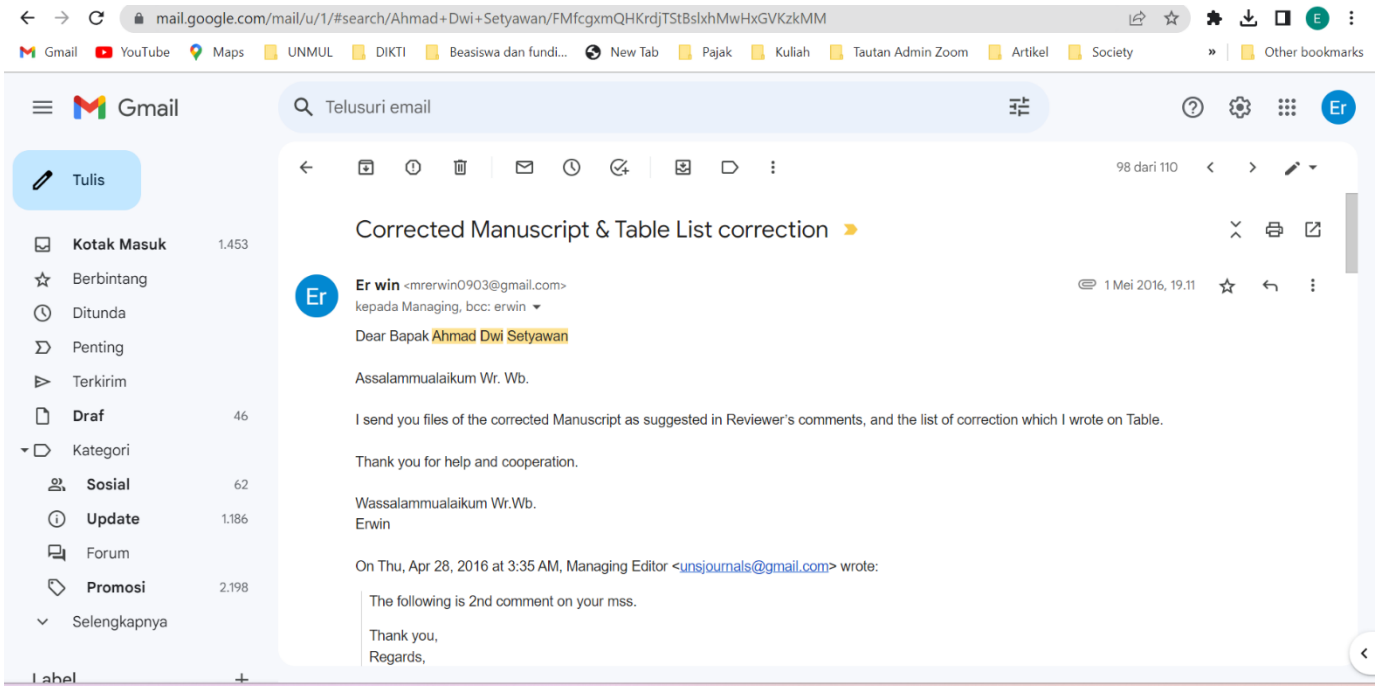
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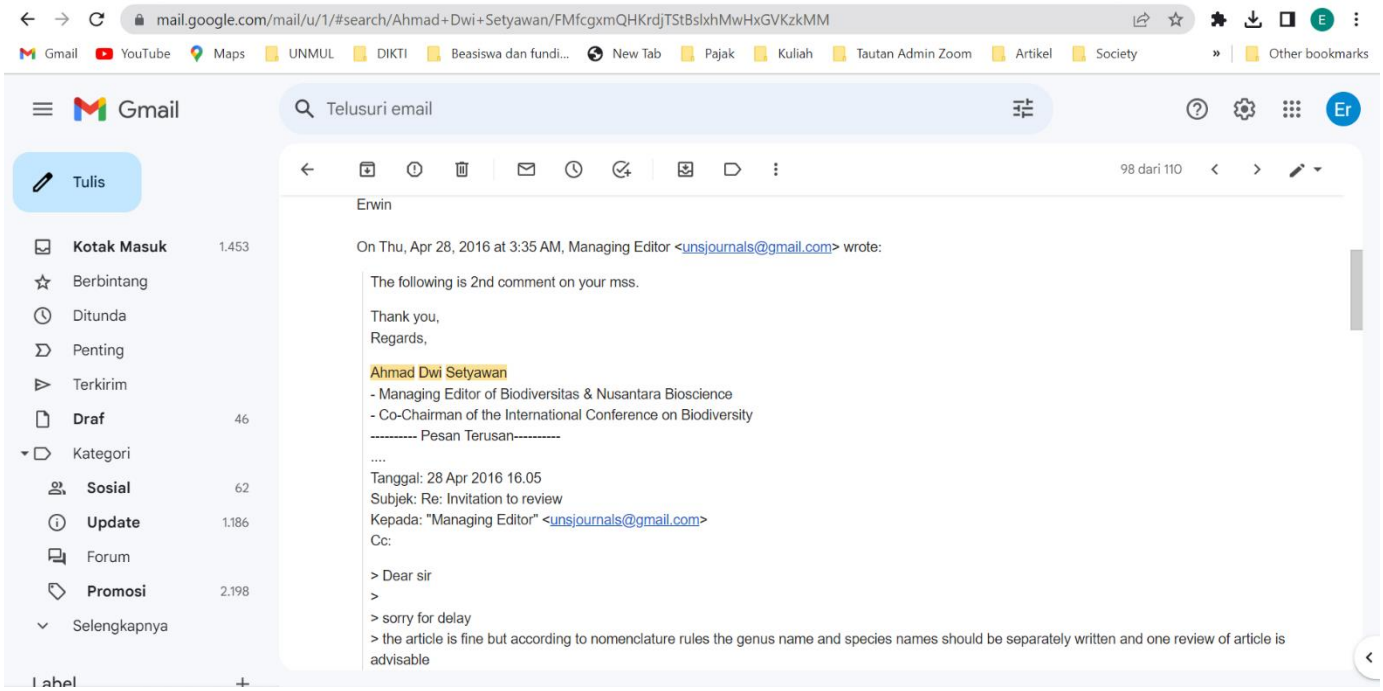
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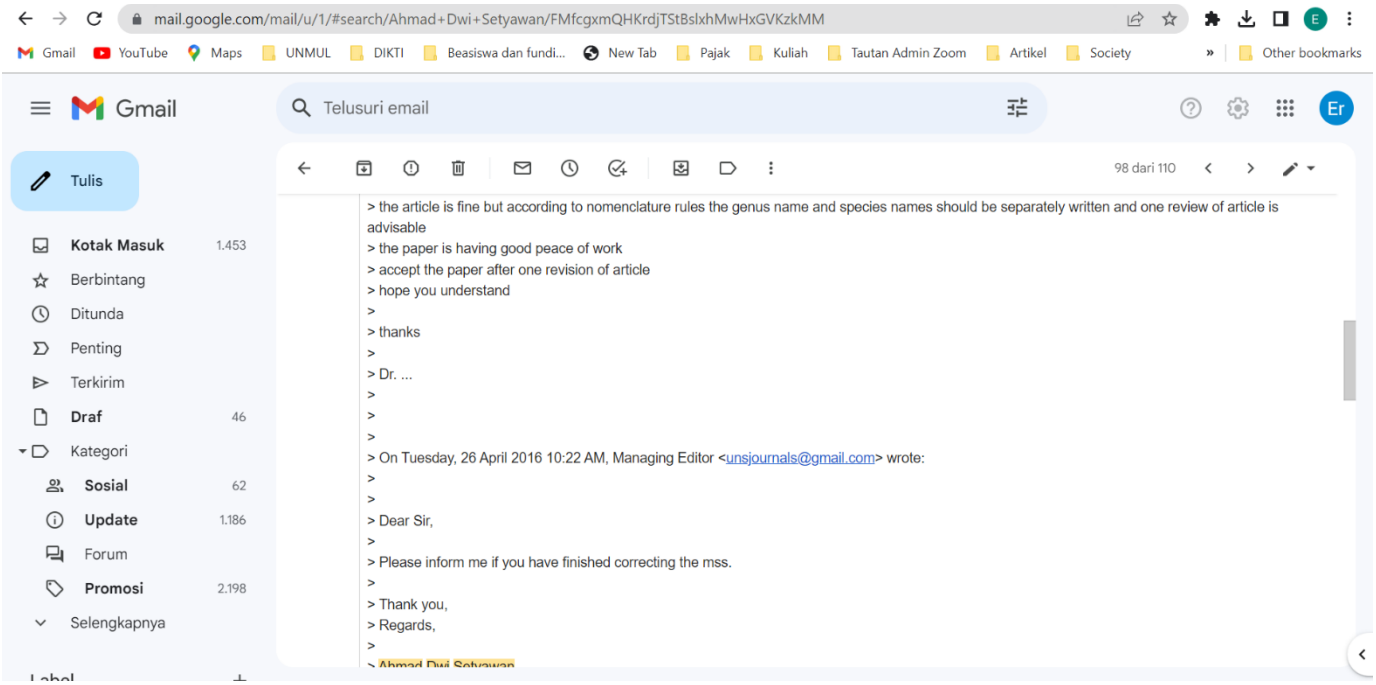
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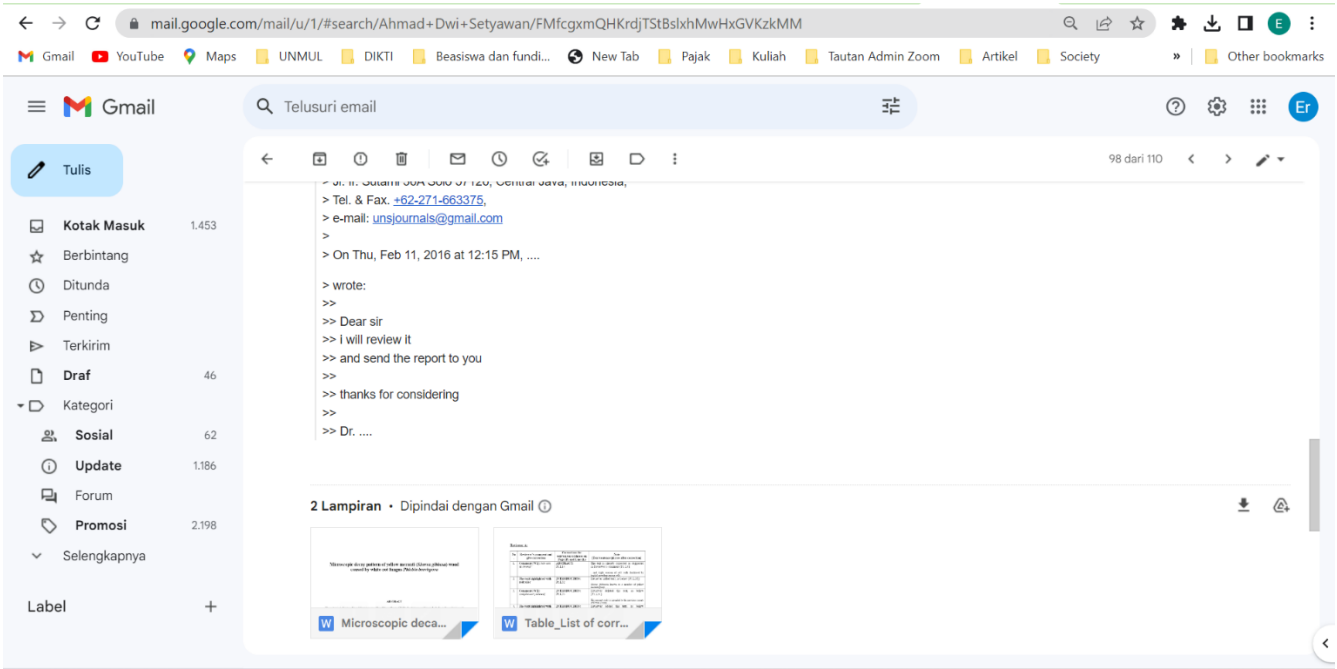
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Reviewer A:

No.	Reviewer's comment and give correction	Corrections for text/sentence/phrase on Page (P) and Line (L)	Note [Text/sentence/phrase after correction]
1.	**Comment [W1]:** *between* or *among?*	**ABSTRACT:** P1.L14	The text is already corrected as suggested in Reviewer's comment [P1.L4]: …and slight erosion of cell walls facilitated by hyphal spreading among cells.
2.	**The text highlighted with red color**	**INTRODUCTION:** P1.L32	Reviewer added text, as below [P1.L32]: *Shorea gibbosa*is known as a member of yellow meranti group…..
3.	**Comment [W2]:** *complement [deleted]*	**INTRODUCTION:** P1.L35	Reviewer deleted the text, as below [P1.L35]: The present study is intended to the previous reports of Erwin (2010)…..
4.	**The text highlighted with red color**	**INTRODUCTION:** P1.L37	Reviewer added the text, as below [P1.L37]: ….microscopic observation techniques are not applicable in the field use…
5.	**Comment [W3]:** please proceed with the description of the timber main axis direction. What is the direction in the dimension of the block?	**MATERIALS AND METHODS** P2.L52	The sentence already corrected as suggested in Reviewer's comment [P2.L51-52]: Twelve sound wood-blocks (20 mm x 20 mm x 10 mm) in radial, tangential, and longitudinal directions, respectively, were obtained from….
6.	**Comment [W4]:** Twelve sound wood blocks- Three blocks- Nine blocks; These explanation are confusing.	**MATERIALS AND METHODS** P2.L52-57	The sentence already corrected as suggested in Reviewer's comment [P2.L51-57]: **Twelve sound wood-blocks** (20 mm x 20 mm x 10 mm) in radial, tangential, and longitudinal directions, respectively, were obtained from uninfected heartwood of the stem disks of *S. gibbosa*. The blocks were oven dried and weighed, then sterilized with gaseous ethylene oxide at 50 ºC for 5 h. The blocks were introduced in four glass jars (each glass jar containing **three blocks**), and inoculated with the liquid fungal culture of the isolated fungus, then aseptically incubated at 26 ± 2 ºC and 70–80% RH for each 2, 4, 6, 8,10 and 12 weeks. The blocks of each incubation period were brushed clean to remove superficial mycelia. **Nine blocks** were oven-dried at 70 ºC until a constant dry weight was reached. Percent weight loss due to decay was then calculated; **three blocks** were reserved for microscopic observations.
7	**Comment [W5]:** Please check this sentence.	**RESULTS AND DISCUSSION** P3.L118-119	The sentence already corrected as suggested in Reviewer's comment [P3.L117-118]: …*S. gibbosa* wood samples, where fungal hyphae became quickly established, …
8	**Comment [W6]:** Process	**RESULTS AND DISCUSSION** P5.L214	The text already corrected as suggested in Reviewer's comment [P5.L212]: This decay process exhibited…

9	**Comment [W7]:** Which word is more appropriate: *appeared* or *visible*?	**RESULTS AND DISCUSSION** P5.L216 & 217	*Appear* is a verb; *visible* is adjective. Synonym of *appear* is become visible. Thus, *appeared* is more appropriate than *visible* on the sentence. [P5.L214]: ...large voids that appeared in transverse sections of… [P5.L216]: …holes appeared in transverse section, …
10	**Comment [W8]:** disintegrated	**RESULTS AND DISCUSSION** P5.L217	The text already corrected as suggested in Reviewer's comment [P5.L216]: ,...where all cell types had already been disintegrated,...
11	**Comment [W9]:** Please check this sentence	**RESULTS AND DISCUSSION** P5.L222-223	The text already corrected as suggested in Reviewer's comment [P5.L221-222]: ,... cell walls facilitated hyphal penetration among cells.

Reviewer B:

I already corrected the genus name and species name [the name of wood species and fungus] that written separately, as suggested in Reviewer's comment.

Page Number	Columns; Line of Paragraph of ...	Uncorrected	Corrected	Note
417	Line 2 of **Abstract**	*Phlebia breviospora*	*Phlebia brevispora*	deleted o
417	Line 3 of **Abstract**	(*Shorea gibbosa*)heartwood	(*Shorea gibbosa*) heartwood	spacing between) and heartwood
417	Line 6 of **Abstract**	of*S. gibbosa*	of *S. gibbosa*	spacing between of and *S. gibbosa*
418	Column 1, Line 5 of Paragraph 3 of **Introduction**	qualitywill	quality will	spacing between words
418	Column 2, Line 2 of Paragraph 5 of **Introduction**	*P. breviospora*	*P. brevispora*	deleted o
418	Column 1, Line 1 of Paragraph 1 of **Results and Discussion**	*P. breviospora*	*P. brevispora*	deleted o
418	Column 1, Line 6 of Paragraph 1 of **Results and Discussion**	*P.breviospora*	*P.breviospora*	deleted o
418	Column 2, Table 1 of **Results and Discussion**	*P. breviospora*	*P. brevispora*	deleted o
421	Column 1, Line 1 of Paragraph 10 of **Results and Discussion**	conclusion,*S. gibbosa*	conclusion, *S. gibbosa*	spacing between comma [,] and *S. gibbosa*
421	Column 1, Line 2 of Paragraph 10 of **Results and Discussion**	*P.brevispora*	*P. brevispora*	spacing between dot [.] and *brevispora*
421	Column 2, Erwin. 2012. of **References**	ayellow	a yellow	spacing between a and yellow

Microscopic decay pattern of yellow meranti (*Shorea gibbosa*) wood caused by white-rot fungus *Phlebia brevispora*

ABSTRACT

The anatomical changes of wood decaying caused by white-rot fungus *Phlebia breviospora* could provide the basis for evaluating and analysis of decay on yellow meranti (*Shorea gibbosa*) heartwood. By using soil-block test procedure of JIS K-1571 and microscopic analysis, a progressive decay *in vitro* of *S. gibbosa* wood caused by *P. brevispora* was well characterized. The percentage of wood weight loss was ranged from 0.91% to 12.34% in 2-12 weeks' incubation. On the first 6 weeks of incubation of *S. gibbosa* infected with *P. brevispora*, the early stages decay, in which pit erosion and slight erosion of cell walls facilitated by hyphal spreading between cells. The intermediate decay features of numerous and conspicuous holes as well as erosion troughs in cell walls were found after 8 weeks' incubation. Furthermore, complete degradation of wood cell components, defined as the advanced stage of decay, was found in some areas of wood after 12 weeks' incubation. The pattern of wood decay was similar to those of the decayed xylem of *S. gibbosa* stem canker in field conditions.

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Keywords: Cell degradation, microscopic, *Phlebia brevispora*, *Shorea gibbosa*, wood decay

Running title: Decay pattern, *Shorea gibbosa*, wood, white-rot fungus, *Phlebia brevispora*

INTRODUCTION

White rot basidiomycetes that cause the decay are especially important in wood decomposition because they are the only fungi capable of degrading all cell wall components (cellulose, lignin, hemicelluloses) of wood (Blanchette 1991; Schmidt 2006; Schwarze 2007). Micromorphological aspects of two main types of white rot, selective delignification and simultaneous rot, have been distinguished (Blanchette 1984; Otjen et al. 1987; Anagnost 1998; Schwarze 2007). In selective delignification, lignin in the secondary wall and middle lamella is almost entirely removed, whereas as large quantities of cellulose in the S2 layer of the cell wall are left intact and are separated from one another. Simultaneous rot is characterized by removal of both cellulose and lignin, leaving cells either riddled with bore holes and erosion troughs, or with extensively thinned secondary walls.

A white-rot fungus has been isolated from decayed xylem of *Shorea gibbosa* stem canker, namely *Phlebia brevispora* (Erwin et al. 2010) and was suspected to cause serious wood decay on this tree species (Erwin 2012).

Shorea gibbosa is known as a member of yellow meranti group (Ogata et al. 2008) which has been long managed for timber production and used for many wood products, therefore, the microbial decay processes go along with a loss of wood quality will affect the lumber value and the wood products in use.

The present study is intended to ~~compliment~~ the previous reports of Erwin (2010) and Erwin et al. (2012) with presented the anatomical features of *S. gibbosa* heartwood infected with *P. brevispora* under laboratory conditions (*in vitro*). Although microscopic observation techniques are not applicable in the field use, however, the decay pattern of the infected wood can clearly be characterized and very useful for providing valuable information and understanding the stages of wood degradation by the fungal attack.

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The aim of this study was to (1) evaluate the ability of *P. breviospora* to degrade *S. gibbosa* heartwood, and (2) confirm the decay pattern of the fungus in artificial laboratory conditions by microscopic observations.

MATERIALS AND METHODS

Fungal strain

The decay fungus isolated from decayed xylem of *S. gibbosa* stem canker, and designated as YM3, was genetically identified by their internal transcribed spacers (ITS) sequence as *Phlebia brevispora* (Erwin et al. 2010). The fungal strains were maintained at 4°C on PDA slants.

Decay test procedure

For this experiment, inoculation procedures followed the JIS K 1571 soil-block test procedure (JIS K 1571 2004). A medium of 250 g quartz sand and 80–85 ml of nutrient solution (4.0% glucose, 0.3% peptone, and 1.5% malt extract) used for culture media. Twelve sound wood-blocks (20 x 20 mm in cross-section x 10 mm in length) were obtained from uninfected heartwood of the stem disks of *S. gibbosa*. The blocks were oven dried and weighed, then sterilized with gaseous ethylene oxide at 50 °C for 5 h. Three blocks were put in each one of four glass jars containing the medium, and inoculated with the liquid fungal culture of the isolated fungus, then aseptically incubated at 26 ± 2 °C and 70–80% RH for each 2, 4, 6, 8,10 and 12 weeks. Nine blocks of each incubation period were brushed clean to remove superficial mycelia, and then oven-dried at 70 °C until a constant dry weight was reached. Percent weight loss due to decay was then calculated; three blocks were used for microscopic observations.

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Microscopic observation

The dried blocks were sectioned with razor blade then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C overnight, washed four times in 0.1 M phosphate buffer at pH 7.2 for 15 minutes each, and rinsed three times in distilled water for 5 minutes each. The blocks were placed in an ethanol dehydration series of 50, 80, 95, 100% each for 20 minutes, then three times in 100% ethanol. The dehydrated blocks were freeze-dried, and mounted on SEM stubs, then coated with gold-palladium using Jeol JFC-1200 Fine Coater. The coated samples were observed under a JEOL Scanning Microscope (JSM-5310) and the EDAX application program used to obtain SEM images of the altered properties of wood.

RESULTS AND DISCUSSION

The weight loss of *S. gibbosa* wood after *P. breviospora* decay over 2–12 weeks is shown in Table 1. Based on classification of natural durability of Indonesian woods (Seng 1990), the infected wood of *S. gibbosa* with 12.34% weight loss, were categorised non-resistant (class IV) against *P. breviospora* attack. The result indicated the fungus capable of attacking the heartwood of *S. gibbosa* under laboratory conditions, thus, it should be taken as a consideration for wood protection, and otherwise, the fungus can produce an extensive degradation into wood under favorable temperature and humidity.

Meanwhile, microscopic observations of this decay showed various stages of decay, as shown in Figures 1-4.

Table 1. Weight loss in *S, gibbosa* wood infected with *P. breviospora* for periods of 2, 4, 6, 8, 10 and 12 weeks

Incubation period (weeks)	Weight loss percentage Mean ± SE
2	0.91 ± 0.10
4	2.24 ± 0.60
6	5.02 ± 1.03
8	8.23 ± 1.22
10	11.80 ± 5.15
12	12.34 ± 2.76

After 2–4 weeks' incubation, the wood blocks had lost 0.91–2.24% in weight. Abundant clamped hyphae colonizing the lumina of vessels were observed in transverse, radial and tangential views (Figs. 1a-d). However, in axial parenchyma cells – rays and fibers adjacent to heavily infected vessels – hyphae were not observed. In this case, hyphae propagated mainly in vessels where they could either grow parallel to the cell axis and diagonally across the lumina, supported at their points of attachment with the cell walls, or in the central part of the lumina, where they are held in place by hyphal branches extending from the main hyphae attached to the cell walls. Hyphae passing through the perforation plates were also detected (Fig. 1e). Despite hyphae being attached deep within the vessel walls, they did not severely damage cell walls (Fig. 1f).

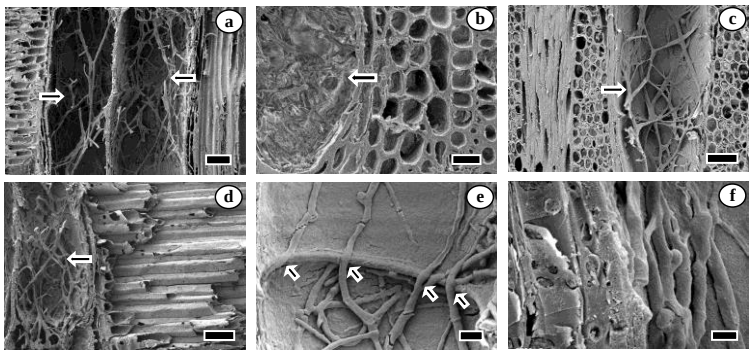

Figure 1. Decay in *S. gibbosa* wood blocks caused by fungus *P. brevispora* after 2–4 weeks' incubation. (a) Hyphal colonization in the lumen of two neighboring vessels at after 2 weeks' incubation (arrows). *Bar* 50 μm; (b) Transverse view of hyphal colonization (arrow) in vessels after 2 weeks' incubation. *Bar* 20 μm; (c) Tangential view of hyphal colonization (arrow) in vessels after 2 weeks' incubation. *Bar* 40 μm; (d) Radial view of hyphal colonization (arrow) in vessels after 2 weeks' incubation. *Bar* 40 μm; (e) Fungal hyphae (arrows) passing through perforation plates of vessels after 4 weeks' incubation. *Bar* 10 μm; (f) Hyphae attached deep within the vessel walls after 4 weeks' incubation. *Bar* 5 μm.

After 6 weeks' incubation, wood blocks had lost 5.02% of their weight. Fungal hyphae had extended from heavily infected vessels into rays, axial parenchyma cells and fibers mainly through pits, causing slight erosion of the cell wall (Figs. 2). In vessels, rounded pit erosion was seen (Fig. 2a). Hyphal penetration into rays, parenchyma cells and fibers could also be seen (Figs. 2b-d).

Initial colonization of vessels by fungal hyphae is the typical decay pattern of simultaneous rot in hardwood caused by white-rot fungi (Zabel and Morrell 1992). Such typical decay appeared in these *S. gibbosa* wood samples where *P. breviospora* hyphae became quickly established, first in vessels (in 2–4 weeks' incubation), then spreading from these vessels into adjacent rays, parenchyma cells and fibers until 6 weeks decay process was reached.

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At the early colonization phase of decay, damage is limited, and any visible evidence is not easily observed on the lumen surfaces as termed. This is the incipient or hidden stage of decay (Zabel and Morrell 1992; Schwarze 2007). Nowadays, this decay stages can be detected within several days by FT-NIR (Fourier transform near-infrared) spectroscopy (Fackler et al. 2006, 2007a,b) and multiplex PCRs methods (Nicolotti et al 2009).

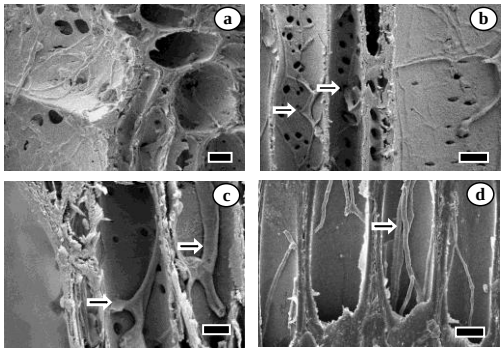

Figure 2. Decay in *S. gibbosa* wood blocks after 6 weeks' incubation. (a) General view of hyphae colonizing vessels, rays and parenchyma cells. *Bar* 10 μm; (b) Hyphae penetrating parenchyma cell through pits (arrows). *Bar* 10 μm; (c) Hyphae begin penetrating ray cell walls (arrows). *Bar* 5 μm; (d) Hyphae present in fibers (arrow). *Bar* 10 μm.

After 8 weeks' incubation, wood blocks had sustained an average weight loss of 8.23%. Rounded pit erosions of vessels were enlarged enzymatically and coalesced to form numerous and conspicuous holes (Fig. 3a). Numbers of fungal hyphae in rays and parenchyma cells increased, and hole formation and cell wall destruction became clear (Fig. 3b-d). Fig.

3e shows the lysis zones that developed around elongated holes and which were frequently observed in parenchyma cell walls. Portions of the secondary walls were removed as well as the compound middle lamella, resulting in erosion troughs within cell walls. Hyphae had also begun to colonize fibers intensively but did not damage cell walls (Fig. 3f).

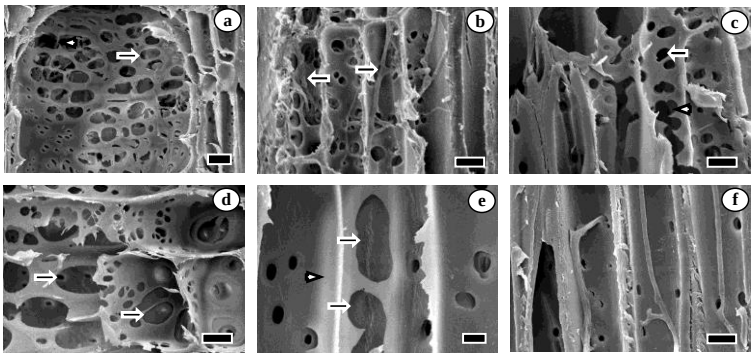

Figure 3. Decay in *S. gibbosa* wood blocks after 8 weeks' incubation. (a) Rounded pit erosion (arrow) and coalesced holes (head arrow) in vessels. *Bar* 20 μm; (b) Hyphae begin to heavily colonize parenchyma cells (arrows). *Bar* 10 μm; (c) Rounded pit erosion (arrow) and coalesced holes (head arrow) in parenchyma cells. *Bar* 10 μm; (d) Enlarged holes in rays (arrows). *Bar* 10 μm; (e) Erosion troughs (arrows) and lyses zone (head arrow) in parenchyma cells. *Bar* 10 μm; (f) Hyphae begin to heavily colonize fibers. *Bar* 10 μm.

Early in the degradation process, depressions could be seen on the inner surfaces of the secondary walls, the S3 layer, under and in the neighborhood of the hyphae, as shown in Figs. 1 and 2. In later stages of degradation (in 8 weeks' incubation), the hyphae caused wide and deep erosion troughs. In this decay stage, the lysis zones that developed around bore holes and axially elongated troughs showed clearly the effects of fungal enzymes on cell walls, which were gradually eroded. Anagnost (1998) and Schwarze (2007) expressed that numerous bore-holes appear between two neighboring cells, showed an intermediate stage of decay had occurred.

Meanwhile, the area of decay in cell walls was found at an extended distance from the hyphae, in accordance to Takano et al. (2006), suggesting that extracellular enzymes of white-rot fungus can diffuse some distance from the fungal cell wall. The lysis zones indicated pre-delignification before the cell walls were completely removed. The extracellular enzymes of *P. brevispora* as reported by Arora and Rampal (2002) and Ponting et al. (2005) were known as laccase, then, Sharma and Arora (2011) identified xylanase and carboxymethyl cellulase could also be released. They were responsible for degradation of lignin and cellulosic materials of wood cell walls. Due to its ability to produce such extracellular enzymes, *P. brevispora* was classified as one of hydrolytic fungi Mtui 2012).

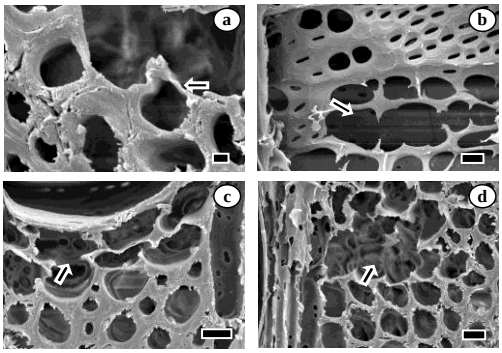

Figure 4. Decay in *S. gibbosa* wood blocks after 10–12 weeks' incubation. (a) Partial thinning of fiber cell wall (arrow). *Bar* 2 μm; (b) Coalesced holes appear enlarged (arrow). *Bar* 10 μm; (c) Erosion channels in parenchyma cells adjacent to infected vessels (arrow). *Bar* 10 μm (d) Complete removal of wood cells (arrow). *Bar* 10 μm.

After 10 and 12 weeks' incubation, wood block sustained an average weight loss of 11.80% and 12.34%, respectively. Partial thinning of fiber walls was frequently observed adjacent to the completely removed cells (Fig. 4a). In some vessels, the rounded and coalesced holes appeared to be enlarged, resulting in severe cell wall damage (Fig. 4b). Parenchyma cell walls adjacent to infected vessels appear partially removed, forming a channel-like appearance (Fig. 4c), and in some decay areas, due to advanced delignification, parenchyma cells have been completely removed. This decay proces exhibited complete degradation of the compound middle lamella and cell corners that recognized as advanced stages of decay (Schwarze 2007). Meanwhile, complete degradation of cell wall components resulted in large voids that appeared in transverse sections of the decayed areas, as shown in Fig. 4d. It seems to be a general sign of this decay stage that large holes appeared in transverse section, where all cell types had already been desintegrated, for instances *Populus* sp decayed by *Trametes trogii* (Levin and Castro 1998) and decaying of *Populus deltoides* by *Pycnoporus sanguineus* (Luna et al 2004).

In conclusion, *S. gibbosa* wood is susceptible to colonization and decay caused by *P. brevispora* under favorable temperature and humidity with a progressive decay pattern that has been well characterized here. The first 6 weeks of incubation was classified as the early stages decay, in which pit erosion and slight erosion of cell walls facilitated hyphal between cells. Numerous and conspicuous holes as well as erosion troughs in cell walls, which were found at the end of 8 weeks' incubation, showed that an intermediate stage of decay had occurred. Furthermore, complete degradation of wood cell components, termed the advanced stage of decay, was found in some areas of wood blocks after 12 weeks' incubation.

The decay pattern *in vitro* that presented in this study was similar to those of the decayed xylem of *S. gibbosa* stem canker as reported in previous work of Erwin (2012). Therefore, a further inoculation experiment is necessary to confirm the pathogenicity of *P. brevispora* to *S. gibbosa* standing trees and also to clarify whether this fungus is one of causal agents of wood decay on the trees.

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