HISTOLOGY

Wood is the most important natural and endlessly renewable source of energy; therefore, it has a major future role as an environmentally cost-effective alternative to burning fossils fuels. The major role of wood is not only the provision of energy but also the provision of energy sufficient material for our buildings and many other products. In addition, developing wood cell represents one of the most important sink for excess atmospheric CO_2, thereby reducing one of the major contributors to the global warming. Wood is the fifth most important product of the world trade. Vast quantities of wood are logged by foresters to provide fuel, fibres (for pulp, paper products and boards) and sawn timber (for house building, and furniture) as commodities. The complex chemical makeup of wood (cellulose, hemicellulose, lignin and pectin) also makes it an ideal raw material for what could be a future lingo-chemical industry that could replace the petrochemical industry, in providing not only plastics and all kind of chemical products, but also food and textile products (Christophe *et al.*, 2001).

Considering the important role that wood is foreseen to play in the near future, it is surprising to see that studies on wood formation, wood quality and health of the

forest trees are completely neglected in India. As far as we are aware, no much attention is paid to research on wood in any of the Indian laboratory. Therefore present study will help to understand the structural variations, properties, quantity and quality of wood on which its utilization depends. It will also help to know the adaptation of particular species, and defence mechanism developed by the timber trees in response to microbial infection.

Fungi that inhabit the forest floor are the most important agents in recycling the carbon stored in wood. Among them white rot Basidiomycetes are especially most important in the forest ecosystem since they are the only fungi capable of degrading all cell wall components of wood (Zabel and Morrell, 1992; Connolly and Jellison, 1997; Maloy and Murray, 2001). On the other hand they also play fundamental role in the large economic losses of timber and forest products by decay and deterioration (Boddy and Watkinson, 1995). Tree decay is the major worldwide cause to damage the timber. Decay causes more damage to the timber than all other destructive agents combined. From 20 to 80 per cent of world timber is lost annually, or used for low-quality products because of decay (Christophe *et al.*, 2001; Maloy and Murray, 2001).

A number of species of fungi live in or on wood, and as a result of their growth anatomical changes may be induced in substrate cell walls. These changes occur in several patterns that can be readily distinguished from one another, and that apparently result from quantitative or qualitative differences in the enzyme complement of the organisms in each group. The patterns resulting from the morphological effects of fungus action tend to correlate closely with taxonomic groupings based upon the morphology of the fungal thallus. Therefore, wood decomposition by fungi usually is seperated into three categories, based on micromorphological and chemical characteristics of decay, which results in different pattern of attack on the middle lamella, S_1, S_2 and S_3 layers: (1) brown

rot, (2) white rot, subdivided into simultaneous rot and selective delignification and (3) soft rot.

Brown rot is often associated with gymnospermous wood whereas white rot occurs predominantly in hardwoods (Gilberstson, 1980; Schwarze and Fink, 1998). The hyphae of brown rot fungi grow within the cell lumina on the surface of the S_3 but causes little alterations in this layer or the compound middle lamella. However, the S_2 and S_1 layers become extensively degraded owing to the removal of polysaccharides (Liese, 1970; Rayner and Boddy, 1988; Erikson *et al.*, 1990; Koyani *et al.*, 2010). The limited ability of these fungi to degrade lignin seems to account for the absence of any localized erosion of the cell wall. This together with the relatively homogenous structure of gymnospermous wood leads to a very uniform mode of degradation.

On the contrary, white rot fungi has a very high lignolytic potential and are adapted to degrade more complex structure of angiosperm wood, thus resulting into very wide range of degradation modes (Schwarz and Fink, 1998). In the past, two broad divisions of white rot have been widely accepted: i) selective delignification and ii) simultaneous rot (Schwarze and Fink, 1998; Schwarze and Baum, 2000; Schwarze, 2007; Lehringer *et al.*, 2010; Koyani *et al.*, 2010). In selective delignification, lignin and hemicelluloses are degraded earlier in the decay process than cellulose. Delignification is initiated by hyphae growing in the lumen and commences within the secondary wall and later into the middle lamella so that the cells tend to seperate (Blanchette, 1980; 1984a, b; Rayner and Boddy, 1988; Eriksson *et al.*, 1990; Schwarze and Fink, 1998; Schwarze and Baum, 2000; Schwarze, 2007; Lehringer *et al.*, 2010; Koyani *et al.*, 2010). In simultaneous rot, lignin and structural polysaccharides including cellulose are degraded in similar rate by fungal hyphae from within the cell lumina towards the middle lamellae. Erosion troughs beneath hyphae extend deeply into the secondary wall

degrading the S1, S2 and S3 layer in succession (Liese, 1970; Rayner and Boddy, 1988; Eriksson *et al.,* 1990; Schwarze and Baum, 2000; Schwarze, 2007; Koyani *et al.*, 2010). In addition to these types Schwarze and Engels (1998) have reported an unusual type of cavity formation reminiscent of soft rot followed by selective delignification for a range of host-fungus combinations.

Characteristic feature of soft rots is their pattern of development, which involves "T" branching or "L" bending and hyphal tunnelling inside lignified cell wall. This distinctive mode of attack was described in the mid-century by Schacht (1863) and was elucidated by Savory (1954) who proposed the term soft rot. Discrete notches of cell wall erosion by hyphae lying within the lumina, in addition to cavities formed by hyphae within the cell wall are also frequently found in wood degraded by soft rot fungi. These erosion troughs, which are indistinguishable from those of white rot fungi, have been attributed to a category of soft rot known as type 2, whereas internal cavity formation is typical of type 1 attack (Corbett, 1965; Hale and Eaton, 1985a, b). Soft rot have generally been attributed to Deuteromycete and Ascomycete fungi and not to Basidiomycetes (Blanchette, 1992).

Although some brown and white rot fungi have been found to produce soft-rot-like cavities within the S2 layer (Duncan, 1960; Courtois, 1965; Liese and Schmid, 1966; Liese, 1970; Nilsson and Daniel, 1988; Schwarze and Fink, 1998; Schwarze, 2007; Lehnringer *et al.,* 2010), these observations were considered not to be conclusive and more likely to be associated with a localised collapse of the cell wall or on obscure white-rot degradation mode (Eriksson *et al.*, 1990). However, recently it has been shown that certain Basidiomycetes such as *Inonotus hispidus* can cause a soft rot in addition, or alternatively, to their more typical mode of attack, i.e. soft rot (Schwarze, 1995; Schwarze *et al.*, 1995; Koyani *et al.*, 2010). In the past cavity formation in the secondary walls of lignified cell wall was regarded

as a distinct and relatively reliable character of soft rot, which can be used readily to differentiate this type of decay from other types of degradation (Zabel *et al.*, 1985; Eriksson *et al.*, 1990; Schwarze and Fink, 1998; Schwarze, 2007). However, above studies clearly indicate that differentiation of white- and soft rot fungi on the grounds of micro-morphological features such as cavity formation and erosion troughs is difficult and may not be used as a key feature to distinguish between both the types and is open question. Fungi associated with different types of wood rot are summarised in Table 34.

Type	Microorganisms	Color	Wood components utilized	Decay characteristics
White	Basidiomycota	± bleached	All cell wall components, some species preferentially attack lignin	Progressive erosion of all cell wall layers. Middle lamella is degraded
Brown	Basidiomycota	± brown	Carbohydrates, some lignin modification	Diffuse depolymerization of cellulose
Soft	Ascomycota and Deuteromycota	bleached or brown	Carbohydrates, some lignin modification	Type 1: cavities form in secondary wall Type 2: progressive erosion of secondary walls but middle lamella is not degraded

Table 34: Fungi that cause wood deterioration and their characteristics.

In the present study one species of white rot Basidiomycetes i.e. *Trichoderma harzianum* and other species of Ascomycetes i.e. *Chrysosporium asperatum* is exploited to study the pattern of delignification and structural alterations in the secondary xylem induced by them. Both the fungal strains occur on living trees as well as on the wood logs of the timber trees growing in different forests of Gujarat

state. Therefore, the main aim of the present study was *invitro* testing isolated fungi for wood decay to investigate: 1) Process of delignification in *Azadirachta indica* and *Tectona grandis* L.f. wood by *Chrysosporium asperatum* and *Trichoderma harzianum* which cause structural alterations in forests and sawmill timbers. 2) Anatomical characterization of their decay pattern and extent of wood damage caused by these fungi which has not been investigated earlier for *Azadirachta indica* and *Tectona grandis* L.f.

MATERIALS AND METHODS

5.1 Collection and preparation of samples (*In vivo* study):

Wood samples of *Azadirachta indica* (L.) Del. and *Tectona grandis* L.f. infected with *Chrysosporium asperatum* and *Trichoderma harzianum* were collected from trees growing in the M. S. University hostel campus as well as from the Gir forest of Junagadh and Pavagadh forest of Gujarat State. Only those trees were sampled from which fruiting bodies of *Chrysosporium asperatum* and *Trichoderma harzianum* were coming out from the stem and branches. Samples disk measuring about 60x60x60 mm in length, width and depth were excised from the main stems and thick branches with the help of chisel and hacksaw. Samples were fixed immediately in Formaldehyde Acetic Acid (Berlyn and Miksche 1976) while some of the unfixed samples were packed in sterile polyethylene bags. After coming back from the field these unfixed samples were inoculated in PDA and malt extract media for isolation and identification of the fungal species.

5.1.1 *In vitro* study

Sound/healthy wood of *Azadirachta indica* (L) Del. and *Tectona grandis* L.f. from the trees growing in the Pavagadh forest of central Gujarat and in the University campus were used to study *in vitro* decay test. Both *Trichoderma harzianum* and *Chrysosporium asperatum* was employed to study the pattern of delignification and to examine their ability to degrade the wood. Both the strains were grown seperately for two weeks in petri plates containing malt extract agar medium prior to inoculating the wood blocks.

For decay test, oven dried cubic blocks (2 x 2 x 2 cm) were exploited. Before weighing some of the blocks were marked to take final weight after each incubation period. After weighing these blocks were soaked in water for 24 hours and then autoclaved for 30 minutes at 120 °C. After cooling, these wood blocks were surface sterilised with 70% alcohol and inoculated with pure cultures of *T.*

harzianum and *C. asperatum*. These samples were incubated for 30, 60, 90 and 120 days at 27±1 °C and 70% relative humidity. After each incubation period the blocks were removed from the petri plates, cleaned to remove mycelia. The marked blocks were weighed to determine final volume while rest of the blocks were fixed in FAA (Berlyn and Miksche, 1976). The percentage of weight loss due to degradation was then calculated whereas un-inoculated blocks were treated as control samples. After 12 hours of fixation in FAA, samples were transferred to 70% alcohol for histological study.

5.2 Microtomy and staining:

After 12 hours of fixation in FAA (formaldehyde, acetic acid, ethyl alcohol (70%); 10:5:85, v/v), samples were cut into smaller pieces and transferred in 70% alcohol. Samples were processed by both sliding and rotary microtome. Transverse, radial and longitudinal sections of 12-15μm thickness were directly cut on the sliding microtome and stained with Safranine and Astra blue combination (Srebotnik and Messner, 1994). For rotary microtome, suitably trimmed samples (5mm^2) were dehydrated with Tertiary Butyl Alcohol (TBA) series (30%, 50%, 70%, 90% followed by 3X100% pure TBA) and processed by routine method of paraffin embedding. After dehydration in ethanol-xylene series the sections were mounted in DPX. Some of the sections were also treated with Potassium Iodide, Commasie Brilliant Blue (CBB), Sudan Black B and ferric chloride for the localisation of starch, proteins, lipids and tannins respectively (Krishnamurthy, 1999). Important results were micro-photographed with Leica DM 2000 trinocular research microscope.

5.3 Isolation and identification of fungi:

For the isolation of fungal pathogen, small pieces of wood blocks were surface sterilized with 95% ethanol for 10-15 seconds followed by 1% sodium hypochlorite for 45-50 seconds. These wood pieces were then aseptically

inoculated on potato dextrose agar (PDA) medium. Inoculated blocks were incubated in an environmental incubator at 28 °C for complete growth. Pure culture was obtained after successive transfer on PDA medium and preserved at 8°C. Both the fungi were isolated from the trees growing the Gujarat forest. Pure cultures were established by routine methods and were identified from Agharkar Research Institute, Pune and Plant Pathology Division, Forest Research Institute Dehradun.

RESULTS

Azadirachta indica (L.) Del.

5.4 Structure of wood:

Secondary xylem of *Azadirachta* was diffuse porous with indistinct growth rings. Annual increment of the xylem may be discerned by wide bands of axial parenchyma. The secondary xylem was composed of vessels, fibres, axial and ray parenchyma cells. Vessels were mostly solitary but radial multiples of 2-6 vessels were also seen frequently. Xylem rays were uni-multiseriate, compound and heterocellular.

5.5 Decay by *Trichoderma harzianum*:

After 30 days of fungal inoculation, there was no appreciable weight loss of wood block (Table 35), but fungal mycelia invaded all the cell types of the secondary xylem. Initially fungal mycelia began to grow on wood block and ultimately covered the whole block within 15 days. In the beginning mycelia invasion was observed through the vessel lumen (Figure 1A). From the vessels, hyphae traversed into the neighboring rays (Figure 1B) and gradually extend in all direction including xylem fibres and adjacent axial parenchyma cells (Figure 1C, D). At this stage no visual damage was observed in the cell walls. Fungal mycelia moved from one cell to the next through the pits present on their walls (Figure 1E). Presence of fungal mycelia in all the cell types of xylem adjacent to vessel elements was a common feature in all the samples studied.

Decay fungi	% Weight loss (60 days)	% Weight loss (90 days)	% Weight loss (120 days)
Trichoderma harzianum	23.11 (± 7.32)	35.73 (± 6.88)	43.39 (± 7.76)
Chrysosporium asperatum	29.87 (± 5.13)	37.08 (± 7.82)	46.73 (± 8.64)

Table 35: Average percent weight loss during each incubation period (60, 90 and 120 days).

Samples exposed to fungi for 60 days showed sign of selective delignification, though the signs were not that distinct in all the cell types, but can be easily observed in fibres. In transverse view, many of them showed concentric delignification starting from middle lamellae towards lumen. As a result the secondary wall adjacent to the middle lamellae stained blue with astra blue instead of red by safranine (Figure 1F). This feature was initially observed in the fibres adjacent to the rays, axial parenchyma cells and vessel elements. Thereafter, it gradually invaded all the cell types.

After 90 days of inoculation, all the cell types of the secondary xylem were invaded by the fungal mycelia. The middle lamellae that were stained blue began to lose the integrity and individual cells became seperated from each other (Figure 2A). As the degradation progressed further, complete seperation of fibres was a common feature and it was observed in most of the sections (Figure 2B). Due to delignification, pits of the ray and axial parenchyma cells became more pronounced and became larger in size and irregular in shape. At the same time, formation of several bore holes on the lateral walls of the rays was a common feature (Figure 2C). Compared to axial elements, ray cells were more affected showing advanced thinning of the cell walls that stained blue colored with astra blue part of the cell wall in which lignified part was relatively unaffected stained red with safranine.

Wood blocks exposed to fungi for 120 days showed similar pattern of delignification but the effects were more pronounced. At this stage, walls of ray cell showed larger erosion troughs (Figure 2D) and the bore holes on the walls were more distinct and became irregular in shape (Figure 2E). With the advancement of decay, many of the ray cells were either partially or completely disintegrated (Figure 2F). Vessel elements began to deform (Figure 3A) due to loss of rigidity while fibres appeared almost completely seperated from each other

(Figure 2D). In longitudinal sections xylem fibres were seen completely seperated while ray cells not only got seperated from each other but also they became completely disintegrated due to the loss of rigidity (Figure 3B). At this stage xylem cells showed characteristic of simultaneous rot i.e. cell-wall thinning, the walls were completely bleached and showed erosion trough across the cell walls. These troughs were irregular in shape and size (Figure 3C). In some instances, the erosion reached the middle lamella completely removing the cell wall in a localized area (Figure 3C). However, cell wall thinning is not much distinct in the axial elements as compared to ray cells (Figure 2F).

5.6 Decay by *Chrysosporium asperatum*:

As seen in case of *Trichoderma harzianum*, *C. asperatum* also showed both selective and simultaneous decay. In the initial stage (60 days) wood blocks inoculated with *C. asperatum* showed selective delignification by defibration due to dissolution of middle lamella whereas erosion troughs and bore holes were also observed occasionally. Fungal hyphae were most abundant within the lumina of vessels, xylem rays and axial parenchyma cells. Similar to former strain, selective delignification by *Chrysosporium* resulted in the degradation of middle lamella which eventually led to seperation of individual cells. Up to 60 days, degradation pattern by *C. asperatum* remained more or less similar to *T. harzianum*. After 90 days of inoculation, considerable variations were observed in the samples investigated. The fibre walls were completely interrupted due to extensive delignification and showed advanced erosion troughs from the lumen surface to middle lamella (Figure 3D). Cell walls were comprehensively degraded and showed larger bore holes and erosion troughs (Figure 3E, F). In longitudinal view, obliquely arranged erosion troughs run parallel with the cellulose microfibrils on the fibre walls, which showed blue staining with astra blue (Figure 4A). Most of the time these troughs were merged to form tunnels of indefinite length and shape. They may be oval, circular, irregular or fusiform shaped.

As compared to wider vessels, narrow vessels present in groups (i.e. radial or diagonal multiples) were not much affected by the fungal invasion, except they were seperated from each other due to crumbling of middle lamella (Figure 4B). On the other hand larger and solitary vessels within the same section showed extensive crumbling of the vessel walls (Figure 4C). These walls were totally degenerated and eventually resulted into complete collapse of the vessel element due to loss of rigidity (Figure 4C). As shown in Figure 4D, in advance stage of decay almost all the fibres were seperated from each other and the walls were crumbled due to lose of rigidity and integrity (Figure 4D). At several places fibre walls were interrupted due to advance erosion troughs (Figure 4D).

Tectona grandis L.f.

5.7 Wood structure:

Secondary xylem of *Tectona grandis* was ring porous with distinct growth rings and generally conspicuous to naked eyes. Sapwood was white or pale yellow while heartwood was light golden brown in fresh and brown to dark brown in dry wood, often with darker streaks. The secondary xylem was composed of vessels, fibres, axial and ray parenchyma cells. Vessels were enclosed in parenchymatous tissue, in early wood they were large, distinctly visible to naked eyes, mostly solitary, oval in outline and partly filled with tyloses. Gradually, vessels became smaller towards the late wood, mostly solitary or in radial pairs and round to oval in outline. Parenchyma formed thin sheath around the vessels, which were distinct in early wood but visible only with hand lens in latewood. Rays were uni-multiseriate with oval to polygonal ray cells.

5.8 Wood colonisation and cell wall degradation:

In the initial stage, both the fungal strains *Trichoderma harzianum* and *Chrysoporium asperatum* showed similar modes of wood colonization and cell wall degradation. Though, *C. asperatum* belongs to Ascomycetes group and was

expected to produce soft rot pattern of wood decay. Initially, seperation of cells from middle lamella was characteristic to it but latter on it showed type 1 pattern of soft rot. There was no appreciable weight loss though the blocks were completely invaded after 30 days of incubation with both the strains (Table 36). Thereafter, weight loss was rapid in the succeeding days and showed 34-38% weight loss after 120 days of inoculation. Initially fungal mycelia completely ramified over the wood blocks within 10-12 days of inoculation. Mycelia of both the strains entered into the wood cells through the vessel lumen and invaded all the cell types of the secondary xylem within 30 days (Figure 5A). From the vessels, hyphae traversed into the neighbouring rays and gradually extend in all direction including xylem fibres and adjacent axial parenchyma cells (Figure 5A, B). No visual damage was observed in the cell walls within 15 days of incubation. Fungal mycelia moved from one cell to the next through the pits present on their walls. Presence of fungal mycelia in all the cell types of xylem adjacent to vessel elements was a common feature in all the samples studied.

5.9 Decay *by Trichoderma harzianum*:

At the end of 30 days, blocks inoculated with *T. harzianum* showed no much appreciable alterations in the cell wall except some of the fibres located at the periphery of the block showed seperation from the middle lamellae. As a result, the secondary wall adjacent to the middle lamellae stained blue with astra blue instead of red by safranine (Figure 5C). This feature was initially observed in the fibres adjacent to the rays, axial parenchyma cells and vessel elements. Thereafter, it gradually invaded all the cell types. In the beginning, mycelial invasion was observed through the vessel lumen. From the vessels, hyphae traversed into the neighbouring rays and gradually extend in all directions including xylem fibres and adjacent axial parenchyma cells. Thus, presence of fungal mycelia in all the cell types of xylem adjacent to vessel elements was a common feature in all the samples studied. At this stage, cell walls of xylem rays and vessels showed no

visible signs of fungal attack but fibres showed the first sign of structural alterations, which were typical of selective delignification i.e. seperation of fibres from the middle lamella were noticed due to its dissolution (Figure 5C). Seperation of xylem cells was more common, which were adjacent to the rays (Figure 5D). At this stage delignification was mostly restricted to the cells located at the periphery of wooden blocks.

At the end of 60 days, blocks inoculated with *T. harzianum* were relatively more delignified than the blocks observed after 30 days. At this stage, delignification was scattered uniformly throughout the wooden blocks. In transverse view, many of the fibres showed concentric delignification starting from middle lamellae towards lumen. As a result, the secondary wall adjacent to the middle lamellae stained blue with astra blue instead of red by safranine (Figure 5E). This feature was initially observed in the fibres and axial parenchyma cells adjacent to the rays. Thereafter, it gradually invaded all the cell types.

At this stage, effect of fungal invasion was more pronounced in xylem rays (Figure 5F). Compared to vessels, rays became more vulnerable to fungal attack. In ray cells, degradation commenced in the corner of the ray cells, along the middle lamellae without any pronounced effect on the primary and secondary wall layers (Figure 5F). Cell corners became discolored due to absence of lignin and stained blue with astra blue. Gradually, ray cells became seperate from each other due to the dissolution of middle lamella (Figure 6A) and the cell walls collapse ultimately (Figure 6B).

Samples exposed to fungi for 90 days, vessel elements also start seperating without any significant effect on the cell wall (Figure 6C). All the cell walls showed pronounced effect on delignification of wall material of all the cell types. Seperation of fibre walls was a common feature and it was observed in most of the sections (Figure 6D). At this stage, xylem cells showed characteristic of

simultaneous rot i.e. cell-wall thinning, the walls were completely bleached and showed erosion trough across the cell walls (Figure 6E). These troughs were irregular in shape and size. In some instances, the erosion reached the middle lamella completely removing the cell wall in a localized area (Figure 6D, E). As the degradation progressed further, pits of the ray and axial parenchyma cells became more pronounced and larger in size and irregular in shape. At the same time, formation of several bore holes on the lateral walls of the rays was a common feature (Figure 6F).

Decay fungi	% Weight loss (60 days)	% Weight loss (90 days)	% Weight loss (120 days)
Trichoderma harzianum	18.65 (± 5.72)	31.18 (± 7.08)	38. 93 (± 5.76)
Chrysosporium asperatum	15.28 (± 7.43)	24.10 (± 5.62)	34. 37 (± 4.64)

Table 36: Average percent weight loss during each incubation period (60, 90 and 120 days).

Wood blocks exposed to fungi for 120 days showed more pronounced effect of delignification on all the cell types of xylem. Xylem fibres were completely bleached and not only stained blue colored with astra blue due to loss of lignin but also showed complete disintegration of cells due to loss of rigidity (Figure 7A, B). With the advancement of decay, not only the xylem fibres were deformed and lost their rigidity but also many of the ray cells were either partially or completely disintegrated (Figure 7C).

5.10 Decay by *C. asperatum*:

Like former fungal species, there was no appreciable weight loss of wood block by *C. asperatum* after 30 days of its inoculation. However, fungal mycelia invaded all the cell types of the secondary xylem. Fungal mycelia invaded the wood block and completely covered the block before completion of 30 days. In the initial stage

mycelial invasion was seen through the vessel lumen and hyphae were most abundant within the vessels lumina, xylem rays and fibres. From the vessels lumen, hyphae entered into the neighbouring rays and gradually spread in all directions including xylem fibres and adjacent axial parenchyma cells. At this stage, no visual damage was observed in the cell walls. Fungal mycelia moved from one cell to the next through the pits present on their walls. Presence of fungal mycelia in all the cell types of xylem adjacent to vessel elements was a common feature in all the samples studied. Xylem rays although showed presence of mycelia but no appreciable alterations were observed in most of the sections. However, initiation of cell wall seperations at the cell junctions was observed occasionally in some of the sections (Figure 7D, E).

After 60 days of inoculation, the degradation pattern of *C. asperatum* resembles selective delignification i.e. at an early stage of decay, degradation commenced in the fibre walls, along the middle lamellae without any pronounced effect on the primary and secondary wall layers. Xylem fibres began to seperate from each other by dissolution of middle lamella (Figure 7F). Similar to previous fungal species, this feature was initially observed in the fibres and axial parenchyma cells adjacent to the rays. Sections stained with safranine and astra blue showed that the discolored secondary wall was delignified and stained blue due to absence of lignin while portion of the cell wall with cellulose was stained blue in color (Figure 7F). As the decay progressed further, localized degradation of lignin, hemicellulose and cellulose resulted in the formation of small cavities running parallel with the cellulose microfibrils of the secondary wall of fibres (Figure 8A, B). Ray cells also showed erosion tunnels and bore holes (Figure 8C).

After 90 days, xylem fibres also showed formation of cavities, due to the fungal activity and formation of distinct erosion troughs across the cell walls (Figure 8D). These troughs were irregular in shape and size. In some instances, the erosion

reached the middle lamella completely removing the cell wall in a localized area (Figure 8D). At this stage, most of the cells in the inoculated wood block showed loss of cell walls rigidity followed by collapse of the walls.

Wood blocks exposed to fungi for 120 days showed more pronounced effect of delignification on all the cell types of xylem. Xylem fibres were completely bleached and not only stained blue coloured with astra blue due to loss of lignin but also showed complete disintegration of cells due to loss of rigidity (Figure 8E, F). Cells with little lignin content showed presence of distinct fungal mycelia (8E) while completely bleached xylem cells showed remnants of fungal filaments as black discrete bodies (Figure 8F). In advance stage of decay, erosion troughs also became indistinct due to loss of rigidity and collapse of the cell wall (Figure 8E, F).

DISCUSSION

Lignin is a highly branched polymer of phenylpropanoid compounds, and is an important component of secondary wall of the plant (particularly secondary xylem). After cellulose, lignin is the second most abundant organic compound in plants, representing approximately 30% of the organic carbon in the biosphere (Boerjan *et al.*, 2003). The functional significance of lignin is associated mainly with the mechanical support allowing plants to stand erect and as a defense against pests and microorganisms (Wainhouse *et al.*, 1990; Boudet, 2000; Lagaert *et al.*, 2009). Despite the importance of lignin in plant as a defense against microorganism, it is most often observed that secondary xylem is invaded by the microbes such as bacteria and fungi. Now a day, white rot fungi are of particular interest because they are one of the few groups of microorganisms that can selectively degrade lignin without appreciable losses of cellulose (Otjen and Blanchette, 1985). Thus, white rot Basidiomycetes are not only extremely attractive for use in biological pulping processes but are also equally imperative in bioremediation of textile dyes, poly aromatic hydrocarbons and xenobiotic compounds.

On the basis of decay pattern, three general types of decay are recognized (Liese, 1970; Nilsson, 1988; Blanchette, 1991; Zabel and Morrell, 1992; Eaton and Hale, 1993). In white rot, all cell wall components (i.e. lignin, cellulose and hemicellulose) are either degraded simultaneously (Blanchette and Reid, 1986) or they may be degraded preferentially, especially in the early stages (Otjen and Blanchette, 1985). There are reports that some white rot fungi are capable to cause both types of decay in the same wood or in different wood species (Blanchette, 1984a, b, 1991). In the present investigation, *Trichoderma harzianum* showed selective delignification pattern and the most remarkable effect is defibration by dissolution of the middle lamella in both *Azadirachta* and *Tectona*. Formation of erosion trough on the cell wall from the lumen surface is a prominent anatomical feature in both the samples exposed to fungi for 120 days. On the contrary, In *Azadirachta* wood samples *Chrysosporium asperatum* showed both forms of delignification characteristic to white rot since beginning of its inoculation. On the other hand, in *Tectona grandis* (teak), pattern of delignification differed from the previous species. In teak, *Chrysosporium asperatum*, though belongs to Ascomycetes it shares the characteristics of both white rot and soft rot type of wood decay. Like *Azadirachta*, initially *C. asperatum* showed selective delignification by seperation of individual xylem elements due to dissolution of middle lamella and later on it showed formation cavities characteristic to soft rot, particularly type-1. Soft rot is characterized by the formation of cavities ("L" bending or "T" branching and hyphal tunneling) around hyphae growing in the secondary cell walls of wood (Nilsson 1974; Blanchette 2000). Formation of these cavities is further classified into two types. In type 1, cavities are formed in the secondary wall whereas in type 2, progressive erosion of secondary walls occur but middle lamella is not degraded in contrast to cell wall erosion by white rot fungi but may be modified in advanced stages (Nilsson *et al.*, 1989; Blanchette, 2000). In addition to cavity formation within the cell wall, development of discrete notches

of cell wall erosion by hyphae lying within the lamina is also observed frequently in wood degraded by soft rot fungi (Schwarze and Fink, 1998).

Available literature indicates that both the forms of white rot may be caused by one fungus in different portions of the same wood or in different wood pieces (Blanchette, 1984a, b, 1991). In the present study, light microscopy showed that *Chrysosporium asperatum* and *Trichoderma harzianum* caused different patterns of decay in *Azadirachta* and *Tectona*. This may be evidenced by the presence of distinctive anatomical features and by the staining technique. Initially, both the strains produced a selective delignification of the tissue, manifested by cell seperation. Seperation of xylem cells owing to the dissolution of middle lamella is considered to be the best indicator of the selective type of decay (Anagnost, 1998; Luna *et al.*, 2004). The staining technique contributed also to seperate the selective delignification from the simultaneous decay, as proposed by Srebotnik and Messner (1994). Delignified tissue of *Azadirachta* wood stained blue with astra blue due to absence of lignin while portion of relatively unaffected cell wall stained red with safranine. Our earlier study also demonstrated similar feature to distinguish the delignified xylem cells in *Ailanthus* (Koyani *et al.*, 2010).

In the advanced stage of decay, other signs of degradation such as formation of bore holes in the fibres of *Azadirachta* and erosion channels in both *Tectona* as well as *Azadirachta* were also detected on the cell wall of xylem fibres and ray cells. Formation of bore holes, erosion troughs and erosion channels are considered to be the characteristic features of simultaneous rot (Liese, 1970; Rayner and Boddy, 1988; Eriksson *et al.*, 1990; Schwarz and Fink, 1998). Our results are in agreement with the earlier reports on selective delignification and simultaneous rot produced by both the fungal strain (Otjen and Blanchette, 1985; Anagnost, 1998; Luna *et al.*, 2004; Koyani *et al.*, 2010). Wood samples inoculated with *Trichoderma harzianum* initially showed selective delignification but later on it showed formation of erosion troughs in samples after 120 days of incubation period. On the other hand, *Chrysosporium asperatum* showed such erosion

troughs within 90 days after the inoculation. Cell wall thinning is another important feature that was observed in the present study. However, it was more conspicuous in xylem rays of both teak and neem as compared to fibres and vessel elements.

Degradation ability of the secondary xylem is considered to be associated with the lignin composition of individual cell type. The libriform fibres and xylem ray parenchyma reported to have relatively high syringyl monomer content (Nakano and Meshitsuka, 1978; Iiyama and Pant, 1998) and it shows peak uv-absorbance at short wave length (Fergus and Goring, 1970a, b). In contrast, fibre-tracheids appear to have high guaiacyl monomer content and show their peak UV-absorbance at longer wave length. Compared to xylem fibres, vessels are found to be more resistant to decay caused by both the strains of fungus in the wood blocks of both the timber species tested. Among the vessels also, narrow vessels were relatively more resistant to fungal attack as compared to wider ones. Wood samples inoculated with both fungi showed that there was no appreciable change in the cell wall of narrow vessels except their seperation. On the contrary, in *Azadirachta*, wider vessels collapsed completely and became deformed in the samples inoculated with both the strains. In *Tectona grandis*, no such collapse of the vessel elements was observed. In hardwoods, vessel walls are considered to be resistant to degradation by white rot Basidiomycetes as has been described in details (Blanchette *et al.*, 1987, 1988). Similar observations are also reported in our earlier study on naturally infected *Ailanthus excelsa* by *Inonotus hispidus*. The persistence of lignin rich vessel elements in *Azadirachta* wood inoculated with *Trichoderma harzianum* and *Chrysosporium asperatum* may be due to high percentage of guaiacyl monomer content as reported in earlier investigations (Blanchette *et al.*, 1987, 1988; Schwarze *et al.*, 2000; Koyani *et al.*, 2010).

Figure 1: Transverse (A–D & F) and tangential longitudinal (E) view of secondary xylem of *Azadirachta indica* showing features of decay by *Trichoderma harzianum.*

A: Fungal hyphae (arrow) passing through vessel lumen, vessel associated parenchyma and ray cells (arrow). Note that all cell types of secondary xylem showing fungal invasion.

B: Fungal hyphae passing through the xylem ray (arrowheads).

C: Invasion of fungal mycelia into axial parenchyma cells (arrowhead).

D: Enlarged view of axial parenchyma cells showing fungal mycelia cut in transverse view (arrowhead).

E: Movement of fungal mycelium from one of the ray cell into neighbouring cell through the pit present on the lateral wall (arrowhead)

F: Initiation of degradation at the cell corners and along the middle lamella of fibre walls (arrowheads). Note the seperation of fibres from the middle lamella (arrow).

Figure 1 (A, C): Scale bar = 75 μm, B, D, E, F: Scale bar = 50 μm

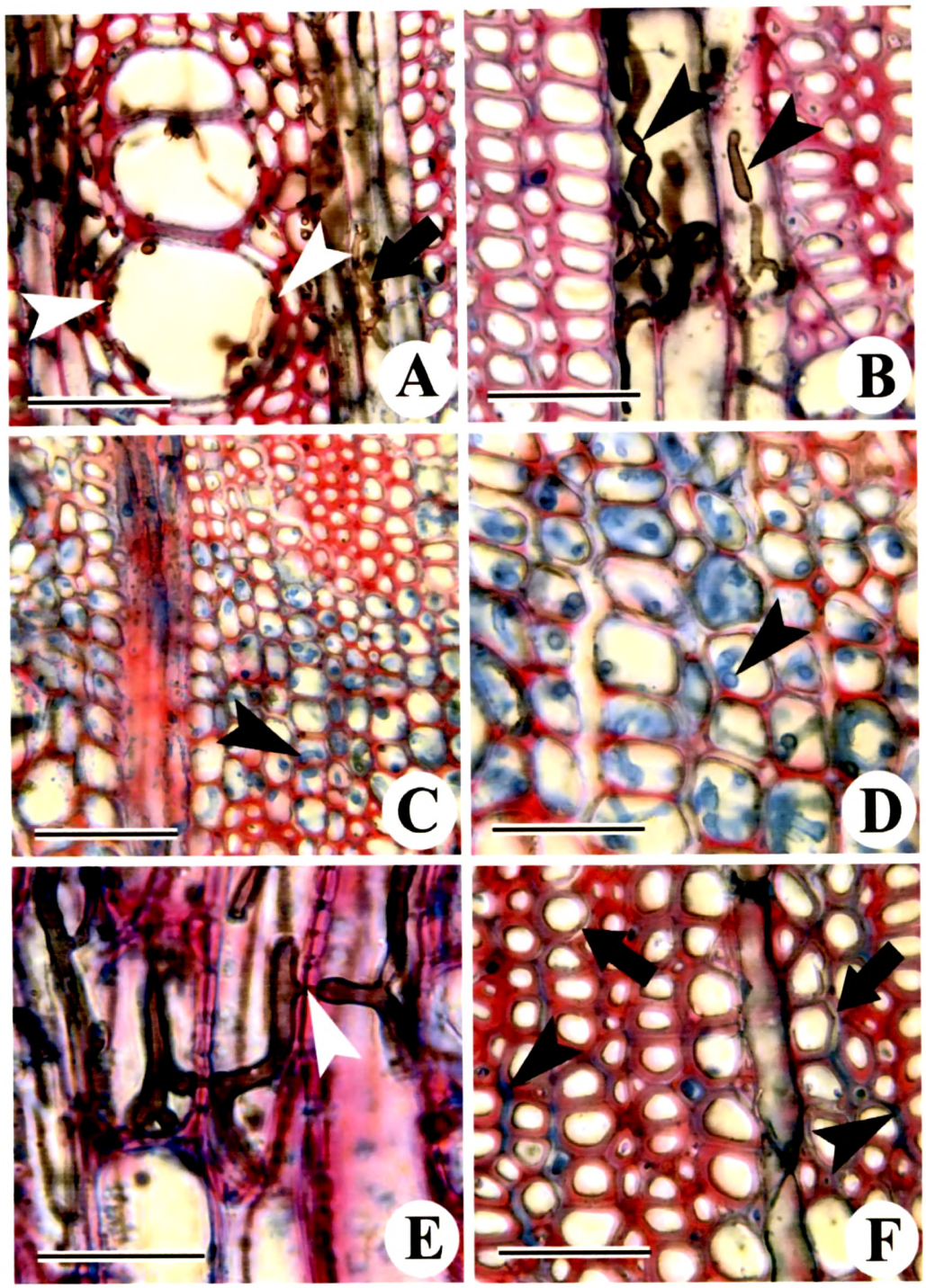

Figure 1

Figure 2: Transverse (A–E) and tangential longitudinal (F) view of secondary xylem of *Azadirachta indica* showing features of decay by *Trichoderma harzianum*.

A: Degradation of middle lamella resulted in the seperation of xylem fibres from each other (arrowheads).

B: Completely seperated xylem fibres (arrowheads) in advanced stage. Note the distinct seperating lines passing through the xylem fibres.

C: Xylem rays showing larger bore holes (dotted structure) on the walls. Note the fungal mycelium passing through one of the bore hole (arrowhead). Arrowhead indicates initiation of degradation at the cell corners and along the middle lamella of fibre walls (arrow).

D: Ray cell wall damaged by fungal attack. Note the eroded wall showing erosion troughs (arrowheads). Arrows indicate completely seperated fibres.

E: Ray cell wall showing large erosion holes on the wall (arrowheads).

F: Complete collapse of ray cells in advance stage of decay (arrows).

Figure 2 (A–F): Scale bar = 50 μm

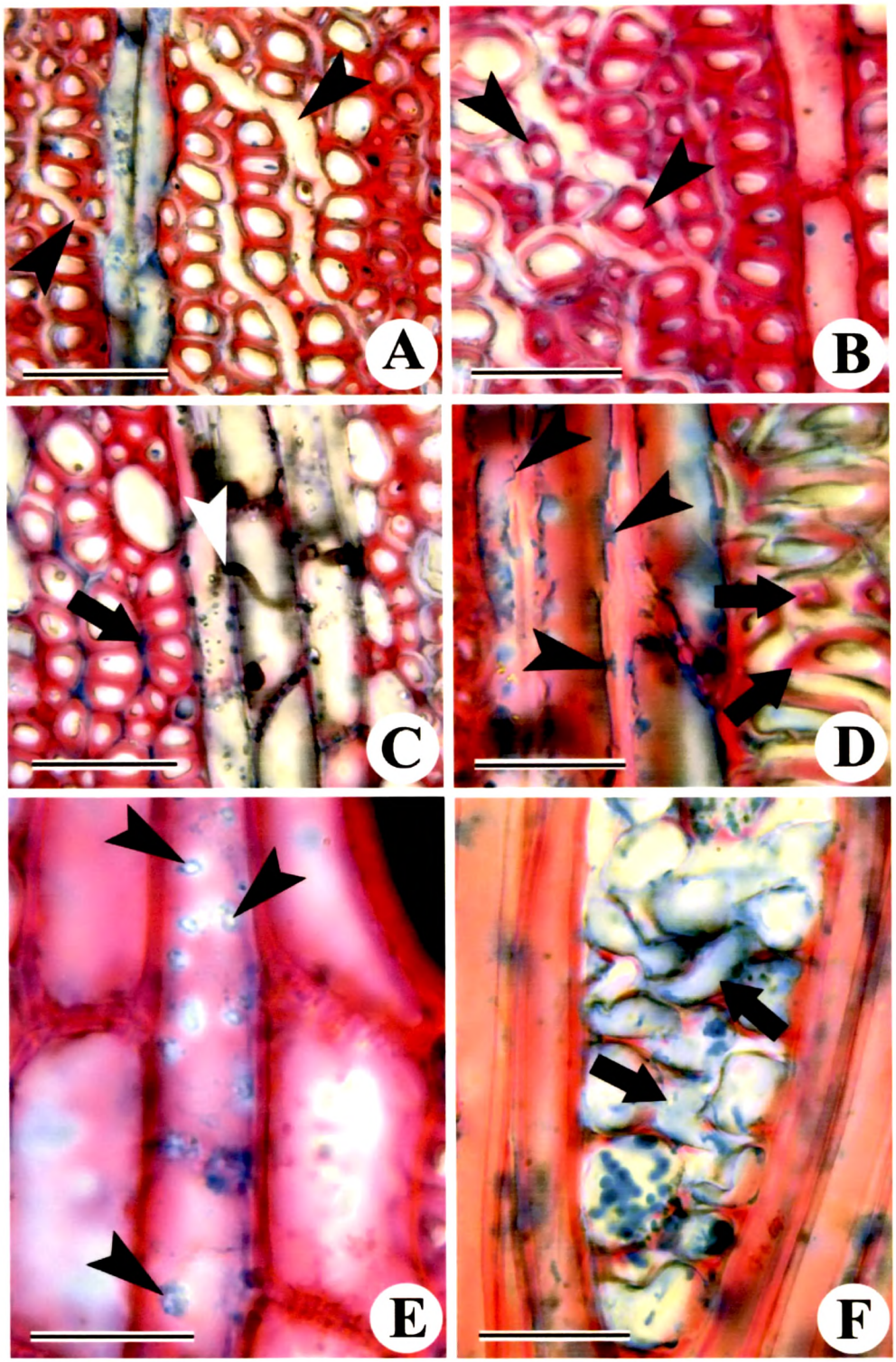

Figure 2

Figure 3: Transverse (A, C–E) and tangential longitudinal (B) view of secondary xylem of *Azadirachta indica* showing features of decay by *Trichoderma harzianum* (A–C) and *Chrysosporium asperatum* (D–F).

A: Phase contrast micrograph showing deformed vessel wall (arrowhead) whereas arrow indicates fungal mycelia in the rays, vessel and vessel associated cells.

B: Completely seperated xylem fibres (arrowhead). Arrows indicates fully disintegrated ray cells.

C: Fibres showing erosion trough across the cell walls (arrowheads). Note that erosion troughs are irregular in shape and sized and continue across the lumen to middle lamella.

D: Completely seperation of xylem fibres showing interrupted walls with erosion troughs (arrowheads).

E: Fibre wall showing erosion trough (arrowhead). Note the distorted fibre wall showing loss of integrity and rigidity (arrows).

F: Fibre walls showing erosion trough (arrowheads).

Figure 3 (A): Scale bar = 100 μm; B–F = 50 μm

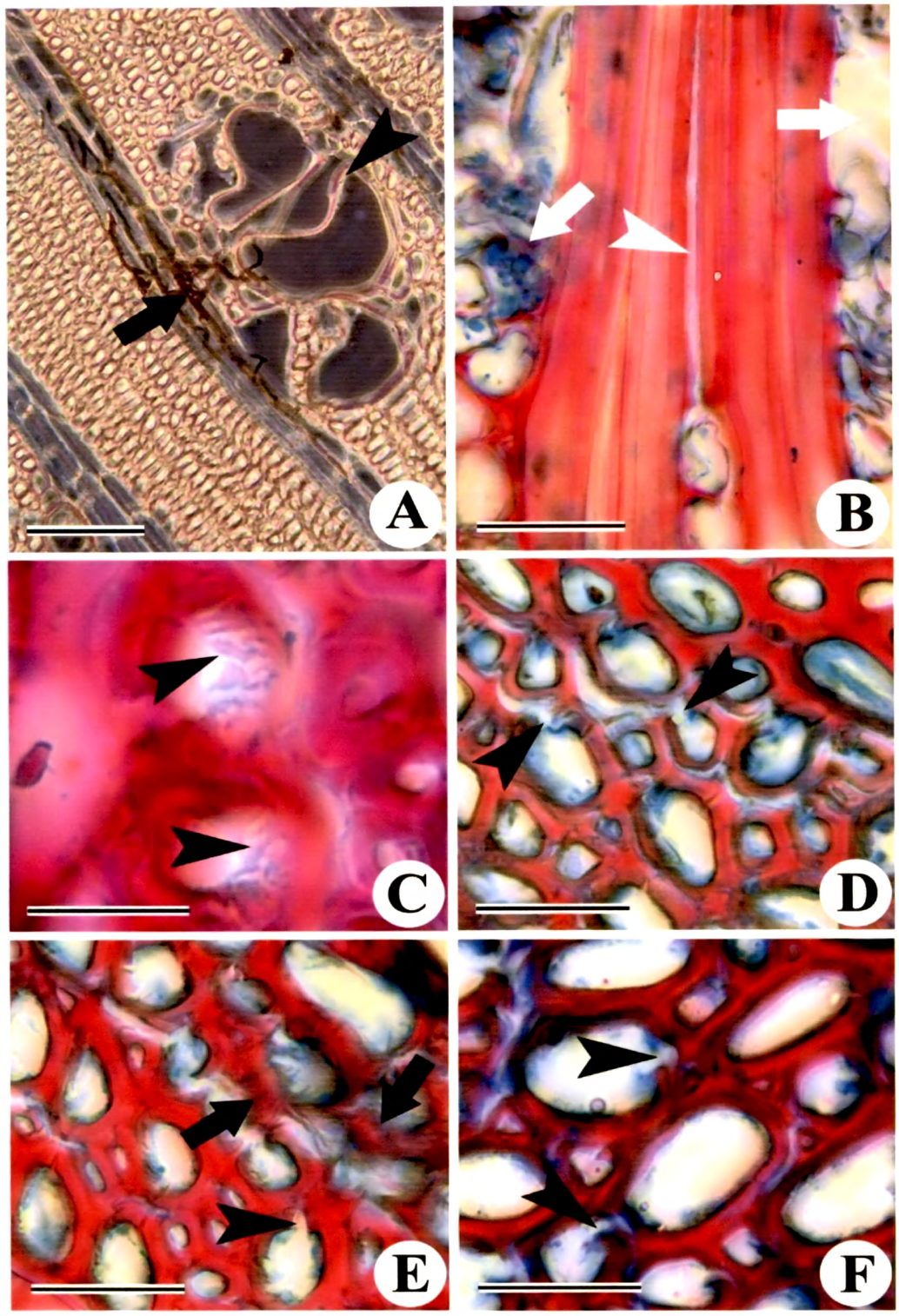

Figure 3

Figure 4: Tangential longitudinal (A) and transverse (B–D) view of secondary xylem of *Azadirachta indica* showing features of decay by *Chrysosporium asperatum.*

A: Fibre wall showing obliquely arranged erosion trough with varying dimensions (arrowheads).

B: Dissolution of middle lamella and seperation of small vessels (arrowheads) while wall remains intact.

C: Deformed vessel element showing loss of rigidity and complete collapse of vessel wall (arrow). Note the other side of the vessel element (arrowhead).

D: Advance stage of decay showing loss of cell wall rigidity and interrupted xylem fibres. Arrowhead indicates breaking of the fibre wall at erosion troughs.

Figure 4 (A–D): Scale bar = 50 μm

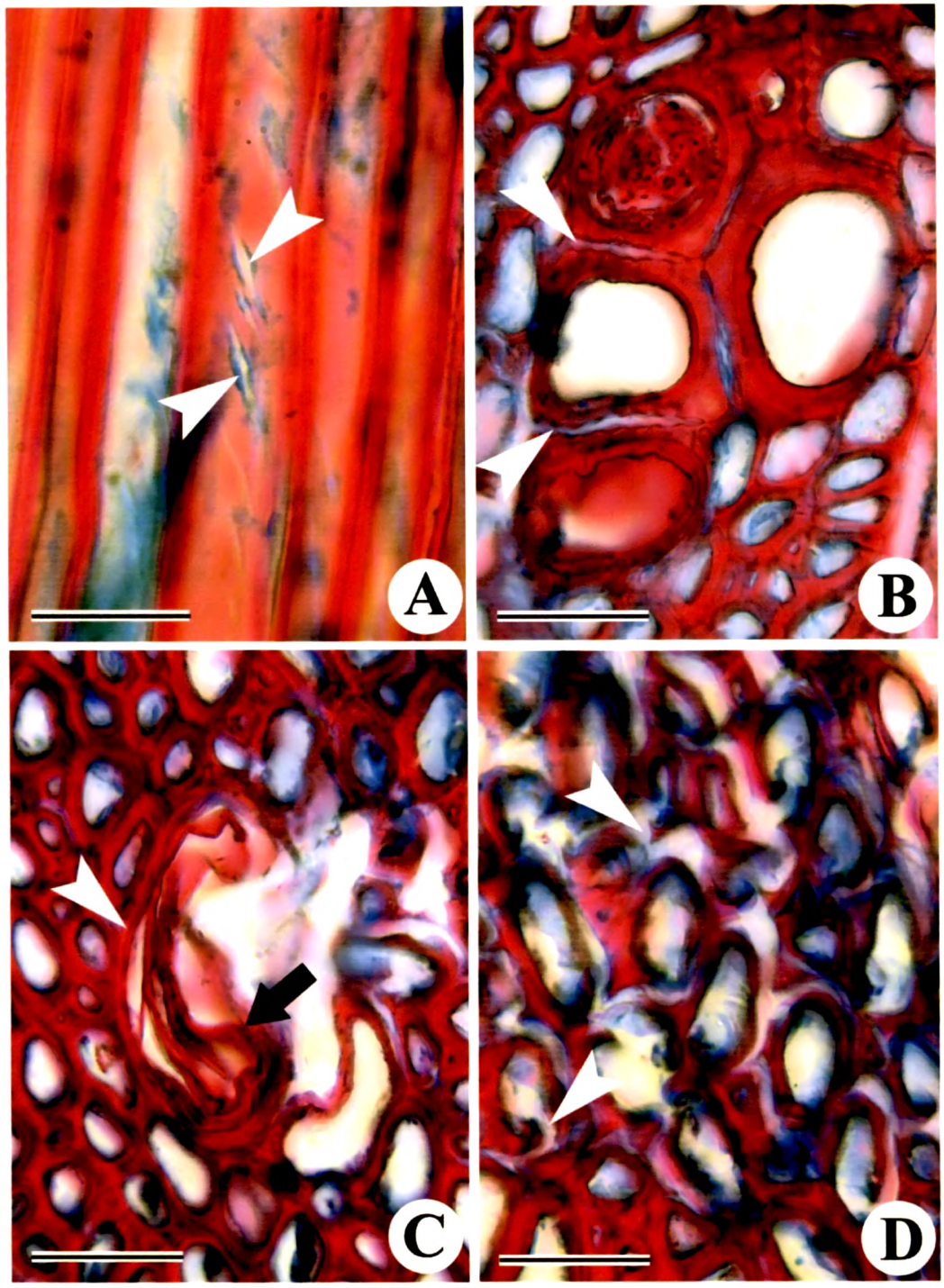

Figure 4

Figure 5: Transverse (A–E) and tangential longitudinal (F) view of secondary xylem of *Tectona grandis* showing features of decay by *Trichoderma harzianum.*

A: Invasion of fungal mycelia showing invasion of ray cells through the vessel lumen and vessel associated axial parenchyma of the secondary xylem. Arrow indicates fungal mycelia in the vessel associated parenchyma while arrowhead showing fungal filaments in the ray cells.

B: Movement of fungal mycelium from one of the ray cell into neighbouring (arrowhead). Arrowhead indicates fungal mycelium cut in transverse view.

C: Initiation of seperation of cell walls along the middle lamella of fibre walls (arrowheads).

D: Seperation of cell walls along the middle lamella of fibre walls (arrowheads). Note the seperation of fibres adjacent to the rays.

E: Completely seperated xylem fibres (arrowhead). Arrows indicates fungal mycelium.

F: Initiation of degradation at the cell corners along the middle lamella of ray cells (arrowheads).

Figure 5 (A): Scale bar = 75 μm; B – D: Scale bar = 50 μm

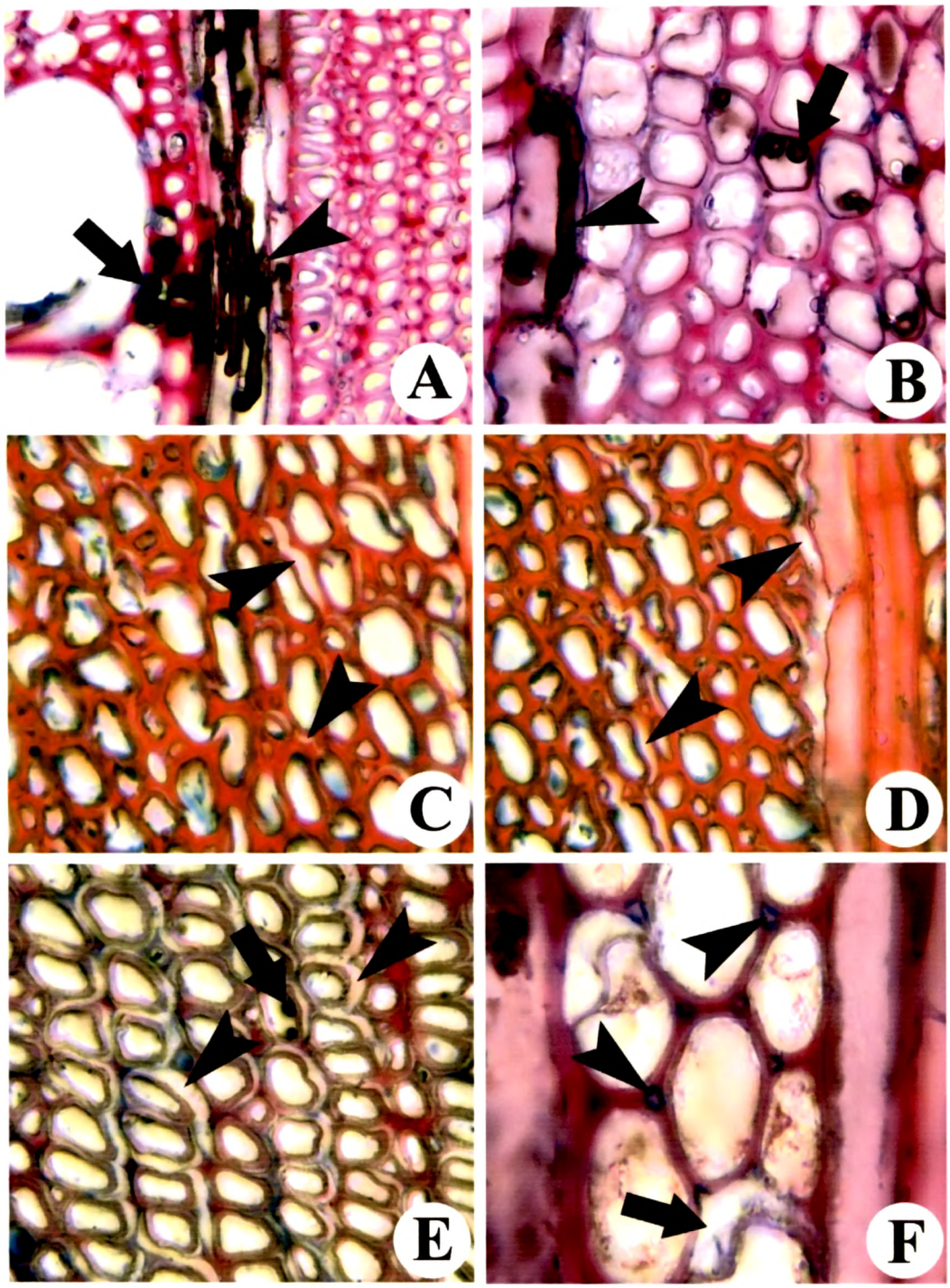

Figure 5

Figure 6: Radial (A), tangential longitudinal and transverse view of secondary xylem of *Tectona grandis* showing features of decay by *Trichoderma harzianum.*

A: Seperation of early wood ray cells (arrowheads) from the middle lamella. Note the latewood ray cells at right corner of the photograph are intact and showing no visible symptoms of degradation.

B: Breakdown of ray cells in relatively advance stage of decay (arrowheads).

C: Dissolution of middle lamella and seperation of vessels elements (arrowheads). Note that the secondary wall of the vessel element remains intact.

D: Seperation of fibre walls and formation of erosion troughs in most of the xylem fibres (arrowhead). Arrow indicates complete breakdown and collapse of cell wall.

E: Enlarged view of Figure 6D showing erosion troughs across the cell wall (arrowhead).

F: Formation of several bore holes on the lateral walls of the ray cells (arrowheads) after 90 days of fungal inoculation.

Figure 6 (A–D): Scale bar = 50 μm; E – F: Scale bar = 45 μm

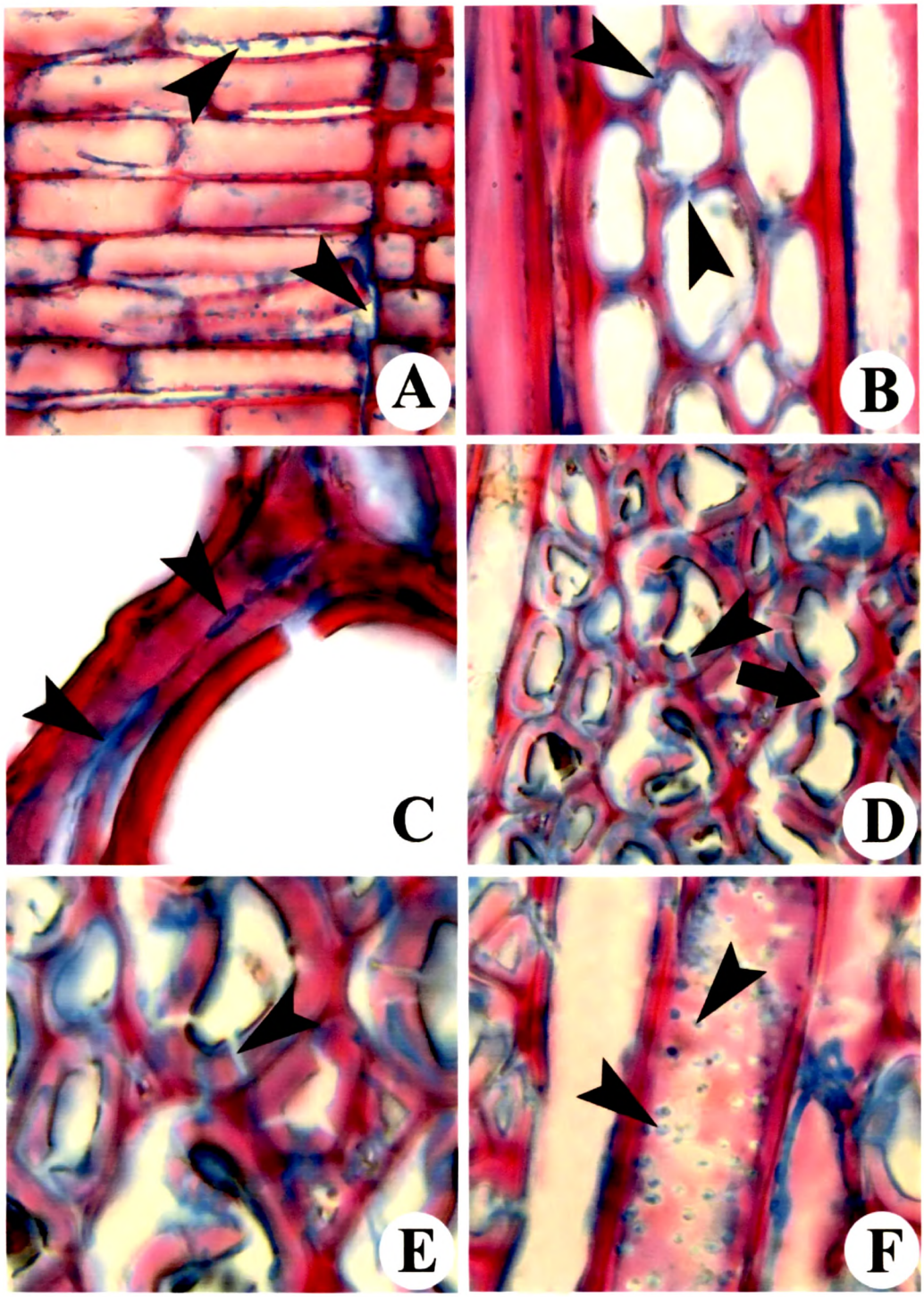

Figure 6

Figure 7: Transverse (A–D) and tangential (E) and radial longitudinal (F) view of secondary xylem of *Tectona grandis* showing features of decay by *Trichoderma harzianum* (A–C) and *Chrysosporium asperatum* (F).

A: Collapse of the cell wall after 120 days of fungal inoculation. Note the seperation and breakdown of the cell walls of the xylem fibres.

B: Advance stage of decay showing loss of cell wall rigidity and interrupted xylem fibres. Note the collapse of the cell walls. Note the distribution of lignin remains restricted to very little part of the cell wall.

C: Complete collapse of ray cells and neighbouring axial elements in advance stage of decay.

D: Seperation of xylem rays in the early part of the fungal invasion (arrowhead).

E: Initiation of degradation at the cell corners along the middle lamella of ray cells (arrowheads).

F: Seperation of cell walls along the middle lamella of fibre walls (arrowheads) adjacent to the rays.

Figure 7 (A–D, F): Scale bar = 50 µm; E: Scale bar = 45 µm

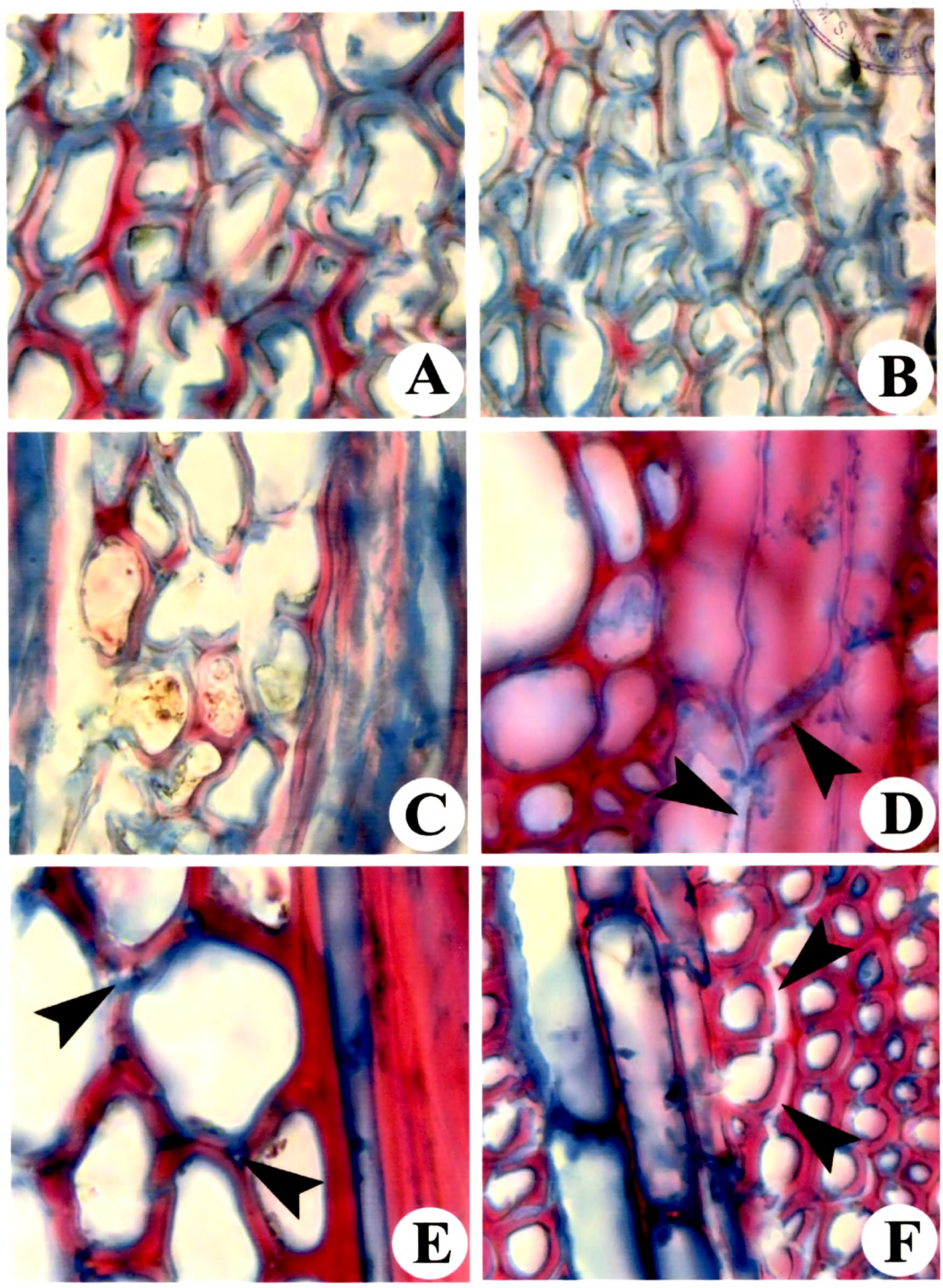

Figure 7

Figure 8: Tangential longitudinal (A–B) and transverse (C–F) view of secondary xylem of *Tectona grandis* showing features of decay by *Chrysosporium asperatum.*

A: Xylem fibres showing erosion troughs oriented parallel to the microfibril angle of the xylem fibre cell wall (arrowhead).

B: Axial parenchyma showing erosion troughs oriented parallel to the microfibril angle of the xylem fibre cell wall (arrowhead).

C: Movement of fungal mycelium from one of the ray cell into neighbouring cell through the pit present on the lateral wall and formation of bore holes on ray cell wall (arrowhead). Arrow indicates fungal hypha.

D: Formation of erosion troughs in most of the xylem fibres showing complete breakdown and collapse of cell wall (arrowhead).

E: Collapse of the fibre walls after 120 days of fungal inoculation. Note the seperation and breakdown of the cell walls of the xylem fibres. Arrowhead indicates erosion troughs while arrow indicates fungal hyphae.

F: Advance stage of decay showing loss of cell wall rigidity and interrupted xylem fibres. Note the collapse of the cell walls. Note the distribution of lignin remains restricted to very little part of the cell wall.

Figure 8 (A–F): Scale bar = 50 μm

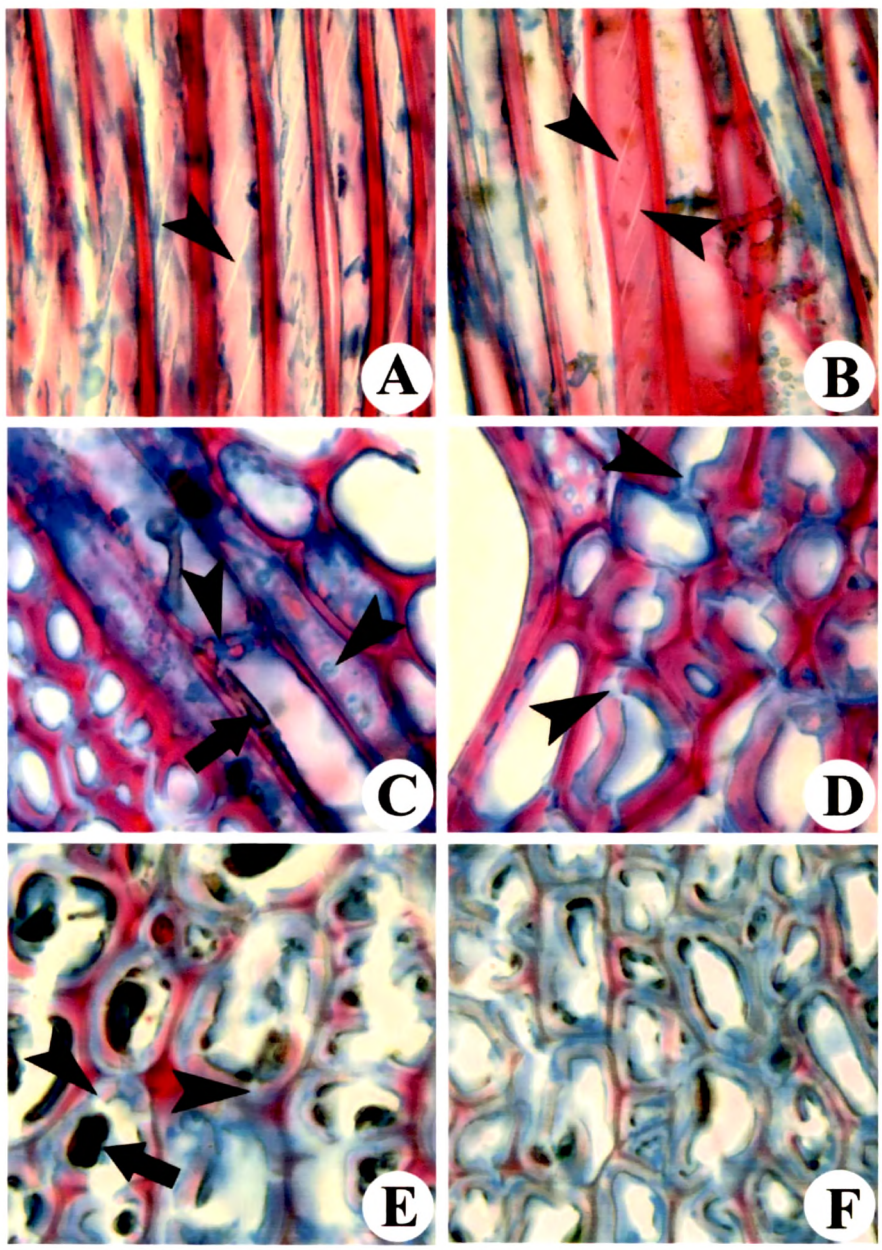

Figure 8.