 100% of samples from both soil types after 18 months from the first bioassay. Recovery of *P. cinnamomi* was unaffected by the simulated rainfall, with recovery declining from 90% after twelve months to 10% after 18 months.

Discussion

This experiment was designed to determine the relative ability of each propagule type to survive under diverse seasonal conditions at two field sites. Zoospore cysts often did not survive longer than the 28 days of the experiment at both field sites. Zoospore survival was also more susceptible to seasonal and site changes than oospores or colonised wood plugs.

For all seasons with the exception of spring, survival of zoospore cysts was significantly affected by site conditions, with survival consistently greater at Dwellingup than Eneabba. The grey sand at Eneabba had a lower soil matric potential than Dwellingup in the dry seasons of autumn and summer, and maintained a higher soil matric potential in winter and spring (Fig. 3.5).

The effect of soil matric potential on sporangial production and zoospore release has been studied for a number of other *Phytophthora* species (e.g. Bernhardt & Grogan 1982; Nesbitt *et al* 1979; Shea *et al* 1978; Pfender *et al* 1976; Duniway 1975). Species vary in their optimal soil moisture requirements for sporangial production, however there is good agreement that few sporangia of any species are formed at saturation, or at soil matric potentials below -4 bars (400 kPa). The increase in numbers of zoospore cysts recovered at Eneabba in spring after day 2 and Dwellingup after day 14 were probably a result of sporangia formation and zoospore release subsequent to placement of zoospore cysts in the field. Rainfall prior to these times increased the soil matric potential to within the optimal range for other *Phytophthora* species, suggesting that sporangial production and zoospore release by *P. citricola* are also favoured by conditions within this

range. No parallel to the day 14 Eneabba increase in zoospore cyst recoveries was observed at Dwellingup, when soil matric potentials only rose to around - 400 kPa.

In winter at Eneabba, zoospore numbers quickly declined in the constantly wet conditions, when soil moisture did not drop below -11 kPa (-0.11 bars) for the period of the trial, and rose to -3 kPa (0.03bars) at day 7. It is possible that zoospore cysts germinated, and resultant hyphae lysed under the wet soil conditions. These results are in agreement with those obtained by Morgan (1990) for *P. cinnamomi*, who found survival of zoospore cysts of *P. cinnamomi* quickly declined under wet conditions. Similarly, Bowers (1990) also could not recover zoospores of *P. capsici* after 2 wk from soil maintained at - 10 kPa. Recovery of zoospores of this species was greatest at -100 kPa, the lowest soil matric potential examined. Nesbitt *et al* (1979) found lysis of hyphae of *P. cinnamomi* in Dwellingup soil to be positively correlated to soil moisture. At field and half-field capacity, lysis of hyphae was complete within 3 days, whereas only 10% of hyphae had lysed after 10 days at twice field capacity.

Survival of zoospore cysts of *P. citricola* in this trial were favoured by soil matric potentials within the range of -200 kPa and -1500kPa. If soil conditions remained outside this range for a significant part of the time cysts were in the field, survival of cysts was adversely affected.

As the design of the experiment was not factorial, an independent test of the effect of temperature alone on zoospore cyst recovery was not possible. However, no obvious adverse effect of temperature on sporangial production by *P. citricola* was apparent, with the lowest average temperature experienced at Dwellingup for winter being 11.8^{o}C. The increase in zoospore cyst recoveries after day 14 at this site would imply that this temperature was not limiting for sporangia formation. Optimum temperature for sporangia production by *P. cinnamomi* has been studied by Nesbitt *et al* (1979). They found sporangia of *P. cinnamomi* were not formed at temperatures below 15^{o}C in soil recovered from the jarrah forest. However Kinal *et al* (1993) suggested that 15^{o}C as a minimum may be too high for sporangial production, as inoculum was recovered from subsurface water flow at lower temperatures. A co-incidence of high soil moisture and high soil temperature favours disease development caused by *P. cinnamomi* in the jarrah forest (Shearer & Shea 1987) and incidence of *P. cinnamomi* outbreaks are often more severe after summer rain (Tippett & Hill, 1983). Such conditions of soil moisture and temperature are optimal for sporulation and consequent infection of hosts. *P. citricola* is recovered from a wider geographical range than *P. cinnamomi* in this region (Stukely pers. comm.), it is possible that local isolates of *P. citricola* are more tolerant of a wider temperature range.

Oospores. Scoring and interpretation of results of oospore viability in the field was confounded by germination of oospores, as it was unknown whether or not the casings of germinated oospores degraded with time. Over-estimation of the number of viable oospores would have occurred if casings degraded with time, as the original number of oospores placed into the field would be impossible to determine.

Additionally if casings did not degrade, or degraded only slowly, overestimation of viability of remaining oospores was also probable if the more obviously visible stained oospores were scored in favour of transparent casings or casing remnants. Consequently harvests of oospores from the field were not conducted after 6 months; initially it was planned for harvests to be conducted for up to 2 years.

A further reason for not assessing oospore viablity longer than 6 months was that the miracloth had started to degrade with the extended period of contact with the soil,. Plant roots had also invaded the nylon pouch and bound pieces of miracloth together, making separation of the individual squares impossible without tearing. Continued qualitative harvests were conducted however, and small numbers of oospores were still viable after 18 months at both sites.

Degradation of oospore casings was most likely occurring as indicated by the increase in the proportion of viable oospores in the autumn harvest of the spring inoculation compared with the summer harvest of the same inoculation (i.e. six month vs three month harvests). This increase in viable oospore numbers is unlikely to be a natural phenomenon. If oospore production was occurring subsequent to placement in the field, it is improbable that the dry summer months favoured this event (see section 4).

Survival of oospores in other field studies has been assessed by determination of viability by plasmolysis and percentage germination of oospores (Pittis & Shattock 1994) and by MTT and percentage death of hosts in a bioassay (Bowers, 1990). Neither of these studies mentioned oospore germination in field soil.

Oospores germinated in equal numbers at both sites seasonally, albeit seasonal factors were important for oospore germination within sites. The fate of germinated oospores and subsequent germ tubes and hyphae was not assessed, however germinated oospores were capable of further development into sporangia if conducive environmental conditions were available. Sporangia were observed on the oospore mats (Fig. 3.7) recovered from both the winter harvest of the autumn inoculation, and the spring harvest of the winter inoculation from both sites. Soil matric potentials were only recorded for the 28 days of the zoospore trials, however rain outside this time was obviously sufficient to raise soil moisture to levels conducive for oospore germination and sporangia production. The majority of these sporangia however failed to germinate or produce zoospores.

Greater numbers of dead oospores at Dwellingup than Eneabba after six months may have been a result of parasitism by soil micro-organisms. Dead oospores recovered from Dwellingup were frequently completely coated with a "furry" covering, and the internal structure of the oospore was not discernable. This phenomena was not often observed on dead oospores retrieved from the Eneabba soil. The organic Carbon content of the Dwellingup soil was more than five times that of the Eneabba sand (Table 3.2), and supported a richer microflora. (Stack & Millar, 1985) also reported parasitism of individual oospores by a filamentous organism resembling an actinomycete, which completely colonised the oospore.

Oospores of other *Phytophthora* species have been implicated in long-term survival in field soil, and the persistence of oospores in the field in this study would confirm the importance of this propagule type for long-term survival. It is possible that the thick wall of the oospore is the means by which resistance to environmental extremes is conferred on the propagule. Thick-walled or dormant oospores of *Pythium ultimum* were insensitive to heat up to 70°C for 30 min, and their ability to become thin-walled and germinate was unaffected by the treatment (Stasz & Martin, 1988). (Lumsden & Ayers, 1975) also reported that thick-walled oospores of the same species survived rapid drying. Dried oospores remained viable for at least 8 months. Oospore walls of *Phytophthora* species undergo changes during germination; the thick inner oospore wall solubilises before the ooplast disappears, followed by the production of one or several germ tubes (Forster *et al*, 1983). During this phase the oospore was most vulnerable to changes in environmental conditions.

The longevity of some propagules in soil is determined in two ways; by endogenous or self-imposed adaptations innate in the propagule, and by exogenous or interactions between the propagule and the immediate environment. A combination of both endogenous and exogenous dormancy ensures nonsynchronous germination of oospores and a reservoir of inoculum. In a study on oospores of *P. capsici* in natural soil (Bowers, 1990) noted a decline in infective ability of oospores after placement in the field. Oospores from culture were able to cause 75-80% disease in a bioassay, however after 1-2 weeks in soil, disease incidence fell to 25-30%. Viability of oospores was maintained, thus inferring some form of inhibitory effect of the soil was operating on oospores. By reducing germination, this inhibition effectively increased the life-span of the oospore in soil over oospores maintained under constant conditions in culture.

P. citricola also exhibited this effect - if oospores were allowed to grow for longer than five weeks under constant temperature (23°C) on V8 juice agar, almost all oospores germinated simultaneously *in situ*. No pretreatment was required for germination. This germination was by the formation of multiple germ tubes, with no further development into sporangia observed on plates. If placed into the field, simultaneous germination did not occur, and a viability of greater than 30% of oospores was maintained after six months. The induction of exogenous dormancy or fungistasis imposed by soil may be in response to variable or inconstant environmental conditions unsuitable for oospore germination.

Colonised wood plugs. P. citricola survival did not decline in colonised wood plugs recovered from both field sites and from cups kept in the laboratory that received simulated rainfall. A significant decline in recovery of *P. citricola* from colonised wood plugs kept in the laboratory under dry soil moisture contents may be due to one of two factors; either the soil moisture at which the colonised wood plugs were maintained was too low for survival of *P. citricola* over an extended period, and population levels of the pathogen had started to decline, or the simulated rainfall to cups in the laboratory and the naturally fluctuating soil moisture levels in the field stimulated further development of the pathogen after placement

n the field or cups, and the pathogen was able to regenerate and build up inoculum levels. The successful colonisation by *P. citricola* of wood plugs initially colonised with *P. cinnamomi*, and recovery of *P. citricola* from soil at the base of field holes would indicate the latter. If survival alone of *P. citricola* (and not further development) had been enhanced by the higher soil moisture levels of the field and rainfall cups, recovery from these sources would not be evident.

P. citricola was probably surviving in soil at the base of holes in the form of oospores. *P. citricola* can produce oospores in both Dwellingup and Eneabba soils at moisture levels likely to be encountered in winter and spring (Bunny, unpublished). As soil was recovered from the field in summer when soil moisture contents were lowest, the likelihood of zoospore cysts or sporangia persisting under these conditions was minimal. Zoospore cysts placed into the field at Eneabba in summer did not persist longer than 14 day, and it is well accepted in the literature that sporangia and zoospores of other *Phytophthora* species do not persist in dry soil (Duniway 1975; Stack & Millar 1985; Bernhardt & Grogan 1982; Nesbitt *et al* 1979).

Colonisation by *P. citricola* of the *P. cinnamomi* infested plugs may have occurred either by zoospores of *P. citricola* swimming to and colonising nearby *P. cinnamomi* infested plugs, or by saprophytic growth of hyphae through the soil. As wood plugs were in separate pouches but in the same hole, neither event can be precluded. Rainfall had occurred prior to all harvests, making interpretation of these findings inconclusive. The ability of *P. citricola* to competitively colonise *P. cinnamomi* infested plugs to the exclusion of *P. cinnamomi* however would imply *P. citricola* was a more successful saprophyte than *P. cinnamomi*.

Temperatures recorded at 20 cm in the field were not lethal to *P. citricola* in any season. Interestingly, in a pilot trial at Eneabba where colonised wood plugs were buried at 10 cm during the summer of 1992/93, no survival was observed after the first harvest at 3 months. Temperatures during this period at this depth regularly exceeded 40°C for up to 4 hours, compared with 30°C at 20 cm in the summer of 1993/94. In a laboratory trial examining the heat tolerance of *P. citricola*, Smith and Davison (1992), using similarly colonised pine plugs, showed less than 50% of samples survived longer than 1 hour at 45°C. Survival after hour at 40°C was 97.5%. The extended periods above 40°C in this present study, combined with the frequency of occurrence of these periods was lethal to *P. citricola* under these conditions.

Baiting of colonised wood plugs was a significantly more effective method of detecting survival of *P. citricola* than plating directly onto selective media. Reasons for this increased detection by baiting may be more intimate contact of the plug with water than with media in which only the face of the plug was in contact, or alternatively the fungus may be persisting within the plug as oospores, which were stimulated to germinate with flooding. Increased recovery of *P. citricola* from colonised wood plugs kept in the laboratory after drying and rewetting suggests the latter. In this situation 45% of recoveries from Dwellingup soil and 4% of recoveries from Eneabba soil yielded *P. citricola* only after the second baiting cycle. If the fungus

was surviving as a propagule other than dormant oospores, the first baiting should have been successful in recovering *P. citricola*.

Premoistening of soil has been used to enhance detection of *P. megasperma* var. *sojae* (Canaday & Schmitthenner 1982) and *P. parasitica* (Ioannou & Grogan 1977). The abbreviated and extended bioassay for detection of *P. megasperma* f. sp. *medicaginis* in alfalfa field soil developed by Stack and Millar (1985) involved baiting flooded field soil with alfalfa seedlings.The extended bioassay was a repeat of the abbreviated bioassay, with 7-10 day period bbetweein bioassays in which soil was allowed to dry to 3-5% moisture. Detection of this *Phytophthora* species was significantly enhanced by drying and rewetting soil, with increased detection of *P. megasperma* f. sp. *medicaginis* after the extended bioassay probably due to enhanced germination of oospores stimulated by drying and rewetting of soil. They hypothesised that the requirement of oospores of *P. megasperma* f. sp. *medicaginis* for a period of drying may be an adaptation to alternating periods of dry and wet soil in a typical growing season in New York. Freezing of soil in winter dries the soil, which is then flooded by thawing in spring.

The sharp decline in recovery of *P. citricola* after 12 months from colonised wood plugs kept in the laboratory would imply that exogenously dormant oospores have a finite life. Sufficient numbers of viable exogenous oospores may have been persisting in the colonised wood plugs up until this time for the initial baiting to be successful in recovering *P. citricola*, after which viability sharply declined. In addition, after the bioassays at 18 months, some of the colonised wood plugs positive for *P. citricola* on the first bioassay did not yield *P. citricola* after the second bioassay. This phenomenon may infer that only small numbers of exogenously dormant oospores remained, or required more extensive manipulation of soil water to break dormancy.

4. Production of oospores by *P. citricola* in two field soils.

Abstract

The production of oospores by four isolates in Dwellingup and Eneabba soils was investigated. All isolates produced oospores in both soils, with significantly greater numbers of oospores produced in Dwellingup soil than Eneabba soil. The isolate from SG 1 (SG confined to jarrah forest) produced only limited numbers in the sandy soil of the northern sandplain.

Introduction

Oospores have been shown to be produced in soil and are the only propagule of *P. citricola* capable of long-term survival in soil (section 3). Oospores are important to survival for two reasons, firstly by maintaining a reservoir of inoculum that enables the fungus to maintain its presence in soil under adverse environmental conditions, and secondly as source of amplification of inoculum by further development into sporangia.

The objective of this section was to further investigate variation between the two SGs (section 2.1) in their ability to produce oospores in two different soil types.

Materials and methods

Inoculum. Four isolates of *P. citricola* representing four ETs of the two major SGs identified in section 2.1 were chosen. Isolate details are given in Table 2.1. Isolates were grown on CMA for four days at 24^{o}C.

Two mm diam stem pieces of three *Banksia* species (*B. attenuata, B. hookerana* and *B. prionotes*) were cut into 2 mm lengths. Care was taken to ensure pieces were of equal size. Stem pieces were sterilised, inoculated with the growing edge of the appropriate culture, and incubated at 24^{o}C for 28 days.

After inoculation period, inividual *Banksia* pieces were folded into a four cm^2 piece of nylon mesh, with a pore size of 25 μ. Mesh pieces were folded only once, to allow maximum hyphal movement from the source of inoculum. A pore size less than the diameter of oospores (30-40μ) was chosen so that any oospores produced would embed onto the mesh. Mesh pieces were then mixed with soil and wrapped into a pouch for easy retrieval from the field. Five pouches with a representative of each isolate on each *Banksia* species were placed in the field.

Field sites. Two field sites selected, one on the northern sandplain in the Moore River National Park and one at Myara near Dwellingup. Both sites were infested with *P. cinnamomi* (see section 3 for description of Dwellingup site). Moore River site had a similar soil type and plant community as described for Eneabba

(section 3). Pouches were placed in the field in mid-autumn for 5 weeks, buried at 10 cm depth. Soil moisutre content was measured at the times of placement into and harvest from the field.

Assessment. In the laboratory, mesh pieces were lightly rinsed with water to remove any superficial soil and placed in 0.5% MTT for 48 hours (see section 3). To assess oospore numbers, an area of the mesh piece 1.5 x 1.5 cm (2.25 cm^2) centred around the source of inoculum was scanned microscopically. Total numbers of oospores were recorded. Results were analysed by ANOVA, and pairwise comparison using Tukeys HSD test.

Results

The energy source for inoculum in the form of three *Banksia* species did not affect numbers of oospores produced (Moore River $P<0.22$, Dwellingup $P<0.27$). Consequently, results for all *Banksia* species were combined for further statistical tests.

Soil moisture contents at both sites was high and varied only slightly over the period of the experiment, with soil moisture contents similar at inoculation and harvest (Table 4.1). Rain fell intermittently at both sites at regular intervals. As soil matric potentials were also similar at both sites, it is improbable that this component affected oospore production between sites (Table 4.1).

Table 4.1. Soil moisture content (% oven dried weight) at two field sites at time of inoculation and harvest. Values are means $\pm$ standard error of five holes. Ψ = soil matric potential, derived from Fig. 3.2.

Site	Inoculation	Harvest	Approx. soil Ψ (-kPa)
Moore River	7 ± 0.7	6 ± 0.4	6
Dwellingup	15 ± 2.2	17 ± 1.9	15

All isolates produced oospores in both soils (Table 4.2). Oospores were often variable in size, in contrast to oospores produced in vitro (Fig. 4.1). Numbers of oospores produced however varied greatly between holes within sites, and between sites (Table 4.2). A significantly greater number of oospores were produced in Dwellingup soil than Moore River soil ($P<0.000$), when all isolates were combined. Individually however, the difference between soils was not significant for isolates 571 and JW 20.

Isolates varied in their ability to produce oospores, with significant differences between isolates at both sites. This difference was affected by site; for example isolate 571 produced the greatest number of oospores at Moore River but the second lowest at Dwellingup. At Dwellingup, 2952 and DP 33 produced the greatest numbers of oospores, with DP 33 producing the highest individual total of oospores in the 2.25 cm^2 area scored.

Fig. 4.1 Variability in size of oospores produced in soil.

2952 (SG confined to forested areas) produced only limited numbers of oospores in the sandy soil of the northern sandplain (Table 4.2). Only eight of the 15 samples (five replicates of each *Banksia* species) of this isolate produced oospores at this site, with an average of 1.2 oospores per sample, compared to 25.7 at Dwellingup. All other isolates produced oospores from at least 13 of the 15 samples at Moore River. Oospore numbers produced by 2952 were not significantly different from two of the other three isolates as a result of the large variation between samples. The largest number of oospores produced by 2952 at Moore River in one sample was six, compared with 58 by isolate 571.

Table 4.2. Numbers of oospores produced in two soils at Moore River and Dwellingup by four *P. citricola* isolates. Results for all holes and *Banksia* species have been combined. Values are means ± standard error. Values with the same letter within and between columns are not significantly different.

Isolate	SG, ET	Moore River	Range	Dwellingup	Range
2952	1, 1	1.2 ± 1.0 a	0-6	25.7 ± 7.0 de	4-57
JW 20	2, 3	4.8 ± 2.5 ab	0-16	13.3 ± 4.5 bc	3--38
DP 33	2, 2	5.3 ± 2.8 ab	0-21	29.3 ± 11.1 e	5-97
571	2, 5	13.8 ± 5.0 bc	0-58	17.7 ± 4.3 cd	9-43

Discussion

Differences in production of oospores between sites by individual isolates may be in response to a number of factors. Dwellingup soil had a higher organic carbon content than the sandy soil of the northern sandplains (Table 3.2). High nutrient contents of soil encourage microbial growth, and it may be that hyphae of *P. citricola* grew further from the source of inoculum at this site. A greater surface area of hyphae would imply greater production of oospores. Production of oospores of pythiacious fungi is often stimulated by stress. Greater parasitism and lysing of fungal hyphae occurs in soils with a high microbial content (Nesbitt *et al* 1979) and stress induced by other soil micro-organisms may have been instrumental in stimulating greater oospore production at this site. The soluble salt content and subsequent electrical conductivity of Moore River soil was greater than Dwellingup soil. As factors stimulating oospore production are not clearly understood, it may be speculated that salts are affecting osmotic regulation of the fungus and subsequent oospore formation.

The water content of Dwellingup soil was far greater than Moore River soil, but the soil matric potential for both soils was similar. As the soil matric potential affects fungal survival more than the total soil moisture content (Morgan, 1990), it is probable that soil moisture was not a factor determining differential production of oospores at each site.

Differences in total numbers of oospores produced by each isolate within a site was not unexpected. Isolates are very variable in the numbers of oospores produced in vitro, with some isolates producing prolific numbers and others only limited numbers. Isolate 571 is one isolate that produces abundant oospores in culture. It is interesting to note that this isolate produced significantly higher numbers at Moore River than any other isolate, despite being isolated from the loamy soil of the forest (Table 2.1).

The isolate from the SG confined to the jarrah forest (2952) was also interesting. This isolate produced the second highest number of oospores in the gravelly loamy sand of Dwellingup, and the lowest at Moore River. Most samples from Moore River in fact did not contain oospores. This result may indicate that this SG is not physiologically suited to sandy soils. The confinement to the jarrah forest of this SG may be a consequence of this physiological inadaptation, rather than a simple geographical confinement. Additionally, the recovery of an isolate of this SG from beneath a forest canopy in N.S.W. would add weight to this suggestion. This hypothesis needs further testing, with an expanded number of isolates from SG 1 included.

Materials and methods

Inoculum production. A total of six isolates were chosen to represent the range of responses to phosphonate shown in vitro (sections 2.5.1 and 2.5.2). These included two isolates from the in vitro insensitive SG 1 (2952 and NX 22), two isolates from SG 2, ET 2 (HSA 1210 and HSA 1211) and one each from SG 2, ET 3 (JW 20) and ET 5 (3398). Cultures were grown on Green Pea Agar. Five mm diam miracloth (Calbiochem Ltd) discs were placed on the agar surface in a circle around the point of inoculum. Plates were incubated at 25^{o}C for 6 days after which the miracloth circles were completely colonised by the fungus.

Phosphonate applicaton. Six month old *B. prionotes* plants were sprayed with 0.25% phosphonate (as Fosject) to run-off, two weeks prior to inoculation. Control plants were untreated.

Inoculation and harvest. Plants were stem inoculated as follows; a small incision into the phloem of the stem was made with a sterile scalpel blade, a colonised miracloth disc was inserted into the incision, sealed over with Vaseline (to prevent dessication of the wound) and bound with nylon tape. Seven replicate plants per isolate per treatment were used. Plants were maintained in a glasshouse at ambient temperature (around 10-22^{o}C). After 28 days, visible lesion lengths were recorded. Sections of stem from one cm behind to 3 cm ahead of the visible lesion were plated on agar selective for *Phytophthora* (Tsao & Guy 1977). Stem sections from which *P. citricola* was recovered were recorded, and the total length of stem infected was calculated.

Statistical analysis. The experiment was a randomised complete blocck design. Total lesion lengths were compared using Fishers Least Significant Difference test.

Results

Plants of *B. prionotes* averaged 5.5 mm diameter and 51 mm in height. Plants used are illustrated in Fig 5.1. Lesions were evident as darkened areas on the stem (Fig. 5.2).

Phosphonate inhibited lesion extension of all six isolates tested (Table 5.1). This inhibition was only significant however for five of the six isolates; lesions of isolate 3398 were not significantly inhibited by phosphonate. Length of lesions induced by this isolate in phosphonate treated plants were 55% of the untreated controls (Table 5.1).

There appears to be no correlation between in vivo and in vitro sensitivity of *P. citricola* isolates to phosphonate. SG 1 was relatively uninhibited by all concentrations of phosphonate in vitro (section 3.5.5 and 3.5.6), but responded to phosphonate in vivo. The two isolates of SG 1 were similarly inhibited by phosphonate. Lesions in the phosphonate treated plants of isolates 2952 and NX 22 were 42 and 34% respectively of the untreated plants (Table 6.1). No significant difference was observed between lesion lengths of treated plants for five of the six isolates. The isolate not significantly inhibited by phosphonate in

5. In vivo effects of phosphonate on *P. citricola* isolates of SG 1 and SG 2.

Abstract

To determine the efficacy of phoshonate against *P. citricola,* phosphonate was applied to *Banksia prionotes.* Stem lesions were induced using a number of *P. citricola* isolates with different in vitro sensitivity to phosphonate. Lesions of all isolates were successfully inhibited by phosphonate, and no correlative relationship between in vivo and in vitro behaviour of isolates was found.

Introduction

Phosphonates have been shown to be important in the control of *Phytophthora* related diseases in both horticultural and agricultural situations (see section 2.5). A number of forms of phosphonate are available, principally as fosetyl salts (Na^{+}, CA^{++}, Al^{+++}). Phosphorous acid is the breakdown product of these salts (Dolan & Coffey, 1988). Four phosphonate compounds (potassium phosphonate and three alkyl-substituted phosphonate compounds) were equally effective in controlling stem rots of avocado caused by *P. citricola.* Since the late eighties, phosphonate has been trialled with great success against *P. cinnamomi* induced dieback in native plant communities in the southwest (Shearer pers comm.).

The mode of action of phosphonate in plant tissue is not clearly understood. Two theories are currently being argued in the literature; firstly phosphonates act directly on the pathogen, inhibiting growth in plant tissue and secondly, a more complex indirect mode of action involving stimulation of host defences and a reduction of growth and sporulation of the pathogen. Correlative in vitro and in vivo behaviour of *Phytophthora* in response to phosphonates is presented as evidence for a direct mode of action (Cohen & Coffey 1986; Dolan & Coffey 1988, Fenn & Coffey 1984). Evidence presented by Guest & Grant (1991) and Guest & Bompeix (1990) for an indirect mode of action include the enhanced responses of plant defences in presence of phosphonates. Both direct and indirect modes of action are probably implicated in control of *Phytophthora* related diseases (Grant *et al* 1990), with concentrations of phosphonic acid in the plant determining the relative importance of each mode (Davis *et al* 1994).

A differential response to phosphonate in vitro between the two major electrophoretic subroups of *P. citricola* has been shown (section 2.5). The objective of this section was to determine the in vivo efficacy of phosphonate in controlling *P. citricola* induced lesions in *Banksia prionotes.* In addition, an assessment of the correlative relationship of in vitro and in vivo behaviour of the two SGs was made.

Fig. 5.1 Plants of *Banksia prionotes* used for determination of efficacy of phosphonate against *Phytophthora citricola*.

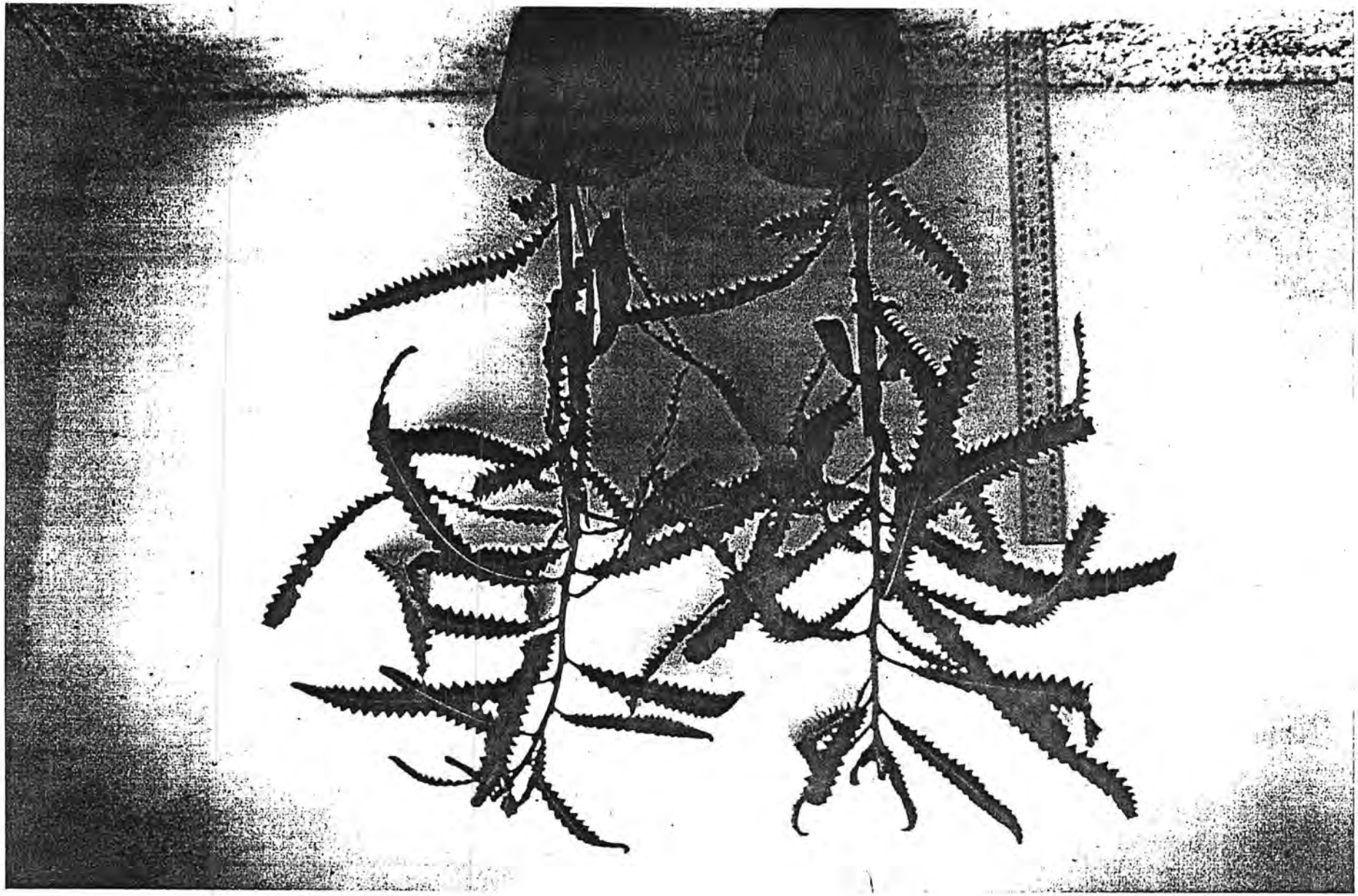
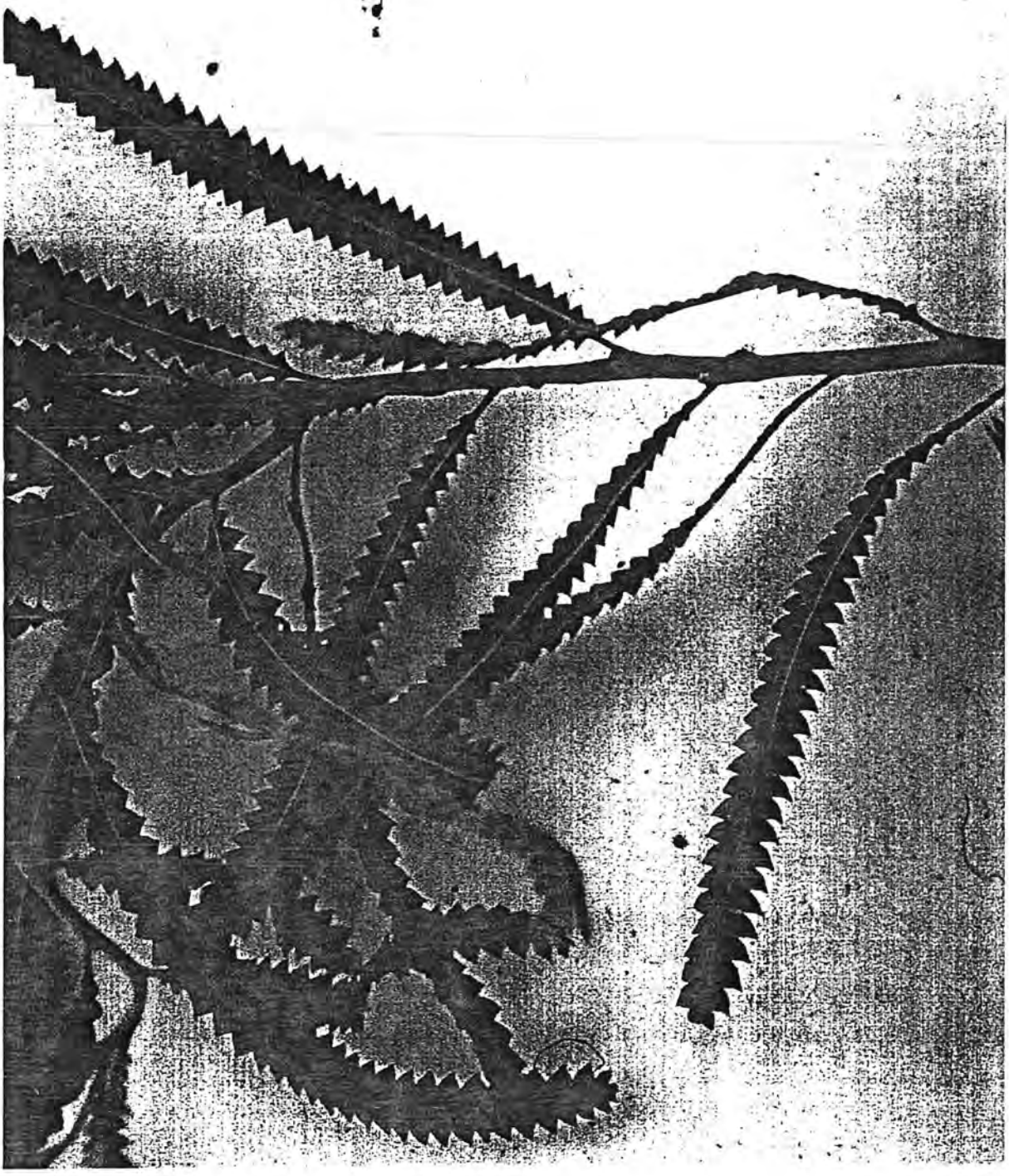


Fig. 5.2 Lesions in *Banksia prionotes* induced by inoculation with *Phytophthora citricola.*



vivo, 3398, was particularly sensitive to phosphonate in vitro, while the in vitro sensitive isolate, JW 20 was also sensitive in vivo.

In untreated plants, both isolates of SG 1 were significantly more aggressive than all isolates of SG 2, with the exception of HSA 1211 (Table 6.1). Isolate NX 22 caused lesions 3.6 times longer than JW 20. This is in agreement with results of section 3.4, where two isolates from SG 1 were generally more aggressive to jarrah clones than isolates from SG 2.

SG, ET	Isolate	Lesion length untreated (cm)	Lesion length treated (cm)	% treated of untreated	In vitro sensitivity
1, 1	NX 22	11.3 ± 1.5^{a}	3.8 ± 0.5^{de}	34	*
1, 1	2952	7.9 ± 0.7^{b}	3.3 ± 0.7^{de}	42	*
2, 2	HSA 1211	6.9 ± 1.6^{bc}	2.2 ± 0.3^{ef}	32	**
2, 2	HSA 1210	5.1 ± 0.8^{cd}	2.4 ± 0.3^{ef}	47	**
2, 5	3398	3.6 ± 0.7^{d}	2.0 ± 0.3^{ef}	55	***
2, 3	JW20	3.1 ± 0.9^{d}	0.5 ± 0.2^{f}	16	***

Table 5.1. Stem lesion lengths in *Banksia prionotes* of phosphonate treated and untreated plants. Values are mean of seven plants, $\pm$ standard error. SG = electrophoretic subgroup, ET = electrophoretic type, * = low sensitivity in vitro, ** moderate sensitivity in vitro , *** high sensitivity in vitro. Values with the same letter within and between columns are not significantly different (Fischers LSD).

P. citricola was rarely recovered from beyond the visible lesion front. This is in contrast to lesions in jarrah coppice in section 3.4. Lesion fronts in this current study were obvious (Fig. 5.2), and the infected area of the stem was clearly defined. Lesion fronts in jarrah coppice were often diffuse, with the margin not always obvious. Lesions caused by isolates of SG 2 in both studies were brown rather than black, as was the case for isolates of SG 1.

Discussion

Phosphonate successfully limited lesion lengths of all *P. citricola* isolates tested. Maximum inhibition of lesion lengths achieved by phosphonate treatment was 16% of untreated plants.

No corresponding relationship between in vitro and in vivo behaviour to phosphonate by isolates was found. Highly tolerant and moderately tolerant isolates in vitro from both SG 1 and SG 2 were similarly inhibited by phosphonate in vivo. The isolate most sensitive in vitro, 3398, was the least sensitive in vivo. It can be concluded that phosphonate is probably acting in an indirect manner on the pathogen in this interaction.

These results contrast with those of Dolan & Coffey (1988), who found a strong correlation between in vitro and in vivo resistance to phosphonate by *P. palmivora*. They used a series of laboratory-derived mutant strains of a *P. palmivora* isolate exhibiting a range of in vitro resistances to phosphonate. The mutants exhibited a similar range of resistance in vivo to phosphonate on tomato seedlings, suggesting a direct mode of action in vivo of the phosphonate fungicides against *Phytophthora*. However Dolan & Coffey (1988) also reported an enhanced level of control of *P. palmivora* in vivo with increasing levels of phosphate. This result was unexpected and they suggested that phosphate (which competes with phosphonate, thereby limiting phosphonate uptake (Griffith *et al* 1989)) influences host and fungal metabolism (Dolan and Coffey, 1988).

In a study using nine *Phytophthora* species and four phosphonate compounds Ouimette & Coffey (1989) showed isolates exhibiting a range of in vitro sensivities did not show similar corresponding sensitivities in vivo. Interestingly however, they did not consider this evidence for an indirect mode of action of phosphonate, but suggested a central role of the phosphonate anion in direct antifungal activity.

Guest and Bompeix (1990) present evidence for an indirect mode of action of phosphonate in tobacco, capsicum and cowpea. They suggest a defence cascade of effects directly inhibiting the pathogen while concomitantly stimulating host plant defences is established by phosphonate. Guest and Bompeix (1990) also noted that complete disease control was often achieved at phosphonate concentrations below those known to be inhibitory in vitro. Griffith *et al* (1989) showed that phosphonate did not provide full control of *P. cinnamomi* in lupins, a plant lacking a dynamic defence system. These results were also interpreted as providing evidence for an indirect mode of action, for if the plant was to halt fungal invasion completely a dynamic defence system was essential.

Perhaps the most relevant investigation to this current study of the effects of phosphonate to native plant species of southwestern Australia is a study by Smith (1994) using *B.coccinea, B. brownii* and *Petrophile biloba*. All three species showed enhanced stimulation of plant reaction zones in phosphonate treated plants when compared to untreated plants. He concluded this was evidence for an indirect mode of action of phosphonate.

The results of this study suggest that phosphonate is a promising method of control of *P. citricola* in native plant communities in the southwest. All *P. citricola* isolates tested responded to phosphonate treatment in vivo. The level of phosphonate applied is considered to be low, when compared to other studies using phosphonate in the southwest flora (Komorek, pers comm.). However there is need for further research using an expanded host range and further concentrations of phosphonate before clear conclusions can be made.

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