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The microscopic examination of *Phytophthora cinnamomi* in plant tissues using Fluorescent In Situ Hybridization (FISH)

**RUNNING TITLE:** Fluorescent In Situ Hybridization of *Phytophthora cinnamomi*

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ABSTRACT

The microscopic examination of *Phytophthora cinnamomi* in plant tissues is often difficult as structures such as hyphae, chlamydospores and oospores are frequently indistinguishable from those of other fungi and oomycetes, with histological stains not enabling species differentiation. This lack of staining specificity makes the localisation of *P. cinnamomi* hyphae and reproductive structures within plant tissues difficult, especially in woody tissues. This study demonstrates that with the use of a species-specific fluorescently labelled DNA probe, *P. cinnamomi* can be specifically detected and visualised directly using fluorescent in situ hybridization (FISH) without damage to plant or pathogen cell integrity or the need for subculturing. This approach provides a new application for FISH with potential use in the detailed study of plant-pathogen interactions in plants.

INTRODUCTION

Studies of plant-pathogen interactions in planta are often inhibited by the inability to specifically identify individual microbial cells within the plant cellular matrix. Given the diversity of symbiotic, endophytic, saprophytic and competing pathogenic microbes within plant systems, the inability to positively identify each species has limited their study microscopically. This is especially true within naturally infected plant tissues. With the advent of molecular techniques species-specific identification is possible, in particular with the application of fluorescent in situ hybridization (FISH). This application has previously been demonstrated to be a valuable tool for the detection of bacteria in a range of samples (Amann et al., 1995). Autofluorescence has been reported to cause severe problems when investigating soil or plant-associated microorganisms with fluorescent probes (Amann et al., 1990, Hahn et al., 1993). Hence precluding the use of the FISH assay with some environmental samples. This problem can be overcome with the
application of dual stains and computer imaging (Franke et al., 2000). For example, FISH has been successfully applied to the detection of the plant pathogen _Ralstonia (Pseudomonas) solanacearum_ within potato tissue samples with the use of dual stains (Wullings et al., 1998). However, FISH has not yet been applied for the detection of oomycetes such as the destructive plant pathogen _Phytophthora cinnamomi_ in plant tissues.

_Phytophthora cinnamomi_ is known to cause extensive root rot on many susceptible hosts whilst in many others it causes the decay of fine surface roots subsequently leading to host vulnerability to seasonal drought stress in over 3000 susceptible plant species worldwide, including many agricultural, ornamental and forest species (Erwin and Ribeiro, 1996, Hardham, 2005). The pathogen is a soil-borne oomycete or “water mould” that infects its hosts primarily via motile zoospores that are attracted to roots (Hardy et al., 2001, Hardy et al., 2007). _Phytophthora cinnamomi_ kills plants by destroying the fine root system and lower stem tissues, restricting the plant’s ability to acquire water and nutrients from the soil (D'Souza et al., 2005, Hardy et al., 2007). Further investigation of the mechanisms of _P. cinnamomi_ development and infection of plant cells requires accurate methods with which to differentiate between the pathogen and the cells.

Isolation and detection techniques currently available for _P. cinnamomi_, both traditional and DNA based, have their limitations for pathogen observational studies. Identification of _Phytophthora_ species via traditional microscopic examination is never easy as the morphological characters are not consistently expressed during isolation culturing (Duncan and Cooke, 2002). This failure for consistent morphological characters has resulted in false-negative _P. cinnamomi_ isolations (Hüberli et al., 2000). Polymerase chain reaction (PCR) that allows for very rapid and
sensitive detection of *Phytophthora* presence (Duncan and Cooke, 2002) fails to allow the visualisation of the pathogen within the surveyed material itself.

Furthermore, there are often difficulties in visualising hyphae and propagules such as oospores and chlamydospores within plant material, and when they are present it is difficult to determine whether the structures observed belong to *P. cinnamomi*, other *Phytophthora* species or other oomycetes such as *Pythium* species. Frequently, it is also difficult to locate hyphae and other structures of *P. cinnamomi* within dark and coarser woody plant tissues using clearing and staining techniques (Shea et al., 1980, Old et al., 1984, Schild, 1995). This lack of confidence in confirming the presence of *P. cinnamomi* in naturally infected plant tissues has hampered the understanding of the biology and ecology of the pathogen in resistant/tolerant and susceptible plant species. Consequently there is a clear need for a detection technique that is species specific and allows for the in situ visualisation of *P. cinnamomi* within naturally infected plant materials.

The objective of this study was to develop a FISH assay for the fast and reliable detection of *P. cinnamomi* as well as a confirmatory tool for detailed microscopic studies of *P. cinnamomi* within plant tissues.

**MATERIALS AND METHODS**

**Design of fluorescent in situ hybridization probe**

The ITS (internal-transcribed spacer) regions of *P. cinnamomi* were selected for the target site of the probe as it showed low intra-species variation and was found to be variable against other *Phytophthora* species (Lee and Taylor, 1992). The probe selected for the present study was adapted from a *P. cinnamomi* specific probe Cin5b reported by Anderson et al. (2006). Mismatch of at least one nucleotide from a probe to other sequences was demonstrated to infer specificity
(Li et al., 1996) and was applied in designing the probe for this study. The probe used was Alcin5F which has a length of 21 oligonucleotide sequence 5’ CTCTTTTTAACCATTCTG and a 38.1% GC ratio, with a melting temperature of 48.8°C for the probe-target duplex. The sequence was checked against the N-BLAST program (PubMed) to assess specificity. The probe was commercially synthesized and was labeled with AlexaFluor350 dye at the 5' end which excites at 350 nm and emits at 442 nm (BioSynthesis, Texas, USA).

**Specificity of probe**

Thirty-seven isolates of *Phytophthora* (n=29), *Pythium* (n=2) and bacterial (n=6) species were sourced from the Vegetation Health Service (VHS, Department of Environment and Conservation, Western Australia), the Centre for *Phytophthora* Science and Management (CPSM, Murdoch University, Western Australia) and from the isolate collection held at the Veterinary Clinical Pathology (VCP) at Murdoch University, Western Australia (Table 1). Of the 29 *Phytophthora* isolates, 16 were *Phytophthora* species from seven different *Phytophthora* clades (see Brasier, 2009), and all isolates from each of these species have been recovered from dead and dying plants in Western Australia. These included species (*P. cambivora* and *P. niederhauserii*) belonging to clade 7 (see Brasier, 2009) that have close phylogenetic affinity to *P. cinnamomi* within the ITS1 region. These isolates were chosen to test the specificity of the probe.

Fresh cultures of the *Phytophthora* and *Pythium* species were regenerated from long-term water storage by plating a single agar plug from water cultures onto corn meal agar (CMA) plates and incubating at 26°C for 3 days in the dark. Sterile, high humidity culture chambers were prepared by moistening filter paper discs within 9 cm diameter Petri-dishes. Two matchsticks were then
placed on top of the filter paper to hold a microscope slide containing the culture slightly off the surface. *Phytophthora* and *Pythium* isolates grown on CMA plates were aseptically cut into 1 cm x 1 cm plugs and mounted face down onto sterile microscope slides. Cultures were incubated at 20°C for 3-5 days in the culture chambers to allow hyphal growth onto the slide. Bacteria species were mounted as dry cell smears on microscope slides and heat fixed by passing the slides briefly over a flame. The FISH assay was then applied.

**Fluorescent in situ hybridization (FISH) assay methodology**

Fluorescent in situ hybridization was performed based on the protocols described by Vandersea et al. (2006) with alterations. Briefly, the growth of the *Phytophthora* and *Pythium* species was examined under a light compound Olympus CH2 microscope (Olympus, Japan) and the agar plugs were carefully removed from the microscope slides leaving the hyphae on the slides. Hyphae were then heat fixed onto the slide by placing the slide on a 50°C hotplate for 15 s and Frame-seal© (BioRad) was placed on the slide around the area of hyphae growth to create a dike on the slide in which successive fixative and staining reactions could be performed. A fixative buffer was added to the dike; 120 µl of 4°C fixative buffer (44 ml of 95% ethanol, 10 ml of deionized H2O, and 6 ml of 25x SET buffer [3.75 M NaCl, 25 mM EDTA, 0.5 M Tris HCl pH 7.8]) and left to incubate at 4°C for 40 min. The fixative buffer was then drawn off with filter paper and rinsed carefully with PBS (800 ml distilled water, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4 and 0.24 g of KH2PO4 [pH 7.4]). Slides were placed on a heating block at 50°C for 5 min. The slides were gradually dehydrated with ethanol by dipping in 50% ethanol solution for 90 s. This process was subsequently repeated with 80% and 96% ethanol solutions for 90 s each and the slides were left to air-dry.
The hybridization procedure was performed on the air-dried slides in a darkened room. A hybridization mix was prepared by mixing 2 µl of probe (20 µM) to 125 µl of preheated 50°C hybridization buffer (5x SET buffer, 0.1% [v/v] Igepal-CA630 [Sigma, Castle Hill, NSW, Australia] and 25 µg/ml polyA potassium salt [Sigma, Castle Hill, NSW, Australia]). This hybridization mix was then added to the air-dried slides. Slides were incubated with the hybridization mix at 50°C for 1.5 h in the dark. The hybridization mix was then removed and 120 µl of 50°C preheated SET buffer was added. The slides were incubated with SET buffer at 50°C for 15 min in the dark. The SET buffer was then drained off and the incubation treatment with preheated SET buffer was repeated. The SET buffer was drained off and the slides were air-dried. To reduce autofluorescence in all the isolates tested, 0.5 ml of 1% toluidine blue was added to the samples for 1 min and then rinsed in PBS until the solution ran clear. The Frame-seal© was removed and a 32 mm coverslip was placed onto the slide with a drop of ProLong® Gold Anti-fade (Invitrogen, Mulgrave, VIC, Australia). Slides were stored in the dark at ambient temperature until viewed.

**In vitro infection of plant material**

Pink Lady apple tissues, commonly used as host for *P. cinnamomi* (Mbaka et al., 2009), were also found to present limited plant autofluorescence. The apples were cored to a depth of 1.5 cm and a five day-old *P. cinnamomi* plug (isolate MP94.48), grown on half-strength Potato Dextrose Agar (PDA) (19.5 g PDA (Becton Dickinson Co.), 7.5 g Difco Agar mixed with 1 liter deionised water) was placed into the core and the apple was then covered in a paper towel moistened with 70% ethanol. The apples were incubated at 20°C for 4 days in the dark for lesions to develop. Tissue sections of approximately 1 mm thickness were sliced free-hand from the infected region of the apple using a sterile scalpel blade. The sections were viewed under the Olympus CH2
compound microscope to confirm \textit{P. cinnamomi} infection. The apple sections were then assayed with FISH as described below. Non-infected apple sections were also assayed accordingly as negative controls.

Three day-old \textit{Trachymene pilosa} and \textit{Lupinus angustifolius} cv. Mandalup seedlings had their tap roots submerged in 800 ml distilled water and 50 ml soil extract. The non-sterile soil extract was obtained by flooding 100 g of commercial composted potting mix (Coles\textsuperscript{®} Reliance Potting Mix) with 1 liter distilled water. After gentle shaking at 150 rpm for 3 h on an orbital shaker, the soil extract was extracted via filtration through Whatman No 1 filter paper (Whatman Ltd, Rydalmere, New South Wales, Australia). Inoculation was set up with 3 day-old cultures of \textit{P. cinnamomi} isolate MP94.48 grown on V8 agar plugs as described by Miller (1955). Five agar plugs were aseptically cut and placed into the distilled water, which contained soil extract. The seedlings were left to allow infection to establish for 5 days at 21°C. Infected roots of \textit{T. pilosa} and \textit{L. angustifolius} were viewed under the Olympus CH2 compound microscope at 100 to 200x magnification for hyphal presence. Sections of roots that had \textit{P. cinnamomi} growth were aseptically removed, squashed between two microscope slides and assayed with FISH as described below. Roots of both species that were not infected were also assayed in parallel as negative controls.

**Analysis of naturally infected plant materials**

Roots of \textit{Chamaescilla corymbosa}, \textit{Paracaleana nigrita}, \textit{Stylidium diuroides} and \textit{T. pilosa} suspected to be infected with \textit{P. cinnamomi} were collected from jarrah (\textit{Eucalyptus marginata}) forest sites highly impacted with \textit{P. cinnamomi} at Willowdale (116.03°E, 32.50°S), Western Australia during the months of July, August and September 2011. The roots were placed onto
NARPH plates a medium selective for *Phytophthora* as described by Hüberli et al. (2000) to confirm infection and colonization prior to the FISH assay. However, due to restriction on the use of Terraclor (PCNB), it was excluded from the medium. The NARH plates were incubated for 3 days at 22°C in the dark. The roots were then viewed using the 100x objective of a CH2 compound microscope (Olympus, Japan) to detect the presence of *P. cinnamomi*. Root regions that showed structures characteristic of *P. cinnamomi* were then aseptically removed and assayed using FISH, as described below.

**Fluorescent in situ hybridization (FISH) assay for infected plant material**

Each of the plant tissues were placed in sterile 32 mm diameter Petri-dishes and 1 ml of fixative buffer with 3% polyoxyethylenesorbitan monolaurate (Tween 20) chilled to 4°C was added and the sections left to incubate at 4°C for 1 h. The fixative buffer was then drained with filter paper and PBS used to wash off any remaining buffer. Petri dishes were placed onto a 50°C heating block for 5 min. The tissue samples were then gradually dehydrated with a series of ethanol washes. One millilitre of 50% ethanol was added and removed after 2 min. This process was repeated with 80% and 96% ethanol solutions for 2 min each and left to air-dry. The hybridization mixture was prepared with 20 µl of probe (20 µM) to 1.25 ml of preheated hybridization buffer (50°C). This hybridization mix was then applied to the air-dried tissue samples and incubated at 50°C for 1.5 h in the dark. The hybridization mix was removed and 1 ml of preheated 50°C SET buffer added. The tissue samples were incubated with SET buffer at 50°C for 15 min in the dark. The SET buffer was drained and repeat incubation with preheated SET buffer was performed before being drained and sections were left to air dry.

**Mounting of plant material onto slides**
To reduce autofluorescence from the plant materials, 0.5 to 1.5 ml of 1% toluidine blue was added to the tissue samples depending on sample size. The tissue samples were stained with toluidine blue for 1 to 5 min and then rinsed in PBS until the solution ran clear. Tissue samples were dried on filter paper and mounted onto microscope slides. A cover slip was placed onto the slide with a drop of ProLong® Gold Anti-fade (Invitrogen). Slides were stored in the dark until they were viewed.

**Microscopy and image acquisition**

Hybridized microscope slides were viewed under an epifluorescence microscope BX51 (Olympus) with violet excitation (330 to 385 nm) with an emission filter at 420 nm under which Alexafluor350 dye appears bright blue. The cellular morphology of both plant and *P. cinnamomi* cells were assessed using the bright field exposure prior to viewing under fluorescent excitation at 200 to 400x magnification. Images were acquired with a DP70 digital camera (Olympus, Japan) and its associated software, DP Controller and DP Manager.

**RESULTS**

**Specificity of probes**

The probe was specific to *P. cinnamomi* and no hybridization was observed with any of the other *Phytophthora*, *Pythium* or bacterial species tested. *Phytophthora cambivora* and *P. niederhauserii* which belong to clade 7 and are closely related to *P. cinnamomi* also showed no hybridization, further highlighting the specificity of the probe (Figure 1B, H). No fluorescence of nuclei was observed for the non *P. cinnamomi* isolates tested even with high exposure of ultraviolet excitation which gives a blue background (Figure 1B, D, F and H). Under
high intensity of ultraviolet exposure, a faint autofluorescence from *P. niederhauserii* hyphae was observed (Figure 1H).

The specificity of the probe was confirmed for *P. cinnamomi* isolates with the nuclei fluorescence of *P. cinnamomi* cells observed following in situ hybridization (Figure 1J, L, N and P). This nuclei fluorescence within the chlamydospores and hyphae was clearly distinct from the dark background as checked under bright field exposure.

**Plant materials**

Non-infected *Lupinus* and *Trachymene pilosa* roots that were treated with toluidine blue showed no fluorescence with UV excitation. Non-infected plant materials that were assayed with FISH also showed no fluorescence, confirming the probe specificity to *P. cinnamomi* as there was no hybridization between probe and the uninfected plant cells (Figure 2B and D). This demonstrated the ability to use the probe to detect *P. cinnamomi* in plant material.

When inoculated plant material was assayed, *P. cinnamomi* mycelium present in the plant tissue hybridized with the probe and formed bright blue fluorescence under UV excitation (Figure 3B, D, F, H and J). The blue fluorescence was observed to be concentrated within the *P. cinnamomi* nuclei present both within plant cells and intercellularly (Figure 3). With adequate quenching of the plant autofluorescence, this blue fluorescence was readily distinguished from any background fluorescence.

Naturally infected field samples that were assayed with FISH showed bright blue fluorescence demonstrating the presence of *P. cinnamomi* and distinguishing it from other fungal or oomycete species present (Figure 3L, M, P, R and T). Oospores of *Pythium* species exhibited a uniform
faint blue autofluorescence but can be readily identified with their “spiky” cell wall appearance (Figure 3Q and R). Fluorescence of *P. cinnamomi* nuclei could also be detected in woody root material of *S. diuroides* when plant cells and hyphal structures were not seen under light magnification (Figure 3S and T).

**DISCUSSION**

The assay described allows for the detection of different *P. cinnamomi* life stages within plant material. The species-specific probe allows *P. cinnamomi* to be readily distinguished from plant cells and other fungal, bacterial or oomycete cells and provides direct visualisation of the pathogen within plant tissues. In comparison to the application of FISH to identify or detect the presence of prokaryotic organisms, where the lack of a nucleolus leads to the fluorescence of the entire cytoplasm, the application of this technique to eukaryotic organisms results in fluorescence being concentrated in the nucleolus only (Figure 3). In contrast to prokaryotic systems for which there is considerably more data available for the 16S and 23S rRNA subunits, the depth of sequence data contrasting *Phytophthora* rRNA is considerably less. Targeting the ITS1 region utilises the depth of sequence data available for the ITS region for *Phytophthora* species (Blair et al., 2008, Cooke et al., 2000). This is critical when designing probes for the analysis of environmental samples in which several species of *Phytophthora* may be present in any given sample. Furthermore, as the ITS region has been used for the development of many species-specific diagnostic assays this assay may be readily adapted to other species across the *Phytophthora* genus (Anderson et al., 2006, O’Brien et al., 2009). As done here, species-specific PCR primers may be adapted for FISH by centralising polymorphic regions within the probe as opposed to having them located at the 3’ end of the primer which is favoured for PCR.
Importantly, the application of FISH on naturally infected field samples demonstrated the ability to identify the different *P. cinnamomi* structures such as hyphae and chlamydospores in plant roots containing structurally analogous hyphal and spore structures of other fungi or oomycetes. Results from the screening of other oomycete and bacterial species confirmed that the probe does not hybridize to other species of *Phytophthora* and *Pythium*, or to common bacterial genera that are found in the environment. This species specificity could be further utilised by designing probes for additional species to allow *P. cinnamomi* growth to be assessed in the presence of competitive pathogens or other endophytic microorganisms. Indeed, the use of multiple species-specific probes could allow for direct comparison of the competitive/synergistic interaction between different *Phytophthora* species in field samples.

Inoculation trials utilised in this study have demonstrated the application of FISH within a range of plant tissues in which different structures of the pathogen were observed. Applying this technique to naturally infected field samples from a range of plant species demonstrated the potential for this assay to rapidly identify *P. cinnamomi* within environmental samples mounted onto microscope slides via direct observation, providing significant benefits to epidemiological studies. This direct approach of identification allows viewing and analysis of the pathogen within the host tissues in their natural state without the need for subculturing and/or isolation. Therefore, application of this assay will facilitate studies on *Phytophthora* species with regards to infection, colonisation, and survival in both horticultural and natural ecosystems.

Although this method is a direct approach for the detection of *P. cinnamomi* nuclei in cells, accurate quantification of *P. cinnamomi* concentration would still largely rely on PCR detection (Eshraghi et al., 2011). The use of more sensitive molecular techniques such as PCR detection however, does not allow the microscopic examination of the pathogen within the host tissue.
PCR results could infer presence or absence of *P. cinnamomi* within root material but does not accurately identify the mycelium and spores of *P. cinnamomi* from other fungi or oomycetes, or provide us with an understanding of how the pathogen might be surviving in plant tissues. The demonstrated species-specificity for the detection of *P. cinnamomi* within host tissue is an advantage that this FISH assay has over current isolation and detection techniques. Mycelium and other fungal or oomycete structures attached to the surface of the plant materials were often observed to wash off during the numerous washing steps involved. This is one limitation of the assay, and precludes the use of it on material that has only mycelium growth on the surface.

The probe was observed to penetrate the layers of plant cell walls and hybridize with *P. cinnamomi* embedded within all the plant materials analysed. The ability of the probe to penetrate several layers of plant cells allows its application to thick sections and to a wider variety of plant material. For example, the FISH assay aided in the detection of *P. cinnamomi* within the woody root tissue of *S. diuroides* when difficulties with viewing the pathogen under bright field magnification were encountered. This advantage allows for the localisation and microscopic examination of infection sites in plant material. The application of the assay to thick sections however, requires the user to focus on specific parts of the magnified view. For example, the fluorescence of *P. cinnamomi* nuclei in Figure 1 can be observed to be blurred in some instances whilst others a clearly defined. This blurring of the fluorescence signals is due to the position of the probe within the image plane, and results from the scattering of the out of focus nuclei fluorescence emission that is diffracted, reflected or refracted on its way to the objective lens (Conchello and Lichtman, 2005). The distinctness of the fluorescence can be resolved when the particular section is set back into focus.
A level of expertise however, is required to be able to discriminate between non-specific and specific fluorescence. Non-specific fluorescence such as autofluorescence from both plant tissues and plant pathogens may cause difficulties in interpreting results from the assay. Therefore, the researcher should also be familiar with structures of plant cells, the organism of interest and other plant pathogens to fully utilise this assay.

In the present study, the major technical difficulty in the application of FISH was overcoming the autofluorescence produced by many plant cells without quenching the fluorescence of the *P. cinnamomi* specific probe conjugated with AlexaFluor350. Numerous treatments were applied to counter this non-target background fluorescence. Infected plant materials were treated with varying concentrations of sodium hydroxide treatments ranging from 0.001% - 1.0% for 4 hours (Shumway et al., 1988). However, no significant differences to the autofluorescence were seen after the treatments. Counterstaining with Evans blue was also trialed (Malajczuk et al., 1975). In the present study, the Evans blue treatment proved to be inadequate as the counterstain often produced red fluorescence from plant cells under UV excitation which at times masked or suppressed the probe fluorescence. Evans blue was particularly unsuccessful in quenching the autofluorescence of xylem vessels in root materials.

In contrast, treatment with toluidine blue completely quenched the autofluorescence across all plant samples analysed, with the probe fluorescence readily distinguished at UV excitation (330-385 nm). Toluidine blue was selected for its properties in quenching plant cell autofluorescence under UV excitation (Sakai, 1973, Biggs, 1985). Fluorescence from the stain excites at 560 nm wavelength, allowing for compatible use with the probe. In addition, non-infected *Lupinus* and *Trachymene pilosa* roots that were treated with toluidine blue showed no fluorescence with UV excitation. This demonstrates the suitability for toluidine blue treatment to be used as a
counterstain with the FISH assay as it quenched the autofluorescence produced from plant tissues under UV excitation. The counterstaining can only be applied post hybridization as toluidine blue is soluble in ethanol and alkaline solutions.

While several studies have investigated the application of FISH in chromosomal mapping studies of Phytophthora species (Moy et al., 2004, Tian et al., 2006), this is to our knowledge the first application of the technique in planta for cytological analysis. This study demonstrates the application of FISH for the detection of *P. cinnamomi* in plants without isolation into pure culture while maintaining the integrity of the pathogen and the plant cells. This assay will improve further investigation of the pathogenic pathways and survival of *P. cinnamomi* within host tissues, as well as detailed plant pathogenic interactions within the root and rhizosphere environments.

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Figure captions


Figure 2: Micrographs of non-infected Trachymene pilosa root (A and B) and Lupinus augustifolius root (C and D) assayed with FISH and AlexaFluor350-labelled, Phytophthora cinnamomi-specific (Alcin5F) probe further stained with 1% toluidine blue. Light micrographs (A and C) and ultraviolet micrographs (B and D).