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# Survival of *Phytophthora cinnamomi* as oospores, stromata, and thick-walled chlamydospores in roots of symptomatic and asymptomatic annual and herbaceous perennial plant species

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ABSTRACT

Studies were conducted to determine how *Phytophthora cinnamomi* survives during hot and dry Mediterranean summers in areas with limited surviving susceptible hosts.
Two Western Australian herbaceous perennials *Chamaescilla corymbosa* and *Stylidium diuroides* and one Western Australian annual *Trachymene pilosa* were collected weekly from a naturally infested site from the *Eucalyptus marginata* (jarrah) forest from winter to spring and less frequently during summer 2011/2012. Selfed oospores, thick-walled chlamydospores, and stromata of *P. cinnamomi* were observed in each species. Oospores and thick-walled chlamydospores germinated *in planta* confirming their viability. This is the first report of autogamy by *P. cinnamomi* in naturally infected plants. Stromata, reported for the first time for *P. cinnamomi*, were densely aggregated inside host cells, and germinated *in planta* with multiple germ tubes with hyphae capable of producing oospores and chlamydospores. *Trachymene pilosa* was completely asymptomatic, *S. diuroides* did not develop root lesions but some plants wilted, whilst *C. corymbosa* remained asymptomatic above ground but lesions developed on some tubers. The presence of haustoria suggests that *P. cinnamomi* grows biotrophically in some hosts. Asymptomatic, biotrophic growth of *P. cinnamomi* in some annual and herbaceous perennials and the production of a range of survival structures have implications for pathogen persistence over summer and its management.



## Introduction

*Phytophthora cinnamomi* is a soil-borne root pathogen with a broad host range and necrotrophic mode of infection (Zentmyer 1980; Cahill *et al.* 2008) and results in the death of many susceptible plant species and the degradation of ecosystems worldwide including 15 global biodiversity hotspots (Dunstan *et al.* 2010). Phylogenetically and taxonomically, this pathogen belongs to the class Oomycetes in which swimming zoospores are produced and released from sporangia (Hardham 2005). This key event in the asexual lifecycle depends on the availability of free water during warm periods.

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As the pathogen is able to quickly produce zoospores it may cause severe disease outbreaks even in Mediterranean areas with only short conducive periods (Cahill *et al.* 2008). One example is in the Western Australian *Eucalyptus marginata* (jarrah) forest, where the spread of the disease increased from approximately 1.5 % of the forest area in 1940 (Dell *et al.* 2005) to 6 % in 1972 (Podger 1972), and more recently 14 % (Davison & Shearer 1989).

Whilst the role of zoospores in the spread of *P. cinnamomi* is well documented (Shearer & Tippett 1989), there is no satisfactory information on the structures used for survival over unfavourable seasons such as the long, hot, and dry summers of Mediterranean ecosystems. It is known that *P. cinnamomi* remains viable in woody tissues of dead susceptible species for up to 34 m (Collins *et al.* 2012), however the type of inoculum was not identified.

*Phytophthora cinnamomi* can also form asexual chlamydospores and sexual oospores. It is commonly accepted that chlamydospores play a role in survival as they can form thick walls and are separated from the hyphae by a septum. However, definitive evidence is lacking (McCarren *et al.* 2005). Oospores are commonly considered unimportant for survival as the heterothallic *P. cinnamomi* requires two mating types for oospore formation. The A1 mating type has a narrow distribution (Zentmyer 1980), has never been reported from the jarrah forest (G. Hardy, pers. comm.), and even where both mating types have been found, there is no evidence of mating (Dobrowolski *et al.* 2003). It has been suggested that genetic isolation of mating types has occurred as a result of a degenerating compatibility system and further, that the A2 type dominates due to the ability to form selfed oospores (Brasier 1975). Selfing of *P. cinnamomi* A2 has been reported to occur in the presence of biotic triggers such as the presence of certain *Trichoderma* species (Reeves & Jackson 1972; Brasier 1975) or suitable plant extracts (Zentmyer 1979). Furthermore, ageing (Ashby 1929) or mechanical injury (Reeves & Jackson 1974) are known abiotic stimuli. However, this autogamy has been only reported under experimental conditions (Zentmyer 1980; Jayasekera *et al.* 2007). The observation of oospores in naturally infected avocado roots obtained from plantations (Mircetich & Zentmyer 1966) are indicative that these could be also formed in natural environments, however no further tests were conducted on the identity of these oospores and relied on the fact that only *P. cinnamomi* was recovered from these roots.

Discussions about alternative survival structures are rare. Lignitubers, also known as papillae have been observed as a response to *P. cinnamomi* infection and are known to form as a general plant response to isolate cells from penetrating hyphae even though these encasements alone do not completely prevent infection (Cahill & Weste 1983) but may protect the embedded hyphal fragments of *P. cinnamomi* from dehydration and microbial attack (T. Jung, pers. comm.). Recently, a survival structure analogous to stromata of true fungi was detected in inoculation experiments of *Phytophthora ramorum* for the first time in a *Phytophthora* species and these stromata were shown to produce dense clusters of chlamydospores and sporangia (Moralejo *et al.* 2006). Persistence of *P. cinnamomi* in 'field-resistant' species was postulated by Phillips & Weste (1984) based on *P. cinnamomi* recoveries from three native monocotyledon species they had inoculated. However, the 10 d experimental period did not aim to assess formation of survival propagules and these species were not tested for *P. cinnamomi* infection in the natural environment.

We have recently reported that *P. cinnamomi* infects at least 15 native annual and herbaceous perennial plant species in the jarrah forest and that most of these were asymptomatic and previously not considered to be hosts (Crone *et al.* 2012). Inoculation experiments (Crone *et al.* 2013) further demonstrated that *P. cinnamomi* colonises a range of annual and herbaceous perennial plant species from the jarrah forest without causing symptoms and that survival propagules are formed. In the present study, we test whether survival structures are formed in the natural environment within annual and herbaceous perennial species, allowing *P. cinnamomi* to persist on highly impacted jarrah forest sites even though susceptible woody plant species have almost been eliminated from such sites.

We challenge the concept that *P. cinnamomi* is solely a necrotroph, discuss the observation of haustoria and provide evidence for the formation of abundant, viable, selfed oospores in nature as well as thick-walled chlamydospores. Further, stromata are reported for the first time for this species.

## Material and methods

### *Collection of* Phytophthora cinnamomi *positive root material*

One native annual, *Trachymene pilosa* (Araliaceae) and two herbaceous perennial plant species, *Chamaescilla corymbosa* (Asparagaceae) and *Stylidium diuroides* (Stylidiaceae) (Western Australian Herbarium 1998) were collected weekly during winter (28th Jun. 2011) to spring 2011 (1st Nov. 2011) and then less frequently at the end of spring and summer 2011/2012 from naturally *P. cinnamomi* infested black gravel sites within the *Eucalyptus marginata* (jarrah) forest of Western Australia (32°50′24.50″S 116°03′50.65″E) in areas prone to temporary waterlogging and some free-draining sites.

Briefly, whole root systems were removed from the soil, cleaned and plated immediately in the field onto a *Phytophthora* selective NARPH medium (Hüberli *et al.* 2000) but without 1,2,3,4,5 Pentachloro-6-nitrobenzene (PCNB). The plates were incubated in the dark at 21 °C (±1 °C) and examined daily for the presence of hyphae typical of *P. cinnamomi*.

### *Microscopic examination*

#### *Examination of root material*

After 2 or more days, as soon as outgrowth of hyphae typical of *Phytophthora cinnamomi* was observed from plated roots, these were removed from the agar and processed in one of four ways as quickly as possible, to minimise the formation of structures which were not present in the roots at the time of harvest. The agar plates were observed for an additional 2–3 d to ensure these hyphae were forming *P. cinnamomi* colonies. For the examination, 65 *Stylidium diuroides*, 74

*Trachymene pilosa* and 51 *Chamaescilla corymbosa* from which *P. cinnamomi* was recovered were available (Crone *et al.* 2012). About half of these were screened microscopically for potential structures of *P. cinnamomi* with representative samples used for each treatment.

*Treatment 1: morphological studies*

For detailed microscopic studies of structures formed by *Phytophthora cinnamomi*, root material was cleared with 10 % potassium hydroxide (KOH) (if suberised) and stained with Trypan blue in 0.05 % w/v lactoglycerol (1:1:1 lactic acid, glycerol, and water) (Brundrett 2008) for one to several hours according to root thickness. The roots were then destained and stored in lactoglycerol. To prepare mounted slides, destained roots were placed between a microscopic slide and a cover slip with 1–3 drops of lactoglycerol. Thicker root material was squashed where necessary and the preparation sealed with nail polish. Material was viewed and documented with a BX51 Olympus microscope and a MicroPublisher 3.3RTV Q Imaging camera and Olysia BioReport (Soft Imaging System 1986–2002) as the associated software.

*Treatment 2: proof of spore identity by germination and sequencing*

To isolate and grow single colonies from single oospores or single thick-walled chlamydospores, root material of *Chamaescilla corymbosa* in which oospores and chlamydospores were observed was cut transversely and placed on a concave microscopic slide with a drop of distilled water. The root material was gently scraped with a razor blade held at a 90° angle along the transversely cut surface. In this species, separation of spores from the root tissue was relatively easy as the already soft tuberous material was softened by some cell destruction due to *Phytophthora cinnamomi* colonisation. Approximately 60 µl of the scraped suspension was taken up with a micro-pipette and placed into a small drop of freshly squeezed mucilaginous gel from the parenchymatous cells of the leaf pulp of *Aloe vera* ('*Aloe vera* gel') that had been placed on a Petri-dish of NARH medium. The Petri-dish was then sealed and stored upside down and during the next 24 h the combined hanging drop of *A. vera* gel and spore suspension diffused into the agar medium. The addition of *A. vera* gel served several functions, it suppressed bacterial growth, provided plant metabolites such as beta sitosterol (Choi & Chung 2003) potentially beneficial for oospore germination and continuous growth, prevented desiccation and improved microscopic visualisation of the spores on agar. Once distal ends of the growing hyphae were confirmed to be derived from a spore and advanced enough to subculture, single spore colonies were prepared for subsequent long-term storage and for sequencing the ITS region (Aghighi *et al.* 2012) to provide molecular proof of identity. During the process of obtaining a single oospore isolate, 38 proliferating putative oospores of *P. cinnamomi* were photographically documented. Further isolations were stopped once the single oospore isolate from naturally infected root material was attained, likewise for germinating thick-walled chlamydospores.

*Treatment 3: fluorescent* in-situ *hybridisation (FISH) to confirm structure identity*

For additional confirmation that the oospores, stromata, and chlamydospores observed within root material were formed by *Phytophthora cinnamomi*, FISH was performed (Li 2012). The methodology was used on material collected over 7 d during winter 2011 on four root systems of *Stylidium diuroides*, three root systems of *Trachymene pilosa* and two of *Chamaescilla corymbosa*. Briefly, roots were immersed in fixative buffer, incubated and gradually dehydrated by a series of ethanol washes. The hybridization procedure was conducted in a darkened room, where 2.0 µl of probe was added as part of a hybridization mix, roots added, and incubated at 50 °C for 1.5 h in the dark, removed and incubated twice with SET buffer at 50 °C for 15 min in the dark. The specificity of the probe only allowed hybridisation with the nuclei of *P. cinnamomi* (Fig 1). Autofluorescence of plant tissues was overcome by staining in 1 % toluidine blue (Fig 1). Root material was fixed and slides viewed at 100 and 200 magnification with an epifluorescence microscope Olympus BX51 under UV light (330–385 nm) with an emission filter of 420 nm under which the labelling dye Alexafluor350 appeared bright blue compared with the bright view.

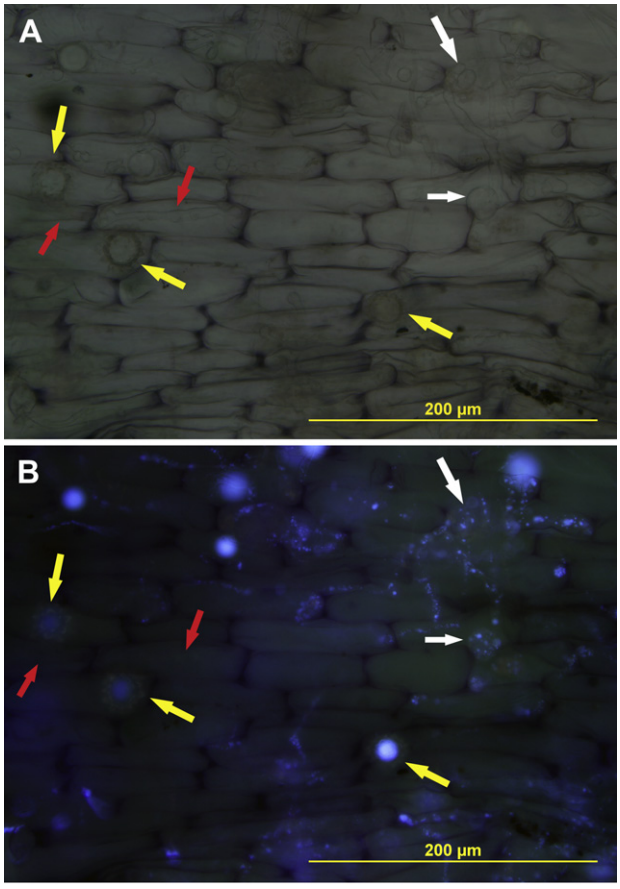


**Fig 1 – Specificity of the FISH assay and elimination of autofluorescence of host material (*Chamaescilla corymbosa*, 2nd Aug. 2011). (A) Root under white light; (B) The same area under UV light. Bright spotted fluorescence is the probe binding to the nuclei of *Phytophthora cinnamomi* in hyphae and chlamydospores (white arrows). Dull uniform fluorescence is seen in spores likely to be oospores of a *Pythium* species (yellow arrows). No fluorescence (and no binding of the probe) is seen in associated hyphae of a *Pythium* sp. (red arrows).**

Images were taken using an Olympus DP70 digital camera (Japan) with DP Controller and DP Manager software.

*Treatment 4: ultrastructural transmission electron microscopic analysis of putative haustoria*

A longitudinally sectioned asymptomatically infected root piece of *Trachymene pilosa* collected on the 19th Jul. 2011 was used for ultrastructural studies to support the microscopic observations of putative haustoria from the three examined species. The material was fixed in 3 % glutaraldehyde in 0.025 M phosphate buffer (pH 7.0) followed by 2 % osmium tetroxide in 0.025 M phosphate buffer then dehydrated through increasing concentrations of acetone (5–100 % acetone), infiltrated with increasing concentrations of Spurr's epoxy resin before embedding in fresh 100 % Spurr's resin. Areas of interest were initially viewed by 1 μm thick light microscope sections stained with 1 % methylene blue and 1 % Azur II in 1 % sodium tetra borate (Richardson *et al.* 1960). Transmission electron microscope sections (80–90 nm thick) were contrasted with uranyl acetate followed by lead citrate and viewed between 1600× to 52 000× with a Philips CM 100 Bio transmission electron microscope.

### *Determination of mating type*

*Isolates*

Out of 219 *Phytophthora cinnamomi* isolations from root systems of annual and herbaceous perennial plant species, eight representative isolates obtained from different host plants, locations within the study sites and collection times during 2011 were sequenced and confirmed to be *P. cinnamomi* (Crone *et al.* 2012). Host plants included *Chamaescilla corymbosa*, *Trachymene pilosa*, *Stylidium diuroides* and two additional jarrah forest species *Pterochaeta paniculata* (Asteraceae, annual) and *Hypocalymma angustifolium* (Myrtaceae, woody perennial). These eight isolates were used to determine the mating type to ascertain whether the oospores were produced by selfing or due to mating with the opposite mating type.

Mating tests were set up with two replicates including a positive control where the tester strains *P. cinnamomi* A1 'MUCC 794' (GenBank Accession number: JX454790) and *P. cinnamomi* A2 'MUCC 795' (GenBank Accession number: JX454791), obtained from the Murdoch University culture collection (sequenced (Aghighi *et al.* 2012) and confirmed to be *P. cinnamomi*), were used for mating the isolates. Both tester strains were used as negative controls by pairing A1 mycelium with A1 and A2 with A2.

*Preparation of isolates*

Isolates were aseptically taken out of long-term water storage and plugs of each isolate as well as the A1 and A2 *Phytophthora cinnamomi* tester strains were inserted approximately 0.5 cm deep into a surface sterilised apple (cv. Granny Smith) to revitalise the isolates. After 10 d all isolates had caused large lesions, from which four pieces (approx. 0.5 $cm^2$) from underneath the apple skin were plated on NARH and incubated. Outgrowing hyphae were subcultured for each isolate after 2 d on V8 agar. Parafilm© sealed plates were stored in the dark at 21 °C (±1 °C) for 7 d.

*Mating*

After 7 d 0.5 $cm^2$ plugs were taken from the edge of each colony and placed approximately 0.5 cm away from a similar plug containing either the *Phytophthora cinnamomi* A1 or A2 tester strain on V8 agar plates amended with 30 mg $l^{-1}$ beta sitosterol (Practical Grade, MP Biomedicals, Ohio, USA) to stimulate oospore formation (Ribeiro 1978). The beta sitosterol was dissolved with approximately 2 ml of 100 % ethanol in a heated water bath before adding to the medium before autoclaving. All plates were sealed with Parafilm©, placed within two zip lock bags and wrapped in aluminium foil to exclude light. The two replicates for each mating combination were kept in two different locations with room temperatures conducive for oospore development (between 20 °C and 26 °C). The plates were inspected after 2 and 6 weeks.

### *Morphologically similar structures in* Phytophthora cinnamomi*-free plants*

As part of a series of inoculation experiments on annual and herbaceous perennial plant species, not-inoculated plants free of *P. cinnamomi* were available for comparison with the infected plants. Microscopic examination allowed to observe whether structures seen in *P. cinnamomi* positive roots were absent in roots of these negative controls. Briefly, seeds of ten annual and herbaceous perennial plant species were sown into pasteurised black gravel soil contained in free-draining square pots (10 cm height × 7 cm) and watered daily to container capacity in an evaporatively cooled glasshouse (9–24 °C ± 2 °C). There was an average of six plants per species as a negative control. The not-inoculated seedlings (on average 39 d-old) were flooded for 24 h and mock-inoculated by exposure to agar plugs free of *P. cinnamomi*, harvested at 56 d and plated on NARH agar to observe hyphal outgrowths. Roots were then fixed and stained for microscopic examination (see Treatment 1).

## Results

### *Microscopic observations of field isolations*

*Overview*

From the end of Jun. to the beginning of Aug., *Phytophthora cinnamomi* was recovered from the naturally infected asymptomatic roots of *Trachymene pilosa*, *Stylidium diuroides*, and *Chamaescilla corymbosa*. However, from Aug. onwards, whilst *P. cinnamomi* infected individuals of *T. pilosa* remained asymptomatic until natural senescence, root lesions were observed for *C. corymbosa* although above ground tissues remained symptomless, and *S. diuroides* wilted without visible root lesions (Crone *et al.* 2012). In all cases, the root tissues appeared to be extensively colonised as evident by hyphal outgrowth onto selective agar from several areas of the root systems, and these areas corresponded with the pathogen detection within the root material. A range of morphological structures of *P. cinnamomi* was observed in all three species.

*Thick-walled chlamydospores and lignitubers*

Thick-walled chlamydospores were detected within root tissues of *Chamaescilla corymbosa*, *Stylidium diuroides*, and

*Trachymene pilosa* and these germinated to produce viable colonies (Fig 2A). Whilst some chlamydospores were observed on the surface of roots, there was evidence that they also occurred within the root tissue. The clearest indicators that chlamydospores were formed within host cells and not on the root surface were that in some cases the chlamydospores were elongated as a result of host cell constriction (Fig 2B), while in other cases the host cell walls had distorted and expanded due to the developing chlamydospore (Fig 2C).

A single thick-walled chlamydospore isolate from *S. diuroides* (collection: 26th Jul. 2011) was obtained by subculturing the proximal end from one out of two proliferating germ tubes. The ITS region was sequenced and Basic Local Sequence Alignment Tool for nucleotides (Blast n) confirmed the identity of the isolate as *Phytophthora cinnamomi* (GenBank Accession number: JX113308; Murdoch University storage MUCC 793).

One germinated chlamydospore with hyphae typical of *P. cinnamomi* was detected in dead root tissue of *C. corymbosa* in which the chlamydospore had persisted over the summer (Fig 2D). Lignitubers were observed rarely in *S. diuroides* and *T. pilosa*, in some cases in close proximity to structures of *P. cinnamomi* (Fig 2E).

*Stromata*

Besides hyphae proliferating through the host tissue, hyphal aggregations variable in density and appearance were observed. These formations are novel to *Phytophthora cinnamomi* and were termed 'stromata' (singular stroma) according to the closest analogous structure in ascomycetes and basidiomycetes. Typically stromata were confined to one cell, but it was also observed that emerging hyphae penetrated new root cells in close proximity to form more stromata (Fig 3B). Within some stromata hyphae formed chlamydospores (Fig S1B, C). Several germ tubes emerged simultaneously from stromata in root material collected during winter and developed into hyphae typical of *P. cinnamomi* (Fig 3A, D). These germ tubes were capable of producing selfed oospores (Fig 3H, Fig S2B, C) and chlamydospores (Fig S2A). The nuclei within these structures and the germ tubes hybridised with the *P. cinnamomi* specific fluorescent probe (Fig 3G). The detection of a darkly pigmented stroma germinating in dead root material (Fig 3C) confirmed that stromata were able to survive summer conditions on the study site.

*Oospores*

Oospores were repeatedly detected in plated *Phytophthora cinnamomi* positive roots of all three species in the absence of other *Phytophthora* species (Fig S3). Their formation was most abundant in the tuberous roots of *Chamaescilla corymbosa*, where they occurred in dense clusters of approximately 300–400 oospores per square millimetre (Fig 4A, Fig S3B–E). For *C. corymbosa*, oospores were mostly found within water soaked lesions which developed in mid Aug., but were also present in asymptomatic root tissue in Jul. Oospores were mainly amphigynous (Fig 4C, K, L); however, a few oospores with paragynous antheridia were also observed (Fig 4N) in accordance with earlier reports (Hüberli *et al.* 1997). Commonly, oospores were plerotic, round in shape and typically between 25 μm and 30 μm in diameter (e.g. Fig 4J), but ranged from 22 to 42 μm with a minority being oval shaped (Fig S4F) or aplerotic (Fig S5J).

Many of the oospores in the root tissue of *C. corymbosa* had germinated and proliferated within the decaying tissue (Fig 4D, E), and it was possible to dissect these proliferating oospores from the root material without damage (Fig 4E, F). However, germinated oospores in this advanced stage rarely continued to grow after transfer to *Phytophthora* selective

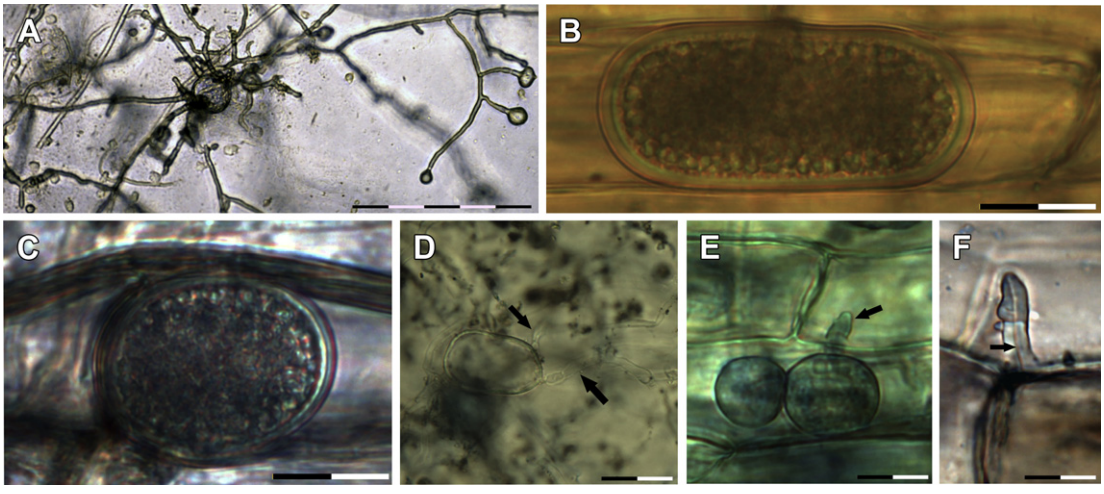


**Fig 2 – Chlamydospores and lignitubers of *Phytophthora cinnamomi*. (A) Chlamydospore separated from naturally infected root tissue of *Chamaescilla corymbosa* showing several germ tubes establishing a colony (23rd Aug. 2011); (B) A thick-walled chlamydospore elongated due to the restriction of the plant cell wall (*Stylidium diuroides*, 30th Aug. 2011); (C) Thick-walled chlamydospore distorting host cell (*S. diuroides*, 13th Sep. 2011); (D) A chlamydospore, which survived in a root over summer and germinated (arrows) the following spring (*C. corymbosa*, 4th Oct. 2011); (E) Germinating chlamydospore with germ tube encapsulated by a lignituber (arrow) (*Trachymene pilosa*, 2nd Aug. 2011); (F) Detail of lignituber with hyphae of *P. cinnamomi* in root of *Podotheca angustifolia*. Scale bars: (A) 100 μm; (B–C), (E–F) 20 μm; (D) 40 μm.**

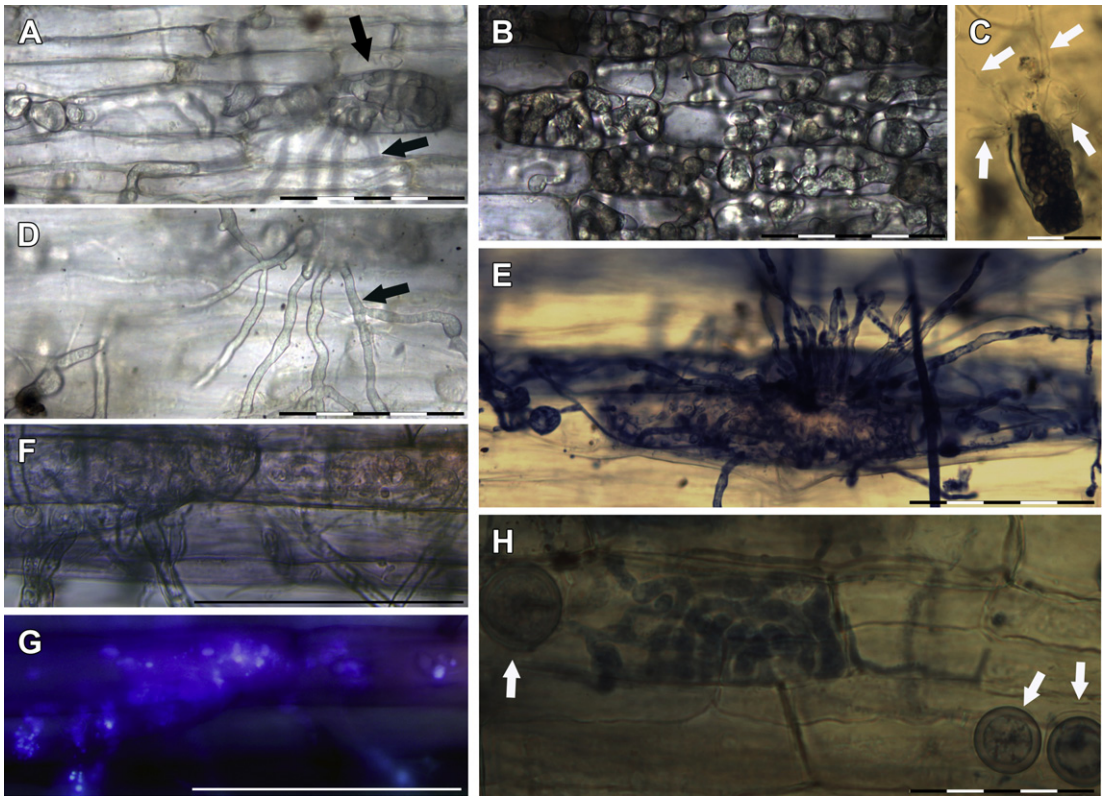


**Fig 3 – Stromata of *Phytophthora cinnamomi* found in annual and herbaceous perennial plant species as a dense hyphal aggregation with septae dividing it into several units and showing growth of multiple germ tubes. (A) and (D) Dense hyphal masses forming stroma (vertical arrow) in *Chamaescilla corymbosa* (13th Sep. 2011) with (D) detail of germ tubes arising from stroma shown in (A) (horizontal arrows); (B) Network of stromata in *C. corymbosa* (2nd Aug. 2011); (C) Pigmented stroma that has over summered and germinated with multiple germ tubes (arrows) the following spring in dead root material of *C. corymbosa* (4th Oct. 2011); (E) Dense stroma with numerous germ tubes in *Stylidium diuroides* (12th Jul. 2011); (F) and (G) Application of FISH to a germinating stroma (F) under bright view and (G) under UV light. Note in (G) the fluorescent probe binding to the *P. cinnamomi* nuclei within the stroma (*S. diuroides*, 12th Jul. 2011); (H) Stroma germinating to produce oospores (arrows) in *S. diuroides* (26th Jul. 2011). Scale bars: (A–B), (D–G) 100 μm; (H) 50 μm; (C) 40 μm.**

NARH agar medium (Fig S4D, E and Fig 4F). In contrast, oospores which had not germinated at the time of transfer to *Aloe vera* gel on NARH produced germ tubes that increased several fold in length within several hours leading to viable colonies. Separated oospores were also floated in water so germinated oospores could be rotated by application of a slight and constant pressure on the cover slip (Fig 4G, H). This enabled the view of the proximal end of the germ tubes (Fig 4H) which was otherwise only occasionally visible (Fig S4F) and also led to the observation that up to four germ tubes are initiated but usually only one developed into a colony (Fig 4H, Fig S4A–C). Oospores could be identified as *P. cinnamomi* with the FISH method at a developmental stage where the amphigynous antheridia and connected hyphae were still in active growth so that the probe binding to the nuclei was very obvious (Fig 4K, L). A single oospore colony was obtained by subculturing the proximal end of a first order side branch (Fig 4I). The ITS region was sequenced and a similarity search against the GenBank database using the Blast n confirmed the identity of the isolate as *P. cinnamomi*. The sequence of the isolate was lodged in GenBank (GenBank Accession number: JX113312) and the isolate was placed in long-term storage at Murdoch University (MUCC 792). This single oospore isolate represents the first oospore isolate obtained *in vivo* from the natural environment. Additionally, for the first time to our knowledge, the ontogeny of selfed oospores within naturally infected plant material was documented (Fig S5).

*Phytophthora cinnamomi* could be recovered from dead root material of *C. corymbosa* in spring 2011, whose deterioration suggested these died the previous year and contained over summering survival propagules. The typical *P. cinnamomi* hyphae could be traced back to thick-walled spores without cytoplasmic content. One of these had an aborted second germ tube initial as was typically observed for oospores and showed two distinct walls equating to the inner oospore wall and outer oogonial wall (Fig 4M). The same material contained clearly identifiable non-germinated oospores.

*Haustoria*

Round, oval or lobed putative haustoria were observed in naturally infected root material of *Chamaescilla corymbosa* (Fig 5B), and *Stylidium diuroides* (Fig 5A) particularly at an early stage of

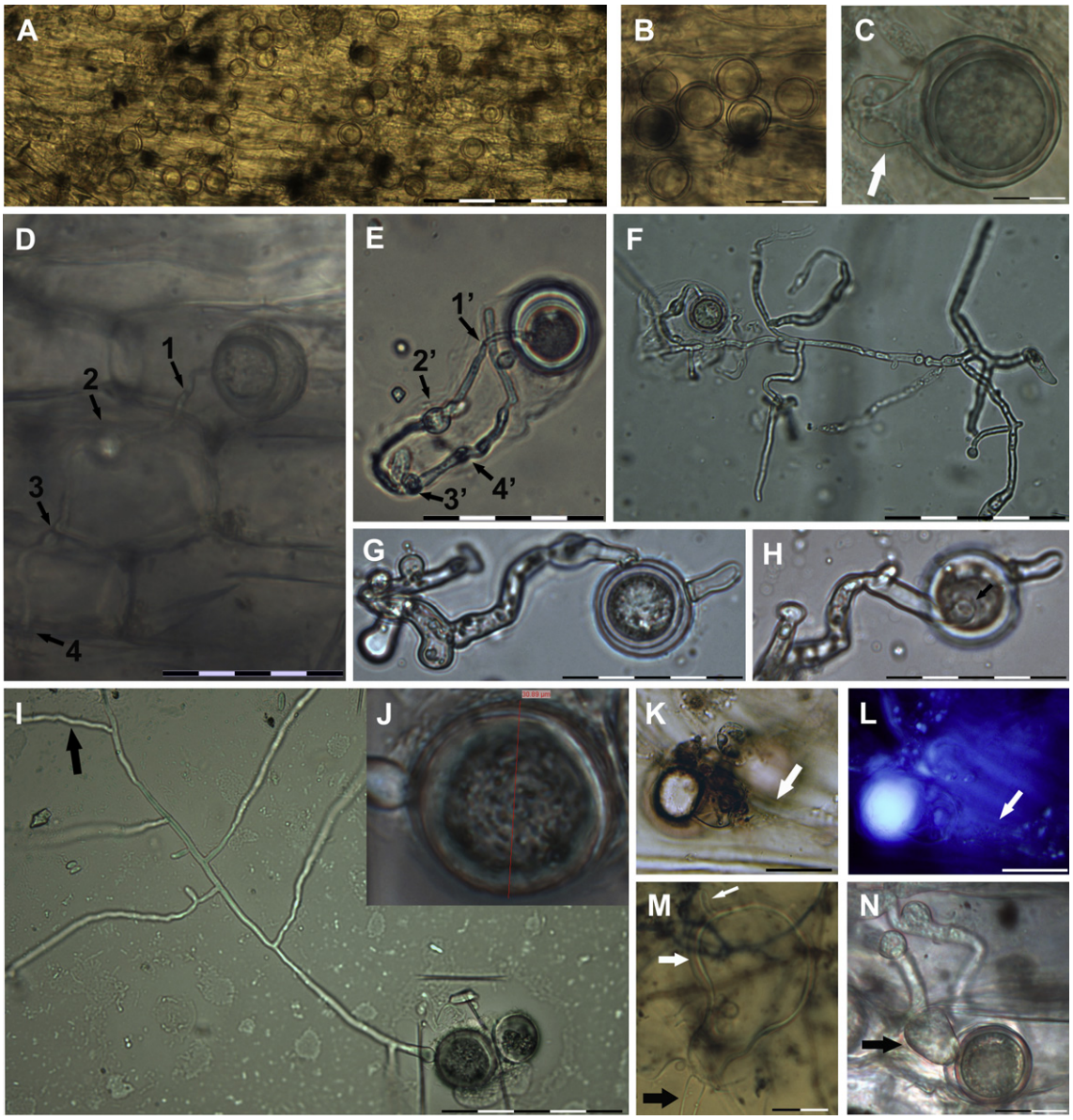


**Fig 4 – Oospores of *Phytophthora cinnamomi*. (A) Oospores in dense clusters inside tuberous roots of *Chamaescilla corymbosa* (26th Jul. 2011). (B) Detail of oospores in (A); (C) Mature plerotic oospore in oogonium (arrow: amphigynous antheridium) in *C. corymbosa* (9th Aug. 2011); (D) and (E) Germinated oospore *in situ* in *C. corymbosa*, (D) and after dissection from the tissue, (E) with hypha showing the four points where plant cell walls have been penetrated; (F) Colony-forming oospore dissected from host tissue and floated in water (*C. corymbosa*, 27th Sep. 2011); (G) and (H) An isolated germinated oospore in two rotations to show the proximal end of the germ tube in (H) (*C. corymbosa*, 6th Sep. 2011); (I) and (J) Germinating oospore, from which the hyphal branch (black arrow) was used to prepare a single oospore colony; (K) and (L) Application of FISH to a developing amphigynous oospore (K) under bright view and (L) under UV light. Note in (L) the fluorescent probe binding to the *P. cinnamomi* nuclei within the young antheridium (fluorescence of the oospore wall obscures nuclei within this cell); (M) Oospore that has over summered and germinated the following spring in dead root material of *C. corymbosa* (4th Oct. 2011). Aborted germ tube (narrow white arrow), oogonial wall (thick white arrow), colony-forming germ tube (black arrow); (N) Paragynous (arrow) antheridium (rare). Scale bars: (A) 200 μm; (B) 40 μm; (F), (I) 100 μm; (C), (M–N) 20 μm; (D–E), (G–H) 50 μm; (K–L) 25 μm.**

infection, and shown to be actual haustoria (Fig 5C–E) in *Trachymene pilosa*. Transmission electron microscopy of the interface between the host cell and *P. cinnamomi* allowed visualisation of the ultrastructure. Most of the typical features of haustoria reported in earlier studies of *P. cinnamomi* and other oomycetes (Enkerli *et al.* 1997; Hardham 2007) were present and included characteristics such as cessation of growth (in contrast to penetrating hyphae increasing in size), ageing of the structures as evident by an increase of vacuole size, and the presence of an extrahaustorial matrix between the hyphal wall and plant plasma membrane (extrahaustorial membrane).

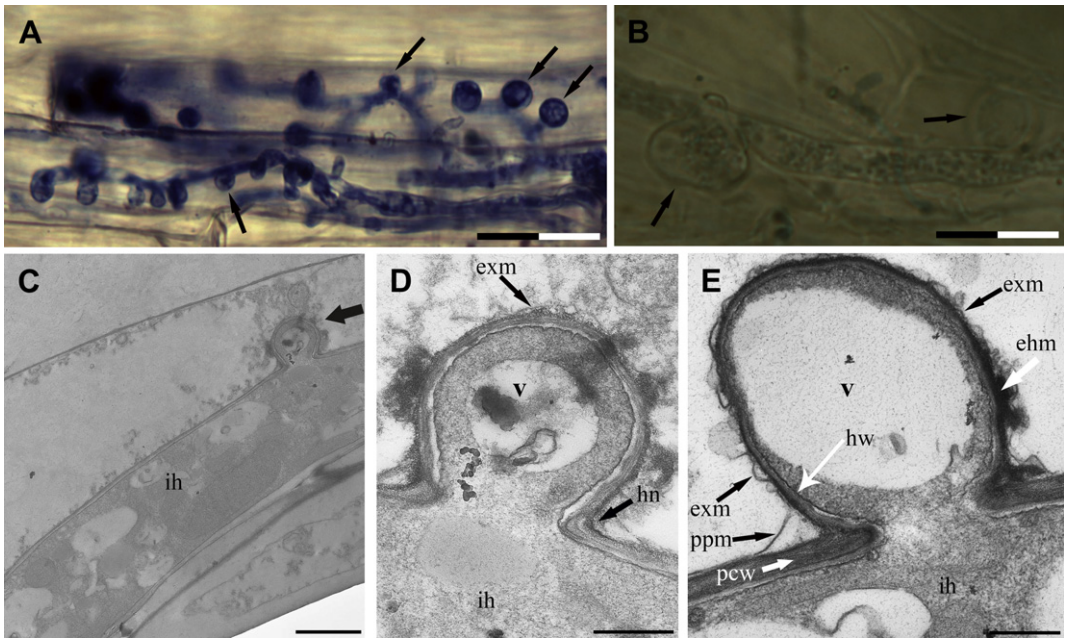


**Fig 5 – Haustoria of *Phytophthora cinnamomi*. (A) Hyphae with several haustoria (arrows show examples) in roots of *Stylidium diuroides* (9th Aug. 2011); (B) Haustoria (arrows) in roots of *Chamaescilla corymbosa* (9th Aug. 2011); (C–E) Transmission electron microscopic pictures of haustoria within roots of asymptomatic *Trachymene pilosa* (19th Jul. 2011); (D) at a higher magnification than same object in (C); (E) An older haustorium, with a large vacuole. ehm, extrahaustorial matrix; exm, extrahaustorial membrane; hn, haustorial neck; hw, hyphal wall; ih, intercellular hyphae of *P. cinnamomi*; pcw, plant cell wall; ppm, plant plasma membrane (becomes extrahaustorial membrane in region of the haustorium); v, vacuole. Scale bars: (A) 40 μm; (B) 20 μm; (C) 2 μm; (D–E) 500 nm.**

### *Mating type*

All eight isolates produced oospores when paired with the known *Phytophthora cinnamomi* A1 tester strain MUCC 794 in both replicates after 2 weeks. The positive control, A1 tester strain paired with the *P. cinnamomi* A2 tester strain MUCC 795 also produced oospores. No oospores were observed when the isolates were paired with the *P. cinnamomi* A2 tester strain, neither did the negative control combinations of the tester strains (A1 with A1; A2 with A2) produce oospores. Therefore, all tested isolates were of the *P. cinnamomi* A2 mating type. Also of interest, after 6 weeks, between one to ten selfed oospores were detected predominantly in close proximity to the cut edges of the agar blocks of the incompatible combinations between the *P. cinnamomi* isolates and A2 tester strain, but never in the zone between the agar blocks indicating that mechanical injury (Reeves & Jackson 1974) and/or ageing (Ashby 1929) has triggered the production of a few selfed oospores. In addition, rare events of selfing in three isolates were observed within the lesion area of the apple tissue when the isolates were passaged (data not presented).

### *Morphologically similar structures in* Phytophthora cinnamomi*-free plants*

No *P. cinnamomi* or other *Phytophthora* species were recovered from the mock-inoculated plants. However, some hyphal outgrowth was observed and demonstrated that the roots within the pasteurised soils were exposed to filamentous organisms other than *Phytophthora*. The microscopic examination showed that whilst some structures were dissimilar to *P. cinnamomi*, others were morphologically comparable (Fig 6).

## Discussion

This study shows for the first time that *Phytophthora cinnamomi* forms abundant selfed oospores, stromata, and thick-walled chlamydospores in naturally infected plant material. These structures were found in naturally infected roots of the Western Australian native herbaceous perennials *Chamaescilla corymbosa*, *Stylidium diuroides*, and the native annual *Trachymene pilosa*. The selfed oospores, stromata, and thick-walled chlamydospores proved to be viable as they could germinate and form colonies. The identity of the pathogen was confirmed by binding of the *P. cinnamomi* specific FISH probe to the nuclei of the hyphae, chlamydospores, oospores, and stromata. Additionally, sequencing of the ITS region of the single oospore and thick-walled chlamydospore isolates further confirmed that these were *P. cinnamomi*. No other *Phytophthora* species was isolated from the root material during the sampling time and therefore excluded oospore formation by intraspecific mating as a possibility. Spores of competing organisms other than *Phytophthora* species were present on several occasions, such as smaller sized oospores or ornamented oospores of *Pythium* species (Fig 1). In areas in which *P. cinnamomi* produced dense oospore clusters (as in the root material of *C. corymbosa*), spores of other organisms were rare. It was concluded that due to the absence of the *P. cinnamomi* A1 mating type or other mating

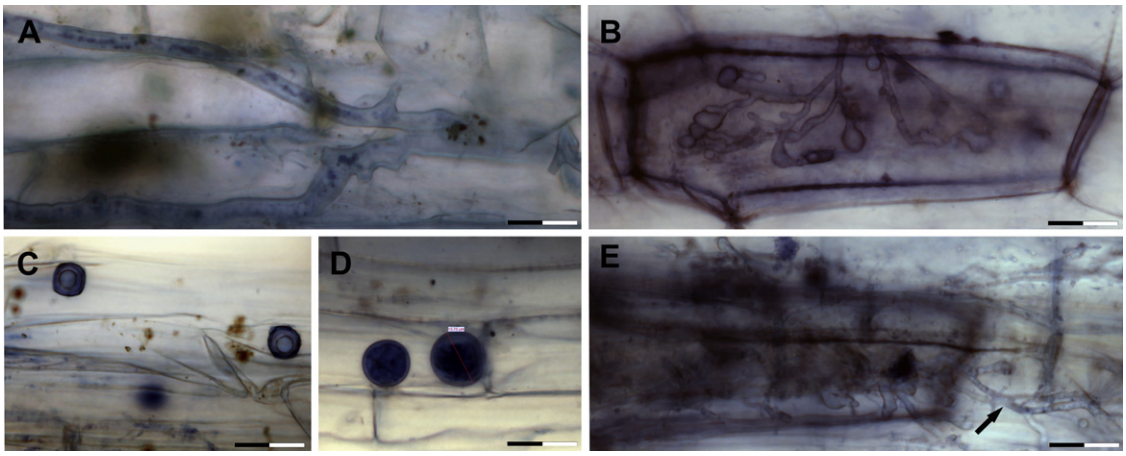


**Fig 6 – Morphologically similar structures to *Phytophthora cinnamomi* from other filamentous organisms in *P. cinnamomi*-free plants grown in pasteurised black gravel soil harvested 56 d after mock-inoculation. None of these negative controls yielded *P. cinnamomi* or other *Phytophthora* species. (A) Very similar coenocytic hyphae to *P. cinnamomi* in roots of *Trachymene pilosa*; (B) and (E) Stromata-like structure with (E) at a focal level where associated coenocytic hyphae are visible (*Dichelachne crinita*, Poaceae); (C) Spores similar to oospores of *P. cinnamomi* in roots of *Dichelachne crinita*; (D) Spores similar to chlamydospores of *P. cinnamomi* (*Amphipogon debilis*, Poaceae). Scale bars: (A), (D) 20 μm; (B), (C), (E) 40 μm.**

compatible *Phytophthora* species, the oospores were formed by selfing and mating tests confirmed that all isolates were A2. This demonstrates that in the natural environment *P. cinnamomi* is facultatively homothallic under certain conditions in some plant species. Additionally, inoculation experiments under controlled conditions produced thick-walled chlamydospores and stromata in the absence of similar filamentous microorganisms within annual and herbaceous perennial plant species (Crone *et al.* 2013); this provided additional evidence of the identity of the structures reported here.

The observation that *P. cinnamomi* proliferates in asymptomatic annual and herbaceous perennial plant species, clearly indicates that it has life strategies other than solely necrotrophic in some species (Serrano *et al.* 2012). This has important implications with regards to its ability to survive long-term in the absence of susceptible host species in which it typically grows as a necrotroph. It has been noted that the pathogen grows as a hemibiotroph under certain circumstances with an initial biotrophic and a later necrotrophic stage (Cahill *et al.* 2008) or found to switch between the modes (Shearer & Crane 2012), but species such as *T. pilosa* remained asymptomatic until natural senescence. Clearly, *P. cinnamomi* is able to grow as a biotroph in these asymptomatic species. In support, Serrano *et al.* (2012) recently described asymptomatic infections of the annual *Vicia sativa* by *P. cinnamomi* from another Mediterranean ecosystem. Although the formation of haustoria *per se* is not a definitive trait of biotrophs, as non-haustorial biotrophs do exist (Spencer-Phillips 1997), globose structures consistent with haustoria of other plant pathogen systems were observed for *P. cinnamomi* in this study. This agrees with previous studies on *P. cinnamomi* structures considered to be haustoria (Cahill & Weste 1983; Wetherbee *et al.* 1985; Cahill *et al.* 1989) although only Wetherbee *et al.* (1985) provided evidence at the level of the electron microscope. In contrast to hyphae penetrating through host cell walls, haustoria as specialised structures do not elongate further but expand and increase the size of their vacuole with age as recorded from haustoria in other species such as from the classical oomycete biotroph *Hyaloperonospora parasitica* (Mims *et al.* 2004). The presence of the extrahaustorial matrix (Coffey & Wilson 1983; Wetherbee *et al.* 1985; Enkerli *et al.* 1997; Mims *et al.* 2004) was also indicative that the observed structures are haustoria. Similar to other studies, not all haustorial structures were clearly differentiated in every region (Bushnell 1972). The formation of haustoria is evidence that *P. cinnamomi* is capable of biotrophic growth; however, we cannot yet discount that in some native annual and herbaceous perennial plant species *P. cinnamomi* lives as an endophyte. Endophytes are microorganisms that grow within the plant tissue without causing any deleterious effects to the plant, and in some cases provide a benefit to the plant. However, under certain conditions some endophytes become pathogens (Schulz & Boyle 2005). In a broader context the plastic behaviour of *P. cinnamomi* is in line with reports on other pathogens which are able to utilise biotrophic growth including other *Phytophthora* species such as *Phytophthora ramorum* and *Phytophthora kernoviae* which are capable of asymptomatic infections (Fichtner *et al.* 2012).

The production in abundance of selfed oospores, stromata, and thick-walled chlamydospores in native annual and herbaceous perennials in nature helps explain the long-term survival of *P. cinnamomi* in harsh natural environments even in the absence of deeper rooting, hence more buffered, susceptible woody plant species. Even though no living root material with oospores, thick-walled chlamydospores, and stromata of *P. cinnamomi* could be found after summer 2011/2012 to test and confirm their survival capacity, on one occasion these were observed in dead root material whose propagules must have persisted over summer from the previous year.

Hyphal aggregations in ascomycetes and basidiomycetes are termed stromata or sclerotia. In distinction to sclerotia, stromata aggregate more randomly and are located inside the host tissue rather than on the host surface (Willetts 1997).

Stromata have been further described as morphologically variable formations in regard to size, compactness, and degree of differentiation and a fungal species might only produce them under certain conditions (Willetts 1997). Functionally, it is suggested that due to the hyphal density of the stromata their capacity to store nutrients acquired from the host material is significant, resulting in the high production of mycelium and spores when conditions are favourable for germination (Willetts 1997). Stromata also act as survival propagules (Willetts 1997). Convergent evolution, resulting in analogous structures has been demonstrated for unrelated taxa occupying the same niche and this is also the case for true fungi and oomycetes (Tyler 2008) as they belong to two different kingdoms. Consequently, it is appropriate to consider that these hyphal aggregates produced by *P. cinnamomi* are stromata.

Stromata formation by a *Phytophthora* species was observed for the first time for *P. ramorum* under experimental conditions and described as small hyphal aggregates formed by repeated branching, budding, swelling, and interweaving (Moralejo *et al.* 2006). These usually darkened with time and were occasionally found to produce sporangia or dense clusters of chlamydospores (Moralejo *et al.* 2006). In the case of *P. cinnamomi*, the stromata produced clusters of oospores and chlamydospores but as yet sporangia have not been observed.

One darkened stroma whose hyphae were typical of *P. cinnamomi* was detected from dead host material from where *P. cinnamomi* was recovered, which is very indicative of their role as direct survival propagules (Fig 3K). Repeated observations are desirable in future to confirm this observation, but detection in degraded host material is difficult as these thin roots are subject to rapid breakdown.

The contribution of stromata to the long-term survival of *P. cinnamomi* is definite based on the observation that stromata occurred commonly, together with oospore clusters within the same area (Fig S3D, E), and from the observation that selfed oospores and chlamydospores (thin and thick-walled) were formed by stromata.

Due to their rare occurrence, no conclusions could be reached about the role of lignitubers in the survival of *P. cinnamomi* in the jarrah forest. Their formation should be also assessed in woody plant species, where they have been observed in larger numbers (T. Jung, pers. comm.). These structures were first described as lignitubers by Fellows (1928) and shown to originate from the plant and are a response to any pathogen attack. As these encapsulate short hyphal strands, they might preserve hyphae during summer. Germination would most likely occur from the proximal end with the least plant cell deposits around the hyphae.

Future studies on isolation and germination of oospores and chlamydospores should consider the use of *Aloe vera* gel as it appeared to have several advantages for *P. cinnamomi* and may have wider application for other oomycetes and true fungi.

## Conclusion

This study has shown for the first time the importance of selfed oospores, thick-walled chlamydospores, and stromata produced by *Phytophthora cinnamomi* in asymptomatic annual and herbaceous perennial species for the long-term survival of *P. cinnamomi*. It has also increased our understanding of a biotrophic and/or endophytic lifestyle of *P. cinnamomi* in these plant species not previously recognised as hosts of this pathogen. The findings suggest future research on whether *P. cinnamomi* grows and survives in these groups of plants similarly in horticulture and other impacted Mediterranean biomes or other conducive ecosystems where it is present. The frequency of the biotrophic/endophytic mode of growth in plant species in its suggested areas of origin where 'pathogen' and host are in relative equilibrium, compared to regions in which it has been recently introduced would also be of interest. Further, the findings might apply to other hemibiotrophic or necrotrophic oomycetes, which could potentially grow asymptomatically as biotrophs in certain hosts. Finally, due to the difficulty of studying microscopically the larger suberised roots of woody perennial species, it is possible that selfed oospores and thick-walled chlamydospores are also produced in these species but have been overlooked. It is postulated that the necrotrophic mode of growth cannot provide enough nutrients to produce the dense clusters of oospores and large numbers of chlamydospores observed in the asymptomatic plants. The biotrophic mode of growth with nutrient acquisition aided through haustoria, followed by formation of dense masses of hyphae in stromata may be a prerequisite to the formation of the high numbers of oospores and chlamydospores. From a disease management perspective, infected small root systems of annual and herbaceous perennial plant species in the surface soil provide a source of propagules easily spread by wind and water movement of soil, and by animal foraging.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2012.12.004.

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