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The Microscopic Examination of *Phytophthora cinnamomi* in Plant Tissues Using Fluorescent *In Situ* Hybridization

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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 localization 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 visualized 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, while 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, 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 visualization of the pathogen within the surveyed material itself.

Furthermore, there are often difficulties in visualizing 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* visualization 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 internal-transcribed spacer (ITS) regions of *P. cinnamomi* were selected for the target site of the probe as it showed low intraspecies 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′ CTCTCTTTTAAACCCATTCTG 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 labelled with AlexaFluor350 dye at the 5′ end which excites at 350 nm and emits at 442 nm (BioSynthesis, Lewisville, TX, 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 × 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.

Table 1 *Phytophthora*, *Pythium* and bacterial isolates used for confirming probe specificity

Genera	Species	Isolate no.	Source	Location of origin	GenBank accession no.
Phytophthora	*P. arenaria*	PAB11-12	CPSM-MU	*Corymbia ficifolia*, Attadale, WA	JX113289
	P. bilorbang	CBS161653	CPSM-MU	*Rubus anglocandicans*, Manjimup, WA	JQ256377
	P. elongata	VHS2952	CPSM-MU	Soil, Nannup, WA	–
	P. elongata	VHS13558	DEC-VHS	*Eucalyptus marginata*, Chipala, WA	JX113301
	P. elongata	VHS13584	DEC-VHS	*E. marginata*, Carder, WA	JX113300
	P. cambivora	DCE5	DEC-VHS	–	JX113292
	P. cambivora	DCE31	DEC-VHS	*Malus domesticus*, Adelaide Hills, SA	JX113293
	P. cambivora	DCE531	DEC-VHS	*Prunus* sp., Michigan, USA	JX113291
	P. cinnamomi	MP94-48	CPSM-MU	*E. marginanta* Willowdale, WA	JX113294
	P. cinnamomi	MP128	CPSM-MU	*Xanthorrhoea preissii* Jarrahdale, WA	–
	P. cinnamomi	VHS15773	DEC-VHS	Fitzgerald River National Park, WA	JX113295
	P. cinnamomi	VHS16441	DEC-VHS	*Banksia cuneata*, Narrogin, WA	JX113298
	P. cinnamomi	VHS16740	DEC-VHS	*Banksia violacea*, Fitzgerald River National Park, WA	JX113296
	P. cinnamomi	VHS16779	DEC-VHS	*Adenanthos barbiger*, North Dandalup, WA	JX113297
	P. constricta	CBS125801	DEC-VHS	Fitzgerald River National Park, WA	HQ013225
	P. crytogea	MU25	CPSM-MU	*P. radiata* Jarrahwood Plantation, WA	–
	P. crytogea	MU28	CPSM-MU	Soil, South Coast, WA	–
	P. gibbosa	CBS127951	DEC-VHS	*Acacia pycnantha*, Scott River, WA	HQ012933
	P. inundata	PAB11-02b	CPSM-MU	*Casuarina obesa*, Shenton Park, WA	JX113302
	P. inundata	VHS25710	DEC-VHS	*Xanthorrhoea platyphylla*, Collets Road, WA	JX113303
	P. litoralis	PAB11-02a	CPSM-MU	*Casuarina obesa*, Shenton Park, WA	JX113304
	P. multivora	MJS	CPSM-MU	*Pinus radiata* Baudin Plantation, WA	–
	P. multivora	VHS14926	DEC-VHS	*E. marginata*, Carder, WA	JX113306
	P. niederhauserii	VHS17577	DEC-VHS	*Banksia prionotes*, Lancelin, WA	JX113307
	P. nicotianae	MP5	CPSM-MU	–	–
	P. nicotianae	MU7	CPSM-MU	–	–
	P. palmivora	MU128	CPSM-MU	–	–
	P. taxon humicola	VHS25241	DEC-VHS	*Casuarina obesa*, Alfred cove, WA	JX113290
	P. thermophila	CBS127954	DEC-VHS	*E. marginata*, Dwellingup, WA	EU301155
Pythium	*Pythium* spp.	MU142	CPSM-MU	–	–
	Pythium irregularis	WAC7678	CPSM-MU	Department of Agriculture WA	–
Bacteria	*Escherichia coli*	–	VCP-MU	–	–
	Enterococcus spp.	–	VCP-MU	–	–
	Proteus mirabilis	–	VCP-MU	–	–
	Pseudomonas aeruginosa	–	VCP-MU	–	–
	Serratia marcescens	–	VCP-MU	–	–
	Staphylococcus aureus	–	VCP-MU	–	–

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, Shinjuku-ku, 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© (Bio-Rad, Gladesville, NSW, Australia) 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. 120 μl of 4°C fixative buffer [44 ml of 95% ethanol, 10 ml of deionized H_2O, and 6 ml of 25× SET buffer (3.75 M NaCl, 25 mM EDTA, 0.5 M Tris HCl pH 7.8)] was added to the dike 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 Na_2HPO_4 and 0.24 g of KH_2PO_4 (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 [5× SET buffer, 0.1% (v/v) Igepal-CA630 (Sigma, Castle Hill, NSW, Australia) and 25 μg/ml polyA potassium salt (Sigma)]. 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 5-day-old *P. cinnamomi* plug (isolate MP94.48), grown on half-strength Potato Dextrose Agar (PDA) [19.5 g PDA (Becton Dickinson Co., North Ryde, NSW, Australia), 7.5 g Difco Agar (Becton Dickinson Co.) mixed with 1 l 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 *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 *Trachymene pilosa* and *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® Reliance Potting Mix, Coles Supermarkets, Tooronga, Vic., Australia) with 1 l 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, NSW, Australia). Inoculation was set up with 3-day-old cultures of *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 *T. pilosa* and *L. angustifolius* were viewed under the Olympus CH2 compound microscope at 100 to 200× magnification for hyphal presence. Sections of roots that had *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 *Chamaescilla corymbosa*, *Paracaleana nigrita*, *Stylidium diuroides* and *T. pilosa* suspected to be infected with *P. cinnamomi* were collected from jarrah (*Eucalyptus marginata*) forest sites highly impacted with *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 100× objective of a CH2 compound microscope (Olympus) 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 was 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 was 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 (330–385 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 was assessed using the bright field exposure prior to viewing under fluorescent excitation at 200 to 400× magnification. Images were acquired with a DP70 digital camera (Olympus) 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 are closely related to *P. cinnamomi* also showed no hybridization, further highlighting the specificity of the probe (Fig. 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 (Fig. 1b,d,f,h). Under high intensity of ultraviolet exposure, a faint uniform autofluorescence from *P. niederhauserii* hyphae was observed (Fig. 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 (Fig. 1j,l,n,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 *T. 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 (Fig. 2b,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 (Fig. 3b,d,f,h,j). The blue fluorescence was observed to be concentrated within the *P. cinnamomi* nuclei present both within plant cells and intercellularly (Fig. 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 (Fig. 3l,m,p,r,t). Oospores of *Pythium* species exhibited a uniform faint blue autofluorescence but can be readily identified with their ‘spiky’ cell wall appearance (Fig. 3q,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 (Fig. 3s,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

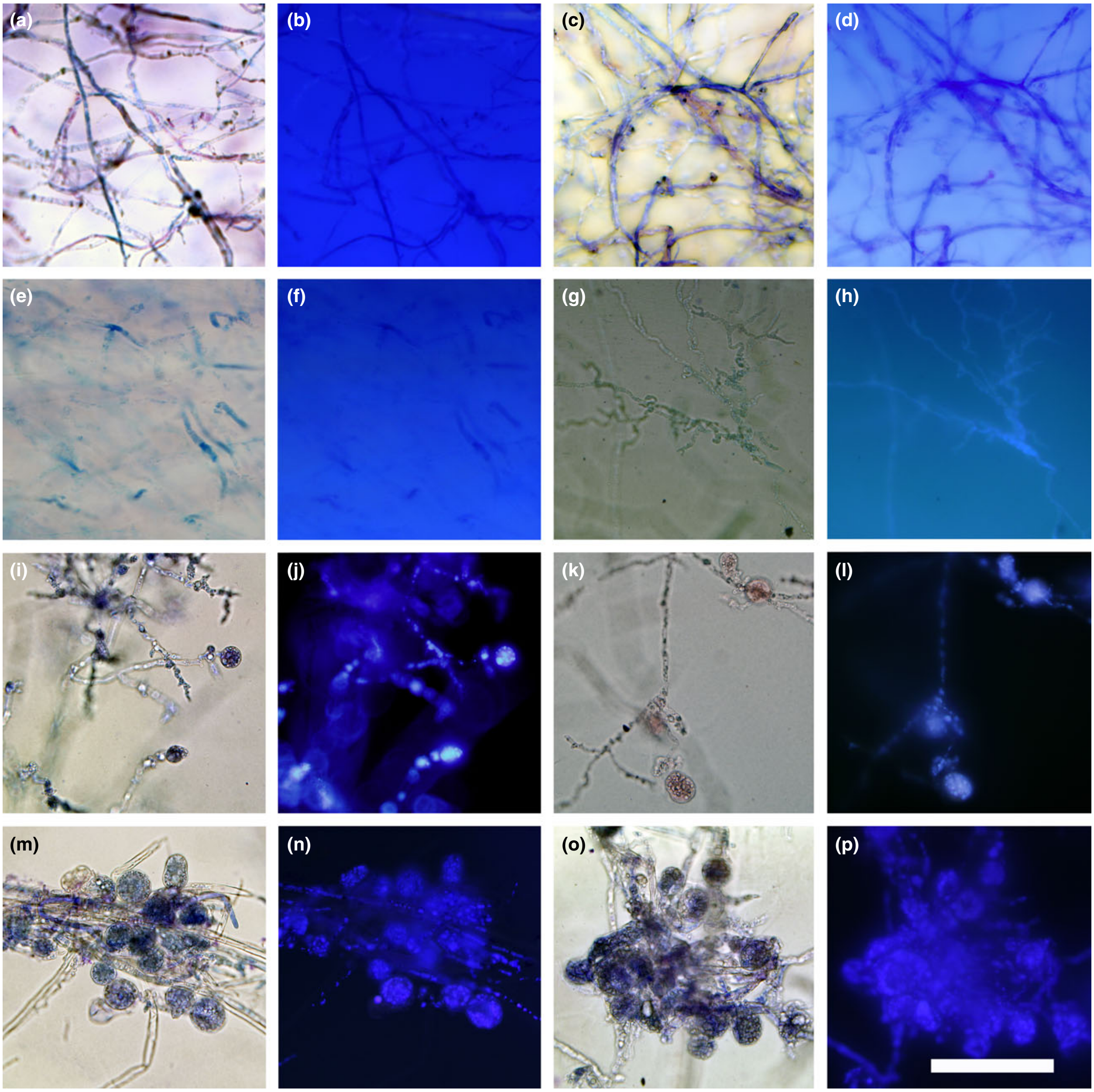

Fig. 1 Micrographs of *Phytophthora* species stained with 1% toluidine blue and tested for probe specificity. Light micrographs (a, c, e, g, i, k, m, o) and ultraviolet micrographs (b, d, f, h, j, l, n, p) are based on fluorescent *in situ* hybridization assays with AlexaFluor350-labelled, *P. cinnamomi*-specific (Alcin5F) probe. (a, b) *P. cambivora* isolate DCE31. (c, d) *P. elongata* isolate VHS13784. (e, f) *P. multivora* isolate VHS14926. (g, h) *P. niederhauserii* isolate VHS17577. (i, j) *P. cinnamomi* isolate VHS15773. (k, l) *P. cinnamomi* isolate VHS16740. (m, n) *P. cinnamomi* isolate VHS16441. (o, p) *P. cinnamomi* isolate VHS16779. Bar represents 50 μm.

visualization of the pathogen within plant tissues. In comparison with 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 (Fig. 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 utilizes the depth of sequence data available for the ITS region for *Phytophthora* species (Cooke et al. 2000; Blair et al. 2008).

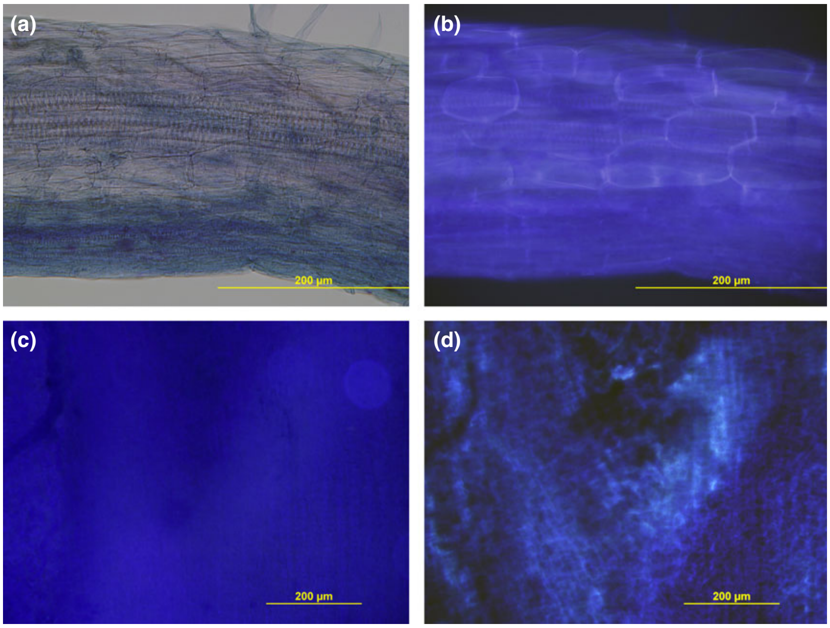

Fig. 2 Micrographs of non-infected *Trachymene pilosa* root (a, b) and *Lupinus augustifolius* root (c, d) assayed with fluorescent *in situ* hybridization and AlexaFluor350-labelled, *Phytophthora cinnamomi*-specific (Alcin5F) probe further stained with 1% toluidine blue. Light micrographs (a, c) and ultraviolet micrographs (b, d).

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 centralizing 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 utilized 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 utilized 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 regard to infection, colonization 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 the 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

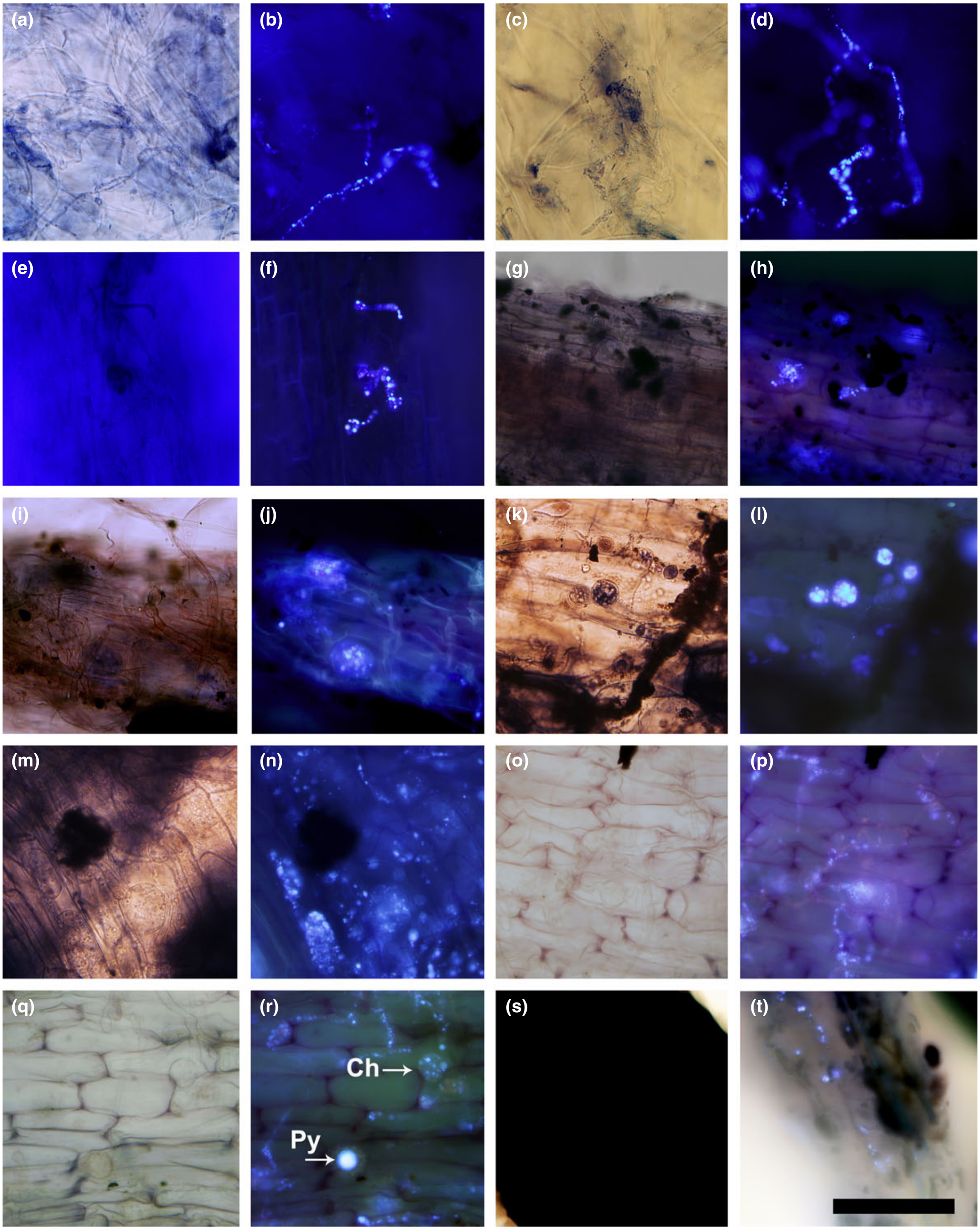

Fig. 3 Micrographs of *Phytophthora cinnamomi* in plant tissue from *in vitro* inoculation and field samples based on fluorescent *in situ* hybridization assays with AlexaFluor350-labelled, *P. cinnamomi*-specific (Alcin5F) probe and stained with 1% toluidine blue. Light micrographs (a, c, e, g, i) and ultraviolet micrographs (b, d, f, h, j) of *in vitro* inoculation; light micrographs (k, m, o, q, s) and ultraviolet micrographs (l, n, p, r, t) of field samples. (a–d) *P. cinnamomi* hyphae in apple tissue. (e, f) *P. cinnamomi* hyphae in *Lupinus angustifolius* root. (g–j) *P. cinnamomi* chlamydospores in *Trachymene pilosa* root. (k, l) *P. cinnamomi* chlamydospores in *Paracaleana nigrita* root. (m, n) *P. cinnamomi* chlamydospore and hyphae in *T. pilosa* root. (o, p) *P. cinnamomi* hyphae and chlamydospore in *Chamaescilla corymbosa* root. (q–t) *P. cinnamomi* hyphae and chlamydospore in *Stylidium diuroides* root. Arrowhead 'Ch' indicates *P. cinnamomi* chlamydospores, and 'Py' indicates oospores of *Pythium* species. Bar represents 50 μm.

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 localization 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 Fig. 1 can be observed to be blurred in some instances, while 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 utilize 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 to 1.0% for 4 h (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 *T. 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 posthybridization 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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