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Visualising endophytic fungi within leaves by detection of (1→3)-ß-D-glucans in fungal cell walls

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ABSTRACT

The presence of endophytic fungi within symptomless leaves of vascular plants is usually recognised indirectly through culturing methods. In order to understand the biology of fungi isolated as endophytes, there is a need to directly observe their hyphae within the leaves of their hosts. Such observations provide information about the mode of infection, the extent of colonisation within the leaf, and the reaction of the plant to infection by the fungus. Many endophytic fungi develop highly localised infections with small amounts of mycelium, making such direct observations difficult. We describe a method adapted from an electron microscopy protocol that labels one of the constituent components of fungal cell walls with a fluorescent dye and enables them to be observed in thin sections under a compound microscope.

1. Introduction

Fungi capable of symptomless infection of apparently healthy leaves of vascular plants are commonly termed 'endophytes' (Stone *et al.* 2004). The presence of fungi within symptomless, living leaves is most commonly detected indirectly, by surface sterilising small pieces of leaf tissue, placing those pieces on agar plates, and waiting for the fungi inside the leaf to grow out on to the agar. Direct observation of endophytic fungi within leaves using histological methods have been reported less often.

The grass-inhabiting endophytes belong to the Clavicipitaceae. They are systemically transmitted from generation to generation through seed, colonise internal leaf tissue extensively, and each host is typically colonised by a single fungal species. In contrast, those in the leaves of non-grass hosts are taxonomically diverse, are transmitted externally by airborne spores, typically show restricted internal colonisation of the host tissue, and each host is usually colonised by several species concurrently (Stone *et al.* 2004).

As well as being highly diverse taxonomically, the non-grass endophytes are diverse biologically. Examples of this diversity given by Rodriguez and Redman (1997) include (1) fungi that grow actively through host tissues resulting in extensive colonisation; (2) fungi that grow actively through host tissues but colonise only a small part of the leaf; (3) fungi that are rapidly isolated by host defence mechanisms and remain metabolically quiescent until host senescence; and (4) fungi that are rapidly isolated by host defence mechanisms but remain metabolically active. Many non-grass endophytes have a saprobic as well as an endophytic phase to their life cycle. For example, three species of Helotiales that form apothecia on dead, fallen leaves of *Metrosideros excelsa* (an undescribed *Torrendiella* sp., an undescribed *Lanzia* sp., and *Lanzia* cf. *griseliniae*) are also frequently isolated into culture from symptomless living leaves (P.R. Johnston, unpubl. data).

To understand the biological interaction between an endophyte and its plant host generally requires direct observation of fungal hyphae within the living leaves. However, attempts to directly observe internal fungal hyphae within leaves of plants

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such as *Metrosideros excelsa* (Myrtaceae), *Kunzea ericoides* (Myrtaceae), and *Dacrydium cupressinum* (Podocarpaceae) have not been successful in our lab. We describe here a fluorescent labelling method that detects (1→3)-β-D-glucan within fungal cell walls through the use of a monoclonal antibody. This method is an adaptation of an electron microscope method (Lemoine *et al.* 1995). In this 2-step protocol, incubation with anti-(1→3)-β-D-glucan is followed by incubation with a secondary antibody conjugated to a fluorochome. The method is particularly valuable for detecting small amounts of fungal hyphae within a leaf or for detecting hyphae within thin sections.

2. Methods

Kunzea ericoides leaves (leaf size approximately 5–10 × 2–3 mm), which were at least 12 months old, were collected from the field and processed immediately. Pieces of leaf tissue approximately 1 mm × 2 mm were cut from one side of a leaf, avoiding the midrib. Leaf pieces were fixed in 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1M phosphate buffer pH 7.2 under vacuum for 1 h, washed in buffer 3 times, dehydrated in an ethanol series and embedded in L R White resin (London Resin, Reading, UK). Sections 200 nm thick were dried onto poly-L-lysine coated slides. Immunolabelling was performed in a Shandon Immunolabeller as described by Sutherland *et al.* (2004). Sections on the slides were rinsed in Phosphate Buffered Saline/Tween (PBS-T), placed in blocking buffer (0.1 % BSA-C (Aurion, Wageningen, The Netherlands) in PBS-T) for 15min, incubated in anti-(1→3)-β-D-glucan antibody (BioSupply, Parkville, Australia) diluted 1:100 in blocking buffer overnight at 4 °C. They were then washed in PBS-T, incubated for 1 h in Alexa goat anti-mouse 488 (Molecular Probes, Eugene, Oreg., USA) diluted at least 1:600 in PBS, washed in PBS-T followed by 2–3 ml of ultrapure water and mounted in Citifluor (Leicester, UK). Plant cell walls can be detected by autofluoresence. Sections were viewed using an Olympus Vanox AHT3 microscope (Olympus Optical, Tokyo) with an interference blue filter set and collected with a CoolSnap colour digital camera.

In addition, 1 μm thick sections were cut from one side of a *K. ericoides* leaf, stained in a 0.05 % solution of toluidine blue in benzoate buffer (pH 4.4), and mounted in Shurmount (Triangle Biomedical).

The plants from which the leaves were collected had earlier been indirectly sampled for the presence of endophytic fungi by surface sterilising whole, apparently healthy leaves, that were more than 12 months old using 95 % ethanol and bleach (1.5 $g\,l^{-1}$ sodium hypochlorite), cutting the sterilised leaves into pieces approximately 2 mm × 2 mm, and incubating the pieces on malt extract agar at about 20 °C. About 80–85 % of these leaf pieces were infected by fungi, which grew on agar under these conditions, with about 10 % of them having more than one kind of fungus present (S. Joshee, unpubl. data).

3. Results and discussion

No structures that could be reliably interpreted as fungal hyphae were seen in the toluidine blue-stained section (Fig. 1).

Chitin and (1→3)-β-D-glucan are the major structural components of the walls of many fungi (Wessels 1993). The (1→3)-β-D-glucan detection protocol highlights both fungal cell walls and callose. Callose, in the form of papillae, is deposited by the plant in response to fungal penetration, as a defence to impede fungal growth (Aist 1993). This antibody will also bind to callose in plasmodesmata and phloem sieve plates in vascular bundle. Labelled fungal hyphae can easily be distinguished visually from these labelled plant cell wall structures (Fig. 3).

Three fungal infections, termed A, B, and C in the discussion and Fig. 2, are present in the leaf section illustrated (Figs 2–6). A similar level of infection was seen in sections from the other leaf pieces sampled. Differences in the mode of penetration of the leaf, the extent of hyphal growth within the leaf, and the reaction of surrounding plant cells to the presence of the hyphae with respect to callose production, suggest three separate species of fungi were responsible for the three infections. Leaf penetration of the infections A and B were both through a stoma. Detail of infection A (Fig. 3) shows callose production in the plant cells surrounding the stomatal cavity, indicating a plant defence reaction to the presence of the fungus, and the fungal hyphae are restricted to the stomatal cavity itself. Infection B (Figs 4–5) has no callose production in response to the fungus, and although fungal hyphae appear to be fewer than those in infection A, the hyphae extend deep within the leaf. In both cases hyphae are restricted to intracellular spaces. Infection C (Fig. 6) shows what appears to be a hypersensitive defence reaction, the fungus being isolated by strong plant defence reactions following the penetration and death of a single plant cell.

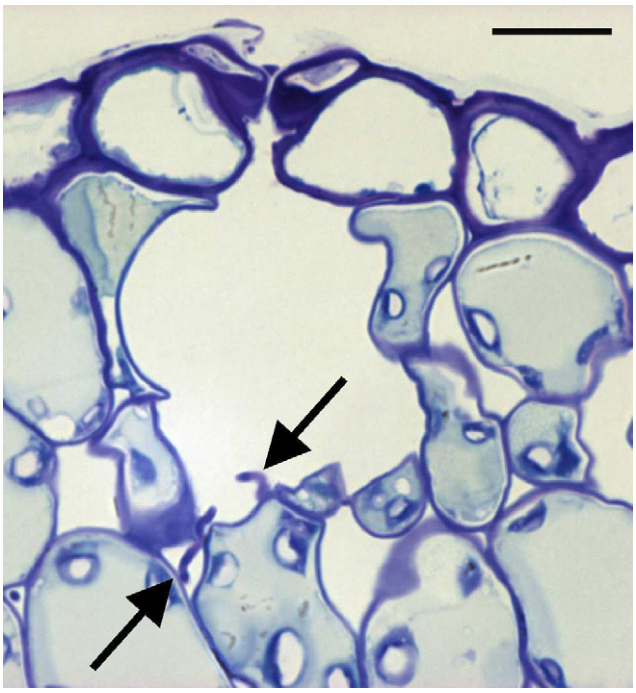
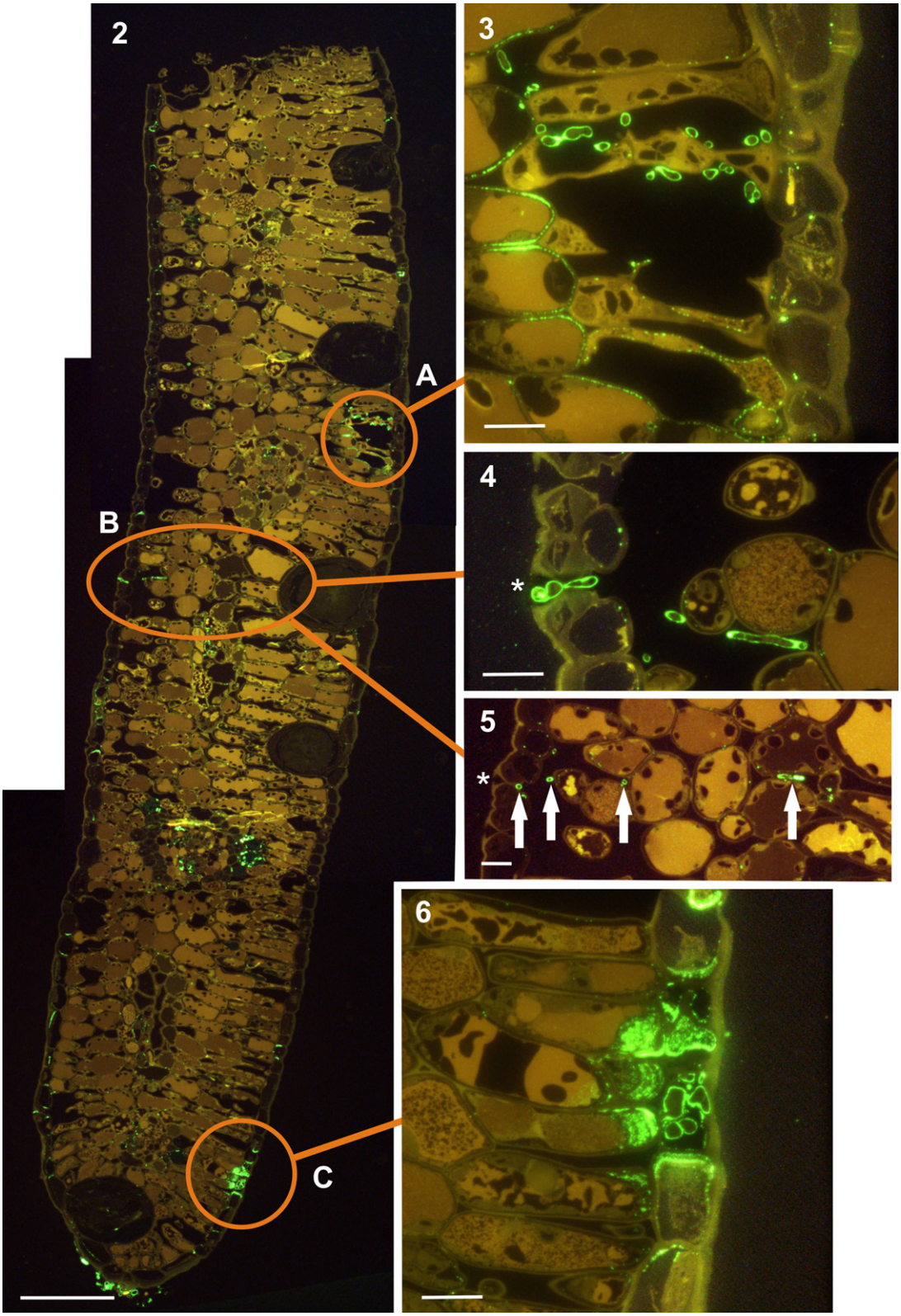


Fig. 1 – Transverse section from a *Kunzea ericoides* leaf, 1μm thick, stained with toluidine blue. The dense lines (arrowed) near the base of the stomatal cavity could possibly represent fungal hyphae. Scale bar = 10 μm.



Figs 2–6 – Section from a *Kunzea ericoides* leaf, 200 nm thick, with fungal cells walls fluorescently labelled to detect (1→3)-β-D-glucan. Walls of fungal hyphae label green, the plant cell walls autofluoresce to appear yellow or brown. Plant cell walls with callose production also appear green. Three separate fungal infections occur in this section, A–C, each illustrated in detail in Figs 3–6. Fig. 3 infection A, entry through stoma, fungal hyphae restricted to stomatal cavity, plant cells around stomatal cavity producing callose (arrowed) in reaction to the fungus. Figs 4–5 infection B, entry through stoma, plant cells surrounding substomatal cavity with no callose production, fungal hyphae extending deep within the leaf tissue (arrow in Fig. 5), asterisk indicates same stoma in Figs 4 and 5. Fig. 6 infection C, fungus directly penetrates epidermal cell wall, extensive callose production in surrounding plant cells restrict the fungus to a single epidermal cell. Scale bar: Fig. 2 = 100 μm, Figs 3–6 = 10 μm.

The fungi seen within the *Kunzea* leaf remain unidentified, but if we assume that the fungal hyphae we observed were of species that can be cultured, then data from the *Kunzea* leaf isolations (S. Joshee, unpubl. data) allows some speculation. The most common fungi isolated from the *Kunzea* leaves were two species of *Mycosphaerella* and a species of *Torrendiella*. Within any one tree, each of these fungi were isolated from up to 33 % of the 200 leaf pieces sampled. Although more than 80 species of fungi were isolated from the eight *Kunzea* trees sampled, no others were found in more than 5 % of leaf pieces from any one tree. *Mycosphaerella* spp. are common leaf pathogens, suggesting that the highly localised pathogenic reaction may have been caused by one of these species. Chance suggests one of the substomatal infection types may have been caused by the discomycete *Torrendiella* sp.

The same technique described and illustrated for *Kunzea* was also attempted for leaf pieces from several New Zealand podocarps (*Dacrycarpus dacrydioides*, *Dacrydium cupressinum*, and *Podocarpus totara*), but internal hyphae were seen in none of these. Isolation following surface sterilisation showed that endophytic fungi were present in all these leaves, although at a lower frequency than *Kunzea*, with around 45–67 % of the leaf pieces sampled containing a culturable fungus. A limitation of the method described is that the volume of leaf being sampled in any one set of sections is very small, extremely thin sections being required. Where fungal infections are relatively infrequent, a large number of sections may need to be examined before any hyphae are seen. The sections from these other hosts did confirm that the fungal endophytes present in these podocarp leaves were not of the type resulting in extensive internal colonisation of the leaves, as have been observed with some Northern Hemisphere conifers (e.g. Deckert *et al.* 2001).

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