

## THE ANATOMY OF BEECH WOOD IN ADVANCED STAGES OF DECAY BY WHITE-ROT FUNGUS *PLEUROTUS OSTREATUS*

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### ABSTRACT

Fruit bodies of *Pleurotus ostreatus* are widely used in food and pharmaceutical industry. Knowledge of its decomposition effects on beech is great importance for the cultivation of mushroom to culinary purposes. Therefore, the aim of this work was to investigate changes in beech wood anatomy after one year of grow the fungus *P. ostreatus* in natural environmental condition. Potential of its growth can affect other colonies of fungi that enter into interactions with *P. ostreatus*. The analyzed beech logs contained areas with exclusive colonization of *P. ostreatus* that were completely decomposed. However, there were also zones with belated wood degradation and with occurrence of the parallel colonization of soft-rot fungi. Differences in the anatomy and histochemical investigations of the individual affected zones are shown and discussed in the paper.

**Key words:** *Pleurotus ostreatus*, *Fagus sylvatica*, Wood anatomy, Mass loss, Light microscopy.

### INTRODUCTION

The hardwood species are often attacked by a wide-range of parasitic and saprophytic white-rot fungi. Some of them immediately start simultaneous erosion of cellulose, lignin, and hemicelluloses (REINPRECHT 2016, SCHMIDT 2006), others prefer selective delignification and consumption of hemicelluloses, whereas cellulose decompose only in the advanced stages of wood decay (KUBICEK 2013).

Simultaneous erosion process progresses from lumen towards S3 and S2 cell-walls layers up to middle lamella. Longitudinal erosion channels often following the pattern of the hyphae growing on the lumen surface. In transverse sections, advanced thinning results in the localized remove of the cell walls. Only portions of the middle lamellae can be remained. Hyphae also pass through the cells via pit apertures and often produce the transverse bore holes in cell walls (ANAGNOST 1998, BLANCHETTE 1991, BLANCHETTE *et al.* 1987).

On the other hand, cell separation is probably the best indicator of selective delignification (ANAGNOST 1998, WORRALL *et al.* 1997), where distinguishing between simultaneous decay can be made by appropriate staining technique (SREBOTNIK and MESSNER 1994).

Simultaneously, the relative rates of decomposition of lignin and cellulose vary greatly according to the species of fungi and the conditions within the wood. Furthermore, there is additional variation related to the preferential decay of different zones within the annual ring (SCHWARZE 2007). Also, the type, concentration and localization of lignin in woody tissues

affects the strategy and rate of decomposition of white-rot fungi. In general, vessels have higher concentrations of guaiacyl lignin, whereas fibres, tracheids and parenchyma cells have low-moderate concentrations of syringyl lignin (BAUM 2001, SCHWARZE 2007).

*P. ostreatus* (basidiomycetes) is white-rot saprophytic fungus but it was also found in living trees as a wounding parasite (BARI *et al.* 2015a). *P. ostreatus* grows from spring until winter. Cultivated oyster mushroom can grow at moderate temperatures, ranging from 18 to 30°C (BELLETTINI *et al.* 2016). For most fungi, the wide humidity range is 20–70% (BELLETTINI *et al.* 2016, PANDEY *et al.* 2001). However, range between 60–75% and 85–97% enable a satisfactory growth of *Pleurotus spp* (BELLETTINI *et al.* 2016, CHANG and MILES 2004, LI *et al.* 2015).

*P. ostreatus* is widely cultivated as a edible fungus for culinary uses. Because of the presence of numerous nutritional compositions and various active ingredients have been reported to have antidiabetic, antibacterial, anticholesterolic, antiarthritic, antioxidant, anticancer, eye health and antiviral activities (DEEPALAKSHMI and MIRUNALINI 2014).

Many of recently and past papers dealt with research of anatomy a strategy of attack and decomposition of beech wood by *P. ostreatus* in vitro (BARI *et al.* 2015a, b, c, d, BARI *et al.* 2016). However, information about the behaviour of this fungus in vivo is largely lacking (BARI *et al.* 2017). Furthermore, the problems have been investigated only from view of early and semi-advanced stages of wood decay (max. 30, 60 and 120 days).

Therefore, the aim of this work was to investigate changes in beech wood anatomy after one years of grow the fungus *P. ostreatus* in natural environmental condition and cultivated for culinary purposes.

## MATERIAL AND METHODS

### Inoculation of fungus *P. ostreatus*

3 fresh logs of diameter 36 cm and length of 80 cm were included longitudinally into the soil up to approx. ½ of their diameters. 12 notches in depth approx. 10 cm were made by chain saw on surface of logs. (Fig. 1 A, B, C). The *P. ostreatus* spore substrate (Jánoš, Jablonka, Slovakia) was inoculated into the prepared holes that were subsequently closed with melted paraffin. Inoculated logs were wetted and covered with a dark foil. The spraying by watter was performing regularly in 7 day intervals during the growth of the fruiting body from mid March to mid November. Samples for microscopic observations were taken in March 2016, accurately one years after substrate inoculation in mid-March 2015.

### Mass loss sample preparation

3 fresh disc and 3 disc in advanced stage of decay were cut from the centre of logs. Discs were dried naturally up to stabilization of their masses (approx. 4% equilibrium moisture content). Wood samples for mass loss determination (15 × 15 × 15 mm) were prepared from the disks. 9 samples were taken from each disc, yielding total of 54 samples. Soft and brittle decayed samples were gently cut with razor blade according to cutting scheme (Fig. 1D). 6 samples from each disc were cut from marginal part, 3 samples from central part. However, mass loss was not determined on the same standardized vitro samples according to (EN 113 1996), but on different samples obtained from the discs before and after decaying process. Because of volumes were not changed, density of wood samples was served as a conversion criterion for determining mass losses that were calculated according to formula:

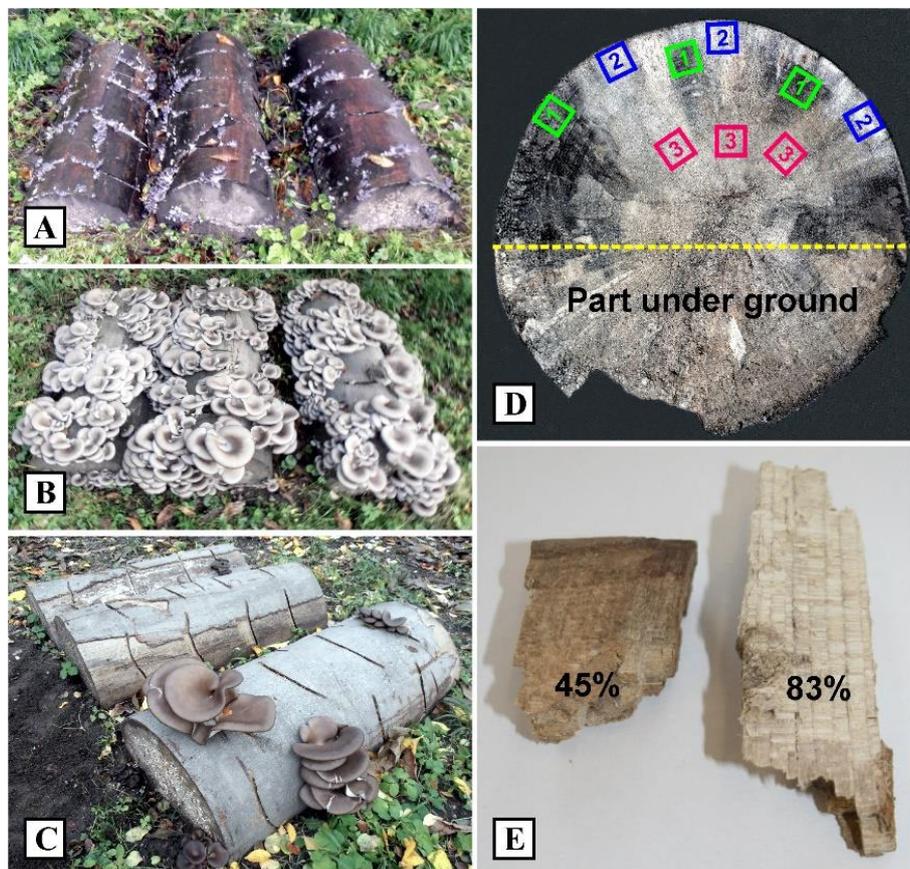
$$\text{Mass loss} = 100(\rho_1 - \rho_2) / \rho_1 \quad [\%] \quad (1)$$

where  $\rho_1$  is the initial density and  $\rho_2$  is the density after the fungal exposure.

### Micropreparation for light microscopy

At first, wooden grafts for light microscopy were separated from samples having defined mass losses (Fig. 1E). 3 specimens ( $5 \times 5 \times 5$  mm) from each graft were prepared by razor blade.

The micropreparation was made according to adjusted methods (BARBOSA *et al.* 2010, RAPP and BEHRMANN 1998), using embedding media polyethylene glycol PEG 1500 (Sigma-Aldrich, St. Louis, United States). The difference from the original methodology was to shorten the penetration time of the PEG 1500 embedding to 12 hours at  $60^\circ$  C and using a reinforcing layer of transparent polish nail gel Essence (Cosnova GmbH, Sulzbach, Deutschland). After penetration of embedding media, samples were oriented in right anatomical positions and stabilized into PEG blocks. Trimming of sample blocks to a flat surface were made by sledge microtome (Reichert, Wien, Austria). Furthermore, reinforcing gel layer was applied on trimmed surface and dried approx 5-7 min. After sectioning, the microsection ( $15\text{--}20\mu\text{m}$ ) was glued on the slide by Mayer's albumin adhesive (Fisher Scientific Company, LLC, Middletown, United States).



**Fig. 1** Fruit body growth of *P. ostreatus* (A-C) and preparation methodology of wooden samples after one year of degradation (D-E). Growth stadium (A) 2 month after inoculation (in mid-May); (B) 4 month after inoculation (in mid-July); (C) 6.5 month after inoculation (in end-September). (D) The scheme of samples preparation. 1- samples from brownish marginal parts; 2- samples from white-colored marginal parts; 3- samples from white-colored central parts. (E) Wooden grafts for preparation of light microscopy samples.

Removal of the gel layer and PEG 1500 from the microsection surface and wood structure was performed with pure acetone during 1 min. Subsequently, microsection was stained with Toluidine blue O (Sigma-Aldrich, St. Louis, United States) during 5 min. Pinkish purple color will appear when the Toluidine reacts with carboxylated polysaccharides such as pectic acid;

green, greenish blue or bright blue with polyphenolic substances such as lignin and tannins (O'BRIEN *et al.* 1964). Subsequently, the dye was removed with a pure acetone, water and 96% ethyl alcohol solution (1:1:1 v/v/v). The microsection was dried by solution of 75% and 96% ethyl alcohol during 2 min. Finally, each microsection was mounted to Euparal (BioQuip Products Inc., Rancho Dominguez, United States).

Finally, the slides were examined with an Axio Lab.A1 microscope (Carl Zeiss Microscopy, Jena, Germany). A set of polarizing filters was used for cellulose localization.

## RESULTS AND DISCUSSION

Different intensity of decay in sampled logs was observed after annual exposure to *P. ostreatus*. The lowest average mass loss value was found in darker (brownish) marginal parts of sampled logs ( $45.3 \pm 10.1\%$ ). On the other hand, the highest values were found in white-coloured marginal ( $78.7 \pm 8.3\%$ ) and white-coloured central parts ( $82.6 \pm 7.8\%$ ) (Fig. 1D).

### **Anatomy and decomposition of white-coloured marginal and central parts**

White-coloured marginal and central parts at the cross-section indicate the most advanced stage of decomposition. The structure of the decayed wood was extremely soft and crumbly. (Fig. 1E). Cell walls were completely decomposed in some parts of the tissues. Lignin was found mainly in the middle lamella, sometimes also in the primary walls, especially in earlywood. Also, early and late wood fibres were decomposed in the close proximity of vessels and rays. In contrast, the fibres, rays and axial parenchyma were distributed less at the early/latewood boundary (Fig. 2A). Although, the delignification and decomposition of cellulose in these zones advanced significantly, there were still zones with higher concentration of lignin and with higher frequency of hyphae in adjacent vessels (Fig. 2B). Also, large multiseriate ray structures were separated from surrounding tissues regularly. On the other hand, some smaller rays remained non-separated (Fig. 2C). Also (BARI *et al.* 2015b) stated that S2 layers of the most fibres in close proximity of rays have not been removed yet in early stages. However, total separation of large rays from ground tissue already was observed at 120 day after inoculation.

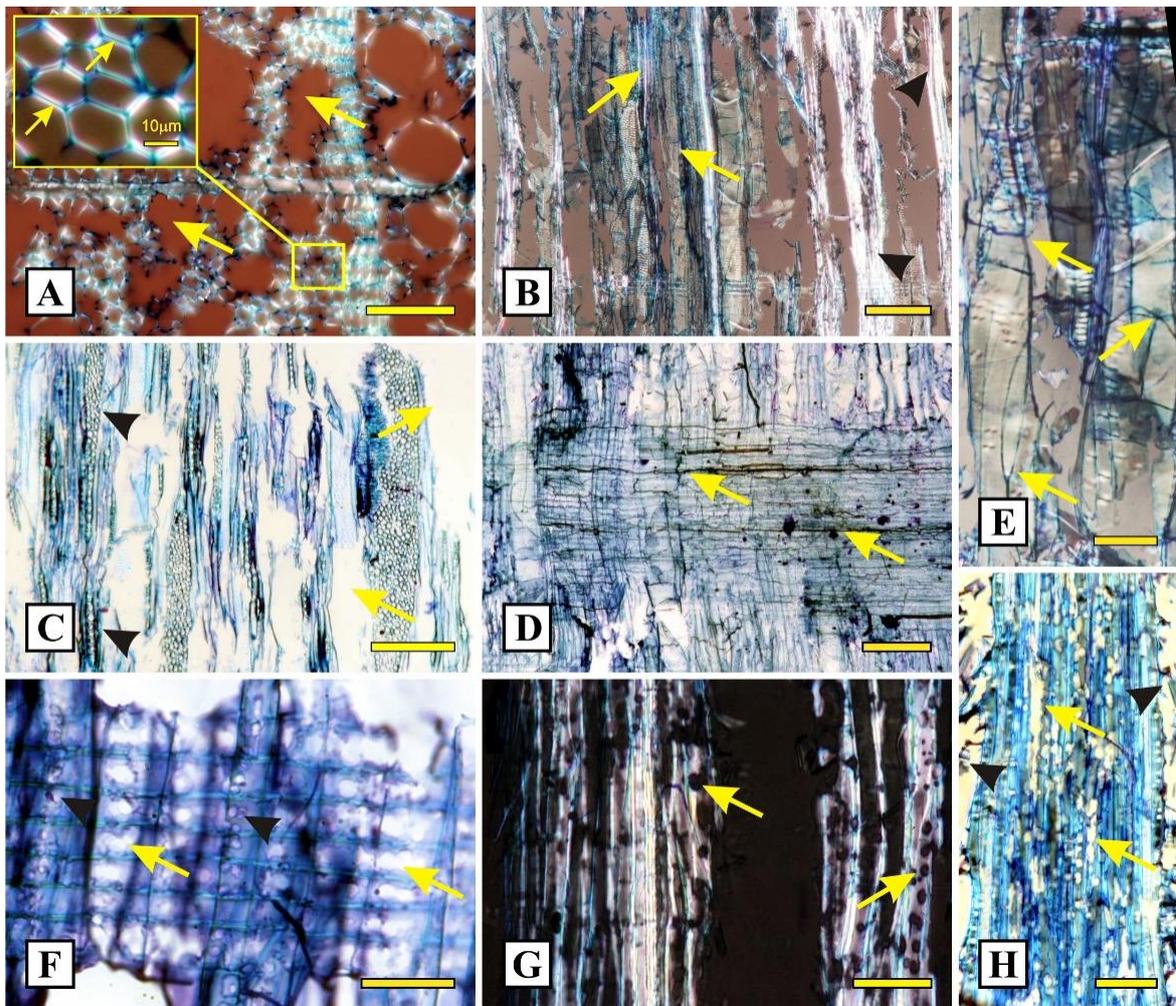
Simultaneous white-rot fungi are able to degrade carbohydrates and lignin at the same rate and uniformly in all stages of their decomposition (REINPRECHT 1991, REINPRECHT *et al.* 2010, SCHWARZE *et al.* 2004). However, the *P. ostreatus* degrade the cell wall components of beech to different degrees and rates. The carbohydrate content decreased rapidly within 30 days, while cellulose and lignin contents displayed a moderate but steady decline (BARI *et al.* 2015d). On the other hand, in more advanced stages (after 120 days), the rays and fiber cell walls intensively attacked by fungi and in fibers become hollow pores (BARI *et al.* 2015a, c, BLANCHETTE and BIGGS 1992).

Although, MARTÍNEZ *et al.* (2005) and KUBICEK (2013) consider *Pluerotus spp.* as lignin selective delignifiers, chemical analysis revealed non-selective white-rot decay of *P. ostreatus* in beech (BARI *et al.* 2015a).

A massive hyphae network that was formed in the rays in our case, allowed the radial propagation toward the pith or toward the log margin. Abundant hyphae branching were occurred. Hyphae grew directly along the rays, but sometimes they turned into next row of parenchyma cells (Fig. 2D). The wider of them penetrated into the vessels closer to the log margin or pith where they were branched (Fig. 2E). From that, they attacked fibers and tracheids mainly, but also the axial parenchyma of unaffected wood rarely.

BARI *et al.* (2015c) stated that, in early stages, hyphae had already colonized the inner parts of wood through the ray parenchyma where first degraded xylem ray parenchyma cell contents (cytoplasm) followed by the cell walls; then, they entered vessel lumina.

BARI *et al.* (2015a), REINPRECHT and LEHÁROVÁ (1997), SCHMIDT (2006), and SCHWARZE (2007) also showed, that hyphae of white-rot fungi in more advanced stages have tend to colonize lumen of the vessels preferentially, to form branched structures and go through simple or bordered pits to easy penetrate into adjacent tissues. Also in our case, the hyphae abundantly penetrated not only through the pits in the crossed fields (Fig. 2 F), but also through the pits between adjacent parenchyma (Figure 2 H) that significantly disrupted. Simultaneously, they penetrated much more often through the fiber walls and formed massive bore (Figure 2 G).



**Fig. 2** The anatomy of the white-coloured marginal and central parts. (A) – Destruction of adjacent tissues around earlywood vessels (arrows). Middle lamellae contained lignin only (arrows in small picture); cross section in polarised light. (B) – Zones containing lignin (arrows), zones containing only cellulose (arrowheads); radial section in polarised light. (C) – Large multiserial rays separated from adjacent tissues (arrows), smaller rays remained non-separated (arrowheads); tangential section. (D) – Massive hyphae network in rays (arrows); radial section. (E) – Branching of hyphae in earlywood vessels (arrows); radial section in polarised light. (F) – Irregular-shaped bore holes (arrows) formed by destruction of walls between adjacent simple pits in cross fields. Cluster of small bore holes (arrowheads); radial section. (G) – Bore holes in fibres created by hyphae (arrows); tangential section in polarised light. (H) – Large bore holes between adjacent parenchyma cells (arrows). Separated ray from adjacent decomposed tissues (arrowheads); cross section. Scale bars: (A) – 200µm; (B) – 200 µm; (C) – 200µm; (D) – 100µm; (E) – 50µm; (F) – 50µm; (G) – 50µm; (H) – 50µm.

The formation of bore holes in the vessel and other cell walls had already been observed in earlier stages of decay ((BARI *et al.* 2015a) that initiated the specialized hyphae with the

initial diameter of 0.5 mm or less (SCHWARZE *et al.* 2004). However, the frequency and size of bore holes after 120 days was lower significantly (BARI *et al.* 2015a) than in our case.

Also, there is a reasonable assumption that the central parts could be decomposed due to a sufficient supply of moisture and additional microbial infection from the soil. However, the microstructure of tissues from central parts did not confirm the symptoms of degradation by other fungal infection. Also, at the most advanced stage, when the anatomy of the wood was significantly disturbed, their presence it cannot be exclude with certainty.

### **Anatomy and decomposition of brownish marginal parts**

Brownish marginal parts show a lower state of decay and higher hardness of wood, than white-coloured marginal and central parts. Simultaneously, the primary cell walls and middle lamellae were compact, without apparent disintegration (Fig. 3A). A lignin content was found compared to the previous examined parts. Also cellulose in the primary cell walls remained intact. However, S2 layers shows attributes of advanced degradation (Fig. 3 B).

The microstructure of the tissues confirmed symptoms of degradation by soft-rot fungi (Type 1) predominantly (Fig. 3 C). Soft-rot cavities in S2 layer are initiated by fine penetration hyphae growing axially in the cellulose-rich S2 layer following the orientation of the cellulose microfibrils (SCHWARZE 2007). Such structures were found mainly in latewood fibres, but were also partly identified in earlywood fibres (Fig. 3C).

On the other hand, degradation by *P. ostreatus* occurred rarely in these zones (Fig. 3D). It occurred in the earlywood parts of the annual growth rings. Simultaneous degradation process was found. Mostly, S2 layers were completely removed. In some parts of the tissues, beginning primary cell walls degradation already have also appeared.

Although the degradation degree of the rays was low, the separation of the surrounding cell structures from the rays was apparent (Fig. 3 E). Starting simultaneous degradation process in some parenchyma cells of rays caused only lignin degradation, but cellulose remained intact (Fig. 3 F). Simultaneously, hyphae frequency in longitudinally oriented cells were abundant, but in parenchyma cell of rays occurred seldom (Fig. 3 G). Although fibres did not contain bore holes in most cases, the erosion of pith membranes were apparent (Fig. 3H). On the other hand, cell wall disturbances were also occurred in some fibres. Therefore, the bore holes indicate the decay process by *P. ostreatus* (Fig. 3 I).

In natural conditions white-rot fungi enter into interactions with other organisms, which may affect their biodegradation capacity. However, the ability of *P. ostreatus* to resist massive bacterial stress was confirmed (VÁLKOVÁ *et al.* 2017). On the other hand, competitive interactions between fungal species can influence on colonization success, and that this can have significant consequences on the outcomes of wood decomposition (SONG *et al.* 2012).

The fact that there were found the features of degradation by soft-rot fungi, apart from *P. ostreatus*, it offer to explain that decay processes could be affected by their mutual interaction. Also, the logs could be infected with other fungal colonies in forest. Subsequently, the latent infection in the logs could triggered under the advantageous conditions concurrent decay processes and thus block their mutually.

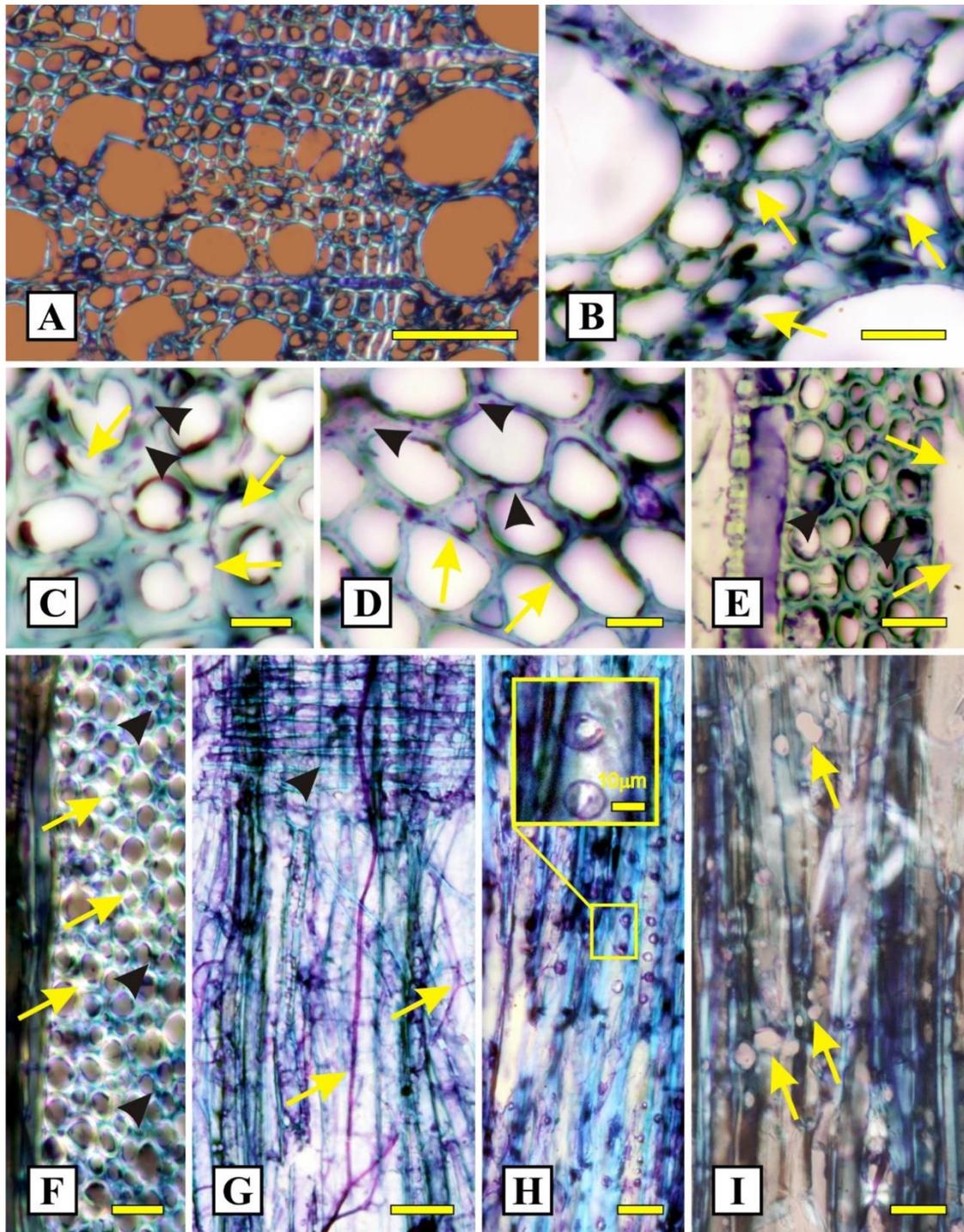


Fig. 3 The anatomy of the brownish marginal parts. (A) – Compact structure of wooden tissues, containing lignified primary cell walls; cross section (B) – Advanced degradation of S2 and S1 layers (arrows); cross section (C) – Soft-rot cavities in S2 layer (arrows), formation of new cavities by thin hyphae (arrowheads); cross section (D) – Simultaneous degradation by *P. ostreatus*. Progress of erosion the secondary (arrows) and primary cell walls (arrowheads); cross section (E) – Separation of ray from adjacent tissues (arrows). Hyphae in parenchyma cells (arrowheads). (F) – Different state of delignification of parenchyma cells in multiseriate ray. Delignification of parenchyma cell walls (arrows). Parenchyma cell walls containing lignin (arrowheads); tangential section in polarised light. (G) – Branched hyphae go through adjacent fibres (arrows); radial section. (H) – Erosion of pith membranes in fibres; radial section. (I) – Frequent occurrence of bore holes in fibres formed by *P. ostreatus* (arrows); radial section. Scale bars: A – 100µm; B – 20 µm; C – 10µm; D – 10µm; E – 20µm; F – 20µm; G – 50µm; H – 20µm; I – 20µm.

## CONCLUSION

- Three zones with different degree of degradation were found on beech logs after one year of grow the fungus *P. ostreatus* in natural environmental condition. Mass loss average value of brownish marginal parts was ( $45.3 \pm 10.1\%$ ), white-coloured marginal part was ( $78.7 \pm 8.3\%$ ) and white-coloured central part was ( $82.6 \pm 7.8 \%$ ).
- Anatomical characteristics of altered woody tissues of white-coloured marginal and central part confirm dominant influence of white-rot fungus *P. ostreatus*. Integrity of the affected tissues was significantly corrupted. Only parts of the compact structures of the secondary cell walls containing predominantly cellulose have been remained. Lignin was located mainly in the middle lamellae, but also in the cell walls parts.
- However, the anatomy of the brownish marginal parts, apart from occurrence of *P. ostreatus* also confirmed the occurrence of soft-rot fungi. The inhibition of the decay process was apparently influenced by interaction and competition of these fungi about the space and nutritional base.

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