Phytophthora root rot of chestnut

by S. M. Chambers

Thesis submitted for the degree of

Doctor of Philosophy

in the

Department of Crop Protection

The University of Adelaide

December 1993

TABLE OF CONTENTS

ABSTRACT

Aspects of *Phytophthora* root rot of chestnut were investigated in this study. The modified excised shoot assay (using micropropagated material) was used, for the first time, to screen micropropagated chestnut cvs, used as rootstocks, for resistance to *Phytophthora* spp. *in vitro*. The cvs "Buffalo Queen" and "Goldsworthy" were susceptible to infection by *P. cinnamomi*, *P. citricola*, *P. cryptogea* and *P. cambivora*. Plantlets of the cvs "Buffalo Queen" and "Goldsworthy" growing *ex vitro* in vermiculite, which were inoculated with zoospores, were also susceptible to these *Phytophthora* spp.. The assay allowed comparison of pathogenicity of *Phytophthora* species on chestnut and indicated that genotypes of "Buffalo Queen" may vary in susceptibility to *Phytophthora* root rot.

Whole plant inoculation methods were also used to screen seedlings of a number of chestnut cvs for resistance to *P. cinnamomi*, *P. citricola* and *P. cryptogea*. Plants were inoculated either by dipping roots in a zoospore suspension or by placing mycelium in a wound made on the tap root. Seedlings of all cvs were susceptible to *P. cinnamomi*, *P. citricola* and *P. cryptogea* when inoculated using either of these methods. This is the first report of *P. citricola* causing root and crown rot of chestnut. Disease symptoms observed on infected plants included wilting, leaf chlorosis, lesion formation on stems and root rot.

Three fungi, isolated from soil from which *Phytophthora* was not obtained, were evaluated for potential biocontrol of *Phytophthora* root rot of chestnut. *Trichoderma hamatum* and *T. pseudokoningii* inhibited *P. cinnamomi* by mycoparasitism, with evidence of parallel growth and coiling, and all three antagonists grew over *P. cinnamomi in vitro*, preventing further growth of this pathogen. Antibiotics produced by young *T. hamatum* cultures and *Gliocladium virens* in cellophane overlay and culture filtrate experiments inhibited growth of *P. cinnamomi* and *P. citricola*, with filtrate from 4 d-old cultures of *G. virens* showing the greatest potential for biocontrol. Volatile antibiotics produced by *T. pseudokoningii* significantly inhibited growth of *P. cinnamomi* and *P. citricola*. All three antagonists

prevented *P. cinnamomi* and *P. citricola* from infecting micropropagated shoots of chestnut cvs "Goldsworthy" and "Buffalo Queen" in a biocontrol excised shoot bioassay *in vitro*. In pot experiments, plant root and shoot weights were generally higher in the presence of the antagonists than in plants inoculated with the antagonists and *P. cinnamomi*.

Infection of micropropagated plantlets of a susceptible cv. of chestnut "Goldsworthy" resulting from zoospore inoculum of *P. cinnamomi* was also followed. Hyphae grew quickly over the roots of plantlets, killing them within 4 d of inoculation. Chlamydospore and sporangium production occurred from 1-2 d after inoculation. Peripheral vesicles, intracellular organelles previously identified in *P. cinnamomi* zoospores, were labelled using monoclonal antibodies, for the first time, in hyphae infecting these chestnut roots from 1 d after inoculation, corresponding with the induction of asexual sporogenesis. Such vesicles have been found previously in nutrient-stressed hyphae *in vitro*.

Oospores of *P. cinnamomi* produced by A1 x A2 mating type crosses, as well as by A1 and A2 isolates alone, were induced in micropropagated chestnut roots for the first time. Large peripheral vesicles were labelled, using monoclonal antibodies, in these oospores as well as in oospores produced axenically in carrot agar culture.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

SIGNED:..... **.....DATE:**15/12/93.....

Acknowledgements

I would like to thank my supervisors Dr Eileen Scott, Dr Adrienne Hardham and Dr Trevor Wicks for their guidance and encouragement during this project,

Ms Lynne Giles for help with statistical analysis,

Mr Andrew Dunbar and Ms Jenny Groom for photographic assistance,

members of the nutgrowers association of Australia, especially Mr Joe Renaudo, Mr David Cumnick, Mr Carmine Russo, Mr Peter Taverna and Mr David Watkins, for supplying me with material and for allowing me sample soil on their properties,

Dr G. Hall and Dr M. Priest for confirming the identification of my fungal isolates,

Dr Margaret Sedgely for allowing me to use her fluorescence microscope,

all the staff and students in the Department of Crop Protection for their friendship, help and support,

my family for their encouragement,

and John, for his patience and for all the help and support he has given me throughout this project.

Abbreviations

BA	Benzyladenine
BSA	Bovine Serum Albumin
CMA	Corn Meal Agar
cv.	Cultivar
cvs	Cultivars
DW	Distilled water
IBA	Indole-3-butyric Acid
kPA	kilo Pascals
LSD	Least Significant Difference
MEA	Malt Extract Agar
MS	Murashige and Skoog
PBS	Phosphate buffered saline
PBS-BSA	Phosphate buffered saline-bovine serum albumen
Pipes	Piperazine-N,N'-bis-[2-ethanesulfonic acid] Sesquisodium salt
PPD	p-Phenylenediamine Dihydrochloride
RFLPs	Restriction fragment length polymorphisms
RO	Reverse osmosis
SAM-FITC	Sheep Anti-Mouse Ig $F(ab')_2$ fragment FITC conjugated
SEM	Scanning electron microscopy

1. INTRODUCTION

The genus *Phytophthora* comprises about 50 species that range from host-specific biotrophs such as *P. infestans* and *P. erythroseptica* to necrotrophs with a very wide host range such as *P. cinnamomi* and *P. cactorum* (Weste, 1992). Species of this genus belong to the Oomycetes, a group of organisms which has been recognized as belonging to the Kingdom Chromista, but is included in the Union of Fungi proposed by Barr (1992). A number of *Phytophthora* spp. cause root, foot and crown rots and blights of foliage and fruits of a wide variety of herbaceous and woody plants (Shaw, 1988).

In Australia, where chestnut blight is absent, root and collar rot of chestnut (*Castanea sativa* Miller) caused by species of *Phytophthora* is probably the most important disease, threatening the viability of the chestnut industry. A number of species of *Phytophthora* have been isolated from diseased chestnut. *P. cinnamomi* Rands causes root rot of chestnut in Europe (Day, 1938; Grente, 1961 a & b), USA (Crandall, Gravatt & Milburn-Ryan, 1945) and Australia (Washington, 1983). This species infects approximately 1000 host species worldwide (Zentmyer, 1980) and has devastated susceptible trees and shrubs in natural forests throughout Australia (Weste, 1992). Economic losses are sustained in a number of horticultural crops annually, for example avocado (Coffey, 1987), as well as chestnut (Washington, 1983).

Another species which causes disease of chestnut in Europe, especially in France and eastern Europe, is *P. cambivora* (Petri) Buism. (Grente, 1961a & b; Reichard & Bolay, 1986). This species has not previously been recorded as a pathogen of this host in Australia, but causes root and collar rot of almond and cherry in South Australia (Wicks & Lee, 1986; Wicks, 1987). *P. cryptogea* Pethybridge & Lafferty has been reported as a pathogen of chestnut in South Australia (Wicks & Volle, 1976) but has not been reported elsewhere on this host. *P. citricola* Sawada has been reported as the causal agent of root rot in a number of woody

plants, for example *Banksia* spp. (Hardy & Sivasithamparam, 1988), walnut (Matheron & Mircetich, 1985), kiwifruit (Stewart & McCarrison, 1991), azalea (Ferrin & Kabashima, 1991), and as a minor pathogen of almond (Wicks, 1987). Prior to this study, *P. citricola* was not known to be associated with chestnut root rot.

Disease is caused by *Phytophthora* spp. when the fungus infects chestnut plants through feeder roots or wounds on the tap root or collar. The pathogen then girdles the roots and stem, eventually killing the plant. Plants often die suddenly at the beginning of summer when water supply is limited (Fig. 1.1). Rates of disease extension vary widely with season, local topography, soil type, the presence of free water and zoospore production. Downhill disease extension, however, has been measured at as much as 400 metres per annum (Weste, 1992). Fungal inoculum is dispersed by running water, as well as by vehicles, machinery, humans and other animals (Weste, 1992). Chestnut yield losses from the disease have not been published, however tree losses are estimated at 2% to 100% of trees in each planting (Washington, 1983). Little is known regarding the disease in Australia and, as the chestnut industry is expanding rapidly here, research in this area is urgently required.

The objectives of the experiments presented in this thesis were, therefore:

(i) to isolate and identify the species of *Phytophthora* causing root rot of chestnut in the Adelaide Hills;

(ii) to screen rootstocks of *C. sativa* commonly used by growers for resistance to *Phytophthora* spp. and to compare the pathogenicity on these rootstocks of *Phytophthora* spp. associated with diseased trees;

(iii) to assess whether a modified excised shoot assay, using micropropagated chestnut shoots, could be used to screen for resistance *in vitro;*

(iv) to isolate and screen potential antagonists for biological control of *Phytophthora* root rot of chestnut both *in vitro* and *in vivo;*

(v) to look at structural features of infection of chestnut by *Phytophthora*, in particular using monoclonal antibodies raised against *P. cinnamomi* to detect the fungus in infected chestnut roots and to study aspects of fungal development.

Fig. 1.1: Diseased chestnut tree (infected with *Phytophthora* spp.) in the Adelaide Hills

2. LITERATURE REVIEW

Introduction

The literature concerning *Phytophthora* root rot of chestnut and other hosts is reviewed in this chapter and it is divided into three sections. The first section deals with the biology of *Phytophthora* spp., the disease they cause on chestnut and the importance of resistance. It also reviews mechanisms involved in plant resistance and the methods used to screen for resistance to *Phytophthora* spp. *in vitro* and *in vivo*. In the second section control of *Phytophthora* root rot is discussed: the cultural practices and types of chemical control used, and the problems associated with them. The use of biological control is reviewed emphasizing research work using species of *Trichoderma* and *Gliocladium*. In the third section the use of monoclonal antibodies to examine infection of plants by *Phytophthora* is reviewed, in particular the use of monoclonal antibodies raised against *P. cinnamomi* in providing information on development of the fungus and on the infection process is discussed.

2.1.1 The biology of *Phytophthora*

The genus *Phytophthora* belongs to the Oomycetes and has a number of features which distinguishes it from other fungi. *Phytophthora* species have cell walls composed primarily of β-1,3 and β-1,6 linked glucans, with some cellulose (Bartnicki-Garcia & Wang, 1983). They are diploid in the vegetative state and meiosis occurs in the gametangia. *Phytophthora* spp. are unable to synthesize sterols, which are needed for sporulation (Zentmyer, 1983), synthesize lysine by the diaminopimelic acid (DAP) pathway (Vogell, Thompson & Shockman, 1970), and have well-defined Golgi bodies (Deacon, 1984). There are over 40 species which cause a wide range of diseases on major food crops, forest trees, subtropical and tropical fruit trees, berries, nut trees, and many ornamental plants (Zentmyer, 1983).

The lifecycle of *Phytophthora* comprises an asexual and a sexual state. In the asexual state, vegetative mycelium give rise to sporangia which release zoospores. These zoospores encyst

on plant roots, germinate, then penetrate the roots causing infection and may eventually cause the death of susceptible plants. Zoospores are attracted to the region of cell elongation on roots of both susceptible and resistant plants (Aveling & Rijkenberg, 1989) but, depending on the host, infect different parts of the root system. *P. cinnamomi* invaded only the small feeder roots of avocado, while in *Banksia* spp. the majority of large roots were infected (Zentmyer, 1980). Roots are infected throughout: Cahill *et al.* (1989) found that both the cortex and stele of eucalypt roots were extensively damaged by *P. cinnamomi* and that hyphae penetrated both inter- and intracellularly. Symptoms of disease include wilting and necrosis in above-ground parts along with lesion formation and girdling of roots (Keen & Yoshikawa, 1983). Sporangium production is favoured by warm, moist and aerobic conditions in soils (Duniway, 1983) and the fungus is dispersed actively as motile zoospores in free water, or passively, as propagules in infested, moist soil or flowing water (Shearer & Tippett, 1989). Optimum soil moisture conditions for the development of *P. cinnamomi* sporangia have been shown to be -8 to -16 kPa in clay and sandy soils (Gisi, Zentmyer & Klure, 1980). The optimum temperature for the development of disease by *P. cinnamomi* and *P. cambivora* on chestnuts in Europe was found to be 24-27°C, with a minimum of 15°C required for the development of infection (Grente, 1961). Optimum temperatures for disease development in Australia, however, have not been determined.

The fungus persists in dead plant material and in soil, sometimes as chlamydospores. These are multinucleate, asexual resting structures produced in a terminal or intercalary position on vegetative hyphae of some species. They have thick walls and food reserves and are capable of surviving under adverse conditions, when water and nutrients are limited. They then germinate to infect plant material when conditions are favourable.

Sexual reproduction occurs when gametangial hyphae mature, then interact to allow plasmogamy and syngamy. Meiosis occurs in the antheridia and oogonia (Sansome, 1965) and then the oosphere within the oogonium is fertilized when a male gametic nucleus enters the oosphere through a fertilization tube linking antheridium and oosphere. The antheridium

may be amphigynous or paragynous. The nuclei associate in the developing oospore and karyogamy occurs (Sansome, 1976). The mature oospore has a thick wall and persists in soil and plant material (Mircetich & Zentmyer, 1966). It serves as a source of primary inoculum (Bowers & Mitchell, 1991; Hord & Ristaino, 1991), germinating by the production of a sporangium or germ tubes (Shaw, 1988). Oospores are produced as a result of inter- and intra-specific crosses between isolates of opposite mating type in heterothallic species (Savage *et al.*, 1968; Boccas, 1981) and by single isolates in homothallic species. They have also been induced by diffusible plant hormone-like substances in heterothallic species, when isolates of opposite mating types were placed in close contact with each other but were physically separated by a polycarbonate membrane (Ko, 1978). *P. cinnamomi* is a heterothallic species, although oospore formation has been induced in A2 isolates exposed to host root extracts and oleic acid (e.g. Zaki *et al.*, 1983; Ho & Zentmyer, 1977; Zentmyer, 1979), other fungi, for example *Trichoderma* spp. (Reeves & Jackson, 1972, Brasier 1975), and mechanical or chemical damage (Reeves & Jackson, 1974).

Sexual reproduction provides a means of genetic variation through recombination. Not all crosses between pairings of isolates of opposite mating type result in recombination however, because both crossing and selfing can occur (e.g. Ko *et al.*, 1978). Galindo & Gallegly (1960) found that A1 and A2 isolates of *P. infestans* could produce both female and male gametangia when paired. Thus a number of different kinds of selfed and crossed progeny can be produced. Poor germination of oospores, difficulties encountered in establishing single-oospore-derived colonies, and a lack of markers other than mating type and virulence have resulted in difficulties in identifying whether progeny produced by pairing isolates of opposite mating type are the result of recombination or selfing (Shattock *et al.*, 1987). Sexual recombination in offspring produced by crosses between opposite mating types has, however, been shown recently, using RFLPs. Förster & Coffey (1990) used RFLPs as genetic markers to investigate sexual recombination during oospore formation in *P. parasitica.* The majority of the oospore progeny carried both of the homozygous parental nuclear DNA markers and few oospores were produced as a result of selfing. Recombination can increase variability in

progeny, e.g. with respect to fungicide resistance in *P. infestans* (Shattock, Tooley & Fry, 1986), which is important to plant pathologists and potato growers.

2.1.2 The chestnut industry in Australia

The chestnut is native to the temperate zone of the northern hemisphere. There are four Asiatic species: *C. henryi* (Kan) Rehd. & Wils.; *C. mollissima* Blume; *C. crenata* Sieb. & Zucc. & *C. seguinii* Dohde, five North American species: *C. dentata* (Marsh.) Borkh.,*C. ozarkhensis* Ashe; *C. pumila* (L.) Mill.; *C. alnifolia* Nutt. and *C. ashei* Sudw., and one European species: *C. sativa* Mill.. The European chestnut occurs naturally in a wide area of southern Europe, and requires a moderately humid, warm, Mediterranean-type climate and rich, neutral soil. It is valued for its timber and its fruit (Vieitez, Vieitez & Vieitez, 1986).

The Australian chestnut industry is an infant but growing industry, producing 537 tonnes of chestnuts in 1991. Production is expected to increase to more than 3500 tonnes by 2001 (Anon, 1992). Victoria produces the majority of Australian chestnuts, with approx. 68% of the trees in Australia being grown in two main areas: the northeast of the state (45%) and the Dandenongs/West Gippsland region (17%). Chestnuts are also produced in the Adelaide Hills region of South Australia and in parts of New South Wales (Anon, 1992).

In Australia, cultivars of *C. sativa* are grown most widely, but a few recent imports of Asian material are also grown (P. Taverna, pers. comm.). Plants are grown as clonal selections of different cultivars selected for nut characteristics, grafted on seedling rootstocks of *C. sativa*. Quarantine restrictions limit the importation of chestnut material, to prevent the introduction of the fungus *Cryphonectria parasitica* (Murr.) Barr., the causal agent of the canker disease chestnut blight. This fungus has ravaged chestnut populations in USA and Europe but, so far, has been excluded from Australia.

2.1.3 *Phytophthora* root rot of chestnut

A number of species of *Phytophthora* cause root and crown rot of chestnut worldwide. *P. cambivora* (Petri) Buiman has been isolated from diseased chestnut in the USA (Milburn & Gravatt, 1932; Crandall, Gravatt & Milburn-Ryan, 1945) and Europe (Day, 1938; Grente, 1961; Reichard & Bolay, 1986). *P. cinnamomi* Rands. has been isolated in the USA (Crandall *et al.*, 1945), Europe (Day, 1938; Grente 1961 a & b) and Australia (Washington, 1983). *P. cryptogea* Pethybridge & Lafferty has been isolated from diseased trees in Australia (Wicks & Volle, 1976), *P. syringae* has been isolated in the UK (Day , 1938), and *P. katsurae* Ko et Chang in Japan (Ko & Chang, 1979).

Above-ground symptoms of the disease include slight leaf chlorosis, wilt, trunk cankers and eventual plant death. *Phytophthora* spp. infect fine roots of chestnut first then spread up to the collar region and form a canker or lesion on the lower trunk. These lesions eventually coalesce to girdle the trunk and kill the tree (Crandall *et al.*, 1945; Grente, 1961 a). Lesions on roots or crowns usually produce an inky-blue exudate that stains the soil close to the root, and thus the disease is often known as "ink disease" (Crandall *et al.*, 1945). Trees are susceptible to the disease at any age (Washington, 1983) and are particularly susceptible when grown on poorly drained soils (Anon., 1992). Losses due to the disease in Australia are estimated to range from 2% to 100% of the trees in each planting (Washington, 1983). Estimated yield losses from the disease have not been published.

2.1.4 Screening for resistance to *Phytophthora*

Most research concerning resistance of chestnuts to plant pathogens has been concerned with resistance to chestnut blight. Species and cultivars of chestnut have been screened for resistance to this pathogen using mycelial inoculations on trunks and branches (e.g. Bazzigher & Miller, 1991; Anagnostakis, 1992).

Very little research has been conducted on *Phytophthora* root rot of chestnuts. Milburn & Gravatt (1932) used soil infested with *P. cambivora* to inoculate seedlings of chestnut species

in the greenhouse to screen for resistance. *C. mollissima* and *C. crenata* were most resistant to *Phytophthora*, with *C. mollissima* more resistant than *C. crenata*, while *C. dentata* and *C. sativa* were found to be susceptible. Crandall *et al.* (1945) inoculated seedlings, in pots in the glasshouse, by inserting a mycelial plug of *P. cinnamomi* into a wound on the main root, just below the collar. Experimental details, for example the age of inoculated plants, the duration of experiments and plant symptoms, were not reported. Inoculation by adding mycelial plugs to soil or using infested soil to infect different seedlings was also tested. They screened a large number of species of chestnut, and confirmed earlier findings that the Asiatic species are highly resistant to *P. cinnamomi*.

Other experiments have been conducted to determine pathogenicity and to compare aggressiveness of isolates of *Phytophthora* on chestnut. Isolates differ in aggressiveness if cv. x isolate interactions are involved. Where there are no such interactions, isolates differ in virulence (Vanderplank, 1978). Grente (1961) found that 4-5 month-old *C. sativa* plants, inoculated with mycelium on the stem or roots, were susceptible to *P. cinnamomi* and *P. cambivora*. Reichard & Bolay (1986) found that infection developed when branches excised from field-grown *C. sativa* were inoculated with mycelium of *P. cinnamomi* and *P. cambivora* and incubated at 20-22°C. Five isolates of *P. cambivora*, from chestnut, inoculated as mycelium on wounds made at the base of trunks of 2 year-old trees in the field also caused infection. Isolates from Geneva, Switzerland, were as aggressive as those from USA and Australia. Robin (1991) inoculated 1 month-old seedlings of *C. sativa* with *P. cinnamomi* using infested soil and found that *P. cinnamomi* isolated from chestnut was a more aggressive pathogen on chestnut than on eucalypt or oak.

In Australia, Wicks & Volle (1976) inoculated mature chestnut trees with *P. cryptogea* by inserting mycelial plugs into wounds cut on branches. Cankers developed on all inoculated branches after 6 months and some of these branches died. Also, seedlings taller than 10 cm, in 1.5 litre pots, were inoculated with sporulating mycelium or placed in soil infested with *P. cryptogea*. Severe wilting of seedlings within 3 weeks of inoculation was recorded.

Washington (1983) inoculated *C. sativa* seedlings at the 4-5 leaf stage, growing in pots, with *P. cinnamomi* grown on a vermiculite/V8 broth substrate. Most plants inoculated with *P. cinnamomi* died within 16 days.

The above screening methods distinguished susceptible from resistant material where the latter was available. There are no published reports on screening different cultivars of *C. sativa* for resistance to *Phytophthora*. This work needs to be done so that growers can be informed of the best cultivars to use as rootstocks in soils infested with *Phytophthora* spp.. Disadvantages of using whole plants to screen for resistance are that they take up a large amount of space and can take from months to years to grow to a size suitable for evaluating. Also, because *Phytophthora* is a persistent fungus which can not be completely eradicated from infested soil using fungicides, it is inadvisable to inoculate trees on growers' properties to screen for resistance. However, trees could be screened by planting in naturally infested soil.

2.1.5 Use of micropropagated plant material to screen for disease resistance.

Studies to identify resistance to *Phytophthora* in a range of plant species *in vitro* have been conducted at the cellular level, using callus, and at the organ level via micropropagation. Phillips, Weste & Hinch (1991) found that callus derived from resistant cultivars of avocado was resistant to *P. cinnamomi*. Resistant callus had sparse, limited mycelial growth over it, while callus from susceptible cultivars had prolific, dense mycelial growth on and within it. Similarly, McComb, Hinch & Clarke (1987) reported that callus of field resistant Australian native plants and horticultural species was resistant to *P. cinnamomi*, using the extent of hyphal growth on callus as an indication of susceptibility. By contrast, Jang & Tainter (1990, 1991) used the number of intracellular hyphae in inoculated callus as an index of resistance. They found that callus derived from species of pine and hybrids with field resistance was resistant to *P. cinnamomi* in culture. The only *in vitro* experiments to evaluate resistance to *Phytophthora* in chestnut to be published were conducted by Borrod (1971). Callus of *C. sativa* and a *C. sativa* x *C. crenata* hybrid was inoculated with mycelium of *P. cinnamomi*.

Growth of mycelium over the callus was sparse and less than 3-4 mm from the point of inoculation in callus derived from the resistant hybrid *C. sativa x C. crenata*, but dense and greater than 10 mm in callus derived from susceptible *C. sativa*. Resistance to *P. cinnamomi* was influenced by light intensity: hybrid callus incubated in the dark was susceptible to *P. cinnamomi*, but when incubated in the light for 3 wk at 500 lux before inoculation, it was resistant to *P. cinnamomi*. Resistance was thought to be due to the production of phenolics, which were probably produced in callus which turned brown after 4 d in the light (Borrod, 1971). The influence of light on susceptibility of chestnut callus needs to be examined further.

Few screening experiments have been conducted using micropropagated plant material. Sharma & Skidmore (1988) observed partial resistance in micropropagated shoots of *Papaya* inoculated with a sporangial suspension of *P. palmivora*. Scott, Wicks & Lee (1992) developed an assay to screen for resistance to *P. cambivora* in almond and peach rootstocks using micropropagated shoots; response of host shoots *in vitro* was correlated with field response. Son (1992) also used this modified excised shoot assay to screen for resistance to *P. citrophthora* in micropropagated citrus rootstocks. Again, a good correlation was observed between field resistance and resistance of micropropagated shoots. This method appears suitable for screening cultivars of different woody hosts for resistance to *Phytophthora* spp., provided that certain conditions are met (see below).

2.1.6 Advantages and disadvantages of using tissue culture techniques to screen for disease resistance

There are number of advantages in using tissue culture techniques to screen for disease resistance. Large amounts of clonal plant material can be screened in a small space and environmental and nutritional conditions can be controlled, so that the accuracy of results is increased. Material can also be screened in the absence of any confounding biotic contaminants. Preliminary resistance trials can be completed more rapidly than if undertaken in the field because material can be micropropagated quickly, screened and then incorporated

into breeding programs. Partial resistance of papaya to *P. palmivora* was detected *in vitro*, whereas it may have been difficult to detect under variable environmental conditions of the field (Sharma & Skidmore, 1988).

There are also some potential disadvantages of using tissue cultures to screen for disease resistance. Environmental conditions must be standardized so that assays are repeatable. Also, pathogens being used in experiments can grow across the culture medium and on the host material and physically obscure any resistant response (Scott *et al.*, 1992; Sharma & Skidmore, 1988). Sharma & Skidmore (1988) transferred inoculated cultures to fresh medium every 3 d so that the pathogen being tested was unable to establish upon the nutrient medium, whereas Scott *et al.* (1992) overcame this problem by assessing response early and frequently. They further recommended that such prompt assessment was necessary as necrosis can develop rapidly. They also emphasized that material showing resistance or partial resistance to *Phytophthora in vitro* must be planted out in soil for subsequent evaluation in glasshouse conditions.

2.1.7 Mechanisms and importance of host resistance

It is important that resistant rootstock material, if available, is grown in areas where the soil is infested with *Phytophthora* spp.. The use of resistant material minimizes losses in crop yield due to infection by the pathogen, and reduces the need to use expensive chemicals to control disease.

Morphological and biochemical barriers to infection may be important in the containment of *P. cinnamomi* within resistant roots. The production of callose in the epidermis and cortex of roots of some resistant species in response to inoculation appears to slow or impede pathogen growth (Cahill & Weste, 1983; Hinch *et al.*, 1985), while the production of lignin and suberin in root tissues provides a mechanical barrier to invasion by the pathogen (Cahill *et al.*, 1989; Tippett & O'Brien, 1976). Similarly, the formation of necrophylactic periderm in an avocado

rootstock (Phillips *et al.*, 1987) and in mature *E. marginata* roots has been shown to restrict pathogen growth (Tippett & Hill, 1984; Tippett *et al.*, 1983).

Resistance of plant roots to *P. cinnamomi* may also depend on biochemical mechanisms such as the production of phytoalexins and enzymes. Phytoalexins are low molecular weight compounds which are synthesized by, and accumulate in, plant cells after microbial infection (Paxton, 1981). The phytoalexin 6,7-dimethoxycoumarin accumulated in roots and stems of resistant citrus seedlings after inoculation with *P. citrophthora*, but not in susceptible seedlings (Sulistyowati, Keane & Anderson, 1990; Afek & Sztejnberg, 1988). Similarly, the enzyme phenylalanine ammonia lyase (PAL) was produced in field resistant *E. calophylla* roots but not in susceptible *E. marginata* roots after inoculation with *P. cinnamomi* (Cahill & McComb, 1992).

In some cases resistant rootstocks are not available or are not used by growers because they are horticulturally unacceptable (Ribeiro & Linderman, 1989), therefore other methods of disease control need to be used, and these are described below.

2.2.1 Control of *Phytophthora*

Diseases caused by *Phytophthora* may be controlled using resistant plant varieties or species (as discussed above), or by cultural or chemical methods. Biological control is also being investigated as a possible means of disease control. These approaches and the advantages and disadvantages associated with their use are discussed below.

2.2.2 Cultural practices used to control *Phytophthora*

Traditional practices used to control *Phytophthora* spp. have involved planting in well-drained sites and avoiding planting in infested soils where possible. Planting apple trees on mounds above the soil level in orchards has been shown to control collar rot due to *P. cactorum* in Australia and California (McIntosh, 1975). Careful soil-water management in orchards, to avoid prolonged and periodic soil saturation, is also important in controlling

Phytophthora diseases (Mircetich, 1981). Companion planting of avocado with citrus in California, bananas in NSW and coffee in Costa Rica has been practised to remove excessive soil moisture, particularly in heavier soils (Coffey, 1991). Similarly, Merwin, Wilcox & Stiles (1992) found that *Phytophthora* infection of young apple trees was prevented in orchards by the use of ground covers such as a crown vetch "living mulch" and sod grass. These prevented prolonged soil saturation and reduced root and crown rot of apple trees.

Soil solarization has also been used to control *Phytophthora*. It was effective in preventing *P. cambivora* root rot of cherry, but not of almonds in South Australia (Wicks, 1987). The lack of success in controlling infection of almonds using this method was attributed to dry soil beneath these trees, which did not conduct heat to its lower depths. Shading by large trees may also prevent increases in soil temperature, making soil solarization ineffective in some systems (T. Wicks, pers. comm.).

A high level of nursery hygiene is also necessary to control *Phytophthora* in production areas (Sivasithamparam & Goss, 1980). Propagation of woody plants in nurseries is often by vegetative cuttings or seeds, whereas rootstocks of some plants are produced in stoolbeds. There are many opportunities for introducing and spreading the fungus during propagation, for example, when non-sterile soil and recycled, contaminated pots are used. This can result in large losses of material in the nursery and when it is planted out in the field. Once the fungus is present in potting mix, control by fungicides is not likely to eradicate it. Bareroot stock, however, can be treated by chemical root dips. Prophylactic treatments of metalaxyl or fosetyl-Al are used by a number of woody plant nurseries to prevent the establishment of *Phytophthora* in soils (Ribeiro & Linderman, 1989), however repeated applications of fungicides are very expensive (Coffey, 1991).

Cultural methods are of limited use in controlling soilborne disease caused by *Phytophthora* because species of this genus are often introduced either with planting material or via contaminated soil or water. Most species of *Phytophthora* persist as dormant propagules in

relatively low densities in soil, yet in the presence of a host inoculum levels build up rapidly by producing cycles of sporangia and zoospores (Coffey, 1991).

2.2.3 Chemical control

A number of fungicides are used to control diseases caused by *Phytophthora* spp.. Metalaxyl is a systemic, fungistatic fungicide, commonly applied as a soil drench to control *Phytophthora* infections. It inhibits RNA synthesis (Davidse, Danial & van Westen, 1983) and is transported predominantly in an acropetal direction in the transpiration stream of plants (Gupta *et al.*, 1985). It affects Oomycete fungi at a number of stages in their lifecycle, including inhibition of sporangium, chlamydospore and oospore formation and hyphal growth (Benson, 1979; Bruck, Fry & Apple, 1980). Metalaxyl has little effect, however, on cyst germination (Coffey, Klure & Bower, 1984).

Malajczuk *et al.* (1983) found that metalaxyl controlled *P. cinnamomi* in soil leachates from sandy, lateritic soil and did not affect antagonist microorganisms. These antagonists increased the lysis of *P. cinnamomi* hyphae, so that control of the pathogen was increased. Ferrin & Kabashima (1991), Ferrin & Wadsworth (1992) and Ferrin & Rohde (1992b) found that metalaxyl inhibited some isolates of *Phytophthora in vitro*. When applied as a soil drench it controlled *P. cactorum* infection of apple (Utkhede, 1987) and apple rootstocks dipped in metalaxyl before planting out were also protected from this fungus (Jeffers, 1992). Metalaxyl applied as a soil drench also controlled *P. cambivora* root rot of European chestnut (Skoudridakis & Bourbos, 1990). Isolates of *P. citricola* and *P. parasitica* resistant to metalaxyl have been identified, however (Ferrin & Kabashima, 1991; Ferrin & Wadsworth, 1992; Ferrin & Rohde, 1992a), and the frequency of resistant isolates will probably increase with continued use of fungicides.

Another fungicide frequently used to control *Phytophthora* is fosetyl-Al. It works by affecting the pathogenicity of *Phytophthora* spp. and by affecting their ability to evade host defence mechanisms (Guest & Grant, 1991). The precise

targets of phosphonate action are still unknown (Griffith, Davis & Grant, 1992). Fosetyl-Al moves in a basipetal direction, from shoot to root (Cohen & Coffey, 1986) and breaks down rapidly in soil (Piedallu & Jamet, 1985) and plant material (Decor, 1985). When applied as a soil drench, fosetyl-Al effectively controlled disease caused by *P. parasitica* in *Catharanthus* (Ferrin & Rohde, 1992 a & b). Its breakdown product, phosphonic acid, has also been used to control disease caused by *Phytophthora*. When injected into the trunk it effectively controlled disease caused by *Phytophthora* in almond and cherry trees (Wicks & Hall, 1990; Schutte, Bezuidehout & Kotze, 1991) and, applied as a soil drench or foliar spray, it controlled disease caused by *P. cambivora* in almond (Wicks & Hall, 1990). Pegg *et al.* (1985) found that phosphonate soil drenches decreased infection of avocado by *Phytophthora*, but that trees injected with phosphonate rapidly recovered from infection. One disadvantage of using the trunk injection method to apply phosphonate, is that it is unsuited to juvenile trees or seedlings, because of their narrow trunks. Lowe, Ryley & Bowdler (1991), however, found that phosphonate did not control *Phytophthora* root rot of lucerne. The fungicide, dimethomorph, is also used to control disease caused by *Phytophthora*. It inhibits sporangium formation and zoospore germination, and can be fungicidal (Kuhn *et al.*, 1989 a & b). Dimethomorph controlled *P. cinnamomi* root rot of eucalypts when applied as a soil drench, but foliar sprays did not control the disease (Marks & Smith, 1991). It is important that the optimum method and dose rate of fungicide application for controlling disease are determined for each plant species infected with *Phytophthora* spp..

Washington (1983) tested the effect of different fungicides for control of *P. cinnamomi* causing root rot of *C. sativa*. He found that either metalaxyl or fosetyl-Al, applied as a soil drench, controlled *P. cinnamomi* infection of *C. sativa* seedlings in pot experiments. *P. cinnamomi* could not be re-isolated from soil treated with metalaxyl, but was recovered from soil treated with fosetyl-Al. Fungicides are of limited use, however, in controlling *P. cinnamomi* infestations in native forests in Australia. Attempts to destroy localized infestations using soil fumigants and fungicides have had little lasting effect on the fungus (Weste, Cooke & Taylor, 1973; Weste & Law, 1973) therefore other methods of controlling

the disease have had to be evaluated. The repeated applications of fungicide necessary to control infection by *Phytophthora* are also expensive and can be phytotoxic to some plant hosts (Sivasithamparam, 1991). Possible contamination of ground and runoff water could also result from repeated applications of fungicides. Acuff (1988) noted that current and future regulation on the restriction of fungicide use will increase losses due to plant disease even further. Control of *Phytophthora*, should therefore involve a combination of the use of resistant host species and limited fungicide use (Fry, 1980) .

2.2.4 Biocontrol of *Phytophthora* using microorganisms

Because of the problems involved in using chemicals to control *Phytophthora*, a number of microorganisms have been isolated from soil and evaluated for use as biological control agents. Biological control is the reduction of inoculum density or disease producing activities of a pathogen in its active or dormant state, by one or more organisms (Baker & Cook, 1974). A number of mechanisms are involved in biocontrol of *Phytophthora* spp.. Competition occurs between microorganisms when space or nutrients are limiting, especially in the rhizosphere (Lewis, Whipps & Cook, 1989). Organic amendments, such as composted hardwood bark, added to container media used to grow some Australian native plants, reduced disease caused by *Phytophthora* spp. (Hardy & Sivasithamparam, 1991). This probably reflects increased competition, especially from antagonistic bacteria and fungi. Competition is difficult to evaluate in soil, however, and further work needs to be done before its importance in controlling pathogens is understood.

The production of secondary metabolites with antibiotic activity has frequently been shown to inhibit pathogen growth *in vitro*. In *Trichoderma* and *Gliocladium* spp. a number of antibiotics have been identified: most isolates or species produce 6-n-pentyl-2H-pyran-2-one (6PP) which has a 'coconut smell' (Ghisalberti & Sivasithamparam, 1991). *Gliocladium* spp. produce gliovirin (Howell & Stipanovic, 1983), gliotoxin (Brian & Hemming, 1945) and viridin (Brian & McGowan, 1945). They also produce the phytotoxin viridiol (Howell & Stipanovic, 1984). Gliovirin is highly active against members of the Oomycetes, but has little

effect on other fungi or bacteria (Howell & Stipanovic, 1983). Species of *Gliocladium*, therefore, may have strong potential in the control of *Phytophthora* spp..

Ectomycorrhizal fungi also protect trees against root pathogens, including *P. cinnamomi*, by producing antibiotics. *Pisolithus tinctorius* produces the antibiotics Pisolithin A and B (p-hydroxybenzoylformic acid and p-hydroxymandelic acid, respectively) which inhibit spore germination and cause hyphal lysis of *Phytophthora* spp. (Tsantrizos *et al.*, 1991). These compounds may disrupt cell turgor (Kope *et al.*, 1991).

Mycoparasitism occurs when one fungus exists in intimate association with another from which it derives some or all of its nutrients while conferring no benefit in return (Lewis *et al.*, 1989). Many studies have shown parasitism of a wide range of fungi by antagonistic *Trichoderma* and *Gliocladium* spp. *in vitro*, usually by coiling, penetration of the host fungus, the formation of haustoria and disorganization of host cell contents (Elad *et al.*, 1983; Howell, 1982; Ridout, Coley-Smith & Lynch, 1988; Dennis & Webster, 1971c).

Many other genera of fungi have been used experimentally to control *Phytophthora* both *in vitro* and *in vivo*. The chytrid *Catenaria anguillulae* invaded oospores of *P. cinnamomi* and *P. parasitica* and chlamydospores of *P. parasitica* in Californian soils, rendering these structures non-viable (Daft & Tsao, 1984), whereas *Myrothecium* spp. protected citrus against infection by *P. nicotianae* var. *parasitica* (Tuset, Hinarejos & Garcia, 1990) and avocado against infection by *P. cinnamomi* in glasshouse tests (Gees & Coffey, 1989), probably using mycoparasitism and antibiotic production to inhibit *Phytophthora* spp.. *Penicillium janthinellum* controlled *P. cinnamomi* in container medium used to grow azalea (Ownley & Benson, 1992). The mechanism of control is not understood, but is probably due to the production of antibiotics. Ectomycorrhizal fungi protected pine and eucalypt roots from *P. cinnamomi*, by competition, physical protection by the fungal sheath and by the production of antibiotics (Marx & Davey, 1968 a & b; Marx, 1970; Ross & Marx, 1972; Marx, 1975; Marais & Kotze, 1976; Malajczuk, 1979). Biological control of *Phytophthora* root rot of

chestnut using ectomycorrhizas has also been investigated (Vrot & Grente, 1985). In preliminary experiments, the mortality of seedlings inoculated with certain strains of *Boletus*, *Scleroderma* and *Hebeloma* was shown to be reduced compared with nonmycorrhizal plants. Further experiments are needed, however, to confirm these findings. Protection of avocado plants against disease caused by *Phytophthora* has also been demonstrated by first inoculating with a nonpathogenic isolate of *Phytophthora parasitica*, a phenomenon termed induced resistance. Prior inoculation of avocado with *P. parasitica* zoospores gave induced protection against *P. cinnamomi* and *P. citricola* (Dolan, Cohen & Coffey, 1986).

In addition, a number of bacteria have been shown to control *Phytophthora* root rot. *Pseudomonas* spp. reduced disease incidence in *Proteas* caused by *P. cinnamomi* (Turnbull *et al.*, 1992) and infection of *Jacaranda acutifolia* (Stirling, Haywood & Pegg, 1992). Similarly, *Enterobacter aerogenes* controlled *P. cactorum in vitro* (Utkhede & Sholberg, 1986) and *P. cactorum* infecting apple in the field (Utkhede & Smith, 1991a & b, 1993). *Rhizobium* spp. have also been found to control *Phytophthora*. They suppressed *P. cinnamomi in vitro*, by reducing sporulation and zoospore survival, and by causing hyphal lysis (Malajczuk, Pearce & Litchfield, 1984). Actinomycetes (*Streptomyces* spp.) isolated from the rhizosphere of *Casuarina* spp. in NSW, Australia, also inhibited the growth of *P. cinnamomi in vitro* by as much as 90%, probably by antibiosis (Gerrettson-Cornell, 1991). These bacteria and actinomycetes were thought to control *Phytophthora* spp. by the production of antibiotics.

2.2.5 Biocontrol of *Phytophthora* using *Trichoderma* and *Gliocladium* spp.

Two of the most common genera of fungi used in the biocontrol of soilborne plant pathogenic fungi are species of *Trichoderma* and *Gliocladium*. The modes of action of these biocontrol agents are discussed in section 2.2.4. The inhibition of *Phytophthora* spp. by these genera has been observed *in vitro* (Dennis & Webster, 1971a, b & c; Kelley & Rodriguez-Kabana, 1976; Lederer, Lorenz & Seemuller, 1992). These fungi have also been shown to control disease caused by *Phytophthora* in glasshouse and field experiments. Reichard & Bolay (1986)

found that when mycelial plugs of *P. cambivora* and *P. cinnamomi* growing on CMA were applied to soil to infect 2 year-old *C. sativa* trees, infection failed to develop. Colonies of *Trichoderma* spp. were observed in the soil and may have been antagonistic to the *Phytophthora* spp.. A large number of *Trichoderma* and *Gliocladium* spp. have been screened for biocontrol of *P. cactorum* collar and root rot of apple seedlings in the glasshouse (Smith, Wilcox & Harman, 1990; Roiger & Jeffers, 1991). A number of isolates protected seedlings against root rot, and these need to be tested further on trees in field.

One *Trichoderma* preparation available commercially to control plant pathogens contains Swedish isolates of *T. harzianum* and *T. polysporum* and is effective against silver leaf disease caused by *Chondrostereum purpureum* in plum trees and other *Prunus* spp. (Ricard, 1981). A number of improved isolates of *Trichoderma*, produced by UV-induced mutation, protoplast fusion and transformation, to induce benomyl-resistance, improved antibiotic production, and improved rhizosphere competence, are also being developed for commercial release (Baker, 1989).

2.2.6 Advantages and disadvantages of using biocontrol agents

There are a number of advantages of using biocontrol agents over conventional chemical control. Such agents are not known to be toxic to animals, including humans, and to crops and the environment. They are also less likely to affect non-target microorganisms. As far as is known, no plant pathogens have become resistant to the action of fungal biocontrol agents, whereas some chemicals have become less effective due to the development of fungal populations insensitive to fungicide (Locke, Marois & Papavizas, 1985). The cost of developing biological controls has also been estimated to be much less than that of chemical controls (approx. US$2 million compared with US$30 million) (Andrews, 1992). Furthermore, the market size required for companies to make a profit on biocontrol agents is thought to be smaller than that required for chemicals (US$5-20 million, compared with US$40 million) (Andrews, 1992). At present there are only a small number of commercially available biocontrol products, for example *Phlebia gigantea* to control root rot of conifers

caused by *Heterobasidion annosum* (Rishbeth, 1975), *Trichoderma viride* to control silver leaf disease of *Prunus* species caused by *Chondrostereum purpureum* (Ricard, 1981) and *Streptomyces griseoviridis* to control *Fusarium* and other soilborne pathogens of various horticultural crops (product name Mycostop ®, Lahdenpera, 1987). A number of *Trichoderma* isolates have been patented and are now being investigated with the aim of product release.

The disadvantages of biocontrol agents are that they are usually slow acting and not expected to eliminate pathogens, whereas most chemicals act quickly to control disease. As mentioned above, biocontrol agents are probably specific to the types of pathogen or diseases they affect, with the disadvantage that they may control a pathogen on one crop but be ineffective against another pathogen of that crop, or that pathogen on another crop. Also, they are living organisms which are affected by environmental extremes. Their shelf life may be limited or they may require special handling to maintain viability (Lumsden & Lewis, 1989). Although these problems must be taken into consideration when developing a biocontrol agent, the advantages of their use over chemicals far outweigh their disadvantages.

2.2.7 The importance of using *in vivo* studies to screen biocontrol agents

In vitro experiments are useful for elucidating the mechanisms of biocontrol agents, but they must be used in conjunction with *in vivo* experiments. Lumsden & Lewis (1989) noted that, ideally, assessment of biocontrol ability should be done in an environment as similar as possible to the environment in which the antagonist will be applied. Lewis & Papavizas (1985) also noted that there is often little correlation between *in vitro* studies and the ability of a potential biocontrol agent to reduce or prevent disease in the greenhouse or the field. Duchesne, Peterson & Ellis (1989) noted, however, that although these factors have to be taken into consideration, screening a large number of microorganisms for biocontrol against pathogens in the field is often unrealistic because of the demands in space, time and manpower. Furthermore, precise environmental conditions required by some pathogens to cause disease may not always be available in field screening conditions. They suggested,

therefore, that initial screening is carried out *in vitro* and that any promising isolates are then selected for glasshouse and field assays.

2.3.1 The use of antibodies to investigate plant-fungal interactions

The mechanical and biochemical responses of plants to infection are discussed in section 2.1.7. These processes are important in controlling infection in resistant plants. In addition to such conventional structural and biochemical approaches, immunological techniques have contributed to a greater understanding of the infection process in fungal pathogen/ host plant interactions.

Antibodies are glycoproteins, produced in lymphocytes, which bind to antigens. Lymphocytes can be fused with myeloma cells, to produce a hybridoma cell. Each hybridoma cell continues to produce and secrete the same antibody made by the lymphocyte cell prior to fusion. Clones of hybridoma cells proliferate *in vitro* and produce monoclonal antibodies (Pelczar, Chan & Krieg, 1988).

Monoclonal and polyclonal antibodies have a number of uses in plant pathology, for example in commercial disease diagnosis kits. They have been used to detect the sap-staining fungus (*Ophiostoma piceae*) and a biocontrol agent (*Gliocladium roseum*) in liquid culture and in wood (Luck, Breuil & Brown, 1990), to detect snow mold fungi (*Pythium* spp.) in wheat plants (Takenaka, 1992), and to detect *Pythium aphanidermatum* in turfgrass (Shane, 1991). Antibodies have also been used to identify taxonomic differences between fungi, for example, between three species of *Armillaria* and *Lentinula edodes* (Burdsall & Banik, 1990), between anastomosis groups of *Rhizoctonia solani* (Kellens & Peumans, 1991), and between species of *Pythium* (Estrada-Garcia *et al.*, 1989).

2.3.2 The use of antibodies to detect *Phytophthora* spp. in plant material and soil

Antibodies have been and are being investigated as a means of detecting *Phytophthora* spp. in infected plant material and soil. Benson (1991) and Pscheidt *et al.* (1992) used commercially

available diagnostic kits to detect *P. cinnamomi* in azalea and *Phytophthora* spp. infecting a large number of woody plants. Hardham and co-workers are currently developing a diagnostic kit to detect *P. cinnamomi* in infested soil (A. Hardham, pers. comm.). ELISA techniques using polyclonal antisera have also been used successfully to study *Phytophthora* spp. in plant material, for example to estimate amounts of *P. infestans* mycelium in leaf tissue of potato (Harrison *et al.*, 1990). This method provides fast, reliable identification of fungal pathogens causing disease, compared with conventional isolation and identification of fungi using morphological features. Monoclonal antibodies have been used to label carbohydrate antigens in the walls of hyphae of *P. megasperma* f. sp. *glycinea* (Wycoff & Ayers, 1990, 1991). Antibodies have also been used to study the ecology of *Phytophthora* spp.. Macdonald & Duniway (1979), for example, used fluorescent polyclonal antibodies to study the survival of *P. megasperma* and *P. cinnamomi* zoospores in soil.

Hardham, Suzaki & Perkin (1986) raised monoclonal antibodies (MAbs) to surface components of glutaraldehyde-fixed zoospores and cysts of *P. cinnamomi* in an attempt to recognize compounds involved in the infection process. Antibodies specific to the zoospore tinsel and whiplash flagella, nuclei, cell membrane, and to components of large peripheral vesicles, ventral and dorsal vesicles were identified and used in ultrastructural studies. They found that the large peripheral vesicles contain three glycoproteins (M_r>300 kD), all with mannosyl/glucosyl residues. After zoospore encystment and wall formation, the large peripheral vesicles migrate to the centre of the cyst. They are degraded during cyst germination and appear to act as a nutrient store (Gubler & Hardham, 1988, 1990). Most of the glycoprotein contents of the dorsal and ventral peripheral vesicles are released within 1-2 min of the induction of encystment. The contents of the ventral vesicles coat the ventral surface of the cyst, allowing it to adhere to the host root. The contents of the dorsal vesicles are released onto the outer surface of the cyst (distal to the root) and the function of this coating is unknown (Gubler & Hardham, 1991). The dorsal coating may play a protective role against desiccation or prevent osmotic swelling during encystment. The coating may also provide a suitable matrix for the assembly of the microfibrillar cell wall of the cyst,

previously thought to be produced by large peripheral vesicles (Sing & Bartnicki-Garcia, 1975a; Bartnicki-Garcia & Hemmes, 1976).

Peripheral vesicles have recently been identified in other parts of the asexual lifecycle of *P. cinnamomi*. Dearnaley & Hardham (pers. comm.) examined vegetative hyphae and, labelling them with monoclonal antibodies and using immunofluoresence and immunodot blotting techniques, found that the three types of vesicles were absent, but that they appeared in hyphae during the development of chlamydospores and sporangia. Peripheral vesicles appeared in germling cysts after they had been grown in mineral salts solution for 6-30 h, after incubation first in nutrient-rich V8 broth. Large peripheral and ventral vesicles appeared in hyphae first, approx. 6 h after transfer to mineral salts, and then dorsal vesicles appeared approx. 24 h later. The production of these three vesicle types was correlated with sporangia production and they probably appeared due to nutrient stress, which induces sporangia and chlamydospore production.

Large peripheral vesicles were observed in hyphae of *P. cinnamomi* but were all degraded by 4 h after germination of cysts on *Eucalyptus seiberi* seedling roots *in vitro* (Gubler & Hardham, 1990). Dorsal and ventral vesicles were degraded by 3.5 min after encystment (Hardham & Gubler, 1990). Investigations of the presence of peripheral vesicles in hyphae, however, were not extended further than this. A study of their presence in the subsequent infection events is required for a further understanding of the role of these vesicles in infection. Evidence to date suggests that peripheral vesicles have an important role in morphogenesis in the fungus and the infection process.

2.4. Summary

A number of species of *Phytophthora* cause disease on *C. sativa* and other plants worldwide. The structural and biochemical mechanisms operating in resistant plants are important in disease control, as are cultural and chemical practices used to control the disease in susceptible plants. There are a number of disadvantages and problems, however, with the use

of these methods. Therefore, biological control, including host plant resistance and the use of antagonistic fungi, is currently being investigated as a means of controlling disease. Species of *Trichoderma* and *Gliocladium* are being developed to control *Phytophthora* spp.. A greater understanding of the infection process of plant roots by *Phytophthora* spp. may facilitate control of the disease, and structural studies, especially those involving the use of monoclonal antibodies, have provided some information on this infection process.

3. GENERAL MATERIALS AND METHODS

The isolates of *Phytophthora* used in this study are shown in Table 3.1. Isolates not obtained from chestnut are indicated with an asterisk.

3.1 Isolation and culture of fungi

Phytophthora spp. were isolated using the pear bait method of McIntosh (1964) from soil collected beneath dead and dying chestnut (*Castanea sativa*) trees in orchards in South Australia. Fresh soil samples were placed in tubs, suspended in tap water, and baited with green "Duchess" pears for 7-10 d. Following incubation at room temperature, small pieces were excised from the leading edge of firm dark lesions which formed on the pear at the water level and plated on 20 ml CMA (17 g l^{-1} Difco Bi-Tek agar) in 90 mm diam. Petri dishes. *Phytophthora* spp. growing out from pear tissue were subcultured for further study. Of the 15 isolates obtained, 14 were identified as *Phytophthora cinnamomi* Rands and one isolate as *P. citricola* Sawada, using morphological criteria (Stamps *et al.* 1990). A fast-growing *P. cinnamomi* isolate (CR6A) and the *P. citricola* isolate (PT4A) were chosen for use in experiments. *Phytophthora* spp. cultures were maintained on CMA at 25°C, and plugs 8 mm in diam. cut from the margin of actively growing 5-7 d-old cultures were used in all *in vitro* experiments. All cultures were grown on 20 ml agar in 90 mm diam. sterile plastic Petri dishes (Disposable Products, South Australia) unless otherwise stated. All media and miracloth were sterilized by autoclaving at 121°C unless otherwise stated.

Many soil samples did not yield *Phytophthora* spp. and when rotted pieces of pear were plated on CMA, colonies of *Trichoderma hamatum* (Bon.) Bain, *T. pseudokoningii* Rifai, and *Gliocladium virens* Miller grew out from them. These isolates were identified using morphological criteria (Rifai, 1969; Bissett, 1991) and were maintained on MEA (20 g l^{-1}, Oxoid) at 25°C. Plugs 8 mm in diam. cut from the margin of actively growing 2 d-old cultures were used in all experiments.

Identifications of isolates of *Phytophthora* spp. were confirmed by Dr G. Hall, CMI, UK and *Trichoderma* spp. and *G. virens* by Dr M. Priest, BCRI, Sydney, Australia.

3.2 Production of zoospores

Zoospores of *Phytophthora* spp. were produced using the method of Hardham *et al.* (1991), in which seven squares of mycelium (2 mm^2) were cut from the margin of a culture growing on V8 agar (10% V8 juice, [Campbell's Soups Pty Ltd, Lemnos, Australia], 0.002% β-sitosterol [Sigma], 0.01% $CaCO_3$ and 1.7% Bitek Agar [Difco]) and placed on a disc of miracloth (Calbiochem Corp., La Jolla, CA, USA) on V8 agar in a Petri dish. The miracloth discs were boiled with three changes of water and autoclaved before use. After 5 d growth at 25°C in the dark, the miracloth and adhering mycelium were transferred to 100 ml of V8 broth (5% V8 juice, 0.01% $CaCO_3$, 0.002% β-sitosterol) in a 250 ml Erlenmeyer flask and shaken overnight at 80 rpm and 25°C. The cultures were then washed three times and incubated in 100 ml mineral salts solution (19 mM $Ca(NO_3)_2$, 5 mM KNO_3, 4 mM $MgSO_4$ and 2 ml l^{-1} of a solution containing 10 mM $FeSO_4$ and 10 mM Na_2EDTA). The cultures were shaken for 48 h at 25°C during which time sporangia developed. Isolate 6BR was only incubated for 24 h. Cleavage of sporangia was induced by transferring the miracloth discs to Petri dishes, rinsing three times in cold sterile DW and submerging the discs in 10 ml sterile DW. The cultures were then placed at 4°C for 15 min and placed on a light box (Medilite. New South Wales) at 18°C for 75 min to induce the release of zoospores.

A method modified from Gees and Coffey (1989) was also used to produce zoospores, in which seven 2 mm^2 plugs of fungus were placed in a Petri dish and flooded with V8 broth. After 24 h incubation at 25°C under white fluorescent lights (Hanimex Statesman), plugs were rinsed three times with sterile soil extract (1% soil mixed with DW, filtered through a No. 1 Whatman filter and then autoclaved) and flooded with soil extract for a further 48-72 h during which time sporangia formed. Cleavage of sporangia was induced by incubating the plates at 4°C for 30 min and zoospores were released during 60-75 min incubation on a light box at 18°C.

3.3 Production of oospores in culture

Plugs were taken from cultures of *Phytophthora* isolates of unknown mating types and placed on one edge of a Petri dish containing 20 ml carrot agar (Ribeiro, 1978), while plugs of either a known A1 or A2 isolate were placed on the other edge of the Petri dish. Cultures were incubated in the dark at 25°C for 8-10 wk after which time oospores had formed between opposite mating types.

3.4 Establishment of plant material

Seeds were obtained from chestnut growers in South Australia and Victoria and dipped in Benlate (Du Pont) fungicide (100 mg l^{-1}) in DW, to prevent rotting, then placed in bags containing moist sterile vermiculite. Dormancy was broken by stratifying seeds at 4°C for 2-3 wk before they were planted in plastic trays (42 x 32 x 12 cm) filled with University of California (UC) mix soil. This consisted of 0.25 m^3 coarse sand and 0.25m^3 peatmoss mechanically mixed with the following nutrients: potassium nitrate 60 g, magnesite 120 g, reverted super 700 g, plaster of paris 460 g, potassium sulphate 60 g, bloodmeal 700 g, hydrated lime 900 g. The mixture was pasteurized at 75°C for 45 min using aerated steam. Seeds were incubated at 25°C with a 16 h day (250 μmol m^{-2} sec^{-1} light) and watered with RO water as needed.

Germinated seeds were transferred to 10 cm diam. pots containing recycled soil (RS) with 50% peat, pH 6.0. Recycled soil consisted of 0.5 m^3 used and composted experimental soil (composted for up to 2 years) mixed with 0.1 m^3 peat moss to which: 500 g bloodmeal, 200 g potassium sulphate, 100 g super phosphate and 200 g ground limestone had been added. The soil was pasteurized as above.

3.5 Micropropagation of chestnut

Nodal explants of *Castanea sativa* (unknown cv., designated "Goldsworthy") and *C. sativa* x *C. crenata* hybrid cv. "Buffalo Queen" were used for micropropagation. Shoots approx. 20 cm long were excised from glasshouse-grown 3 month-old seedlings and washed, with

brushing, in a dilute solution of Tween 20 (Sigma) in DW to increase surface wetting during sterilization. Shoots were surface sterilized with 0.6% White King bleach (4% avail. chlorine) for 15 min, rinsed three times in sterile DW, then soaked in sterile DW for 3 h. Chevre *et al.* (1983) found that soaking reduced the amount of black exudate released by shoots into the medium. Shoots were then cut into nodal sections of approx. 1 cm and placed in an initiation medium (modified from Vieitez *et al.*, 1986). This consisted of 1/2 MS medium (Murashige & Skoog, 1962) with all mineral salts at half strength, 30 mg l^{-1} sucrose and 1 mg l^{-1} BA. The pH was adjusted to 5.7 with 1N NaOH. Ten ml was dispensed into 8 x 3 cm polycarbonate universal tubes (Disposable Products, South Australia) and autoclaved for 20 min at 121°C. Due to the release of a black exudate into the medium, uncontaminated explants were transferred to fresh medium one week later.

Shoots were subcultured every 4 wk to fresh 1/2 MS medium and after three transfers the concentration of BA was reduced to 0.5 mg l^{-1} to reduce the growth of callus at the base of the shoot. This was further reduced to 0.2 mg l^{-1} before rooting was attempted. Good rates of multiplication were obtained in the presence of 0.2 mg l^{-1} BA. Shoots were grown in a controlled environment room at 25°C, with a 16 h day and under cool white fluorescent lights (approx. 35 μmol m^{-2} s^{-1}).

3.6 Rooting of micropropagated shoots

Shoots, 2 cm long, were excised from cultures multiplied on 1/2 MS with 0.2 mg l^{-1} BA and five were placed in 50 ml 1/2 MS medium in each 250 ml polycarbonate tub (Disposable Products, South Australia) with 2 mg l^{-1} IBA (Sigma). Shoots were incubated as above and rooting was assessed after 28 d.

Table 3.1: Isolates of *Phytophthora* spp. used in this study

isolate and code	mating type	origin (locality)	date of isolation	source
P. cinnamomi CR6A	A2	Norton Summit, South Australia	February 1991	S. Chambers
P. citricola PT4A	homothallic	Upper Sturt, South Australia	February 1991	S. Chambers
P. cryptogea P173	A2	Lenswood, South Australia	1985	T. Wicks
P. cambivora P6414	A2	Bordeaux, France	1989	H. Förster (isolated by C. Robin)
P. cinnamomi 6BR* (DAR52646)	A2	Brisbane Ranges, Victoria	1986	A. Hardham
P. cinnamomi PT1B	A2	Upper Sturt, South Australia	February 1991	S. Chambers
P. cinnamomi PT2A	A2	Upper Sturt, South Australia	February 1991	S. Chambers
P. cinnamomi PT2B	A2	Upper Sturt, South Australia	February 1991	S. Chambers
P. cinnamomi PT5A	A2	Upper Sturt, South Australia	February 1991	S. Chambers
P. cinnamomi PT5B	A2	Upper Sturt, South Australia	February 1991	S. Chambers
P. cinnamomi AT1A	A2	Basket Range, South Australia	February 1991	S. Chambers
P. cinnamomi AT1B	A2	Basket Range, South Australia	February 1991	S. Chambers
P. cinnamomi CR6B	A2	Norton Summit, South Australia	February 1991	S. Chambers
P. cinnamomi DK3A	A2	Lenswood, South Australia	February 1991	S. Chambers
P. cinnamomi DK3B	A2	Lenswood, South Australia	February 1991	S. Chambers

P. cinnamomi QJ2A	A2	Heathfield, South Australia	February 1991	S. Chambers
P. cinnamomi QJ2A	A2	Heathfield, South Australia	February 1991	S. Chambers
P. cinnamomi P63	A2	Carey Gully, South Australia	1988	E. Scott
P. cambivora P5*	A1	South Australia	1983	T. Lee
P. cinnamomi P1*	A1	Coromandel Valley, South Australia	1986	E. Scott
P. cryptogea Q138*	A1	Wayne Co., NY USA	1978	S. Jeffers

* Isolates not obtained from chestnut.

4. SCREENING CHESTNUT ROOTSTOCKS FOR RESISTANCE TO *PHYTOPHTHORA* USING MICROPROPAGATED SHOOTS

4.1 Introduction

Methods used to screen for resistance to *Phytophthora* root rot are discussed in Chapter 2. Rapid screening methods to evaluate response to pathogens are important to plant breeders, so that resistant material can be identified and incorporated into breeding programs. One such method, the excised twig assay, was used to study root and collar rot of apple caused by *P. cactorum* (Jeffers *et al.*, 1981). Dormant shoots, excised from orchard trees, were surface-sterilised, exposed to *P. cactorum* , and then the extent of necrosis measured. Cultivars were ranked according to their relative resistance to *P. cactorum*, and cv. x isolate reactions compared. Wicks & Lee (1986) and Scott *et al.* (1992) used modifications of this excised twig assay to screen almond, peach and peach x almond hybrid rootstocks for resistance to *P. cambivora.* The peach cv. "Nemaguard" was resistant to *P. cambivora*, while almond cvs "Mission" and "Chellaston" were susceptible, and these responses reflected the field situation.

Initially, in experiments conducted in this study, chestnut twigs excised from different cvs of *C. sativa*, *C. crenata* and *C. mollissima*, used as rootstocks, were screened for response to *P. cinnamomi* using the excised twig assay (Scott *et al.*, 1992). Shoots of different cvs and species of *Castanea* were exposed to *P. cinnamomi* for 14 d and then the length of necrosis measured. Discolouration extended along the entire length of twigs of all rootstocks screened, and, therefore, this method was considered unsuitable for use in screening cvs and species of chestnut for differences in susceptibility to *P. cinnamomi.*

Tissue culture techniques, in which plantlets are produced by micropropagation by axillary shoot proliferation or via callus, can also be used, in conjunction with conventional breeding programs, to screen for disease resistance and facilitate production of disease resistant material (Daub, 1986). Many studies have been conducted using callus (e.g. McComb *et al.*,

1987; Jang & Tainter, 1990; Phillips *et al.*, 1991). It would not be practical to use chestnut callus to screen for resistance to *Phytophthora* because "normal" plants have not been regenerated from chestnut callus. Other methods of screening for resistance need to be considered. A few studies have recently been conducted using tissue culture methods to screen for disease resistance in shoot cultures of woody plants. Sharma & Skidmore (1988) identified partial resistance in micropropagated shoots of *Carica papaya* L. to *P. palmivora*, while Scott *et al.* (1992) used a modified excised twig assay to screen micropropagated shoots of almond and peach rootstocks for resistance to *P. cambivora*. Resistance of micropropagated shoots to *P. cambivora in vitro* was correlated with field resistance. Son (1992) also used micropropagated shoots of citrus rootstocks, to screen for resistance to *P. citrophthora*. Again, a good correlation was observed between the *in vitro* response of shoots to infection and the field response. There are a number of advantages and disadvantages of using tissue culture material to screen for disease resistance and these are discussed in section 2.1.6. Nevertheless, screening for resistance to *Phytophthora* spp., using micropropagated shoots of almond and citrus has been successful, and this technique could potentially be used to screen for disease resistance of other woody plant species.

The excised shoot assay system described in this chapter was used before whole plant inoculations (see chapter 6), for screening rootstocks for differences in susceptibility to *Phytophthora* spp.. In the first two years of the project, seed germination rates were consistently less than 50%, due in part to storage rot fungi, and only small quantities of seed were available, thus it was difficult to obtain enough seedlings for adequate replication in inoculation experiments. Also, chestnut seeds were available only in the autumn, immediately after the nuts were harvested. Due to spoilage fungi, it was very difficult to maintain the storage conditions required to keep seeds viable and, therefore, seeds had to be stratified immediately after the harvest and then planted. Because of these difficulties, alternative methods for screening clonal material were evaluated. The principal aims of the experiments presented here were to investigate whether a modified excised shoot assay, using micropropagated plant material, could be used as a rapid screening technique to assess

differences in susceptibility of chestnut cvs to *Phytophthora* spp.. When this method was established for chestnut, experiments were conducted:

(i) to determine whether isolates of *Phytophthora* spp. vary in pathogenicity;

(ii) to determine whether any resistant chestnut genotypes could be identified within a susceptible cv.;

(iii) to confirm the results of the excised shoot assays, by inoculating plantlets of chestnut cvs, growing in vermiculite, to assess response to *Phytophthora* spp..

4.2 Materials and Methods

4.2.1 Micropropagation of chestnut

An unknown cv. of *Castanea sativa* "Goldsworthy" and a *C. sativa* x *C. crenata* hybrid cv. "Buffalo Queen" were micropropagated (Fig. 4.1) as described in section 3.5. Attempts were made to establish resistant *C. crenata* and *C. mollissima* material in culture from mature trees, however, these were unsuccessful. Seedling material of these species was unavailable for use in this project because strict quarantine regulations do not allow seeds from most countries to be imported (R. Van Velsen, pers. comm.). Only seeds from Washington State, USA, could be imported and these were unobtainable because of local demand in the USA (M. Dolan, pers. comm.). Therefore, only shoots of "Goldsworthy" and "Buffalo Queen" were used in experiments.

4.2.2 The modified excised twig assay

The modified excised twig assay (Scott *et al.*, 1992) was used to determine whether differences in susceptibility existed between cvs of chestnut "Goldsworthy" and "Buffalo Queen". Polycarbonate tubs (250 ml, Disposable Products, South Australia), containing 50 ml CMA (Oxoid), were inoculated in the centre with an 8 mm diam. plug of an isolate of *Phytophthora* spp. taken from the edge of an actively growing culture on CMA. Inoculated tubs were incubated in the dark at 25°C until the fungus had reached the edge of the tub (approx. 5-7 d, depending on the isolate). Shoots, 2 cm in length, were then excised from 4 wk-old chestnut cultures growing on 1/2MS medium containing 0.5 mgl^{-1} BA, partially

Fig. 4.1: Micropropagated shoot of chestnut cv. "Buffalo Queen" (3 wk after last transfer) growing on 1/2MS medium containing 0.5 mg l^{-1} BA. Bar = 0.7 cm.

defoliated by cutting the leaves off close to the stem with a sterile scalpel, and five were placed upright around the margin of each tub. There were 20 shoots for each pathogen x cultivar combination for all experiments and material was incubated in the dark at 25°C for 2 d, after which time lesions were observed along inoculated shoots (Fig. 4.2). One assessment of lesion length along shoots was made after 2d. Experiments were conducted twice and the fungus was re-isolated from one randomly chosen shoot per tub, by plating the shoot on CMA in a 90 mm diam. Petri dish and incubating in the dark at 25° C for 4 d.

4.2.3 Effect of BA on response to inoculation

Initial experiments were conducted to determine whether residual BA in the shoot had any effect on susceptibility to *Phytophthora* spp.. Shoots, 4 wk-old and 2 cm in length were taken from 1/2MS medium with 0.5 mg l^{-1} BA and placed on 1/2MS medium without BA for 10 d. Shoots from this medium without BA and shoots of equivalent age taken directly from medium containing BA were then placed in tubs pre-inoculated with *P. cinnamomi* (isolate CR6A) as above. Control shoots were placed in tubs containing uninoculated CMA.

4.2.4 Response of excised shoots to different *Phytophthora* spp.

CMA in tubs, as above, was inoculated with *P. cinnamomi* (isolate CR6A), *P. citricola* (isolate PT4A), *P. cambivora* (isolate P6414) or *P. cryptogea* (isolate P173) and incubated as in section 4.2.2. Excised shoots of cvs "Goldsworthy" and "Buffalo Queen" were placed around the margin of the tub and incubated as in section 4.2.2.

The growth of the four isolates of *Phytophthora* was also measured, for comparison with its pathogenicity on shoots *in vitro*. Cultures were grown on CMA agar at 25°C in the dark, and colony diam. (the mean of two measurements taken at right angles) was measured after 3, 5 and 7 d. There were five replicate plates of each isolate.

Fig. 4.2: The excised shoot assay system. Five excised shoots of chestnut were placed around the margin of a polycarbonate tub containing 50 ml CMA preinoculated with *Phytophthora* spp., incubated for 2 d at 25°C in the dark and then lesion length measured. Susceptibility was assessed by the length of necrosis along shoots. Shoots of "Buffalo Queen" BQ34, 2 d after inoculation with *P. cinnamomi* and *P. citricola*. The tub on the left contains healthy, green control shoots. Necrosis is visible on shoots incubated with *Phytophthora* spp. (arrows). Bar = 1.5 cm.


BQ34
P. cinnamomi
P. citricola

4.2.5 Response of different *Castanea* genotypes

Six genotypes of the cv. "Buffalo Queen" were inoculated to determine whether genotypes differed in susceptibility to isolates of *Phytophthora*. Shoots of each genotype were obtained by micropropagation of material from individual seedlings of "Buffalo Queen". CMA was pre-inoculated with *P. cinnamomi* (isolate CR6A) or *P. citricola* (isolate PT4A) as in section 4.2.2 and then excised shoots of "Buffalo Queen" genotypes BQ25, BQ30, BQ34, BQ40, BQ41 and BQ56 were incubated with the fungus for 2 d.

4.2.6 Effect of inoculum type on response

The effect of two types of inoculum on response was also examined. Shoots were placed in tubs pre-colonized with *P. cambivora* (isolate P6414) and control shoots were inserted into uninoculated CMA as above. Other excised shoots were defoliated and inoculated at the base by injecting a drop (approx. 10 μl) of a zoospore suspension (10^4 zoospores ml^{-1}, produced using the method of Hardham *et al.* (1991)) from a hypodermic syringe (Sharma & Skidmore, 1988). These inoculated shoots were then placed in CMA. *P. cambivora* was used because zoospores of this species were produced most reliably. Control shoots were inoculated with a drop of sterile DW. Shoots were then inserted in CMA in tubs and incubated as in section 4.2.2.

4.2.7 Inoculation of plantlets *in vitro*

Plantlets (Fig. 4.3) were produced as described in section 3.6. Two micropropagated plantlets were placed on opposite sides of CMA in a 250 ml polycarbonate tub, pre-inoculated, as in section 4.2.2, with *P. cinnamomi* (isolate CR6A), with the roots sitting on the surface of the agar. Control plants were placed on uninoculated CMA in tubs. There were ten replicate tubs of each pathogen x cultivar combination. Lesion length was measured daily.

4.2.8 Transfer of chestnut plantlets to vermiculite

Chestnut plantlets, which had been incubated for 28 d on 1/2MS medium containing IBA (see section 3.6), were taken from this medium, dipped in Benlate (100 mg l^{-1} in tap water) and

Fig. 4.3: Rooted shoot of chestnut cv. "Goldsworthy" (RG7), after 2 wk incubation on 1/2MS medium containing 2 mg l^{-1} IBA. Bar = 0.7 cm.


RG7

placed into plastic root trainers (5 x 5 cm in diam.) which had been sprayed with ethanol, air-dried and containing approx. 150 ml moistened, sterilized vermiculite. Seedling trays were supported in rows in plastic trays (42 x 32 x 12 cm). Plantlets were sprayed with sterile DW and then trays were covered with clear plastic sheets to maintain high humidity. Plants were incubated at room temperature (approx. 25°C) under white fluorescent light with approx. 10 h daylength and the plastic sheeting was removed daily, for gradually increasing periods, to harden the plants off. Plantlets were then transferred to a growth room at 25°C under cool, white fluorescent lights (approx. 250 μmol m^{-2} sec^{-1}) with a 16 h daylength for inoculation with *P. cinnamomi*. Forty plants of chestnut "Goldsworthy" clone RG7, and 19 plants of "Buffalo Queen" clone BQ30, 56 of clone BQ34 and 39 of clone BQ40 were transferred to vermiculite and survival assessed after 4 wk. BQ41 was not included, as insufficient shoots developed roots.

4.2.9 Inoculation of plantlets *ex vitro*

Thirty-three rooted plantlets of clone RG7, 41 of clone BQ34 and 27 of clone BQ40 were used in an inoculation experiment. Plants were first watered with tap water, then inoculated with 2 ml of a zoospore suspension (10^4 zoospores ml^{-1} in soil extract), produced using the modified method of Gees & Coffey (1989) (described in section 3.2). Control plantlets were inoculated with 2 ml of sterile soil extract. Inoculated and control plantlets were assessed for disease symptoms after 7 and 14 d. After 14 d, one root was randomly chosen from three plantlets per treatment and plated on CMA to re-isolate the pathogen.

4.2.10 Statistical Analysis

Means of treatments for all excised shoot assay experiments were compared by Analysis of Variance and significant differences were detected between the means using Least Significant Differences. A binomial model was fitted, using data for plant rooting and survival after inoculation with *P. cinnamomi*, to test for differences between genotypes. A null hypothesis, that the expected number of survivors was the same for each genotype, was tested for the plantlet inoculation and survival experiments.

4.3 Results

4.3.1 Micropropagation of chestnut

Chestnut grew quickly in culture and shoots suitable for use in excised shoot assays were produced after a 4 wk multiplication cycle. Table 4.1 shows the percentage of rooted shoots of different clones of "Goldsworthy" and "Buffalo Queen" after 4 wk on rooting medium. A binomial model was fitted to the rooting data and the resulting analysis of deviance showed the effects of genotype to be highly significant ($P<0.001$). Shoots of "Goldsworthy" (RG7) consistently rooted more easily than those of "Buffalo Queen". Two "Buffalo Queen" clones (BQ34 and BQ40) also rooted more easily than BQ30, BQ41, and BQ56, with 14 and 15% rooting, respectively. One "Buffalo Queen" clone (BQ56) did not produce roots and less than 2% of BQ41 shoots rooted in the conditions tested.

4.3.2 Effect of BA on response to inoculation

Lesion length was scored only after 2 d in the excised shoot assays. Further assessments were not made because the fungus overgrew plant material after this. Only *Phytophthora* spp. were re-isolated from material plated on CMA. Fig. 4.4 shows mean lesion length after 2 d on shoots of "Goldsworthy" and "Buffalo Queen", taken from 1/2MS medium without BA or from medium containing 0.5 mg l^{-1} BA, and inoculated with *P. cinnamomi* or *P. citricola*. For both cvs, there was no significant difference between shoots cultured on medium without BA for 10 d prior to inoculation and those taken directly from medium with BA and so further experiments were conducted using shoots taken directly from BA medium.

4.3.3 Response of excised shoots to different *Phytophthora* spp.

The response of shoots of cultivars "Goldsworthy" and "Buffalo Queen" to four isolates of *Phytophthora* is shown in Fig. 4.5. Shoots of chestnut cvs "Buffalo Queen" and "Goldsworthy" were equally susceptible to *P. cinnamomi* and *P. citricola*, with lesions extending along almost the entire length of shoots. Lesion length differed significantly ($P<0.025$), however, on "Goldsworthy" shoots incubated with *P. cambivora* and *P. cryptogea*, with lesions caused by *P. cryptogea* smaller than those by *P. cambivora*. There was, however, no

Table 4.1: Percentage of rooted shoots of different clones of micropropagated shoots of chestnut cvs "Goldsworthy" (RG7) and "Buffalo Queen" (clones BQ30, BQ34, BQ40, BQ41 & BQ56). n = >70.

clone	% rooted shoots
RG7	37
BQ30	2.8
BQ34	14.67
BQ40	15.67
BQ41	1.67
BQ56	0

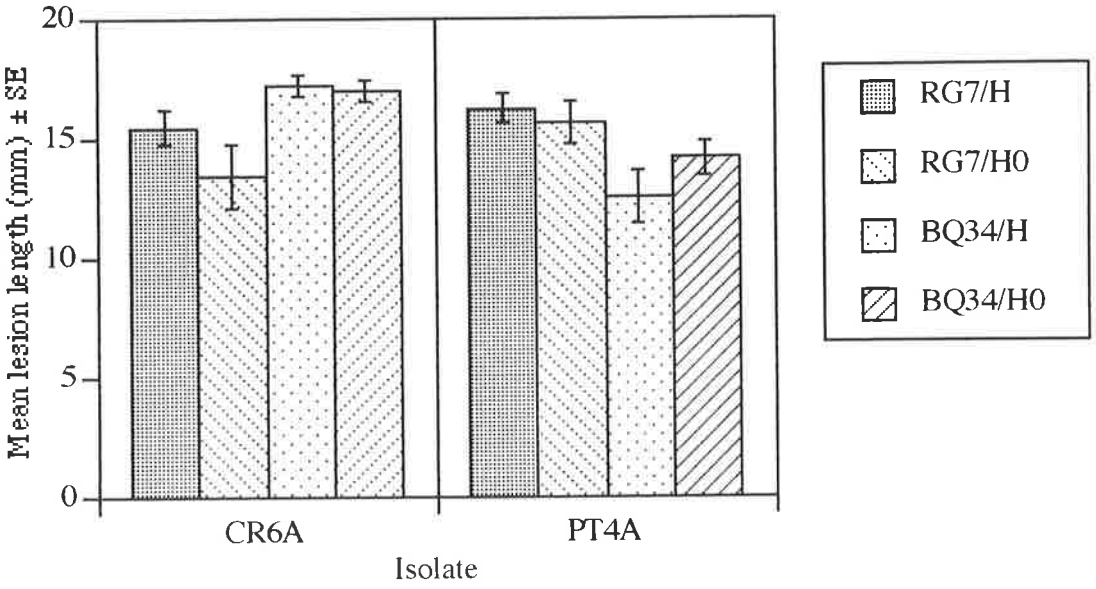


Fig. 4.4: Mean lesion length along micropropagated shoots of chestnut cvs "Goldsworthy" (RG7) and "Buffalo Queen" (BQ34) excised from 4 wk-old cultures on 1/2MS medium containing 0.5 mg l^{-1} BA (H), or cultured for 10 d on medium without BA (H0), and incubated with *P. cinnamomi* (CR6A) and *P. citricola* (PT4A) for 2 d. Bars indicate SE. LSD = 1.63, P<0.025, n = 20.

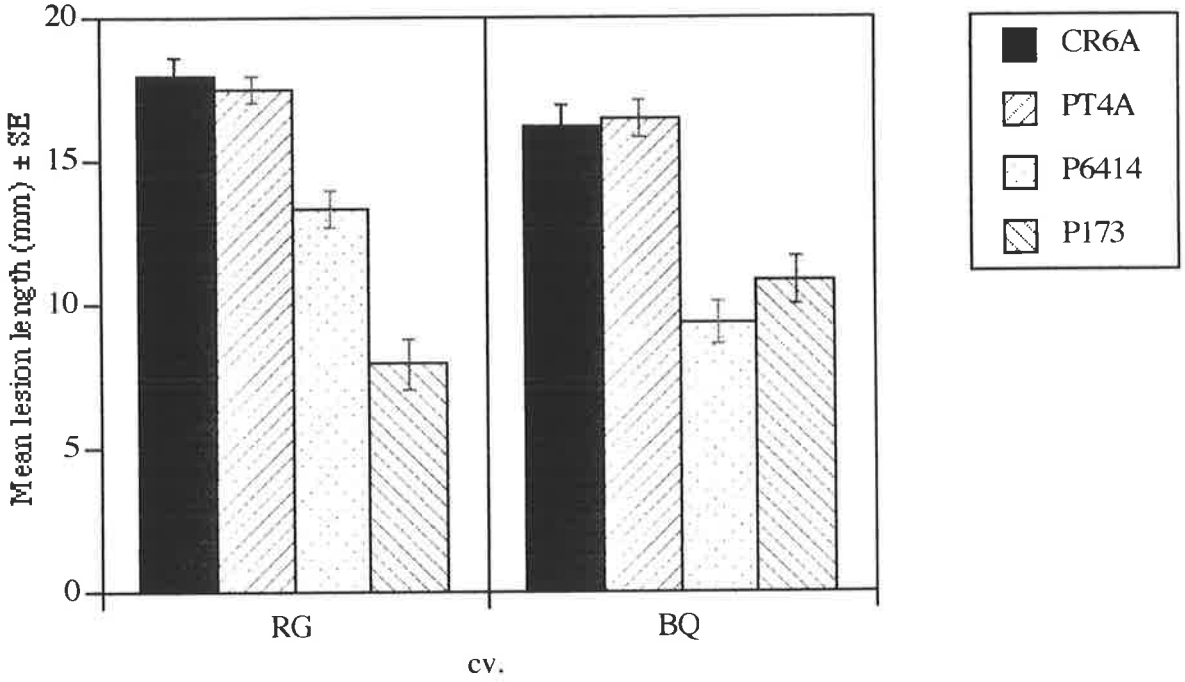


Fig. 4.5: Mean lesion length along micropropagated shoots of "Goldsworthy" (RG7) and "Buffalo Queen" (BQ30) incubated with four species of *Phytophthora* for 2 d. Bars indicate SE. LSD = 1.5, P<0.025, n = 20. CR6A = *P. cinnamomi*, PT4A = *P. citricola*, P6414 = *P. cambivora*, P173 = *P. cryptogea*.

significant difference in lesion length caused by these two isolates in "Buffalo Queen" shoots. *P. cryptogea* and *P. cambivora* caused significantly less necrosis on "Goldsworthy" and "Buffalo Queen" shoots than did *P. cinnamomi* and *P. citricola*.

The growth of the four isolates of *Phytophthora* used in experiments presented here is shown in Table 4.2. Although *P. cinnamomi* grew significantly faster ($P < 0.025$) than *P. citricola* after 3 and 5 d, both had reached the edge of the agar plate by 7 d. *P. cryptogea* and *P. cambivora* grew at significantly different rates at 3, 5 and 7 d. They both grew much more slowly than *P. cinnamomi* and *P. citricola.*

4.3.4 Response of different *Castanea* genotypes

The susceptibility of shoots of six different genotypes of "Buffalo Queen" to *P. cinnamomi* and *P. citricola* is shown in Fig. 4.6. Lesions extended along almost the entire length of shoots of five "Buffalo Queen" genotypes after 2 d. There was no significant difference in lesion length between the five genotypes, inoculated with either *P. cinnamomi* or *P. citricola.* Only lesions on shoots of BQ41, inoculated with either isolate of *Phytophthora*, were significantly smaller than those on the other genotypes (approx. 10 mm cf. approx. 17 mm).

4.3.5 Effect of inoculum type on response

The effect of mycelium or zoospores, as inoculum, on response of shoots to *P. cambivora* (isolate P6414) is shown in Fig. 4.7. There was a significant difference in lesion length on "Buffalo Queen" shoots between inoculum types ($P<0.001$), with zoospores causing only minor lesion development compared to mycelium. No significant difference in lesion development occurred between the two types of inoculum on "Goldsworthy" shoots.

4.3.6 Inoculation of plantlets *in vitro*

Plantlets inoculated *in vitro* were assessed daily for infection. However, because plants had root systems of different lengths and morphology, it was difficult to measure the response to

Table 4.2: Mean colony diameter ± SE of four *Phytophthora* spp. grown on CMA in a 90 mm diam. Petri dishes at 25°C in the dark for 3, 5 & 7 d. CR6A = *P. cinnamomi*, PT4A = *P. citricola*, P173 = *P. cryptogea*, P6414 = *P. cambivora*. LSD (3 d) = 2.26, LSD (5 d) = 3.91, LSD (7 d) = 4.60, P<0.025, n = 5.

d / Isolate	CR6A	PT4A	P173	P6414
3	36.1 ± 0.40	31.8 ±0.72	11.5 ±1.22	17.7 ±0.30
5	63.6 ±0.58	51.7 ±0.20	21.4 ±2.43	32.4 ±0.73
7	74.9 ±0.80	68.9 ±0.43	35.2 ±2.72	47.3 ±1.08

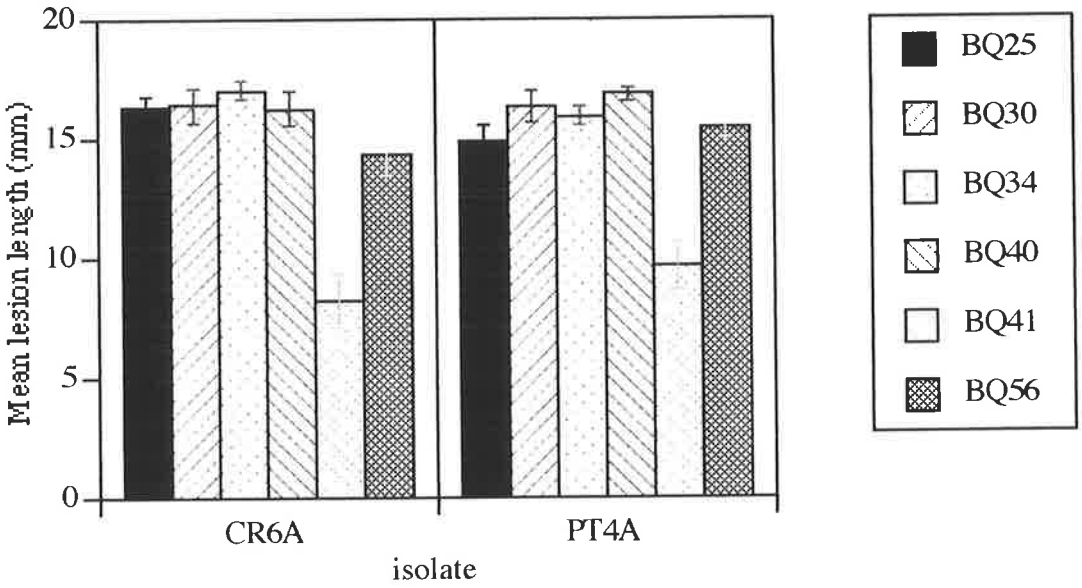


Fig. 4.6: Mean lesion length along micropropagated shoots of six different genotypes of "Buffalo Queen" (BQ25, BQ30, BQ34, BQ40, BQ41 & BQ56) incubated with *P. cinnamomi* (CR6A) or *P. citricola* (PT4A) for 2 d. Bars indicate SE. LSD = 2.64, P<0.025, n = 20.

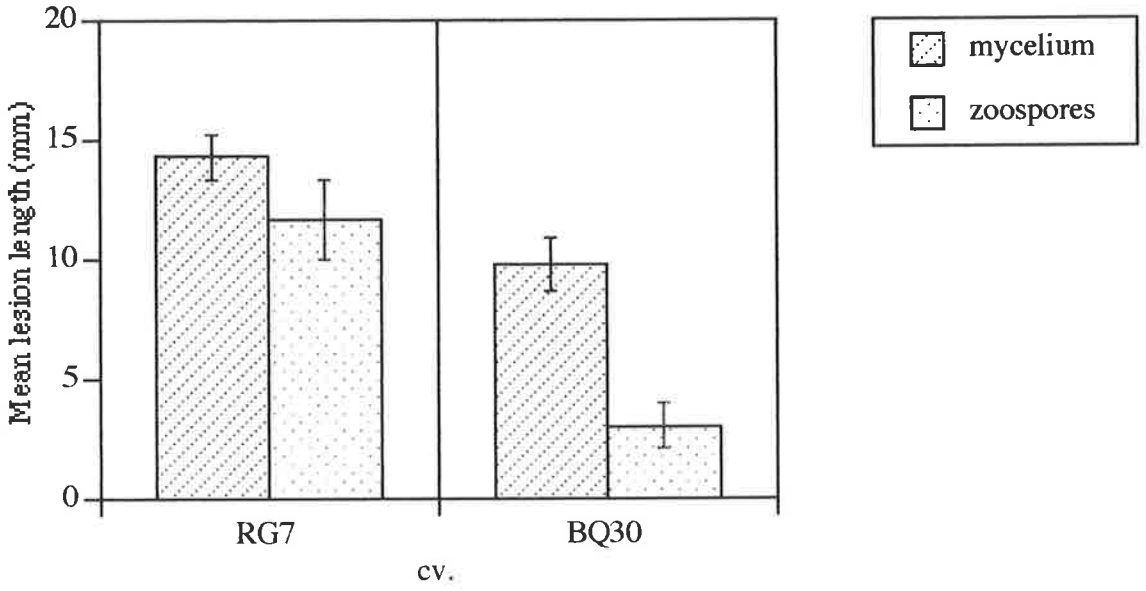


Fig. 4.7: Mean lesion length along micropropagated shoots of "Buffalo Queen" (BQ30) or "Goldsworthy" (RG7) incubated with mycelium or inoculated at the base with a zoospore suspension of *P. cambivora* (P6414) and incubated for 2 d. Bars indicate SE. LSD = 3.23, P<0.025, n = 20.

inoculation. Also, plantlets often lay on the agar surface at different angles, depending on the orientation of the roots, and lesions often formed first on parts of the roots other than the root tips. Comparative assessment between plants and treatments was, therefore, not possible and the results of this experiment are not shown.

4.2.7 Transfer of chestnut plantlets to vermiculite

The survival of chestnut plantlets of "Goldsworthy" and "Buffalo Queen" after transfer to vermiculite is shown in Table 4.3. Survival fitted a X^2 distribution, so the null hypothesis (that the expected number of survivors would be the same for each genotype) was rejected ($P<0.05$). Fewer "Buffalo Queen" clone BQ30 plantlets survived (42%), compared with the other "Buffalo Queen" and "Goldsworthy" clones (69-82%).

4.2.8 Inoculation of plantlets *ex vitro*

The results of the experiment in which plantlets were inoculated *ex vitro* are shown in Table 4.4. Both cvs of chestnut were susceptible to *P. cinnamomi* when inoculated with zoospores. Stem lesions were apparent from 7 d after inoculation and plants were killed when lesions spread up the shoots. *P. cinnamomi* was re-isolated from all material plated on CMA. Although BQ41 showed the least lesion development in excised shoot assays, only 2% of shoots rooted on the medium tested, therefore, this genotype could not be tested in plantlet inoculation experiments.

4.4 Discussion

The modified excised shoot assay was used here to screen chestnut cvs, used as rootstocks, for the first time, for resistance to *Phytophthora* spp.. The cv. "Buffalo Queen" was as susceptible as "Goldsworthy" to *Phytophthora* spp.. Seedlings of the cvs "Buffalo Queen" and "Goldsworthy", growing in soil and inoculated with zoospores, were also susceptible to *Phytophthora* spp. (measured using a root rot index, see table 6.2). The response of adult trees of these cvs to infection by *Phytophthora* spp. in the field has not been tested. The assay

Table 4.3: Percentage survival of micropropagated plantlets of chestnut "Goldsworthy" (RG7) and "Buffalo Queen" clones (BQ30, BQ34 & BQ40) grown in vermiculite for 4 wk at 25°C. a and b indicate significant differences between cvs or clones at the $P<0.05$ level.

clone	% survival	initial no. of plantlets
RG7	82.50 b	40
BQ30	42.11 a	19
BQ34	75.00 b	56
BQ40	69.23 b	39

Table 4.4: Survival of plantlets of chestnut "Goldsworthy" (RG7) and "Buffalo Queen" clones (BQ34 & BQ40), growing in vermiculite, 2 wk after inoculation with zoospores of *P. cinnamomi* (CR6A).

clone	no. of dead plants	total no. plants inoculated
RG7	11	17
BQ34	15	15
BQ40	17	18

allowed comparison of pathogenicity of *Phytophthora* species on chestnut and indicated that genotypes of "Buffalo Queen" may vary in susceptibility.

Differences in pathogenicity between isolates of *Phytophthora* were observed in the modified excised shoot assay, using micropropagated chestnut material. The growth rate of each isolate of *Phytophthora* on CMA appeared to be correlated with pathogenicity on chestnut shoots *in vitro*. This correlation has also been noted for isolates of *P. cambivora* infecting almond and peach shoots (E. Scott, pers. comm.), and by Son (1992), for isolates of *P. citrophthora* infecting micropropagated citrus shoots. Scott *et al.* (1992) correlated this *in vitro* aggressiveness of isolates of *P. cambivora* with aggressiveness on almond trees in the field. Similarly, Robin (1991) correlated the growth rates of some isolates of *P. cinnamomi* with aggressiveness of infection of seedlings of a number of forest trees in glasshouse experiments. In the present study, however, aggressiveness *in vitro* was not correlated with aggressiveness on seedlings using zoospore inoculation and collar inoculation techniques (see section 6.4).

Residual BA appeared to have no effect on the susceptibility of chestnut shoots to *P. cinnamomi*. Scott *et al.* (1992) found that residual BA in shoots of almond rootstocks had inconsistent effects on susceptibility to *P. cambivora*, whereas Son (1992) found that shoots taken directly from medium containing 0.2 or 2 mg l^{-1} BA were significantly more susceptible to *P. citrophthora* than those placed on basal medium for 10 d prior to inoculation (to dilute residual BA). Uptake of BA by micropropagated shoots of woody plants may differ and, therefore, shoots of the same age may contain differing amounts of residual BA. Apical necrosis and gradual dieback of many shoots of chestnut occurred after incubation for 10 d on basal medium without BA, perhaps indicating that residual amounts of BA in shoots was low. Apical necrosis of shoots of almond rootstocks was also observed when they were incubated on medium without BA (E. Scott, pers. comm.), whereas citrus shoots remained healthy (C. Son, pers. comm.).

Susceptible cvs or species can contain resistant genotypes and tissue culture can be used to multiply this material for use by growers. For example, Cahill *et al.* (1992) identified resistant seedlings within the susceptible species *E. marginata* in collar inoculation experiments with *P. cinnamomi.* Seedlings, 9-12 months old, were inoculated by placing mycelium in a shallow wound in the stem. Resistant plants, showing restricted lesion development, were then multiplied by micropropagation, and planted out in sand and vermiculite for further testing. When inoculated with zoospores, 80% of these micropropagated plantlets were resistant to *P. cinnamomi*, compared with susceptible *E. marginata* plants. The excised shoot assay, conducted to examine differences between genotypes of chestnut, identified BQ41 as less susceptible to *P. cinnamomi* and *P. citricola* than five other genotypes of the cv. "Buffalo Queen". The excised shoot assay may therefore be able to identify resistant material within a susceptible cv. or species of woody plant. This "superior" material, after further evaluation, could then be multiplied and made available to growers. Although BQ41 showed some resistance to *Phytophthora* spp. in the modified excised shoot assay, this genotype could not be induced to root satisfactorily *in vitro*, and therefore could not be tested further. Material would need to be planted out and inoculated with *Phytophthora* spp. to confirm the findings of the excised shoot assay.

The type of inoculum used to infect chestnut shoots *in vitro* significantly affected their response in experiments reported here. Zoospores caused smaller lesions than did mycelium on "Buffalo Queen" shoots, after incubation for 2 d, and many shoots did not develop lesions. A resistant response may have been induced in "Buffalo Queen" shoots, which prevented colonization and growth on this plant material. Cahill *et al.* (1989) and Miller & Maxwell (1984 a & b) found that zoospores were equally attracted to, encysted on and germinated to penetrate roots of both susceptible and resistant species. Zoospores applied to susceptible "Goldsworthy" material germinated in all instances to infect shoots, so it appears that some sort of resistant response was operating in shoots of "Buffalo Queen". Shoots of "Buffalo Queen" may have been producing phytoalexins which prevented cyst germination. The inoculation of shoots using zoospores of *Phytophthora* spp. requires further investigation.

There were a number of advantages of using mycelium rather than zoospore inoculum to infect excised shoots in this study. It was difficult to produce zoospores of *P. cinnamomi*, *P. citricola*, *P. cryptogea* and *P. cambivora* consistently, whereas mycelium was easy to subculture and use as inoculum. The inoculation of chestnut shoots with zoospores of *P. cambivora* was labour intensive, while it was easier and less time consuming to pre-inoculate CMA with mycelium of this isolate. Lesion development along shoots was also more consistent when they were inoculated with mycelium rather than zoospores.

Differences in response to infection by *Phytophthora* spp. were observed in shoots of chestnut 2 d after inoculation. One problem with the excised shoot assay method of screening for resistance in micropropagated material, however, was the rapid spread of the fungus up the shoots. The fungus overwhelmed inoculated shoots after 2 d. Son (1992) also noted this problem for citrus shoots inoculated with *P. citrophthora*, although lesions took 5 d to spread up shoots in that system. The rapid development of necrosis on micropropagated shoots, compared to necrosis on shoots excised from orchard-grown material, may occur because excised micropropagated shoots lack the structural and biochemical barriers to infection operating in woody material (Scott *et al.*, 1992). To overcome this difficulty shoots could be assessed at 24 or 36 h after inoculation. It would be interesting to compare the response of shoots to *Phytophthora* spp. if the assay was conducted at different temperatures. Helgeson *et al.* (1972) found that incubation of tobacco callus inoculated with *P. parasitica* var. *nicotianae* at 20°C produced the biggest difference between known resistant and susceptible hosts, whereas Jang & Tainter (1991) found that callus of different species of pine incubated on medium containing 10^{-2} mM 2,4-D, at 26°C, gave the greatest differential response to infection when inoculated with *P. cinnamomi*. McComb *et al.* (1987) screened calli of Australian native plants for resistance to *P. cinnamomi* at a range of temperatures. The optimum temperature for fungal growth on the calli was 30°C. Light may also have some effect on susceptibility. Borrod (1971) found that callus of a *C. sativa* x *C. crenata* hybrid was susceptible to *P. cinnamomi* when incubated in the dark, but that when callus was

incubated in the light at 25°C for 3 wk before inoculation, it was resistant to *P. cinnamomi*. The field response of this material was not reported.

It is unfortunate that material known to be resistant to *Phytophthora* in the field, ie. *C. crenata* and *C. mollissima*, could not be included in these experiments. If resistant and susceptible responses can indeed be distinguished, lesion formation on shoots of these species incubated with *Phytophthora* spp. would be expected to be small. Lesions caused by *Phytophthora* spp. have been observed to be smaller on shoots of resistant peach and citrus than on shoots from susceptible rootstocks screened using this method (Scott *et al.*, 1992; Son, 1992). Avirulent isolates could be used in an attempt to simulate a resistant response in chestnut. Avirulent or less aggressive isolates, however, have been reported to change with age in culture (E.S. Scott, pers. comm.).

Vegetative propagation of chestnut by conventional methods is difficult, therefore tissue culture has been developed for the production of plants with desirable traits, including disease resistance (Vieitez *et al.*, 1986). A few researchers have multiplied shoots of chestnut *in vitro* (e.g. Vieitez & Vieitez, 1980; Rodríguez, 1982; Chauvin & Salesses, 1988; Vieitez, Sánchez & San-José, 1989), however successful rooting of chestnut *in vitro* has been much more difficult to achieve. Rooting differed significantly (0 to 37%) between the cvs "Goldsworthy" and "Buffalo Queen" and also within the cv. "Buffalo Queen" in experiments reported here. Vieitez *et al.* (1989) also found large differences in rooting ability between clones of chestnut; depending on the medium used and the clone tested, rooting of shoots varied from 0 to 94%. Similarly, Chauvin & Salesses (1988) found rooting varied from 34 to 84% in different clones of *C. sativa*.

Necrosis of the apical bud was observed in many rooted shoots of both "Goldsworthy" and "Buffalo Queen" cvs in this study. Apical bud necrosis was also reported by Vieitez *et al.* (1986) in rooted *C. sativa* shoots and has been attributed to calcium deficiency, lack of cytokinins and the presence of auxin in the culture medium. Vieitez *et al.* (1989) found that the necrosis could be prevented by applying a drop of BA, in water agar, to the cut end of excised shoot tips. Axillary bud growth was then induced. This method, however, is not

practical for the large scale production of chestnut by micropropagation because it is time consuming and does not produce "normal" plants. Further work, therefore, needs to be conducted to improve rooting of chestnut *in vitro*.

No plantlets survived in initial experiments conducted here, where chestnut plantlets were transferred to sterile RS soil or sterile RS soil mixed 1:1 with vermiculite. In subsequent experiments plantlets were transferred directly to sterile vermiculite. Survival of plantlets was similar to that reported by Vieitez *et al.* (1986), who obtained survival of 34% in *C. sativa*. Chauvin & Salesses (1988) transferred rooted shoots to a peat/perlite mix and kept them under conditions of high humidity in the glasshouse. Good acclimatization of plantlets was reported, however, the percentage of surviving plantlets was not given. Recently, chestnut plantlets were inoculated with mycorrhizal fungi prior to transfer to soil. Chestnuts are ectomycorrhizal, and mycorrhizal plantlets were reported to survive much better than non-mycorrhizal plantlets, although again, percentages of surviving plantlets were not given (Martins & Pais, 1992). Vidal, Azcón-Aguilar & Barea (1992) found that avocado plantlets inoculated with VA mycorrhizal fungi improved the formation of roots *in vitro* and increased survival after transfer to soil. Inoculation of chestnut shoots, which have developed root primordia, with ectomycorrhizal fungi may also improve their rooting *in vitro* and survival after transfer to soil. This might also help to produce "normal" plants .

The pilot assay in which plantlets were inoculated *in vitro* was unsuccessful in screening chestnut plants for resistance to *Phytophthora* spp. The major problem with the system was that plantlets, with roots of different lengths and morphology, did not sit flat on the agar surface and initial infection developed on different parts of the root system. Comparisons of infection between isolates or clones, therefore, could not be made. If the chestnut roots could have been inserted into the agar this problem may have been avoided to some extent, however, the roots were fragile, and easily broken off if the plants were inserted into the medium. These problems were overcome by planting material in vermiculite before inoculation and the results of these inoculation experiments confirmed the results of the

excised shoot assays with plantlets of both "Buffalo Queen" and "Goldsworthy" being susceptible to *P. cinnamomi.*

In conclusion, the excised shoot assay was used to evaluate chestnut cvs for response to *Phytophthora* spp. *in vitro.* Plantlets established in vermiculite and inoculated with *P. cinnamomi* confirmed the results of the assay. Micropropagated material of *C. crenata* and *C. mollissima* also needs to be screened in order to determine the efficacy of the excised shoot assay in screening for resistance *in vitro.* This technique could be used to screen other woody plants for resistance to *Phytophthora* spp..

5. ANTAGONISM AND BIOCONTROL OF *PHYTOPHTHORA* SPP. BY *TRICHODERMA* SPP. AND *GLIOCLADIUM VIRENS*

5.1 Introduction

In woody plants, root and collar rot caused by *Phytophthora* spp. can be controlled, to some extent, using resistant plant species or cvs, or by cultural and chemical practices. In Australia, resistant varieties of chestnut rootstocks are not available, therefore cultural and chemical methods of preventing and controlling disease are used. Fungicides such as metalaxyl, fosetyl-Al (Taylor & Washington, 1984), and phosphonate (Pegg *et al.*, 1985; Wicks & Hall, 1990) can prevent disease on plants including chestnut, however, there are a number of problems with their use. Because of these problems (see sections 2.2.2 & 2.2.3) there is currently great interest in developing microorganisms as biocontrol agents of *Phytophthora* rots and other plant diseases (see section 2.2.4). Only a few biocontrol products are available commercially, however, a large number are currently being developed for release (Baker, 1989; see section 2.2.5).

The potential of species of *Trichoderma* and *Gliocladium* as biocontrol agents of root and collar diseases of woody plants are being investigated for control of *P. cactorum* root and collar rot of apple trees (Roiger & Jeffers, 1991; Smith, Wilcox & Harman, 1990). Smith *et al.* (1990) added isolates of *Trichoderma* and *Gliocladium*, as a colonized mixture of peat and wheat bran, to soil in which apple seedlings were growing. These apple seedlings had been inoculated with vermiculite colonized with *P. cactorum*, in soil in the glasshouse. Some isolates of *Trichoderma* and *Gliocladium* spp. significantly reduced root damage caused by *P. cactorum* and increased plant weight of apple seedlings, 14 days after inoculation, compared with seedlings inoculated with *P. cactorum* alone. In the absence of *P. cactorum*, the growth of apple seedlings was also significantly increased by the presence of some isolates of the biocontrol fungi.

Roiger & Jeffers (1991) also used a glasshouse bioassay to evaluate isolates of *Trichoderma* spp. for biocontrol of *P. cactorum* root and collar rot of apple seedlings growing in

pasteurized soil. Isolates of *Trichoderma* spp. were added to soil infested with *P. cactorum* using either a colonized mixture of peat and wheat-bran ("peat-bran") before planting apple seedlings, or were applied to seedling roots as a viscous suspension of conidia in an aqueous gel preparation before planting in soil infested with *P. cactorum*. One isolate of *T. virens*, applied in peat-bran to seedlings, consistently increased survival of plants compared with plants inoculated with vermiculite colonized by *P. cactorum* alone. Three other isolates also increased survival time of seedlings in most trials, depending on the method of application.

Both glasshouse bioassays showed that species of *Trichoderma* and *Gliocladium* controlled root and collar rot of apple caused by *P. cactorum*, and thus isolates of these genera have potential for the control of disease caused by *Phytophthora* on other woody plant hosts. Field studies are needed, however, to confirm these results.

In vitro experiments can be used to elucidate the mechanisms of such disease control. Smith *et al.* (1990) initially screened isolates of *Trichoderma* for production of antibiotics active against *P. cactorum*, however, other possible mechanisms of control were not investigated. Mycoparasitism of *P. cinnamomi* hyphae by *T. harzianum* hyphae has been observed *in vitro* (Finlay & McCracken, 1991), and antibiosis by *T. harzianum* cultures, inhibiting the growth of *P. parasitica* f. sp. *nicotianae* (= *P. nicotianae*, Hall, 1993) has also been observed (Bell, Wells & Markham, 1982). Further work investigating the mechanisms of biocontrol of *Phytophthora* spp. by fungi is required.

A different method of application of pathogen and antagonist was used by Tuset *et al.* (1990), who investigated biocontrol of root and collar rot of citrus, caused by *P. nicotianae* var. *parasitica* (= *P. nicotianae*, Hall, 1993), using *Myrothecium roridum*. Mycelium of *M. roridum* was added to wounds made in the lower stem and roots of citrus seedlings growing in the glasshouse or to trees in the field. Mycelium of *P. nicotianae* var. *parasitica* was added, 8-9 d later, to wounds made above those for the biocontrol agent. After 7-10 d, canker development by this pathogen was assessed and was found to be limited compared with plants inoculated with the pathogen alone. This was probably limited by the production of toxins

which moved through the vascular system, inhibiting lesion extension by the pathogen. The collar inoculation method, used to evaluate *M. roridum* for biocontrol of root rot, merits investigation for other potential biocontrol fungi which may control *Phytophthora* spp..

In another study, stems of *Persea* seedlings were inoculated with zoospores of a citrus isolate of *P. parasitica* (non-pathogenic on avocado) and then inoculated with zoospores of the pathogenic fungi *P. cinnamomi* or *P. citricola* 2 or 48 h later (Dolan et al., 1986). Lesion development along stems inoculated with *P. cinnamomi* or *P. citricola* alone was much greater than on plants inoculated with either of these pathogens and *P. parasitica*. Induced protection was thought to protect plants from disease by acting systemically.

When soil samples, collected from beneath dead and dying chestnut trees in orchards in the Adelaide Hills, were baited to isolate *Phytophthora* spp., some samples did not yield pathogens. Instead, *Trichoderma hamatum, T. pseudokoningii* and *Gliocladium virens* were obtained (see section 3.1). The aims of the experiments reported here were, therefore, to evaluate the potential of these isolates of *T. hamatum, T. pseudokoningii* and *G. virens* as biocontrol agents of *Phytophthora* root and crown rot of chestnut. In particular:

(i) to use *in vitro* screening to detect antagonism to *P. cinnamomi* and *P. citricola* by these three fungi and to elucidate the mechanisms involved;

(ii) to assess the ability of these three antagonists to control *Phytophthora* infection of micropropagated chestnut shoots; and

(iii) to screen these fungi for biocontrol of *Phytophthora* root and collar rot of chestnut seedlings in pasteurized soil in pots in the glasshouse.

5.2 Materials and Methods

5.2.1 Fungal cultures

P. cinnamomi (isolate CR6A), *P. citricola* (isolate PT4A), *T. hamatum, T. pseudokoningii* and *G. virens* were isolated, cultured and maintained as described in section 3.1.

5.2.2 Dual culture experiment

An experiment was conducted *in vitro* to observe interactions between colonies of *P. cinnamomi* and the three possible antagonists, based on the method of Dennis & Webster (1971c). *P. cinnamomi* was used because it has previously been reported as a serious pathogen of chestnut. A sterile piece of porous uncoated cellophane (80 x 30 mm; Australia Cellophane, Victoria) was placed on either 20 ml DW agar (15 g l^{-1} Difco Bi-tek agar) or 1/5M32 (soil mimic agar, Sivasithamparam, Parker & Edwards, 1979) in the centre of a 90 mm diam. Petri dish. Agar plugs of *P. cinnamomi* (CMA) and either *T. hamatum*, *T. pseudokoningii* or *G. virens* (MEA), 8 mm in diam. cut from the edge of an actively growing culture, were placed 50 mm apart on the cellophane. There were ten replicates of each pathogen x antagonist combination. Plates were sealed with parafilm and incubated at 25°C in the dark. In order to determine whether growth of the antagonists was stimulated in the presence of *P. cinnamomi*, growth of *T. hamatum*, *T. pseudokoningii* and *G. virens* toward the *P. cinnamomi* cultures was measured daily and compared to control treatments in which plates were inoculated with only *T. hamatum*, *T. pseudokoningii*, *G. virens* or *P. cinnamomi*.

After 4-5 d, when the two fungal colonies had grown together, pieces of cellophane (5 x 5 mm) supporting the mycelium, were cut from the interaction zone, mounted on slides and stained with lactophenol cotton blue or ammoniacal congo red for light microscopy. Interactions between the fungi were photographed using a Leitz Orthoplan photomicroscope.

In order to study the interactions using SEM, plugs, similar to those used above, were placed 20 mm apart on sterile 0.2 μm Millipore filters on 1/5M32 agar, since preliminary experiments indicated that these filters maintained their shape better than cellophane during preparation for SEM. After 2 d, when the colonies had grown together, 5-10 mm^2 pieces were cut from the interaction zone and fixed in 2.5% glutaraldehyde in 0.025 M phosphate buffer (pH 7.0) at room temperature for 30 min. Samples were dehydrated through an ethanol series to 100% (each increase of 10% for 10 min), critical point dried, mounted on stubs and sputter coated with gold. Samples were viewed with a Cambridge S250 SEM, with an accelerating voltage of 20 kV.

5.2.3 Cellophane antibiosis

To investigate the effect of antibiotics produced by potential antagonists on the growth of *Phytophthora* spp., a method modified from Dennis & Webster (1971a) was used. Sterile cellophane circles (80 mm diam.) were placed on 20 ml DW or 1/5M32 agar in 90 mm diam. Petri dishes. Mycelial plugs, as described in section 5.2.2, were cut from cultures of *T. hamatum, T. pseudokoningii* or *G. virens* and placed on the cellophane in the centre of each plate and plates sealed with parafilm. After 2 d, when mycelium covered 50% of the plate, the cellophane along with all of the mycelium was removed and replaced with a plug of either *P. cinnamomi* or *P. citricola.* Plates were incubated in the dark at 25°C. There were ten replicate plates of each pathogen x antagonist combination. Control plates of DW or 1/5M32 agar were inoculated with sterile plugs of 2% MEA on cellophane which was removed after 2 d and replaced with a plug of either *P. cinnamomi* or *P. citricola* as above. *Phytophthora* spp. colony diameter, determined as the mean of two measurements at right angles, was measured after 7 d.

5.2.4 Culture filtrate antibiosis

Liquid cultures of *T. hamatum, T. pseudokoningii* and *G. virens* were prepared by transferring an 8 mm diam. plug from the margin of a fungal culture on MEA to 50 ml 1/5M32 broth in a sterile 250 ml polycarbonate tub (Disposable Products, South Australia). Cultures were shaken in the dark at 110 rpm at 25°C. A preliminary experiment to determine the effect of age of filtrate on growth of *Phytophthora* spp. was conducted using *T. hamatum* culture filtrate harvested at 2 wk, 3 wk, 6 wk and 7 wk after inoculation. Because 2-7 week-old filtrates did not reduce the growth of P. cinnamomi and P. citricola significantly, the effect of *T. hamatum, T. pseudokoningii* and *G. virens* culture filtrates at 2 d, 4 d and 7 d harvests on growth of *Phytophthora* spp. was assessed. Cultures were harvested by passing them through four layers of muslin cloth, and the filtrate centrifuged for 10 min at 4°C and 1000 g. The supernatant was then filter-sterilized through a 0.45 μm Millipore filter. The cell-free culture filtrate was incorporated at 250 ml l^{-1} into cooled 1/5M32 agar after autoclaving. Plugs of either *P. cinnamomi* or *P. citricola* were placed in the centre of 90 mm diam. Petri dishes containing 20 ml 1/5M32 + culture filtrate agar and incubated in the dark at 25°C. There were ten replicates of each pathogen x antagonist combination for each culture

harvest. Sterile 1/5M32 broth was incorporated into 1/5M32 agar for controls. Colony diameters were measured as above after 7 d.

5.2.5 Volatile antibiosis

To investigate whether antagonists produced volatile antibiotics which inhibited the growth of *Phytophthora* spp., the method of Dennis & Webster (1971b) was used. Plugs of either *T. hamatum*, *T. pseudokoningii* or *G. virens* were placed in the centre of 90 mm diam. Petri dishes containing 20 ml 1/5M32 agar and incubated in the dark at 25°C for 2 d. Fresh plates of 1/5M32 agar were then inoculated with plugs of either *P. cinnamomi* or *P. citricola* and the bases of these plates inverted and taped to the bases of the plates containing the 2 d-old antagonists. Plates were incubated, with *Phytophthora* spp. uppermost, at 25°C in the dark and colony diameters measured after 5 d. Colony diam. was measured at 5 d because by 7 d *Phytophthora* spp. plates had become contaminated by spores of the antagonist. There were ten replicates for each pathogen x antagonist combination. Controls consisted of *Phytophthora* spp. inverted over plates of 1/5M32 inoculated with sterile plugs of 2% MEA.

5.2.6 Biocontrol shoot assay

In order to determine whether the three antagonist fungi could reduce lesion development on shoots of micropropagated chestnut incubated with *Phytophthora* spp., a biocontrol shoot assay, modified from the excised shoot assay of Scott *et al.* (1992) (section 4.2.2), was used. Plugs of *T. hamatum*,. *T. pseudokoningii* or *G. virens* and *P. cinnamomi* or *P. citricola*, taken from actively growing cultures on MEA or CMA, respectively, were placed on opposite sides of 50 ml CMA agar in 250 ml polycarbonate tubs (Disposable Products, South Australia). Inoculated tubs were incubated at 25°C in the dark until the pathogen and potential antagonist had just grown together. Three micropropagated shoots of chestnut cvs "Goldsworthy" or "Buffalo Queen" (produced as described in section 3.5), 2 cm long, were excised from tissue cultures 4 wk after the last transfer, defoliated and placed in the interaction zone between the two fungi. Control shoots were placed in uninoculated tubs, in tubs inoculated only with *P. cinnamomi* or *P. citricola*, and in tubs inoculated only with *T. hamatum*, *T. pseudokoningii* or *G. virens*. Material was incubated in the dark at 25°C for 2 d, then one assessment of lesion

length along the shoots was made (after this time *Phytophthora* spp. overwhelmed chestnut shoots, see section 4.3.2). There were three replicate tubs for each antagonist x pathogen x cultivar combination. Fungi were re-isolated from one randomly chosen shoot per tub, by plating a shoot on CMA in 90 mm diam. Petri dishes and incubating in the dark at 25°C for 2d.

5.2.7 Inoculation of seedlings in soil with *P. cinnamomi* and the antagonist fungi

5.2.7.1 Inoculation of seedlings with zoospores of *P. cinnamomi*

Four plugs, cut from the margin of an actively growing culture of *T. hamatum*, *T. pseudokoningii* or *G. virens* on MEA, were placed in 100 ml 1/5M32 broth in a 250 ml polycarbonate tub (Disposable Products, South Australia). Cultures were shaken at 110 rpm in the dark at 25°C for 2 d, then blended for 60 s at medium speed using a Black and Decker Hand Held Food Processor. Five ml of macerated culture was placed in a 250 ml polycarbonate tub, containing 50 g of oat grain cv. "Echidna", which had been covered with 62 ml DW and sterilized at 121°C for 1 h. There were three replicate tubs of each fungus. Inoculated grain cultures were incubated in the dark at 25°C for 7 d and cultures were shaken every second day to ensure thorough colonization of the grain.

Chestnut seeds cv. "Sword" were germinated as described in section 3.4 and seedlings transferred to 10 cm pots containing RS soil with 50% peat and grown in a glasshouse at 15-25°C with ambient daylight (approx. 10 h daylength) for 3 wk. Twenty g of grain pre-colonized with *T. hamatum, T. pseudokoningii* or *G. virens* was mixed with the top 7 cm of soil in each pot, without disturbing the plants. Sterile oat grain was mixed with soil in pots for controls. Pots containing seedlings and antagonists or controls were returned to the glasshouse in conditions described in section for 2 wk. Twenty ml of a zoospore suspension of *P. cinnamomi* (isolate CR6A) in sterile soil extract (10^4 zoospores per ml), produced using the method modified from Gees & Coffey (1989), as described in section 3.2, was placed in each pot pre-inoculated with either sterile oat grain (positive controls), oat grain colonized by *T. hamatum, T. pseudokoningii* or by *G. virens*. Negative controls were plants inoculated with sterile soil extract. There were seven replicate plants for each treatment and pots were

arranged randomly within two separate trays, for *Phytophthora*-inoculated or negative control plants. Pots were flooded with tap water in these trays for 24 h after inoculation with zoospores, then watered as required. Plants were monitored twice weekly for disease symptoms including wilting, lesion development and death (see table 6.1) for 3 wk, then harvested and the roots examined for signs of necrosis.

5.2.7.2 Collar inoculation of seedlings with mycelium of *P. cinnamomi*

Seedlings in 10 cm diam. pots containing RS soil with 50% peat were grown for 7 wk in the glasshouse under the conditions described in section 5.7.2.1. Seedlings were then inoculated at the top of the tap root using a method modified from Tuset *et al.* (1990). Soil from around the collar region was gently removed and, using a scalpel, a wound was made on the tap root *ca* 2 cm below the soil surface to expose the cambium, creating a flap of bark about 1 cm long. A plug of either *T. hamatum*, *T. pseudokoningii* or *G. virens*, 7 mm in diam., was cut from the margin of an actively growing culture on MEA, placed in this wound and the flap of bark closed and soil replaced. Control plants were inoculated with sterile plugs of MEA. After 14 d, a wound was made at the top of the tap root, about 2 cm above the antagonist inoculum, and a plug of *P. cinnamomi*, 7 mm in diam., cut from a culture on CMA, was placed in it. Control plants were inoculated with a sterile plug of CMA. Cotton wool, moistened with tap water, and then aluminium foil, were wrapped around each wound to protect the inoculum plug from desiccation. There were six or seven replicate plants of each pathogen x antagonist combination and for controls. Plants were watered as needed with tap water, and assessed twice weekly, for 14 d, for disease symptoms, using a shoot symptom index (see table 6.1). At the final assessment, plants were harvested and the roots were scored for necrosis using a root rot index (see table 6.2). Shoots and roots were then separated, fresh weights obtained and material dried in an oven at 105°C for 24 h before obtaining shoot and root dry weights.

5.2.8 Analysis of data

Each *in vitro* experiment was carried out twice, and the results pooled. Analysis of Variance was used to compare the effect of the treatments on colony diameter or lesion length and

means were compared using the Least Significant Difference (LSD) method. Final colony diameter values from *in vitro* growth inhibition experiments had 8 mm subtracted before means were calculated to give net mycelial growth values. Pot experiments were conducted once, and the shoot symptoms, root rot index and plant weight data were analysed using Analysis of Variance.

5.3 Results

5.3.1 Dual culture experiment

None of the antagonist fungi grew significantly faster in the presence of *P. cinnamomi* compared to controls (data not shown). The hyphae of *P. cinnamomi* were easily distinguished from the antagonist hyphae, being larger in diameter and more coralloid with abundant swellings. Growth of *P. cinnamomi* was not inhibited by the antagonists before colonies were overgrown. When fungal colonies grew together, a number of features were noted in the interaction zone. Most commonly, parallel growth of either *T. hamatum*, *T. pseudokoningii* or *G. virens* along *P. cinnamomi* hyphae was observed (Figs 5.1A, 5.1B and 5.1C). Scanning electron micrographs showed that the hyphae of the antagonist were tightly appressed to *P. cinnamomi* hyphae (Fig. 5.1D); occurring most frequently with *T. hamatum* and *T. pseudokoningii* hyphae, and less commonly with *G. virens* hyphae. Appressorium-like structures produced by *T. hamatum* and *T. pseudokoningii* hyphae extending off regions of parallel growth were frequently observed (Fig. 5.1E); these were never observed in *G. virens* hyphae.

Coiling of *T. hamatum* hyphae around *P. cinnamomi* hyphae was common (Fig. 5.1F). Large areas of coiling by *T. pseudokoningii* hyphae along single *P. cinnamomi* hyphae were also observed frequently (Fig. 5.1G). Coiling by *G. virens* hyphae was also observed (Fig. 5.1H), but was less common than with *Trichoderma* spp.. Following an initial interaction period of approximately 1 d, the antagonist fungus always grew over the *P. cinnamomi* colony and filled the plate, preventing further growth of *P. cinnamomi*.

Fig. 5.1: Light and scanning electron micrographs of hyphal interactions of *T. hamatum*, *T. pseudokoningii* and *G. virens* with *P. cinnamomi* hyphae in dual culture.

Fig. 5.1A-C: light micrographs of hyphae stained with lactophenol cotton blue. Bars = 20 μm.

Fig. 5.1A: parallel growth of *T. hamatum* hypha (a) and *P. cinnamomi* hypha (p) (arrowed). Appressorium-like structure indicated by arrowhead.

Fig. 5.1B: parallel growth of *T. pseudokoningii* hypha (a) and *P. cinnamomi* hypha (arrowed).

Fig. 5.1C: parallel growth of *G. virens* hypha (a) on *P. cinnamomi* hypha (p) (arrowed).

Figs 5.1D-E: scanning electron micrographs. Bars = 10 μm.

Fig. 5.1D: *T. pseudokoningii* hypha (a) tightly appressed to *P. cinnamomi* hypha (p).

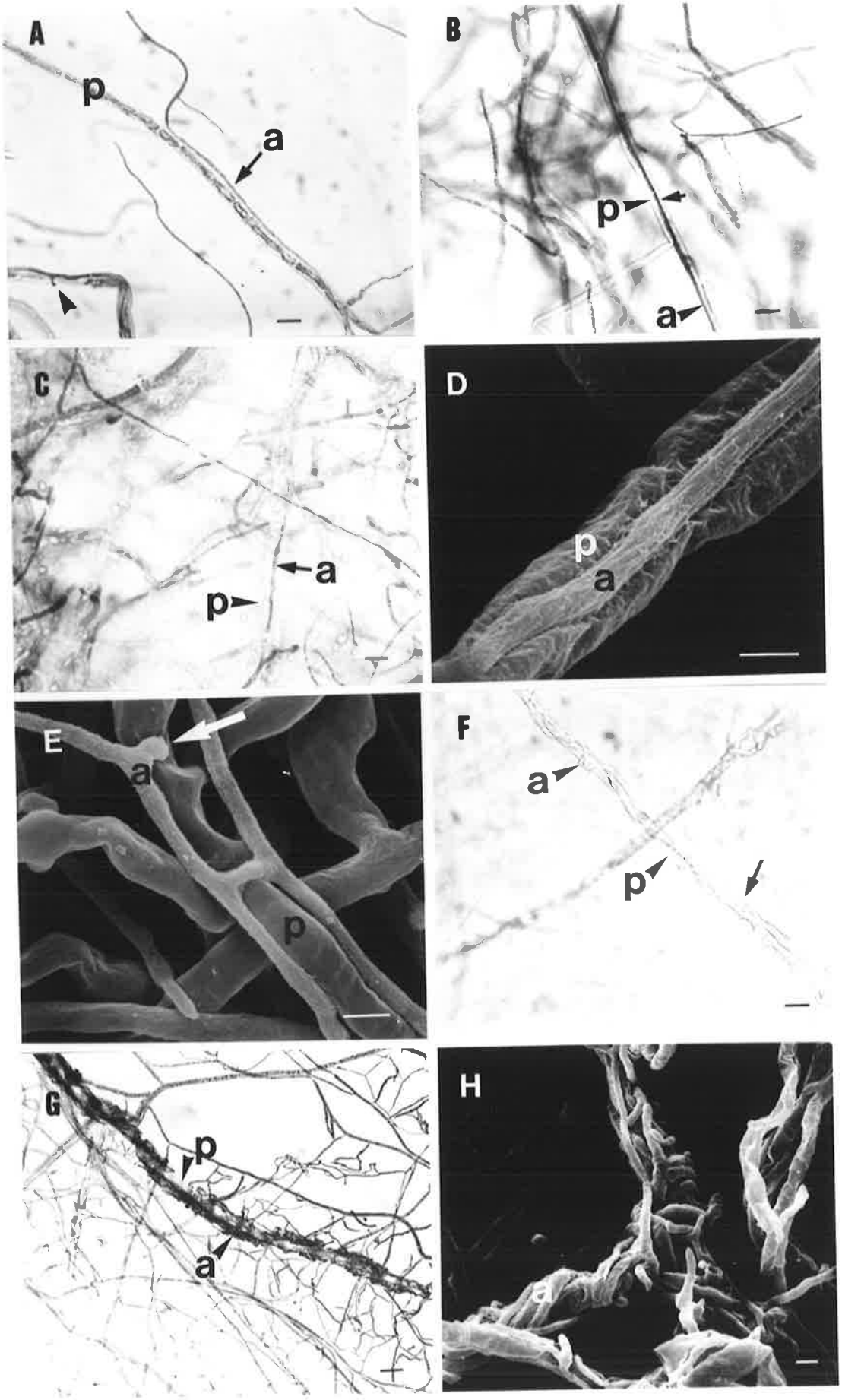
Fig. 5.1E: *T. hamatum* hypha (a) on *P. cinnamomi* hypha (p). Appressorium-like structure (arrowed) in region of parallel growth.

Fig. 5.1F-G: light micrographs of hyphae stained with lactophenol cotton blue.

Fig. 5.1F: *T. hamatum* hypha (a) coiling around *P. cinnamomi* hypha (p) (arrowed). Bar = 20 μm.

Fig. 5.1G: *T. pseudokoningii* hyphae (a) tightly coiled around *P. cinnamomi* hypha (p). Bar = 4 μm.

Fig. 5.1H: scanning electron micrograph of *G. virens* hypha (a) coiled tightly around *P. cinnamomi* hypha (p). Bar = 10 μm.


A
p
a
B
p
a
C
a
p
D
p
a
E
a
p
F
a
p
G
p
a
H
a

5.3.2 Cellophane antibiosis

All antagonists significantly (P<0.1%) reduced the growth of *P. cinnamomi* and *P. citricola* compared to controls (Fig. 5.2), as determined by an unbalanced analysis of variance. In the DW medium treatments, *G. virens* caused the greatest inhibition in growth of *Phytophthora* spp., completely inhibiting *P. citricola* and reducing colony growth of *P. cinnamomi* to less than half that of controls (Fig. 5.2A). *T. hamatum* and *T. pseudokoningii* significantly inhibited growth of both *P. cinnamomi* and *P. citricola*, but to a lesser extent than did *G. virens*. There was also a significant difference in colony diameter between *P. cinnamomi* and *P. citricola*, with *P. citricola* consistently growing faster than *P. cinnamomi*.

Similar trends were observed in inhibition of growth of *Phytophthora* spp. by the antagonists in the 1/5M32 medium treatments (Fig. 5.2B). *P. citricola* was inhibited more by *T. hamatum* than was *P. cinnamomi*, and *G. virens* inhibited the growth of both *Phytophthora* spp. completely. Inhibition in the growth of *P. cinnamomi* by potential antagonist fungi was generally greater in the 1/5M32 medium treatments than in the DW medium treatments, whereas inhibition of *P. citricola* was generally similar on both media.

5.3.3 Culture filtrate antibiosis

The effect of *T. hamatum* culture filtrate at 2 wk, 3 wk, 6 wk and 7 wk harvests on growth of *Phytophthora* spp. is shown in Fig. 5.3. Antagonist and age of culture filtrate significantly affected inhibition of growth of both *Phytophthora* spp. (P<0.1%). Filtrate harvested at 2 wk and 3 wk significantly inhibited the growth of *P. cinnamomi* compared with controls, however 6 wk filtrate did not inhibit *P. cinnamomi* growth at all and filtrate from 7 wk-old cultures stimulated growth of *P. cinnamomi*. Culture filtrates of *T. hamatum*, harvested at 2-7 wk, had little effect on growth of *P. citricola*.

Mean colony diameter of *Phytophthora* spp. after 7 d on agar media supplemented with filtrates from 2, 4 and 7 d-old cultures is shown in Fig. 5.4A & B. Under the conditions used in these experiments, the younger the *T. hamatum* culture, the more the filtrate inhibited growth of both *P. cinnamomi* and *P. citricola*. Filtrate from 2 d-old cultures almost halved

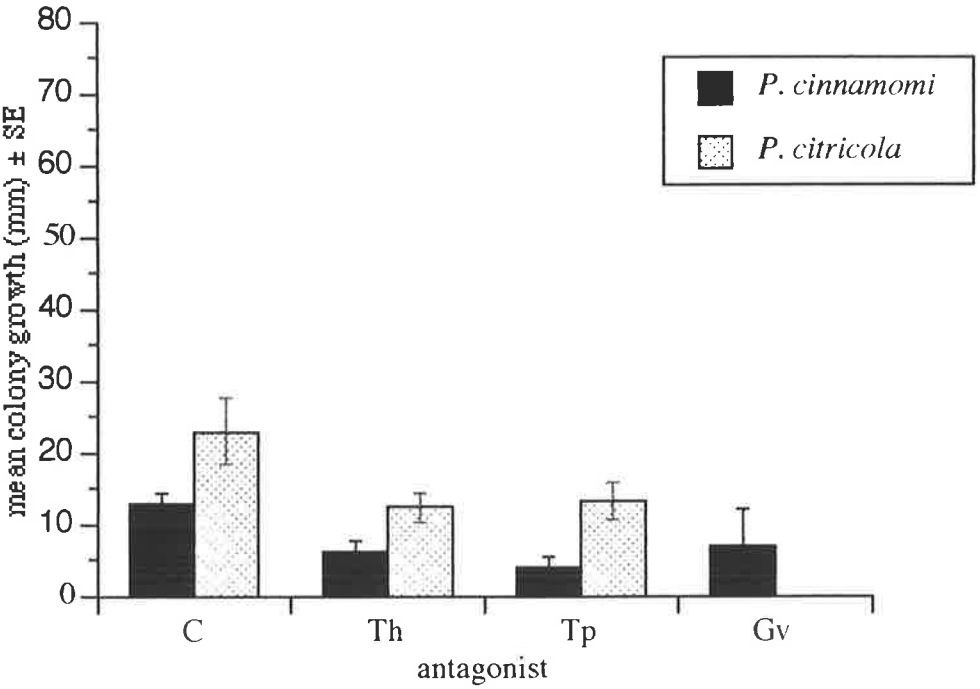
Fig. 5.2: Growth of *P. cinnamomi* and *P. citricola* after 7 d on DW or 1/5M32 medium after removal of cellophane on which one of the three possible antagonists had been grown. Bars represent SE.

Th = *T. hamatum*, Tp = *T. pseudokoningii*, Gv = *G. virens*, C= control.

Fig. 5.2A: DW agar medium.

Fig. 5.2B: 1/5M32 medium

A


80
70
60
50
40
30
20
10
0
mean colony growth (mm) ± SE
P. cinnamomi
P. citricola
C
Th
Tp
Gv
antagonist

B

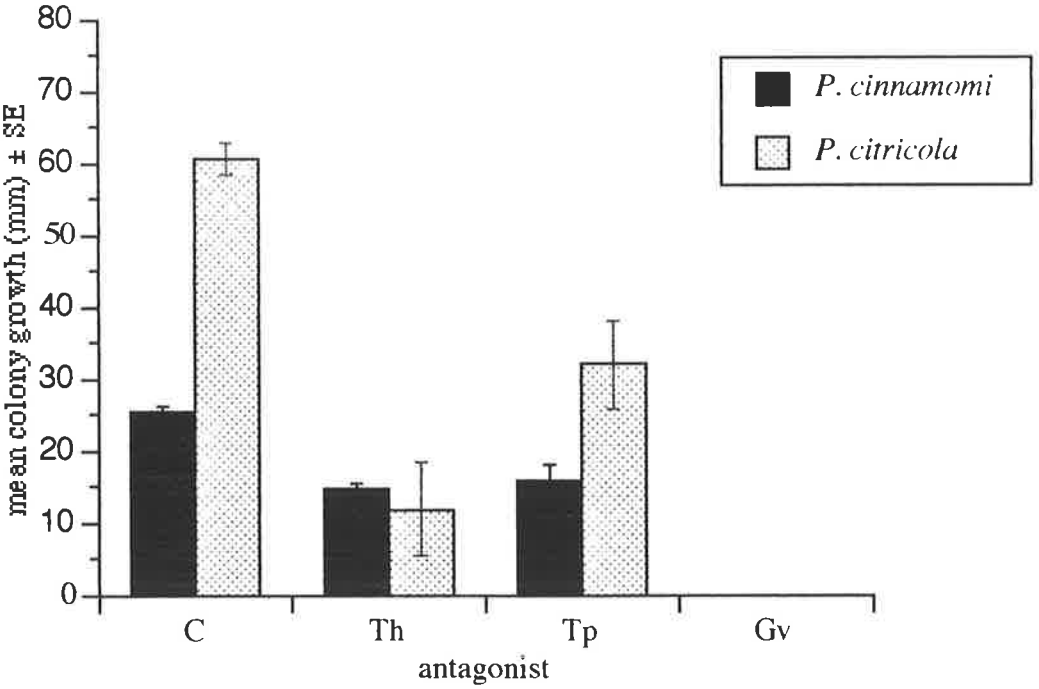

80
70
60
50
40
30
20
10
0
mean colony growth (mm) ± SE
P. cinnamomi
P. citricola
C
Th
Tp
Gv
antagonist

Fig. 5.3: Growth of *P. cinnamomi* and *P. citricola* after 7 d on 1/5M32 culture medium with and without filtrate from 2-7 wk-old cultures of *T. hamatum*. Bars represent SE. LSD = 1.83, P<0.001, n = 10.

c = control, cf = culture filtrate.

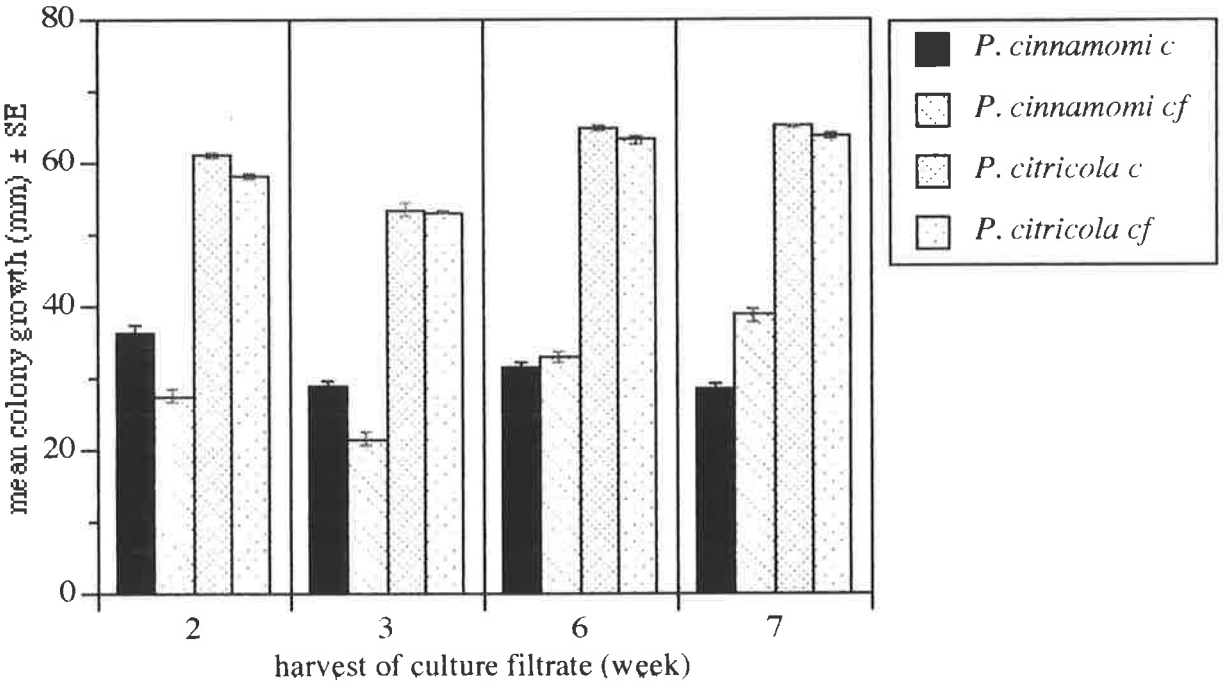

80
60
40
20
0
mean colony growth (mm) ± SE
2
3
6
7
harvest of culture filtrate (week)
P. cinnamomi c
P. cinnamomi cf
P. citricola c
P. citricola cf

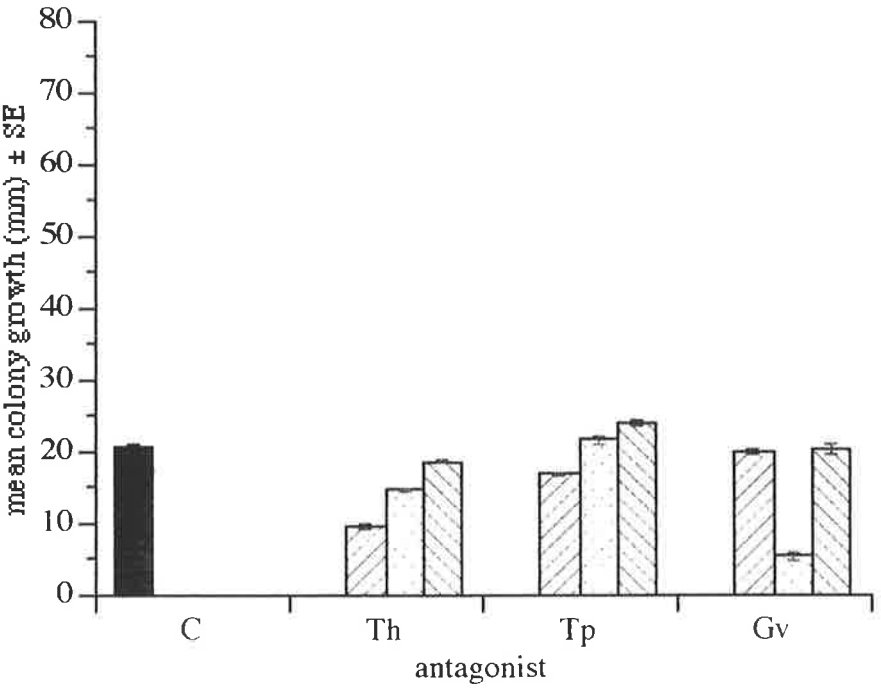
Fig. 5.4: Growth of *P. cinnamomi* or *P. citricola* after 7 d on 1/5M32 culture medium containing filtrate from 2, 4 & 7 d-old cultures of *T. hamatum*, *T. pseudokoningii* or *G. virens*. Bars represent SE. LSD = 2.517, P<0.001, n = 10.

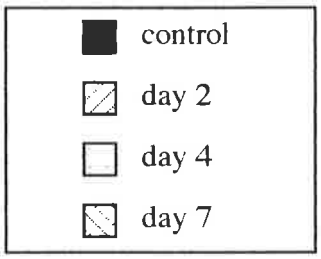
c = control, Th = *T. hamatum*, Tp = *T. pseudokoningii*, Gv = *G. virens*.

Fig. 5.4A: *P. cinnamomi*.

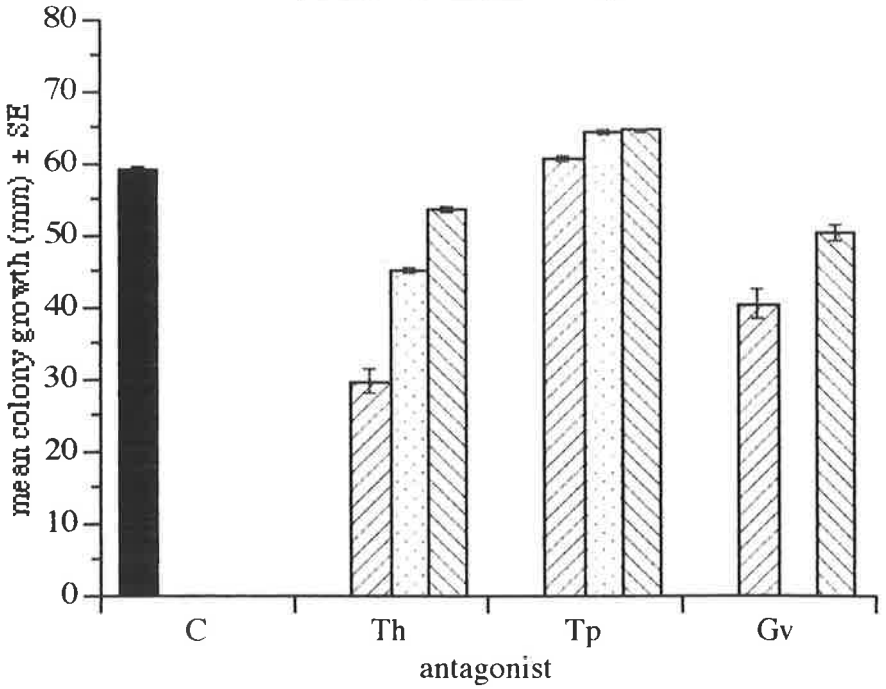
Fig. 5.4B: *P. citricola*.

A


mean colony growth (mm) ± SE
80
70
60
50
40
30
20
10
0
C
Th
Tp
Gv
antagonist


control
day 2
day 4
day 7

B


mean colony growth (mm) ± SE
80
70
60
50
40
30
20
10
0
C
Th
Tp
Gv
antagonist

the growth of both *Phytophthora* spp. compared to controls. *T. pseudokoningii* filtrate harvested at 2, 4 or 7 d did not inhibit the growth of *P. citricola*, however the growth of *P. cinnamomi* was significantly inhibited by the 2 d filtrate compared to controls.

The effect of *G. virens* culture filtrate on *Phytophthora* spp. colony growth varied with time of harvest, with 4 d filtrate completely inhibiting the growth of *P. citricola*, and strongly inhibiting growth of *P. cinnamomi* compared to controls. *G. virens* filtrate harvested at 2 d and 7 d also significantly reduced *P. citricola* growth compared to controls, but to a lesser extent, with less inhibition by the older filtrate. *G. virens* cultures harvested at 2 d and 7 d caused changes in *P. cinnamomi* colony morphology. Sparse, aerial mycelial growth was commonly observed and hyphae were also more vesicular in appearance. No marked effects on the colony morphology of *P. citricola* were observed. As in the cellophane antibiosis experiment, *P. citricola* controls grew significantly faster than *P. cinnamomi* controls.

5.3.4 Volatile antibiosis

Mean colony diam. of *P. cinnamomi* and *P. citricola* in the volatile antibiosis experiment is shown in Fig. 5.5. *T. hamatum* and *G. virens* had little effect on the growth of either *P. cinnamomi* or *P. citricola*. Exposure to *T. pseudokoningii*, however, significantly inhibited both *Phytophthora* spp.; colony diam. of *P. cinnamomi* was less than half that of controls (*ca* 5 mm and 16 mm respectively) and just over half that of controls for *P. citricola* (*ca* 28 mm and 47 mm respectively). As in the experiments above, *P. citricola* grew visibly faster over 5 d than *P. cinnamomi* (about 2.5 times faster).

5.3.5 Biocontrol shoot assay

After 2 d, lesions had developed along almost the entire length of shoots of chestnut cvs "Goldsworthy" and "Buffalo Queen" incubated with only *P. cinnamomi* or *P. citricola*, whereas shoots incubated with the antagonist alone had slight basal discolouration. Likewise, shoots incubated with the pathogen and one of the three antagonists developed only minor lesions (Fig. 5.6). No lesions developed on shoots incubated on uninoculated CMA. There was no visible difference between lesions on shoots caused by *P. cinnamomi* or *P.*

Fig. 5.5: Growth of *P. cinnamomi* and *P. citricola* after 5 d on 1/5M32 culture medium inverted over cultures of possible antagonists to test for inhibition by production of volatile antibiotics. Bars represent SE. LSD = 2.41, P<0.001, n = 10.

Th = *T. hamatum*, Tp = *T. pseudokoningii*, Gv = *G. virens*.

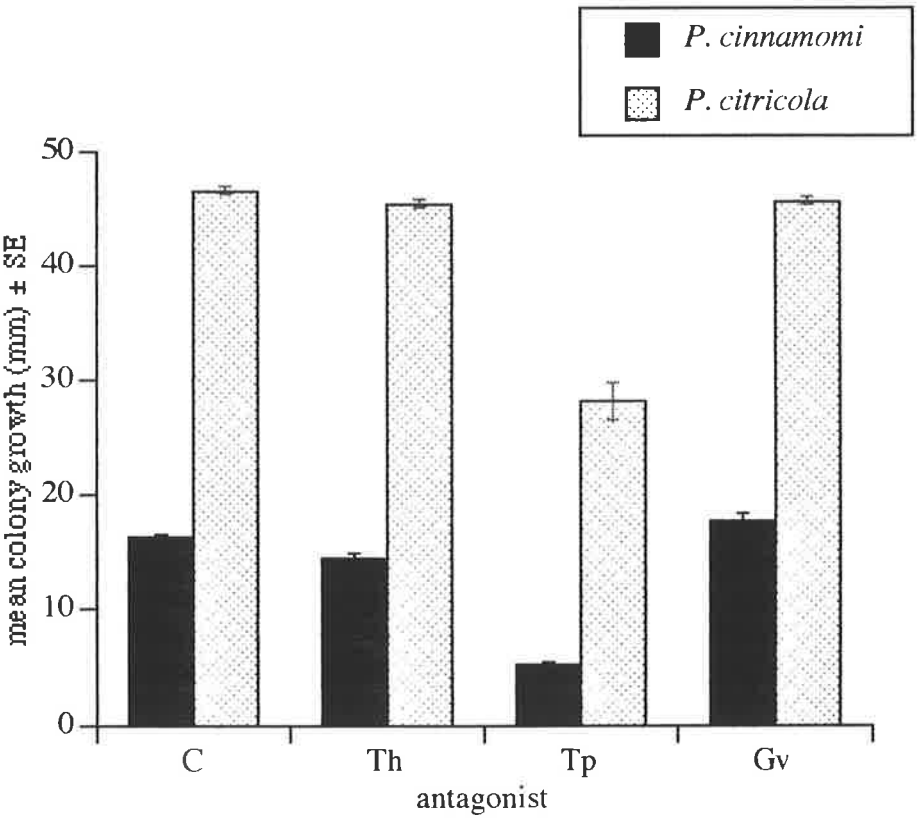
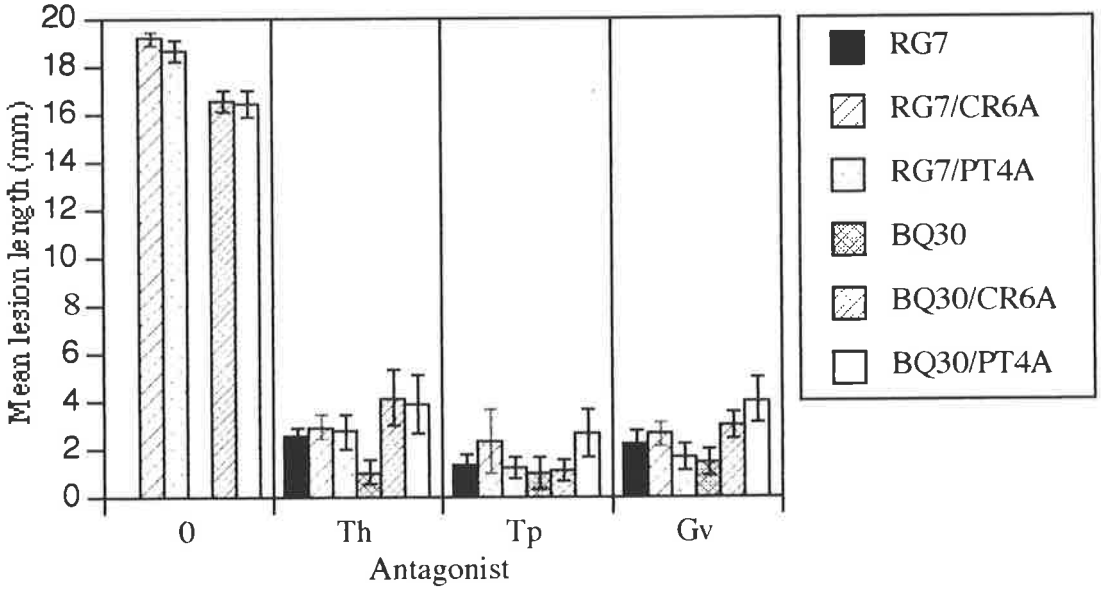

P. cinnamomi
P. citricola
mean colony growth (mm) ± SE
50
40
30
20
10
0
C
Th
Tp
Gv
antagonist

Fig. 5.6: Mean lesion length along micropropagated shoots of chestnut cvs "Goldsworthy" (RG7) and "Buffalo Queen" (BQ30) incubated for 2 d with co-cultures of *P. cinnamomi* (CR6A) or *P. citricola* (PT4A) and *T. hamatum* (Th), *T. pseudokoningii* (Tp), or *G. virens* (Gv) on CMA in polycarbonate tubs at 25°C in the dark. Controls were shoots incubated on uninoculated CMA, or inoculated with either the antagonist or pathogen alone. Bars represent SE. LSD = 3.27 (calculated with n = the minimum number of replicates, therefore is the most conservative value), $P<0.01$, n = >9.


Mean lesion length (mm)
20
18
16
14
12
10
8
6
4
2
0
0
Th
Tp
Gv
Antagonist
RG7
RG7/CR6A
RG7/PT4A
BQ30
BQ30/CR6A
BQ30/PT4A

citricola, however in all cases the biocontrol agent significantly reduced lesion development ($P<0.01$). There was no significant difference between cvs, however, in lesion lengths on shoots inoculated with the pathogen and antagonists. Only the antagonist fungi were re-isolated from shoots incubated in the presence of the antagonist with or without *Phytophthora* spp., whereas in control treatments in which shoots were incubated only with *P. cinnamomi* or *P. citricola*, only *Phytophthora* spp. were isolated.

5.3.7 Inoculation of seedlings in soil with *P. cinnamomi* and the antagonist fungi

5.3.7.1 Inoculation of seedlings with zoospores of *P. cinnamomi*

The shoots of inoculated seedlings were monitored for 3 wk for signs of disease, however, no disease symptoms appeared on plants in this time. When plants were removed from the soil at this time no necrotic roots were observed on plants from any treatment, including controls inoculated with *P. cinnamomi* alone and, therefore, results are not shown.

5.3.7.2 Collar inoculation of seedlings with mycelium of *P. cinnamomi*

Lesions developed after 3 d on some plants from all treatments and on controls inoculated with *P. cinnamomi*. Lesions spread up and around the shoot, ultimately girdling the stem of some plants. Leaf chlorosis and wilting were often associated with lesion extension, and some plants eventually died. After 14 d, there were no significant differences in symptoms on plants inoculated with *T. hamatum*, *T. pseudokoningii* or *G. virens* and *P. cinnamomi*, or *P. cinnamomi* alone (Table 5.1). Control plants remained healthy throughout the experiment, except for one plant inoculated with *T. hamatum* and one with *T. pseudokoningii*, which showed some leaf chlorosis toward the end of the experiment.

The results of the root assessment, measured using the root rot index 14 d after inoculation with the pathogen, are shown in Table 5.2. The tap roots of plants inoculated with both *T. pseudokoningii* or *G. virens* and *P. cinnamomi* were significantly less necrotic than those inoculated with *P. cinnamomi* alone, however, there was no significant difference in necrosis

Table 5.1: Mean shoot (± SE) assessments for plants inoculated with *P. cinnamomi* (CR6A) and *T. hamatum* (Th), *T. pseudokoningii* (Tp) or *G. virens* (Gv). (Assessment scores: 0 = healthy plant; 1 = small lesion and/or leaves wilted; 2 = large lesion and/or top of shoot collapsed; 3 = plant collapsed; 4 = plant dead). There were six or seven replicate plants for each treatment and plants were assessed twice weekly for 14 d for disease symptoms. d 10 LSD (antagonist) = 1.07. P<0.01. d 14 LSD (antagonist) = 1.33. P<0.01.

pathogen	antagonist	d 3	d 7	d 10	d 14
CR6A	**0**	0.71 ±0.18	0.71 ±0.18	1.29 ±0.18	1.57 ±.43
	Th	1.14 ±0.34	1.57 ±0.48	2.57 ±0.53	3.29 ±0.47
	Tp	0.57 ±0.20	1.00 ±0	1.14 ±0.14	1.57 ±0.43
	Gv	0.29 ±0.18	0.57 ±0.20	0.86 ±0.34	1.00 ±0.31
0	**0**	0	0	0	0
	Th	0	0	0	0.17 ±0.17
	Tp	0	0	0	0.17 ±0.17
	Gv	0	0	0	0

Table 5.2: Mean assessment of rot of tap and feeder roots (±SE) measured using a root rot index described in section 6.1. Roots were assessed 2 wk after collar inoculation of chestnut seedlings cv. "Sword" with *P. cinnamomi* (CR6A). Plants were inoculated with *T. hamatum* (Th), *T. pseudokoningii* (Tp) or *G. virens* (Gv) 2 wk prior to inoculation with *P. cinnamomi*. Controls were plants inoculated with sterile plugs of CMA and MEA or with the pathogen or antagonist alone.

LSD (feeder) = 1.34. LSD (tap) = 0.79. P<0.001.

pathogen	antagonist	mean root rot of feeder roots	mean root rot of tap root
CR6A	0	1.71 ±0.57	2.86 ±0.40
	Th	4.43 ±0.57	4.71 ±0.29
	Tp	1.29 ±0.18	2.00 ±0
	Gv	1.14 ±0.14	2.00 ±0
0	0	1.00 ±0	1.00 ±0
	Th	1.00 ±0	1.00 ±0
	Tp	1.00 ±0	1.00 ±0
	Gv	1.00 ±0	1.00 ±0

of feeder roots inoculated with *T. pseudokoningii* or *G. virens* and the pathogen compared with those inoculated with *P. cinnamomi* alone. Plants inoculated with *P. cinnamomi* and *T. hamatum* had significantly more necrotic tap and feeder roots than did plants inoculated with the pathogen alone or with *P. cinnamomi* and *T. pseudokoningii* or *G. virens*. No plants inoculated with *G. virens* and *P. cinnamomi* were dead by the end of the experiment, whereas in treatments where plants were inoculated with *T. pseudokoningii* or *T. hamatum* and *P. cinnamomi*, some were dead. Necrosis did not develop on the roots of control plants, whether uninoculated or inoculated with biocontrol fungi alone.

Root weights (Fig. 5.7A) differed significantly between treatments. Control treatments (not inoculated with *P. cinnamomi*) generally had significantly larger root fresh and dry weights than those inoculated with *P. cinnamomi*. Within these control treatments, there was no significant difference between the fresh and dry root weight of plants inoculated only with sterile CMA and MEA plugs and plants inoculated with *T. pseudokoningii*. Plants inoculated with *G. virens* and *T. hamatum* alone, however, had significantly smaller weights than those inoculated with sterile MEA and *T. pseudokoningii*, respectively. In the treatments inoculated with *P. cinnamomi*, plants inoculated previously with *T. pseudokoningii* or *G. virens* had significantly larger fresh and dry root weights than those inoculated with *T. hamatum*, or those inoculated with *P. cinnamomi* alone, with no significant difference in root weight between plants inoculated with *T. pseudokoningii* or *G. virens*.

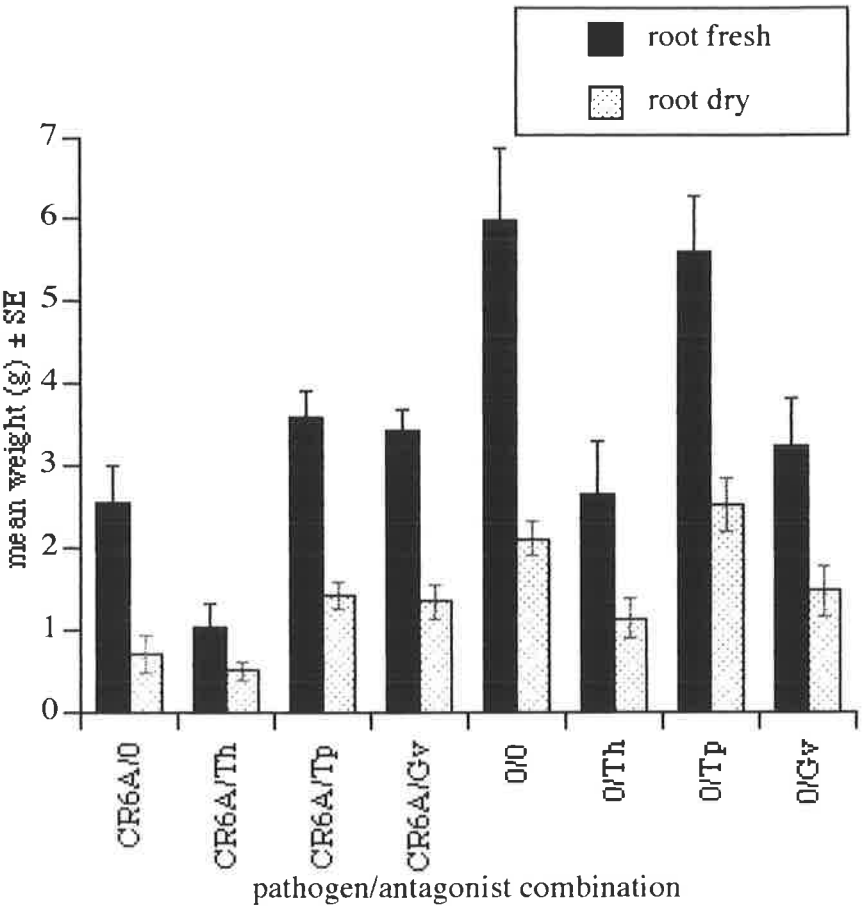
There was a significant difference in shoot weights between treatments (Fig. 5.7B), with most shoot fresh weights significantly higher in plants from treatments not inoculated with *P. cinnamomi* than in those inoculated with *P. cinnamomi*. An exception was plants inoculated with *G. virens*, in which shoot fresh weights were less than that of plants inoculated with *G. virens* and *P. cinnamomi*. In the control treatments, plants inoculated with *T. pseudokoningii* and uninoculated plants had significantly higher fresh and dry shoot weights than plants inoculated with *T. hamatum* and *G. virens*. There was no significant difference, however, in shoot dry weight, between plants inoculated with the biocontrol agents alone and those inoculated with biocontrol agents and *P. cinnamomi*. In treatments inoculated with *P.*

Fig. 5.7: Mean root and shoot weights of plants harvested 14 d after collar inoculation of chestnut seedlings cv. "Sword" with P. cinnamomi (CR6A). Plants were inoculated with *T. hamatum* (Th), *T. pseudokoningii* (Tp) or *G. virens* (Gv) 14 d prior to inoculation with *P. cinnamomi*. Controls were plants inoculated with sterile plugs of CMA (0) and MEA (0) or with the pathogen or antagonist alone. Bars indicate SE.

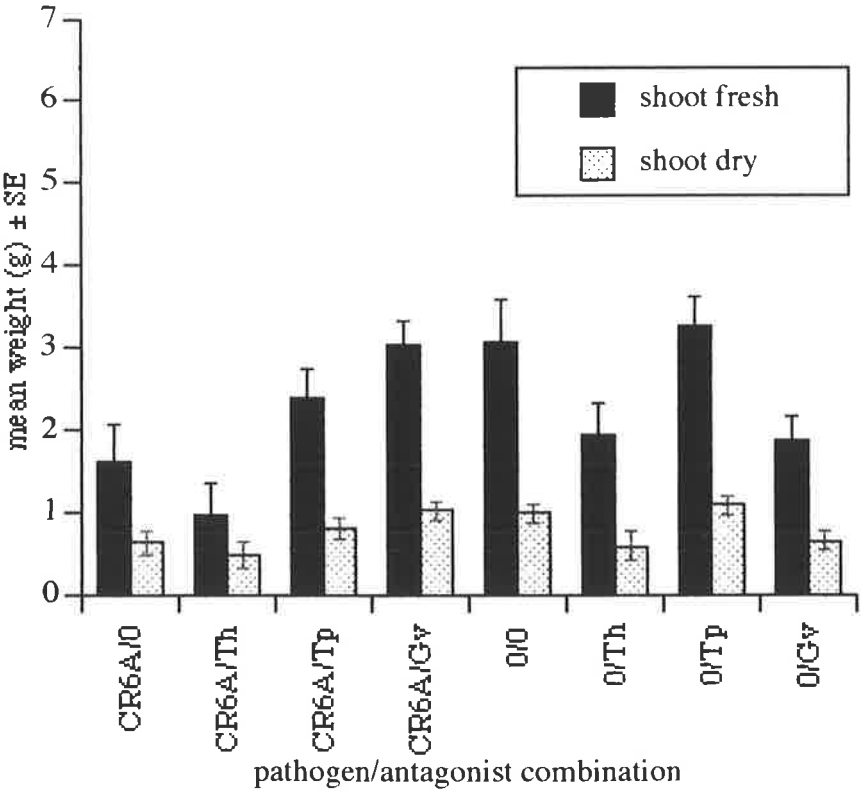
A: Mean root weight. LSD (fresh weight) = 1.65. LSD (dry weight) = 0.69. $P<0.05$.

B: Mean shoot weight. LSD (fresh weight) = 1.18. $P<0.01$. LSD (dry weight) = 0.41. $P<0.05$.

A


root fresh
root dry
mean weight (g) ± SE
0
1
2
3
4
5
6
7
CR6A/0
CR6A/Th
CR6A/Tp
CR6A/Gv
0/0
0/Th
0/Tp
0/Gv
pathogen/antagonist combination

B


shoot fresh
shoot dry
mean weight (g) ± SE
0
1
2
3
4
5
6
7
CR6A/0
CR6A/Th
CR6A/Tp
CR6A/Gv
0/0
0/Th
0/Tp
0/Gv
pathogen/antagonist combination

cinnamomi, plants inoculated with *G. virens* had the highest shoot fresh and dry weight, followed by those inoculated with *T. pseudokoningii*, *P. cinnamomi* alone, and *T. hamatum*, respectively.

5.4 Discussion

Both *Trichoderma* spp. inhibited *P. cinnamomi* by mycoparasitism, with evidence of parallel growth and coiling, and all three antagonists grew over *P. cinnamomi in vitro*, preventing further growth of this pathogen. Antibiotics produced by 2-4 d-old *T. hamatum* cultures and *G. virens* in the cellophane antibiosis and culture filtrate experiments inhibited growth of *P. cinnamomi* and *P. citricola*, with 4 d filtrate of *G. virens* showing the greatest potential for biocontrol. Volatile antibiotics produced by *T. pseudokoningii* significantly inhibited growth of *P. cinnamomi* and *P. citricola*. All three antagonists prevented *P. cinnamomi* and *P. citricola* from infecting shoots of chestnut cvs "Goldsworthy" and "Buffalo Queen" in a biocontrol shoot bioassay. In pot experiments, root and shoot weights of seedlings were generally higher in the presence of the antagonists than with the antagonists and *P. cinnamomi*.

Both *Trichoderma* spp. inhibited the *Phytophthora* spp. by direct antagonism (i.e. hyphal coiling and mycoparasitism) with minor inhibition by antibiosis. Inhibition by antibiosis, although statistically significant, was slight, and there was still significant fungal growth. It is unlikely that antibiotics produced by these *Trichoderma* spp. would prevent *Phytophthora* spp. from growing in the field.

The observations recorded in experiments here add to the list of pathogens upon which *Trichoderma* spp. are mycoparasitic. Dennis & Webster (1971c) found that two isolates of *T. viride* penetrated hyphae of *P. cactorum* and *P. erythroseptica*, and prevented further growth of these *Phytophthora* spp. by growing over the colony. Chet, Harman & Baker (1981) also observed parasitism by *T. hamatum* hyphae to control hyphal growth of *Pythium* spp. and *Rhizoctonia solani*. Parasitism was thought to be accompanied by the production of lytic enzymes rather than antibiosis. Elad *et al.* (1983) observed coiling and penetration of hyphae

of *R. solani* and *S. rolfsii* by *T. hamatum* and *T. harzianum*. Appressorium-like structures and hooks, similar to those reported here, were observed using SEM. When these hooks were peeled off, hyphal entry points were observed. Chet *et al.* (1981) also observed formation of appressorium-like structures, by *T. hamatum* hyphae on *Pythium* spp. and *Rhizoctonia solani* hyphae. Entry of antagonist hyphae into *Phytophthora* spp. hyphae was not observed using light microscopy here, however entry points into hyphae of *P. cinnamomi* may have been beneath appressorium-like structures observed using SEM.

Sivan, Elad & Chet (1984) noted that culture filtrate of *T. harzianum* strongly inhibited growth of *Pythium aphanidermatum* in culture, however, *T. hamatum* filtrates caused only minor inhibition in growth. *T. hamatum* does not appear to produce non-volatile antibiotics.

The results presented here show that culture filtrate of G. virens and T. hamatum significantly inhibited growth of both *Phytophthora* spp.. Generally, the younger the antagonist culture, the more the filtrate inhibited pathogen growth, although 4 d-old filtrate of *G. virens* inhibited the growth of *Phytophthora* spp. most strongly. Both *Trichoderma* spp. and *G. virens* grew rapidly, reaching the edge of a 90 mm diam. Petri dish in approximately 3 d. Filtrate from actively growing mycelium (i.e. younger than 4 d-old) inhibited colony growth strongly, while older mycelium (1-7 wk old), yielded filtrates which had little or no effect in inhibiting the pathogen; in fact, filtrate from 7 wk-old cultures stimulated growth of *P. cinnamomi* significantly. The antagonist fungus is likely to have been degenerating at this stage, with hyphal lysis releasing cell contents into the medium, providing additional nutrients for pathogen growth.

Of the three antagonists screened, *G. virens* caused the greatest inhibition of both species of *Phytophthora*. Colony growth of both *Phytophthora* spp. was strongly or completely inhibited in the cellophane antibiosis and culture filtrate experiments, whereas hyphal coiling was rare and no appressoria were observed in dual culture with *P. cinnamomi*. This lack of physical interaction suggests that *G. virens* inhibits *P. cinnamomi* by antibiosis rather than by mycoparasitism.

G. virens inhibits *Pythium ultimum* by antibiosis, as found for *P. cinnamomi* and *P. citricola* in experiments here, but has been found to inhibit *R. solani* by mycoparasitism (Howell, 1982). Isolates and species of *Trichoderma* and *Gliocladium* vary in effectiveness of biocontrol of different isolates and species of fungal pathogens (e.g. Dennis & Webster, 1971a; Bell *et al.*, 1982; Howell, 1982; Lynch, 1987). When larger amounts of the enzymes β-1,3-glucanase, chitinase and cellulase were produced by isolates of *T. harzianum in vitro*, mycoparasitism of *Sclerotium rolfsii*, *Rhizoctonia solani* and *Pythium aphanidermatum* was greater (Elad, Chet & Henis, 1982). Enzyme production was not investigated here, but it is possible that the isolate of *G. virens* tested was unable to produce sufficient quantities of these enzymes to lyse the walls of *Phytophthora* spp. under the conditions tested .

Although Dennis and Webster (1971a) found greater antibiotic production in media such as malt extract agar than in DW agar, the results of experiments presented here differed from this. Inhibition was generally inconsistent on the DW agar treatments, compared to 1/5M32 agar treatments, in the present study. Only antibiosis from *T. pseudokoningii* produced in DW medium was more effective in inhibiting *P. cinnamomi* growth than on 1/5M32 medium compared to controls. Nutrient limitations in media are known to promote secondary metabolite production (see Franco & Coutinho, 1991). Age of the culture was also important in inhibition of the growth of *P. cinnamomi* and *P. citricola.* In experiments reported in this study, using culture filtrate incorporated into agar medium, antibiosis by 2-7 d-old cultures produced the greatest inhibition in pathogen growth. Greater antibiotic production by young cultures was also observed in *Trichoderma* isolates inhibiting the growth of *Sclerotium rolfsii* (A. Na Lampang, pers. comm.). Lederer *et al.* (1992), however, using the cellophane overlay technique, observed greatest inhibition in the growth of *P. cactorum* by isolates of *Trichoderma* in the presence of substances derived from 3 d-old, sporulating cultures rather than from younger cultures. Peptide antibiotics (peptaibols) were thought to be concentrated in the conidiophore tufts formed in older cultures. Antibiotics other than these peptaibols may have been produced by young cultures of *Trichoderma* which inhibited the growth of *P. cinnamomi* and *P. citricola* in this study.

Most isolates of *Trichoderma* and *Gliocladium* produce the antibiotic 6-n-pentyl-2H-pyran-2-one (Ghisalberti & Sivasithamparam, 1991) and gliovirin (Howell & Stipanovic, 1983), gliotoxin (Brian & Hemming, 1945) and viridin (Brian & McGowan 1945). A phytotoxin, viridiol, has also been identified (Howell & Stipanovic, 1984). Gliovirin is highly active against members of the Oomycetes, but has little effect on other fungi or bacteria (Howell & Stipanovic, 1983). Gliotoxin also inhibits mycelial growth, sporangium production and zoospore motility of *Phytophthora* spp. (Wilcox, Harman & Di Pietro, 1992). Filtrate from 4 d-old *G. virens* cultures consistently inhibited *P. citricola* completely and resulted in a 74% inhibition of *P. cinnamomi* growth in this study. Filtrates need to be analysed for the presence of antibiotics, especially gliovirin and gliotoxin, which may be of greatest use in biocontrol of *Phytophthora* spp..

Dennis & Webster (1971b) identified a volatile antibiotic produced by *Trichoderma* sp. which inhibited growth of *Rhizoctonia solani* and other test fungi, with activity being related to a 'coconut' odour. This antibiotic activity and odour have been characterized as 6-n-pentyl-2H-pyran-2-one, with pentenyl pyrone as a minor component (Claydon *et al.*, 1987). The isocyanide compounds have also been grouped by Ghisalberti & Sivasithamparam (1991) into this category of volatile metabolites. Lynch (1987) recognized the potential of these volatile antibiotics as fungicides for use as soil drenches; they would leave no harmful residues and would not be toxic to workers. The potential of volatile antibiotics produced here by *T. pseudokoningii*, which significantly inhibited both species of *Phytophthora*, needs to be further examined.

In the dual culture experiments reported here, all three isolates of *Trichoderma* and *Gliocladium* overgrew mycelium of *P. cinnamomi*, preventing further growth. Bell *et al.* (1982) found that most isolates of *Trichoderma*, screened *in vitro* against *P. parasitica* f. sp. *nicotianae* (= *P. nicotianae*, Hall, 1993), completely overgrew the pathogen and covered the entire surface of V8 agar medium. When discs from two of these co-cultures were used to inoculate "Hicks" cv tobacco seedlings, by placing a mycelial plug against the base of the stem of each plant, no disease developed after 12 d. Seedlings of wheat and capsicum, placed

on plugs of co-cultures of *Sclerotium rolfsii* and *Trichoderma* spp., were also protected from basal stem rot in *in vitro* bioassays (A. Na Lampang, pers. comm.). *Trichoderma* spp. and *G. virens* protected excised shoots of chestnut against necrosis by *P. cinnamomi* and *P. citricola* *in vitro* in experiments here. In the biocontrol shoot assay used in this study, all three antagonists reduced lesion development by *P. cinnamomi* and *P. citricola* on chestnut shoots in comparison with controls after 2 d. The three antagonist fungi grew over the *Phytophthora* spp. mycelium, as in dual culture experiments (section 5.3.1), inhibiting the growth of *Phytophthora* spp. and preventing *Phytophthora* spp. from causing lesions on chestnut shoots. The biocontrol shoot assay could, therefore, be used as a rapid screening method for potential biocontrol agents before verification of biocontrol capabilities in soil experiments.

The results of dual culture experiments, antibiosis experiments and the biocontrol shoot bioassay, indicated that these fungi have potential as biocontrol agents of chestnut root rot. The *in vitro* experiments reported here also indicated the mode of action of each antagonist. Smith *et al.* (1990) also used preliminary *in vitro* experiments to screen cultures of *Trichoderma* spp. for biocontrol potential against *P. cactorum.* Isolates were selected for antibiotic production (inhibition of growth of *P. cactorum* in dual culture) and for cold-temperature tolerance, as *P. cactorum* primarily infects apple during the cooler months of Spring and autumn. These isolates were then screened further using apple seedlings in pot experiments in the glasshouse and a number of them gave effective biocontrol of disease. Similarly, Lederer *et al.* (1992) used a cellophane antibiosis experiment, similar to that described by Dennis & Webster (1971a), to screen isolates of *Trichoderma* spp. for antibiosis toward *P. cactorum.* Encysted zoospores were killed or their growth was retarded within 24 h of being placed on a medium from which a cellophane overlay and 2-3 d-old mycelium of *Trichoderma* spp. had been removed. This was thought to be due to the production of antibiotics and cysts were killed predominantly in the presence of substances derived from old cultures. Antibiotic production by cultures of *Trichoderma* spp. also inhibited the growth of *P. cactorum* in dual culture experiments.

Lewis & Papavizas (1985) noted that there is often little correlation between plate studies and the ability of a potential biocontrol agent to reduce or prevent disease in the greenhouse or field. It is important that pot and field experiments, therefore, are conducted in the investigation of possible biocontrol agents. Duchesne *et al.* (1989) noted, however, that to screen isolates using pot experiments in the glasshouse and field experiments requires large amounts of space and time. In comparison, initial *in vitro* experiments can be used to screen a large number of isolates in a small amount of space. They can also be used to elucidate the mechanisms of antagonism. Evaluation of the biocontrol potential of isolates also takes much longer using pot and field experiments than *in vitro* experiments, because plants have to be grown to a suitable size for screening in pot and field experiments and the pathogen can often take much longer to cause disease than *in vitro*. Duchesne *et al.* (1989) suggested, therefore, that any promising biocontrol isolates first be identified using *in vitro* experiments, then further tested in pot and field experiments.

Having elucidated the interactions between the antagonists and *P. cinnamomi* or *P. citricola*, experiments were conducted in the glasshouse in this study to determine whether *Trichoderma* spp. and *G. virens* protected chestnut from *P. cinnamomi* infection. The lack of infection of chestnut seedlings by *P. cinnamomi*, in the initial experiment in which plants were inoculated using zoospores, may have occurred because insufficient inoculum was applied to soil or because soil used was not conducive to zoospore movement towards host roots. Pasteurized soil may have contained bacterial or fungal antagonists which were inhibitory to *Phytophthora* spp.. Further experiments are needed to determine the optimum conditions for infection of chestnut seedlings by zoospores of *P. cinnamomi* in pot experiments. Zoospores of *P. cinnamomi* caused disease in chestnut seedlings, using the same soil type as above, when applied as a root dip in other experiments in this study (see section 6.3.3.2). Zoospores of *P. cinnamomi*, applied (at 10^4 zoospores ml^{-1}) as a drench to sandy soil in another study, caused disease on *Banksia* spp. plants of a similar size to the chestnut plants used in this study (K. Tynan, pers. comm.). Because of the larger pore size of the sandy soil, zoospores may have been able to reach the roots faster before encysting, than

in the soil composed of peat and loam used in this study. Different soil types, therefore, need to be investigated to establish the soil type required for optimum infection.

Alternatively, other types of inoculum could be used to infect seedlings. Roiger & Jeffers (1991) and Smith *et al.* (1990) used vermiculite colonized by *P. cactorum* to infect apple seedlings in experiments designed to screen biocontrol fungi. Vermiculite colonized by *P. cinnamomi* might have caused infection in chestnut in experiments here and the method of inoculation needs to be further investigated.

Two formulations were used in this study to assess antagonists for potential biocontrol of root rot caused by *P. cinnamomi* on chestnut in pot experiments. The first was the oat grain colonized by antagonists. Preliminary experiments, assessing the growth of antagonists on wheat bran and oat grain, showed the antagonists colonized oat grain more extensively than wheat bran and, therefore, oat grain was used for experiments. Zoospores were used to infect plants pre-inoculated with this antagonist formulation. In view of the lack of success with the zoospore inoculation method, a second formulation was used, in which mycelium was added to wounds made on the root and collar.

In the experiment in which the collar of seedlings was inoculated with mycelium of the antagonists and *P. cinnamomi*, shoot and root weights of plants inoculated with the antagonists *T. pseudokoningii* and *G. virens* were generally higher than those of plants inoculated with *P. cinnamomi* alone. Although these weights were lower than in control plants inoculated with the biocontrol agents alone, *T. pseudokoningii* and *G. virens* possibly reduced the development of disease to some extent. Root necrosis on plants inoculated with these fungi was less than on plants inoculated with *P. cinnamomi* alone. Also, no deaths occurred among plants inoculated with *G. virens* and *P. cinnamomi*. The lack of protection given by the three antagonists, in collar inoculation experiments, against lesion extension by *P. cinnamomi* on some plants may have been due to the type of inoculum used. Roots were inoculated with the antagonist fungi for 14 d prior to inoculation with *P. cinnamomi*, however, a longer period of establishment may have been required by these antagonists.

It is unclear why T. hamatum caused symptoms on both roots and shoots compared to controls in that experiment.

Also, once infection by *P. cinnamomi* occurred in experiments here, via a wound, it would have been difficult for *Trichoderma* spp. and *G. virens* to contain disease. Plant deaths often occurred when lesions girdled the shoots. *Trichoderma* and *Gliocladium* spp. would not have been able to prevent this spread of disease.

Tuset *et al.* (1990) used this collar inoculation method to evaluate the fungus *Myrothecium roridum* for control of root disease on citrus caused by *P. nicotianae* var. *parasitica* (= *P. nicotianae*, Hall, 1993). Plugs of *M. roridum* were placed on the root 8-9 d prior to inoculation with *P. nicotianae* var. *parasitica.* Canker development by *P. nicotianae* var. *parasitica* was limited by *M. roridum* on citrus seedlings in experiments in the glasshouse and field. Metabolites which may have been produced by the biocontrol agent, thought to be toxic to the pathogen, were thought to move acropetally through the vascular system, into the roots or stems of the plants, and inhibit the growth of *P. nicotianae* var. *parasitica.* Culture filtrate of *M. roridum* also suppressed the growth of *P. nicotianae* var. *parasitica* in *in vitro* experiments. Dubos & Ricard (1974) found that the injection of a paste of *T. viride* in holes in the trunks of peach and nectarine trees controlled silver leaf disease caused by *Chondrostereum purpureum*, although the mechanism of disease control was not understood. Biocontrol may have occurred because of the production of metabolites toxic to *C. purpureum*, which were transported through the vascular system, as proposed for *M. roridum*, and prevented establishment or transfer of the pathogen. Metabolites produced by liquid cultures of *T. hamatum, T. pseudokoningii* and *G. virens* inhibited the growth of *P. cinnamomi in vitro* in experiments reported here. These may have been produced by the antagonists in chestnut roots in this study, but were ineffective in preventing the spread of *P. cinnamomi*. There was no evidence of induced resistance in seedlings inoculated with any of the antagonists and P. cinnamomi.

While growth enhancement has been observed in many plants inoculated with strains of *Trichoderma* and *Gliocladium* (Chet, 1987), no such response was observed in this study. There was no significant difference in the weight of plants inoculated with *T. pseudokoningii* compared to uninoculated plants, whereas plants inoculated with *T. hamatum* and *G. virens*

weighed less than the uninoculated controls. Other formulation and delivery methods may result in a growth response.

Formulation of a biocontrol agent is known to be important if a disease is to be effectively controlled. Biocontrol by isolates of *Trichoderma* and *Gliocladium* spp. in general was best if preparations contained chlamydospores, the survival propagules of the fungus (Lewis & Papavizas, 1984). Kelley (1976) found that *T. harzianum*, impregnated in clay granules amended with molasses and applied to soil in pots of pine seedlings, did not control damping-off caused by *P. cinnamomi*. The molasses acted as a food source for *P. cinnamomi*, increasing the infection of pine seedlings by this pathogen. Preparations of the antagonists, *T. hamatum*, *T. pseudokoningii* and *G. virens*, used in this study, need to be further investigated. The optimum formulation for each strain, using either dusts, granules, pellets, wettable powders, or emulsifiable liquids needs to be determined (Lumsden & Lewis, 1989). Effective delivery must be employed for control of disease and, for commercial production, a formulation with reasonable shelf-life and marketing potential must also be developed (Lumsden & Lewis, 1989). Effective biocontrol can thus depend upon a number of factors, and these need to be investigated thoroughly for each system.

In this study, three antagonistic fungi, evaluated *in vitro* and *in vivo*, showed some promise in biocontrol of *Phytophthora* root rot of chestnut. The mechanisms of control *in vitro* were elucidated for each isolate and preliminary pot experiments conducted in pasteurized soil. In these preliminary pot experiments, *T. pseudokoningii* and *G. virens* showed the greatest potential for reducing the extent of disease.

6. SCREENING SEEDLINGS OF CHESTNUT ROOTSTOCKS FOR RESISTANCE TO *PHYTOPHTHORA* SPP.

6.1 Introduction

Very little research has been conducted on the root rot disease caused by *Phytophthora* spp. on chestnut: the methods researchers have used to screen for resistance of chestnut to *Phytophthora* spp. are discussed in section 2.1.4. Briefly, Milburn & Gravatt (1932) inoculated seedlings of different species of chestnut in the greenhouse with soil infested with *P. cambivora*, whereas Crandall *et al.* (1945) inoculated seedlings by inserting a plug of mycelium of *P. cinnamomi* into a wound on the main root, just below the collar. Plant mortality was used as a measure of susceptibility in these studies and *C. sativa* was found to be susceptible to *P. cinnamomi* and *P. cambivora*, whereas *C. mollissima* and *C. crenata* were resistant to infection.

In Australia, resistant species of chestnut (*C. crenata* and *C. mollissima*) are not readily available for growers to use as rootstocks due to quarantine regulations which restrict their importation. Therefore, a number of *C. sativa* cvs including "Sword" and "Marone" and a few *C. sativa* x *C. crenata* hybrid cvs, for example "Colossal" and "Buffalo Queen", which have been selected for their nut characteristics, are used. They are grown mainly as clonal selections of cvs grafted on rootstocks of *C. sativa*. There are no known published reports investigating differences in susceptibility of these cvs to *Phytophthora* spp. and chestnut cvs need to be screened to identify any with resistance present in Australia. These could then be used as rootstocks by growers in *Phytophthora*-infested soils.

P. cinnamomi and *P. cambivora* are severe pathogens of *C. sativa* worldwide (Crandall *et al.*, 1945; Grente, 1961; Washington, 1983; Reichard & Bolay, 1986) and *P. cryptogea* is pathogenic on chestnut in Australia (Wicks & Volle, 1976). The biology of *Phytophthora* spp. and the symptoms of disease on chestnut caused by these species of *Phytophthora* are described in sections 2.1.1 & 2.1.3, respectively.

A number of isolates of *Phytophthora* spp. were obtained from soil beneath dead and dying chestnut trees in orchards in the Adelaide Hills, using the pear baiting method (section 3.1). Most isolates were identified as *P. cinnamomi*, however, one isolate of *P. citricola* was obtained. This species has not previously been reported in the literature as a pathogen of chestnut, although it is a pathogen of other nut trees such as walnut (Matheron & Mircetich, 1985), and a minor pathogen of almond (Wicks, 1987). Studies are needed to determine whether *P. citricola* is pathogenic on chestnut.

The principal aim of these experiments was, therefore, to screen different cvs of chestnut, used as rootstocks by growers, for resistance to locally isolated *Phytophthora* spp. by inoculating seedlings using a zoospore drench, zoospore dip and collar inoculation method. The experiments were also designed to provide information on :

(i) the pathogenicity of *P. citricola* on chestnut; and

(ii) on differences in virulence of the isolates.

6.2 Materials and Methods

6.2.1 Inoculation of the tap root of chestnut seedlings with mycelium of *Phytophthora* spp.

Seeds of *C. sativa* cvs "Goldsworthy", "Sword" and "Marone" and *C. sativa* x *C. crenata* hybrid cvs "Colossal" and "Buffalo Queen", were germinated as described in section 3.4. Seedlings were transferred to 10 cm diam. pots containing RS soil with 50% peat for 4 wk in a growth cabinet (16 h day) with 25°C day and 18°C night temperatures under white fluorescent light (approx. 250 μmol m^{-2} s^{-1}) (Fig. 6.1). Plants, with shoots approx. 21 cm tall, were inoculated using a technique modified from Crandall *et al.* (1945). Soil from around the collar region was gently removed and, using a scalpel, a wound was made down to the cambium on the main tap root, 2 cm below the soil surface. The flap of bark produced was approx. 2 cm long. A plug of mycelium, 6 mm in diam., was taken from the edge of an actively growing culture of *P. cinnamomi* (isolate CR6A), *P. citricola* (isolate PT4A), or *P. cryptogea* (isolate P173) on CMA and placed in each wound. The flap of bark was then

Fig. 6.1: Four wk-old chestnut seedlings in growth cabinet with 16 h day (250 μmol m^{-2} sec^{-1}), 25°C day and 18°C night. Seedlings were grown in 10 cm diam. pots containing RS soil with 50% peat.

closed and soil replaced. Control plants were inoculated with a plug of sterile CMA. Plants were arranged randomly within separate plastic trays for each isolate in the growth cabinet. They were watered as required with tap water and shoots scored twice weekly, using a shoot symptom index (Table 6.1), for 21 d for signs of disease. At the end of this time plants were harvested and roots scored for disease using a root rot index (Table 6.2; Grimm & Hutchison, 1973). Shoots and roots were then separated, fresh weights obtained and material placed in an oven at 105°C for 24 h before obtaining shoot and root dry weights. There were 5-7 replicate plants per cv. x isolate interaction. Two roots, from one randomly chosen plant per treatment were plated on CMA in 90 mm diam. Petri dishes and incubated in the dark at 25°C for 2 d to re-isolate the pathogen.

6.2.2 Inoculation of chestnut seedlings with zoospores of *Phytophthora* spp.

6.2.2.1 Drench method

Chestnut seeds were germinated as in section 3.4. Seedlings of chestnut cvs "Buffalo Queen", "Colossal", "Sword" and "Goldsworthy" were transferred to 10 cm diam. pots containing RS soil with 50% peat and grown for 4 wk in a glasshouse with ambient daylight (daylength approx. 10 h) and a temperature range of 15-25°C. Seedlings were inoculated using the zoospore drench method. Seedlings in pots were first placed in individual plastic tubs 10 x 7 cm in diam. (Detpack, Australia) and watered with tap water. A zoospore suspension (10^4 zoospores ml^{-1}) of *P. cinnamomi* (CR6A), *P. citricola* (PT4A) or *P. cryptogea* (P173) was produced using a method modified from Gees and Coffey (1989) (section 3.2). Twenty ml of this suspension in sterile soil extract was poured on the soil surface around the base of each seedling. *P. cambivora* (P6414) was not used in seedling inoculation experiments because it was imported from the U.S.A. and, therefore, could only be used under quarantine conditions. Control plants were inoculated with sterile soil extract. Inoculated and control plants were placed in tubs filled with tap water to a depth of 5 cm for 24 h. Plants were then watered from above as required with tap water. There were 5 replicate plants of cv. "Sword" per treatment, 7 of cvs "Goldsworthy" and "Colossal" and 10 of the cv. "Buffalo Queen" per treatment. Seedlings of the cv. "Sword" were not inoculated with *P.*

Table 6.1: Index of shoot symptoms used to score plants for disease

rating	shoot symptoms
0	healthy plant
1	leaves flaccid and/or small lesion on stem
2	leaves flaccid and/or chlorotic, large lesion on stem, top of shoot wilted
3	leaves dry, large lesion on stem, stem collapsed
4	plant dead

Table 6.2: Index of root rot used to score plant roots for necrosis

rating	feeder roots	tap roots
1	no visible necrosis	no visible necrosis
2	a few necrotic roots	necrosis only at tip
3	majority of root system necrotic	diseased from tip to 1/4 length of root
4	all roots infected, cortex sloughed	disease for more than 1/4 length of root from major tap roots
5	roots dead	tap root dead

cryptogea and the cv. "Marone" was not used in this experiment because insufficient seeds germinated for adequate replication. Plants were scored for disease symptoms as in section 6.2.1 and then plant fresh and dry weights were obtained as in section 6.2.1.

6.2.2.2 Dip method

Chestnut seedlings, of cvs used in the zoospore drench experiment, were grown in the glasshouse for 8 wk under the conditions described as in section 6.2.2.1. Plants were gently removed from the soil and roots washed with tap water. The roots of plants of each cv. were dipped in a zoospore suspension (10^4 zoospores ml^{-1}) of *P. cinnamomi* (CR6A), *P. citricola* (PT4A) or *P. cryptogea* (P173) in sterile soil extract, produced as in section 3.2, in 2 l beakers for 24 h. Roots of control plants were dipped in sterile soil extract. Plants were then placed in fresh soil in pots as above and placed in a glasshouse under the conditions described above. Pots were placed in tubs filled with tap water to a depth of 5 cm for 24 h and then watered from above as needed. Plants were scored for disease symptoms and harvested and roots scored as in section 6.2.1. Plant fresh and dry weights were then obtained as in section 6.2.1. There were 5-10 replicate plants per treatment and two roots, from one randomly chosen plant per treatment were plated on CMA in 90 mm diam. Petri dishes and incubated in the dark at 25°C for 2 d to re-isolate the pathogen.

6.2.3 Analysis of data

Means of shoot symptoms, root rot and plant weight data were compared using Analysis of Variance and Least Significant Differences were used to identify significant differences between means.

6.3 Results

6.3.1 Inoculation of the tap root of chestnut seedlings with mycelium of *Phytophthora* spp.

Some plants from all treatments showed symptoms by 21 d after inoculation, except for plants inoculated with sterile CMA plugs (controls) which remained healthy throughout the experiment (Fig. 6.2.1). These symptoms varied from plant to plant and included flaccid, chlorotic and dry leaves (Fig. 6.2.2, 6.2.3) and wilting of the shoot (Fig. 6.2.4). Heavily infected plants had dry, wilted leaves and often a large lesion on the stem (Fig. 6.3). This led to plant death. There was a large amount of variation in response of seedlings to infection within a treatment, however, some plants from all treatments inoculated with *Phytophthora* spp. showed disease symptoms at 21 d (Fig. 6.4). Most cvs were equally susceptible to *P. cinnamomi*, *P. citricola* and *P. cryptogea* compared to controls 7, 14 and 21 d after inoculation. Shoots of seedlings of the cv. "Goldsworthy" showed the most severe disease symptoms when inoculated with *P. cryptogea* and the largest number of plants of this cv. were dead after 21 d compared with seedlings inoculated with *P. cinnamomi* or *P. citricola*.

When the plants were harvested and roots examined, necrotic tap and feeder roots were evident on dead or dying seedlings inoculated with *P. cinnamomi*, *P. citricola* or *P. cryptogea* compared to plants from control treatments (Fig. 6.5). The majority of plants often showing only slight shoot symptoms had necrotic roots. These plants mostly had completely rotted roots with only a few healthy feeder roots at the soil surface. Tap and feeder roots of seedlings from all cvs were equally susceptible to infection (Fig 6.6). There was no significant difference in necrosis on roots of the seedlings of cvs infected with any of these isolates.

Shoot fresh and dry weights are shown in Fig. 6.7. Shoot fresh and dry weights were generally significantly higher for seedlings of the cv. "Buffalo Queen" inoculated with *P. cinnamomi* than for seedlings of this cv. inoculated with *P. citricola*, *P. cryptogea* or sterile soil extract ($P<0.01$). There was no significant difference, however, between the weights of

Fig. 6.2: Typical symptoms displayed by chestnut seedlings 21 d after inoculation with a mycelial plug of *Phytophthora* spp. to a wound made on the tap root.

Fig. 6.2.1: Healthy control plant (inoculated with a sterile plug of CMA). Bar = 4 cm.

Fig. 6.2.2: Dry, chlorotic leaves of chestnut seedling following inoculation with *P. cinnamomi*. Bar = 3.3 cm.

Fig. 6.2.3: Dry, withered leaves of chestnut seedling following inoculation with *P. citricola*. Bar = 3.2 cm.

Fig. 6.2.4: Wilted leaves and shoot of chestnut seedling following inoculation with *P. cryptogea*. Bar = 2.2 cm.

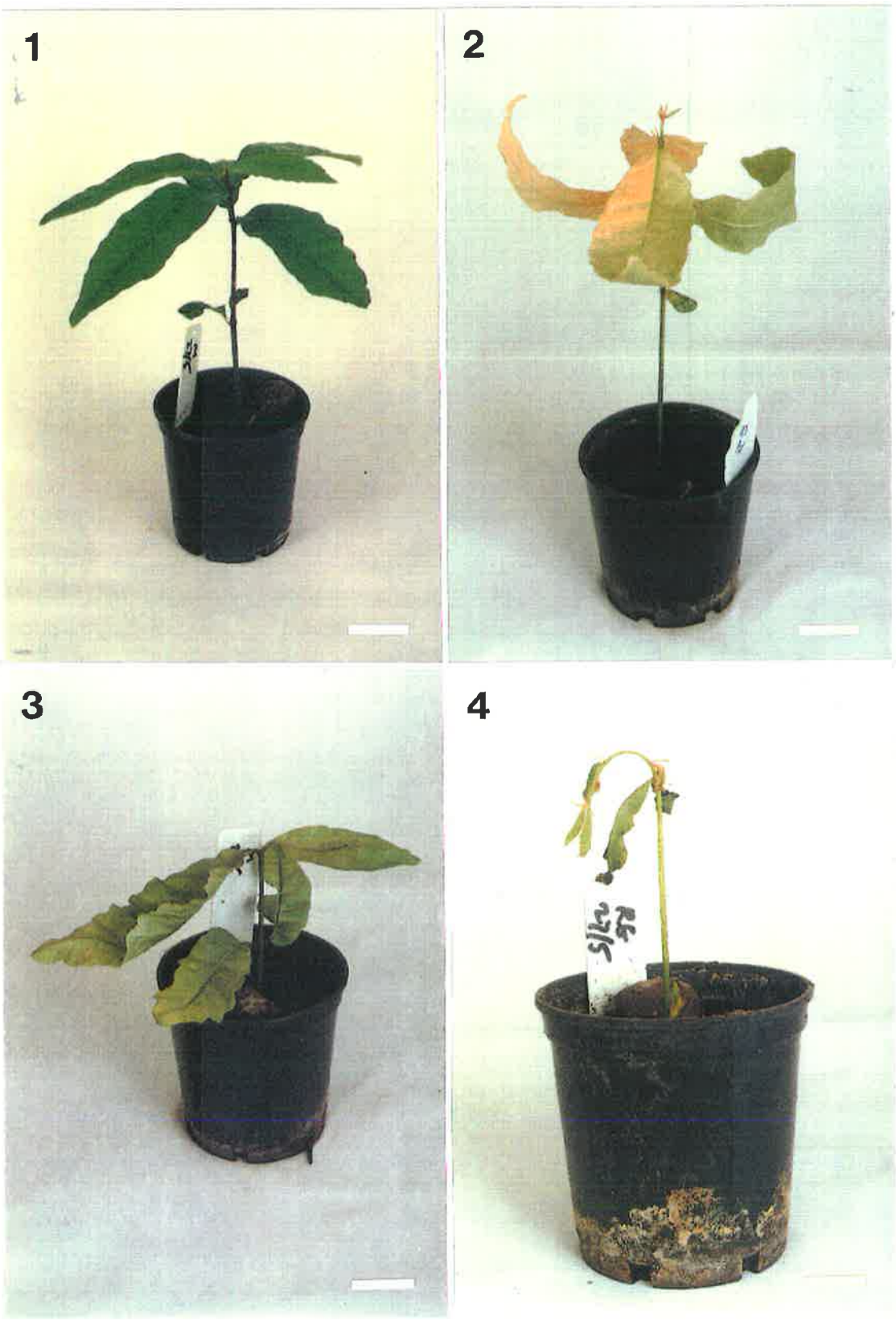

1
2
3
4

Fig. 6.3: Lesion development on the shoot of a chestnut seedling 21 d after inoculation with *P. cryptogea*.

Fig. 6.3.1: Seedling showing lesion on shoot (arrowed). Plant looks healthy otherwise. Bar = 3.7 cm.

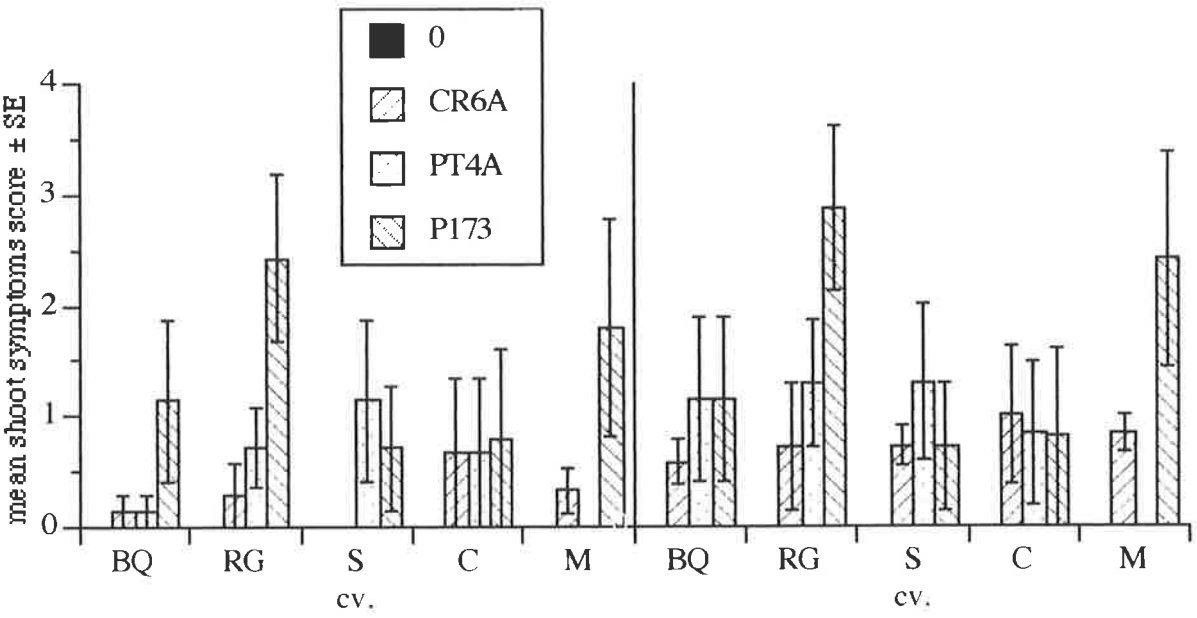
Fig. 6.3.2: Closer view of lesion on stem of seedling shown in Fig. 6.3.1. Lesion has girdled stem (arrowed). Bar = 0.8 cm.


1

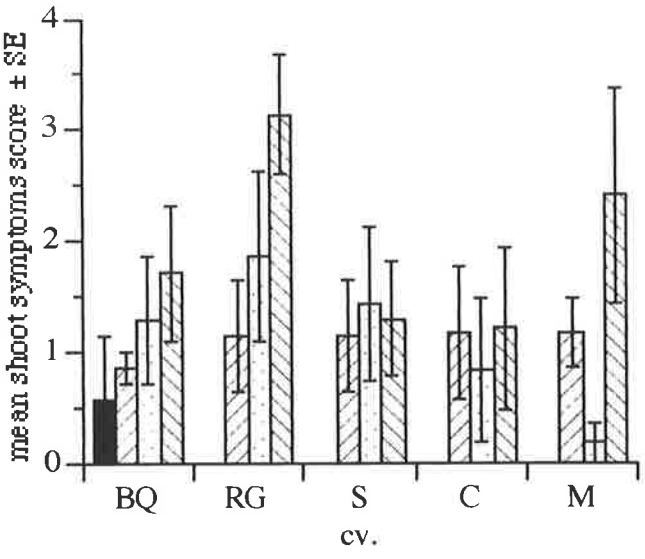

2

Fig. 6.4: Symptoms displayed by shoots of chestnut seedlings cvs "Buffalo Queen" (BQ), "Goldsworthy" (RG), "Sword" (S), "Colossal" (C) and "Marone" (M), 7, 14 and 21 d after inoculation with mycelium of *Phytophthora* spp. to a wound made on the tap root. Symptoms were assessed using a shoot symptoms index (see Table 6.1).

0 = control plants (inoculated with sterile CMA), CR6A = *P. cinnamomi*, PT4A = *P. citricola*, P173 = *P. cryptogea*. LSD, 7 d = 2.5. LSD, 14 d = 1.41. LSD, 21 d = 1.41. ($P<0.001$)



day 7 **day 14**



day 21

Fig. 6.5: Symptoms on roots of chestnut seedlings 21 d after inoculation with *P. cinnamomi*. (a) healthy control plant, inoculated with a plug of sterile CMA on a wound made on the tap root. Note the healthy, brown tap root (arrowed) and many healthy, white feeder roots (arrowed). (b) plant inoculated with a plug of *P. cinnamomi* on a wound made on the tap root. Note the wilted, dry leaves. This plant has a black, necrotic tap root (arrowed) and feeder roots (arrowed). Bar = 4.3 cm.

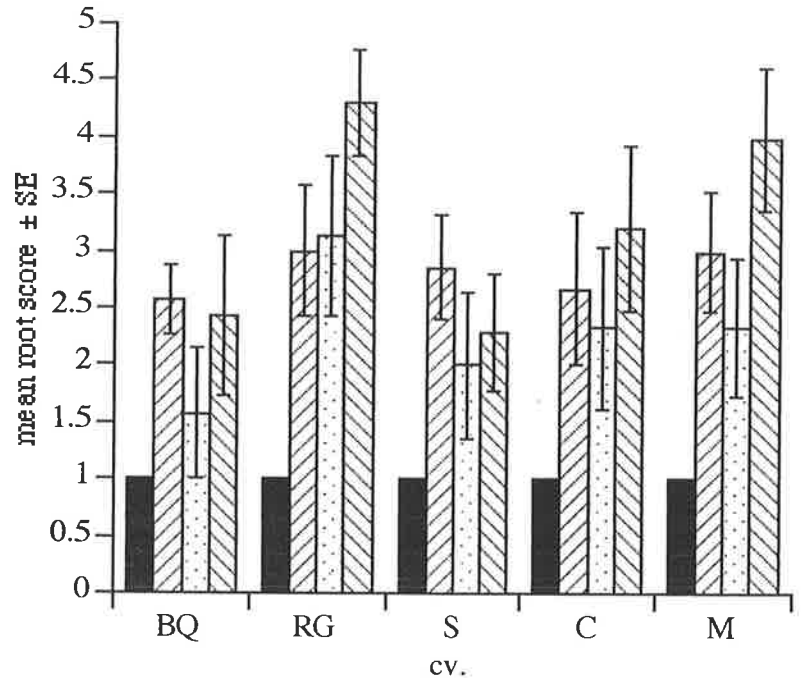

a
b

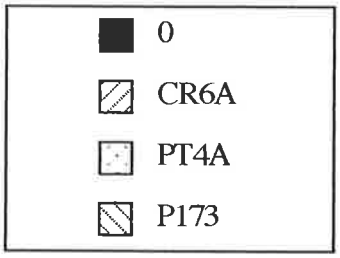
Fig. 6.6: Root score for plants of cvs "Buffalo Queen" (BQ), "Goldsworthy" (RG), "Sword" (S), "Colossal" (C) and "Marone" (M), 21 d after inoculation of a wound made on the tap root with either a sterile CMA plug (0) or a mycelial plug of *P. cinnamomi* (CR6A), *P. citricola* (PT4A) or *P. cryptogea* (P173). Roots were scored according to a root rot index (see table 6.2).

A: feeder roots. LSD (isolate) = 1.58. $P<0.001$.

B: tap root. LSD (isolate) = 0.63. $P<0.001$.

feeder roots





tap roots

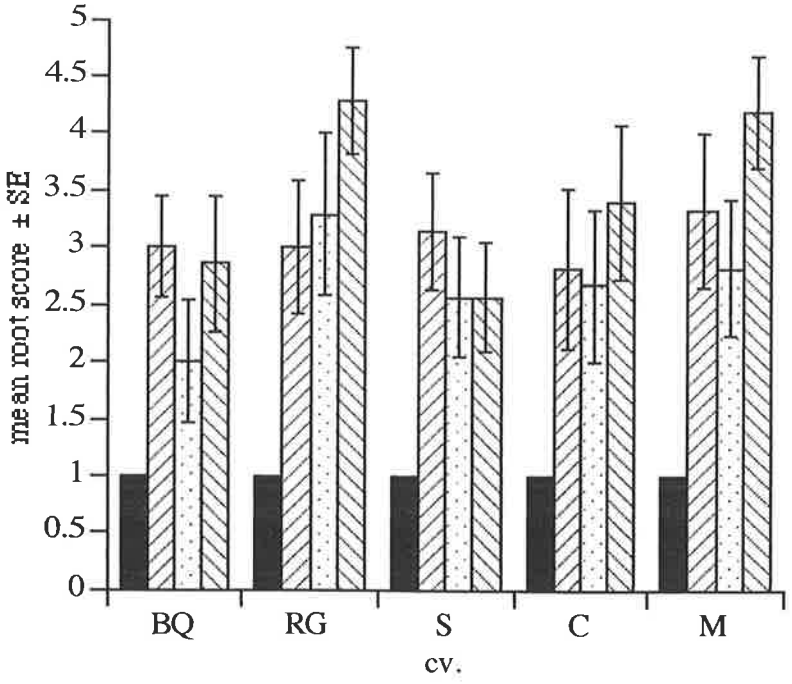
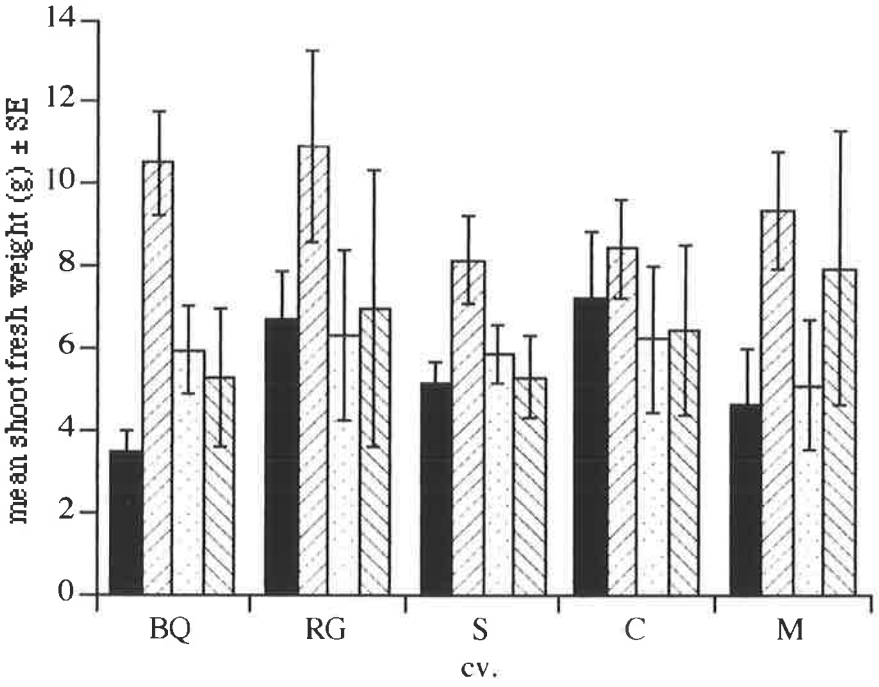


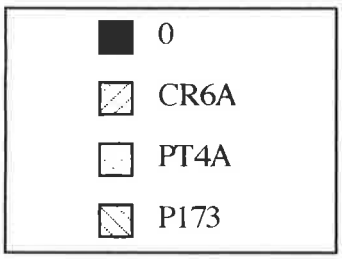
Fig. 6.7: Mean shoot weights of plants of cvs "Buffalo Queen" (BQ), "Goldsworthy" (RG), "Sword" (S), "Colossal" (C) and "Marone" (M) harvested 21 d after inoculation with either a sterile CMA plug (0) or a mycelial plug of *P. cinnamomi* (CR6A), *P. citricola* (PT4A) or *P. cryptogea* (P173). Shoot fresh weights were obtained immediately after harvest and shoots were placed at 105°C for 24 h before dry weights were obtained.

A: Mean shoot fresh weight. LSD (isolate) = 2.67. $P<0.01$.

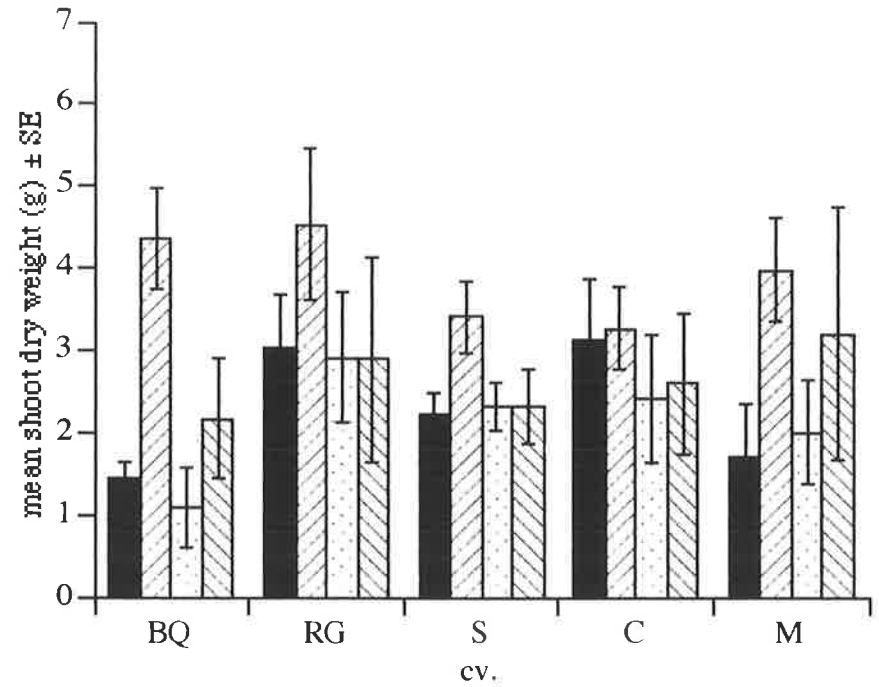
B: Mean shoot dry weight. LSD (isolate) = 0.88. $P<0.01$.

A





B



shoots of other cvs inoculated with *P. cinnamomi*, *P. citricola* or *P. cryptogea*. There was also no significant difference in the weights of shoots of all cvs inoculated with *P. cinnamomi*; likewise for all cvs inoculated with *P. citricola* and *P. cryptogea*.

Root weights also differed significantly between treatments, with fresh weights of the cvs "Buffalo Queen" and "Goldsworthy" inoculated with *P. cinnamomi* higher than those of the same cvs inoculated with *P. citricola*, *P. cryptogea* or sterile CHA plugs ($P<0.001$; Fig. 6.8A). There was no significant difference between root dry weights, however, for any treatment (Fig. 6.8B).

6.3.2 Inoculation of chestnut seedlings with zoospores of *Phytophthora* spp.

6.3.2.1 Drench method

No shoot symptoms were observed on plants 21 d after inoculation with *P. cinnamomi*, *P. citricola* or *P. cryptogea* and when plants were harvested no necrotic roots were evident on plants inoculated with zoospores or sterile soil extract. Results from this experiment are. therefore, not shown.

6.3.2.2 Dip method

All cvs of chestnut, inoculated with *P. cinnamomi*, *P. citricola* or *P. cryptogea*, showed some symptoms of infection during the course of this experiment, although there was a large amount of variation in response of seedlings to infection within a treatment (Fig. 6.9). Plants of the cvs "Goldsworthy" and "Buffalo Queen" inoculated with *P. cinnamomi* showed fewer symptoms after 21 d than other cvs inoculated with this isolate. Symptoms at 21 d of plants of the cvs "Colossal", "Sword" and "Goldsworthy" inoculated with *P. citricola* or *P. cryptogea* were generally similar to each other. Symptoms were similar to those observed on plants inoculated on the tap root with mycelium. Control plants inoculated with sterile soil extract, remained healthy throughout the experiment. Poor germination rates of seeds of the cv. "Sword" meant there were insufficient plants for adequate replication, therefore seedlings of this cv. were not inoculated with *P. cryptogea*.

Fig. 6.8: Mean root weights of plants of cvs "Buffalo Queen" (BQ), "Goldsworthy" (RG), "Sword" (S), "Colossal" (C) and "Marone" (M) harvested 21 d after inoculation with either a sterile CMA plug (0) or a mycelial plug of *P. cinnamomi* (CR6A), *P. citricola* (PT4A) or *P. cryptogea* (P173). Root fresh weights were obtained immediately after harvest and roots were placed at 105°C for 24 h before dry weights were obtained.

A: Mean root fresh weight. LSD (isolate) = 3.2. $P<0.001$.

B: Mean root dry weight. No significant differences.

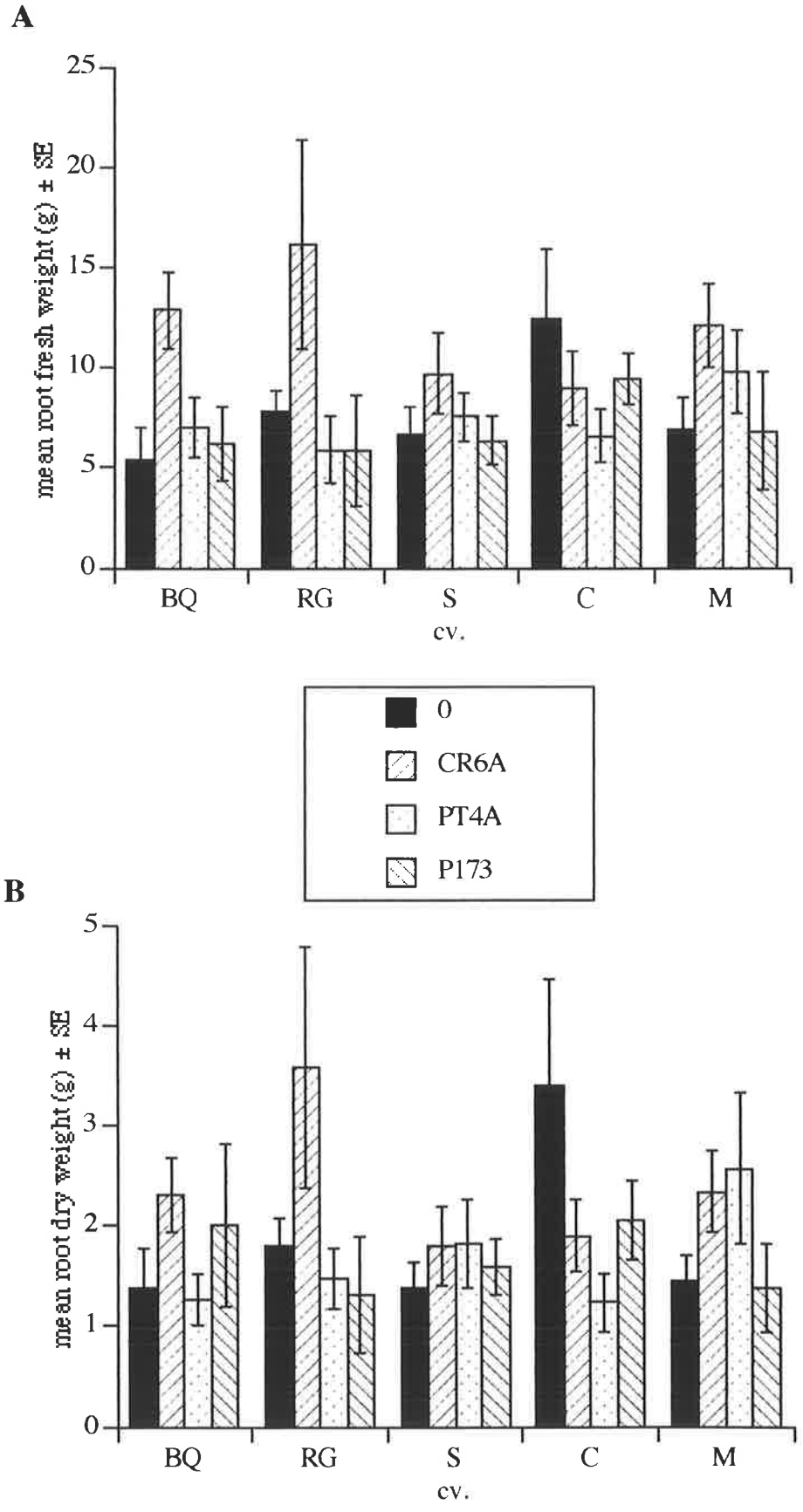
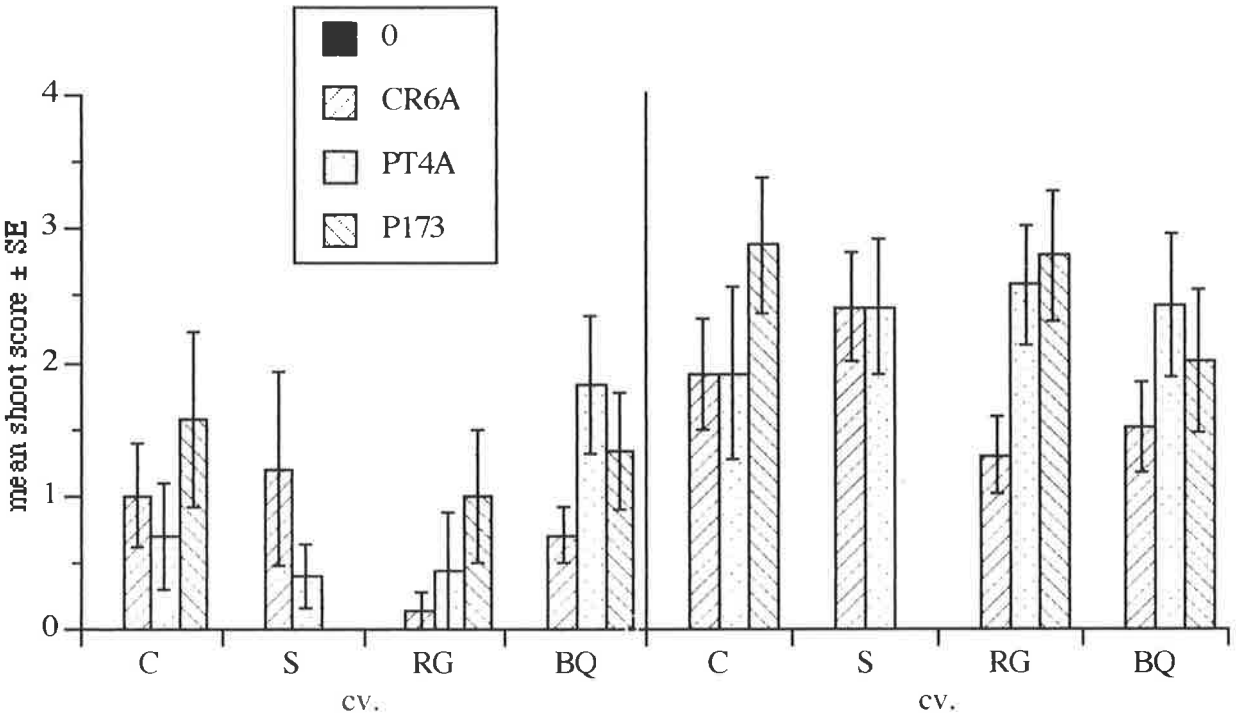
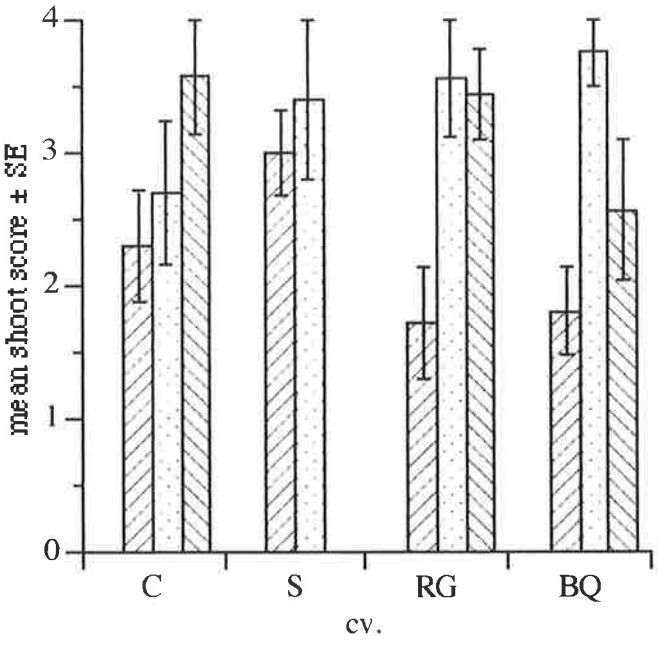

A
mean root fresh weight (g) ± SE
25
20
15
10
5
0
BQ
RG
S
C
M
cv.
0
CR6A
PT4A
P173
B
mean root dry weight (g) ± SE
5
4
3
2
1
0
BQ
RG
S
C
M
cv.

Fig. 6.9: Symptoms displayed by shoots of chestnut seedlings cvs "Buffalo Queen" (BQ), "Goldsworthy" (RG), "Sword" (S) and "Colossal" (C) , 7, 14 and 21 d after inoculation with either sterile soil extract (0) or zoospores of *P. cinnamomi* (CR6A), *P. citricola* (PT4A) or *P. cryptogea* (P173) using the zoospore dip method. The cv. "Sword" was not inoculated with *P. cryptogea* as insufficient seedlings were available. Shoots were scored according to a shoot symptoms index (table 6.1).

7 d = no significant differences, 14 d = no significant differences. LSD (isolate) 21 d = 1.38. $P<0.001$.



day 7 **day 14**



day 21

There was no significant difference between the susceptibility of tap or feeder roots of most cvs to infection (Fig. 6.10) and necrosis of roots of most cvs of chestnut inoculated with *P. cinnamomi*, *P. citricola* or *P. cryptogea* was similar. Tap and feeder roots of plants of all cvs inoculated with sterile soil extract remained healthy throughout the experiment. Roots of seedlings of the cv. "Buffalo Queen" displayed greater necrosis after inoculation with *P. citricola* than after inoculation with *P. cinnamomi* or *P. cryptogea*.

Shoot weights are shown in Fig. 6.11. Shoot fresh weights were generally significantly higher for plants inoculated with *P. cinnamomi* and sterile soil extract than for plants inoculated with *P. citricola* or *P. cryptogea* (Fig. 6.11A), with no significant difference between the weights of plants of cvs inoculated with *P. citricola* or *P. cryptogea*. Shoot dry weights followed a similar pattern to fresh weights for all cvs in all treatments (Fig. 6.11B). Shoots of seedlings of "Buffalo Queen" inoculated with *P. cinnamomi* weighed significantly more than plants of this cv. inoculated with *P. citricola* or *P. cryptogea.*

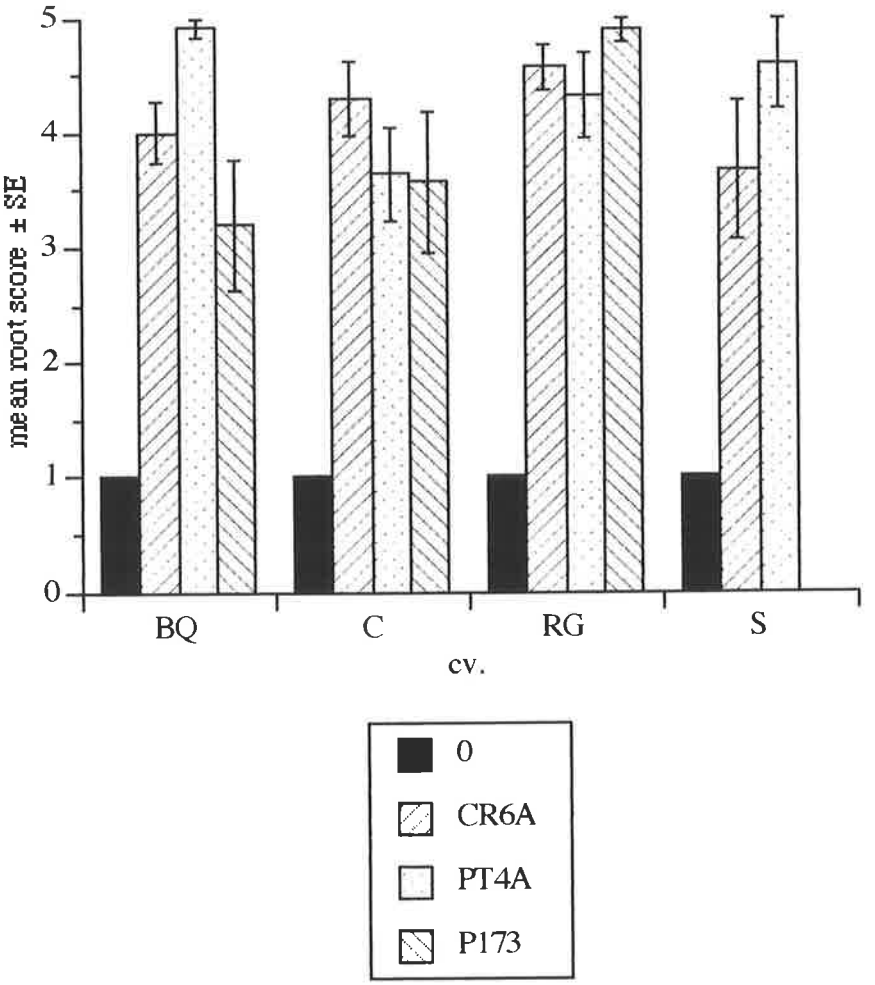
Root weights are shown in Fig. 6.12. Root fresh weights of plants of cvs from all treatments were generally similar (Fig. 6.12A), except for roots of the cvs "Colossal" and "Goldsworthy" inoculated with *P. citricola* and *P. cinnamomi* which weighed more than those inoculated with *P. cryptogea*. Root fresh weights of the cv. "Buffalo Queen" inoculated with *P. cinnamomi* were also higher than for seedlings of this cv. inoculated with *P. citricola* or *P. cryptogea*. Root dry weights were generally lower for plants of all cvs inoculated with *P. cryptogea* than those inoculated with *P. cinnamomi*, *P. citricola* or sterile soil extract (Fig. 6.12B) although this was not always statistically significant. Root dry weights of cvs "Goldsworthy" and "Colossal" inoculated with *P. citricola* had higher dry weights than those inoculated with *P. cinnamomi* or *P. cryptogea*. Root dry weights of all other treatments were generally similar.

Fig. 6.10: Mean root score for plants of cvs "Buffalo Queen" (BQ), "Goldsworthy" (RG), "Sword" (S) and "Colossal" (C), 21 d after inoculation with either sterile soil extract (0) or zoospores of *P. cinnamomi* (CR6A), *P. citricola* (PT4A) or *P. cryptogea* (P173) using the zoospore dip method. The cv. "Sword" was not inoculated with *P. cryptogea* as insufficient seedlings were available. Roots were scored according to a root rot index (table 6.2).

A: feeder roots. LSD (isolate x cv.) = 0.45. $P<0.05$.

B: tap root. LSD (isolate x cv.) = 0.95. $P<0.05$.

A


mean root score ± SE
0
1
2
3
4
5
BQ
C
RG
S
cv.
0
CR6A
PT4A
P173

B

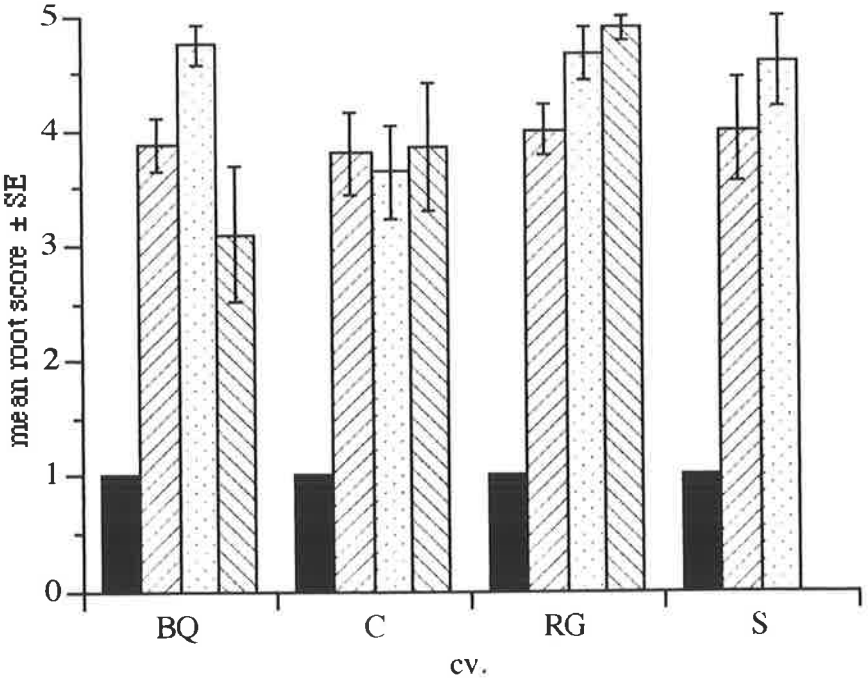
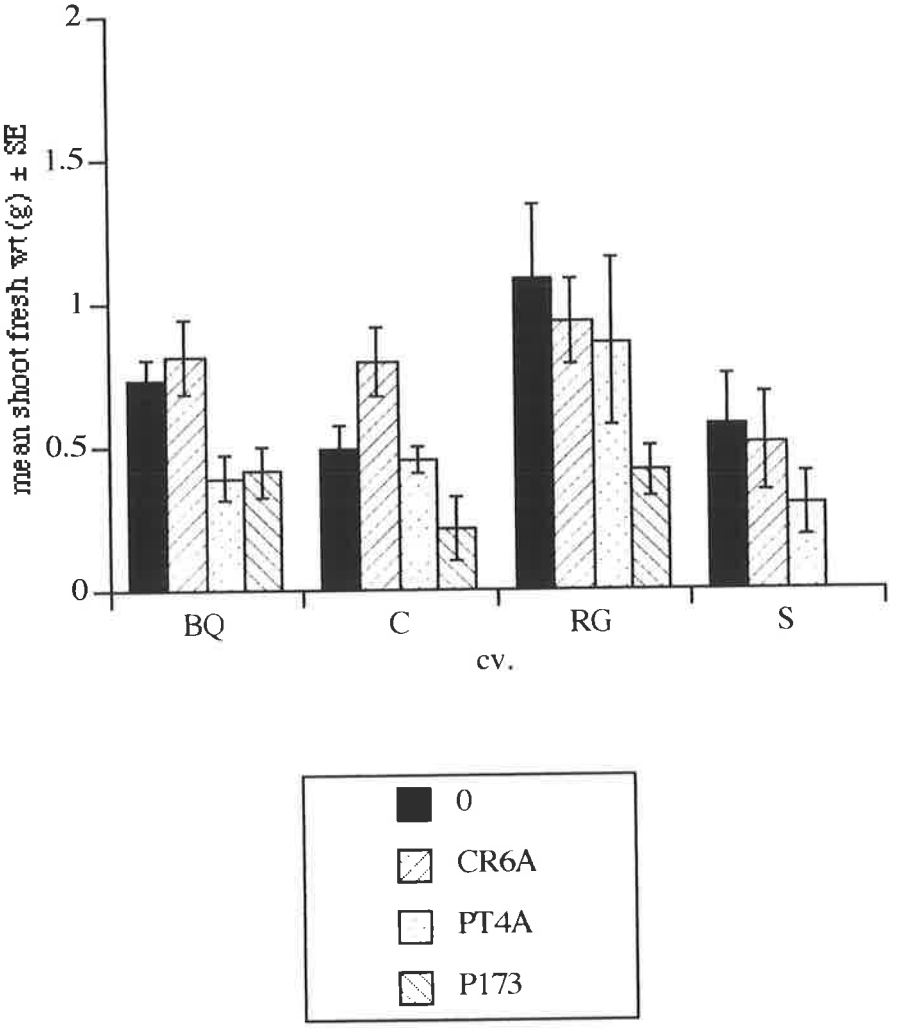

mean root score ± SE
0
1
2
3
4
5
BQ
C
RG
S
cv.

Fig. 6.11: Mean shoot weights of plants of cvs "Buffalo Queen" (BQ), "Goldsworthy" (RG), "Sword" (S) and "Colossal" (C) harvested 21 d after inoculation with either sterile soil extract (0) or zoospores of *P. cinnamomi* (CR6A), *P. citricola* (PT4A) or *P. cryptogea* (P173) using the zoospore dip method. The cv. "Sword" was not inoculated with *P. cryptogea* as insufficient seedlings were available. Shoot fresh weights were obtained immediately after harvest and shoots were placed at 105°C for 24 h before dry weights were obtained.

A: Mean shoot fresh weight. LSD (isolate) = 0.79. $P<0.001$.

B: Mean shoot dry weight. LSD (isolate) = 0.49. $P<0.001$.

A


mean shoot fresh wt (g) ± SE
2
1.5
1
0.5
0
BQ
C
RG
S
cv.
0
CR6A
PT4A
P173

B

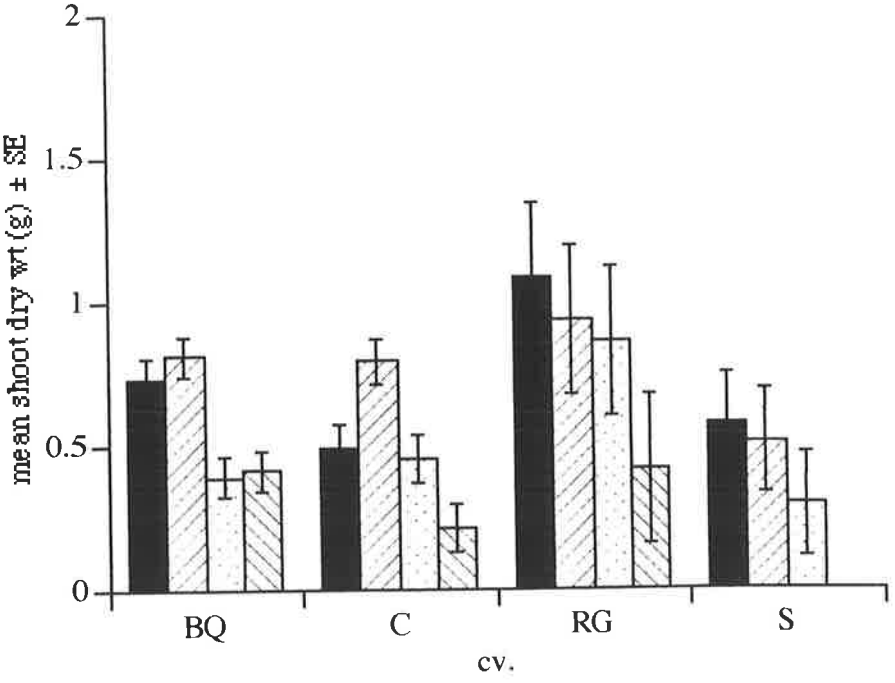
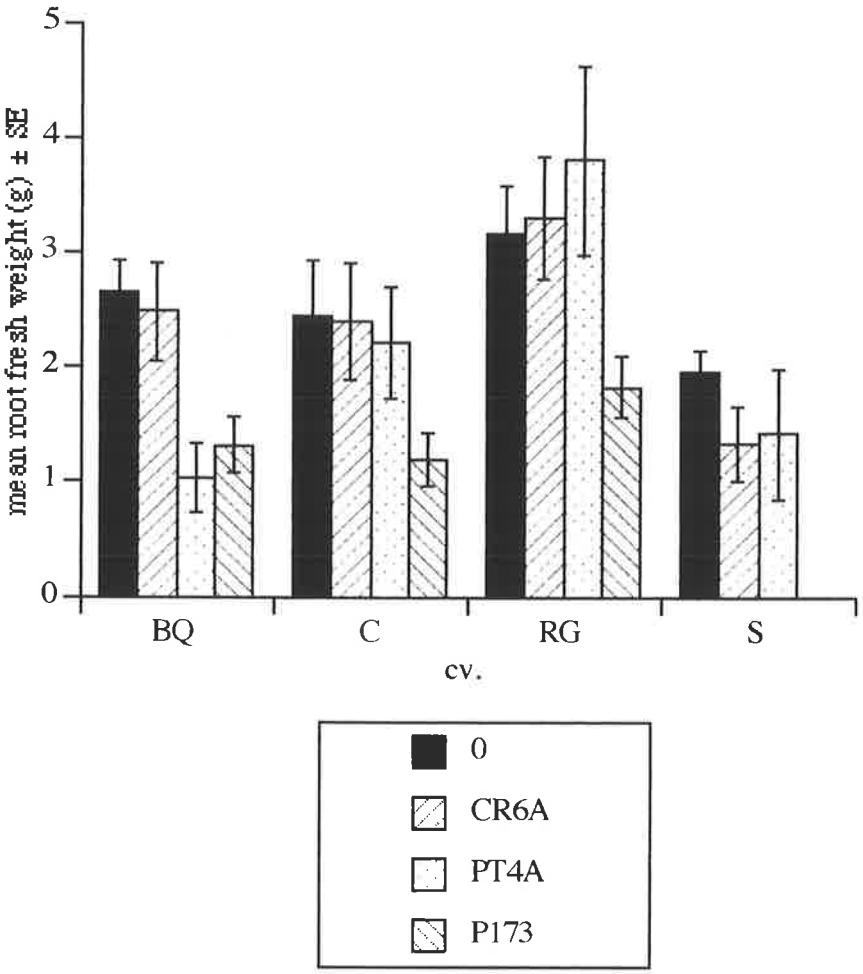

mean shoot dry wt (g) ± SE
2
1.5
1
0.5
0
BQ
C
RG
S
cv.

Fig. 6.12: Mean root weights of plants of cvs "Buffalo Queen" (BQ), "Goldsworthy" (RG), "Sword" (S) and "Colossal" (C) harvested 21 d after inoculation with either sterile soil extract (0) or zoospores of *P. cinnamomi* (CR6A), *P. citricola* (PT4A) or *P. cryptogea* (P173) using the zoospore dip method. The cv. "Sword" was not inoculated with *P. cryptogea* as insufficient seedlings were available. Root fresh weights were obtained immediately after harvest and roots were placed at 105°C for 24 h before dry weights were obtained.

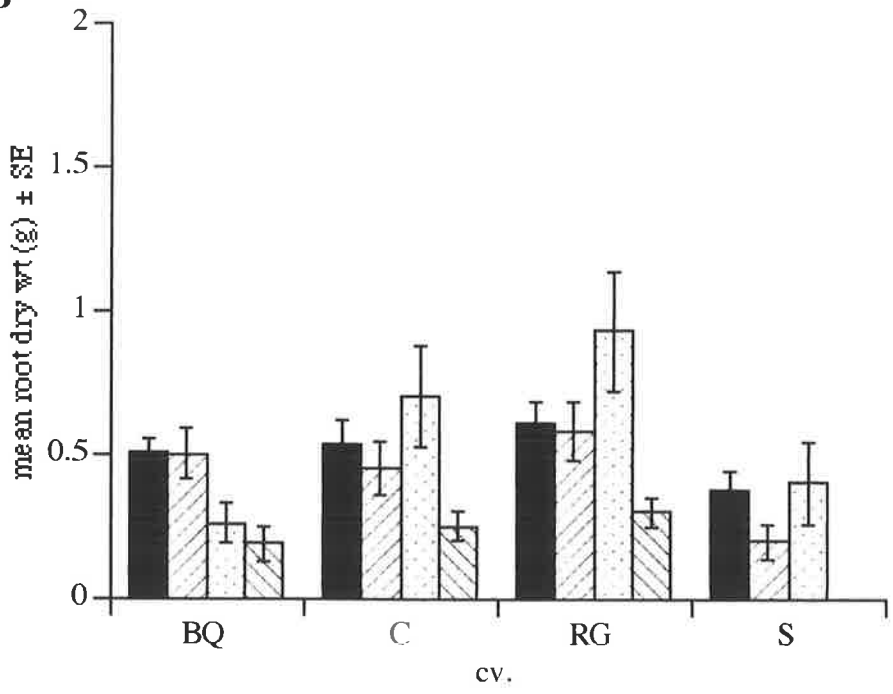
A: Mean root fresh weight. LSD (cv.) = 0.79, LSD (isolate) = 0.69. $P<0.001$.

B: Mean root dry weight. cv. LSD (cv.) = 0.61, LSD (isolate) = 0.14. $P<0.001$.

A


mean root fresh weight (g) ± SE
5
4
3
2
1
0
BQ
C
RG
S
cv.
0
CR6A
PT4A
P173

B


mean root dry wt (g) ± SE
2
1.5
1
0.5
0
BQ
C
RG
S
cv.

6.4 Discussion

Three methods of inoculation were used to screen chestnut cvs for resistance to *P. cinnamomi* (CR6A), *P. citricola* (PT4A) and *P. cryptogea* (P173). The first method involved inoculation of a wound made on the tap root with mycelium of *Phytophthora* spp.. Two other techniques involved inoculation of plants with zoospores, using either a zoospore drench or dip. Seedlings of all cvs were susceptible to *P. cinnamomi, P. citricola* or *P. cryptogea* when inoculated using the root dip method (with zoospores) or when inoculated on the tap root with mycelium, however no infection occurred on plants when they were inoculated using the zoospore drench method. Disease symptoms observed on infected plants in this study were similar to those observed previously on plants infected with *P. cinnamomi* and *P. cambivora* (Crandall *et al.*, 1945; Grente, 1961), with wilting, leaf chlorosis and lesion formation observed on stems of infected plants.

All cvs of chestnut were susceptible to *P. cinnamomi, P. citricola* and *P. cryptogea* 21 d after inoculation on the tap root with mycelium or using a zoospore dip in this study. Although plants of all treatments showed some symptoms of disease after inoculation, variation among seedlings was almost as great as variation between treatments, therefore no significant differences in susceptibility between cvs were consistently observed. This variation in response of seedlings to infection was also observed by Son (1992), when citrus plants were inoculated with *P. citrophthora* and was thought to reflect the field situation. Scott *et al.* (1992) also observed variation of response of almond trees to inoculation with *P. cambivora* in the orchard. Since all are outcrossing species, seedling material is evidently heterogeneous for response to *Phytophthora* diseases as well as other characteristics.

Excised shoots and plantlets of cvs "Goldsworthy" and "Buffalo Queen", growing *in vitro* and in vermiculite (section 4.3), were also susceptible to *P. cinnamomi, P. citricola* and *P. cryptogea* when inoculated with the fungus. The cvs "Colossal" and "Buffalo Queen" are thought to be *C. sativa* x *C. crenata* hybrids and, therefore, were expected to be less susceptible to *P. cinnamomi, P. citricola* and *P. cryptogea* than the *C. sativa* cvs "Sword",

"Marone" and "Goldsworthy" but this was not observed. Seedlings screened were up to 8 wk-old at the time of inoculation and, therefore, roots may not have developed secondary thickening. Juvenile material is thought to lack the structural and biochemical barriers to infection that are present in woody material (Scott et al., 1992). Older plants in glasshouse and field conditions need to be inoculated with *Phytophthora* spp. in order to determine whether these hybrid cvs show any resistance to this pathogen. It may be that these hybrid cvs are mostly *C. sativa*, therefore not displaying a resistant response. A wider range of hybrid cvs needs to be screened, as other genotypes may be resistant to infection.

Chestnut plants have a main tap root with feeder roots and the root rot index used in this experiment measured the susceptibility of these two different root types. There were no significant differences between the susceptibility of these tap and feeder roots for any cv. x pathogen interaction, with both root types equally susceptible to infection by *P. cinnamomi*, *P. citricola* or *P. cryptogea*. In contrast, Son (1992) found feeder roots of 10 wk-old citrus more susceptible than tap roots to infection by *P. citrophthora*. In that system, cells of the tap root were thought to be more difficult to penetrate than feeder roots due to suberization of hypodermal cells. The roots were also thought to have had better regenerative ability than feeder roots. The *P. citrophthora* isolate in that system may have been less aggressive than the isolates of *P. cinnamomi*, *P. citricola* or *P. cryptogea* used in this study. Alternatively, chestnut tap roots may lack the suberized hypodermal walls that citrus tap roots are thought to have, thus increasing their susceptibility to infection.

When the results of the zoospore-dip and tap root inoculation experiments were pooled, *P. cinnamomi*, *P. citricola* and *P. cryptogea* were found to have caused, in general, similar levels of disease on chestnut. Virulence, however, did not appear to be correlated with growth rate on CMA at 25°C: *P. cinnamomi* and *P. citricola* had similar growth rates after 7 d whereas *P. cryptogea* grew more slowly (section 4.3.3). In contrast, Robin (1991) found that isolates of *P. cinnamomi* from chestnut had similar growth rates *in vitro* but differed in

pathogenicity on 1 month-old seedlings inoculated with infested soil. Isolates from walnut and pine, however, had similar growth rates and aggressiveness.

Infection by *P. cinnamomi* caused less symptoms in the cvs tested than did *P. citricola* or *P. cryptogea* and plants inoculated with this isolate of P. cinnamomi grew better, in terms of fresh and dry weights of shoots and roots in the tap root inoculation experiment. However, in the zoospore dip inoculation experiment, this isolate of *P. cinnamomi* caused as much disease as did the other species tested. Further experiments are needed to determine whether there are consistent differences in virulence between the isolates. More extensive glasshouse experiments and field inoculation trials may reveal differences between these isolates: Scott *et al.* (1992) identified differences in the virulence of isolates of *P. cambivora* infecting almond in field inoculation experiments.

Modifications of the root inoculation technique could improve reliability, for example inoculation of a wound made on the collar with a plug of mycelium. Son (1992) used this method to inoculate citrus seedlings with *P. citrophthora* and differences in the susceptibility of seedlings of citrus rootstocks to an isolate of *P. citrophthora* were identified. She used a root dip method also and found the method less likely to discern subtle differences between susceptible and resistant cvs than was the collar inoculation technique. One advantage of the collar inoculation method over the root inoculation method used in experiments in this study, is that lesion extension from either side of the point of inoculation can be seen more easily than on inoculated roots.

Another method which has been used to detect differences in susceptibility of a number of plant species is the inoculation of branches of rootstock cvs with mycelium of *Phytophthora* spp.. This method was used to detect differences in the susceptibility of rootstocks of citrus to *P. citrophthora* (Afek & Sztejnberg, 1990) and *Banksia* spp. to *P. cinnamomi* (Dixon, Thinlay & Sivasithamparam, 1984). This method could easily be used for chestnut and may

reduce variation within a treatment in response to infection. It therefore warrants further investigation as a screening method for chestnut.

Both the tap root inoculation and zoospore dip inoculation methods detected the susceptibility of chestnut to species of *Phytophthora*, however, there were a number of advantages and disadvantages with each technique. The production of zoospores in sufficient quantities for infection in experiments in this study, was labour intensive and time-consuming in comparison with the production of mycelial inoculum. It was also difficult to produce zoospores of any one isolate consistently and reliably for use in experiments presented here. The tap root inoculation method would, therefore, be more useful in screening large numbers of cultivars for resistance to a number of isolates. This method has been used previously to screen seedlings of *C. sativa*, *C. crenata* and *C. mollissima* for resistance to *P. cinnamomi* (Crandall *et al*., 1945) and successfully distinguished *C. sativa* seedlings as susceptible and *C. mollissima* and *C. crenata* seedlings as resistant to *P. cinnamomi.* Infection by zoospores, however, is more comparable to that occurring in the field than is infection caused by mycelium. Quantification of mycelial inoculum is also difficult compared to zoospores which can be quantified readily.

The inoculation of plants using dip and drench methods with zoospores of *P. citrophthora* has also been used to screen citrus rootstocks for resistance to *P. citrophthora* (Son, 1992). The root-drenching method with zoospores was less effective at causing infection of citrus cvs than was the root-dipping method. Root-drenching also gave more variable results than did root-dipping. The application of zoospores using the root drench method failed to cause infection of chestnut plants in this study. Zoospores of this isolate of *P. cinnamomi*, applied to chestnut seedlings using the drench method, also failed to cause disease in other experiments in this study (section 5.3.7.1). An increase in the volume of zoospore suspension used as inoculum may have caused disease. Also, an alternative soil type or potting mix could be used (see section 5.4). This method of inoculation requires further evaluation before use in screening chestnut for resistance to *Phytophthora* spp..

The root-dip inoculation method and tap root inoculation method evaluated the response of chestnut to isolates of *P. cinnamomi*, *P. citricola* and *P. cryptogea* in experiments reported here. Further inoculation studies are needed and may identify small differences in the susceptibility or partial susceptibility of cvs to infection or differences in the virulence of isolates. The results of the experiments on seedlings in this study also need to be confirmed by inoculating older trees of each cv. with these isolates in pot or field experiments and assessing the response to infection.

7. PERIPHERAL VESICLES IN HYPHAE OF *P. CINNAMOMI* INFECTING CHESTNUT

7.1 Introduction

Soilborne *Phytophthora* spp. generally infect host plants by producing zoospores which encyst on plant roots and germinate to penetrate the plant (Weste & Marks, 1987). The ultrastructure of zoospores and cysts of *Phytophthora* spp. produced *in vitro* has been studied in detail (e.g. Ho *et al.*, 1968; Reichle, 1969; Sing & Bartnicki-Garcia, 1975 a & b). These researchers identified vesicles in the peripheral cytoplasm of the mature zoospore. Hardham *et al.* (1991) studied the ultrastructure of zoospores of *P. cinnamomi* and identified four types of vesicles in the peripheral cytoplasm: large peripheral vesicles; peripheral flattened cisternae; and two types of small vesicles, named ventral and dorsal vesicles.

Monoclonal antibodies (MAbs) were raised by Hardham *et al.* (1986) to localized, antigenic components on the surface of glutaraldehyde-fixed zoospores and cysts of an isolate of *P. cinnamomi* (6BR). Different MAbs reacted specifically with components of large peripheral vesicles, or ventral and dorsal vesicles and these MAbs have been used to elucidate the function of the vesicles. After encystment and wall formation, the large peripheral vesicles migrate to the centre of the cyst and are thought to act as nutrient sources that are degraded during subsequent cyst germination (Gubler & Hardham, 1988, 1990). The contents of the dorsal and ventral vesicles are released within 1-2 min of encystment: the contents of the ventral vesicles adhere the cyst to the root surface, whereas the contents of the dorsal vesicles coat the outer surface of the cyst. The function of the dorsal coat is unknown (Gubler & Hardham, 1991). Dearnaley and Hardham (pers. comm.) observed large peripheral vesicles and dorsal and ventral vesicles in hyphae of *P. cinnamomi* producing sporangia and chlamydospores *in vitro*. These vesicles were induced when vegetative hyphae were nutrient-stressed (see section 2.3.2).

The structural response of roots of different plant species to infection by species of *Phytophthora* has been examined by many authors (e.g. Malajczuk *et al.*, 1977; Miller & Maxwell, 1984 a & b; Palloix *et al.*, 1988 a & b). When susceptible seedlings of a number of Australian native plant species were infected by *P. cinnamomi*, lesions, sporangia and chlamydospores developed on the roots, shoots wilted and the plants eventually died (Weste & Cahill, 1982; Cahill & Weste, 1983; Cahill *et al.*, 1989).

Little is known, however, about the presence of vesicles in hyphae of *P. cinnamomi* during infection of plant roots. Large peripheral vesicles were observed in germinating cysts, but all were degraded by 4 h after inoculation of 5-7 d-old *Eucalyptus seiberi* roots with zoospores (Gubler & Hardham, 1990). The contents of dorsal and ventral vesicles were secreted within 2 min of encystment, with only a few ventral vesicles remaining in the cyst (Hardham & Gubler, 1990).

The time-course of infection of micropropagated chestnut plants by *P. cinnamomi* (isolate 6BR) was studied with the following aims:

(i) to examine hyphae of *P. cinnamomi* in infected chestnut roots, up to 4 d after inoculation with zoospores, for the presence of peripheral vesicles;

(ii) to determine whether the availability of nutrients affects the appearance of peripheral vesicles in hyphae in infected roots;

(iii) to determine whether there was a lag in the appearance of vesicles after initial infection, coinciding with the development of sporangia and chlamydospores.

7.2 Materials and Methods

7.2.1 Inoculation of entire root systems on plantlets

This preliminary experiment was designed to test the system of inoculation and infection.

Zoospores of *P. cinnamomi* (6BR) were produced using the method of Hardham *et al.* (1991) (see section 3.2). Plantlets of *C. sativa* "Goldsworthy" were produced as described in section 3.6. Micropropagated plants were used in the expectation that clonal material would allow a uniform response to be observed in infected material. Plantlets were transferred from 1/2 MS

medium containing 2 mg l^{-1} IBA (see section 3.6) to 1/2MS medium without IBA 10 d before each experiment was initiated. Plantlets were removed from agar medium, taking care not to damage roots, and the entire root system was immersed in a zoospore suspension (10^4 zoospores ml^{-1}) in sterile DW for 1 h. Plantlets were then placed on 10 ml of either 1/2MS or DW agar medium (7 g l^{-1} Difco Bitek agar), so that the roots were sitting on the agar surface, in 8 x 3 cm polycarbonate tubes (Disposable Products, South Australia). Control plants were inoculated with sterile distilled water. There were five replicate plantlets per treatment and they were incubated at 25°C under cool white fluorescent lights (approx. 35 μmol m^{-2} s^{-1}) with a 16 h day.

7.2.2 Harvest and labelling of infected roots of plantlets

Plantlets were harvested at d 0 (1 h after inoculation), d 1, d 2 and d 3. At each harvest one root from each treatment was cut into 5 mm pieces, from the tip back, and fixed in 4% paraformaldehyde (Polysciences Inc., Warrington, PA) in 50 mM PIPES buffer (pH 7.0) for 1 h at room temperature. Control roots, inoculated with sterile distilled water, were harvested and fixed as above. After fixation, roots were rinsed three times in 50 mM Pipes buffer, embedded in Tissue Tek OCT compound (Miles Inc., Elkhart, IN) in plastic moulds (Miles Inc., IN) and frozen by dipping in liquid nitrogen. Samples were stored at -18°C until sections were cut. Longitudinal sections 10 μm thick were cut on a Reichert-Jung 1800 Cryocut cryotome and dried on poly-L-lysine (Sigma) coated slides. Slides were placed on moistened filter paper in 12 cm^2 plastic boxes and at least six sections per treatment immunolabelled using the methods of Dearnaley & Hardham (pers. comm.).

For all treatments, sections were labelled for 45 min at 37°C with 1 μg ml^{-1} purified Vsv-1 antibody, 0.1 μg ml^{-1} purified Cpa-2 antibody or 1:10 Lpv-1 hybridoma supernatant in PBS-BSA (0.08 M $Na_2HPO_4.2H_2O$, 0.02 M $NaH_2PO_4.2H_2O$, 0.75 M NaCl, containing 1% BSA). Negative controls were root sections from all treatments stained with Mouse $IgG1_{,K}$ (Sigma). Sections were washed three times in PBS, then incubated as above with SAM-FITC (Silenus, Victoria) diluted 1:60 in PBS-BSA. Sections were then washed three times in PBS

and once in DW. They were then air dried and mounted in Mowiol (Hoechst) containing 1% PPD (Sigma). Positive controls were samples of sporulating hyphae grown *in vitro* in the absence of the host. These samples were obtained by cutting 2 mm^2 pieces of mycelium from the growing edge of a colony on V8 agar, placing them in a Petri dish and flooding them with V8 broth for 12 h, then rinsing three times with mineral salts solution (section 3.2) and incubating in the mineral salts solution for 8 h (Dearnaley, 1993). Pieces of agar with adhering mycelium were removed after 8 h and fixed, sectioned and stained as above. At least six labelled sections per treatment were examined and photographed using a Zeiss Axiophot photomicroscope with an F1 filter cube (excitation 450-490 nm: dichroic mirror 510 nm: barrier 515-565 nm).

7.2.3 Inoculation of root tips on plantlets

Zoospores were produced by a method modified from Gees & Coffey (1989) (see section 3.2) and suspended in sterile soil extract. Plantlets, produced as described in section 3.6, were placed flat on the surface of 10 ml DW agar in a Petri dish (100 mm x 20 mm, Surgical and Medical, South Australia) (Fig. 7.1). Root tips were inoculated with 10 μl of zoospore suspension (10^4 zoospores ml^{-1}) while control roots were inoculated with 10 μl sterile soil extract. There were ten replicate plants for each treatment. Inoculated plants and controls were incubated under cool white fluorescent lights (approx. 35 μmol m^{-2} s^{-1}) with a 16 h day at 25°C and roots were harvested on d 0 - d 3, as above, and also on d 4. Three roots per day were harvested from 2 plants, cleared in 10% KOH for 1.5 h at 80°C, bleached for 2-3 min in 1N HCl, then stained for 25 min in 0.05% Trypan blue in lactoglycerol (Phillips & Hayman, 1970). Another three roots per day were harvested, cut into 5 mm pieces from the tip back, as far as the lesion extended, and fixed, sectioned and immunolabelled with antibodies as described in section 7.2.2.

Fig. 7.1: Chestnut "Goldsworthy" plantlet 2d after inoculation of root tips with zoospores of *P. cinnamomi*.

Plantlets were placed flat on DW agar in a Petri dish and 10 μl of a zoospore suspension (10^4 zoospores ml^{-1}) was placed on each root tip. Plantlets were incubated under cool white fluorescent lights (approx. 35 μmol s^{-1} m^{-2})with a 16 h day at 25°C. The arrows show lesion development on roots. Bar = 12 mm.

7.3 Results

7.3.1 Inoculation of entire root systems on plantlets

Lesions appeared on the entire root surface of plantlets one day after inoculation with zoospores. Hyphae of *P. cinnamomi* grew out from inoculated roots on all replicates; growth was dense on the surface of the 1/2MS medium (with 30 g l^{-1} sucrose), but sparse on DW agar. When sections, labelled with MAbs, were viewed using fluorescence microscopy, large peripheral vesicles were labelled in hyphae along most of the roots of plantlets incubated on DW agar at d 1, whereas there was no labelling in hyphae in roots from 1/2MS medium (see Table 7.1 a & b). At d 2 and d 3 harvests, large peripheral vesicles were labelled in hyphae along the entire root of plantlets incubated on DW agar, whereas vesicles were only labelled in hyphae 5-10 mm behind the tip of roots of plantlets incubated on 1/2MS medium.

Ventral and dorsal vesicles were not labelled in hyphae infecting roots harvested from DW agar after 1 d, but were labelled in hyphae in roots harvested after 2 and 3 d. Dorsal vesicles were labelled in hyphae in all root sections 10-15 mm behind the tip after 2 d and 15-20 mm behind the tip after 3 d. Dorsal vesicles were only labelled 15-20 mm behind the root tip in roots harvested from 1/2MS medium after 3 d. Ventral vesicles were labelled in hyphae 15-20 mm behind the root tip after 2 and 3 d on DW agar but were not labelled in hyphae in any roots harvested at d 0 - 3 from 1/2MS medium.

7.3.2 Inoculation of root tips on plantlets

Dark brown lesions were clearly visible on inoculated roots as early as 1 d after inoculation with zoospores of *P. cinnamomi* (Fig. 7.1). These lesions spread as the fungus grew along the root. Control plants remained healthy throughout the experiment (Fig. 7.2.1). Descriptions of plant symptoms after inoculation and fungal development on roots are given in Table 7.2.

Large peripheral, dorsal and ventral vesicles were labelled in germinating cysts at the root tip in two of the replicates at d 0 (Tables 7.3, 7.4 & 7.5), however they were not labelled in any

Table 7.1: Presence of large peripheral vesicles in hyphae infecting roots after dipping in a zoospore suspension for 1 h and then incubating on DW or 1/2MS agar for up to 3 d. Roots were cut into 5 mm pieces, sectioned, labelled with the monoclonal antibody Lpv-1 and SAM-FITC, and viewed using fluorescence microscopy. One root was sectioned per treatment. Controls were roots inoculated with sterile soil extract. + denotes presence, - denotes absence of peripheral vesicles.

Table 7.1a: DW agar medium.

root section (mm) behind tip	control	d 0	d 1	d 2	d 3
0 - 5	-	-	-	+	+
5 - 10	-	-	+	+	+
10 - 15	-	-	+	+	+
15 - 20	-	+	+	+	+

Table 7.1b: 1/2MS agar medium

root section (mm) behind tip	control	d 0	d 1	d 2	d 3
0 - 5	-	-	-	-	-
5 - 10	-	-	-	+	+
10 - 15	-	-	-	-	-
15 - 20	-	-	-	-	-

Figs 7.2.1 - 7.2.6: Infection of chestnut "Goldsworthy" roots up to 4 d after inoculation of root tips with zoospores of *P. cinnamomi*.

Micropropagated chestnut plantlets were placed flat on the surface of DW agar in Petri dishes. Root tips were inoculated with 10 μl of a zoospore suspension (10^4 zoospores ml^{-1}) or sterile soil extract and the plantlets were incubated under cool white fluorescent lights (approx. 35 μmol s^{-1} m^{-2}) with a 16 h day at 25 °C for up to 4 d. Roots were harvested at 0 d (1 h after inoculation),1 d, 2 d, 3 d and 4 d after inoculation, and control roots were harvested also. Roots were cleared in 10% KOH for 1.5 h at 80°C, bleached in 1N HCl for 2-3 min, then stained in 0.05% Trypan blue in lactoglycerol for 25 min. Roots were then squash mounted in lactoglycerol and viewed using light microscopy.

Fig. 7.2.1: Healthy control root at 4 d inoculated with sterile soil extract. Root hairs (arrowed) were abundant over the surface of the root. Bar = 300 μm.

Fig. 7.2.2: Hyphal front (hyphal tips arrowed) on 2 d-infected root. Root hairs indicated by large arrow. Bar = 75 μm.

Fig. 7.2.3: Hyphae growing over the entire root surface 3 d after inoculation with zoospores (arrowed). Bar = 150 μm.

Fig. 7.2.4: Sporangium on the edge of a root 2 d after inoculation with zoospores. Cleavage of contents into zoospores is evident (arrowed). Bar = 100 μm.

Fig. 7.2.5: Empty sporangium (arrowed) on infected root 4 d after inoculation with zoospores. Bar = 50 μm.

Fig. 7.2.6: Chlamydospores (arrowed) in cells of root 4 d after inoculation with zoospores. Bar = 50 μm.

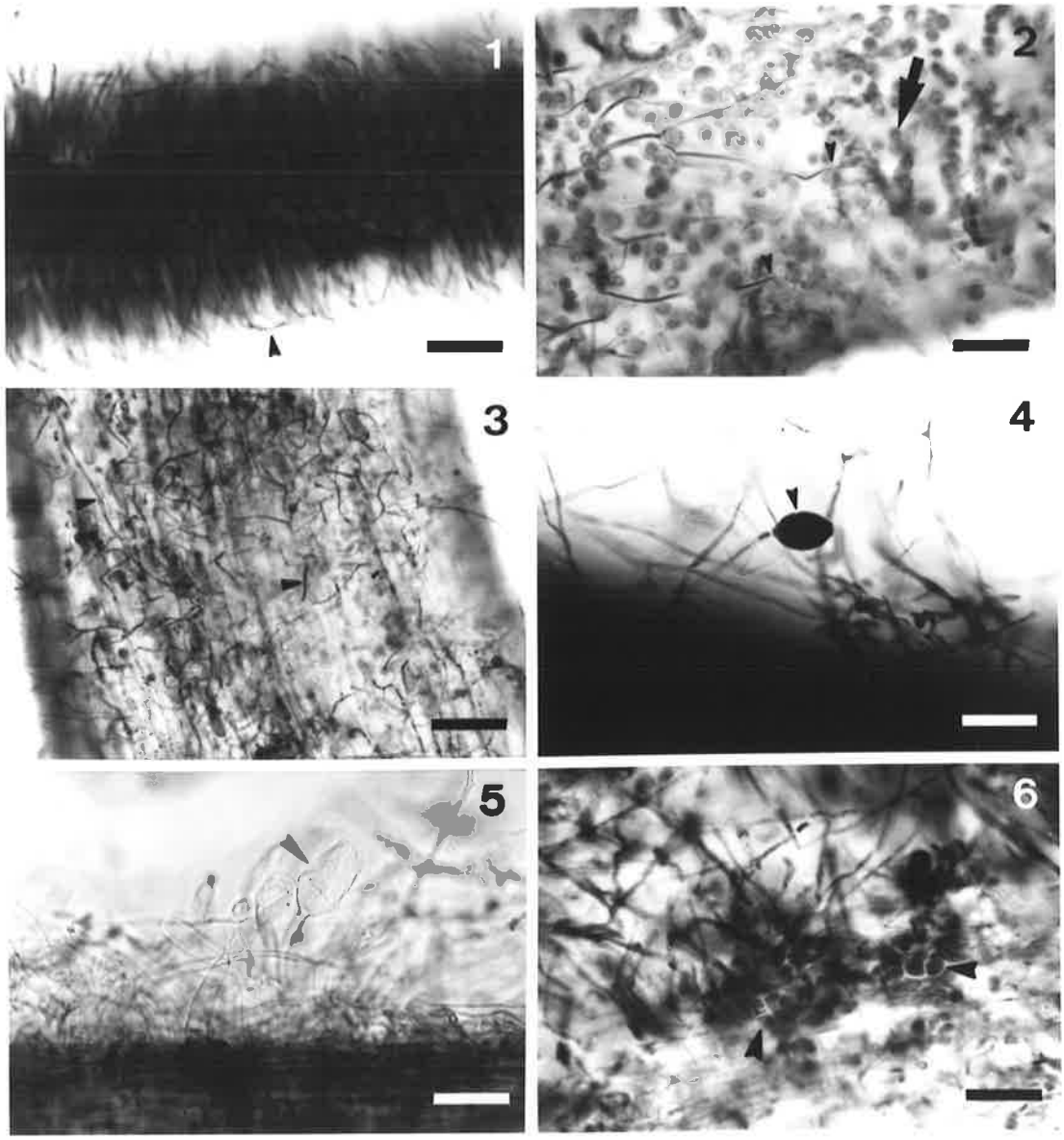

1
2
3
4
5
6

Table 7.2: Symptoms displayed by micropropagated chestnut inoculated with *P. cinnamomi*. Symptoms displayed over 4 d by micropropagated plantlets of chestnut "Goldsworthy" inoculated on the root tip with 10 μl of a zoospore suspension and subsequently cleared with 10% KOH, bleached for 2-3 min in 1N HCl and stained for 25 min with 0.05% Trypan Blue in lactoglycerol. Control plants were inoculated with sterile soil extract and remained healthy throughout the experiment.

d	symptoms displayed by plantlets. n = 2 per treatment.
0	All plantlets healthy
1	Small lesions along roots (approx. 10 mm). No shoot symptoms. Sparse hyphal growth over the surface of the root. A few hyphal lobes visible.
2	Lesions to 20 mm. No symptoms visible on shoots. Hyphal growth thicker over root. First chlamydospores visible in cells near the root tip and on the surface of the root. First sporangia observed near the root tip.
3	Lesions over entire roots of plantlets, some lesions on stems. Shoots chlorotic. Hyphae growing thickly over the entire root. Hyphal lobes, chlamydospores and sporangia common.
4	Plantlets dead. Entire root and shoot necrotic. Many mature and discharged sporangia on root surface. Chlamydospores abundant.

Table 7.3: Presence of large peripheral vesicles in hyphae along roots up to 4 d after inoculation of root tips of "Goldsworthy" plantlets with zoospores.

Root pieces approx. 5 mm long were sectioned, stained with the monoclonal antibody Lpv-1 and SAM-FITC, then viewed using fluorescence microscopy. Three roots were sectioned per treatment, except where roots were short. Sections were cut as far as lesions extended along roots harvested at d 1-4. Controls were roots inoculated with sterile soil extract. + denotes presence, - denotes absence of large peripheral vesicles in each of the three replicates per treatment.

root section (mm)	control	d 0	d 1	d 2	d 3	d 4
0 - 5	- - -	- + +	- - -	+ + +	+ + +	+ + +
5 - 10	- - -	- - -	- -+	+ + +	+ + +	+ + +
10 - 15	- - -	- - -	- - -	+ + +	+ + +	+ + +
15 - 20	- - -	- - -	- - -	- + +	+ + +	+ + +
20 - 25		-	- -	- - +	+ + +	+ + +
25 - 30				- -	+ + +	+ + +
30 - 35					+ + +	+ + +
35 - 40					+ +	+
40 - 45					+ +	+
45- 50					+ +	

Table 7.4: Presence of ventral vesicles in hyphae along roots up to 4 d after inoculation of root tips of "Goldsworthy" plantlets with zoospores.

Root pieces approx. 5 mm long were sectioned, labelled with the monoclonal antibody Vsv-1 and SAM-FITC, then viewed using fluorescence microscopy. Three roots were sectioned per treatment, except where roots were short. Sections were cut as far as lesions extended along roots harvested at d 1-4. Controls were roots inoculated with sterile root extract. + denotes presence, - denotes absence of ventral vesicles in each of the three replicates per treatment.

root section (mm)	control	d 0	d 1	d 2	d 3	d 4
0 - 5	- - -	- + +	- - -	+ + +	- + +	+ + +
5 - 10	- - -	- - -	- - +	+ + +	- + +	+ + +
10 - 15	- - -	- - -	- - -	- + +	+ + +	+ + +
15 - 20	- - -	- - -	- - -	- - -	+ + +	- + +
20 - 25		-	- -	- - -	- + +	- + +
25 - 30				- -	- + +	- + +
30 - 35					- + +	- - -
35 - 40					- +	-
40 - 45					- -	-
45 - 50					- -	

Table 7.5: Presence of dorsal vesicles in hyphae along roots up to 4 d after inoculation of root tips of "Goldsworthy" plantlets with zoospores.

Root pieces (approx. 5 mm long) were sectioned, labelled with the monoclonal antibody Cpa-2, SAM-FITC, then viewed using fluorescence microscopy. Three roots were sectioned per treatment, except where roots were short. Sections were cut as far as lesions extended along roots harvested at d 1-4. Controls were roots inoculated with sterile soil extract. + denotes presence, - denotes absence of dorsal vesicles in each of the three replicates per treatment.

root section (mm)	control	d 0	d 1	d 2	d 3	d 4
0 - 5	- - -	- + +	- - -	+ + +	- + +	+ + +
5 - 10	- - -	- - -	- - +	+ + +	+ + +	+ + +
10 - 15	- - -	- - -	- - -	+ + +	+ + +	+ + +
15 - 20	- - -	- - -	- - -	- - -	- + +	+ + +
20 - 25			- -	- - -	- + +	+ + +
25 - 30				- -	- + +	+ + +
30 - 35					- + +	- - +
35 - 40					- - -	-
40 - 45					- - -	-
45 - 50					- -	

other root piece. Hyphae had not grown through these pieces 1 h after inoculation of zoospores at the tip. All plantlets appeared healthy at this harvest.

After 1 d, lesions extended to 15-20 mm behind the tip. Hyphae, sometimes lobed, were observed growing sparsely over the surface of the root (Table 7.2). Large peripheral, dorsal and ventral vesicles were labelled in hyphae, however this was only 5-10 mm behind the tip of one of the root pieces sectioned (Tables 7.3, 7.4 & 7.5). There was a lag in the appearance of labelled vesicles in hyphae behind the growing front.

After 2 d, lesions extended to approx. 30 mm behind the tip and hyphae grew more densely on roots (Table 7.2; Fig. 7.2.2). Large peripheral vesicles were labelled in hyphae to approx. 20-25 mm behind the tip and dorsal and ventral vesicles to approx. 15 mm (Tables 7.3, 7.4 & 7.5). The labelling of peripheral vesicles at d 2 corresponded with the first appearance of chlamydospores and sporangia 5-10 mm behind the root tip (Table 7.2). There was a lag in the appearance of labelling of the three vesicle types. This was more pronounced for dorsal and ventral vesicles than for large peripheral vesicles.

Lesions extended along the entire root system 3 d after inoculation and hyphal lobes, sporangia and chlamydospores were common (Table 7.2). Large peripheral vesicles were labelled in hyphae along the whole length of the root, whereas dorsal and ventral vesicles were labelled only as far as 35 and 40 mm from the tip, respectively (Tables 7.3, 7.4 & 7.5; Figs 7.2.3, 7.3.3 & 7.4.3 respectively). Lesions extended along the entire root and shoot systems 4 d after inoculation and plants were dead. Large peripheral vesicles were again labelled along the entire root while dorsal and ventral vesicles were still labelled only to 30-35 mm and 15-30 mm along the root respectively (Tables 7.3, 7.4 & 7.5). Many developing and discharged sporangia, and chlamydospores were observed at this stage (Figs 7.2.5 & 7.2.6). Large peripheral vesicles, dorsal and ventral vesicles were labelled in sporangia and chlamydospores (Figs 7.3.4, 7.3.5, 7.3.6, 7.4.4 & 7.5.4).

Figs 7.3.1 - 7.3.6: Large peripheral vesicles in hyphae of *P. cinnamomi* infecting roots of micropropagated chestnut plantlet "Goldsworthy".

Root tips were inoculated with 10 μl of a zoospore suspension (10^4 zoospores ml^{-1}) or sterile soil extract (controls). Plantlets were incubated at 25°C under cool white fluorescent lights (approx. 35 μmol s^{-1} m^{-2}) with a 16 h day for up to 4 d. Roots were harvested, cut into 5 mm long sections, fixed, and embedded in Tissue Tek OCT compound. Longitudinal sections were cut, 10 μm thick, and labelled with the monoclonal antibody Lpv-1 or Mouse IgG1,k (controls) and with SAM-FITC, then mounted and viewed using fluorescence microscopy (excitation 450-490 nm).

Fig. 7.3.1: Section of control root (inoculated with sterile soil extract) labelled with Lpv-1 and SAM-FITC. There was no binding of the antibody to antigens in the root. Bar = 100 μm.

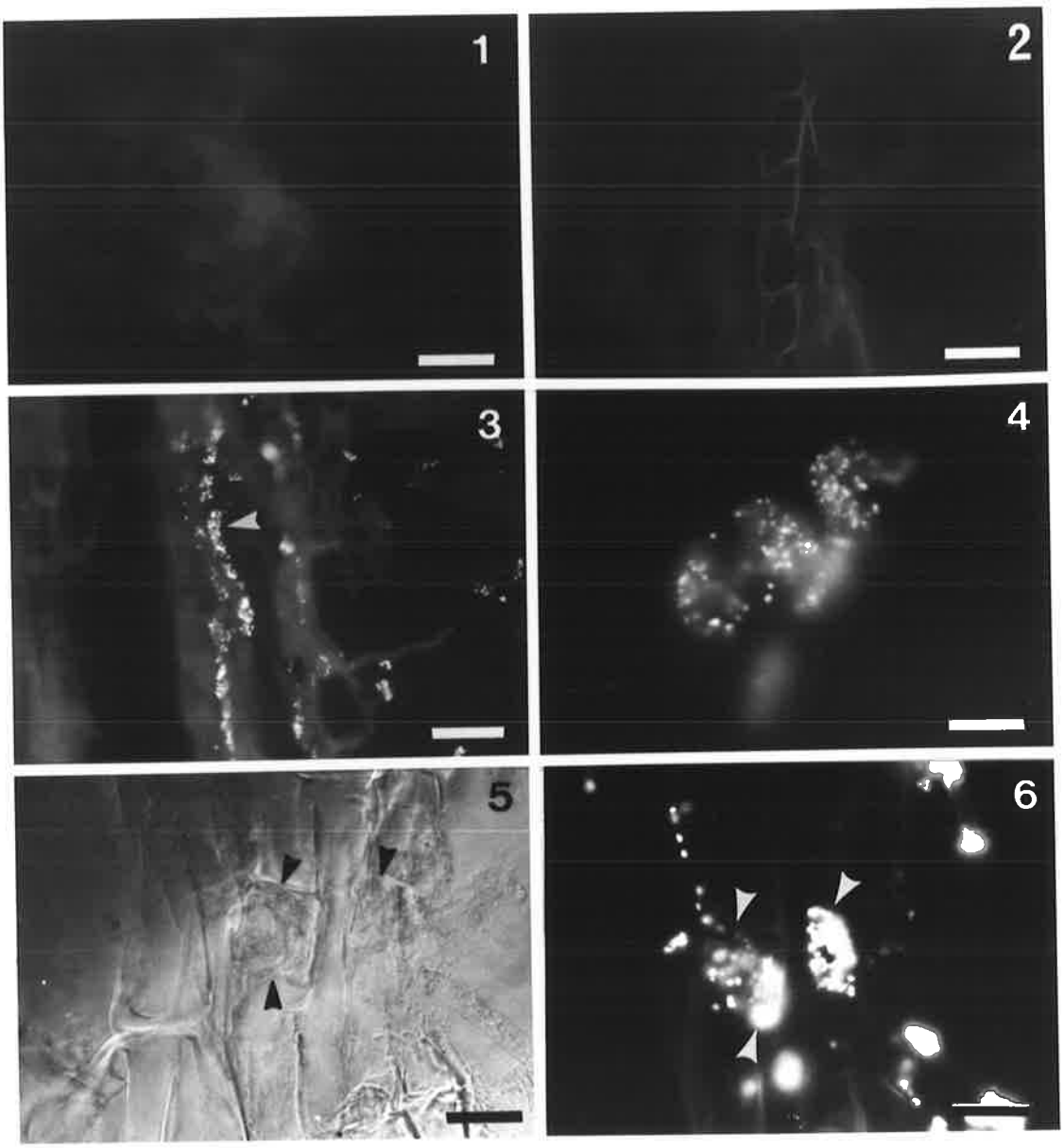
Fig. 7.3.2: Section of inoculated root stained with Mouse IgG1,k (for control) showing no labelling of large peripheral vesicles. Bar = 100 μm.

Fig. 7.3.3: Labelling of large peripheral vesicles in hyphae (arrowed) infecting root 4 d after inoculation. Bar = 100 μm.

Fig. 7.3.4: Large peripheral vesicles in sporangium on a root 2 d after inoculation. Bar = 12.5 μm.

Fig. 7.3.5: Differential interference contrast (DIC) micrograph of hyphae and chlamydospores (arrowed) in root cells. Bar = 30 μm.

Fig. 7.3.6: Large peripheral vesicles in hyphae and chlamydospores (arrowed). Bar = 30 μm.


1
2
3
4
5
6

Figs 7.4.1 - 7.4.4. Dorsal vesicles in hyphae of *P. cinnamomi* infecting roots of micropropagated chestnut.

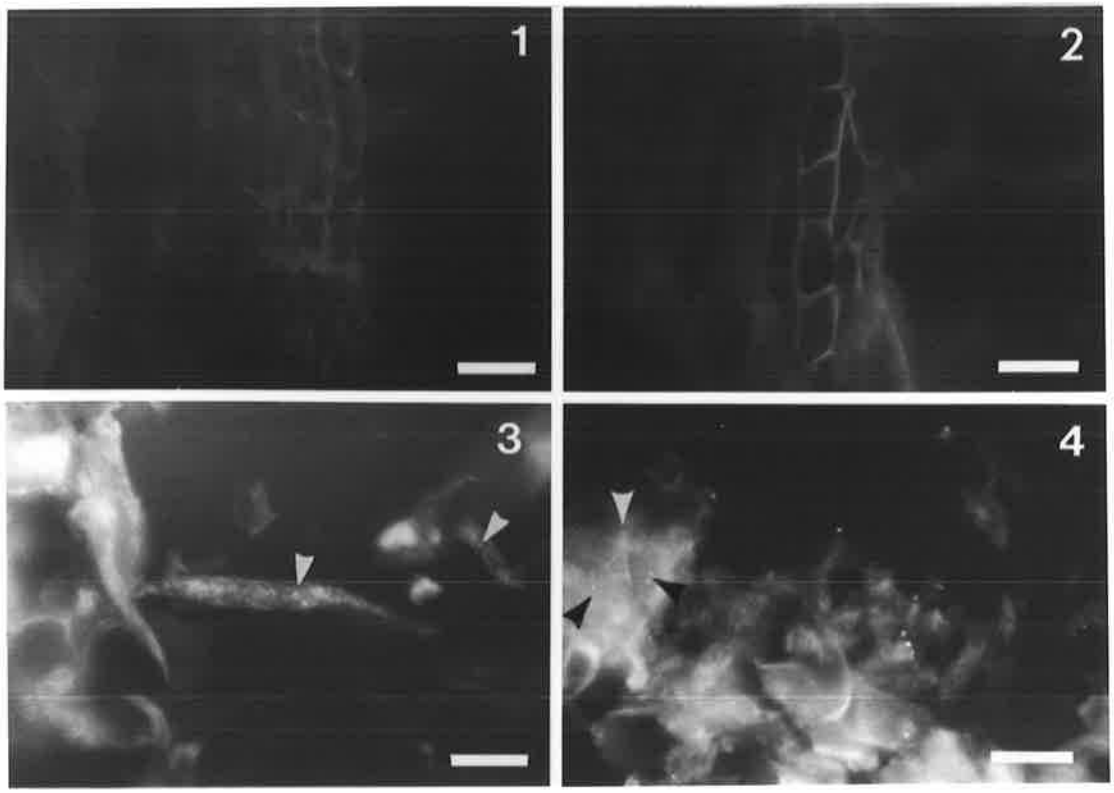
Root tips were inoculated with 10 μl of a zoospore suspension (10^4 zoospores ml^{-1}) or sterile soil extract (controls). Plantlets were incubated at 25°C under cool white fluorescent lights (approx. 35 μmol s^{-1} m^{-2}) with a 16 h day for up to 4 d. Roots were harvested, cut into 5 mm long sections, fixed, and embedded in Tissue Tek OCT compound. Longitudinal sections were cut, 10 μm thick, and labelled with the monoclonal antibody Cpa-2 or Mouse IgG1,k (controls) and with SAM-FITC, then mounted and viewed using fluorescence microscopy (excitation 450-490 nm).

Fig. 7.4.1: Section of control root (inoculated with sterile soil extract) labelled with Cpa-2. There was no binding of the antibody to antigens in the root. Bar = 125 μm.

Fig. 7.4.2: Section of inoculated root labelled with Mouse IgG1,k (for control). There was no labelling of dorsal vesicles in hyphae. Bar = 100 μm.

Fig. 7.4.3: Dorsal vesicles in hyphae (arrowed) 4 d after inoculation of chestnut roots. Bar = 25 μm.

Fig. 7.4.4: Dorsal vesicles in chlamydospores (arrowed) formed on infected root. Bar = 30 μm.


1
2
3
4

Figs 7.5.1 - 7.5.4: Ventral vesicles in hyphae of *P. cinnamomi* infecting roots of micropropagated chestnut "Goldsworthy".

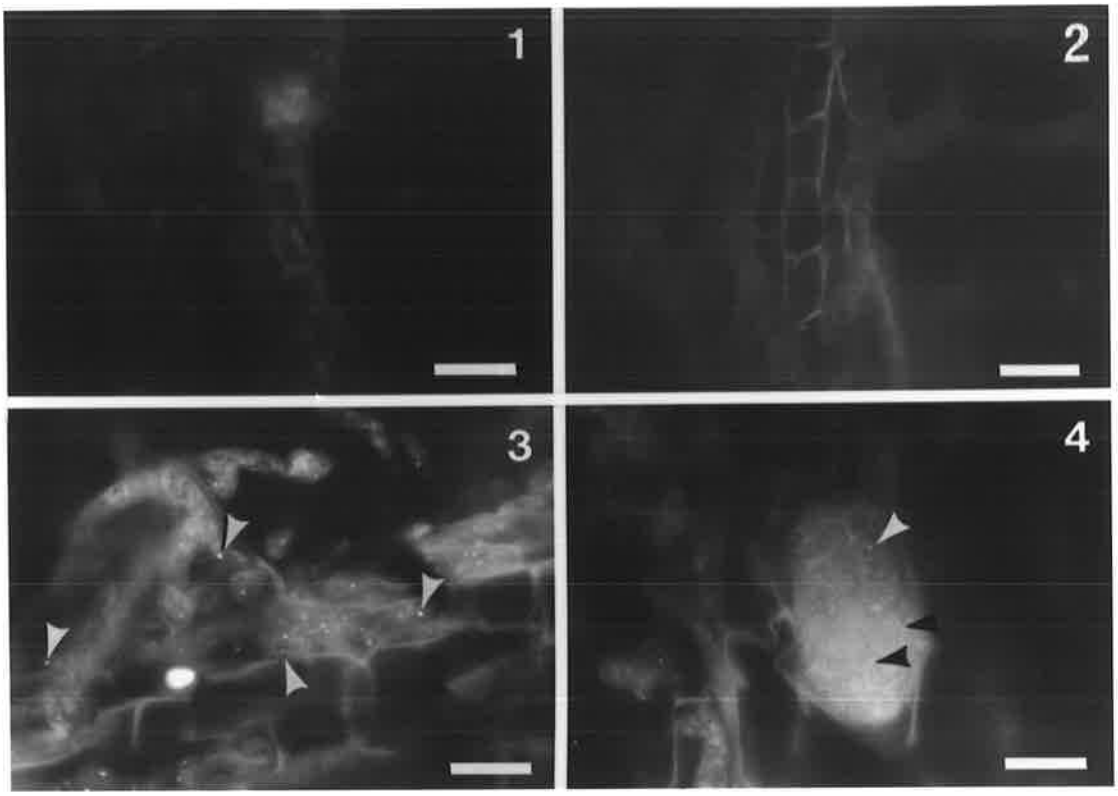
Root tips were inoculated with 10 μl of a zoospore suspension (10^4 zoospores ml^{-1}) or sterile soil extract (controls). Plantlets were incubated at 25°C under cool white fluorescent lights (approx. 35 μmol s^{-1} m^{-2}) with a 16 h day for up to 4 d. Roots were harvested, cut into 5 mm long sections, fixed, and embedded in Tissue Tek OCT compound. Longitudinal sections were cut, 10 μm thick, and labelled with the monoclonal antibody Vsv-1 or Mouse IgG1,k (controls) and with SAM-FITC, then mounted and viewed using fluorescence microscopy (excitation 450-490 nm).

Fig. 7.5.1: Section of control root (inoculated with sterile soil extract) labelled with Vsv-1. There was no binding of the antibody to antigens in the root. Bar = 100 μm.

Fig. 7.5.2: Section of inoculated root labelled with Mouse IgG1,k (for control). There was no labelling of ventral vesicles in hyphae. Bar = 100 μm.

Fig. 7.5.3: Ventral vesicles in hyphae (arrowed) 4 d after inoculation of chestnut roots. Bar = 30 μm.

Fig. 7.5.4: Ventral vesicles in chlamydospores (arrowed) formed on infected root. Bar = 10 μm.


1
2
3
4

There was no labelling of sections cut from roots inoculated with sterile soil extract and stained with Lpv-1, Cpa-2 and Vsv-1, indicating that there was no binding of the antibody to antigens in the root (Fig 7.3.1, 7.4.1 and 7.5.1). There was, likewise, no labelling of sections stained with Mouse IgG1,κ (Figs 7.3.2, 7.4.2 & 7.5.2).

7.4 Discussion

In this study, infection of micropropagated chestnut from zoospore inoculum of *P. cinnamomi* was followed. Hyphae grew quickly over the entire root, killing the plantlets within 4 d of inoculation. Chlamydospore and sporangium production occurred 1-2 d after inoculation. Peripheral vesicles were labelled in hyphae infecting chestnut roots from 1 d after inoculation, corresponding with the induction of sporogenesis.

The present study documents, for the first time, the production of peripheral vesicles of *P. cinnamomi* in hyphae infecting plant roots. Peripheral vesicles were labelled in hyphae infecting roots after incubation for 1d on DW agar, but were not labelled in hyphae infecting roots incubated on 1/2MS medium. This suggested that hyphae could obtain sufficient nutrients to maintain vegetative growth because of the high nutrient status of 1/2MS medium (containing 30 g l^{-1} sucrose) and so chlamydospores and sporangia were not produced. Hyphae infecting roots incubated on DW agar, however, may have exhausted nutrients quickly and so peripheral vesicles might have been expected to occur in hyphae infecting these roots. Because the entire root inoculation experiment was an initial experiment, only one root per sample was examined for vesicle production. This showed that vesicles were present in hyphae infecting some root pieces. Dorsal and ventral vesicles, which were present in roots incubated on DW only in a few root pieces, would have been expected to be present in all root sections 3 d after infection. When infected root pieces were stained and examined in the root tip inoculation experiment, dorsal and ventral vesicles were present throughout most infected root pieces by d 4. More root samples need to be examined from treatments in the entire root system inoculation experiment to determine whether this pattern of vesicle production in hyphae is consistent.

In the experiments in which zoospores were applied to root tips, the lag in the appearance of vesicles in hyphae behind the hyphal front probably occurred because hyphae at the growing front would have had access to nutrients from healthy roots. The exhaustion of nutrients by hyphae behind the growing front resulted in the production of peripheral vesicles in hyphae and also in production of sporangia and chlamydospores in response to the nutrient stress.

This root tip inoculation experiment had a number of advantages over the experiment in which entire root systems of plantlets were inoculated with zoospores of *P. cinnamomi*. Inoculation at the root tip with zoospores made it possible to follow infection as the fungus grew up the root. This form of infection was comparable with that which occurs in the field, where zoospores would probably not encyst on the entire surface of a root. The use of three replicate samples in the root tip inoculation experiment also meant that more reliable results were obtained than in the experiment where entire roots were inoculated. Trends in the appearance of vesicles before sporulation and the lag in appearance of vesicles in hyphae behind the growing front of the fungus were, therefore, able to be identified.

This study extends the analysis of the appearance of peripheral vesicles from axenic (Dearnaley & Hardham, pers. comm.) to *in planta* conditions. The results presented here correlated with those obtained by Dearnaley & Hardham (pers. comm.) who found that peripheral vesicles were absent from hyphae grown in nutrient-rich V8 broth, but appeared in nutrient-stressed hyphae after incubation in a low-nutrient solution. They also found that large peripheral and ventral vesicles appeared first in hyphae, followed by dorsal vesicles. In the experiments reported here, large peripheral vesicles appeared in hyphae first, then, after a lag period, dorsal and ventral vesicles appeared together. The results presented in this study also support the theory that peripheral vesicles are produced in hyphae under nutrient stress and move into developing sporangia and chlamydospores.

Chlamydospores were produced abundantly in root cells and on the root surface after 2-4 d. These structures persist after nutrients have been exhausted and subsequently germinate in

favourable conditions to infect plant material (Weste, 1983). Peripheral vesicles are thought to occur in chlamydospores so that, after germination, sporangia can be produced rapidly (Dearnaley & Hardham, pers. comm.). Large peripheral vesicles are used as a nutrient source for germinating cysts (Gubler & Hardham, 1990) and are probably present in chlamydospores in preparation for the production of sporangia when they germinate. Dorsal and ventral vesicles probably play this role in chlamydospores too (Hardham, pers. comm.). Experiments examining the presence of peripheral vesicles in germinating chlamydospores need to be conducted. Chlamydospores at all stages of maturity would probably contain these vesicles, because they would be transported into the developing chlamydospore from nutrient-stressed hyphae. Vesicle contents would probably be released after chlamydospore germination and sporangium production and upon zoospore encystment and germination, as found in studies of zoospores and cysts of *P. cinnamomi* released by sporulating hyphae.

The infection of roots of micropropagated chestnut by *P. cinnamomi* followed a similar pattern to that observed in susceptible seedlings by other authors (e.g. Weste & Cahill, 1982; Cahill *et al.*, 1989). It would be interesting to examine the development of peripheral vesicles in hyphae infecting resistant plant material over time. Resistant seedling material could not be obtained because of the strict quarantine regulations in Australia, which restrict the importation of most chestnut material to prevent the introduction of chestnut blight (R. Van Velsen, pers. comm.) and, therefore, it was impossible to produce plantlets of resistant species by micropropagation (see section 4.4).

Cahill *et al.* (1989) reported that zoospores of *P. cinnamomi* encysted, germinated and penetrated the roots of a range of susceptible and resistant plant species. Root growth of all plants ceased within 24 h of inoculation, but began again by 48 h after inoculation in resistant species. The response to infection varied depending on the plant species, however, in resistant species lesions were contained. No anatomical feature or histological change was consistently associated with this resistance, however the lignification of cell walls, deposition of phenolics, and the formation of callosic papillae in some plants may have been responsible.

Resistance was probably due to the production of phytoalexins, one of which, 6,7-dimethoxycoumarin has been isolated from citrus seedlings inoculated with *P. citrophthora* (Afek & Sztejnberg, 1988; Sulistyowati *et al.*, 1990). Resistance due to the increased activity of the phenylalanine ammonia lyase (PAL) enzyme, has been identified in roots of seedlings of *Eucalyptus calophylla* inoculated with *P. cinnamomi* (Cahill *et al.*, 1992).

Milholland & Daykin (1993), examining the colonization of roots of strawberry cvs by *P. fragariae*, also found that no reproductive structures were produced on the roots of resistant strawberry cv. "Climax" inoculated with *P. fragariae*, even up to 10 d after inoculation. In contrast, dense mycelial growth, sporangial and oospore formation were observed 8 d after inoculation of roots of the susceptible cv. "Tennessee Beauty" with *P. fragariae*.

Palloix *et al.* (1988 a), similarly, found that root exudates from resistant varieties of *Capsicum annuum* inhibited sporogenesis in every *P. capsici* isolate tested. Root exudates from susceptible plants, however, either had no influence on, or stimulated sporogenesis. Because reproductive structures were not produced by hyphae infecting resistant species of *C. annuum*, peripheral vesicles would not be expected to occur in hyphae of *P. capsici*.

In the above investigations, researchers have found that sporulation of *Phytophthora* spp. in resistant plant cvs is inhibited. Sporangial production by *P. cinnamomi* would probably have been inhibited in resistant species of chestnut and peripheral vesicles, therefore, would not be expected to be present in hyphae infecting these plants. Further work needs to be done to examine the response of resistant chestnut plantlets to inoculation with *P. cinnamomi*.

The use of clonal material produced by micropropagation in experiments reported here increased the likelihood of a uniform response of plant material to infection by zoospores of *P. cinnamomi*. Scott *et al.* (1992) also used clonally propagated plant material to screen for resistance in almond rootstocks to *P. cambivora*. A uniform response was observed in shoots of particular cvs 2 d after inoculation with *P. cambivora*, compared with variable lesion

development on shoots of inoculated plants in the field. Excised, micropropagated shoots of rootstocks of *Citrus* spp. inoculated with *P. citrophthora* also displayed resistance *in vitro* which correlated with field resistance (Son, 1992). In both systems, roots of susceptible rootstocks were colonized rapidly (E. Scott, pers. comm.).

In this study, the course of *P. cinnamomi* infection, of micropropagated plantlets of a susceptible cv. of chestnut was followed. MAbs, specific for peripheral vesicles in zoospores of *P. cinnamomi*, were used to label vesicles, for the first time, in hyphae infecting chestnut roots, therefore extending the analysis of the appearance of peripheral vesicles from *in vitro* to *in planta*. These vesicles appeared in nutrient-stressed hyphae about to undergo sporogenesis, with large peripheral vesicles appearing first, then dorsal and ventral vesicles appearing after a lag period. The evidence suggests that peripheral vesicles have an important role in the infection process and this needs to be further investigated.

8. OOSPORES OF *P. CINNAMOMI*

8.1 Introduction

The role of oospores in the lifecycle of *Phytophthora* spp. is discussed in Chapter 2. Oospore formation in amphigynous species of *Phytophthora* such as *P. cinnamomi* occurs when the mature oogonial initial contacts and penetrates a slightly swollen, mature antheridial initial. Cytoplasm flows into the developing oogonium from the underlying mycelium during the rapid oogonial expansion phase and vesicles appear, budding from the dictyosomal cisternae. These vesicles underlie the entire oogonial plasma membrane during expansion. The oogonial initial then ruptures through the other side of the antheridial initial (Hemmes, 1983). The fertilisation tube ruptures the oogonial wall and deposits the antheridial nucleus in the oogonial cytoplasm (Hemmes, 1983) and the single male and female gametic nuclei associate in the developing oosphere and eventually fuse (Brasier & Brasier, 1978). The mature oospore has a lipid-rich vacuole (ooplast), a thick inner wall composed largely of carbohydrate and an impermeable outer wall (Shaw, 1988).

During the germination of mature oospores, the inner wall of the aplerotic oospore, rich in glucans, is degraded by the process of activation. The biochemical factors triggering the dissolution of the inner wall are unknown, however, the removal of endogenous inhibitors of glucanase activity or activation of a latent enzyme may be involved (Jiang & Erwin, 1993). The inner wall probably acts as an energy reserve for the germinating oospore (Hohl, 1991). The single diploid zygotic nucleus divides mitotically many times and one or more germ tubes are produced (Shaw, 1988). These may form mycelium or germ tubes terminating in sporangia (Ribeiro, 1983). The germination rate of oospores produced by homothallic species is generally high (up to 90%) (Zentmyer & Erwin, 1970; Förster, Ribeiro & Erwin, 1983; Jiang & Erwin, 1993), whereas in heterothallic species it is usually low (up to 40%) (Ribeiro, Erwin & Zentmyer, 1975a; Kaosiri, Zentmyer & Erwin, 1980).

Germination may be induced by a number of factors. Root extracts and exudates, and soil extracts (Förster *et al.*, 1983; El-Hamalawi & Erwin, 1986) induce germination in *P. megasperma* f. sp. *medicagensis*. The effect of light on oospore germination varies depending on the species. Hord & Ristaino (1991) found that incubation under white light reduced germination of *P. capsici* oospores *in vitro* compared to germination in the dark, whereas Ann & Ko (1988) found oospore germination in *P. parasitica* was reduced when incubated in darkness rather than white light. Germination of several species of *Phytophthora* was enhanced by blue light (400-480 nm) (Ribeiro *et al.*, 1975 b, 1976 ; Banihashimi & Mitchell, 1976, 1980). The optimum incubation temperature required for oospore germination also varies depending on the species of *Phytophthora*. The optimum temperature for germination of *P. cactorum* oospores was 20°C, with 88% of oospores germinating (Banihashimi & Mitchell, 1976), whereas in *P. megasperma* f. sp. *glycinea* 78% germination was obtained at 27°C (Erwin & McCormick, 1971). Enzymes, particularly helicase and glusulase derived from snails, have been found to induce germination of *P. erythroseptica* and *P. megasperma* oospores (Gregg, 1957; Salvatore *et al.*, 1973). Similarly, oospore maturity affects germination *in vitro*, with consistently high germination frequencies obtained with oospores from cultures at least 30 d-old. (Ribeiro *et al.*, 1975b; Förster *et al.*, 1983).

Three categories of vesicles that are peripherally located in zoospores have been identified, using monoclonal antibodies, in zoospores and cysts, hyphae, sporangia and chlamydospores of *P. cinnamomi* (Hardham *et al.*, 1986; Gubler & Hardham, 1988; Dearnaley & Hardham, pers. comm.). Dearnaley & Hardham (pers. comm.) observed these types of vesicles in nutrient-starved hyphae of *P. cinnamomi* producing sporangia and chlamydospores and these vesicles were thought to move into developing sporangia and chlamydospores by cytoplasmic streaming. Ultrastructural studies have identified vesicles in the cytoplasm of *Phytophthora* spp. oospores (Hemmes, 1983), however, their function remains unclear.

The aims of the present study were to investigate the formation and germination of oospores of *P. cinnamomi in vitro*, and to examine whether peripheral vesicles were present in mature

and germinating oospores of *P. cinnamomi*. MAbs raised against components of glutaraldehyde-fixed zoospores and cysts of *P. cinnamomi* were used to identify peripheral vesicles in this material.

8.2 Materials and Methods

8.2.1 Production of oospores in carrot agar culture

Oospores were produced as described in section 2.3 by crossing *P. cinnamomi* isolate P1 (A1 mating type) with *P. cinnamomi* isolate 6BR (A2 mating type), or by incubating plugs of 6BR and P1 alone (controls).

8.2.2 Production of oospores in micropropagated chestnut

Plantlets of chestnut (*C. sativa* "Goldsworthy") were placed, with the roots inserted into 10 ml DW agar (7 g l^{-1} Difco Bitek agar) in 8 x 3 cm polycarbonate vials (Disposable Products, South Australia). Plugs of *P. cinnamomi* isolates 6BR (A2 mating type) and P1 (A1 mating type), 5 mm in diam., taken from the margin of actively growing cultures on carrot agar, were placed on either side of the base of the shoot. Controls consisted of plantlets placed in DW agar and not inoculated, or inoculated with isolates 6BR or P1, paired with plugs of the same culture, only. There were five replicate plantlets of each treatment and plantlets were incubated in the dark at 25°C.

Three plantlets were harvested after 10 wk, and roots cleared in 10% KOH for 1.5 h at 80°C, bleached in 1N HCl for 2-3 min and stained in 0.05% Trypan Blue in lactoglycerol for 25 min (Phillips & Hayman, 1970). Roots were mounted in lactoglycerol then viewed and photographed using a Leitz Orthoplan compound microscope. Roots of a further two plantlets per treatment were harvested, cut into 5 mm pieces and fixed, sectioned and labelled with antibodies as in section 7.2.2. The experiment was conducted twice.

The dimensions of ten oospores and ten oogonia from each treatment were measured under 250 x magnification on a Leitz Orthoplan compound microscope. Mean length and breadth

of oogonia and oospores were compared using Analysis of Variance and Least Significant Difference was calculated for treatments where statistically significant interactions were observed.

8.2.3 Oospore germination

Mature oospores, that had developed 10 wk after crossing, produced in carrot agar culture, were germinated using a modified method of Ann & Ko (1988). Twelve 1 cm^2 pieces of carrot agar containing oospores were cut from the interaction zone of 6BR x P1 crosses and blended in 100 ml sterile DW at high speed for 60 s using a Black and Decker Hand Held Food Processor. The macerated suspension was passed through sterile 53 μm pore-size nylon mesh, and the oospores collected on 20 μm mesh. They were rinsed with 1 l sterile DW on the 20 μm mesh, then suspended in 100 ml 0.025% $KMnO_4$, and stirred for 20 min. The oospores were then washed with 2 l sterile DW on the 20 μm mesh, collected in 3 ml sterile DW and 20 μl of this suspension (containing approx. 150 oospores) was spread on 20 ml S & L medium (Ann & Ko, 1988) in a 90 mm diam. Petri dish. Ten replicate plates were incubated in the dark at 25°C and germination was assessed after 10 d. The experiment was conducted twice. No attempts were made to germinate oospores produced on micropropagated chestnut.

8.2.4 Labelling of mature and germinated oospores

Mycelium and mature oospores were scraped from the surface of a carrot agar culture using a razor blade and fixed and embedded as described in section 7.2.2. Sections 10 μm thick were cut, labelled with antibodies and viewed as in section 7.2.2. Germinated oospores, that had developed colonies 1-2 mm in diam. or 1 cm in diam., were also cut from S&L medium and fixed. Plugs, 1 mm thick, and adhering mycelium were placed with mycelium downward in moulds for ease of sectioning. In addition, five oospore-derived colonies (2 $mm^{2)}$ were placed in 90 mm diam. Petri dishes and flooded with V8 broth (section 2.2) for 36 h. Agar plugs were incubated in V8 broth for 36 h because very little mycelial growth occurred before this time. They were then rinsed three times with mineral salts solution (section 2.2), flooded

with fresh solution and incubated under white fluorescent lights (Hanimex, Statesman) for 8 h. Colonies were harvested from V8 broth and from mineral salts solution at 2 h, 3 h, 5 h, 6 h, 7 h and 8 h after flooding. Mycelium was fixed, sectioned and labelled as in section 7.2.2. One plug with adhering mycelium per treatment was sectioned and stained.

8.3 Results

Oospores were abundant in carrot agar cultures of *P. cinnamomi* 6BR x P1 crosses which had been incubated in the dark at 25°C for 10 wk, but were absent from selfed cultures. Oogonia. with two-celled, amphigynous antheridia, were abundant in roots of chestnut plantlets inoculated with *P. cinnamomi* 6BR x P1 harvested at 10 wk (Figs 8.1.1 & 8.1.2), but were less common in roots of controls inoculated with *P. cinnamomi* 6BR (Figs 8.1.3 & 8.1.4) or *P. cinnamomi* P1 alone (Fig. 8.1.5). No oospores formed in uninoculated roots or in shoots of plantlets. Few (0.03%) of the oospores isolated from agar cultures germinated on S&L medium (Fig. 8.1.6).

There was a statistically significant interaction between oogonial lengths and breadths measured between treatments ($P<0.01$). The length of oogonia formed in carrot agar culture and in micropropagated chestnut inoculated with 6BR alone was larger than those formed in the other cultures on micropropagated chestnut (Table 8.1). The breadth of oogonia formed in carrot agar cultures was significantly greater than that of oogonia formed in any of the cultures on micropropagated chestnut (Table 8.1); there was no significant difference in oogonial breadth between these cultures on micropropagated chestnut. Similarly, there was no significant difference in the length or breadth of oospores in any treatment. Few antheridia were observed in oospores formed in micropropagated chestnut inoculated with P1 alone (<10) and, therefore, measurements of them were not made. Germination was by germ tubes and mycelial growth only, either from one point or multiple points in the oospore (Fig. 8.1.6).

Only large peripheral vesicles were labelled with the antibody Lpv-1 in oospores formed in carrot agar culture between *P. cinnamomi* 6BR x P1 crosses (Fig. 8.2.1); no labelling of

Figs 8.1.1-8.1.4: Light micrographs of oospores of *P. cinnamomi* in roots of micropropagated chestnut "Goldsworthy" after incubation with *P. cinnamomi* at 25°C in the dark for 10 wk. Roots were cleared and stained with 0.05% Trypan Blue in lactoglycerol, squash mounted and viewed using light microscopy.

Fig. 8.1.1: Oogonium (large arrowhead) with two-celled amphigynous antheridium (small arrowheads) in chestnut root co-inoculated with isolates 6BR and P1. Bar = 25μm.

Fig. 8.1.2: Abundant oogonia (arrowed) in chestnut root co-inoculated with isolates 6BR and P1. Bar = 230 μm.

Fig. 8.1.3: Oogonia (arrowed) formed in chestnut root inoculated with isolate 6BR only. Antheridia in different focal plane (not shown). Bar = 33 μm.

Fig. 8.1.4: Oogonia as shown in Fig. 8.1.3, with antheridia in focus (arrowed). Bar = 33 μm.

Fig. 8.1.5: Oogonia (arrowed) formed in chestnut root inoculated with isolate P1 only. Bar = 23 μm.

Fig. 8.1.6: Oospore germination (o) to produce mycelium (m), 1 d after isolation from carrot agar cultures and incubation on S&L medium at 25°C in the dark. Bar = 80 μm.

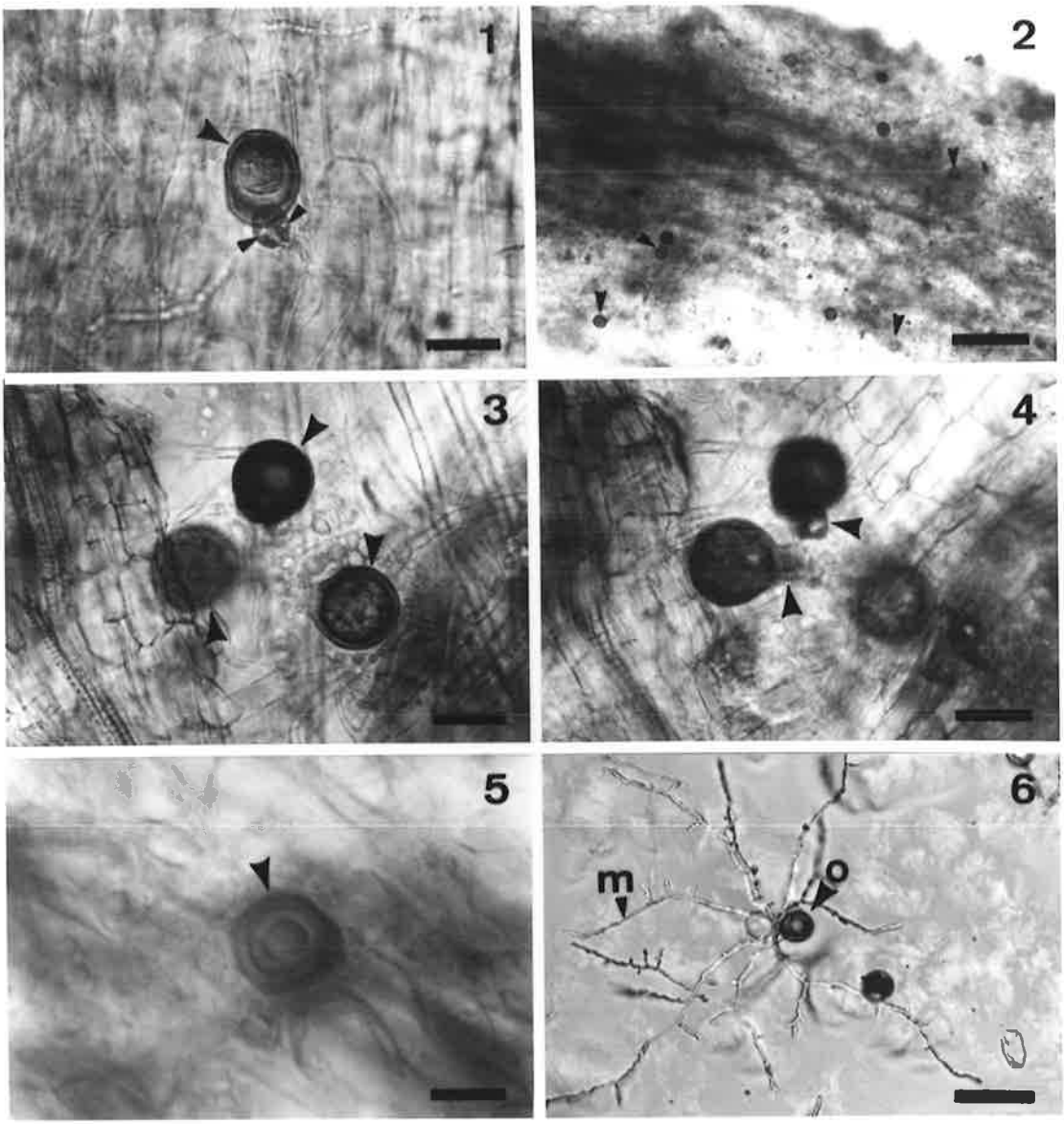

1
2
3
4
5
6
m
o

Table 8.1: Dimensions of oogonia and oospores of *P. cinnamomi* produced using four different methods (means ± standard errors are presented). Ten oogonia were measured per treatment. Oospores were produced by crossing isolate 6BR and P1 on carrot agar (= carrot agar 6BR/P1), or by inoculating micropropagated chestnut "Goldsworthy" plantlets with isolates 6BR and P1 (= chestnut 6BR/P1), 6BR only (= chestnut 6BR), or P1 only (= chestnut P1). A comparison between length and breadth was not made. Oogonial length LSD = 4.10 ($P<0.05$), oogonial breadth LSD = 3.70 ($P<0.05$), different letters indicate a significant difference between treatments for length or breadth (rows).

	dimension	carrot agar 6BR/P1	chestnut 6BR/P1	chestnut 6BR	chestnut P1
oogonia	length	42.09 ± 1.71^a	36.80 ± 1.45^b	39.55 ± 0.89^a	34.69 ± 1.52^b
	breadth	38.92 ± 1.63^a	34.05 ± 0.38^b	34.90 ± 1.15^b	32.36 ± 1.67^b
oospores	length	19.67 ± 0.71	18.19 ± 1.42	19.884 ± 1.19	17.98 ± 0.85
	breadth	17.77 ± 0.72	16.07 ± 1.10	17.45 ± 0.74	16.71 ± 0.74

Fig. 8.2.1: Large peripheral vesicles in oospores of *P. cinnamomi* 6BR and P1 co-inoculated on carrot agar and incubated at 25°C in the dark for 10 wk. Cryosections, 10μm thick, were labelled with the MAb Lpv-1 and SAM-FITC, then viewed using fluorescence microscopy. Bar = 13 μm.

Figs 8.2.2-8.2.4: Oospores produced by *P. cinnamomi* in micropropagated chestnut "Goldsworthy" roots after incubation in the dark at 25°C for 10 wk. Roots were cut into 5 mm pieces, cryosections 10μm thick cut, then labelled with Lpv-1 and SAM-FITC and viewed using fluorescence microscopy.

Fig. 8.2.2: Large peripheral vesicles in oospore formed in a chestnut root inoculated with *P. cinnamomi* isolates 6BR and P1. Bar = 9 μm.

Fig. 8.2.3: Large peripheral vesicles in oospore and antheridium (arrowed) formed in a chestnut root inoculated with *P. cinnamomi* isolate 6BR. Bar = 11.5 μm.

Fig. 8.2.4: Large peripheral vesicles in oospore formed in a chestnut root inoculated with *P. cinnamomi* isolate P1. Bar = 8.3 μm.

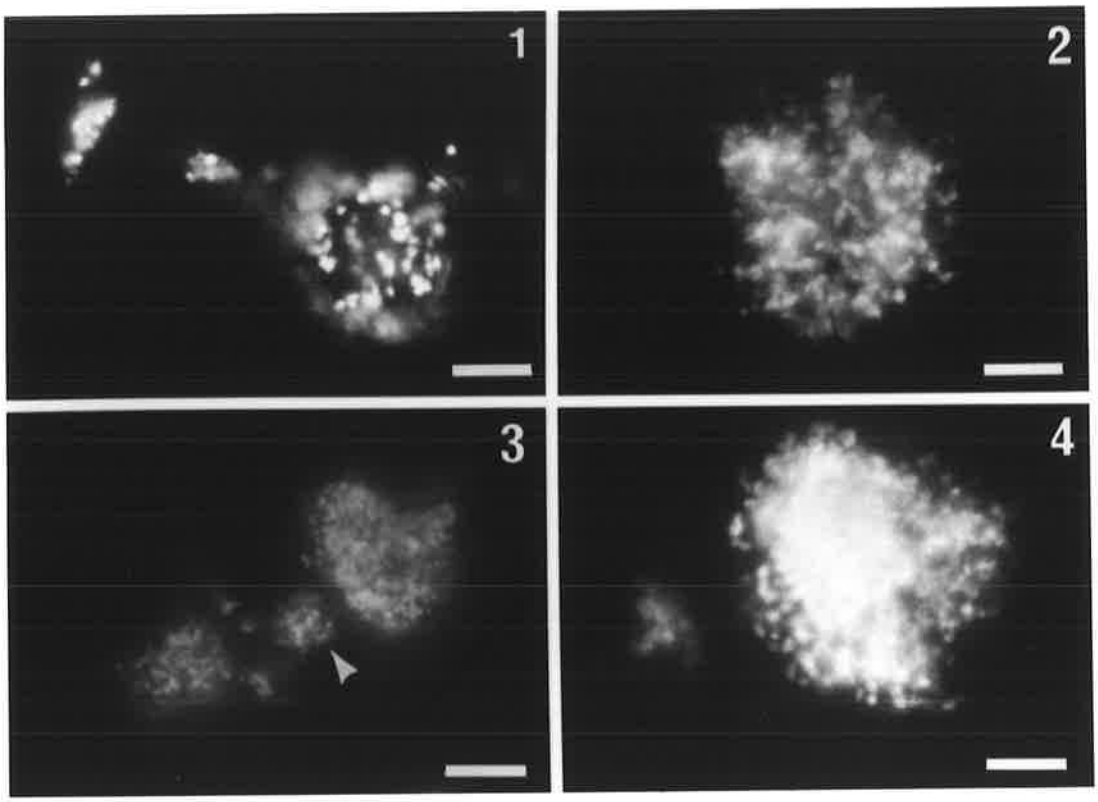

1
2
3
4

Table 8.2: Presence of peripheral vesicles in hyphae after incubation at 25°C under white fluorescent light in V8 broth for 36 h and then for up to 8 h in mineral salts. + denotes presence of peripheral vesicles, - denotes absence of peripheral vesicles. Lpv-1 = large peripheral vesicles, Cpa-2 = dorsal vesicles, Vsv-1 = ventral vesicles.

treatment	Lpv-1	Cpa-2	Vsv-1
V8 broth	-	-	-
mineral salts 2 h	+	-	-
" 3 h	+	-	-
" 5 h	+	+	+
" 6 h	+	+	+
" 7 h	+	+	+
" 8 h	+	+	+

vesicles by the antibodies Cpa-2 and Vsv-1 occurred. Vesicles were labelled abundantly throughout the oospore. Large peripheral vesicles were also labelled in hyphae surrounding these oospores, as well as in hyphae of P1 and 6BR isolates in carrot agar cultures of the same age. Likewise, only large peripheral vesicles were labelled in oospores present in chestnut roots inoculated with *P. cinnamomi* 6BR x P1, 6BR, and P1 (Figs 8.2.2, 8.2.3, 8.2.4). None of the three vesicle types were labelled in hyphae sectioned from 1-2 mm^2 or 1 cm^2 colonies derived from germinated oospores, or in hyphae harvested after 36 h incubation in V8 broth. Large peripheral vesicles were labelled in hyphae from 2 h incubation onwards in mineral salts solution (Table 8.2). Sporangia formed between 4 and 5 h after transfer to mineral salts solution and ventral and dorsal vesicles were labelled with Vsv-1 and Cpa-2 from 5 h onwards.

8.4 Discussion

Oospores of *P. cinnamomi* were produced by A1 x A2 mating type crosses or by inoculating micropropagated chestnut roots with either A1 or A2 isolates alone. Oogonial shape and dimensions produced in carrot agar and in micropropagated chestnut roots were typical of those reported for *P. cinnamomi*, however, oospore dimensions were slightly smaller than those previously reported (approx. 16-19 μm cf. > 20 μm) (Stamps *et al.*, 1990). Oospores of *P. citrophthora* have previously been observed in the agar around inoculated, micropropagated citrus plantlets, but only by A1 x A2 crosses (Son, 1992). There was no evidence of homothallism in *P. citrophthora*, however, her experiments were preliminary and only the agar around roots was examined for oospores. Oospores may have been produced by selfing in roots of citrus and this needs to be examined further.

Oospores were only produced in agar where rooted shoots of citrus were incubated with *P. citrophthora*; they were not observed where excised shoots of citrus were incubated with the fungus. Root exudates, produced by rooted shoots of citrus, may have stimulated oospore formation in *P. citrophthora*. Oospores, which formed in roots of chestnut, were probably also induced by root exudates. Oospores have previously been induced, in A2 isolates of *P.*

cinnamomi, by root extracts of avocado, myrtle, apricot, macadamia, chestnut, heath and rhododendron (Zentmyer, 1979). Oospore formation was also induced by root extract in A2 isolates of *P. capsici* and *P. drechsleri*, but not in A2 isolates of several other *Phytophthora* spp. (Zentmyer, 1979). Zaki *et al.* (1983) found that it was the lipid fraction of the avocado root extracts, as well as authentic oleic acid and triolein, which induced oospore formation in A2 isolates of *P. cinnamomi*. This is the first published report of oospore formation in an A1 isolate of *P. cinnamomi*, induced in roots of chestnut. Avocado root extracts did not induce oospore formation in A1 isolates of *P. cinnamomi* (Zentmyer, 1979) and neither the lipids or the oleic acid and triolein, obtained from these extracts, stimulated oospore formation in A1 isolates of *P cinnamomi* (Zaki *et al.*, 1983).

Ko (1978) found that oospores of *P. cinnamomi*, *P. parasitica* and *P. palmivora* were formed by selfing, when different mating types were paired on opposite sides of polycarbonate membranes. Diffusible plant hormone-like substances, designated α_1 and α_2, were thought to control sexual reproduction. Heterothallism in species of *Phytophthora* thus appears to be chemically mediated (Ashby, 1929; Ko, 1978). Although *P. cinnamomi* has traditionally been regarded as a heterothallic species (Stamps *et al.*, 1990), a number of stimuli induce homothallism in this species. Reeves & Jackson (1972) found that plugs of an A2 isolate of *P. cinnamomi* mycelium, growing on *C. sativa* root pieces in different soil types, produced oospores within 6-8 d of inoculation. Oospore formation was associated with the presence of *Trichoderma viride* mycelium, on and in the root pieces. Oospores also formed when *T. viride* was grown on carrot agar where *P. cinnamomi* mycelium was growing. Similarly, Brasier (1975) found that all of the 41 A2 isolates of *P. cinnamomi* and *P. cambivora* examined, produced oospores *in vitro* in response to volatile compounds produced by *Trichoderma* spp. (after 24 h) and contact with (after 15 h) *Trichoderma* spp. Similarly, O'Brien (1991) found that volatile compounds from the culture filtrate of 6 d-old cultures of *T. koningii* growing in 1/4 Czapek-Dox medium induced oospore formation in an A2 isolate of *P. cinnamomi*. Oospore formation was not induced by culture filtrates of *T. hamatum*, *T.*

pseudokoningii or *G. virens* in other experiments in this study, although these isolates were grown on DW or 1/5M32 medium (see section 5.3.3).

It would be interesting to determine whether oospores formed in dual culture were the result of recombination or selfing. Researchers have used a number of genetic markers to identify whether oospores are the result of recombination or selfing. For example, Shattock *et al.* (1986), using isozyme markers, found that the majority of oospore-derived progeny in *P. infestans*, from parents homozygous for different alleles, were heterozygous. Förster & Coffey (1990) used RFLPs as genetic markers to examine oospore-derived progeny of *P. parasitica* for recombination. The majority of the 23 progeny examined were the result of recombination, with only two of the progeny being the result of selfing. The majority of oospore-derived progeny of *P. cinnamomi*, formed in dual culture in experiments presented here, would probably, therefore, be the result of recombination.

Oospore germination in experiments reported here was low (0.03%), consistent with that reported most for heterothallic species (Ribeiro *et al.*, 1975a; Kaosiri *et al.*, 1980), even using the medium of Ann & Ko (1988), in which >90% of oospores of the heterothallic species *P. parasitica* germinated after 10 d. Germination of oospores of *P. cinnamomi* has previously been induced by incubation of oospores on water agar at 24°C in the dark. Few oospores which had been produced on synthetic medium and V8 agar, germinated on this water agar medium (5% and 2% respectively) (Ribeiro *et al.*, 1975a). Son (1992) also found low incidence of germination of oospores of *P. citrophthora* on S & L medium after 42 d (up to 13%). In experiments reported here, germination was only assessed up to 10 d after incubation of oospores on S&L medium. The germination rate may have been higher if assessments had been carried out over a longer period of time, or if oospores had been incubated in the light. Oospores of *P. citrophthora* produced in citrus tissue cultures were isolated but did not germinate (Son, 1992), however the sample size was very small. It would be interesting to compare the germination rate of oospores of *P. cinnamomi* produced by A1 x A2 crosses on chestnut plantlet roots with that of oospores produced by A2 and A1 isolates

alone. A more detailed study of the factors affecting germination of *P. cinnamomi* oospores *in vitro* needs to be conducted.

Ultrastructural studies have shown the presence of many vesicles in the cytoplasm of *Phytophthora* spp. oospores (Hemmes, 1983). The immunofluorescence labelling with MAbs directed towards large peripheral vesicles of zoospores reported here has demonstrated, for the first time, the presence of these large peripheral vesicles within the oospores. These vesicles were labelled abundantly throughout the cytoplasm. They probably provide nutrients needed for germination, as they do in germinating cysts (Gubler & Hardham, 1988, 1990). It is interesting that dorsal and ventral vesicles were not observed in oospores. Ventral and dorsal vesicles are present in zoospores and hyphae about to undergo sporulation. The contents of the ventral vesicles are released after the induction of encystment and allow the cyst to adhere to the root surface (Hardham & Gubler, 1990), whereas the contents of the dorsal vesicles coat the outer surface of the cyst, possibly preventing desiccation. It is not surprising, therefore, that these vesicles were not detected in oospores; they would have no role in oospore germination, as the oospore has a thick wall to prevent desiccation and does not adhere to the host prior to germination. Oospores in this study germinated by germ tubes only, hence ventral and dorsal vesicles would not be expected to be present in the oospore. Dearnaley & Hardham (pers. comm.) found that peripheral vesicles formed in nutrient-stressed hyphae and were probably moved into sporangia and chlamydospores by cytoplasmic streaming. Large peripheral vesicles were also observed in hyphae around developing oospores in experiments here and were probably moved into the developing oospores in this way.

The appearance of peripheral vesicles in oospores during their development and maturation also needs to be investigated further, although it was difficult to determine the stage of maturation of oospores of *P. cinnamomi* in experiments here. It would also be interesting to study peripheral vesicles in oospores germinating by the production of sporangia. Large

peripheral, dorsal and ventral vesicles would be expected to be present in hyphae germinating from oospores and then move into sporangia.

9. GENERAL DISCUSSION

In this study various aspects of root rot of chestnut caused by *Phytophthora* spp. were investigated. A modified excised shoot assay, using micropropagated material, was evaluated as a screening method for chestnut cvs *in vitro*, and gave promising results, although shoots of resistant chestnut species do need to be tested before the method can be fully assessed. Recent, locally-obtained isolates of *P. cinnamomi* and *P. citricola*, and an isolate of *P. cryptogea*, previously found to cause root rot of chestnut in South Australia (Wicks & Volle, 1976), were used in screening experiments to inoculate pot-grown seedlings of a number of cvs used as rootstocks by growers. All cvs were susceptible to *Phytophthora* spp., with *P. citricola* and *P. cryptogea* found to be more virulent on chestnut than was *P. cinnamomi*. This is the first report of *P. citricola* causing root rot of chestnut. Biocontrol of *Phytophthora* root rot of chestnut was also investigated: three fungi were evaluated for antagonism of *P. cinnamomi* and *P. citricola* and mycoparasitism, involving parallel growth and coiling, and antibiosis were observed *in vitro*. In preliminary pot experiments, *T. pseudokoningii* and *G. virens* showed some potential in protecting chestnut seedlings from disease caused by *P. cinnamomi*. In structural studies of the infection of micropropagated chestnut by *P. cinnamomi*, hyphae of *P. cinnamomi* had completely overgrown roots of susceptible plantlets 3 d after inoculation. Peripheral vesicles in these hyphae, labelled using monoclonal antibodies, appeared from 1 d after inoculation, in conjunction with sporangia and chlamydospore production. Large peripheral vesicles were also observed in oospores formed on carrot agar and in infected chestnut roots *in vitro*.

A number of methods were used to screen chestnut plants for response to *Phytophthora* spp.. Seedlings were inoculated with zoospores of *P. cinnamomi*, *P. citricola* and *P. cryptogea* using a zoospore-dip or drench method or by application of mycelium to a wound on the tap root and shoot, and disease symptoms were recorded and plant material weighed 21 d after inoculation (Chapter 5). The zoospore drench method failed to cause infection of seedlings, whereas none of the cvs of chestnut tested using the tap root and zoospore-dip inoculation

methods showed resistance to *P. cinnamomi*, *P. citricola* or *P. cryptogea* in the conditions tested. It was surprising that *C. sativa* x *C. crenata* hybrid cvs "Buffalo Queen" and "Colossal" were as susceptible to infection by *Phytophthora* spp. as *C. sativa* cvs, as the former were expected to show some resistance derived from the resistant parent *C. crenata*. Presumably, these hybrid cvs do not contain the genes for resistance which the parent species has.

Although the root rot and shoot symptoms indices were useful in quantifying disease on seedlings in inoculation experiments in this study, these techniques are somewhat subjective. Other methods of disease assessment, which may be more accurate for measuring colonization of material, have been developed and could be used with chestnut. Kellam & Coffey (1985) quantified infection of three avocado rootstocks by *P. cinnamomi* using a dilution plate method. They mixed samples of infested roots with water then plated aliquots of this mixture on *Phytophthora*-selective medium. Colonies of *P. cinnamomi* were counted 2 or 3 d later and differences in the susceptibility of rootstocks were inferred using this method. Another technique to quantify infection caused by *P. cinnamomi* on avocado rootstocks was reported by Gabor & Coffey (1991). Samples of diseased shoot sections of a number of inoculated rootstocks were plated on *Phytophthora*-selective medium, incubated for 72 h and plates were then scored for the percentage of segments yielding colonies of *P. cinnamomi*. These methods could be used in conjunction with symptom assessment, so that colonization and disease could be correlated.

Further whole plant inoculation experiments need to be conducted under conditions similar to those in the field. Differences in virulence between isolates might then become more apparent: Scott *et al.* (1992) identified differences in aggressiveness between isolates of *P. cambivora* on almond when orchard trees were inoculated *in situ*.

The excised shoot assay was tested as a screening method for detecting response of micropropagated chestnut shoots to *Phytophthora* spp. *in vitro* (Chapter 4) and compared to

inoculation of seedlings in pot experiments. Shoots of the chestnut cvs "Goldsworthy" and "Buffalo Queen" were susceptible to *P. cinnamomi, P. citricola, P. cryptogea* and *P. cambivora* in the excised shoot assay (*in vitro* assay), developing lesions along their length 2 d after inoculation.

The major advantage of the *in vitro* screening method was that it detected the susceptibility of cvs faster than whole plant inoculations in the glasshouse or field could. One of the disadvantages with the seedling inoculation method used in this study was the large variation in response of individual plants to infection, making it difficult to detect differences in response of cvs. The use of more replicates in future experiments, however, might reduce this problem. An advantage of the excised shoot assay method using micropropagated material over pot and field inoculation experiments using seedling material is the expected uniform response to infection of clonally propagated plants *in vitro*. This assay could also be developed further to screen different species or cvs of other woody plants for resistance to various soilborne plant pathogens.

In this study, the response of chestnut plantlets growing in vermiculite *ex vitro*, following inoculation with zoospores of *P. cinnamomi*, was investigated and found to be similar to that of excised shoots inoculated *in vitro*. Experiments in which *ex vitro* material and seedlings were inoculated confirmed the results of the excised shoot assay for susceptible cvs. Validation of the results obtained in the excised shoot assay extended the work of Scott *et al.* (1992) and Son (1992), in which excised micropropagated shoots were screened *in vitro* but material was not planted out for further study. Although the excised shoot assay is an artificial system, in which roots of cvs being tested are not inoculated with the pathogen, the inoculation of plantlets *ex vitro* confirmed that this excised shoot assay can detect different responses to infection. Indeed, in species such as chestnut, in which root formation *in vitro* is unreliable, the method provides an alternative to traditional whole plant screening methods.

All of the screening methods showed that chestnut clones available to growers in Australia are generally susceptible to *P. cinnamomi*, *P. citricola* and *P. cryptogea.* These clones are, therefore, not suitable for use as rootstocks by growers in areas where the soil is infested with this pathogen. There were indications from the excised shoot assay that some clones of chestnut cvs may be less susceptible to infection by *P. cinnamomi* than others. These less susceptible clones, however, should be multiplied by micropropagation and further tested for resistance under pot and field conditions before being considered as superior rootstocks.

Resistant species of chestnut, *C. crenata* and *C. mollissima*, were not available and, therefore, could not be used in screening experiments. These species would have been valuable in all experiments and would probably have shown minor lesion development in the excised shoot assay. Resistant species need to be screened using this assay before a full evaluation of the method can be made. Sharma & Skidmore (1988) detected partial resistance of papaya to *P. palmivora*; this response may be expressed in chestnut also and might be detected if experimental conditions were adjusted to include different temperatures or levels of inoculum. *C. crenata* and *C. mollissima* would have been expected to show resistance in pot experiments. It is unfortunate that these resistant species of chestnut are not readily available to growers at present for use as rootstocks. A combination of cultural and chemical methods. therefore, is necessary at present to control disease on chestnut in Australia caused by *Phytophthora* spp..

It would be useful to conduct a survey of root rot disease incidence in chestnut orchards throughout Australia, so that the full extent of disease could be determined. Such a survey might also identify the importance of *P. citricola,* compared with other *Phytophthora* spp., as a pathogen of chestnut, both in South Australia and in other states. Growers in *Phytophthora*-infested areas, particularly those propagating planting material commercially, could then practise cultural and chemical control methods to try to prevent the spread of disease to other growers. This is especially important because of the lack of resistant material. Resistant species should become more available to growers in the future as material is gradually

imported, through quarantine, into Australia. This material could then be planted in *Phytophthora*-infested soil.

Because of the problems associated with the cultural and chemical methods of controlling disease caused by *Phytophthora* spp. (see Chapter 2), there is growing interest in the development of biological control agents. Three fungi isolated from soil from which *Phytophthora* spp. were not obtained, *T. hamatum*, *T. pseudokoningii* and *G. virens*, showed some promise in biocontrol of *Phytophthora* root rot of chestnut. *In vitro* experiments identified mechanisms of inhibition of *P. cinnamomi* and *P. citricola*, however, further work could be conducted, for example, to identify antibiotics produced *in vitro* by these fungi.

These antagonist fungi were also evaluated for prevention of lesion development on chestnut shoots in an *in vitro* biocontrol shoot assay, modified from the excised shoot assay used in this study. This assay showed the potential of these antagonist fungi as biocontrol agents. in that small lesions developed on "Goldsworthy' and "Buffalo Queen" shoots 2 d after inoculation with *P. cinnamomi* or *P. citricola* and *T. hamatum*, *T. pseudokoningii* or *G. virens*, whereas large lesions developed on shoots inoculated with *P. cinnamomi* or *P. citricola* alone. It could be developed further, to screen large numbers of fungal isolates for potential biocontrol of disease caused by *Phytophthora* spp. on other woody plants. These isolates could be co-inoculated with the pathogen on agar in tubs and micropropagated shoots of the test plant could then be incubated with these dual cultures. The tubs take up a small amount of space compared to pots used in conventional screening experiments, and an indication of the biocontrol potential of an isolate can be gained after only a few days incubation with the test plant. The assay could also be used to screen bacteria, including actinomycetes, for biocontrol against pathogens, by streaking the test bacterium on one half of the agar surface in a tub and placing a plug of the fungal pathogen on the other.

In preliminary pot experiments, *T. pseudokoningii* and *G. virens* showed promise in the biocontrol of root rot of chestnut seedlings caused by *P. cinnamomi*. The weights of plants

inoculated on the tap root with mycelial plugs of one of these antagonist fungi, and then inoculated 14 d later with plugs of *P. cinnamomi*, were higher than those of plants inoculated with *P. cinnamomi* alone. However, convincing evidence of reduction of disease was not obtained and further pot experiments need to be conducted to evaluate a wider range of antagonist formulation and delivery methods, in both glasshouse and field experiments, before the full potential of these fungi for biocontrol of *Phytophthora* root rot of chestnut is known. The method of application of the pathogen is important also, with zoospore inocula being most like the natural situation.

A number of criteria need to be addressed when developing biocontrol formulations with agricultural potential, for example they must be easy to prepare and apply, be stable, with adequate shelf life, have abundant viable propagules and have a low cost (Lumsden & Lewis, 1989). Formulations must also be developed to aid the competitive ability of the microbial antagonists when added to the soil (Renwick & Poole, 1989).

Isolates of *Trichoderma* spp. and *Gliocladium* spp. are being developed for use in the control of root rot of apple caused by *P. cactorum* (e.g. Smith *et al.*, 1990; Roiger & Jeffers, 1991) and a number of other pathogens. Formulations of *Trichoderma* and *Gliocladium*, produced in the USA, are now on sale in Australia and a large number of isolates are being patented and developed for release.

Researchers have produced a number of improved strains which exhibit, for example, benomyl-resistance, greater rhizosphere competence and the production of greater amounts of antibiotics *in vitro* compared to original isolates. These modified strains are thought to have greater disease control capabilities than wild type isolates, so that they may be useful, in the future, instead of, or as well as, chemicals to prevent disease (see Baker, 1989). Indeed, with increasing regulations likely to restrict fungicide use further in the future (Acuff, 1988), the need for biocontrol agents to control plant disease will increase. Integrated control of disease, involving the use of fungicides and biocontrol agents, may provide the best method for

controlling root rot of chestnut in Australia. For example, treatment with the fungicide pentachloronitrobenzene (PCNB) combined with *T. harzianum* gave significantly greater control of disease on radish caused by *Rhizoctonia solani* than did fungicide or antagonist alone (Henis, Ghaffar & Baker, 1978).

Structural investigations of the infection of plants by pathogens such as *Phytophthora* spp. have led to a greater understanding of disease development. Many studies have been undertaken to examine the mechanisms associated with the resistant response of plants to infection by *Phytophthora* spp. (e.g. Cahill & Weste, 1983; Hinch *et al.*, 1985; Cahill *et al.*, 1989). Less is known about changes occurring in the fungus during disease development. Hardham *et al.* (e.g. 1986, 1991) have developed a system for microscopic investigations of structural and biochemical aspects of development of *P. cinnamomi* and of early stages of infection of host (*Eucalyptus* sp.) roots.

Monoclonal antibodies, raised against components of zoospores and cysts of *P. cinnamomi* (6BR) have been used to label, for example, the contents of the dorsal, ventral and large peripheral vesicles. Recently, these vesicles were labelled in nutrient-stressed hyphae growing in axenic culture, their presence corresponding with the formation of sporangia and chlamydospores (Dearnaley & Hardham, pers. comm.).

This study extended the work of Hardham's group, to label peripheral vesicles, for the first time, in hyphae infecting susceptible chestnut roots (Chapter 7). Vesicle appearance coincided with the production of sporangia and chlamydospores in and on infected chestnut roots, thereby extending the analysis of the appearance of peripheral vesicles from axenic to *in planta* conditions. Future research could involve investigating whether peripheral vesicles are produced in hyphae of *P. cinnamomi* infecting roots of resistant plantlets and also the sequence of events in inoculated seedlings, both resistant and susceptible. Sporangia are rarely produced when *P. cinnamomi* infects resistant plants (e.g. Cahill *et al.*, 1992) and, therefore, peripheral vesicles might be present in smaller numbers or be absent from hyphae

infecting these plants. Inoculated seedlings of susceptible and resistant plants, as opposed to micropropagated plantlets, could also be investigated.

Large peripheral vesicles were also labelled in oospores of *P. cinnamomi* isolates (6BR x P1) produced on carrot agar axenically, as well as in oospores formed in roots of chestnut plantlets inoculated *in vitro* with *P. cinnamomi* (6BR x P1) and with *P. cinnamomi* (6BR) or *P. cinnamomi* (P1) alone (Chapter 8). Future work might include, for example, observations of oospores germinating by germ tubes resulting in sporangia, rather than by germ tubes leading to mycelial growth only, as the former might be expected to contain all three vesicle types. These vesicles would then move into the sporangia and be compartmentalized in the zoospores.

The evidence provided in this and earlier studies suggests that these vesicles have an important role in the infection process and this role needs to be investigated further. The contents of the ventral vesicles, which are secreted on encystment, adhere the cyst to the plant root. Further studies could investigate the effect of different stimuli on the secretion of this material, with the aim of preventing cyst adhesion. The function of the material secreted from dorsal vesicles onto the outer surface of the cyst is unknown and further work is needed to identify the role of this material in the infection process.

The first report of oospores of *P. cinnamomi* being formed in the roots of micropropagated chestnut plantlets by individual isolates of A2 and A1 mating types was also made in this study (Chapter 8). *P. cinnamomi* is a heterothallic species, although a number of stimuli, for example root extracts (Zaki *et al.*, 1983), *Trichoderma* spp. (Reeves & Jackson, 1972) and chemical or mechanical damage (Reeves & Jackson, 1974), have been found to induce oospore formation in A2 isolates of *P. cinnamomi* (see Chapter 6). There are no known published reports of oospore formation in an A1 isolate of *P. cinnamomi* alone. Oospore formation may have been induced (in isolates 6BR and P1) in these chestnut roots by plant

hormones, similar to fungal hormones such as those produced by *P. cinnamomi* (Ko, 1978), although more research is needed to understand fully the mechanism of induction.

It would be interesting to induce germination of those oospores formed in chestnut roots. The parent culture(s) and progeny could then be examined, using molecular techniques such as those used by Förster & Coffey (1990), to determine whether they were the result of selfing or recombination. Genetic variation is known to be produced during sexual reproduction, through recombination, and fungicide-resistant strains of *P. infestans* are thought to have developed in this way (e.g. Shattock *et al.*, 1986). These pose a major threat to crops such as potato. Such fungicide resistant strains might, in future, be controlled by biocontrol agents, as discussed above.

In conclusion, this study has provided information on a number of aspects of *Phytophthora* root rot of chestnut in Australia. *P. citricola*, not previously recorded as a pathogen of chestnut has been found to cause disease on cvs grown in Australia. This study has highlighted the problems faced by growers due to the lack of resistant material which can be used for rootstocks. Methods such as the excised shoot assay using micropropagated material, could facilitate evaluation of material in breeding programs for resistance to *Phytophthora* spp.. Biological control, using antagonist fungi, is a possible alternative means of disease control, but further development of these antagonist fungi is needed before their potential in preventing disease can be properly assessed. A greater understanding of the infection process of susceptible chestnut plants by *P. cinnamomi* and of the developmental biology of this important pathogen was also gained through structural studies. These studies have opened a number of avenues for future research on the stimuli affecting pathogen development and reproduction in the host plant.

11. REFERENCES

Acuff, G. (1988). New rules will affect pesticide use. *American Vegetable Grower* **36**, 11-14.

Afek, U. & Sztejnberg, A. (1988). Accumulation of scoparone, a phytoalexin associated with resistance of citrus to *Phytophthora citrophthora*. *Phytopathology* **78**, 1678-1682.

Afek, U. & Sztejnberg, A. (1990). A rapid method for evaluating citrus seedlings for resistance to foot rot caused by *Phytophthora citrophthora*. *Plant Disease* **74**, 66-68.

Anagnostakis, S. L. (1992). Measuring resistance of chestnut trees to chestnut blight. *Canadian Journal of Forest Research* **22**, 568-571.

Andrews, J. H. (1992). Biological control in the phyllosphere. *Annual Review of Phytopathology* **30**, 603-635.

Ann, P. J. & Ko, W. H. (1988). Induction of oospore germination of *Phytophthora parasitica*. *Phytopathology* **78**, 335-338.

Anonymous (1992). Chestnut production tipped to rise but prices may fall. *Successful Horticulture* January-February 1992.

Ashby, S. F. (1929). Strains and taxonomy of *Phytophthora palmivora* Butler (*P. faberi* Maubl). *Transactions of the British Mycological Society* **14**, 18-38.

Aveling, T. A. S. & Rijkenberg, F. H. J. (1989). Behaviour of *Phytophthora cinnamomi* zoospores on roots of four avocado cultivars. *Journal of Phytopathology* **125**, 157-164.

Bailey, A. M. & Coffey, M. D. (1984). A sensitive bioassay for quantification of metalaxyl in soils. *Phytopathology* **74**, 667-669.

Baker, R. (1989). Some perspectives on the applications of molecular approaches to biocontrol problems. In: *Biotechnology of Fungi for Improving Plant Growth*. (ed. J. M. Whipps & R. D. Lumsden). pp 219-234. Cambridge University Press: Cambridge.

Baker, K. F. & Cook, R. J. (1974). *Biological Control of Plant Pathogens*. W. H. Freeman & Co.: San Francisco.

Banihashimi, Z. & Mitchell, J. E. (1976). Factors affecting oospore germination in *Phytophthora cactorum*, the incitant of apple collar rot. *Phytopathology* **66**, 443-448.

Banihashimi, Z. & Mitchell. J. E. (1980). Photobiology of oospores of *Phytophthora cactorum* and *P. citricola* (Abstr.). *Proceedings of the American Phytopathological Society* **14**, 88.

Barr, D. J. S. (1992). Evolution and kingdoms of organisms from the perspective of a mycologist. *Mycologia* **84**, 1-11.

Bartnicki-Garcia, S. & Hemmes, D. E. (1976). Some aspects of the form and function of Oomycete spores. In *The Fungal Spore: Form and Function* (ed. D. J. Weber & W. M. Hess) pp. 593-639. Wiley: New York.

Bartnicki-Garcia, S. & Wang, M. C. (1983). Biochemical aspects of morphogenesis in *Phytophthora*. In: *Phytophthora: its Biology, Taxonomy, Ecology and Pathology*. (ed. D. C. Erwin, S. Bartnicki-Garcia & P. H. Tsao). pp. 121-137. The American Phytopathological Society: St. Paul, Minnesota.

Bazzigher, G. & Miller, G. A. (1991). Blight-resistant chestnut selections in Switzerland: a valuable germ plasm source. *Plant Disease* **75**, 5-9.

Bell, D. K., Wells, H. D. & Markham, C. R. (1982). *In vitro* antagonism of *Trichoderma* species against six fungal plant pathogens. *Phytopathology* **72**, 379-382.

Benson, D. M. (1979). Efficacy and *in vitro* activity of two systemic acylalanines and ethazole for control of *Phytophthora cinnamomi* root rot of azalea. *Phytopathology* **69**, 174-78.

Benson, D. M. (1991). Detection of *Phytophthora cinnamomi* in azalea with commercial serological assay kits. *Plant Disease* **75**, 478-482.

Bissett, J. (1991). A revision of the genus *Trichoderma*. III. Section *Pachybasium*. *Canadian Journal of Botany* **69**, 2373-2417.

Boccas, B. R. (1981). Interspecific crosses between closely related heterothallic *Phytophthora* species. *Phytopathology* **71**, 60-65.

Borrod, G. (1971). Contribution à l'étude de la résistance au *Phytophthora cinnamomi* chez les cultures de tissues de Châtaignier. *Comptes Rendus Hebdomaires des Seances, Academie des Sciences Serie D- Sciences Naturelle* **272**, 1511-1514.

Bowers, J. H. & Mitchell, D. J. (1991). Relationship between inoculum level of *Phytophthora capsici* and mortality of pepper. *Phytopathology* **81**, 178-184.

Brasier, C. M. (1975). Stimulation of sex organ formation in *Phytophthora* by antagonistic species of *Trichoderma*. I. The effect *in vitro*. *New Phytologist* **74**, 183-194.

Brasier, C. M. & Brasier, C. (1978). *Transactions of the British Mycological Society* **70**, 297-302.

Brian, P. W. & Hemming, H. G. (1945). Gliotoxin, a fungistatic metabolite product of *Trichoderma viride*. *Annals of Applied Biology* **32**, 214-220.

Brian, P. W. & McGowan, J. G. (1945). Viridin: a highly fungistatic substance produced by *Trichoderma viride*. *Nature* **156**, 144.

Bruck, R. I., Fry, W. E. & Apple, A. E. (1980). Effect of metalaxyl, an acylalanine fungicide, on developmental stages of *Phytophthora infestans*. *Phytopathology* **70**, 597-601.

Burdsall, H. H. & Banik, M. (1990). Serological differentiation of three species of *Armillaria* and *Lentinula edodes* by enzyme-linked immunosorbent assay using immunized chickens as a source of antibodies. *Mycologia* **82**, 415-423.

Cahill, D. M., Bennett, I. J. & McComb, J. A. (1992). Resistance of micropropagated *Eucalyptus marginata* to *Phytophthora cinnamomi*. *Plant Disease* **76**, 630-632.

Cahill, D., Legge, N., Grant, B. & Weste, G. (1989). Cellular and histological changes induced by *Phytophthora cinnamomi* in a group of plant species ranging from fully susceptible to fully resistant. *Phytopathology* **79**, 417-424.

Cahill, D. M. & McComb, J. A. (1992). A comparison of changes in phenylalanine ammonia-lyase activity, lignin and phenolic synthesis in the roots of *Eucalyptus calophylla* (field resistant) and *E. marginata* (susceptible) when infected with *Phytophthora cinnamomi*. *Physiological and Molecular Plant Pathology* **40**, 315-332.

Cahill, D. & Weste, G. (1983). Formation of callose deposits as a response to infection with *Phytophthora cinnamomi*. *Transactions of the British Mycological Society* **80**, 23-29.

Chauvin, J. E. & Salesses, G. (1988). Advances in chestnut micropropagation (*Castanea* sp.). *Acta Horticulturae* **222**, 340-345.

Chet, I. (1987). Trichoderma-application, mode of action, and potential as a biocontrol agent of soilborne plant pathogenic fungi. In: *Innovative Approaches to Plant Disease Control*. (ed. I. Chet). pp. 137-160. John Wiley & Sons: New York.

Chet, I., Harman, G. E. & Baker, R. (1981). *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microbial Ecology* **7**, 29-38.

Chevre, A.-M., Gill, S. S., Mouras, A. & Salesses, G. (1983). *In vitro* vegetative micropropagation of chestnut. *Journal of Horticultural Science* **58**, 23-29.

Claydon, N., Allan, M., Hanson, J. R. & Avent, A. G. (1987). Antifungal alkyl pyrones of *Trichoderma harzianum*. *Transactions of the British Mycological Society* **88**, 503-513

Coffey, M. D. (1987). *Phytophthora* root rot of avocado: an integrated approach to control in California. *Plant Disease* **71**, 1046-1052.

Coffey, M. D. (1991). Strategies for the integrated control of soilborne *Phytophthora* species. In: *Phytophthora* (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw & L. R. Cooke). pp. 411-432. Cambridge University Press: Cambridge.

Coffey, M. D. & Joseph, M. C. (1985). Effects of phosphorous acid and fosetyl-Al on the lifecycle of *Phytophthora cinnamomi* and *Phytophthora citricola*. *Phytopathology* **75**, 1042-1046.

Coffey, M. D., Klure, L. J. & Bower, L. A. (1984). Variability in sensitivity to metalaxyl of isolates of *Phytophthora cinnamomi* and *Phytophthora citricola*. *Phytopathology* **74**, 417-422.

Cohen, Y. & Coffey, M. D. (1986). Systemic fungicides and the control of Oomycetes. *Annual Review of Phytopathology* **24**, 311-338.

Crandall, B. S., Gravatt, G. F. & Milburn-Ryan, M. (1945). Root disease of *Castanea* species and some coniferous and broadleaf nursery stocks caused by *Phytophthora cinnamomi*. *Phytopathology* **35**, 162-180.

Daft, G. C. & Tsao, P. H. (1984). Parasitism of *Phytophthora cinnamomi* and *P. parasitica* spores by *Catenaria anguillulae* in a soil environment. *Transactions of the British Mycological Society* **82**, 485-490.

Daub, M. E. (1986). Tissue culture and the selection of resistance to pathogens. *Annual Review of Phytopathology* **24**, 159-186.

Davidse, L. C., Danial, D. S. & van Westen, C. J. (1983). Resistance to metalaxyl in *Phytophthora infestans* in the Netherlands. *Netherlands Journal of Plant Pathology* **89**, 1-20.

Day, W. R. (1938). Root rot of sweet chestnut and beech caused by species of *Phytophthora*. *Forestry* **12**, 101-116.

Deacon, J. W. (1984). *Introduction to Modern Mycology*. Blackwell Scientific Publications: Oxford.

Dearnaley, J. D. W. (1993). *The formation and transport of peripheral vesicles in Phytophthora cinnamomi*. Ph.D. thesis, The Australian National University.

Decor, J. P. (1985). Strategie d'etude de nouveaux fongicides systemiques: exemple de phosethyl-Al. In: *Fungicides for Crop Protection, 100 Years of Progress*. Monograph No. 31. (ed. I. M. Smith). pp. 119-125. British Crop Protection Council Publication: Croydon, England.

Dennis, C. & Webster, J. (1971a). Antagonistic properties of species-groups of *Trichoderma*. I. Production of non-volatile antibiotics. *Transactions of the British Mycological Society* **57**, 25-39.

Dennis, C. & Webster, J. (1971b). Antagonistic properties of species-groups of *Trichoderma*. II. Production of volatile antibiotics. *Transactions of the British Mycological Society* **57**, 41-48.

Dennis, C. & Webster, J. (1971c). Antagonistic properties of species-groups of *Trichoderma*. III Hyphal interactions. *Transactions of the British Mycological Society* **57**, 363-369.

Dixon, K. W., Thinlay, & K. Sivasithamparam (1984). Technique for rapid assessment of tolerance of *Banksia* spp. to root rot caused by *Phytophthora cinnamomi*. *Plant Disease* **68**, 1077-1080.

Dolan, T. E., Cohen, Y. & Coffey, M. D. (1986). Protection of *Persea* species against *Phytophthora cinnamomi* and *P. citricola* by prior inoculation with a citrus isolate of *P. parasitica*. *Phytopathology* **76**, 194-198.

Dubos, B. & Ricard, J. L. (1974). Curative treatment of peach trees against silver leaf disease (*Stereum purpureum*) with *Trichoderma viride* preparations. *Plant Disease Reporter* **58**, 147, 150.

Duchesne, L. C., Peterson, R. L. & Ellis, B. E. (1989). The future of ectomycorrhizal fungi as biological control agents. *Phytoprotection* **70**, 51-58.

Duniway, J. M. (1983). Role of physical factors in the development of *Phytophthora* diseases. In: *Phytophthora. It's Biology, Taxonomy, Ecology and Pathology*. (ed. D. C. Erwin, S. Bartnicki-Garcia & P. H. Tsao). pp. 175-187. The American Phytopathological Society: St. Paul, Minnesota.

El-Hamalawi, Z. A. & Erwin, D. C. (1986). Components in alfalfa root extract and root exudate that increase oospore germination of *Phytophthora megasperma* f. sp. *medicagensis*. *Phytopathology* **76**, 508-513.

Elad, Y., Chet, I. & Henis, Y. (1982). Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Canadian Journal of Microbiology* **28**, 719-725.

Elad, Y., Chet, I., Boyle, P. & Henis, Y. (1983). Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium rolfsii* - scanning electron microscopy and fluorescence microscopy. *Phytopathology* **73**, 85-88.

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

Estrada-Garcia, M. T., Green, J. R., Booth, J. M., White, J. G. & Callow, J. A. (1989). Monoclonal antibodies to cell surface components of zoospores and cysts of the fungus *Pythium aphanidermatum* reveal species-specific antigens. *Experimental Mycology* **13**, 348-355.

Farih, A., Tsao, P. H. & Menge, J. A. (1981a). Fungitoxic activity of efosite aluminium on growth, sporulation, and germination of *Phytophthora parasitica* and *P. citrophthora*. *Phytopathology* **71**, 934-936.

Farih, A., Tsao, P. H. & Menge, J. A. (1981b). *In vitro* effects of metalaxyl on growth, sporulation, and germination of *Phytophthora parasitica* and *P. citrophthora*. *Plant Disease* **65**, 651-654.

Ferrin, D. M. & Kabashima, J. N. (1991). *In vitro* insensitivity of isolates of *Phytophthora citricola* and *P. parasitica* from ornamental hosts in southern California. *Plant Disease* **75**, 1041-1044.

Ferrin, D. M. & Rohde, R. G. (1992a). *In vivo* expression of resistance to metalaxyl by a nursery isolate of *Phytophthora parasitica* from *Catharanthus roseus*. *Plant Disease* **76**, 82-84.

Ferrin, D. M. & Rohde, R. G. (1992b). Population dynamics of *Phytophthora parasitica*, the cause of root and crown rot of *Catharanthus roseus*, in relation to fungicide use. *Plant Disease* **76**, 60-63.

Ferrin, D. M. & Wadsworth, M. L. (1992). Effect of metalaxyl on sporulation and growth of metalaxyl-resistant and metalaxyl-sensitive isolates of *Phytophthora parasitica in vitro*. *Plant Disease* **76**, 492-495.

Finlay, A. R. & McCracken, A. R. (1991). Microbial suppression of *Phytophthora cinnamomi*. In: *Phytophthora*. (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw & L. R. Cooke). pp. 383-398. Cambridge University Press: Cambridge.

Förster, H. & Coffey, M. D. (1990). Mating behaviour in *Phytophthora parasitica*: evidence for sexual recombination in oospores using DNA restriction fragment length polymorphisms as genetic markers. *Experimental Mycology* **14**, 351-359.

Förster, H., Ribeiro, O. K. & Erwin, D. C. (1983). Germination of oospores of *Phytophthora megasperma* f. sp. *medicagensis*. *Phytopathology* **73**, 442-448.

Franco, C. M. M. & Coutinho, L. E. L. (1991). Detection of novel secondary metabolites. *Critical Reviews in Biotechnology* **11**, 193-276.

Fry, W. E. (1980). Integration of host resistance and pesticides to manage disease. *Protection Ecology* **2**, 259-264.

Gabor, B. K. & Coffey, M. D. (1991). Comparison of rapid methods for evaluating resistance to *Phytophthora cinnamomi* in avocado rootstocks. *Plant Disease* **75**, 118-120.

Gabor, B. K., Guillemet, F. B. & Coffey, M. D. (1990). Comparison of field resistance to *Phytophthora cinnamomi* in twelve avocado rootstocks. *HortScience* **25**, 1655-1656.

Galindo, A. J. & Gallegly, M. E. (1960). The nature of sexuality in *Phytophthora infestans*. *Phytopathology* **50**, 123-128.

Gees, R. & Coffey, M. D. (1989). Evaluation of a strain of *Myrothecium roridum* as a potential biocontrol agent against *Phytophthora cinnamomi*. *Phytopathology* **79**, 1079-1084.

Gerretson-Cornell, L. (1991). Actinomycetes interaction with *Phytophthora cinnamomi* Rands. *Phytophthora Newsletter* **17**, 31.

Ghisalberti, E. L. & Sivasithamparam, K. (1991). Antifungal antibiotics produced by *Trichoderma* spp. *Soil Biology and Biochemistry* **23**, 1011-1020.

Gisi, U., Zentmyer, G. A. & Klure, L. J. (1980). Production of sporangia of *Phytophthora cinnamomi* and *P. palmivora* in soils at different matric potentials. *Phytopathology* **70**, 301-306.

Gregg, M. (1957). Germination of oospores of *Phytophthora erythroseptica*. *Nature* (London) **180**, 150.

Grente, J. (1961a). La maladie de l'encre du chataignier. I. Etiologie et biologie. *Annales des Epiphyties* **12**, 1-24.

Grente, J. (1961b). La maladie de l'encre du chataignier. II. Les agents pathogenes. *Annales des Epiphyties* **12**, 25-29.

Griffith, J. M., Davis, A. J. & Grant, B. R. (1992). Target sites of fungicides to control Oomycetes. In: *Target Sites of Fungicide Action*. (ed. W. Köller). pp. 69-100. CRC Press: Boca Raton.

Grimm, G. R. & Hutchison, D. J. (1973). A procedure for evaluating resistance of citrus seedlings to *Phytophthora parasitica*. *Plant Disease Reporter* **57**, 669-672.

Gubler, F. & Hardham, A. R. (1988). Secretion of adhesive material during encystment of *Phytophthora cinnamomi* zoospores, characterized by immunogold labelling with monoclonal antibodies to components of peripheral vesicles. *Journal of Cell Science* **90**, 225-235.

Gubler, F. & Hardham, A. R. (1990). Protein storage in large peripheral vesicles in *Phytophthora* zoospores and its breakdown after cyst germination. *Experimental Mycology* **14**, 393-404.

Gubler, F. & Hardham, A. R. (1991). The fate of peripheral vesicles in zoospores of *Phytophthora cinnamomi* during infection of plants. In: *Electron Microscopy of Plant Pathogens*. (ed. K. Mengden & D.-E. Lesemann). pp. 197-210. Springer-Verlag: Berlin.

Gupta, J. P., Erwin, D. C., Eckert, J. W. & Zaki, A. I. (1985). Translocation of metalaxyl in soybean plants by *Phytophthora megasperma* f. sp. *glycinea*. *Phytopathology* **75**, 865-869.

Hardham, A. & Gubler, F. (1990). Polarity of attachment of zoospores of a root pathogen and pre-alignment of the emerging germ tube. *Cell Biology International Reports* **14**, 947-956.

Hall, G. (1993). An integrated approach to the analysis of variation in *Phytophthora nicotianae* and a redescription of the species. *Mycological Research* **5**, 559-574.

Hardham, A. R., Gubler, F., Duniec, J. & Elliott, J. (1991). A review of methods for the production and use of monoclonal antibodies to study zoosporic plant pathogens. *Journal of Microscopy* **162**, 305-318.

Hardham, A. R., Suzaki, E. & Perkin, J. L. (1986). Monoclonal antibodies to isolate-, species- and genus-specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*. *Canadian Journal of Botany* **64**, 311-321.

Hardy, G. St. J. & Sivasithamparam, K. (1991). Suppression of *Phytophthora* root rot by a composted *Eucalyptus* bark mix. *Soil Biology & Biochemistry* **39**, 153-159.

Harrison, J. G., Barker, H., Lowe, R. & Rees, A. E. (1990). Estimation of amounts of *Phytophthora infestans* mycelium in leaf tissue by enzyme-linked immunosorbent assay. *Plant Pathology* **39**, 274-277.

Helgeson, J. P., Kemp, J. O., Haberlach, G. T. & Maxwell, D. P. (1972). A tissue culture system for studying disease resistance: the black shank disease in tobacco callus cultures. *Phytopathology* **62**, 1439-1443.

Hemmes, D. E. (1983). Cytology of *Phytophthora*. In: *Phytophthora: its Biology, Taxonomy, Ecology and Pathology*. (ed. D. C. Erwin, S. Bartnicki-Garcia & P. H. Tsao). pp. 41-54. The American Phytopathological Society: St. Paul, Minnesota.

Henis, Y., Ghaffar, A. & Baker, R. (1978). Integrated control of *Rhizoctonia solani* damping-off of radish: effect of successive planting, PCNB and *Trichoderma harzianum* on pathogen and disease. *Phytopathology* **68**, 900-907.

Hinch, J. M., Wetherbee, R., Mallett, J. E. & Clarke, A. E. (1985). Response of *Zea mays* roots to infection with *Phytophthora cinnamomi*. I. The epidermal layer. *Protoplasma* **126**, 178-187.

Ho, H. H., Zachariah, K. & Hickman, C. J. (1968). The ultrastructure of zoospores of *Phytophthora megasperma* var. *sojae*. *Canadian Journal of Botany* **46**, 37-41.

Ho, H. H. & Zentmyer, G. A. (1977) Morphology of *Phytophthora cinnamomi*. *Mycologia* **69**, 701-703.

Hohl, H. R. (1991). Surface-related host-pathogen interactions in *Phytophthora*. In: *Phytophthora*. (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw & L. R. Cooke). pp. 41-54. Cambridge University Press: Cambridge.

Hord, M. J. & Ristaino, J. B. (1991). Effects of physical and chemical factors on the germination of oospores of *Phytophthora capsici in vitro*. *Phytopathology* **81**, 1541-1546.

Howell, C. R. & Stipanovic, R. D. (1983). Gliovirin, a new antibiotic from *Gliocladium virens* and its role in the biological control of *Pythium ultimum*. *Canadian Journal of Microbiology* **29**, 321-324.

Howell, C. R. & Stipanovic, R. D. (1984). Phytotoxicity to crop plants and herbicidal effects on weeds of viridiol produced by *Gliocladium virens*. *Phytopathology* **74**, 1346-1349.

Howell, C. R. (1982). Effect of *Gliocladium virens* on *Pythium ultimum, Rhizoctonia solani*, and damping-off of cotton seedlings. *Phytopathology* **72**, 496-498.

Jang, J. C. & Tainter, F. H. (1990). Hyphal growth of *Phytophthora cinnamomi* on pine callus tissue. *Plant Cell Reports* **8**, 741-744.

Jang, J. C. & Tainter, F. H. (1991). Optimum tissue culture conditions for selection of resistance to *Phytophthora cinnamomi* in pine callus tissue. *Plant Cell Reports* **9**, 488-491.

Jeffers, S. N. (1992). Preplant root treatments to reduce the incidence of *Phytophthora* species on dormant apple rootstocks. *Plant Disease* **76**, 12-19.

Jeffers, S. N., Aldwinckle, H. S., Burr, T. J. & Arneson, P. A. (1981). Excised twig assay for the study of apple tree crown rot pathogens *in vitro*. *Plant Disease* **65**, 823-825.

Jiang, J. & Erwin, D. C. (1993). The effects of nutrients on germination of cold-treated oospores of *P. cactorum in vitro*. *Mycological Research* **97**, 293-298.

Kaosiri, T. Zentmyer, G. A. & Erwin, D. C. (1980). Oospore morphology and germination in the *Phytophthora palmivora* complex from cacao. *Mycologia* **72**, 888-907.

Keen, N. T. & Yoshikawa, M. (1983). Physiology of disease and the nature of resistance in *Phytophthora*. In: *Phytophthora: its Biology, Taxonomy, Ecology and Pathology.* (ed. D. C. Erwin, S. Bartnicki-Garcia & P. H. Tsao). pp. 279-287. The American Phytopathological Society: St. Paul, Minnesota.

Kellam, M. K. & Coffey, M. D. (1985). Quantitative comparison of the resistance to *Phytophthora* root rot in three avocado rootstocks. *Phytopathology* **75**, 230-234.

Kellens, J. T. & Peumans, W. J. (1991). Biochemical and serological comparison of lectins from different anastomosis groups of *Rhizoctonia solani*. *Mycological Research* **95**, 1235-1241.

Kelley, W. D. (1976). Evaluation of *Trichoderma harzianum*-impregnated clay granules as a biocontrol for *Phytophthora cinnamomi* causing damping-off of pine seedlings. *Phytopathology* **66**, 1023-1027.

Kelley, W. D. & Rodriguez-Kabana, R. (1976). Competition between *Phytophthora cinnamomi* and *Trichoderma* spp. in autoclaved soil. *Canadian Journal of Microbiology* **22**, 1120-1127.

Ko, W. H. (1978). Heterothallic *Phytophthora*: evidence for hormonal regulation of sexual reproduction. *Journal of General Microbiology* **107**, 15-18.

Ko, W. H. & Chang, H. S. (1979). *Phytophthora katsurae*, a new name for *P. castaneae*. *Mycologia* **71**, 840-844.

Kope, H. H., Tsantrizos, Y. S., Fortin, J. A. & Ogilvie, K. K. (1991). p-Hydroxybenzoylformic acid and (R)-(-)-p-hydroxymandelic acid, two antifungal compounds isolated from the liquid culture of the ectomycorrhizal fungus *Pisolithus arhizus*. *Canadian Journal of Microbiology* **37**, 258-264.

Kuhn, P. J., Albert, G., Wyness, L. E., Lee, S. A. & Lindsley, M. C. (1989a). Studies on the antifungal activity *in vitro* of dimethomorph, a novel fungicide active against downy mildews and *Phytophthora*. (Abstr.). *9th International Symposium on Systemic Fungicides and Antifungal Compounds*, Reinhardsbrunn, GDR.

Kuhn, P. J., Westwell, J. M., Albert, G., Lee, S. A. & Wyness, L. E. (1989b). Effects of dimethomorph on the growth and development of *Phytophthora infestans in vitro* and *in vivo*. (Abstr.). *International Symposium on Phytophthora*. Dublin, Eire.

Lahdenpera, M. L. (1987). Streptomyces - a new tool for controlling plant diseases. Product information brochure.

Lederer, W., Lorenz, K.-H. & Seemuller, E. (1992). Studies on the antagonistic effects of *Trichoderma* isolates against *Phytophthora cactorum*. *Journal of Phytopathology* **136**, 154-164.

Lewis, J. A. & Papavizas, G. C. (1985). Characteristics of alginate pellets formulated with *Trichoderma* and *Gliocladium* and their effect on the proliferation of the fungi in soil. *Plant Pathology* **34**, 517-577.

Lewis, K., Whipps, J. M. & Cooke, R. C. (1989). Mechanisms of biological disease control with special reference to the case study of *Pythium oligandrum* as an antagonist. In: *Biotechnology of Fungi for Improving Plant Growth*. (ed. J. M. Whipps & R. D. Lumsden). pp. 191-218. Cambridge University Press: Cambridge.

Locke, J. C., Marois, J. J. & Papavizas, G. C. (1985). Biological control of *Fusarium* wilt of greenhouse-grown chrysanthemums. *Plant Disease* **69**, 167-169.

Lowe, K. F., Ryley, M. J. & Bowdler, T. M. (1991). Lack of control of *Phytophthora* root rot in lucerne (*Medicago sativa*) by foliar applications of phosphonate. *Australian Journal of Experimental Agriculture* **31**, 503-507.

Luck, B. T., Breuil, C. & Brown, D. L. (1990). Immunological discrimination between a sap-staining fungus and a biological control fungus. *Canadian Journal of Botany* **68**, 1578-1588.

Lumsden, R. D. & Lewis, J. A. (1989). Selection, formulation and commercial use of plant disease biocontrol fungi: problems and progress. In: *Biotechnology of Fungi for Improving Plant Growth*. (ed. J. M. Whipps & R. D. Lumsden). pp. 171-190. Cambridge University Press: Cambridge.

Lynch, J. M. (1987). Biological control within microbial communities of the rhizosphere. In: *Ecology of Microbial Communities*. (ed. M. Fletcher, T. R. G. Gray & J. G. Jones). Cambridge University Press: Cambridge.

MacDonald, J. D. & Duniway, J. M. (1979). Use of fluorescent antibodies to study the survival of *Phytophthora megasperma* and *Phytophthora cinnamomi* zoospores in soil. *Phytopathology* **69**, 436-441.

Malajczuk, N. (1979). The microflora of unsuberized roots of *Eucalyptus calophylla* R.Br. and *E. marginata* Donn. ex Sm. seedlings grown in soils suppressive and conducive to *Phytophthora cinnamomi* Rands. II. Mycorrhizal roots and associated microflora. *Australian Journal of Botany* **27**, 255-272.

Malajczuk, N., Boughton, T. J., Campbell, N. A. & Litchfield, R. T. (1983). Interactions between the fungicide metalaxyl and soil microorganisms on survival of *Phytophthora cinnamomi*. *Annals of Applied Biology* **103**, 57-61.

Malajczuk, N., McComb, A. J. & Parker, C. A. (1977). Infection by *Phytophthora cinnamomi* of roots of *Eucalyptus calophylla* and *Eucalyptus marginata*. *Australian Journal of Botany* **25**, 483-500.

Malajczuk, N., Pearce, M. & Litchfield, R. T. (1984). Interactions between *Phytophthora cinnamomi* and *Rhizobium* isolates. *Transactions of the British Mycological Soc*iety **82**, 491-500.

Marais, L. J. & Kotze, J. M. (1976). Ectomycorrhizae of *Pinus patula* as biological deterrents to *Phytophthora cinnamomi*. *South African Forestry Journal* **99**, 35-39.

Marks, G. C. & Smith, I. W. (1991). *The Cinnamon Fungus in Victorian Forests: History, Distribution, Management and Control.* Lands and Forests Bulletin No. 31. Department of Conservation and Environment: Victoria.

Martins, A. & Pais, M. S. (1992). Growth parameters of mycorrhized micropropagated *Castanea sativa* plants after transplanting to field conditions. (Abstr.) *The International Symposium on Management of Mycorrhizas in Agriculture, Horticulture and Forestry*, Perth, Aust.

Marx, D. H. (1970). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. V. Resistance of mycorrhizae to infection by vegetative mycelium of *Phytophthora cinnamomi*. *Phytopathology* **60**, 1472-1473.

Marx, D. H. (1975). The role of ectomycorrhizae in the protection of pine from root infection by *Phytophthora cinnamomi*. In: *Biology and Control of Soilborne Plant Pathogens*. (ed. G. W. Bruehl). pp. 112-115. American Phytopathological Society.

Marx, D. H. & Davey, C. B. (1968a). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. III. Resistance of aseptically formed mycorrhizae to infection by *Phytophthora cinnamomi*. *Phytopathology* **59**, 549-558.

Marx, D. H. & Davey, C. B. (1968b). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. IV. Resistance of naturally occurring mycorrhizae to infections by *Phytophthora cinnamomi*. *Phytopathology* **59**, 559-565.

Matheron, M. E. & Mircetich, S. M. (1985). Relative resistance of different rootstocks of english walnut to six *Phytophthora* spp. that cause root and crown rot in orchard trees. *Plant Disease* **69**, 1039-1041.

McComb, J. A., Hinch, J. M. & Clarke, A. E. (1987). Expression of field resistance in callus tissue inoculated with *Phytophthora cinnamomi*. *Phytopathology* **77**, 346-351.

McIntosh, D. L. (1964). *Phytophthora* spp. in soils of the Okanagan and Similkameen valleys of British Columbia. *Canadian Journal of Botany* **42**, 141-145.

McIntosh, D. L. (1975). Proceedings of the 1974 APDW workshop on crown rot of apple trees. *Canadian Plant Disease Survey* **55**, 109-116.

Merwin, I. A., Wilcox, W. F. & Stiles, W. C. (1992). Influence of orchard ground cover management on the development of *Phytophthora* crown and root rots of apple. *Plant Disease* **76**, 199-205.

Milburn, M. & Gravatt, G. I. (1932). Preliminary note on a *Phytophthora* root disease of chestnut. *Phytopathology* **22**, 977-978.

Miller, S. A. & Maxwell, D. P. (1984a). Light microscope observations of susceptible, host resistant, and nonhost resistant interactions of alfalfa with *Phytophthora megasperma. Canadian Journal of Botany* **62**, 109-116.

Miller, S. A. & Maxwell, D. P. (1984b). Ultrastructure of susceptible, host resistant, and nonhost resistant interactions of alfalfa with *Phytophthora megasperma. Canadian Journal of Botany* **62**, 117-128.

Millholland, R. D. & Daykin, M. E. (1993). Colonization of roots of strawberry cultivars with different levels of susceptibility to *Phytophthora fragariae*. *Phytopathology* **83**, 538-542.

Mircetich, S. M. (1981). *Phytophthora* root and crown rot of deciduous fruit trees in California. *Proceedings of the Annual British Colombia Fruit Growers Association* **13**, 1-14.

Mircetich, S. M. & Zentmyer, G. A. (1966). Production of oospores and chlamydospores of *Phytophthora cinnamomi* in roots and soil. *Phytopathology* **56**, 1076-1078.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 475-97.

O'Brien, I. G. (1991). Induced homothallism in *P. cinnamomi*: a bioassay for volatile bioactive compounds. *Phytochemistry* **30**, 3545-3548.

Ownley, B. H. & Benson, D. M. (1992). Evaluation of *Penicillium janthinellum* as a biological control agent of *Phytophthora* root rot of azalea. *Journal of the American Society of Horticultural Science* **117**, 407-410.

Palloix, A., Daubeze, A. M. & Pochard, E. (1988a). Time sequences of root infection and resistance expression in an artificial inoculation method of pepper with *P. capsici*. *Journal of Phytopathology* **123**, 12-24.

Palloix, A., Daubeze, A. M. & Pochard, E. (1988b). *Phytophthora* root rot of pepper: influence of host genotype and pathogen strain on the inoculum density-disease severity relationships. *Journal of Phytopathology* **123**, 25-33.

Paxton, J. D. (1981). Phytoalexins-a working redefinition. *Phytopathologische Zeitschrift* **101**, 106-109.

Pegg, K. G., Whiley, A. W., Saranah, J. B. & Glass, R. J. (1985). Control of *Phytophthora* root rot of avocado with phosphorous acid. *Australasian Plant Pathology* **14**, 25-29.

Pelczar, M. J., Chan, E. C. S. & Krieg, N. R. (1988). *Microbiology*. 5th ed. McGraw-Hill Book Co.: New York.

Phillips, D. & Weste, G. (1985). Growth rates of four Australian isolates of *Phytophthora cinnamomi* in relation to temperature. *Transactions of the British Mycological Society* **84**, 183-85.

Phillips, D., Weste, G. & Hinch, J. M. (1991). Resistance to *Phytophthora cinnamomi* in callus tissue derived from three avocado cultivars. *Canadian Journal of Botany* **69**, 2026-2032.

Phillips, J. M. & Hayman, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* **55**, 158-161.

Piedallu, M.-A. & Jamet, P. (1985). Cinetique de disparition et degradation du phosethyl-Al dans le sol en conditions controlees. In: *Fungicides for Crop Protection, 100 Years of Progress*. Monograph No. 31. (ed. I. M. Smith). pp. 297-300. British Crop Protection Council Publication: Croydon.

Pscheidt, J. W., Burket, J. Z., Fischer, S. L. & Hamm, P. B. (1992). Sensitivity and clinical use of *Phytophthora* -specific immunoassay kits. *Plant Disease* **76**, 928-932.

Reeves, R. J. & Jackson, R. M. (1972). Induction of *Phytophthora cinnamomi* oospores in soil by *Trichoderma viride*. *Transactions of the British Mycological Soc*iety **59**, 156-159.

Reeves, R. J. & Jackson, R. M. (1974). Stimulation of sexual reproduction in *Phytophthora* by damage. *Journal of General Microbiology* **84**, 303-310.

Reichard, M. & Bolay, A. (1986). La maladie de l'encre du châtaignier dans le canton de Genève. *Revue suisse de viticulture arboriculture horticulture* **18**, 243-250.

Reichle, R. E. (1969). Fine structure of *Phytophthora parasitica* zoospores. *Mycologia* **61**, 30-51.

Renwick, A. & Poole, N. (1989). The environmental challenge to biological control of plant pathogens. In: *Biotechnology of Fungi for Improving Plant Growth*. (ed. J. M. Whipps & R. D. Lumsden). pp. 277-290. Cambridge University Press: Cambridge.

Ribeiro, O. K. (1978). *A Sourcebook of the Genus Phytophthora*. J. Cramer: Lehre, Germany.

Ribeiro, O. K. (1983). Physiology of asexual sporulation and spore germination in *Phytophthora*. In: *Phytophthora: its Biology, Taxonomy, Ecology and Pathology*. (ed. D. C.

Erwin, S. Bartnicki-Garcia & P. H. Tsao). pp. 55-70. The American Phytopathological Society. St. Paul, Minnesota.

Ribeiro, O. K., Erwin, D. C. & Zentmyer, G. A. (1975a). An improved synthetic medium for oospore production and germination of several *Phytophthora* species. *Mycologia* **67**, 1012-1019.

Ribeiro, O. K., Gallegly, M. E. & Young, R. J. (1971). Oospore production, viability and germination in relation to establishment of F2 cultures of *Phytophthora infestans*. (Abstr.) *Phytopathology* **61**, 907-908.

Ribeiro, O. K. & Linderman, R. G. (1989). Chemical and biological control of *Phytophthora* species in woody plants. In: *Phytophthora*. (ed. J. A. Lucas, R. C. Shattock, D. S. Shaw & L. R. Cooke). pp. 399-410. Cambridge University Press: Cambridge.

Ribeiro, O. K., Zentmyer, G. A. & Erwin, D. C. (1975b). Comparative effects of monochromatic radiation on the germination of oospores of the three *Phytophthora* species. *Phytopathology* **65**, 904-907.

Ribeiro, O. K., Zentmyer, G. A. & Erwin, D. C. (1976). The influence of qualitative and quantitative radiation on reproduction and spore germination of four *Phytophthora* species. *Mycologia* **68**, 972-977.

Ricard, J. L. (1981). Commercialization of a *Trichoderma* based mycofungicide: some problems and solutions. *Biocontrol News and Information* **2**, 95-98.

Ridout, C. J., Coley-Smith, J. R. & Lynch, J. M. (1988). Fractionation of extracellular enzymes from a mycoparasitic strain of *Trichoderma harzianum*. *Enzyme & Microbial Technology* **10**, 180-187.

Rifai, M. (1969). A revision of the genus *Trichoderma*. *Mycological Papers*, CMI. Association of Applied Biologists: Kew, Surrey, UK.

Rishbeth, J. (1975). Stump inoculation: a biological control of *Fomes annosus*. In: *Biology and Control of Soilborne Plant Pathogens*. (ed. G. W. Bruehl). pp. 158-162. American Phytopathological Society: St Paul, Minnesota.

Robin, C. (1991). Comparative study of strains of *Phytophthora cinnamomi*: growth rates, pathogenicity & RFLP. *Phytophthora Newsletter* **17**, 34.

Rodríguez, R. (1982). *In vitro* propagation of *Castanea sativa* Mill. through meristem-tip culture. *HortScience* **17,** 888-889.

Roiger, D. J. & Jeffers, S. N. (1991). Evaluation of *Trichoderma* spp. for biological control of *Phytophthora* crown and root rot of apple seedlings. *Phytopathology* **81**, 910-917.

Ross, E. W. & Marx, D. H. (1972). Susceptibility of sand pine to *Phytophthora cinnamomi*. *Phytopathology* **62**, 1197-1200.

Salvatore, M. A., Gray, F. A. & Hine, R. B. (1973). Enzymatically induced germination of oospores of *Phytophthora megasperma*. *Phytopathology* **63**, 1083-1084.

Sansome, E. R. (1965). Meiosis in diploid and polyploid sex organs of *Phytophthora* and *Achlya*. *Cytologia* **30**, 103-117.

Sansome, E. R. (1976). Gametangial meiosis in *Phytophthora capsici*. *Canadian Journal of Botany* **54**, 1535-1545.

Savage, E. J., Clayton, C. W., Hunter, J. H., Brenneman, J. A., Laviola, C. & Gallegly, M. E. (1968). Homothallism, heterothallism and interspecific hybridization in the genus *Phytophthora*. *Phytopathology* **58**, 1004-1021.

Schutte, G. C., Bezuidenhout, J. J. & Kotze, J. M. (1991). Timing of application of phosphonate fungicides using different application methods as determined by means of gas-liquid-chromatography for *Phytophthora* root rot control of citrus. *Phytophylactica* **23**, 69-71.

Scott, E. S., Wicks, T. J. & Lee, T. C. (1992). The development of an assay for resistance to *Phytophthora cambivora* in almond rootstocks using shoots excised from tissue cultures. *Plant Pathology* **41**, 639-645.

Shane, W. W. (1991). Prospects for early detection of *Pythium* blight epidemics of turfgrass by antibody-aided monitoring. *Plant Disease* **75**, 921-925.

Sharma, N. K. & Skidmore, D. I. (1988). *In vitro* expression of partial resistance to *Phytophthora palmivora* by shoot cultures of papaya. *Plant Cell, Tissue & Organ Culture* **14**, 187-196.

Shattock, R. C., Tooley, P. W. & Fry, W. E. (1986). Genetics of *Phytophthora infestans*: characterization of single-oospore cultures from A1 isolates induced to self by intra-specific stimulation. *Phytopathology* **76**, 407-410.

Shattock, R. C., Tooley, P. W., Sweigard, J. & Fry, W. E. (1987). Genetic studies of *Phytophthora infestans*. In: *Genetics and Plant Pathogenesis*. (ed. P. R. Day & G. J. Jellis). Blackwell Scientific Publications: Oxford.

Shaw, D. C. (1988). The *Phytophthora* species. *Advances in Plant Pathology* **6**, 27-51.

Shearer, B. L. & Tippett, J. T. (1988). Distribution and impact of *Armillaria luteobubalina* in the Eucalyptus forest of south-western Australia. *Australian Journal of Botany* **36**, 433-445.

Sing, V. O. & Bartnicki-Garcia, S. (1975a). Adhesion of *Phytophthora palmivora* zoospores: electron microscopy of cell attachment and cyst wall fibril formation. *Journal of Cell Science* **18**, 123-132.

Sing, V. O. & Bartnicki-Garcia, S. (1975b). Adhesion of *Phytophthora palmivora* zoospores: detection and ultrastructural vizualization of Concanavalin A-receptor sites appearing during encystment. *Journal of Cell Science* **19**, 11-20.

Sivan, A., Elad, Y. & Chet, I. (1984). Biological control effects of a new isolate of *Trichoderma harzianum* on *Pythium aphanidermatum*. *Phytopathology* **74**, 498-501.

Sivasithamparam, K. (1991). Management of root rot. In: *Proceedings of the 6th International Protea Association Biennial Conference*, Perth, Australia. pp. 283-287.

Sivasithamparam, K. & Goss, O. M. (1980). Jarrah dieback-a threat to horticulture. *Journal of Agriculture of Western Australia* **21**, 88-91.

Sivasithamparam, K., Parker, C.A. & Edwards, C. S. (1979). Rhizosphere microorganisms of seminal and nodal roots of wheat grown in pots. *Soil Biology and Biochemistry* **11**, 155-160.

Skoudridakis, M. T. & Bourbos, V. A. (1990). Comparative effectiveness of metalaxyl and phosethyl Al to *Phytophthora cambivora* (Petri) Buism.. *Medelingen van Faculeit Landbouwwetenschappen, Rijksuniversiteit Gent* **55**, 963-966.

Smith, G. S., Hutchison, D. J. & Henderson, C. T. (1991). Comparative use of soil infested with chlamydospores to screen for relative susceptibility to *Phytophthora* foot rot in citrus cultivars. *Plant Disease* **75**, 402-405.

Smith, V. L., Wilcox, W. F. & Harman, G. E. (1990). Potential for biological control of *Phytophthora* root and crown rots of apple by *Trichoderma* and *Gliocladium* spp.. *Phytopathology* **80**, 880-885.

Son, C. (1992). *The use of tissue culture and molecular techniques to assess variation within Phytophthora citrophthora and screen for resistance in its citrus host.* Ph.D. thesis, University of Adelaide.

Stamps, D. J., Waterhouse, G. M., Newhook, F. J. & Hall, G. S. (1990). Revised tabular key to the species of *Phytophthora*. *Mycological Papers* **62**, 1-28.

Stewart, A. & McCarrison, A. M. (1992). The role of *Phytophthora cryptogea* and waterlogging in root rot of kiwifruit. *Journal of Phytopathology* **136**, 194-198.

Stirling, A. M., Hayward, A. C. & Pegg, K. G. (1992). Evaluation of the biological control potential of bacteria isolated from a soil suppressive to *Phytophthora cinnamomi*. *Australasian Plant Pathology* **21**, 133-141.

Sulistyowati, L., Keane, P. J. & Anderson, J. W. (1990). Accumulation of the phytoalexin, 6,7-dimethoxycoumarin, in roots and stems of citrus seedlings following inoculation with *Phytophthora citrophthora*. *Physiological and Molecular Plant Pathology* **37**, 451-461.

Takenaka, S. (1992). Use of immunological methods with antiribosome serum to detect snow mold fungi in wheat plants. *Phytopathology* **82**, 896-901.

Taylor, P. A. & Washington, W. S. (1984). Curative treatments for *Phytophthora cactorum* in peach trees using metalaxyl and phosethyl Al. *Australasian Plant Pathology* **13**, 31-33.

Tidball, C. J. & Linderman, R. G. (1990). *Phytophthora* root and stem rot of apple rootstocks from stool beds. *Plant Disease* **74**, 141-146.

Tippett, J. T. & Hill, T. C. (1984). Role of periderm in resistance of *Eucalyptus marginata* roots against *Phytophthora cinnamomi. European Journal of Forest Pathology* **14**, 431-439.

Tippett, J. T. & O'Brien, T. P. (1976). The structure of eucalypt roots. *Australian Journal of Botany* **24**, 619-632.

Tippett, J. T., Shea, S. R., Hill, T. C. & Shearer, B. L. (1983). Development of lesions caused by *Phytophthora cinnamomi* in the secondary phloem of *Eucalyptus marginata. Australian Journal of Botany* **31**, 197-210.

Tsantrizos, Y. S., Kope, H. H., Fortin, J. A. & Ogilvie, K. K. (1991). Antifungal antibiotics from *Pisolithus tinctorius*. *Phytochemistry* **30**, 1113-1118.

Turnbull, L. V., Ogle, H. J., Stirling, A. M. & Dart, P. J. (1992). Preliminary investigations into the influence of *Pseudomonas cepacia* on infection and survival of proteas in *Phytophthora cinnamomi* infected potting mix. *Scientia Horticulturae* **52**, 257-263.

Tuset, J. J., Hinarejos, C. & Garcia, J. (1990). *Phytophthora* foot rot control in citrus with *Myrothecium roridum. EPPO Bulletin* **20**, 169-176.

Utkhede, R. S. (1987). Chemical and biological control of crown and root rot of apple caused by *Phytophthora cactorum. Canadian Journal of Plant Pathology* **9**, 295-300.

Utkhede, R. S. & Sholberg, P. L. (1986). *In vitro* inhibition of plant pathogens by *Bacillus subtilis* and *Enterobacter aerogenes* and *in vivo* control of two postharvest cherry diseases. *Canadian Journal of Microbiology* **32**, 963-967.

Utkhede, R. S. & Smith, E. M. (1991a). Biological and chemical treatments for control of *Phytophthora* crown and root rot caused by *Phytophthora cactorum* in a high density apple orchard. *Canadian Journal of Plant Pathology* **13**, 267-270.

Utkhede, R. S. & Smith, E. M. (1991b). Effects of fosetyl-Al, metalaxyl, and *Enterobacter aerogenes* on crown and root rot of apple trees caused by *Phytophthora cactorum* in British Columbia. *Plant Disease* **75**, 406-409.

Utkhede, R. S. & Smith, E. M. (1993). Long-term effects of chemical and biological treatments on crown and root rot of apple trees caused by *Phytophthora cactorum*. *Soil Biology and Biochemistry* **25**, 383-386.

Vanderplank, J. E. (1978). *Genetic and Molecular Basis of Plant Pathogenesis*. Springer-Verlag: Berlin.

Vidal, M. T., Azcón-Aguilar, C. & Barea, J. M. (1992). Mycorrhizal inoculation enhances growth and development of micropropagated plants of avocado. *HortScience* **27**, 785-787.

Vieitez, A. M., , Sánchez, C. & San-José, C. (1989). Prevention of shoot-tip necrosis in shoot cultures of chestnut and oak. *Scientia Horticulturae* **41**, 151-159.

Vieitez, A. M. & Vieitez, E. (1980). Plantlet formation from embryonic tissue of chestnut grown *in vitro*. *Physiologia Plantarum* **50**, 127-130.

Vieitez, A. M., Vieitez, M. L. & Vieitez, E. (1986). Chestnut (*Castanea* spp.). In: *Biotechnology in Agriculture and Forestry. I. Trees.* (ed. Y. P. S. Bajaj). pp. 393-414. Springer-Verlag: Berlin.

Vogell, H. J., Thompson, J. S. & Shockman, G. D. (1970). Characteristic metabolic patterns of prokaryotes and eukaryotes. In: *Organization and Control in Prokaryotic and Eukaryotic Cells* (20th Symposium of the society for General Microbiology). pp. 107-119. Cambridge University Press: Cambridge.

Vrot, F. & Grente, J. (1985). Recherche d'un moyen de lutte biologique contre la maladie de l'encre par utilization de la symbiose mycorrhizienne. *European Journal of Forest Pathology* **15**, 379-383.

Washington, W. S. (1983). The occurrence and control of *Phytophthora* root and crown rot of chestnut in Victoria. *Australasian Plant Pathology* **12**, 57-58.

Weste, G. (1983). The dynamics of chlamydospore formation and survival. *Phytopathologische Zeitschrift* **106**, 163-176.

Weste, G. (1992). *Phytophthora* root rot and dieback of forest trees. In: *Plant Diseases of International Importance. IV. Diseases of Sugar, Forest and Plantation Crops.* (ed. A. N. Mukhopadhyay, J. Kumar, U. S. Singh & H. S. Chaube). pp. 346-368. Prentice Hall: New Jersey.

Weste, G. & Cahill, D. (1982). Changes in root tissue associated with infection by *Phytophthora cinnamomi*. *Phytopathology Zeitschrift* **103**, 97-108.

Weste, G. M., Cooke, D. & Taylor, P. (1973). The invasion of native forest by *Phytophthora cinnamomi*. II. Regeneration patterns, declining pathogenicity and attempted control. *Australian Journal of Botany* **21**, 13-29.

Weste, G. M. & Law, C. (1973). The invasion of native forest by *Phytophthora cinnamomi*. III. Threat to the National Park, Wilsons Promontory. *Australian Journal of Botany* **21**, 31-51.

Weste, G. & Marks, G. C. (1987). The biology of *Phytophthora cinnamomi* in Australasian forests. *Annual Review of Phytopathology* **25**, 207-229.

Wicks, T. J. (1987). *Phytophthora crown rot of almond and cherry trees*. Ph.D. thesis, University of Adelaide.

Wicks, T. J. (1989). Susceptibility of almond and cherry rootstocks and scions to *Phytophthora* species. *Australian Journal of Experimental Agriculture* **29**, 103-109.

Wicks, T. J. & Hall, B. (1990). Control of *Phytophthora* canker with phosphonate in artificially inoculated almond and cherry trees. *Australian Journal of Experimental Agriculture* **30**, 413-420.

Wicks, T. J. & Lee, T. C. (1986). *Phytophthora* crown rot of almond trees. *Australian Journal of Agricultural Research* **37**, 277-287.

Wicks, T. J. & Volle, D. (1976). *Phytophthora* wilt of chestnuts in South Australia. *Plant Disease Reporter* **60**, 700-702.

Wilcox, W. F., Harman, G. E. & Di Pietro, A. (1992). Effect of gliotoxin on growth, sporulation, and zoospore motility of seven *Phytophthora* spp. *in vitro*. *Phytopathology* **82**, 1121.

Wycoff, K. L. & Ayers, A. R. (1990). Monoclonal antibodies to surface and extracellular antigens of a fungal plant pathogen, *Phytophthora megasperma* f. sp. *glycinea*, recognize specific carbohydrate epitopes. *Physiological and Molecular Plant Pathology* **37**, 55-79.

Wycoff, K. L. & Ayers, A. R. (1991). Localization of carbohydrate antigens in the walls of *Phytophthora megasperma* f. sp. *glycinea* by monoclonal antibodies. *Physiological and Molecular Plant Pathology* **39**, 95-109.

Zaki, A. I., Zentmyer, G. A., Sims, J. J. & Keen, N. T. (1983). Stimulation of sexual reproduction in the A2 mating type of *Phytophthora cinnamomi* by oleic acid and lipids from avocado roots. *Phytopathology* **73**, 199-203.

Zentmyer, G. A. (1979). Stimulation of sexual reproduction in the A2 mating type of *Phytophthora cinnamomi* by a substance in avocado roots. *Phytopathology* **69**, 1129-1131.

Zentmyer, G. A. (1980). *Phytophthora cinnamomi and the Diseases it Causes*. Monograph 10. The American Phytopathological Society: St. Paul, Minnesota.

Zentmyer , G. A. (1983). The world of *Phytophthora*. In: *Phytophthora: its Biology, Taxonomy, Ecology and Pathology*. (ed. D. C. Erwin, S. Bartnicki-Garcia & P. H. Tsao). pp. 1-7. The American Phytopathological Society: St. Paul, Minnesota.

Zentmyer, G. A. & Erwin, D. C. (1970). Development and reproduction in *Phytophthora*. *Phytopathology* **60**, 1120-1127.

Thesis errata

The named genotypes of chestnut may be termed cultivars or improved selections (pers. comm., Dr A. Allan, Institute for Horticultural Development, Victoria).

p. 35, section 4.2.1, line 6. Dr R. J. Van Velsen, Chief Quarantine Officer (Plants), South Australian Department of Agriculture.

p. 35, section 4.2.1, line 8. Shoots from 5-10 year-old trees were used for micropropagation attempts. Micropropagation from mature trees is known to be difficult and the attempts in this study were unsuccessful.

p. 111, para. 2, line 9. It is not clear why isolates of *Phytophthora* sp. caused an increase in shoot and root weight compared to controls in the collar inoculation experiment. Although plants were chosen randomly for use in experiments, it is possible that control plants were smaller than plants inoculated with *Phytophthora* spp. at the beginning of the experiment. There were no signs of infection by other organisms and no cankers beyond the wound response on these control plants.

p. 116, section 7.2.2, line 11. Monoclonal antibodies were raised against aldehyde-fixed zoospores and cysts of *P. cinnamomi* isolate DAR52646. The contents of two vesicles, designated dorsal and ventral vesicles because of their distribution within the zoospores, are secreted during encystment (Hardham & Gubler, 1990). These are labelled by the antibodies Cpa-2 and Vsv-1, respectively. Large peripheral vesicles, also found in zoospores, are labelled by the antibody Lpv-1 (Gubler & Hardham, 1988).

p. 130, para. 3, line 8. Low nutrient status of mycelium of *Phytophthora* spp. is known to promote asexual reproduction (Ribeiro, 1983). It may be that the fungus takes up nutrients from agar media or infected plants in order to reproduce, and does so when these nutrients are exhausted. Free water is also known to promote reproduction (Shearer & Tippett, 1989). The stimulus for reproduction in *Phytophthora* spp. is not well understood and further work is required to elucidate this.

References

Guest, D. I. & Grant, B. (1991). The complex action of phosphonates as antifungal agents. *Biological Reviews* **66**, 159-187.

Shearer, B. L. & Tippett, J. T. (1989). Jarrah dieback: the dynamics and management of *Phytophthora cinnamomi* in the Jarrah (*Eucalyptus marginata*) forest of south-western Australia. *Research Bulletin* No. **3**. Department of Conservation and Land Management, Como, Western Australia.