ANATOMICAL CHARACTERIZATION OF *EUCALYPTUS GLOBULUS* WOOD DECAY BY TWO WHITE ROT SPECIES OF *TRAMETES*

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SUMMARY

Application of ligninolytic enzymes in paper and pulp industry has opened a new avenue to overcome the problems associated with mechanical and chemical pulping. The present study was therefore aimed to investigate the anatomical alterations in the cell wall of eucalyptus sap wood inoculated with *Trametes hirsuta* (Fr.) Pilat and *T. versicolor* (Fr.) Pilat. *T. hirsuta* caused cell separation, formation of oval shaped cavities rich in cellulosic polysaccharides and pit erosion during early stages of decay, suggesting selective delignification pattern. However, degradation of carbohydrate components resulting in large void areas was detected during advanced stages of decay. Thinning of cell wall was detected in the fibres of wood blocks colonized by *T. versicolor*, indicating simultaneous white rot decay. The degradation occurred preferentially to the middle layers of secondary wall, while compound middle lamellae and S_3 layer resisted degradation during early stages of decay. Confocal microscopy revealed strong delignification from vessel wall and separation of fibres following delignification of compound middle lamellae. The degradation of lignin rich vessel wall during advanced stages of decay by both species suggests their strong ligninolytic properties suitable for its use in paper and pulp industry.

Keywords: Simultaneous wood rot, wood decay, white rot, *Trametes hirsuta*, *Trametes versicolor.*

INTRODUCTION

The genus *Eucalyptus* (Myrtaceae) is mainly cultivated for paper, pulp, pharmaceutical and cosmetics industries (Silva *et al*., 2003). In recent years, many studies have revealed the potential of *E. globuluus* in the production of timber, pulpwood and bioethanol (Whittock *et al*., 2007; Romani *et al*., 2012). Due to the growing economic importance, *Eucalyptus* spp. are also used in the fungal decay studies (Fernandes *et al*., 2005). The propensity of trees to wood decay is important as decay leads to breakdown of cellular structure of wood and the defense response of plant leads to increased deposition of lignin content in the wood which will reduce pulp yield (Pearce, 1996). The non-specific enzyme system of wood-degrading fungi with the ability to completely degrade lignin have tremendously contributed the biotechnological researches with their potential in bioremediation of organic pollutants, ethanol production, bio-pulping of wood in paper industry (Maciel *et al*., 2010).

Three types of fungi are generally responsible for decay: soft rot, brown rot and white rot fungi (Rio *et al*., 2002). Among these, decay in angiosperms is mainly due to white rot fungi, which have the potential to attack all wood components and the ability to degrade lignin, which is of interest in wood pretreatment processes for paper pulping industries (Jennings, 1995; Ferraz *et al*., 1998; Breen and Singletan, 1999; Fernanades *et al*., 2005). Depending upon the rate at which white-rot fungi decay all structural cell wall constituents, two forms of white rot are distinguished. Selective (or preferential) white rots which degrade hemicellulose and lignin at an early stage of decay while non- selective (simultaneous) white rots results in homogenous cell wall decay as they eliminate carbohydrates and lignin at similar rate (Davis *et al*., 1994; Blanchette, 1995; Enoki *et al*., 1998; Pandey and Pitman, 2003).

Heterogeneity in decay pattern was shown by several white rot fungi, i.e. the same species cause selective degradation of lignin in one tree species, and simultaneous degradation in another (Agosin *et al*., 1990). The genus *Trametes* belongs to white rot fungi group and is known to show both selective and simultaneous mode of decay with respect to species variation within genera and wood type (Anagnost, 1998; Levin and Castro, 1998). These two species *viz.* T. *hirsuta* and *T. versicolor* are known to possess strong ligninolytic activity such as manganese peroxidase, laccase and lignin peroxidase. The present work is therefore aimed to study the structural changes occurring in the wood of *E. globulus* associated with colonization of *T. hirsuta* and *T. versicolor* and to provide perspective of wood decay capabilities of both species for their application in paper and pulp industry.

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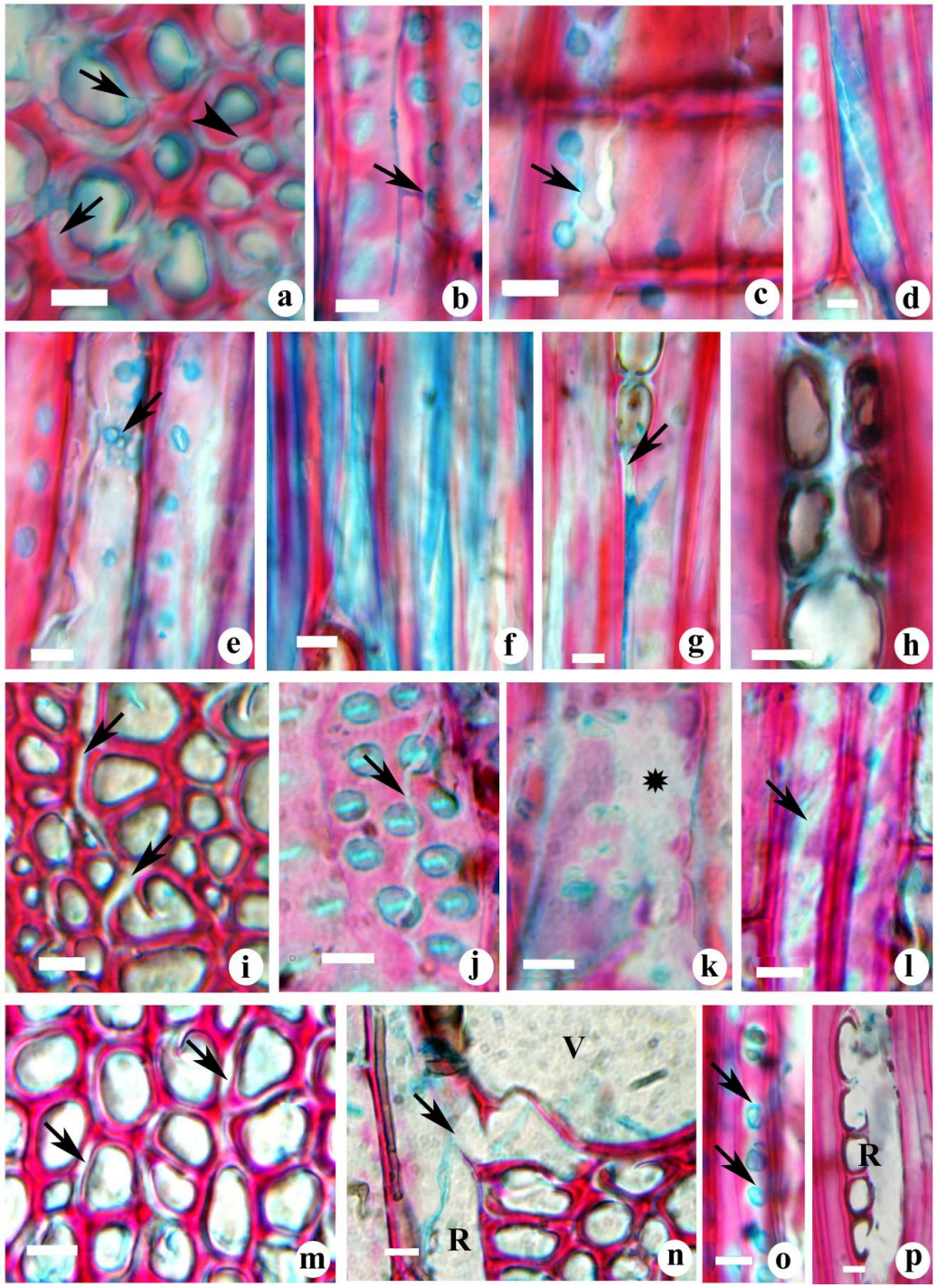

Fig. 1. Transverse, tangential and radial longitudinal sections of *E. globulus* wood blocks infected with *Trametes hirsuta* for 30, 60, 90 and 120 days.
(a) Separation of cells (arrowhead) and blue colored regions (cellulose stained with astra blue) of secondary wall (arrows) indicating the selective delignification. (b) Branching of mycelia and migration into adjacent fibre through the pit (arrow). (c) The erosion channels showing blue to white color due to selective delignification (arrow). (d) Fibre wall showing selective delignification. (e) The secondary wall of fibres adjacent to pit showing selective delignification (arrow). (f) Extensive delignification of fibre wall showing cellulose rich secondary wall. (g) The ray cell showing separation (arrow). Note the selective delignification of adjacent fibre. (h) Separation of ray cells. (i) Fibre showing dissolution of middle lamellae followed by cell separation (arrow). (j) The erosion channels formed across the bordered pits in the vessel (arrow). (k) The advanced stage of vessel wall erosion (asterisk). (l) Fibre wall showing patches of unstained regions representing the degraded wall (arrow). (m) The middle layer of secondary wall showing whitish space formed after delignification (arrows). (n) Collapse of vessel (V) during advanced stage of decay. Note the fungal mycelia (arrow) passing through the erosion channels in the contact rays (R). (o) Pits in the fibre showing enlargement following erosion and joining of adjacent ones (arrows). (p) Ray cells (R) showing separation from the axial elements and formation of erosion channels. Scale bar = 10 µm.

MATERIALS AND METHODS

The pure strains of *Trametes hirsuta* (Fr.) Pilat (NTCC 729/C) and *Trametes versicolor* (Fr.) Pilat (NTCC 165/S) were obtained from the fungal depository of Forest Research Institute, Dehradun, India. Defect free sapwood portion of sound wood of *Eucalyptus globulus* Labill. trees growing in the arboretum of the M.S. University of Baroda, Gujarat, India were used for *in vitro* decay test. Wood disks were obtained at breast height from 12-15 years old trees and cut into small cubic blocks measuring 2×2×2 cm. Total 128 blocks were oven dried at 60ºC for 48 hours and stored in sterile polyethylene bags at dry place with 40-45% relative humidity. At the time of experiment, these wood blocks were soaked overnight in water to obtain optimum moisture level to facilitate the fungal growth. Subsequently, these test blocks were autoclaved at 120ºC for 30 min and surface sterilized with 70% ethanol. Four test blocks were kept in each autoclaved petri plate containing Malt Extract Agar media (MEA) and inoculated with plugs of mycelium taken from 15-days-old pure cultures of *T. versicolor and T. hirsuta* (one fungi/petri dish). These samples were incubated for 30, 60, 90 and 120 days at 27±1°C and 70% relative humidity. After each incubation period, 16 test blocks (12 treated and 4 controls) for each fungus were removed and cleaned with a brush to take out mycelia. The marked blocks were weighed after oven drying to determine per cent weight loss while r
ests of the blocks were fixed in Formaldehyde-Acetic acid-Alcohol (FAA) (Berlyn and Miksche, 1976). After 12 hours of fixation, these samples were transferred in 70% ethanol. The experiment was performed in triplicates.

Samples were processed for paraffin embedding to obtain 10 to 12 µm thick sections from experimental wood blocks. Suitably trimmed samples were dehydrated with tertiary butyl alcohol series and processed by routine method of paraffin embedding (Berlyn and Miksche, 1976). Transverse, radial and longitudinal sections of 10-12 µm thickness were taken with a rotary microtome (Leica RM 2035, Germany). The sections were de-waxed in xylene-alcohol series and stained with safranin-Astra blue (Sigma, Germany) combinations (Srebotnik and Messner, 1994). After dehydration in ethanol-xylene series (Berlyn and Miksche, 1976), the sections were mounted in DPX (Dibutyl Phathalate Xylene). Samples were micro-photographed at 40× and 100× magnifications using Leica DM 2000 microscope (Germany) equipped with a digital camera (Cannon S70D, Germany). For confocal laser scanning microscopy (CLSM), FAA fixed samples were washed in water followed by 0.01 M phosphate buffer (pH 9.0). Hand sections (approximately 40-80 µm thickness) taken from the wood block were stained with 0.001% acridine orange for 2 hours in dark and mounted in buffered glycerol (pH 8-9). Slides were examined with Zeiss confocal laser scanning microscope (LSM 710) using a Krypton/argon laser emitting at wavelength of 488 (excitation) and 568 nm (emission; Donaldson and Lausber, 1998).

RESULTS

Anatomical changes induced by *T. hirsuta*. The transverse sections obtained from the wood blocks after 30 days of inoculation revealed separation of wood cells by dissolution of middle lamellae (Fig. 1a). Fungal mycelia migrated into adjacent cells through the bordered pits in the fibre tracheids (Fig. 1b). The wall erosion channels were observed between the pits (Fig. 1c). The longitudinal sections also showed blue colored tangential wall of fibres due to cellulose rich cell wall following selective delignification (Fig. 1d). Extensive pit erosion in fibre tracheids was noticed after 60 days of inoculation (Fig. 1e). The selectively delignified, eroded regions in the wall often appeared oval-oblong in shape (Fig. 1e). Majority of fibre tracheids were stained with astrablue because of advancement of selective delignification (Fig. 1f). The separation of ray cells and fibres due to delamellation became more frequent after 60 days of incubation (Fig. 1g, 1h, 1i). Vessel wall damage initiated with erosion channels between bordered pits and the complete erosion of larger areas was observed in the later stages of decay (Fig. 1j, 1k).

After 90 days post inoculation, fibres often showed merging of erosion patches and formed large unstained areas (Fig. 1l). The complete removal of cell wall polymers in the S_2 region of the secondary wall resulted in large hollow space in the fibre tracheids (Fig. 1m). At an advanced stage (i.e. after 120 days) migration of fungal mycelia from contact rays into vessel led to collapse of vessels and subsequent degradation of their wall (Fig. 1n). Wall erosion around the bordered pits was common in the secondary wall of fibre tracheids (Fig. 1o). The degradation of both radial and tangential wall of ray cells resulted in large hollow region in the tangential sections (Fig. 1p).

Anatomical changes induced by *T. versicolor*. Contrary to the selective delignification pattern showed by *T. hirsuta*, the wood colonized by *T. versicolor* showed thinning of fibre cell wall in two patterns. In first case, formation of localized erosion occurred from inner wall layer (S_3) and progressed further into middle layer of the wall, while the second pattern showed initiation of delignification from outer layers of secondary wall (S_1+S_2) leaving compound middle lamellae and S3 layer intact (Fig. 2a). Fungal mycelia moved through the cell lumen and invaded adjacent cells by erosion of bordered pits located on the lateral walls of fibre tracheids (Fig. 2b, 2c, 2e). In tangential sections, the radial walls of fibres also showed separation (Fig. 2d). The analysis of pit erosion under high magnification revealed mycelial penetration through the pit (Fig. 2e), which may then enter into adjacent cell (Fig. 2f) or bend and pass vertically through middle lamella (Fig. 2g), leading to the separation of cells (Fig. 2h). Simultaneous degradation of wall material around and between pits in the fibres appeared as patches of unstained region in their tangential wall (Fig. 2i, 2j). One of the characteristic of simultaneous

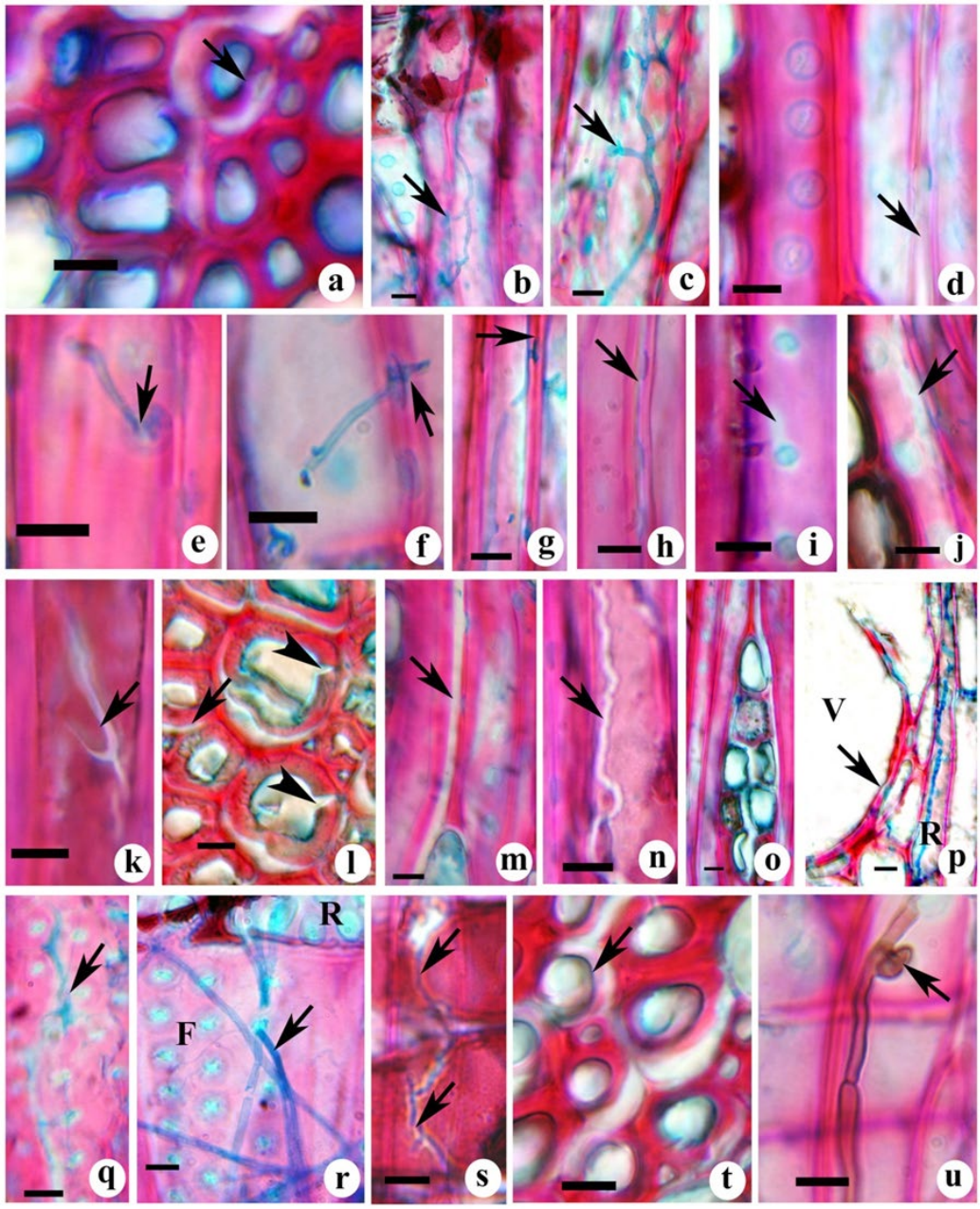

Fig. 2. Transverse, tangential and radial longitudinal sections of *E. globulus* wood blocks inoculated with *Trametes versicolor* for 30, 60, 90 and 120 days.
(a) Thinning of secondary wall (arrow) and separation of fibres. Note the preferential degradation of outer layers of fibre secondary wall and its lateral expansion. (b) Mycelia migrating into adjacent cells through pits in the tangential wall (arrow). (c) Branching of mycelia with thin cell lumen of fibre (arrow). (d) Erosion channels formed in the radial wall of fibre (arrow). Note the large bordered pit on the tangential wall of adjacent fibre tracheid. (e) Fungal mycelia entering into pit (arrow). (f) Mycelia passing through the pit (arrow). (g) Vertical growth of fungus within cell wall through middle lamellae (arrow). (h) Separation of cells after dissolution of middle lamellae (arrow). (i) The wall around the pit showing erosion of secondary wall (arrow). (j) Enlargement of erosion channels in the fibre tracheids (arrow). (k) The U-notch appearance of erosion channels in tangential sections of fibre tracheids (arrow). (l) Separation of cells (arrow) and formation of large erosion channels (arrowheads) in the fibre secondary wall. (m) Separation of wall between fibre tracheids and ray cell beneath the fibre (arrow). (n) Progression of erosion of fibre wall in vertical direction (arrow). (o) Separation of ray cells after dissolution of middle lamellae in both radial and tangential walls (arrow). (p) Delignification followed by collapse of vessel wall (V). Arrow indicates eroded region of secondary wall of vessel. Note the mycelia passing through the contact rays (R) and erosion channels in the parenchyma cell. (q) Vessel wall showing erosion channels formed pit between adjacent bordered pits. Note the blue colored mycelia within the erosion channel (arrow). (r) Mycelia (arrow) passing through the bordered pits in the fibre tracheids (F) and simple pits in the ray cell (R). (s) The fungal mycelia passing through erosion channel in the ray cell wall (arrow). (t) The thinning of secondary wall (arrow) and separation of fibre tracheids during advanced stage of decay. (u) The chlamydospore formation in the fungal mycelia within the cell lumen (arrow). Scale bar = 10 µm.

degradation (i.e. the U-notch and appearance of erosion channels) was apparent in the tangential wall of fibres (Fig. 2k). Separation of cells and erosion channels from cell lumen became more evident after 60 days of inoculation (Fig. 2l). Separation of both axial and radial elements by dissolution of middle lamellae in all the cell types was also observed (Fig. 2m). Vertical tunneling along the cell axis appeared as unstained regions due to simultaneous pattern of decay (Fig. 2n). In case of ray cells, dissolution of middle lamellae in both radial and tangential wall resulted in separation and isolation of cells (Fig. 2o). The fungal mycelia passing through contact rays into vessels and paratracheal cell and vessel wall often showed erosion near the bordered pit regions (Fig. 2p). The presence of fungal mycelia within the erosion channels formed between bordered pits in the vessel wall was also seen frequently at several sections (Fig. 2q). Migration of fungal hyphae between rays and fibre tracheids through simple and bordered pits respectively was a common feature (Fig. 2r). Presence of artificially blue colored hyphae was often observed in the erosion channels in the radial wall of rays (Fig. 2s). After 120 days of inoculation, thinning of the wall and dissolution of middle lamellae became more pronounced (Fig. 2t) and fungal mycelia also formed chlamydospores (Fig. 2u).

Confocal Laser Scanning Microscopy (CLSM). The vessel wall and compound middle lamellae of fibres showed high intensity of fluorescence even after 120 days incubation. At this stage, wood blocks inoculated with *T. hirsuta* showed gradual loss of fluorescence from the inner secondary wall layer of vessel (Fig. 3a, 3b) while compound middle lamellae of fibres showed separation and absence of autofluorescence due to strong delignification in this region (Fig. 3c). Ray parenchyma also showed enlarged pit fields due to extension of pit erosion from adjacent lignified region of the cell wall (Fig. 3d). Delignification of vessel walls and compound middle lamellae of fibres were also apparent in the wood infected with *T. versicolor* (Fig. 3f, 3g). Delignification of cell wall adjacent to pits in the fibre wall appeared as non-fluorescent regions in middle lamellae and outer layers of secondary wall (Fig. 3h).

DISCUSSION

Both the species of *Trametes* are classified under white rot group of fungi. The present study documents the sequential stages of entry and colonization. Entry of fungal hyphae occurs mainly through pits on lateral walls and enters either into cell lumen or moves laterally by dissolving the middle lamellae. According to Schwarze (2007), access to adjacent xylem cells occurs via pit apertures or direct penetration through the cell wall. The present study also shows that once mycelia enters inside the cell lumen, multiple branching of hyphae produce penetration hyphae which pass through the pits and produce erosion channels leading to the extensive growth of fungi. Ray cells also showed separation and pit erosion during the initial stages fungal invasion suggesting they function as the radial channels during the fungal colonization within the tissue. The axial alignment xylem fibres, vessels and the radial arrangement of xylem rays make easy access to mycelia into the wood and allow widespread distribution of hyphae within the xylem (Schwarze *et al*., 2000). Eslyn and Lombard (1983) reported the occurrence of chlamydospores in *T. versicolor* growing in coal mining timbers. Robles *et al*. (2014) correlated formation of chlamydospures in the vessel lumen and xylem rays by *Inonotus rickii* as a mechanism of spread and efficiency to infect new individuals. Similar mechanism may be followed by *Trametes* as observed in the present study. Chlamydospore formation has also been observed during the advanced stage of decay in *Eucalyptus* wood by *T. versicolor* suggesting its potential of spread and capacity of new individual of the same species for colonization (Robles *et al*., 2014).

Wood decay pattern by *T. hirsuta*. Light microscopic analysis revealed two different patterns of white rot in eucalyptus wood by *T. hirsuta* and *T. versicolor*. *T. hirsuta* exhibited selective delignification causing dissolution of middle lamella of the xylem cells followed by cell separation at an early stage of degradation. This separation of cells is the best sign of selective delignification (Anagnost, 1998). A similar pattern of wood degradation by *Inonotus hispidus* in *Ailanthus* is reported wherein, dissolution of middle lamellae commences at the cell corners in xylem fibres which is remarkable feature for selective delignification (Koyani *et al*., 2010). Advancement in the selective delignification of secondary wall of fibre tracheids has been evident from its blue colored appearance after safranin-astra blue staining. Astra blue is specific for cellulosic polysaccharides and widely used as a best indicator for delignification studies (Srebotnik and Messner, 1994; Kraus *et al*., 1998).

Complete removal of lignin in the secondary wall and middle lamellae, leaving large quantities of cellulose in the S_2 layer of the cell wall is evident in case of selective delignification. After 90 days of inoculation, the formation of erosion channels and tunnels has been observed in the secondary wall of fibres indicating a transition from selective delignification to removal of cellulosic polysaccharides. Although internal cavity formation in cell walls is generally regarded as characteristic of soft rots, there are some evidences that it is also associated with white rot type of decay (Schwarze *et al*., 1995). *Ganoderma pfeifferi* Bres., caused both selective and simultaneous rot in beech and oak wood suggesting simultaneous and soft rot mode of decay can be caused by same species and it may take place side by side (Blanchette, 1980; Schwarze, 2007). The present study demonstrates that these processes occur during different stages of degradation (initial and advanced) rather than occurring side by side. Our results are

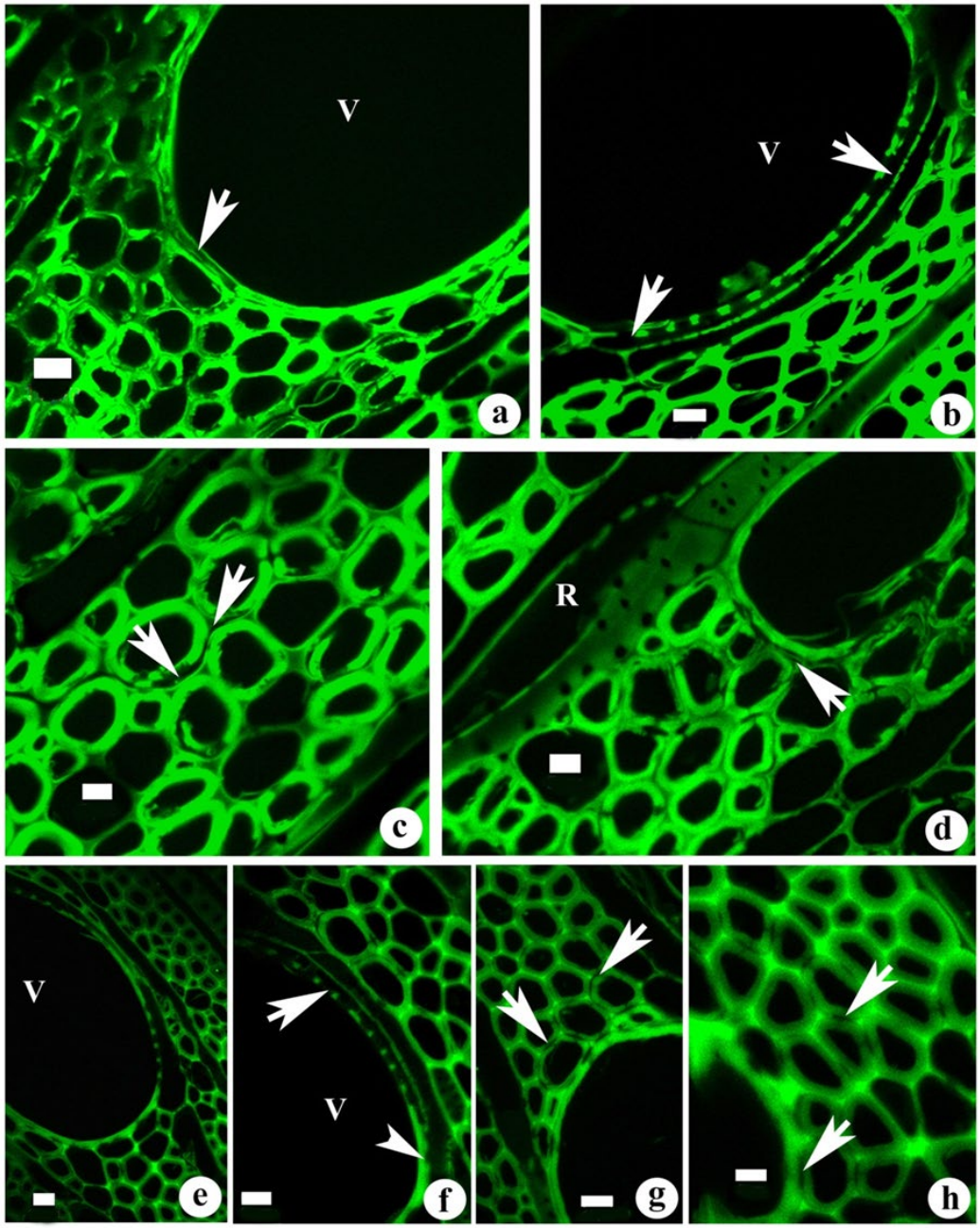

Fig. 3. Confocal images of transverse sections from the wood of *Eucalyptus globulus* inoculated with *Trametes hirsuta* (a-d) and *Trametes versicolor* (e-h).
(a) Lignin degradation in the inner secondary wall layer of vessel (V) wall (arrow). (b) The advanced stage of delignification of vessel (V) wall showing disappearance of auto-fluorescence from S_2 and S_3 layers. (c) The middle lamellae region of fibre (arrows) showing delignification indicated by absence of auto-fluorescence. (d) Lignin degradation leading to collapse of vessel and separation of its associated cells (arrow). (e) Vessel (V) wall showing delignified wall with dotted appearance of fluorescence. (f) Enlarged view of vessel (V) wall delignification (arrow). Arrowhead indicates the lignin rich secondary wall showing fluorescence. (g, h) Fibres showing absence of auto-fluorescence from compound middle lamellae and cell corners following delignification. Scale bar: a, b, e, f, g = 10 µm; c, d, h = 5 µm.

in agreement with Levin and Castro (1998) who observed a combined pattern of selective and simultaneous white rot in poplar by *Trametes trogii* during early and late stages of decay respectively. Erosion in oval patches extending from lumen to the middle lamellae has been demonstrated to be a specific feature associated with selective delignification in birch wood by *Phellinus pini* (Anagnost, 1998). Present study also shows oval shaped, selectively delignified (blue stained) regions in the fibre wall during early stages of decay while these regions coalesced to form larger patches of erosion which is characterized by complete removal of wall polymers (unstained regions) during advanced stage of decay. This further suggest loss of oval shape of erosion channels could be an anatomical feature during transition

from selective to simultaneous mode of decay by *T. hirsuta* in *E. globulus*.

T. versicolor demonstrated characteristics of a typical simultaneous rot fungus (Eriksson *et al*., 1990). Present study also shows that it causes simultaneous degradation of cell wall components in *Eucalyptus* wood. Early stage of decay by *T. versicolor* revealed erosion channels around the pit chamber which extended laterally forming tunnels and often forms U-notch in tangential sections. Localized thinning of cell wall has also been noticed in the fibres. The major anatomical features during simultaneous white rot decay includes erosion channels with U-shaped notches, localized removal of cell wall resulting in cell separation and general thinning of wall (Anagnost, 1998). In the present study also thinning of cell wall through degradation of lignin from outer layer of secondary wall during initial stages of decay was conspicuous feature. Ruel *et al.* (1981) observed similar pattern during degradation of cell wall in spruce wood (*Picea abies*) by *Phanerochaete chrysosporium*. These authors observed that an inward attack of the cell originated at the pit membrane and subsequently followed the degradation across the transition between the S_1 and S_2 layers. Although majority of these structural features shown in the present study are of simultaneous rot, separation of cells by dissolution of middle lamellae during advanced stages of decay is a typical feature associated with selective mode of decay and indicates the ability of the *T. versicolor* to cause selective delignification. This feature also forms a common link between *T. hirsuta* and *T. versicolor* for their ability of selective delignification process where former retains the selective mode of decay for extended period. Apart from these features, the present study also showed the multiple branching of hyphae within the cavities which is reported to be a characteristic of soft rot decay (Schwarze *et al*., 2000). Worall *et al*. (1997) postulated that wood decay evolution began with soft rot fungi from which white rot and subsequently brown rot fungi have been evolved. Therefore, similarities in features of different groups by the two related species of fungi could plausibly due to their phylogenetic relationship with ancestral group.

According to Robles *et al*. (2014), high concentration of lignin in the vessel wall makes it resistant to attack by some white rot fungi. The present study shows the collapse of the vessels in the advance stage of decay by both *T. hirsuta* and *T. versicolor* by erosion channels formed between the bordered pits. Large voids in the tangential wall formed by lateral merging of erosion channels has also been noticed in the vessel during advanced stages of decay indicating removal of all the polysaccharides and strong ligninolytic activity during this stage. Confocal microscopy also confirms the substantial depletion of lignin from middle lamellae and secondary wall of vessels. The compound middle lamellae of fibres also showed absence of fluorescence indicates removal of lignin from this region. Both vessel wall and compound middle lamellae of fibres generally resist degradation due to the presence of more condensed guaiacyl lignin monomers in these regions (Blanchette, 1984). The delignification of these guaiacyl lignin rich regions of cell wall supports the ability of fungus for selective mode of decay using strong activity of its ligninolytic enzymes.

In conclusion, the present study demonstrates the decay pattern of two species of *Trametes* in the wood of *E. globulus*. *T. hirsuta* induced selective delignification which is evident from the typical anatomical features like cell separation, formation of oval shaped cavities rich in cellulosic polysaccharides and pit erosion while degradation of all wall constituents was noticed in advanced stages of decay resulting in transformation of oval cavities into large void areas. *T. versicolor* showed distinct simultaneous white rot decay leading to formation of erosion channels across the wall and tunnels within the secondary wall. Thinning cell wall by preferential degradation of outer layers of secondary wall was conspicuous feature detected during early stages of degradation. Although *T. versicolor* showed simultaneous rot, its ability for selective delignification was evident from the dissolution of middle lamellae and separation of cells, and this also forms a similar white rot feature exhibited by both the species of *Trametes* investigated in this study. Hence our results suggest that both the species of *Trametes* possess ability for selective and simultaneous modes of decay with a specific difference in its duration of selective delignification mode in the wood of *E. globulus*. Looking at the pattern of delignification and ability of lignin removal, both the species are potential species for utilization in paper industry.

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