MICROMORPHOLOGY OF A NOVEL FUNGAL DECAY IN PRESERVATIVE-TREATED *PINUS RADIATA* WOOD IN WET ACIDIC SOILS

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ABSTRACT

Light microscopy of *Pinus radiata* D. Don field test stakes (20 × 20 × 500 mm) exposed in wet acidic (pH4.5) soil for 12–24 months showed predominance of an unusual type of decay characterised by tunnelling attack of wood cell walls. After 2 years, decay was moderate to severe in wood treated to ground contact CCA specifications and also equivalent retentions of creosote, and a number of new generation preservatives. Relative to other New Zealand temperate test sites and also an Australian tropical site, the New Zealand acidic soil test site was very aggressive.

Correlative microscopy, including light microscopy (LM), confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM), was used to elucidate the micromorphology of this attack. Tunnels of diameter 0.2–5 μm were present throughout all layers of the cell wall, and their orientation was not related to the cellulose microfibril orientation. They also showed no preference for particular cell wall layers, indicating a lignin-degrading capability. CLSM images showed that living, connecting fungal hyphae were present in the cell lumina and tunnels, and this was also confirmed by other forms of microscopy used. This type of attack was predominant in wood that was highly saturated with water whereas wood that was less moist was attacked predominantly by classical white rot at the same site. Ongoing isolation and incubation studies in conjunction with further microscopy should enable identification of the fungal species involved.

Keywords: tunnelling; wood decay; wet acidic soils; confocal laser scanning microscopy; transmission electron microscopy; *Pinus radiata*.

INTRODUCTION

From a study of the effects of field test site parameters, on the type of decay that occurs in variously preservative-treated wood, an unusual type of decay micromorphology was observed during routine light microscopy. This decay was characterised by a reticulum of tunnels, of highly variable size and orientation, throughout wood cell walls. A similar type of decay was recently reported for belian (*Eusideroxylon zwageri* T. & B.) wood which was caused by hyphal tunnelling of wood cell walls (Singh & Wong 1996; Wong *et al.* 1996). Work at FRI has suggested that this type of decay is very aggressive in wet peaty soil, causing decay in variously preservative-treated and untreated *Pinus radiata* and European beech (*Fagus sylvatica* L.). For example, *P. radiata* stakes (20 × 20 mm) treated at 10 kg/m^3 with

a preservative containing copper, chromium, and arsenic (CCA) had established decay after 18 months of soil contact.

Light microscopy (LM), confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) were used to investigate this decay. Of these, CLSM proved most valuable, providing information on fungal morphology and distribution, and characterisation of the decay micromorphology. Sequential optical sectioning of relatively thick wood samples using techniques of CLSM can ultimately produce a true three-dimensional image of decay morphology. The techniques of computer-assisted imaging and the use of the most appropriate methods of sample preparation are still being perfected. However, serial sectioning has already given insight into the three-dimensional interactions between tunnelling hyphae and the wood cell wall and has shown the great potential of CLSM for advancing our understanding of wood decay morphology.

MATERIALS AND METHODS

Preservative-treated Test Stakes

Pinus radiata test stakes (20 × 20 × 500 mm) were variously preservative-treated using water-borne preservatives with a Bethel process, and Light Organic Solvent Preservatives (LOSP) with a Lowry process. Stakes were installed in peaty soil at Peppers blueberry orchard along the length of a drainage ditch. They were buried vertically to two-thirds of their length. Stakes were randomly assessed for the extent and type of decay at approximately 12-monthly intervals. Whole stakes were removed from the site just before chips were taken for light microscopy.

Light Microscopy (LM)

Hand-held sections approximately 20 μm thick were prepared using a disposable microtome blade and mounted in glycerol. Bright field, phase contrast, and polarised light techniques were employed using a Zeiss Axioplan light microscope to examine the wood cell walls.

Confocal Laser Scanning Microscopy (CLSM)

Sections of approximately 20 and 100 μm thickness were cut using a sliding microtome and then fixed in 3% glutaraldehyde (in 0.05M sodium cacodylate buffer) overnight. The fixed sections were washed thoroughly in distilled water and mounted in water. A TCS/NT CLSM was used to examine the sections at the excitation wavelength of 488/568 nm, and a SGI Indy workstation was employed to process the images obtained. Thin sections of resin-embedded samples were also examined. Briefly, after glutaraldehyde fixation as described above, samples were dehydrated in acetone and embedded in Spurr's low-viscosity resin (Spurr 1969). Sections of 3–5 μm thickness were cut on an LKB ultramicrotome and then examined.

Scanning Electron Microscopy (SEM)

Sections approximately 100 microns in thickness were cut using a microtome and then fixed in 3% glutaraldehyde overnight. After being dehydrated in acetone and air dried,

sections were coated using a gold sputter coater and examined with a Cambridge Stereoscan SEM.

Transmission Electron Microscopy (TEM)

Small sections were fixed in 3% glutaraldehyde (in 0.05M sodium cacodylate buffer) and post-fixed in 2% osmium tetroxide also prepared in the same buffer. They were then dehydrated in acetone and embedded in Spurr's low-viscosity resin (Spurr 1969). Ultrathin sections were cut on an LKB ultramicrotome, stained with 1% potassium permanganate (in 0.1% citrate buffer) and examined in a Philips 300 TEM.

RESULTS AND DISCUSSION

Light microscopy showed the presence of a complex reticulation of tunnels of variable diameter (Fig. 1). Smaller tunnels were less than 1 μm in diameter and larger tunnels were about 5 μm in diameter. However, it was not possible to distinguish reliably between single large tunnels and tunnels formed by coalescence of smaller tunnels. Septate hyphae were occasionally associated with tunnels but it was not possible to confirm that these hyphae were responsible for causing tunnels. The tunnels were highly variable in their orientation within the cell wall and appeared to show no preference for direction of penetration.

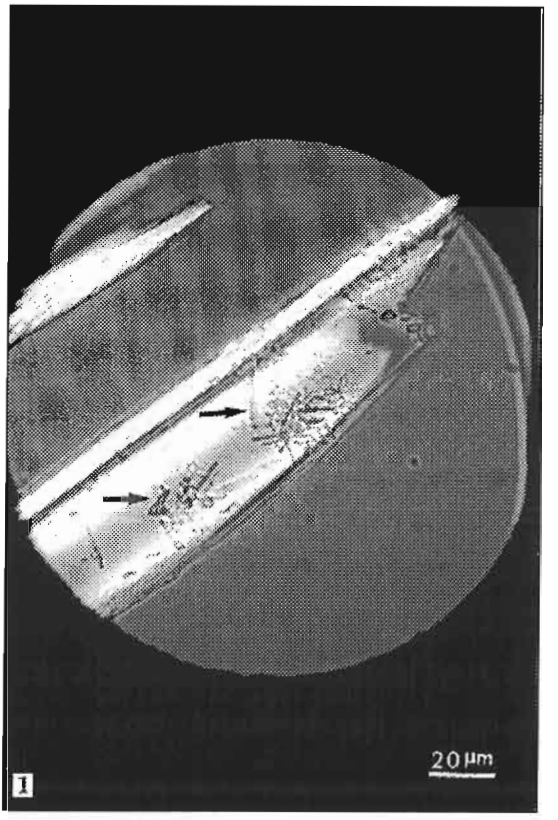

FIG. 1–Light micrograph of a tracheid wall showing tunnels of variable sizes and forms (arrows).

The major finding of LM studies concerned the distribution of different patterns of decay in test stakes. Sections taken from below the ground-line region usually had exclusive tunnelling-type attack. Sections taken from the ground-line region (5 cm above and below the ground-line) contained decay patterns as shown in Fig. 1, and also patterns typical of white rot such as erosion troughs, cell-wall thinning, and bore holes (Blanchette *et al.* 1990). These two distinctive patterns were often mixed up in the same field of view. Sections taken from above the ground-line region typically did not contain tunnelling patterns but often contained typical white rot. Soft rot was also common in many of the test stakes and was intermediate between the tunnelling attack and the white rot in terms of positioning on the stake.

The test stakes were situated immediately adjacent to a drainage ditch. During the winter the water table in the ditch was approximately 20 cm from the surface (ground-line) and the high water-holding capacity and wicking effect of peat ensured that the stake below ground was in a highly saturated condition. During the driest months of summer the water table sank to approximately 50 cm below the surface. Even under these conditions the lower one-third of the stake remained in a highly saturated condition. The complete absence of decay of any type in this region in most stakes, suggests that the availability of oxygen was below a threshold required for decay within the time frame of this study (24 months).

The pattern of distribution of white rot and tunnelling attack suggests that the degree of water saturation of the wood is important, indicating that the tunnelling attack is particularly tolerant of highly saturated wood and concurrently low oxygen concentration.

Wood exhibiting exclusively this type of decay, had a fibrous texture and its colour had typically not changed greatly from its original colour. It was therefore difficult to distinguish macroscopically between tunnelling attack and early white rot attack.

CLSM, SEM, and TEM confirmed that the tunnelling-type wood decay observed under LM was caused by fungal hyphae and therefore this type of decay will be referred to as hyphal tunnelling. Hyphae were of highly variable diameter, measuring 0.2–5 μm (Fig. 2–8). Hyphae were common in the lumen of wood cells, from where they penetrated cell walls by producing thinner hyphal branches (Fig. 2–5). Hyphal branches were produced after (Fig. 2–5), and occasionally during (Fig. 8), penetration, and subsequent to penetration the hyphae ramified through the cell wall moving in all directions and into all parts of the wall.

Tunnels were present in all parts of the wood cell wall, including the highly lignified middle lamella (Fig. 9). The direction of tunnelling was highly variable, and there did not seem to be any correlation between the direction of tunnelling and the orientation of cellulose microfibrils. This is in sharp contrast to the pattern seen during soft-rot attack where a close correspondence between the long axis of cavities and that of cellulose microfibrils is found (Eaton & Hale 1993). The tunnels varied greatly in their orientation (Fig. 7, 9, 10); consequently, tunnels were cut transversely, obliquely, and longitudinally within the plane of a single section, and correspondingly were seen in their circular, oval, and long-channel profiles (Fig. 7, 9). When present within tunnels, hyphae completely filled them (Fig. 7, 8). This is unlike erosion-type white rot attack where normally there is a gap between hyphae and the margins of the troughs they produce (Blanchette *et al.* 1990). The pattern of fungal tunnelling described, instead parallels that seen during bacterial tunnelling of wood cell walls (Singh & Butcher 1991), and is very similar to fungal tunnelling described for belian wood (Singh & Wong 1996). Some aspects of this decay also resemble that described earlier

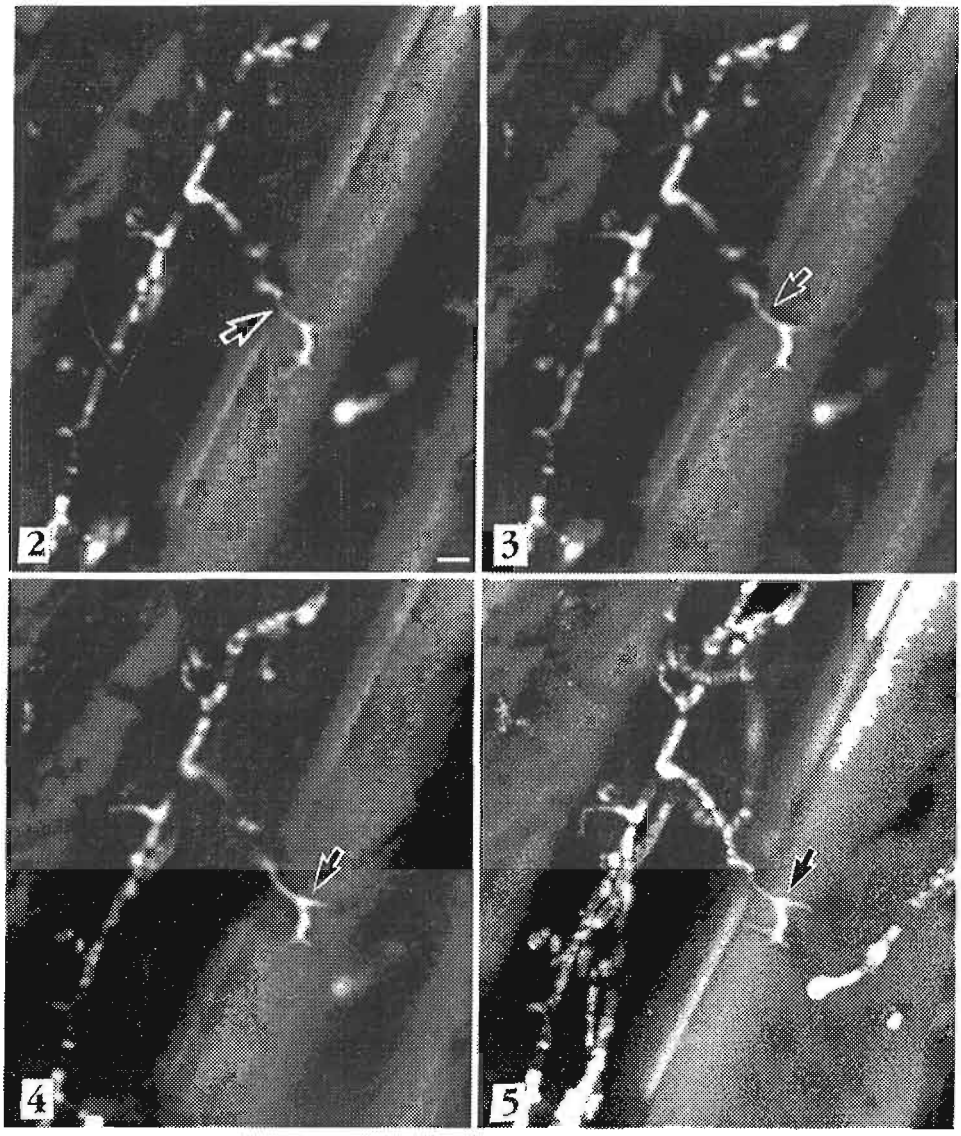

FIG. 2–5–Confocal laser scanning microscope images of hyphae in the lumen of tracheids, and penetration of tracheid walls by hyphal branches (arrows). Figures 2–4 are images of individual sequential optical sections showing hyphal penetration into tracheid wall. The image in Fig.5 is a composite projection of the images in Fig. 2–4. Bar = 5 μm.

as soft rot and multiple-branching by basidiomycetes (Daniel *et al.* 1992; Schwarze *et al.* 1995), and that produced during the degradation of wood cell walls by actinomycetes (Nilsson *et al.* 1990).

Although there may be an initial preference for the secondary wall, as the decay becomes more severe all regions of wood cell walls, including the highly lignified middle lamella, are

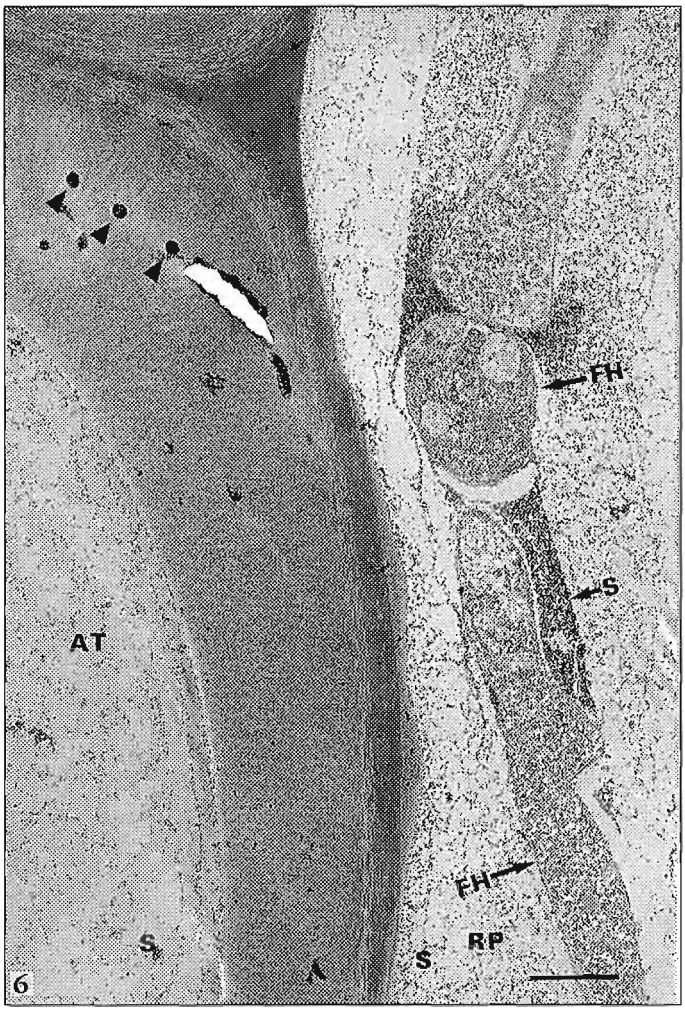

FIG. 6–Transverse section through an axial tracheid (AT) and a ray parenchyma cell (RP). Large fungal hyhae (FH) are present in the ray parenchyma cell and small hyphae, which are cut transversely, are present in the S2 layer of the tracheid (arrowheads). S = slime. Transmission electron micrograph. Bar = 2 μm.

attacked and degraded. Tunnelling of all areas of the wood cell wall may thus result in loss of the integrity of wood cells and in their collapse. As the decay progresses and the tunnel number increases, adjoining tunnels often coalesce, resulting in the formation of larger irregular voids (Fig. 11). The tunnels and voids appear empty of any contents except for the occasional presence of hyphae, hyphal residues, or lignin degradation products (Fig. 7, 8).

TEM revealed the presence of a "halo" around the hyphae in the early stages of tunnelling (Fig. 9), which was particularly prominent in the S2 layer. The cell wall in the "halo" region appeared more electron lucent, indicating a loss in lignin, as the sections viewed in the TEM were stained with $KMnO_4$, a reagent widely used in botanical (Hapler

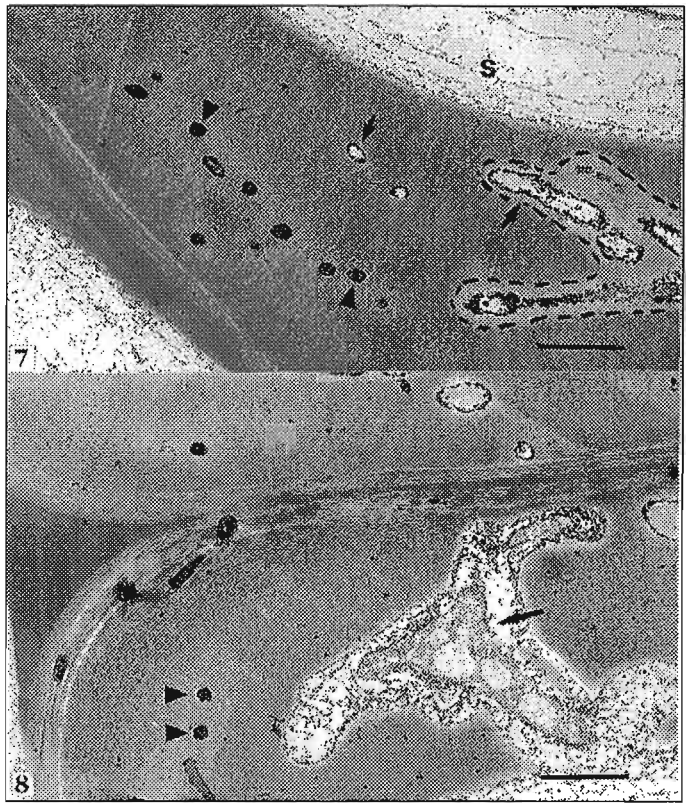

FIG. 7–Transverse section through part of an axial tracheid wall containing fungal hyphae in the S2 layer, which vary in their size and orientation. The hyphae indicated by arrows appear largely empty. The arrowheads indicate small dense hyphae. A translucent zone present in the S2 wall around hyphae is marked by broken lines. S = slime. Transmission electron micrograph. Bar = 2 μm.

FIG. 8–Transverse section through part of an axial tracheid. A large branched hypha (arrow) is tunnelling into the S2 wall of the tracheid from the lumen side. The arrowheads indicate small dense hyphae present in the S2 wall. Transmission electron micrograph. Bar = 2 μm.

et al. 1970) and wood science work (Maurer & Fengel 1990; Donaldson 1994; Singh 1997) to contrast lignin. The appearance of the S2 wall around fungal hyphae was similar to that observed during white rot attack of wood where lignin is removed in preference to and in advance of polysaccharides (Eriksson *et al.* 1990). As the "halo" was not confined to the immediate vicinity of the hyphal wall and could extend into the S2 wall to some distance from where the hyphae were present, it is likely that tunnelling hyphae initially produce degrading elements small enough to penetrate the intact wood cell wall. This suggestion is consistent with the current thinking that early stages of white-rot attack of wood cell wall is a radical-mediated process (Blanchette *et al.* 1990).

The morphology of tunnelling hyphae suggests that they are likely to be of fungal origin. The tunnelling pattern of decay reported here is very different from soft rot and brown rot,

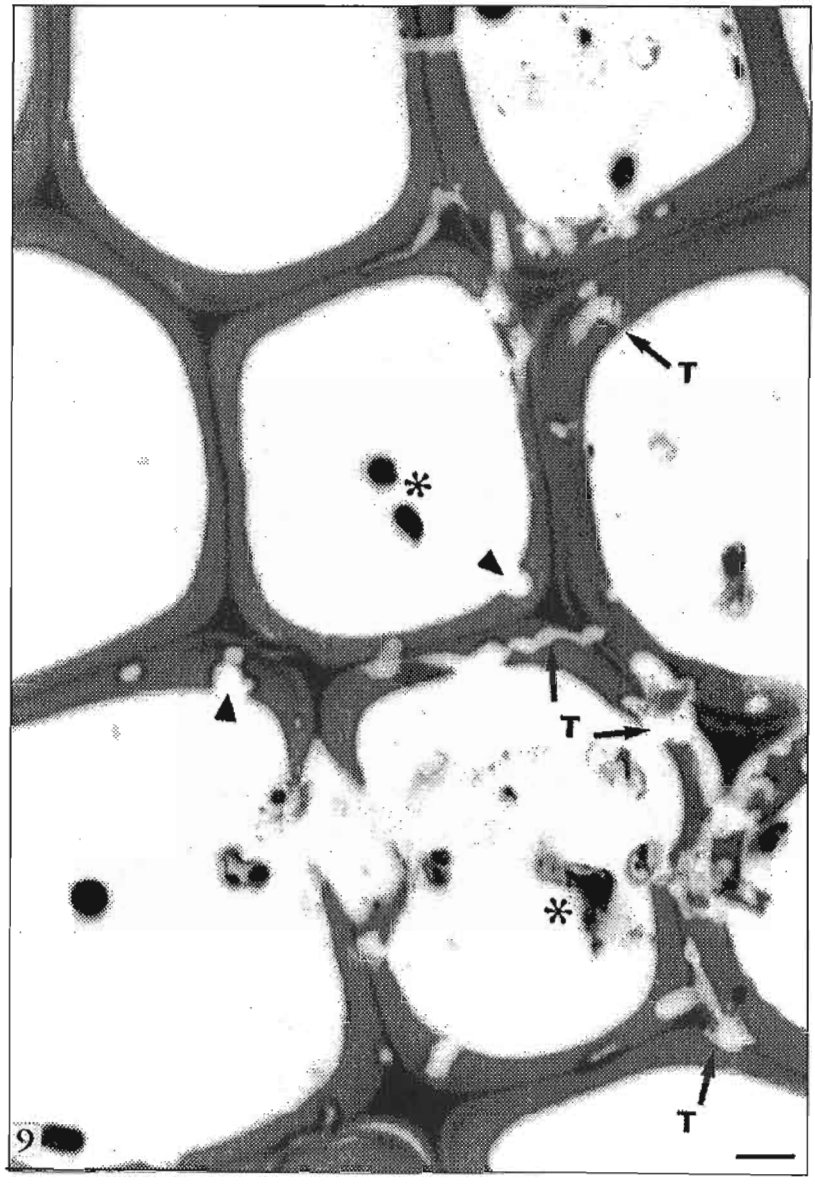

FIG. 9–Transverse section through axial tracheids showing the presence of tunnels (T) in the secondary walls as well as middle lamellae. The points of fungal entry into tracheid walls are indicated by arrowheads. The asterisks show the location of hyphae in the lumen. Confocal laser scanning micrograph. Bar = 5 μm.

and is also different from the reported white-rot decay. However, at least in one respect, hyphal tunnelling decay is similar to the white rot in that in addition to secondary walls the tunnelling hyphae can attack the middle lamellae, and no apparent wall residues are left in the tunnels or voids.

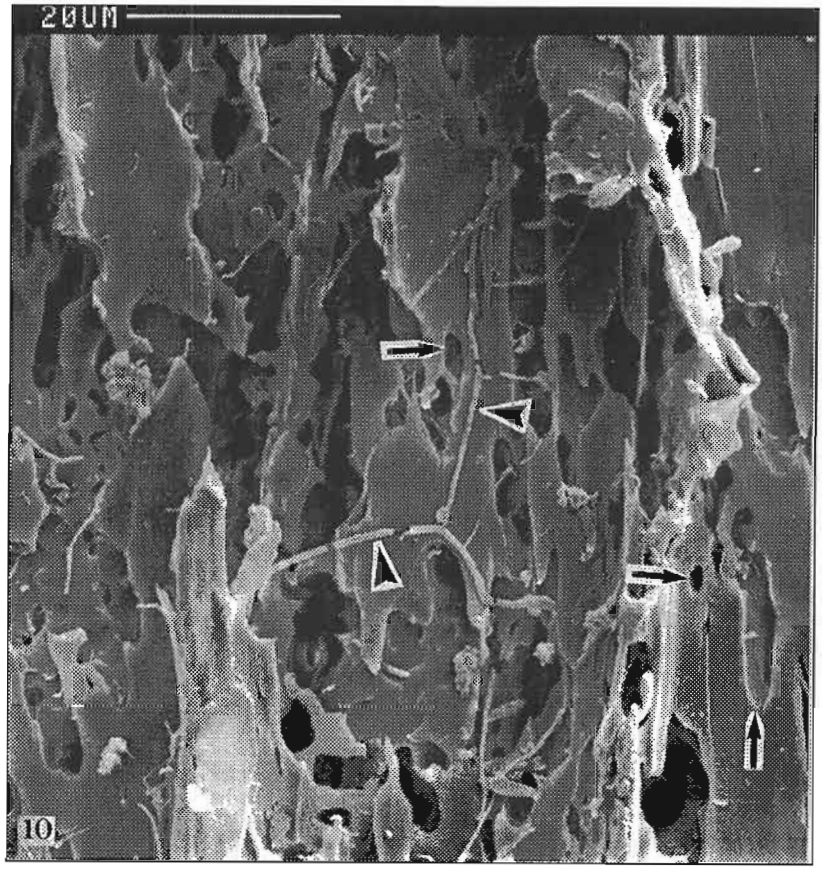

FIG. 10–Scanning electron micrograph showing presence of very small diameter hyphae in wood (arrowheads) and tunnels in wood cell walls (arrows).

Hyphal tunnelling thus appears to be a highly specialised form of wood decay, which may be adapted to certain wood characteristics, such as a high degree of cell wall lignification and very high extractives content as in belian wood (Syafii *et al.* 1987; Syafii, Samejima & Yoshimoto 1988; Syafii, Yoshimoto & Samejima 1988), and/or soil conditions, e.g., pH, moisture content, and aeration. Further studies are needed to characterise and identify the organism(s) involved in the decay process, which should also enable controlled laboratory studies to be undertaken in order to obtain more specific information on factors that may be unique to this form of wood decay.

REFERENCES

BLANCHETTE, R.A.; NILSSON, T.; DANIEL, G.F.; ABAD, A. 1990: Biological degradation of wood. Pp.141–174 *in* Rowell, R.M.; Barbour, R.J. (Ed.) "Archaeological Wood: Properties, Chemistry and Preservation". American Chemical Society, Washington, DC, *Advances in Chemistry Series No. 225.*

DANIEL, G.F.; VALE, J.; NILSSON, T. 1992: Soft rot and multiple T-branching by the basidiomycete *Oudemansiella mucida. Mycological Research 96*: 49–54.

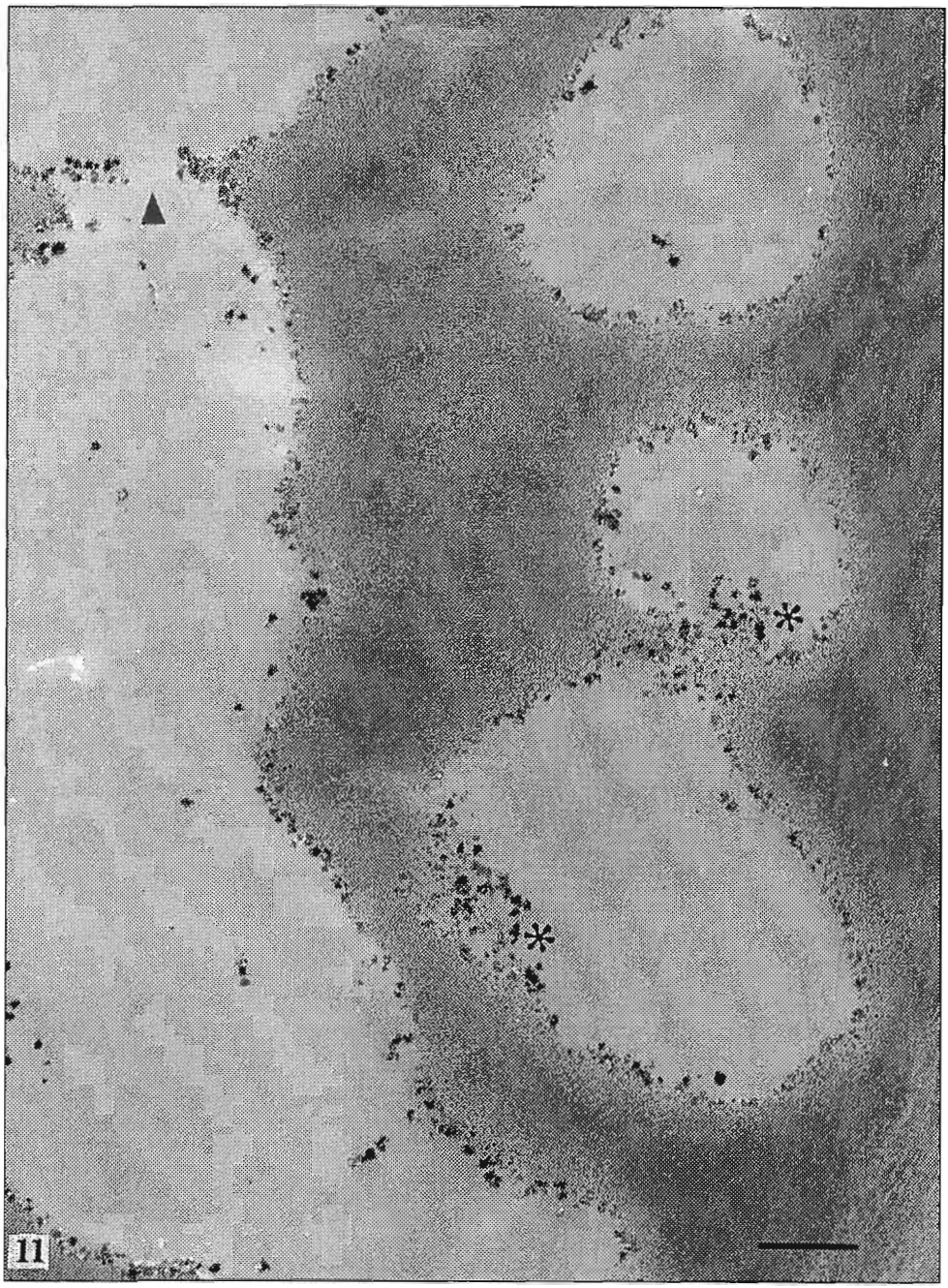

FIG. 11–Transverse section through part of an axial tracheid wall containing tunnels. The arrowhead indicates the region of coalescence between adjacent tunnels. Dense, granular material present in the tunnels is indicated by asterisks. Transmission electron micrograph. Bar = 250 nm.

DONALDSON, L.A. 1994: Mechanical constraints on lignin deposition during lignification. *Wood Science & Technology 28*: 111–118.

EATON, R.A.; HALE, M.D.C. 1993: "Wood: Decay, Pests and Protection". Chapman and Hall, London.

ERIKSSON, K.-E.; BLANCHETTE, R.A.; ANDER, P. 1990: "Microbial and Enzymatic Degradation of Wood and Wood Components". Springer-Verlag, Berlin.

HEPLER, P.K.; FOSKET, D.E.; NEWCOMB, E.G. 1970: Lignification during secondary wall formation in coleus: an electron microscope study. *American Journal of Botany 57*: 85–96.

MAURER, A.; FENGEL, D. 1990: A new process for improving the quality and lignin staining of ultrathin sections from wood tissue. *Holzforschung 44*: 453–460.

NILSSON, T.; DANIEL, G.F.; BARDAGE, S. 1990: Evidence for actinomycete degradation of wood cell walls. The International Research Group on Wood Preservation, Document No. IRG/WP/1444. Stockholm, Sweden.

SCHWARZE, F.W.M.R.; LONSDALE, D.; FINK, S. 1995: Soft rot and multiple T-branching by the basidiomycete *Inonotus hispidus* in ash and London plane. *Mycological Research 99*: 813–820.

SINGH, A.P. 1997: Initial pit borders in *Pinus radiata* are resistant to degradation by soft rot fungi and erosion bacteria but not tunnelling bacteria. *Holzforschung 51*: 15–18.

SINGH, A.P.; BUTCHER, J.A. 1991: Bacterial degradation of wood cell walls. A review of degradation patterns. *Journal of the Institute of Wood Science 12*: 143–157.

SINGH, A.P.; WONG, A.H.H. 1996: Hyphal tunnelling of belian (*Eusideroxylon zwageri*) wood cell walls. The International Research Group on Wood Preservation, Document No.: IRG/WP/96-10152. Stockholm, Sweden.

SPURR, A.R. 1969: A low viscosity embedding medium for electron microscopy. *Journal of Ultrastructural Research 26*: 31–43.

SYAFII, W.; SAMEJIMA, M.; YOSHIMOTO, T. 1987: The role of extractives in decay resistance of Ulin (*Eusideroxylon zwageri* T. Et B.) wood. *Bulletin of Tokyo University Forests 77*: 1–8.

SYAFII, W.; SAMEJIMA, M.; YOSHIMOTO, T. 1988: The role of lignin on decay resistance of Ulin (*Eusideroxylon zwageri* T. Et B.) to wood-rotting fungi. *Bulletin of Tokyo University Forests 77*: 119–126.

SYAFII, W.; YOSHIMOTO, T.; SAMEJIMA, M. 1988: The effect of lignin structure on decay resistance of some tropical woods. *Bulletin of Tokyo University Forests 80*: 69–77.

WONG, A.H.H.; SINGH, A.P.; LING, W.C. 1996: Major decay types recognised in an extremely durable timber species belian (*Eusideroxylon zwageri*) from East Malaysia. Pp.162–173 *in* Proceedings of the TRTTC (Timber Research and Technical Training Centre)/STA (Sarawak Timber Association) Forest Products Seminar '96, 11–13 March 1996, Kuching, Sarawak, Malaysia.