EVALUATION OF WOOD CHEMICAL CONSTITUENTS OF *HEVEA BRASILIENSIS* AND *CUPRESSUS* DECOMPOSED BY *GLOEOPHYLLUM STRIATUM* USING CP/MAS ^{13}C NMR AND HPLC TECHNIQUES

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OKINO EYA, RESCK IS, SANTANA MAE, CRUZ CL da SC, SANTOS PHO & FALCOMER VAS. 2010. Evaluation of wood chemical constituents of *Hevea brasiliensis* and *Cupressus* decomposed by *Gloeophyllum striatum* using CP/MAS ^{13}C NMR and HPLC techniques. The aim of this study was to evaluate chemical changes in wood constituents of six rubberwood *Hevea brasiliensis* clones and two cypress *Cupressus glauca* and *Cupressus* spp., exposed to brown-rot fungus *Gloeophyllum striatum*. Wood constituents were analysed before and after deterioration by HPLC and CP/MAS ^{13}C NMR. Extractive contents increased, on average, 1.3 and 1.7 times in attacked rubberwood and cypress respectively. Ash content increased 12.5% in attacked rubberwood. Fungus in rubberwood relatively decreased cellulose as much as 8.9% and an increase of 16% in total lignin. However, there was no clear trend in hemicelluloses. Cypress showed a relative decrease of 3.6% in cellulose, 15.1% in hemicelluloses and an increase of 14.6% in total lignin. Mannan was removed faster than xylan, and both faster than glucan. Cypress showed higher content of xylan than mannan. Nevertheless, rubberwood showed higher acetyl groups than cypress. *Gloeophyllm striatum* corroborated the biodeterioration pattern where more carbohydrate was removed than lignin. Spectroscopic and chromatographic techniques are shown to be complementary tools in assessing chemical changes in wood. HPLC is time consuming in sample preparation but the results are quantitative and accurate. On the other hand, CP/MAS uses non-destructive samples, demands high end machine and the results are qualitative but suitable for monitoring structural organic changes.

Keywords: Rubberwood, cypress, brown rot decay, wood polymers

OKINO EYA, RESCK IS, SANTANA MAE, CRUZ CL da SC, SANTOS PHO & FALCOMER VAS. 2010. Penilaian komposisi kimia kayu *Hevea brasiliensis* dan *Cupressus* yang direput oleh *Gloeophyllum striatum* menggunakan teknik CP/MAS ^{13}C NMR dan HPLC. Kajian ini bertujuan untuk menilai perubahan komposisi kimia enam klon kayu getah *Hevea brasiliensis* serta kayu *Cupressus glauca* dan *Cupressus* spp. yang didedah kepada kulat reput perang *Gloeophyllum striatum*. Komposisi kayu dianalisis sebelum dan selepas kemerosotan menggunakan teknik HPLC dan CP/MAS ^{13}C NMR. Purata kandungan ekstrakan bertambah sebanyak 1.3 kali dan 1.7 kali masing-masing dalam kayu getah dan kayu *Cupressus* yang diserang. Kandungan abu naik 12.5% dalam kayu getah yang diserang. Kulat yang menyerang kayu getah mengurangkan kandungan selulosa sebanyak 8.9% tetapi menaikkan kandungan lignin sebanyak 16%. Namun, kandungan hemiselulosa tidak menunjukkan trend yang jelas. Kayu *Cupressus* menunjukkan pengurangan relatif kandungan selulosa sebanyak 3.6%, hemiselulosa sebanyak 15.1% dan pertambahan jumlah lignin sebanyak 14.6%. Manan disingkirkan lebih cepat daripada xilan manakala kedua-dua manan dan xilan disingkirkan lebih cepat daripada glukan. Kayu *Cupressus* menunjukkan kandungan xilan yang lebih tinggi daripada manan. Sebaliknya, kayu getah menunjukkan kandungan kumpulan asetil yang lebih tinggi daripada kayu *Cupressus*. *Gloeophyllum striatum* menunjukkan corak biodegradasi yang biasa iaitu karbohidrat disingkirkan lebih banyak daripada lignin. Teknik spektroskopi dan kromatografi merupakan teknik yang saling melengkapi untuk menilai perubahan kimia di dalam kayu. Penyediaan sampel dalam HPLC memakan masa yang lama tetapi keputusannya adalah kuantitatif dan tepat. Sebaliknya, CP/MAS tidak memusnahkan sampel, memerlukan mesin yang canggih dan keputusannya adalah kualitatif tetapi alat ini sesuai bagi memantau perubahan struktur organik kayu.

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INTRODUCTION

Hevea brasiliensis is a native hardwood species of the Amazon basin. It was introduced into several tropical countries and nowadays it is being cultivated for latex and for wood. It is a potential substitute for commercial timbers.

In Brazil, São Paulo state accounts for 50 000 ha of planted rubberwood, whereby 80% is RRIM 600 clone. Mato Grosso state has 44 000 ha of planted area yielding 35 000 tonne year^{-1} of natural rubberwood with IAN 3044, IAN 873, FX 3864, RRIM 600, PB217, GT1 clones. Despite this, there is a regional programme of incentives for rubberwood plantations, whereby the target is to reach 269 000 ha in the next 20 years.

Brazil imports 63% of its demand for natural rubberwood, yielding only 1% of the total worldwide production. In 2006, the yield of natural rubberwood in the country was 178 665 tonnes (13.5% lower than that of 2005), whereby Amazon and Acre states were the largest producers of native rubberwood, with outputs of 51.9 and 35.7% respectively (IBGE 2009).

Cypress is an exotic species in Brazil but native in some countries of Central and South America. Cypress wood has great potential as an alternative wood species (Pereira and Higa 2003, Okino *et al.* 2006).

The study of biodeterioration of lignocelluloses is of importance because of the need for decay prevention in wood and its by-products, and to aid prognosis of decay development in living trees for hazard assessment. It can be applied to facilitate the identification of tree rings in diffuse porous wood of angiosperms, effectively induce permeability changes in gymnospermous heartwood, and as potential use in biotechnological applications (Schwarze 2007).

Discoloration caused by fungi is a major loss in value for both timber products and wood in service, causing 15–25% of value loss in standing timber and 10–15% in wood products during storage and utilisation. Prevention of the oxidative degradation of cellulose offers an excellent target for developing methods of protecting wood in an environmental-friendly manner (Ritschkoff 1996).

Tree species differ in their chemical composition and wood anatomy, which partially determine the severity and nature of strength loss due to fungus. Similarly, fungal species differ in their biochemical system loss of degrading components of the woody cell wall, in their morphology and in their tolerance to environmental extremes (Schwarze 2007). Brown-rot fungi modify lignin, as indicated by demethylation and accumulation of oxidised polymeric lignin-degradation products. These fungi cause the wood to darken, shrink and break into brick-shaped pieces that crumble easily into brown powder (Ritschkoff 1996, Green III & Highley 1997, Highley & Dashek 1998).

Decay caused by brown-rot fungi is the most prevalent and destructive type of wood deterioration because it can cause rapid structural failure. Rapid decrease of arabinan, xylan and rhamnan was detected in yellow pine blocks, exceeding 30% after four weeks of exposure to *Postia placenta* (Clausen & Kartal 2003). Kirk and Highley (1973) determined quantitative changes in lignin, glucan, mannan and xylan during decay of conifer woods by white- and brown-rots. The brown-rot fungi remove the polysaccharides, mannan and usually xylan faster than glucan but not lignin. Okino *et al.* (2008) exposed *Cupressus glauca* to two white- and brown-rot fungi for 12 weeks. There was a relative decrease of 14.2% cellulose and 35.3% hemicelluloses, and an increase of 43.0% total lignin due to brown-rot fungi.

Curling *et al.* (2002a) exposed southern pine to *Gloeophyllum trabeum* and *P. placenta* and detected, during incipient decay, a direct relationship between weight loss and strength loss which was related to changes in the hemicellulose sugars. Curling *et al.* (2002b) reported that early strength loss (up to 40% weight loss) was associated with loss of arabinan and galactan components. Subsequent strength loss (greater than 40%) was associated with loss of mannan and xylan components. Significant loss of glucan (representing cellulose) was only detected above 75% modulus of rupture loss.

CP/MAS ^{13}C NMR spectra of sawdust sample allow direct information about organic groups without any chemical or physical sample fractionations (Preston 1996). Nevertheless, one of the drawbacks is the long time needed to obtain ^{13}C experiments. This technique has been used to characterise residues of white-rot decay of *Eucryphia cordifolia* in Chilean rain forests (Martínez *et al.* 1991) as well as fungal wood decomposition in laboratory incubations (Pérez *et al.* 1993, Davis *et al.* 1994a, b, c, Gilardi *et al.* 1995, Kim & Newman 1995).

The aim of this study was to investigate the relative changes in the amounts of chemical constituents such as lignin, extractives, ash, polysaccharides, by-products and degradation products of six clones (IAN 717, IAN 873, GT 711, AVROS 1301, Tjir 16, RRIM 600) of rubberwood *H. brasiliensis* and two cypress *C. glauca* and *Cupressus* spp. in control samples and those attacked by brown-rot fungus *Gloeophyllum striatum*. Changes were studied using CP/MAS ^{13}C NMR and HPLC techniques.

MATERIALS AND METHODS

Materials

Resistance class data and colour changes were reported in a previous paper (Okino *et al.* 2009), using six clones of rubberwood *H. brasiliensis* harvested between 17 and 41 years old, with diameter at breast height (dbh) of approximately 200 to 400 mm. The trees of cypress were between 25 and 17 years old, with dbh of 150 to 200mm. Three trees from each species were randomly selected. One disc from each tree was processed into blocks of 25 × 25 × 9 mm.

The accelerated laboratory testing was conducted according to ASTM D 2017-05 (ASTM 2005) with few modifications such as the use of malt extract media to the brown-rot *G. striatum*. Twelve repetitions were employed for each treatment, totalling 192 samples tested. The samples were conditioned, weighed, sterilised and exposed to fungus for 12 weeks in a room with controlled temperature at 26.7 ± 1 °C and relative humidity 70 ± 4%. After conditioning, the samples exposed to fungal growth were re-weighed. The conditioning of the samples, before and after fungal treatment, was achieved in an air-forced oven at 50 ± 1 °C until they attained constant weight. Wood resistance to fungal attack was evaluated based on percentage of weight loss.

General methods

Sample preparation was based on TAPPI T-257 cm-85 (TAPPI 1996a) standards with slight modifications. Decayed surfaces were withdrawn and the gross material was ground through a Willey mill to pass through a 42 mesh (350 µm) and retained on 80 mesh (177 µm) screen.

In order to obtain extractive-free samples to determine the percentage of alcohol–toluene extractive and ash contents, TAPPI T-264 om-88 (TAPPI 1996b), TAPPI T-204 om-88 (TAPPI 1996c) and TAPPI T-211 om-93 (TAPPI 1996d) were used.

Wood acid hydrolysis

Approximately 0.3 g of extractive-free wood meal of known moisture content was weighed in a glass test tube to the nearest 0.1 mg. About 3 ml of 72% sulphuric acid were added to the wood meal and the slurry was mixed for 1 min to ensure sample wetting. The test tubes were placed in a water bath set at 30 ± 1 °C for 2 hours and samples were stirred every 15 min. The samples were transferred to 100 ml serum bottles and diluted to 4% by adding 84 ± 0.04 ml deionised water. The serum bottles were capped with rubber stoppers, sealed with aluminum caps and placed in an autoclave for 1 hour at 121 ± 3 °C.

Sugar analysis

The procedure was based on the methodology described by Kaar *et al.*(1991), and Ruiz and Ehrman (1996a) with slight modifications. Details are described in previous studies by Santana and Okino (2007), and Okino *et al.* (2008). This procedure is labour intensive and time consuming.

The neutralised hydrolysate was injected two times, either to sound or decayed samples. A series of sugar calibration standards in deionised water was performed from a set of 12 multi-component standards containing glucose, xylose, galactose, arabinose and mannose in the range of 0.01 to 3.00 mg ml^{-1}.

The conditions used for carbohydrate were Bio-Rad Aminex HPX-87P column (300 mm × 7.8 mm Ø); 50 µl sample volume; 0.20 µm filtered, degassed and deionised water (18 MΩ) eluent; 0.6 ml min^{-1} flow rate; 85 °C column temperature; refractive index detector and 25 min run time data collection plus 15 min post-run.

Determination of acid-lignin in wood

Acid-insoluble lignin was determined according to Templeton and Ehrman (1995). After completion of the autoclave cycle in the wood

acid hydrolysis, samples were allowed to cool for about 20 min. The hydrolysed samples were vacuum filtered through a de-ashed and weighted filtering crucible. The crucible and contents were dried at 105 ± 3 °C until constant weight.

Soluble lignin, on an extractive-free basis, was determined by UV spectrophotometer at 205 nm, based on Ehrman (1996). The absorbance of the hydrolysed samples was measured at 205 nm using the 1 cm light path curvette, so the resulting absorbance reading should fall between 0.2 and 0.7. A 4% solution of sulphuric acid was used as a reference blank, which was diluted to the same ratio as the sample.

Determination of acetyl groups and other by-products

The acetic acid, 4-*O*-methylglucuronic acid, glucuronic acid, levulinic acid, 2-furaldehyde, and HMF (5-hydroxymethyl-2-furaldehyde) followed the methodology suggested by Ruiz and Ehrman (1996b), and Ehrman and Ruiz (1998), with a few modifications as described by Santana and Okino (2007).

The instrumental conditions used for acetyl groups and other by-products were Bio-Rad Aminex HPX-87H column (300 × 7.8 mm Ø); 50 µl sample volume; 0.6 ml min^{-1} flow rate; 35 °C column temperature; 0.20 µm filtered, degassed and deionised H_2SO_4 0.01N; refractive index detector and 40 min run time data collection plus 10 min post-run.

A series of standards was prepared from a nine-component solution of acetic acid (0.2–12.0 mg ml^{-1}), levulinic acid (0.02–0.50 mg ml^{-1}), and HMF (0.02–0.50 mg ml^{-1}), all in deionised water while furaldehyde (0.02–0.50 mg ml^{-1}) was in ethanol.

Calculations

The percentage of each of the monomeric sugars (glucose, xylose, galactose, arabinose, mannose) and by-products (acetic acid, 4-*O*-methylglucuronic acid, glucuronic acid) or degradation products (HMF, furaldehyde, levulinic acid), on an extractive-free basis, were retrieved from HPLC chromatograms. The equations described by Ruiz and Ehrman (1996a), Ruiz and Ehrman (1996b), and Ehrman and Ruiz (1998) were applied. Each percentage had to be multiplied by a conversion factor (Kaar *et al.* 1991) to obtain the yield of the original homopolymer in the sample.

Determination of uronic anhydrides

The procedure used to determine 4-*O*-methylglucuronic and glucuronic acids were determined colorimetrically according to Scott (1979) and the results expressed as percentage of uronic anhydride. About 0.125 ml hydrolised aliquot containing 20–80 µg ml^{-1} of the uronic acid to be analysed was mixed with 0.125 ml of NaCl-H_3BO_3 solution in a reaction tube. About 2 ml of concentrated sulphuric acid were immediately added to the mixture and the tube placed in a heat block at 70 °C for 40 min. Then the test tube was cooled to room temperature. About 0.1 ml of the colorimetric reagent solution (0.1 g of 3,5-dimethylphenol in 100 ml of glacial acetic acid) was added to the mixture and shaken. Absorbance was measured between 10 and 15 min after adding the colorimetric reagent at 450 and 400 nm against a water reference with 100% transmission at 450 nm. The uronic anhydride was calculated based on the 450–400 nm difference. A calibration curve was prepared by subjecting 0.125 ml of hydrolysed solutions containing 12.5, 25.0, 37.5, 50.0, 62.5, 75.0, 87.5 and 100 µg ml^{-1} of D-glucofuranurono-6,3-lactone (glucuronolactone) to the procedure above. The percentage relative change was calculated (decayed – undecayed)/undecayed (Karppanen *et al.* 2008).

Solid-state CP/MAS ^{13}C NMR analysis

Solid-state ^{13}C NMR spectra were obtained with the CP/MAS technique and experiments performed on a VARIAN MERCURY plus 300 spectrometer with a 7.05 T wide-bore Oxford superconducting magnet, operating at 76.46 MHz for carbon 13. The spectrometer was equipped with a 7 mm CP/MAS probe and sawdust samples were spun at the magic angle at 6 kHz packed in 7 mm diameter zirconium dioxide rotors. All spectra were attained with a 100 kHz spectral width, 4.7 µs single pulse length, 1 s recycle delay time (dl), 0.05 s acquisition time, 1 ms contact time, 2000 scans were accumulated and externally referenced to the hexamethylbenzene signal at 17.3 ppm. All spectra were processed with line broadening (lb) 50 Hz and Gaussian function (gf) 0.003 s.

The NMR spectra of lignocellulosic materials were divided into chemical shift regions as follows: 0 to 45 ppm, alkyl C; 45 to 93 ppm, methoxyl and *O*-alkyl C; 93 to 112 ppm, di-*O*-alkyl C and some aromatics; 112 to 140 ppm, aromatic C; 140 to 165 ppm, phenolic C; and 165 to 190 ppm, carboxyl C (Preston & Forrester 2004). Areas of the chemical shift regions were determined after integration and expressed as percentages of total area (relative intensity).

RESULTS AND DISCUSSION

The amount of neutral sugars clearly showed important differences in the relative abundance of the main polysaccharides. To sound wood, the amounts of glucan, xylan, mannan contents decreased in this sequence. Variation in the amounts of neutral sugars was in accordance with brown-rot fungus behaviour that attack cellulose and hemicelluloses as a source of nourishment.

The mean values of glucan were 37.6 and 32.4% in control and attacked rubberwood samples respectively (Table 1). Glucan decreased an average of 13.8% due to *G. striatum*. In cypress, the average glucan amount was 42.4% in control and attacked samples, although it is known that brown-rot fungus prefers conifer wood.

The mean values of xylan were 10.0 and 8.7% in control and attacked rubberwood respectively (Table 1). The effect of *G. striatum* on rubberwood was on average 13.0% reduction. Cypress wood showed 7.1 and 5.7% xylan content in control and attacked samples respectively, meaning a relative decrease of 19.7%.

The mean values of mannan were 1.0 and 0.6% in control and attacked rubberwood respectively, meaning a relative reduction of 40.0% in mannan content. In cypress, the average mannan values were 5.3 and 4.8% in control and attacked samples respectively, with 9.4% reduction due to

Table 1 Chemical composition of control and attacked rubber and cypress woods

Sample	Neutral sugar[a] (%)			Lignin[b] (%)	
	Glucan	Xylan	Mannan	Insoluble	Soluble
Specimens unexposed to fungus (control)					
IAN 717	37.21 (1.13)	10.35 (1.11)	0.75 (0.53)	22.13 (0.05)	1.98 (0.07)
IAN 873	39.06 (0.75)	10.62 (0.40)	0.76 (0.03)	22.51 (0.04)	1.99 (0.08)
GT 711	36.23 (0.42)	10.39 (0.37)	0.90 (0.02)	22.92 (0.13)	1.89 (0.01)
AVROS 1301	33.12 (0.71)	9.87 (1.08)	1.11 (0.42)	25.59 (0.14)	1.60 (0.03)
RRIM 600	41.78 (0.22)	9.58 (0.10)	1.22 (0.03)	21.19 (0.11)	2.54 (0.02)
Tjir 16	38.27 (1.01)	9.31 (0.32)	1.30 (0.03)	22.78 (0.17)	1.78 (0.33)
Cupressus spp.	41.40 (1.12)	7.47 (0.13)	5.10 (0.39)	32.29 (0.06)	0.53 (0.00)
C. glauca	43.40 (3.09)	6.74 (0.60)	5.50 (0.05)	31.06 (0.05)	0.58 (0.08)
Specimens exposed to *Gloeophyllum striatum*					
IAN 717	33.46 (0.23)	9.90 (1.04)	0.81 (0.01)	25.13 (0.22)	2.31 (0.07)
IAN 873	32.02 (1.03)	8.40 (0.12)	0.43 (0.01)	26.85 (0.04)	2.58 (0.07)
GT 711	32.44 (0.98)	8.83 (0.28)	0.45 (0.03)	27.60 (0.71)	2.34 (0.08)
AVROS 1301	28.30 (2.53)	7.67 (0.92)	0.69 (0.10)	28.61 (0.07)	2.00 (0.01)
RRIM 600	30.01 (2.47)	7.92 (0.67)	0.51 (0.07)	25.67 (0.12)	2.79 (0.07)
Tjir 16	37.93 (1.53)	9.37 (0.49)	0.65 (0.11)	24.45 (0.08)	2.23 (0.09)
Cupressus spp.	41.43 (0.38)	5.57 (0.07)	5.51 (0.05)	34.26 (0.03)	0.51 (0.01)
C. glauca	43.37 (1.54)	5.77 (1.36)	4.09 (1.66)	38.50 (0.00)	0.58 (0.06)

[a] Mean values based on four repetitions, extractive-free samples and dry weight. [b]Mean values based on two repetitions, extractive-free samples and dry weight. Values in parentheses are standard deviations. Samples were exposed to the brown-rot *G. striatum* for 12 weeks.

deterioration. Rubberwood showed higher rate of mannan deterioration than cypress.

The mean values of insoluble lignin were 22.9 and 26.4% in control and attacked rubberwood respectively, showing a relative increase of 15.3% in insoluble lignin contents due to *G. striatum*. Anoymous (1974) reported 23.3% of lignin content in rubberwood. In this study, the mean values of insoluble lignin were 31.7 and 36.4% in control and attacked cypress samples respectively, meaning a relative increase of 14.8%. Rubberwood and cypress showed similar behaviour and magnitude in relation to insoluble lignin content.

The increase in lignin content can be attributed to the loss of hemicelluloses or fragile pentoses and hexoses during biodeterioration. The apparent increase in lignin contents was merely a remnant of the systematic decrease in holocellulose. It did not imply the formation of lignin during the process but the reduction of other wood components.

Amounts of about 1% of acid-soluble lignin are generally found in softwoods while amounts up to 4% of acid-soluble lignin are reported in hardwoods. Sjöström (1981) reported that the contents of Klason lignin were 26.8 to 32.1% for eight softwoods and 20.8 to 26.1% for 11 hardwoods species.

While the mechanism of brown-rot fungus on lignin is little known, some studies state that there is no enzymatic system involved. The effect of the fungi on lignin is demethylation of aryl methoxyl groups, although it is firstly oxidative and results in the formation of polymeric lignin fraction, including some cleavage of aromatic rings (Highley & Dashek 1998, Kirk 1975).

The mean values of soluble lignin were 2.0 and 2.4% in control rubberwood and attacked samples respectively, indicating an increase of 20.0% in soluble lignin content (Table 1). Cypress showed mean values of 0.6% in control and 0.5% in attacked samples.

In rubberwood, xylan was the most abundant hemicellulose and mannan, the least. RRIM 600 clone showed the highest glucan content among all the clones and consequently cellulose content. After deterioration in RRIM 600, the amounts of glucan, xylan and mannan were lower than the control, conversely to soluble and insoluble lignins.

In cypress, mannan content was lower than xylan. In the attacked samples, the amount of glucan and soluble lignin remained the same, xylan decreased and insoluble lignin increased. The absolute values of glucan, mannan and insoluble lignin were higher in cypress than in rubberwood. Many studies have suggested that hemicelluloses, due to their accessibility to enzymatic attack, are firstly utilised by decay fungi, which is characterised by degradation of their side chains, such as mannose and xylose followed by the hemicelluloses main chain (Highley 1987, Kirk & Highley 1973).

The fungus *G. striatum* aggressively degraded rubberwood hemicellulose side chains. About 40.0% reductions in mannan and 13.0% in xylan were observed, suggesting that mannan was degraded faster than xylan. Brown-rot fungus reduced glucan to 13.8%. This result agrees with the statement that glucomannans are removed faster than xylan.

Galactose and arabinose were not detected (Table 2). According to Santana and Okino (2007), it was unclear if both sugars were not present or were lost during the acid hydrolysis process and neutralisation. Furfuraldehyde was also not detected or else evaporated (volatised) probably because the xylose stabilised under the current experimental conditions. The undetected peak may be attributed to low concentration of the by-products which can be detected by the equipment.

After brown-rot fungus exposure, the acetyl groups, levulinic acid and HMF increased (Table 2). Mckibbins *et al.* (1962) reported the action of hot mineral acids in which one mole each of HMF, levulinic acid and formic acid was produced per mole of initial glucose. It was also found that the yields of HMF increased as the initial glucose concentration decreased. It seems that fungus facilitates the cleavage of the acetyl side chains, strengthened by acid hydrolysis of glucose, as shown by relative increase in acetyl groups, levulinic acid and HMF compounds in rubberwood.

Table 3 lists the summative analysis. The estimated hemicelluloses were the sum of xylan, mannan, uronic anhydride and acetyl groups. The estimated cellulose was the sum of glucan, HMF and levulinic acid. The mean values of estimated hemicelluloses and cellulose were adjusted to 100% in the summative analysis.

Since the above polymers are the sum of each specific homopolymer, the general behaviour will

Table 2 Mean values of by-products or degradation products of rubberwood *Hevea brasiliensis* and cypress *Cupressus* spp. and *C. glauca* exposed to brown-rot fungus *Gloeophyllum striatum*

Sample	Percentage[a]			
	Acetyl group	Levulinic acid	HMF	Uronic anhydride
Specimens unexposed to fungus attack (control)				
IAN 717	4.30 (0.01)	0.40 (0.01)	0.24 (0.00)	2.26 (0.04)
IAN 873	3.99 (0.05)	0.42 (0.01)	0.27 (0.01)	2.52 (0.07)
GT 711	3.94 (0.02)	0.43 (0.00)	0.26 (0.01)	2.27 (0.09)
AVROS 1301	4.23 (0.02)	0.41 (0.01)	0.25 (0.00)	2.63 (0.01)
RRIM 600	3.72 (0.02)	0.45 (0.00)	0.28 (0.01)	2.40 (0.05)
Tjir 16	3.29 (0.05)	0.43 (0.00)	0.28 (0.01)	2.44 (0.06)
Cupressus spp.	1.83 (0.02)	0.51 (0.01)	0.27 (0.18)	2.49 (0.07)
C. glauca	2.48 (0.04)	0.53 (0.06)	0.38 (0.01)	2.45 (0.07)
Specimens exposed to *Gloeophyllum striatum* attack				
IAN 717	6.30 (0.02)	0.51 (0.01)	0.33 (0.01)	2.29 (0.04)
IAN 873	5.74 (0.04)	0.54 (0.01)	0.33 (0.01)	2.55 (0.07)
GT 711	5.75 (0.03)	0.54 (0.01)	0.34 (0.01)	2.26 (0.05)
AVROS 1301	5.42 (0.03)	0.54 (0.02)	0.33 (0.01)	2.66 (0.01)
RRIM 600	6.06 (0.02)	0.54 (0.00)	0.34 (0.01)	2.37 (0.05)
Tjir 16	5.14 (0.04)	0.53 (0.01)	0.38 (0.01)	2.39 (0.06)
Cupressus spp.	1.18 0.02)	0.52 (0.01)	0.37 (0.00)	2.34 (0.02)
C. glauca	1.42 (0.01)	0.51 (0.01)	0.33 (0.00)	1.68 (0.26)

[a]Mean values are based on four repetitions, extractive-free samples and dry weight. Values in parentheses are standard deviations. HMF= 5-hydroxymethyl-2-furaldehyde. Samples were exposed to the brown-rot *G. striatum* for 12 weeks.

be about the same as the homopolymer already discussed. This is valid for total lignin as well. Therefore, minor detail will not be discussed.

There was a relative decrease of 8.9% cellulose content in rubberwood (Table 3). This phenomenon is due to fungus production of extracellular hydrogen peroxide, which breaks the glycoside bonds between the chains, where end products consist mainly of carbonyl and carboxyl groups (Ritschkoff 1996). Cypress showed a relative decrease of 3.6% cellulose.

The mean values of estimated hemicelluloses in rubberwood clones showed no clear tread. This may be because rubberwood is not the preferred wood to brown-rot. Only GT 711, AVROS 1301 and Tjir 16 showed a relative decrease of 1.3%. Once the estimated hemicelluloses are the sum of four individual components, the final behaviour is very complex due to relative changes in the contents of degraded products.

Mean values of extractive content were 3.0 and 6.9% in control and attacked rubberwood respectively. The brown-rot fungus radically changed the extractives concentration; there was a relative increment of 1.3 fold.

The increase in extractives constitutes a characteristic of the brown-rot decay pattern as observed by Martinez *et al.* (1991). All decayed wood samples showed higher percentages of ethanol–toluene extractives than sound wood. Similar findings were reported by Karppanen *et al.* (2008) where the concentration of stilbenes, resin acids and free fatty acids decreased but total phenolics increased, which obviously reflected chemical changes in cell wall constituents other than extractives. Other possible constituents can be fungal sugars or degradation products of hemicelluloses, cellulose and lignin.

Species with high contents of extractives are important because chemical compounds from the crude extracts can be used for food, pharmaceutical, dyes, cosmetics, perfumes, metal scavengers, friendly environment preservatives and natural antioxidants. On the other hand,

Table 3 Estimated values of chemical constituents of rubberwood *Hevea brasiliensis* clones and cypress *Cupressus* spp. and *C. glauca* exposed to brown-rot fungus *Gloeophyllum striatum*

Sample	Summative result (%)			Extraneous wood component (%)	
	Cellulose	Hemicelluloses	Total lignin	Extractives	Ash
Specimens unexposed to fungus attack (control)					
IAN 717	51.78	24.12	24.10	3.19	1.76
IAN 873	52.08	23.43	24.49	2.86	0.84
GT 711	51.01	24.18	24.81	2.68	0.45
AVROS 1301	47.66	25.15	27.20	3.15	0.81
RRIM 600	54.56	21.71	23.33	2.91	0.32
Tjir 16	51.90	21.77	24.56	3.45	0.77
Cupressus spp.	47.51	19.67	32.82	3.50	0.15
C. glauca	50.95	17.41	31.64	1.61	0.38
Specimens exposed to *Gloeophyllum striatum* attack					
IAN 717	46.09	26.48	27.43	8.31	1.59
IAN 873	46.40	24.17	29.43	6.84	1.36
GT 711	46.11	23.95	29.93	7.49	0.32
AVROS 1301	44.35	25.04	30.61	7.12	0.84
RRIM 600	46.24	25.30	28.46	6.19	0.60
Tjir 16	52.08	21.24	26.68	5.61	0.80
Cupressus spp.	47.99	17.25	34.77	7.20	0.20
C. glauca	46.87	14.05	39.08	6.61	0.16

Samples were exposed to the brown-rot *G. striatum* for 12 weeks.

high amounts of extractives in the production of pulp and cellulose are negatively affected by pith formation, and in wood finishing it affects the application of varnish and time of curing.

Sugars and starch are the most critical compounds in wood which cause a retarding effect on the setting of cement. Fresh rubberwood contains 1.1 to 2.3% of free sugars and 7.5 to 10.2% of starch, based on oven-dried wood (Azizol & Rahim 1989). Rubberwood retards the setting of the inorganic binder in gypsyum-bonded particleboards due to 4.6% water-soluble extractives, a mixture of free amino acids such as glutamine, arginine, alanine and asparagines (Simatupang & Schmitt 1994). The co-occurrence of rather high amounts of free carbohydrates and amino acids is the major factor governing the high susceptibility of freshly cut wood to sapstain by microfungi belonging to the fungi imperfecti.

The mean values of alcohol–toluene extractives, were 2.6% in control cypress and 6.9% in attacked samples respectively, indicating a relative increment of 1.7 fold due to fungus attack (Table 3). The increment in the extractive content may be explained by (1) rapid brown-rot depolymerisation and (2) some of the non-metabolised low molecular weight (mannose, arabinose and galactose) carbohydrate fragments becoming soluble and were leached out by the solvents during sample preparation to obtain extractive-free sample. In addition to the changes in the structure of the cell walls, Jones and Worrall (1995) found about 9.0% of fungal biomass in birch decayed by *Bjerkandera adusta* and *G. trabeum*, and Karppanen *et al.* (2008) reported the existence of fungal sugars in decayed heartwood by the presence of mycelia within the tracheids, which was verified with the SEM. However, in this study, care was taken to remove the superficial mycelia from the blocks.

Mean values of ash content were 0.8 and 0.9% in control and attacked rubbberwood samples respectively (Table 3). In cypress, ash contents were 0.3 and 0.2% in control and attacked cypress samples respectively. Simatupang

and Schmitt (1994) reported that starch- and calcium-containing crystals were also present in rubberwood vessels. Since inorganic material is not a preferential nourishment to fungus, the presence of non-organic material can be detected.

The integrations of the specific areas corresponding to the different carbon types in the CP/MAS ^{13}CNMR spectra (Figures 1 and 2) were calculated and presented in Table 4.

The brown-rot patterns under accelerated laboratory conditions were evidenced by the solid state ^{13}C NMR spectra of the degraded sample (Table 4) and by the chemical analyses presented in Tables 1 to 3. The main features of the spectra such as line shapes were mildly changed after 12 weeks of fungus exposure. The chemical shifts of the attacked samples showed a slight displacement compared with the control and changes in the relative intensities.

There are limitations in the quantitative reliability of CP/MAS spectra, mainly due to a difference in the cross-polarisation dynamics of carbons in different environments, but it is appropriate to use the spectra to compare intensity distributions among similar samples (Lorenz *et al.* 2000, Preston & Forrester 2004).

The intensity data should be regarded as semi-quantitative once their distributions in magic-angle spinning spectra are also distorted by spinning sidebands. This effect is more severe for carbons in highly anisotropic environments (aromatic, carbonyl and carboxyl carbons) and with increasing field (Preston *et al.* 1997).

During sample preparation, the carbohydrate may be leached out from the surface of the sample. Once the CP/MAS ^{13}C NMR analysis in solid state characterises the functional organic groups, this may cause a mild difference between control and attacked spectra. There was no such interference using HPLC because the extractive-free sample went through acid hydrolysis in two stages.

Comparing the spectra of rubberwood and cypress in the alkyl C (0–45 ppm), there was a peak at 20 ppm, which refers to acetyl group either in control and attacked rubberwood samples. There was 12% increment in relative intensities of alkyl C regions. In cypress, this peak almost disappeared in attacked specimens, which showed a 25% decrease. This behaviour is due to hardwoods containing more acetyl groups than softwoods, and this is corroborated by HPLC data, whereby the acetyl content of control rubberwood was 3.9% and cypress 2.2%, indicating 44% reduction compared with rubberwood.

The samples (control and decayed) were dominated by signals of polysaccharides (cellulose and hemicellulose) in the 45–112 ppm region. For control samples, cellulose peaked at 60

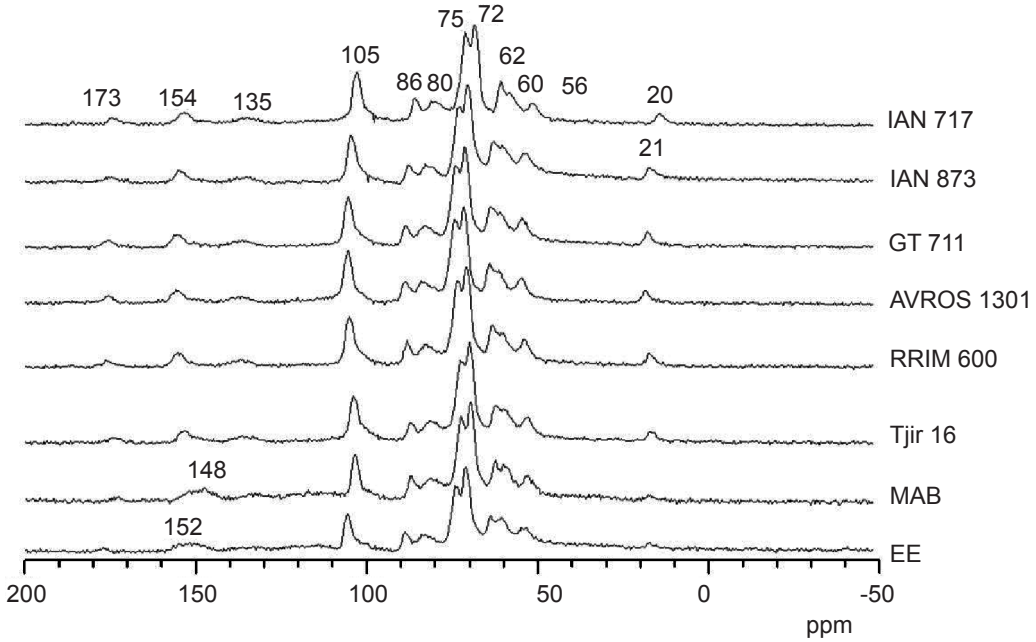

Figure 1 CP/MAS ^{13}C NMR spectra of the control sample of *Hevea brasiliensis, Cupressus* spp. (MAB) and *Cupressus glauca* (EE)

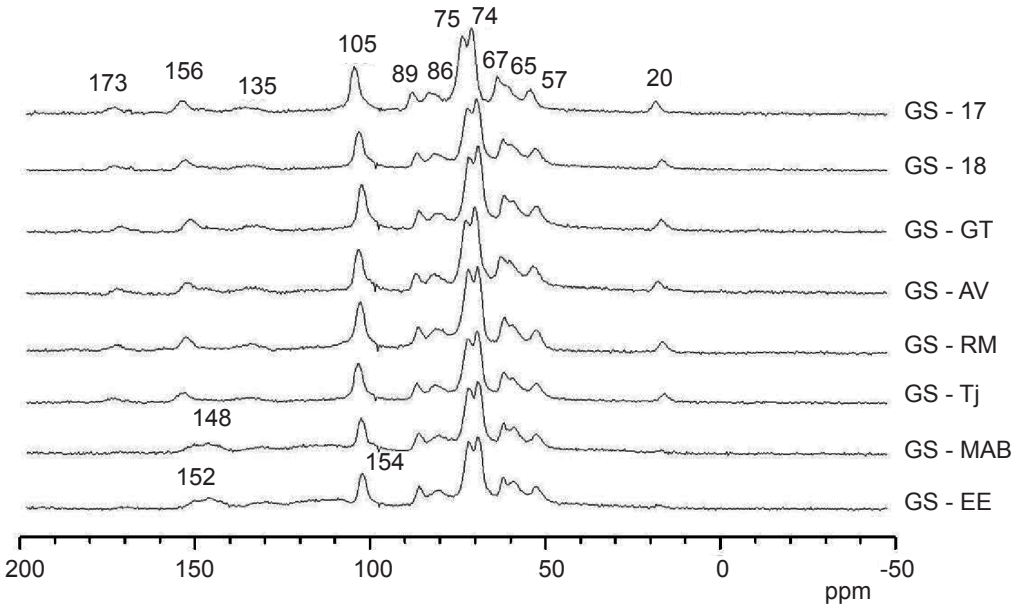

Figure 2 CP/MAS ^{13}C NMR spectra of the attacked sample of *Hevea brasiliensis, Cupressus* spp. (MAB), and *Cupressus glauca* (EE) by brown-rot fungus *Gloeophyllum striatum* (GS). Samples were exposed to the brown-rot *G. striatum* for 12 weeks.

Table 4 Relative intensities (as percentage of total area) of chemical shift integration regions of CP/MAS ^{13}C NMR spectra of rubberwood *Hevea brasiliensis* and cypress *Cupressus* spp. and *C. glauca*, before and after *Gloeophyllum striatum* attacks

Sample	Carbon type and NMR chemical shift region (ppm)					
	Carboxyl C	Phenolic C	Aromatic C	Di-*O*-alkyl C and some aromatics	Methoxyl and *O*-alkyl C	*A*lkyl C
	190–165	140–165	112–140	93–112	45–93	0–45
Specimens unexposed to fungus attack (control)						
IAN 717	5.27	7.14	10.73	11.26	48.40	17.20
IAN 873	1.54	3.91	3.66	12.33	67.76	10.79
GT 711	3.82	5.87	7.80	11.98	63.73	6.80
AVROS 1301	4.03	6.42	6.40	12.89	63.66	6.60
RRIM 600	6.28	7.30	8.25	11.07	52.69	14.42
Tjir 16	3.85	5.99	7.61	11.63	63.38	7.54
Cupressus spp.	6.53	8.65	11.29	8.67	46.73	18.13
C. glauca	6.24	8.45	11.69	9.23	48.18	16.20
Specimens exposed to *Gloeophyllum striatum* attack						
IAN 717	5.59	7.32	9.43	10.94	50.07	16.66
IAN 873	5.95	7.86	10.44	11.29	48.04	16.42
GT 711	0.99	4.24	3.54	12.18	67.37	11.69
AVROS 1301	3.54	6.50	6.82	12.54	62.41	8.19
RRIM 600	3.48	5.64	5.77	12.14	64.78	8.18
Tjir 16	5.16	7.90	7.55	13.13	56.45	9.81
Cupressus spp.	4.58	9.07	11.15	8.44	59.82	6.94
C. glauca	6.46	10.02	10.80	8.85	45.22	18.65

Samples were exposed to the brown-rot *G. striatum* for 12 weeks.

and 62 ppm (for C6 in non-crystalline and crystalline forms respectively), a doublet at 72 and 75 ppm (C2, C3 and C5), at 80 and 86 ppm (for C4 in non-crystalline and crystalline forms respectively), and at 105 ppm assigned to non-crystalline or amorphous cellulose from the anomeric C1. Broader and weaker signals from lignin could be seen at 56 ppm for methoxyl carbon, and at 112 to 165 ppm for aromatic and phenolic carbons, more specifically at 135 and 154 ppm for rubberwood, and at 148 and 152 ppm for cypress species respectively. The C1, C2, C5 and C6 of lignin contributed to the aromatic region with broad maxima at 115 ppm, not appearing here, but at 135 ppm. Signals from the 3-carbon side chain of lignin at 45 to 93 ppm were masked by the strong peaks of cellulose and hemicelluloses. The weak features at 20 and 173 ppm can be attributed to CH_3 and C=O of acetate in hemicelluloses. The acetyl peak of cypress samples at 20 ppm was not very intense, as observed in rubberwood samples, and for the first time there was a little change in this signal after fungal decay by *G. striatum*.

The selective removal of polysaccharides by brown-rot fungi caused an accumulation of lignin. As polysaccharides decreased, the relatively sharp peak at 72 ppm was influenced by a broader feature, due mainly to the 3-carbon side chain of lignin, resulting in the resonance signal at 74 ppm and shoulders at 65 and 86 ppm. Highest relative intensities at 45 to 93 ppm were observed on methoxyl and *O*-alkyl C in control and attacked rubberwood with 59.9 and 58.2% respectively. Cypress showed 47.5 and 52.5% respectively in the same region. The intensities in this region were affected by cellulose, hemicelluloses and 3-carbon side chain of lignin, where decayed cypress samples showed an increment of 11%.

Preston *et al.* (1990) reported that Klason lignin from well decomposed wood caused loss of side chains and increased condensation of aromatic rings, leading to loss of *O*-alkyl C intensity and broadening of the aromatic region, but this behaviour was not observed in Figures 1 and 2. There was also some quantitative increment in the alkyl C region, except GS-I7, GS-RM and GS-MAB, possibly due to CH_2 methylene groups bridging aromatic rings. Preston *et al.* (1998) stated that methoxyl region is particularly difficult to determine accurately due to overlapping with the alkyl and *O*-alkyl regions. The carboxyl peaks at 173 ppm showed that there had been some oxidation, while the broad, weak signal in the alkyl region probably arose from waxes and resins which were concentrated by the decay process. The principal changes verified by Kirk (1975) in rotted lignin of Spruce wood include decrease in methoxyl content, oxidations of some alcohol and aldehyde groups to carboxyls, and introduction of some phenolic hydroxyls. Nevertheless, major signals of these aspects were not observed in the studied spectra and the lack of unique signals for specific biopolymers (carbohydrate, lignin) made it difficult to use NMR in determining their proportions (Preston *et al.* 1997).

For guaiacyl lignin, the phenolic region of attacked samples had a slight peak at 148 ppm, and a shoulder at around 152 ppm. These displacement shifts showed little deviation from those shown by Preston and Forrester (2004), while the peak positions were affected by the degree of etherification at C-4.

In our study, the resonance signal at 154 ppm for rubberwood species displaced to 156 ppm but did not disappear. Such behaviour could arise from cleavage of the β-*O*-4 linkage from demethylation as observed by Davis *et al.* (1994c), and Hemmingson and Newman (1985) in attacked samples.

Kim and Newman (1995) reported that the spectra of *Pinus koraiensis* degraded by *G. trabeum* showed no evidence for preferential degradation of the non-crystalline cellulose, loss of hemicelluloses and detectable loss of lignin. The most prominent peaks were at 72, 74 and 105 ppm for both rubberwood and cypress species. These peaks showed that on further attacked species a clear split peak occurred at 65–67 ppm, different from those hardwood lignins spectra shown in Martínez *et al.* (1999). NMR spectra emphasised the complex understanding of the lignocellulosic material compound.

There were increments in relative intensities of di-*O*-alkyl C, some aromatics (93–112 ppm) and phenolic C (140–165 ppm) in the rubberwood samples. In cypress, there was decrease of 3, 4 and 14% in di-*O*-Alkyl C, some aromatics (93–112 ppm), to aromatic C (112–140 ppm) and to carboxyl C (190–165 ppm) respectively. Conversely, cypress also showed 12% in phenolic C (140–165 ppm) due to *G. striatum* attack which was related to lignin polymer contribution in the sample.

CONCLUSIONS

Gloeophyllum striatum fungus caused an increment in the percentage of ethanol–toluene extractives. This brown-rot fungus also affected the amounts of detected monosaccharide, showing a higher rate of deterioration for mannan, xylan and glucan. A relative increment in total lignin was observed.

Cupressus glauca showed higher amount of xylan than mannan. *Hevea brasiliensis* showed higher amounts of acetyl groups and xylan contents. Rubberwood showed a relative decrease of 8.9% in cellulose content, no clear trend in hemicelluloses and an increase of 16.0% in total lignin. Cypress showed a slight decrease of 3.6% in cellulose content, a relative decrease of 15.1% in hemicelluloses and an increase of 14.6% in total lignin.

HPLC was confirmed as a feasible and accurate technique to evaluate the fungus response to micro-chemical changes in wood. Semi-quantitative CP/MAS ^{13}C NMR data corroborated the chromatographic results, showing that both techniques are complementary in monitoring degradation changes.

The main features of the CP/MAS ^{13}C NMR spectrum for attacked samples were found at 20 ppm (acetyl), 56/57 ppm (methoxyl), 72/74 and 75 ppm (*O*-alkyl C including carbohydrates), 105 ppm (di-*O*-alkyl C including anomeric C of carbohydrates), 112–165 ppm (aromatic and phenolic) and a broad, weak region for acyl derivatives at 173 ppm.

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