Penetration of suberised periderm of a woody host by *Phytophthora cinnamomi*

O'Gara E[1], Howard K[1], McComb J[1], Colquhoun IJ[2] and Hardy GEStJ[1*]

[1]Centre for Phytophthora Science and Management, School of Veterinary and Life Sciences, Murdoch University, Murdoch, Western Australia, 6150.

[2]Alcoa World Alumina Australia, Environmental Department, PO Box 242, Booragoon, WA 6953, Australia.

*Corresponding author. Email: G.Hardy@murdoch.edu.au

ABSTRACT

The mechanisms by which *Phytophthora cinnamomi* zoospores infect inundated, aboveground woody stem tissue is are described. Using 4-6 and 18-month month-old jarrah seedlings, the infection courts were identified and the invasion of the stems at sites of zoospore-cyst binding described. Stems were inoculated with a suspension of motile zoospores on the green stem/young periderm region and light microscopy was used to examine penetration at sites of taxis, and fluorescent microscopy to examine penetration sites of seedlings with intact periderm. Two main infection courts were identified on stems: the emerging axillary shoot and the region of stem of immediately surrounding an axillary shoot, where the periderm was thin or discontinuous. Invasion also occurred at sites where the developing shoot had not yet emerged but was at the stem surface. At these sites the pathogen also directly invaded through the thin-walled phellem of the periderm surrounding

the shoot. Zoospores of *P. cinnamomi* were not attracted to mature leaf or green stem stomata. Penetration of the epidermal cell layers of the axillary bud leaf primordia was inter- and intra-cellular; growth of hyphae in periderm surrounding the shoot was intercellular; while in collenchyma it was inter- and intra-cellular being intercellular between polyphenolic rich cells. Exposed stem collenchyma was also directly invaded immediately adjacent to the young axillary shoot. Zoospores demonstrated taxis to sites of discontinuous periderm, similar to wounded areas where the outer protective layers of the plant are breached. This study presents first evidence that *P. cinnamomi* is capable of intercellular penetration of suberised periderm.

Key words: encystment, periderm, phellem, taxis, *Eucalyptus marginata*

Introduction

Phytophthora cinnamomi causes plant diseasehas been found on all continents except Antarctica, causing important diseases in ornamental and native plants, in agriculture, horticulture and forestry. It is almost certainly introduced to most of these regions (Cahill *et al*., 2008), and has a wide host range as many species are unable to mount a timely defence against this pathogen. In Australia, this soil-borne plant pathogen causes major floristic and structural changes in native vegetation (McDougall *et al.* 2005). It was estimated that by 2009 a million ha of south-western Australia was infested with *P. cinnamomi*, mainly *Eucalyptus marginata* (jarrah) forest Forests (Department of Environment and Conservation, 2012). Jarrah is Western Australia's most important hardwood timber.

The infection of susceptible hosts by *P. cinnamomi* through primary roots has been described, and the processes of zoospore chemotaxis, encystment and penetration are well documented (Tippett *et al*., 1976; Ho & Zentmyer, 1977; Otaye, 2005). Infection of secondarily thickened root and stem collars by *P. cinnamomi* is known to occur at naturally infested sites (Dell & Wallace, 1981; Shearer *et al*., 1981; Shea *et al*., 1982; Hardy *et al*., 1996) but it is unlikely that these collar infections result from initial entry and invasion via root tips (Marks & Smith, 1980). In mine-site rehabilitation in south-western Australia, the stems and the leaves from the lower branches of lignotuberous jarrah seedlings are often found immersed in surface water ponding in riplines, exposing them to motile zoospores of *P. cinnamomi*. While the infection of the collar and lower stem of jarrah has been demonstrated (O'Gara *et al*., 1996; O'Gara *et al*., 1997), the mechanisms by which *P. cinnamomi* invades woody tissue are unknown.

Wounds, growth cracks and lenticels are some of the possible infection courts reported to be utilised by *Phytophthora* to invade woody host tissue (Weste & Marks 1987; Harris 1991). Dell & Malajczuk (1989) suggested that infection of jarrah by *P. cinnamomi*

may also occur through the lignified and suberised layered periderm., and O'Gara *et al*. (2009) demonstrated temporary discontinuity of the periderm and/or cuticle occurring at sites of axillary growth during shoot emergence. However, there is little evidence that fungal and oomycete pathogens, including *P. cinnamomi*, can directly invade the tissues of the periderm or rhytidome (Marks *et al*., 1981; Kolattukudy & Crawford, 1987; Lindberg and Johansson, 1991; Heneen *et al*., 1994).

The aim of this study was to determine the taxis of *P. cinnamomi* zoospores to woody stems and fully expanded leaves, identify infections courts and describe the subsequent invasion of the stems.

Materials and methods

Experimental Design

Stems of four 18-month -old jarrah plants were inoculated, and the *P. cinnamomi* zoospore behaviour, patterns of cyst binding, germination and germ-tube growth were examined. The stems of ten 4-6-month month-old, and stems and leaves of a further ten 18-month month-old seedlings, were also inoculated to examine sites of infection and subsequent invasion process.

Plant Material

The 4-6-month-old jarrah seedlings used in this study were raised from seed until 4-6 months old, while 18-month-old seedlings with a lignotuber were obtained from Alcoa of Australia Ltd. All plants were maintained in an evaporatively cooled glasshouse on raised benches.

87

88 ***P. cinnamomi* zoospore production**

89 *P. cinnamomi* isolate MP94/48 (A2 mating type, isolated from jarrah at Willowdale, Western
90 Australia), which has been shown to be highly virulent in jarrah (Hüberli, 1995) was used.
91 Axenic zoospore suspensions were produced according to the methods described by O'Gara
92 *et al*. (1998). Briefly, *P. cinnamomi* was grown on cheesecloth overlain on V8 agar for 3
93 days. The colonised cheesecloth was incubated in 100 mL of sterile V8 broth for 18 h,
94 washed in sterile mineral salts, and then incubated in minerals salts for 20 h to induce
95 sporangia formation. For experiments in which fluorescent zoospores were required, 2 µg/mL
96 Calcofluor White M2R Fluorescent Brightener 28 (Sigma Chemical Co., St Louis, Montana,
97 USA) was added to the mineral salts. To encourage zoospore release, the culture was washed
98 thoroughly in sterile deionised water and then cold-shocked at 4°C for 30 min. Zoospore
99 concentration was adjusted to 6000-8000 zoospores mL^{-1} immediately before each
100 inoculation.

101

102 **Inoculation and Examination**

103 ***Motile zoospore behaviour and taxis***

104 The mid/lower stem of the four 18-month month-old jarrah plants was inoculated at 24°C
105 with *P. cinnamomi* using a modification of the method described by O'Gara *et al.* (1996).
106 The intact potted plants were positioned horizontally immediately prior to inoculation. A
107 watertight 10 cm dia. receptacle was attached to the stem to include the transition from green
108 stem to stem with periderm. The receptacle was filled with water for 1 h to moisten the stem
109 and then replaced with a motile zoospore suspension, and the behaviour of the zoospores in
110 relation to the stems was observed for 1 h using an Olympus stereomicroscope Model
111 SZH10.

Inoculum receptacles were also constructed around the mid/lower stem of ten upright 18-month-old plants so as to immerse a single fully expandedmature leaf from each stem. This was inoculated and incubated for 8 12 or 24 h at 24°C. The inoculated region of the stems and the leaves were then dissected, fixed in a 2% paraformaldehyde/2.5% glutaraldehyde mixture in 0.1 M phosphate buffer (PF/G), and processed for SEM examination by dehydration in an ascending series of ethanol–deionised water solutions. This was , followed by infiltration in amyl acetate, critical point drying and sputter coating with gold. Samples were examined with a Philips Model XL20SEM (Phillips, Eindhoven, Netherlands). Zoospore taxis was defined as preferential attraction, accumulation and encystment of zoospores, and orientation of germ-tube emergence at localised sites on the stem and leaf.

Light microscopy (LM) to examine penetration at sites of taxis

Stems of 18-month-old plants were inoculated as above, incubated for 24 h, and then dissected with sections fixed in a PF/G mixture and embedded in Spurr's medium. Briefly, the tissue was dehydrated in an ascending series of acetone–deionised water solutions and, infiltrated in an ascending series of Spurr's–acetone solutions. , Tissue was embedded in Spurr's epoxy resin (ProSciTech Microscopy PLUS, Qld, Australia), polymerised at 70^{o}C for 24 h, and sectioned at 1-4 mm with glass knives on a Sorvall JB-4 microtome (Du Pont Instruments, Newtown, CT, US). Sections (1 μm thick) were dried onto slides treated with saturated KOH (32 g KOH in 100% Etoh) for 5 sec and rinsed in deionised water prior to staining with toluidine blue (TBO) for polyphenolics and as a general stain. Treatment of sections with saturated KOH enhanced staining contrast and reduced wrinkling in mounted sections.

Fluorescent microscopy (FM) to examine penetration of sites of young seedlings with intact periderm.

The main stem of 4-6 month month-old seedlings was inoculated using small open-ended glass vials (4 mL) to contain the zoospore suspension. These were fixed into place around the mid/lower stem, which did not contain leaf scars or axillary buds, with warm molten wax and Parafilm (American National Can. Greenwich, Ct. 06836). During inoculation, the seedlings were laid horizontally with the stem supported. The top of the pot was covered with plastic to minimise soil and root disruption. Calcofluor-treated zoospore suspension was added to the vial and the seedlings incubated at 24°C for 24 h. Seedlings were then dissected, fixed and processed as for LM.

Results

Motile zoospore behaviour, encystment and germ-tube growth

Initially, zoospores swam very quickly, and those in close proximity to the stem frequently collided with it, often bouncing along its length, but over after 40 min they became sluggish, settled and encysted on the stem. On green stems (Fig. 1a and 1b) and stems with periderm, zoospores of *P. cinnamomi* showed preferential attraction to areas of leaf abscission and axillary shoot emergence (Fig. 1c and 1d). Zoospores moved toward these sites, then commenced small 'jerky' movements in the immediate vicinity of the attractant and rapidly encysted. In the absence of leaf abscission scars or axillary buds, zoospores swam and encysted in a random pattern and showed no particular attraction to either the green stem or periderm tissue.

Sites of taxis (leaf scars and axillary bud emergence)

By 12 h, tTaxis were evident from the concentration of cysts and their docking with the side of germ-tube emergence directed towards the attractant. Where a shoot had emerged, zoospores were attracted to the area between the leaf primordia (Fig. 1d) and/or to the crevices that resulted from the emergence of the shoot from the main body of the stem (Fig. 1e). Occasionally in localised areas on green stems, apparently distant from axils, small clumps of cysts were observed with germ-tubes orientated toward the host tissue (Fig. 1f) suggesting that zoospore taxis had occurred. However, it was unclear why they were attracted to these areas as the zoospores obscured the surface of the stem.

Sites of random binding

As distance from the site of an attractant increased, aAt sites where the lateral shoot had not broken through the stem surface (Fig. 2a), or in the absence of features to which taxis was demonstrated (Fig. 2b), cysts bound randomly, often individually, and apparently non-specifically to either green stem or periderm tissue. Zoospores became 'trapped' in the rough topography of the periderm, where they encysted and germinated (Fig. 2b).

Germ-tube orientation of non-specifically bound cysts was random, with some germ-tubes emanating from the side furthest from the host: some of which changed direction to head towards the host. However, most germ-tubes only grew across the surface of the host. Some germ-tubes grew for extended periods before attempting penetration (Fig. 3a), while others terminated in a swelling, which appeared to be attached to the stem surface (Fig. 3b). *P. cinnamomi* sometimes took a torturous path across the surface of the stem, and numerous swellings occurred along the length of the hypha. Within 12 h of inoculation, *P. cinnamomi* had produced abundant hyphal growth on the surface of jarrah stems from randomly bound cysts, and within 24 h hyphal swellings and sporangia were observed.

Hyphae from randomly bound cysts of *P. cinnamomi* often entered breaches in disintegrating cuticle of old green stem at the green stem/periderm boundary (Fig. 4a). Hyphae also often penetrated between phellem cells of the periderm (Fig. 4b). *P. cinnamomi* was not preferentially attracted to stomata on either stems or leaves of jarrah. Hyphae grew across the mature immersed leaf (Fig. 4c) or stem surface with no attempt to penetrate open stomata, with one exception on a green stem, where hyphae were seen to enter the stomata. Similarly, zoospores showed no taxis to lenticels on the stem.

Invasion of Host Tissue

At sites of taxis

From concentrations of cysts at the site of axillary bud emergence the pathogen invaded through the leaf primordia of the newly emerged lateral shoots (Fig. 5a and 5b). Penetration of the epidermal cell layers of the leaf primordia was inter- and intra-cellular (Fig. 5b); hyphae were intercellular in the periderm surrounding the axillary shoot; while in collenchyma hyphae were intracellular with intercellular growth most often observed in areas of polyphenolic rich cells. Although the pathogen entered the stem via the axillary shoot, as well as through the mature tissue surrounding the shoot, colonisation was most rapid and extensive in new shoot tissue. The pathogen extended rapidly and predominantly in a radial direction in tissues of the new shoot. Within 24 h of inoculation *P. cinnamomi* had completely colonised the tissues of the new shoot including the vascular tissue (Fig. 5c), and had progressed to the mature stem tissue internal to the shoot. It had also as well as destroyeding a buried axillary bud (Fig. 5d). Tissue damage was extensive and ranged from an apparent shrinkage of the cell contents (Fig. 5b) to complete maceration of the cells.

Collenchyma of the stem, which became exposed as a result of periderm rupture as the axillary shoot emerged, was also invaded. A halo of zoospore-cysts was observed

infecting the secondary phloem of a jarrah stem through a periderm discontinuity associated with an emerging bud (Fig. 6a). Invasion also occurred at sites where the developing shoot had not yet emerged but was at the surface of the stem. At these sites the pathogen directly invaded through periderm discontinuities, but also through the thin-walled phellem (TnP) of the periderm surrounding the shoot (Fig. 6b). Intracellular penetration occurred in apparently empty collenchyma cells, where the hyphae became constricted as they passed through the cell wall. In contrast, there was no visible shrinkage of cells contents or cell maceration in infected collenchyma ground tissue surrounding the shoot at 24 h (Fig. 6a and 6b).

At sites of random binding

Calcofluor-treated, randomly bound cysts of *P. cinnamomi* were readily located on the surface of jarrah stems in sectioned material. However, they were infrequently observed attempting to penetrate intact periderm or cuticle. Randomly bound propagules never resulted in extensive infections as did those at sites of taxis.

Although individual *P. cinnamomi* propagules were never observed directly penetrating phellem cells, they occasionally grew between the cells of the TnP layer immediately external to the living tissue (Fig. 7a). They, and were sometimes observed within TnP cells (Fig. 7b). Cortical tissue which was moribund and fragmented as a result of the formation of the first periderm internal to the primary phloem fibres was extensively colonised by 24 h. The pathogen directly penetrated intact phenolic rich cells in the moribund cortex, and was occasionally observed in the lumen and within the thick walls of the isolated primary phloem fibres.

Cysts were often observed attached to the intact cuticle of green stems with a swollen germ-tube (Fig. 7c). However, the pathogen was never observed to directly penetrate intact cuticle.

Discussion

The results of this study demonstrated that rRegions of axillary shoot emergence in jarrah stems are major infection courts for *P. cinnamomi* if stems are inundated and the threshold of host resistance is overcome. Infection of jarrah stems at these sites was facilitated by: taxis of zoospores to the region; the absence of cuticle on the leaves of emerging shoots; rapid proliferation of the pathogen in the immature tissues of the new shoot tissue; the access gained to the axial vascular system through infection of lateral shoots; and the fragmentation and discontinuity of periderm in the region immediately surrounding the site of shoot emergence. There is evidence of sites of lateral shoot growth also being a region for plant host infection by other fungal pathogens. For example, apple canker resulted from the infection of leaf scars by *Nectria galligena* (Crowdy, 1952). R, rot in apple stems occurred from infection of buds or broken laterals by *Phytophthora syringae* (Sewell & Wilson, 1964) and, infection of larch caused by the canker pathogen *Trichoscyphella willkommii*, occurred in needle abscission zones where the periderm was discontinuous (Buczacki, 1973).

It is possible that exudates leaking from sites of axillary bud emergence attract zoospores. It is known that even intact internodes release carbohydrates into water (O'Gara *et al*., 1998) and that exudate leakage, from regions of lateral root growth in alfalfa, could be responsible for *Phytophthora megasperma* utilising these areas as major infection courts (Marks & Mitchell, 1971).

Zoospores also demonstrated taxis to sites where the periderm was discontinuous, adjacent to the emerging shoot. This area is similar to a wound as the outer protective layers of the plant are breached. Chemotaxis of *Phytophthora* zoospores to deliberately wounded roots has been demonstrated previously (Zentmyer, 1960; Dukes & Apple, 1961). Pathogens

can avoid contact with phenols in the periderm and rhytidome by entering a host through pre-existing wounds and growing intercellularly (Tattar & Rich, 1973). However, in our study we observed hyphae of *P. cinnamomi* colonising intercellularly colonising collenchyma cells rich in phenols. It is unclear how the pathogen is able to do this and further work is required to understand the mechanisms involved. Reactivation of old infection sites in plant tissue has been attributed to discontinuities in necrophylactic periderm (Tippett & Hill, 1984; Biggs, 1986; Cahill *et al*., 1989).

Zoospores also demonstrated taxis to regions where periderm was present but was reduced to a single layer TnP, due to disruption and fragmentation of the outer periderm layers. It is unclear why zoospores accumulated at sites where only a thin periderm was present. The behaviour of zoospores is often density-dependent although the underlying mechanisms are poorly understood (Kong & Hong, 2010). Zoospores are quickly immobilised and encyst when they encounter a changing environment (van West *et al*., 2002; Gow, 2004; Otaye, 2005). For instance, induced or natural changes in the electrical field around a root predicated changes in where *P. palmivora* zoospores accumulated (Gow, 2004). Otaye (2005) suggests that a diffusible factor (autoinducer) released by the zoospore can arise after a random collision, which starts the aggregation process, thus increasing the inoculum potential. In the current study, aggregation in areas of single layer TnP may have been a function of diffuse exudation of chemotactic compounds from the region of bud development, or from the sites where periderm was discontinuous, or it may have resulted from random collision. Determining the pathogen's autoinducer responsible for zoospore aggregation could allow the development of new control strategies for *Phytophthora* species (Kong & Hong, 2010).

In the peripheral portion of jarrah stems, collenchyma constituted the ground tissue surrounding the developing shoot. Where the periderm was discontinuous the pathogen

directly invaded the collenchyma but at a slower rate compared to the rapid colonisation of the young tissue of the emerging shoot. Intercellular invasion was commonly associated with polyphenolic-rich cells, while intracellular penetration was more common in apparently empty cells. Although fungal pathogens may utilise the pectin-rich primary walls of collenchyma as a source of nutrients, the thickness of the walls may hamper proliferation of the pathogen.

P. cinnamomi was never observed to directly penetrate intact cuticle. Similarly, the mycelium of *P. citricola*, which were later described as *P. mengei* by Hong *et al.* (2009), in an aqueous suspension was unable to penetrate the intact cuticle of avocado stems (El-Hamalawi & Menge, 1994). In contrast, *P. sojae* has been observed penetrating the cuticle of soybean stems (Sugimoto *et al*., 2009). More research on the cuticle as a barrier to penetration is needed.

While extensive host infection was observed at sites where taxis were was demonstrated, little infection was observed in association with randomly bound propagules. Other researchers have reported that where inoculum density is low the development of *Phytophthora* is slow and penetration takes longer, compared to sites where zoospore-cysts have massed (Marks & Mitchell, 1971; Tippett & Malajczuk, 1979; Hinch *et al*., 1985). Brasier and Kirk (2001) demonstrated a critical threshold in host resistance that can be reduced by external influences, such as ponding in the case of the current study. Hinch *et al.* (1985) suggested that synergism in amassed propagules may enable the pathogen to overcome the capacity of a plant to impede the growth of a single hyphae. Kong & Hong (2010) showed that chemical communication for *P. nicotianae* zoospores is an important factor in the infection process, whereby autoinducers regulate zoospore aggregation, taxis and infection, allowing the zoospores to work synchronically.

Infection courts used by *Phytophthora*

Germ-tube swellings were often observed in association with randomly bound zoospores. Although from SEM examination they appeared to be firmly attached, LM examination of sections through the tissue revealed that they were often not adhered to the host surface. Infection pegs were never observed in association with germ-tube swellings. The currentOther studies y have shownis in agreement with Tippett *et al*. (1976) that swellings formed where penetration of *P. cinnamomi* (Tippett *et al*., 1976) or growth of *P. plurivora* (Jung and Burgess, 2009) was impeded. . Stress has also been implicated as inducing appressoria formation in fungal pathogens (Emmett & Parbery, 1975; Swiecki & MacDonald, 1988). Given that *P. cinnamomi* is soil-borne and root-invading pathogen, the aerial environment may present stresses that result in the formation of appressoria-like swellings.

Zoospores of *P. cinnamomi* were not attracted to leaf or green stem stomata of jarrah, and rarely seen to enter a stem through an open stomata. In contrast, stomata on the hypocotyls of chickpea were a preferred infection court for *P. megasperma* f. sp. *medicaginis* (Dale & Irwin, 1991). *P. cinnamomi* showed no attraction or evidence of invasion through lenticels, Similarly, avocado stems did not become infected by *P. citricolamengei* when lenticels were exposed to mycelium in an aqueous suspension for 15 days (El-Hamalawi & Menge, 1994). However, this is in contrast to the *Alnus* where the lenticels in the collar region are a primary infection court for *P. alni* during flooding (Jung and Blaschke, 2004). Penetration and infection of unwounded stems has also been reported in *Fagus sylvatica* by *P. ramorum*, and *P. kernoviae* (Brown and Brasier, 2007) which suggests penetration via lenticels. ~~Similarly, avocado stems did not become infected by *P. citricola* when lenticels were exposed to mycelium in an aqueous suspension for 15 days (El-Hamalawi & Menge, 1994).~~

In conclusion, *P. cinnamomi* zoospores utilised sites of axillary shoot emergence as major infection courts on the stems of jarrah seedlings. Sites of discontinuous or thin periderm were also vulnerable to infection. This study presents the first evidence that *P. cinnamomi* is capable of intercellular penetration of the suberised periderm.

Acknowledgements

E. O'Gara acknowledges financial support from an ARC SPIRT scholarship and from Alcoa World Alumina Ltd. Dr Joanna Young's assistance with the interpretation of micrographs and the technical assistance of Mr Gordon Thomson is much appreciated

References

Biggs AR, 1986. Comparative anatomy and host response of two peach cultivars inoculated with *Leucostoma cincta* and *L. persoonii*. *Phytopathology* **76**, 905-12.

Brasier CM, Kirk SA, 2001. Comparative aggressiveness of standard and variant hybrid alder phytophthoras, *Phytophthora cambivora* and other *Phytophthora* species on bark of *Alnus*, *Quercus* and other woody hosts. *Plant Pathology* **50**, 218-229.

Brown AV, Brasier CM, 2007. Colonization of tree xylem by *Phytophthora ramorum, P. kernoviae* and other *Phytophthora* species. *Plant Pathology*, **56**, 227-241.

Buczacki ST, 1973. Observation on the infection biology of larch canker. *European Journal of Forest Pathology* **3**, 228-32.

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**, 417-24.

359 Cahill DM, Rookes JE, Wilson BA, Gibson L, McDougall KL, 2008. *Phytophthora*
360 *cinnamomi* and Australia's biodiversity: impacts, predictions and progress towards
361 control. Turner review No. 17. *Australian Journal of Botany* **56**, 279–310.
362 Crowdy SH, 1952. Observations on apple canker. 4. The infection of leaf scars. *Annals of*
363 *Applied Biology* **39**, 569-80.
364 Dale ML, Irwin JAG, 1991. Stomata as an infection court for *Phytophthora megasperma* f.
365 sp. *medicaginis* in chickpea and a histological study of infection. *Phytopathology* **81**,
366 375-79.
367 Dell B, Malajczuk N, 1989. Jarrah dieback - A disease caused by *Phytophthora cinnamomi*.
368 In: Dell B, Havel JJ, Malajczuk N, eds. *The Jarrah Forest*. Dordrecht; Kluwer
369 Academic Publishers, 67-87.
370 Dell B, Wallace IM, 1981. Recovery of *Phytophthora cinnamomi* from naturally infected
371 jarrah roots. *Australasian Plant Pathology* **10**, 1-2.
372 Department of Environment and Conservation, 2012. *Phytophthora* Dieback. Perth, Western
373 Australia: Department of Environment and Conservation.
374 http://www.dec.wa.gov.au/management-and-protection/land/managing-dieback.html
375 Dukes PD, Apple JL, 1961. Chemotaxis of zoospores of *Phytophthora parasitica* var.
376 *nicotianae* by plant roots and certain chemical solutions. *Phytopathology* **51**, 195-97.
377 El-Hamalawi ZA, Menge JA, 1994. Avocado trunk canker disease caused by *Phytophthora*
378 *citricola*: Investigation of factors affecting infection and disease development. *Plant*
379 *Disease* **78**, 260-64.
380 Emmett RW, Parbery DG, 1975. Appressoria. *Annual Review of Phytopathology* **13**, 147-67.
381 Gow NAR, 2004. New angles in mycology: studies in directional growth and directional
382 motility. *Mycological Research* **108 (1)**, 5–13.

Hardy GEStJ, Colquhoun IJ, Nielsen P, 1996. The early development of disease caused by *Phytophthora cinnamomi* in *Eucalytpus marginata* and *Eucalyptus calophylla* growing in rehabilitated bauxite mined areas. *Plant Pathology* **45**, 944-54.

Harris DC, 1991. The *Phytophthora* diseases of apple. *Journal of Horticultural Science* **66**, 513-44.

Heneen WK, Gustafsson M, Brismar K, Karlsson G, 1994. Interactions between Norway spruce (*Picea abies*) and *Heterobasidion annosum*. 2. Infection of woody roots. *Canadian Journal of Botany* **72**, 884-89.

Hinch JM, Wetherbee R, Mallett JE, Clarke AE, 1985. Response of Zea mays roots to infection with *Phytophthora cinnamomi*. 1. The epidermal layer. *Protoplasma* **126**, 178-87.

Ho HH, Zentmyer GA, 1977. Infection of avocado and other species of Persea by *Phytophthora cinnamomi*. *Phytopathology* **67**, 1085-89.

Hong CX, Gallegly ME, Browne GT, Bhat RG, Richardson PA, Kong P, 2009. The avocado subgroup of *Phytophthora citricola* constitutes a distinct species, *Phytophthora mengei* sp. nov. *Mycologia* **101**, 833-840.

Hüberli D, 1995. *Analysis of Variability Among Isolates of* Phytophthora cinnamomi *Rands from* Eucalyptus margintata *Donn Ex. Sm. and* E. calophylla *R. Br. Based on Cultural Characteristics, Sporangia and Gametangia Morphology, and Pathogenicity*. Perth, Australia: Murdoch University, Hons. thesis

Jung T, Blaschke M, 2004. Phytophthora root and collar rot of alders in Bavaria: distribution, modes of spread, and possible management strategies. *Plant Pathology* **53**, 197-208.

Jung T, Burgess TI, 2009. Re-evaluation *of Phytophthora citricola* isolates from multiple woody hosts in Europe and North America reveals a new species, *Phytophthora plurivora* sp. nov. *Persoonia* **22**, 95-110.

Kolattukudy PE, Crawford MS, 1987. The role of polymer degrading enzymes in fungal pathogenesis. In: Nishimura S, Vance CP, Doke N, eds. *Molecular Determinants of Plant Diseases*. Berlin; Springer-Verlag.

Kong P, Hong C, 2010. Zoospore density-dependent behaviors of *Phytophthora nicotianae* are autoregulated by extracellular products. *Phytopathology* **100**, 632-37.

Lindberg M, Johansson M, 1991. Growth of *Heterobasidion annosum* through bark of *Picea abies*. *European Journal of Forest Pathology* **21**, 377-88.

Marks GC, Mitchell JE, 1971. Penetration and infection of alfalfa roots by *Phytophthora megasperma* and the pathological anatomy of infected roots. *Canadian Journal of Botany* **49**, 63-7.

Marks GC, Smith IW, 1980. A new approach to the *Phytophthora cinnamom*i problem. *Australian Forestry* **43**, 261-63.

Marks GC, Smith IW, Kassaby FY, 1981. Trunk infection of *Eucalyptus* species by *Phytophthora cinnamomi* Rands: A preliminary report. *Australian Forest Research* **11**, 257-67.

McDougall KL, Hobbs RJ, Hardy GEStJ, 2005. Distribution of understorey species in forest affect by *Phytophthora cinnamomi* in south-western Australia. *Australian Journal Botany* **53**, 813-19.

O'Gara E, Hardy GEStJ, McComb JA, 1996. The ability of *Phytophthora cinnamomi* to infect through unwounded and wounded periderm tissue of *Eucalyptus marginata*. *Plant Pathology* **45**, 955-63.

O'Gara E, McComb JA, Colquhoun IJ, Hardy GEStJ, 1997. The infection of non-wounded and wounded periderm tissue at the lower stem of *Eucalyptus marginata* by zoospores of *Phytophthora cinnamomi*, in a rehabilitated bauxite mine. *Australasian Plant Pathology* **26**, 135-41.

O'Gara E, 1998. *Infection and disease of* Eucalyptus marginata *(jarrah), caused by* Phytophthora cinnamomi *in rehabilitated bauxite mines in the south-west of western Australia.* Perth, Australia: Murdoch University, PhD thesis

O'Gara E, Howard K, Colquhoun IJ, Dell B, McComb J, Hardy GStEJ, 2009. The development and characteristics of periderm and rhytidome in *Eucalyptus marginata* *Australian Journal of Botany* **57**, 221–28.

Otaye DO, 2005. *Repeated emergence, motility, and autonomous dispersal by sporangial and cyst derived zoospores of* Phytophthora *Spp.* Oklahoma, USA: Oklahoma State University, PhD thesis.

Sewell GWF, Wilson JF, 1964. Death of maiden apple trees caused by *Phytophthora syringae* Kleb. and a comparison of the pathogen with *P. cactorum* (L. & C.) Schroet. *Annals of Applied Biology* **53**, 275-80.

Shea SR, Shearer B, Tippett J, 1982. Recovery of *Phytophthora cinnamomi* Rands from vertical roots of jarrah (*Eucalyptus marginata* Sm). *Australasian Plant Pathology* **11**, 25-8.

Shearer BL, Shea SR, Fairman RG, 1981. Infection of the stem and large roots of *Eucalyptus marginata* by *Phytophthora cinnamomi*. *Australasian Plant Pathology* **10**, 2-3.

Sugimoto T, Watanabe K, Furiki M, Walker DR, Yoshida S, Aino M, Kanto T, Irie K, 2009. The effect of potassium nitrate on the reduction of *Phytophthora* stem rot disease of soybeans, the growth rate and zoospore release of *Phytophthora sojae*. *Journal Phytopathology* **157**, 379–89.

Swiecki TJ, MacDonald JD, 1988. Histology of chrysanthemum roots exposed to salinity stress and *Phytophthora cryptogea*. *Canadian Journal of Botany* **66**, 280-88.

Tattar TA, Rich AE, 1973. Extractable phenols in clear, discoloured and decayed woody tissue and bark of sugar maple and red maple. *Phytopathology* **63**, 167-69.

458 Tippett JT, Hill TC, 1984. Role of periderm resistance of *Eucalyptus marginata* roots against
459 *Phytophthora cinnamomi*. *European Journal of Forest Pathology* **14**, 431-39.
460 Tippett J, Malajczuk N, 1979. Interaction of *Phytophthora cinnamomi* and a resistant host,
461 *Acacia pulchella*. *Phytopathology* **69**, 764-72.
462 Tippett JT, Holland AA, Marks GC, O'Brien TP, 1976. Penetration of *Phytophthora*
463 *cinnamomi* into disease tolerant and susceptible eucalypts. *Arch. Microbiol.* **108**, 231-
464 42.
465 van West P, Morris BM, Reid B, Appiah AA, Osborne MC, Campbell TA, Shepherd S J,
466 Gow NAR, 2002. Oomycete plant pathogens use electric fields to target roots.
467 *Molecular Plant-Microbe Interactions* **15 (8)**, 790–98.
468 Weste G, Marks GC, 1987. The biology of *Phytophthora cinnamomi* in Australasian forests.
469 *Ann. Rev. Phytopathol.* **25**, 207-29.
470 Zentmyer GA, 1960. Chemotaxis of zoospores for root exudates in relation to infection by
471 *Phytophthora cinnamomi*. (Abstr.). *Phytopathology* **50**, 660.
472
473
474
475

Figure 1. *Phytophthora cinnamomi* zoospores exhibiting chemotaxis and binding to areas of the stems of *Eucalyptus marginata* seedlings at 12 h after inoculation. SEM. **a)** at the stem-leaf-scar (SLS) and surrounding green stem**.** Bar = 500 µm. **b)** enlargement of section of 1**a** showing uniform orientation of the zoospore germ-tubes towards the host in the region of the SLS. Bar = 50 µm. **c)** the region of the axillary shoot (AS) that has emerged through the periderm (P) above the stem-leaf-scar. Bar = 500 µm. **d)** enlargement of AS in 1c showing zoospores on and between the new leaves. Bar = 200 µm. **e)** site of an emerging axillary shoot (AS; large arrow) where the periderm (P) has ruptured. Hyphae are growing across the surface of the periderm (small arrow). **f)** an area of green stem between two oil glands (OG) but point of taxis obscured by tight grouping of zoospores zoospore-cysts. Bar = 50 µm.

Figure 2. Sites of random binding of *Phytophthora cinnamomi* zoospores to the stem of stems of *Eucalyptus marginata* seedlings 12 h after inoculation. SEM. Bar = 50 µm. **a)** site of imminent axillary shoot emergence where a cap of corky cells still protects the protruding tissue. Zoospore-cysts have bound randomly to the area around the protrusion and are growing across the surface of the periderm. **b)** zoospore-cysts bound randomly and germinated between layers of phellem.

Figure 3. Zoospore-cysts of *Phytophthora cinnamomi* bound to the stem of *Eucalyptus marginata* at 24 h post inoculation. SEM. **a)** randomly and individually bound zoospore-cysts (arrows) on the periderm surface with the central germ tubes having only one apparent attempt at penetration. Bar = 50 µm. **b)** zoospore-cyst bound to green stem, where the germ

Formatted: Font: Bold

tube has terminated in a swelling (arrow) which appears firmly attached to the host surface. Bar = 5 µm.

Figure 4. Zoospore-cysts of *Phytophthora cinnamomi* bound to different tissues of *Eucalyptus marginata* 24 h after inoculation. SEM. Bar = 5 µm. **a)** hyphae growing into ruptured cuticle on green stem nearat the green stem/periderm boundary. **b)** germ-tubes entering between phellem cells on the periderm of stems. **c)** hypha growing across an open stoma on the abaxial surface of a fully expanded leaf making no attempt at penetration.

Figure 5. Axillary shoot (AS) which has emerged through the periderm (P) on a *Eucalyptus marginata* stem colonised by *Phytophthora cinnamomi* zoospores within 24 h of inoculation. LM, LS, KOH treated/TBO stained. **a)** a shoot was colonised by *P. cinnamomi* within 24 h of inoculation with (b) leaf primordia and (c) vascular tissue of the axillary shoot and a buried axillary bud (d) indicated. Bar = 100 µm. **b)** empty zoospore-cysts at the surface, inter- and intra-cellular penetration (little arrow and big arrow, respectively) of the epidermal cells of the leaf primordia, showing ingress of pathogen (arrows) and the contraction of the cell contents. Bar = 20 µm. **c)** infection of the vascular tissue of a new shoot (arrow). Bar = 20 µm. **d)** infected buried axillary bud where all the cells have collapsed after infection (stained purple). Bar = 20 µm.

Figure 6. Invasion and colonisation of *Eucalyptus marginata* stems by *Phytophthora cinnamomi* zoospores at regions of periderm discontinuity 24 h after inoculation. Bar = 50 µm. TS, TBO stained. **a)** a halo of zoospores-cysts invading the stem through a periderm

discontinuity (between the black arrows) which is associated with axillary shoot development nearby. Hyphal ramification of the host tissue is shown (white arrow). **b)** an axillary bud shoot (AS) immediately prior to its emergence showing invasion of the stem beneath the cap (capCap) of corky cells, and through a periderm discontinuity (between the black arrows). Invasion of the mature tissue surrounding the shoot has also occurred between the thin-walled phellem (TnP) of the periderm (white arrows). The new shoot tissue is completely colonised compared to the ramification of the hyphae in the collenchyma of the mature tissue.

Figure 7. Penetration of thin walled phellem (TnP) and stoma of *Eucalyptus marginata* seedling by zoospores of *Phytophthora cinnamomi*, 24 h after inoculation. Bar = 10 µm. TS, FM, Calcofluor. **a)** zoospore-cyst (blue) attempting intracellular penetration of the thin-walled phellem (TnP) of 5-month-old seedling. **b)** germinated zoospore-cyst (large arrow) at the surface of thin-walled phellem (TnP) and hyphae within the phellem cells (small arrows) of 6-month-old stem. **c)** zoospore-cyst (blue) attached to the cuticle of the 5-month-old green stem but without evidence of penetration.