Detection of *Ophiostoma Piceae* in Radiata Pine using Immunofluorescence Labeling and Confocal Laser Scanning Microscopy

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The use of fluorescence microscopy to investigate fungal growth in wood often causes interference due to the strong autofluorescence of wood lignin, unless fluorescent probes which specifically react to fungal hyphae, are used. Techniques to enable differentiation of hyphae from wood have been recently reported (Singh *et al.*, 1997; Xiao *et al.*, 1997). The authors demonstrated that while glutaraldehyde can be used to detect fungal native proteins, wheat germ agglutinin (WGA) which reacts with cell wall chitin of hyphae, considerably improved detection of fungi growing in wood.

Confocal laser scanning microscopy (CLSM), a new technique in wood biodeterioration research, has recently been employed because it produces blur free images and allows optical sectioning across the thickness of the specimen (Xiao *et al.*, 1998). CLSM also enables multi-channel, fluorescent imaging which can be monitored on a big screen and controlled by computer.

In the current study, an immunofluorescence technique was developed to detect *Ophiostoma piceae*, a common sapstain fungus in New Zealand, in radiata pine wood using a monoclonal antibody, 1F3(1)), and CLSM. Production and characterisation of the monoclonal antibody used in this investigation, has been described previously (Banerjee *et al.*, 1994).

Wood wafers which were infected with *0. piceae*, were cut into 20 µm thick sections using a microtome prior to incubation in 1% (w/v) casein in phosphate buffered saline (PBS, pH 7.4) for 20 minutes to block non-specific antibody binding. Wood sections were then incubated for 1 hour in monoclonal antibody diluted 1:500 in PBS containing 0.01% Triton X-100 (Breuil *et al.*, 1992). Sections were repeatedly washed in water and then incubated in *0.5* mg/mL Oregon green 514 Goat anti-mouse IgG (H+L) fluorescent dye (excitation 511 nm, emission 530 nm; Molecular Probes, Inc., Eugene, OR) in the dark for 1.5-2 hours. After four washes in water, sections were individually mounted in glycerol on glass slides with a cover slip and the edges were sealed with nail vanish. Microscopic examination was performed using a Leica TCS NT CLSM with an Argon-Krypton laser excitation source (488/568/647 nm). Images were captured in green channel through a band pass filter BP 530/30 nm and in red channel through a long pass filter LP 590 nm using a 63x oil lens with numerical aperture setting of 1.4. Digital image stacks obtained on

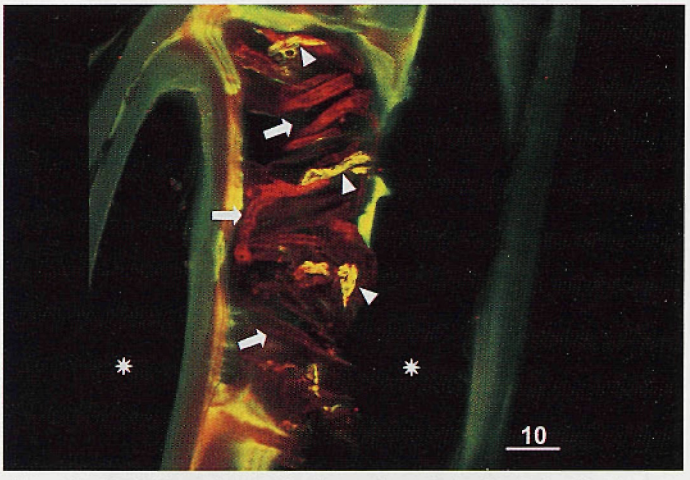

Figure 1: Duel channel projection in maximum intensity mode of an image stack with 2.4 µm thickness. Because the colour of the green and red channel were inverted fungal hyphae appear in red (arrows), and wood cell walls in yellow-green. Arrowheads indicate wood extractives and asterisks wood lumina. Bar = 10 µm.

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CLSM were transferred to a SGI Indy workstation and processed using Image Space™ software (Molecular Dynamics Inc.). Dual channel projection was obtained by combining the images collected of two channels and image slices were composed together using Volume Workbench.

This investigation showed that immunofluorescent labeling yielded strong fluorescence of *0. piceae* in the green channel whereas wood strongly autofluoresced in both, green and red channels. Therefore it was possible to differentiate hyphae of *0. piceae* from wood using a duel channel projection (Figure 1). Furthermore, the immunofluorescence labeling technique described enabled us to distinguish readily between *0. piceae* hyphae and any autofluorescing of wood extractives which were associated with ray parenchyma cells. This latter differentiation is of particular importance in investigations on the early stages of colonisation when *0. piceae* preferentially invades ray parenchyma cells where readily accessible nutrients are present.

Immunofluorescence labeling of additional sections which were infected respectively with other sapstain fungi, such as *Diolodia.* sp, *0. piliferum 0. floccosum*, was performed using the procedure described above. It failed to detect fungal hyphae in these sections. However WGA staining, which was subsequently performed on the same sections, confirmed their presence.

In conclusion, the immunofluorescence labeling technique described in this paper does not involve any fixation and dehydration of wood sections; therefore damage which often occurs during sample preparation is minimized and the inconvenience of reducing possible autofluorescence induced by glutaraldehyde, avoided. Furthermore, the technique is easy to carry out and provides a useful tool for specific detection of *0. piceae* in radiata pine. The method described herein is currently being used to investigate fungal interactions between *0. piceae* and other sapstain fungi as well as the effect of fungicides on development of *0. piceae*.

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