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ORIGINAL

Chemical alterations induced by *Pycnoporus sanguineus*/*Aspergillus flavipes* co-cultures in wood from different tree species

Andrea van Heerden · Niel J. le Roux · Jan Swart · Tim Rypstra · Sugnet Gardner-Lubbe · Alfred Botha

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Abstract Chemical alterations following inoculation of *Acacia mearnsii*, *Eucalyptus dunnii*, *E. grandis*, and *E. macarthurii* with a *Pycnoporus sanguineus*/*Aspergillus flavipes* co-culture were investigated. Several wood chemical parameters were measured using standard methods from the pulp and paper industry. The data were described and analyzed using univariate as well as multivariate statistical techniques. Boxplots and in particular biplots show clearly how the chemical composition of each tree species was differently affected by the co-culture. Lignin content was significantly decreased in *A. mearnsii*, while *E. dunnii* showed a decrease in cellulose content. The results, therefore, indicate that the manner in which wood is degraded by a specific fungal co-culture depends on the tree species involved. This phenomenon should be considered when selecting fungi for biopulping.

Introduction

Among the microorganisms responsible for wood degradation, fungi are the dominant group in terrestrial ecosystems (Schwarze et al. 2000). They use various processes to degrade woody tissues resulting in three forms of decay: brown-rot,

A. van Heerden · A. Botha (✉)
Department of Microbiology, Stellenbosch University, Private Bag X1, Stellenbosch, South Africa
e-mail: abo@sun.ac.za

N. J. le Roux · S. Gardner-Lubbe
Department of Statistics and Actuarial Science, Stellenbosch University, Private Bag X1, Stellenbosch, South Africa

J. Swart · T. Rypstra
Department of Forest and Wood Science, Stellenbosch University, Private Bag X1, Stellenbosch, South Africa

soft-rot, and white-rot. The latter form of decay is caused by a group of fungi that is able to degrade lignin in addition to cellulose and hemicelluloses (Otjen and Blanchette 1986; Myneni et al. 2001). White-rot fungi, for example *Fomes fomentarius*, simultaneously degrade cellulose, hemicelluloses, and lignin, while *Ganoderma pheifferi* selectively degrades lignin and hemicelluloses first (Schwarze et al. 2000). *Pycnoporus sanguineus*, a known white-rot fungus, shows a selective delignification pattern on poplar wood (*Populus deltoides*), while exerting a simultaneous delignification pattern when cultured on *Eucalyptus grandis* wood (Ferraz et al. 1998; Luna et al. 2004). This indicates that this white-rot fungus may exhibit different delignification patterns depending on the wood substrate it grows on.

The majority of studies on delignification of wood focused on pure cultures of white-rot fungi (Luna et al. 2004). The effect of co-cultures on wood is, however, closer to the situation in nature, as consortia of microbes are known to degrade lignocellulosic material (Watanabe et al. 2003). Studies by Dommisse in 1998 indicate that a co-culture of *P. sanguineus* and *Aspergillus flavipes* enhanced the pulping properties of *E. grandis*. *Aspergillus flavipes* is a known pioneer fungus on wood and utilizes the readily available sugars (Schwarze et al. 2000). Studies by Van Heerden et al. (2008) indicated that the *P. sanguineus/A. flavipes* co-culture caused less cellulose degradation in *E. grandis* compared to the mono cultures of this white-rot fungus.

Since literature indicates that *P. sanguineus* may have the ability to exhibit different degradation patterns, it could be asked whether the effect of a *P. sanguineus/A. flavipes* co-culture on the chemical composition of wood differs between tree species. For possible implementation in bio-pulping, the aim of this study was to investigate chemical alterations in three *Eucalyptus* and one *Acacia* species originating from one geographical area, as induced by a *P. sanguineus/A. flavipes* co-culture. Knowledge of this will play a significant role in matching tree species and fungal co-cultures for bio-pulping in the pulp and paper industry.

Materials and methods

Preparation of wood samples and *P. sanguineus/A. flavipes* co-cultures

This investigation was part of an extensive pulpwood evaluation of four, 12-year old plantation species, i.e. *Acacia mearnsii*, *Eucalyptus dunni*, *E. grandis,* and *E. macarthurii*, from the eastern Highveld geographical area in South Africa. For each species, 10 trees were selected in such a way as to get the largest possible biological variation in the research material. For every species, five localities (East, West, North, South, and Center) were identified within their distribution area. Furthermore, in each locality, two trees within a radius of 50 m from each other were randomly selected. All debarked logs of each of the forty sampled trees (ten trees and four species) were chipped with a Wigger pilot-plant chipper, and the chips screened and classified into thickness classes. The chips were stored in sample bags at −4°C.

Only wood chips with a thickness greater than 6 mm and less than 9 mm were used for experimentation. To enhance fungal degradation, some of the chips were pre-treated with a hot water wash at 150°C for 2 h (unpublished results) in an oscillating, 15-dm^3 pressure vessel. At 150°C, the vessel degassed automatically from 800 to 0 kPa, thereafter, the pressure was increased again to 800 kPa where it remained for 25 min until the end of the pre-treatment period.

The moisture content of the wood was determined by drying sub-samples of the chips (±20 g each) in an oven for 14 h at 102°C, before weighing them. To obtain a final moisture content of 60% for the chips, a nutrient supplement [5% (w/v) molasses and 0.28% (w/v) urea] as well as an appropriate volume of fungal inoculum was added to the wood chips (Wolfaardt et al. 2004).

A strain representing *P. sanguineus* was obtained from the fungal culture collection of the ARC-PPRI (Agricultural Research Council—Plant Protection Research Institute), Pretoria, South Africa. This white-rot fungus (*Pycnoporus sanguineus* PPRI 6762) as well as *Aspergillus flavipes* J11904 is being maintained at 22°C on malt extract-agar (MEA, Biolab) in the fungal culture collection of the Department of Microbiology, University of Stellenbosch, South Africa.

Inocula of *A. flavipes* J11904 and *P. sanguineus* PPRI 6762 were made by growing each strain at 30°C for 1 week in a 5% (w/v) molasses broth, whereafter each culture was homogenized using a blender (Pineware) for 30 s. The homogenized fungal biomass was then used to inoculate wood chips from different tree species (Table 1). One kilogram wood chip samples, representing different tree species, were then each incubated at 30°C in a closed, cylindrical bio-reactor (17 cm high and 23 cm in diameter), while being aerated from the bottom of the reactor with 10 L min^{-1}, sterile, moist air (Van Heerden et al. 2008). Inoculated bio-reactors each received 1.8×10^{-4} g and 1.7×10^{-4} g dry fungal biomass per gram oven-dried wood of *A. flavipes* and *P. sanguineu*, respectively. Cultures were harvested after 2 weeks.

Chemical analyses of wood chips

Chemical analyses were performed on the following: the residual wood chips obtained after hot water wash and fungal cultivation, the un-inoculated, hot water-washed chips (to provide a base-line), and on the un-inoculated, non-hot water-washed wood chips. The various treatment combinations used together with their acronyms are shown in Table 1. To prevent loss of extractives, wood chips were not rinsed after the 2-week incubation period and prior to chemical analyses. The chemical analyses performed were:

1. Soxhlet extractions (TAPPI Standard Methods T 264 om-88), to determine both percentage [%] of alcohol-benzene (solvent-borne) extractive content (EBE) and percentage [%] of water-soluble (polar or water-borne) extractive content (EH2).
2. The percentage [%] of lignin content (Lig) was determined as Klason lignin (TAPPI Standard Methods T 222 om-88) and

Table 1 Different treatments of wood chips originating from the different tree species including inoculation with *P. sanguineus* PPRI 6762/*A. flavipes* J11904 co-cultures

Tree species and treatment of wood chips	Acronym	Number of replicate chemical analyses
Acacia mearnsii; no hot water wash; no inoculum	AmUN	27
Acacia mearnsii; hot water wash; no inoculum	AmWO	3
Acacia mearnsii; hot water wash; co-culture inoculum	AmWB	3
Eucalyptus dunnii; no hot water wash; no inoculum	EdUN	30
Eucalyptus dunnii; hot water wash; no inoculum	EdWO	3
Eucalyptus dunnii; hot water wash; co-culture inoculum	EdWB	3
Eucalyptus grandis; no hot water wash; no inoculum	EgUN	30
Eucalyptus grandis; hot water wash; no inoculum	EgWO	3
Eucalyptus grandis; hot water wash; co-culture inoculum	EgWB	3
Eucalyptus macarthurii; no hot water wash; no inoculum	EmUN	30
Eucalyptus macarthurii; hot water wash; no inoculum	EmWO	3
Eucalyptus macarthurii; hot water wash; co-culture inoculum	EmWB	3

3. the percentage [%] of cellulose content (Cel) was determined using the Seifert method (Browning 1967).

All the results of the chemical analyses are reported as percentages based on oven-dry wood mass. This does not influence the results since care was taken to ensure that the differences among corresponding gram values were negligible.

Results and discussion

Statistical analyses

As shown in Table 1, three replicate chemical analyses were performed on a single co-culture-treated sample of each species, as well as on an un-inoculated sample obtained for each tree species after the hot water wash. The untreated data for each tree species consisted of ca. 30 observations: three chemical analysis replicates of chip samples originating from the two trees randomly selected from the five locations to represent the area of largest biological variation. The complete dataset consists of the four measurements, % cellulose content (Cel), % lignin content (Lig), % alcohol-benzene-soluble extractive content (EBE), and % water-soluble extractive content (EH2), made on each of the 141 chemical analyses indicated in Table 1.

The dataset can be written in the form of a species × treatment table. Therefore, the first question to be addressed concerned the interaction between these two factors. This was done by performing a two-way multivariate analysis of variance (MANOVA). This is a standard statistical technique for analyzing the differences between treatment combinations when there are more than one

response variable. For a detailed discussion of MANOVA, see for example Johnson and Wichern (2007). From Table 1, it follows that each cell in the two-way table representing the dataset did not contain the same number of replicates; therefore, the order of terms entering the MANOVA model had to be taken into account. This MANOVA resulted in a highly statistically significant species × treatment interaction with an associated P value approaching zero. Accordingly, the MANOVA was followed by performing two-way univariate analysis of variance (ANOVA) procedures treating in turn each of Cel, Lig, EBE, and EH2 as the response variable. The ANOVAs resulted in the following: highly statistically significant species × treatment interactions in the case of Cel (P value approaching zero), EBE (P value approaching zero) and Lig (P value = 0.03) but a negligible interaction in the case of EH2 (P value = 0.27). In the latter case, both the species and treatment main effects are statistically highly significant with P values < 0.002. Following all the above significant ANOVAs, simultaneous Tukey 99% confidence intervals (Scheffé 1959) were calculated for all pair-wise comparisons between the effects concerned. An interval excluding zero indicates a statistically significant difference between the two means at a 1% significance level. The results of these analyses substantiate the graphical display of the dataset in the form of notched box plots in Fig. 1 and a biplot in Fig. 2. Discussion of these figures is deferred to the next section.

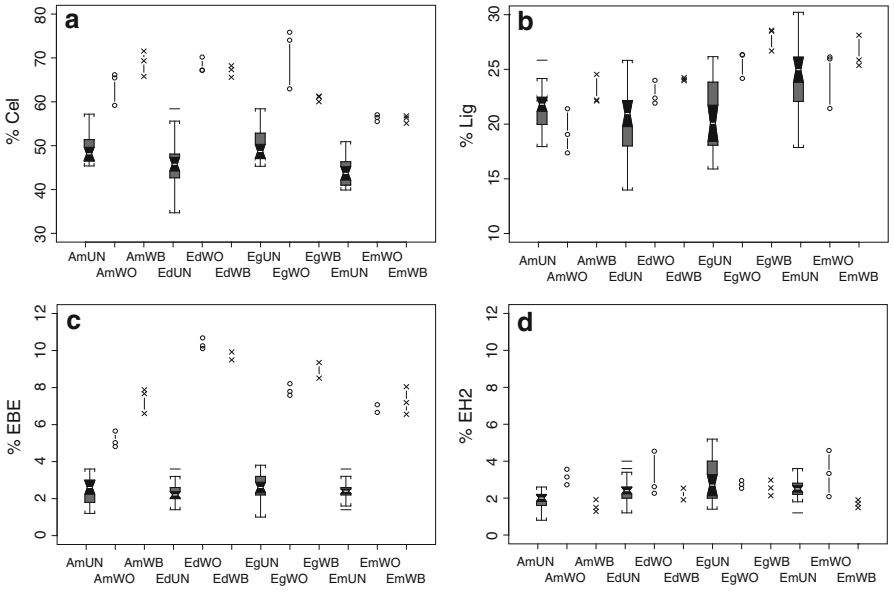

Fig. 1 Notched box plot representation of differences between tree species. **a** Percentage cellulose in wood after treatments (% Cel). **b** Percentage lignin in wood after treatments (% Lig). **c** Percentage alcohol-benzene extractive content in wood after treatments (% EBE). **d** Percentage water-soluble extractive content in wood after treatments (% EH2). Explanations of the acronyms on the *horizontal axes* are listed in Table 1

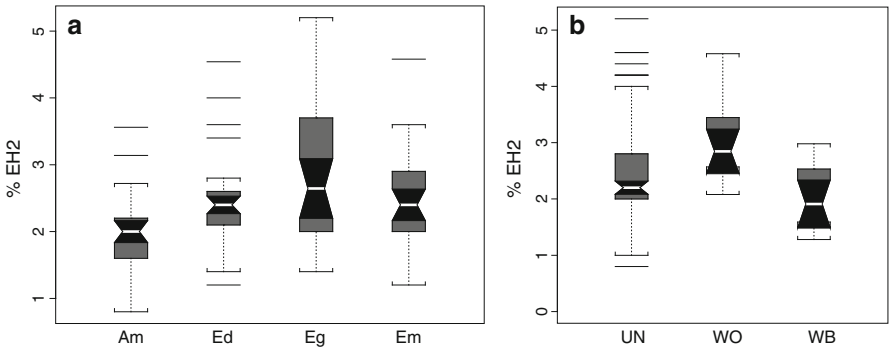

Fig. 2 Notched box plots representation of differences in water soluble extractives. **a** Main effect of tree species. Am = *A. mearnsii*; Ed = *E. dunnii*; Eg = *E. grandis*; Em = *E. macarthurii.* **b** Main effect of treatment. Un = Untreated; WO = Hot water-washed with no inoculum; WB = Hot water-washed with inoculum

Degradation of wood chemical components by *P. sanguineus/A. flavipes* co-cultures

The results of Seifert analyses conducted on untreated wood chips, on hot water-washed wood chips, and on residual wood chips after a 2-week incubation period of a *P. sanguineus/A. flavipes* co-culture are depicted in Fig. 1a. Interestingly, the cellulose content of all four wood species, which were all similar in the untreated state, increased after the 2 h hot water wash at 150°C. These findings were supported by Tukey's simultaneous confidence intervals. This increase may be a result of the hot water extraction of some of the hemicelluloses during the latter process with the concomitant increase in the relative cellulose content. It is known that some hemicelluloses are hydrolyzed by hot water and steam, leading to leaching of the resultant monosaccharides from the wood (Rowell et al. 2002; Williams 2005).

Fungal treatment of *A. mearnsii*, *E. dunnii*, and *E. macarthurii* hot water-washed wood chips resulted in no significant change in the cellulose content of the wood (Fig. 1a and supported by Tukey's simultaneous confidence intervals that include zero). Interestingly, although the results depicted in Fig. 1a suggested that the fungal co-culture decreased the cellulose content of hot water-washed *E. grandis* wood chips, these findings were not supported by Tukey's simultaneous confidence intervals.

The results of Klason lignin analyses conducted on untreated wood chips, on hot water washed wood chips, and on residual wood chips after a 2-week incubation period of a *P. sanguineus/A. flavipes* co-culture are depicted in Fig. 1b. Although a tendency was noted in Fig. 1b for the hot water wash to increase the lignin content of untreated *E. dunnii* and *E. grandis* wood chips, which may be ascribed to the removal of the hemicelluloses during the hot water wash with the concomitant increase in the relative lignin content (Nuopponen et al. 2004; Garcia et al. 2006), these findings were not supported by Tukey's simultaneous confidence intervals.

Figure 1b shows a tendency for fungal growth to increase the lignin content of the wood. However, this observation is not supported by Tukey's simultaneous confidence. This tendency may be ascribed to modification of the lignin polymer during the hot water wash as a result of excessive heat, rendering it recalcitrant against fungal degradation (Weiland and Guyonnet 2003), thus increasing the relative content of this polymer during degradation of other wood components. The perceived increased lignin content during fungal growth may also have been a result of plant protein, as well as protein from fungal biomass that remain insoluble during the extraction process (Hatfield and Fukushima 2005).

Analyses of the alcohol-benzene-soluble extractives obtained from the treated wood revealed significant increases in all the samples after the hot water wash (Fig. 1c) and is also supported by the Tukey's simultaneous confidence intervals excluding zero. These extractives mainly consist of aliphatic ketones, alkanes, fatty acids, sitosterol esters, triglycerides, and waxes (Gutiérrez et al. 1999). The hot water wash may have removed some of the hemicelluloses thereby, increasing the relative quantities of alcohol-benzene-soluble extractives and/or may have increased the availability of the lipophilic compounds during analysis (TAPPI Tests Methods, T264 om-88).

Fungal treatment of *A. mearnsii* wood chips resulted in an increase in the alcohol-benzene-soluble extractives present in hot water-washed wood (Fig. 1c and supported by the corresponding Tukey's simultaneous confidence intervals). Though not supported by Tukey's simultaneous confidence intervals, a tendency was noted for these extractives to increase during fungal treatment of hot water-washed *E. grandis* wood chips (Fig. 1c). As pointed out by Van Heerden et al. (2008), these increases may be the result of fungal anabolism or the degradation of wood components by the fungi. Fungal treatment of *E. macarthurii* had no effect on the alcohol-benzene-soluble extractives (Fig. 1c) while this treatment tended to decrease the alcohol-benzene-soluble extractives in *E. dunnii* wood chips. The latter was not supported by Tukey's simultaneous confidence intervals. Nevertheless, these results on alcohol-benzene-soluble extractives indicate differences in the lipid metabolism and interactions within the fungal co-culture when growing on different wood species.

The data obtained after analyses of the water-soluble extractives in the wood were displayed in the form of notched box plots in Fig. 1d. These water-soluble compounds include amino acids, phenols, simple sugars, and starches (Martin and Aber 1996). No obvious difference between the treatment classes was revealed after the hot water wash, except for an increase in the quantities of these water-soluble compounds after the hot water wash of *A. mearnsii* wood chips. Since the two-way ANOVA, performed with "% Water-soluble extractives" as response variable, revealed negligible interaction between "species" and "treatment", the highly significant main effects could be considered separately (see Fig. 2). Simultaneous pair-wise 95% confidence intervals associated with the species main effect revealed that differences do exist between some tree species regarding the water-soluble extractive content. Thus, as shown in Fig. 2a, the concentrations of these water-soluble compounds were significantly lower in *A. mearnsii* than in wood chips from all the other species. The concentration of water-soluble compounds in *E. dunnii*

was significantly lower than that of *E. macarthurii* although it is not reflected in the median differences in Fig. 2a.

Furthermore, when untreated wood chips were hot water washed, a significant increase in the water soluble extractives generally occurred in all the wood species (Fig. 2b and supported by Tukey's simultaneous confidence intervals). The treatment main effect applies to all the wood species since there was no statistically significant interaction found. The water-soluble extractives, which after fungal cultivation may include degradation products of wood components and the water extractable fraction of fungal biomass, generally decreased statistically significantly upon fungal treatment (Fig. 2b and supported by Tukey's simultaneous confidence intervals).

Although the boxplots in Figs. 1 and 2 provided important insights into the diverse ways that the chemical properties of the different wood species were affected by the fungi, they suffer from a critical drawback: they are univariate displays of the data not able to show graphically the interactions which were shown to exist in the dataset. The biplots provided in Figs. 3, 4, and 5 overcome this shortcoming.

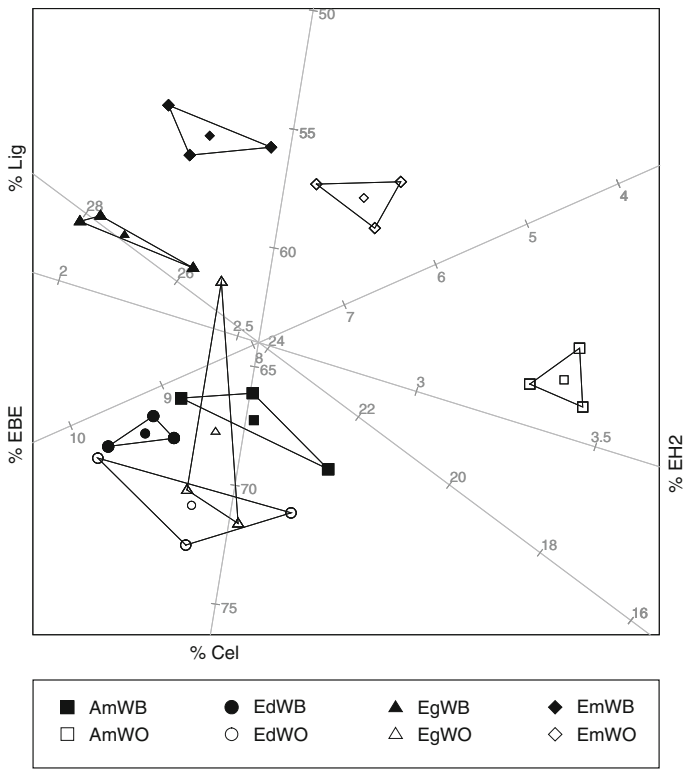

Fig. 3 PCA biplot showing differences among the class means in the center of each group. *Solid symbols* = Fungal-treated wood chips; *Open symbols* = Untreated wood chips; % Cel = % cellulose in wood after treatments; % Lig = % lignin in wood after treatments; % EBE = % alcohol-benzene extractive content in wood after treatments; % EH2 = % water-soluble extractive content in wood after treatments

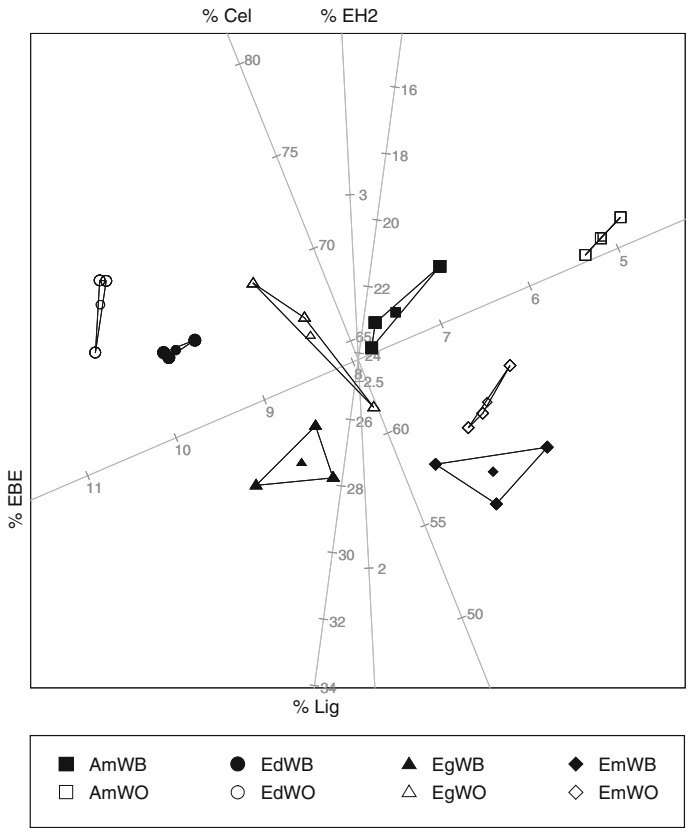

Fig. 4 CVA biplot displaying differences between the culture combinations. *Solid symbols* = Fungal-treated wood chips; *Open symbols* = Untreated wood chips; % Cel = % cellulose in wood after treatments; % Lig = % lignin in wood after treatments; % EBE = % alcohol-benzene extractive content in wood after treatments; % EH2 = % water-soluble extractive content in wood after treatments

To investigate chemical alterations in the different wood species induced by the fungi while growing on the hot water-washed wood chips, a series of biplots were constructed (Figs. 3, 4, 5). A principle component analysis (PCA) biplot of the data revealed that despite the relative large variation observed within some treatments (*A. mearnsii* inoculated with the co-culture and hot water-washed *E. dunnii* and *E. grandis* wood chips), the fungal co-culture altered the chemical composition of all the tree species (Fig. 3). Notice that the biplot provides a detailed display of the differences in chemical composition. Using this biplot as a multivariate scatterplot, it is clear that each pair of untreated and fungal-treated groups do not overlap. The *E. dunnii* groups are close to one another, but the fungal-treated samples are lower in % cellulose content. These two groups overlap on the other three chemical characteristics. The pairs for *A. mearnsii* and *E. macarthurii* differ most notably on % lignin content. The biplots in Figs. 4 and 5 are constructed to optimally separate the class means but can be interpreted similarly as multivariate scatterplots. To investigate whether the *P. sanguineus/A. flavipes* co-culture impacted differently on

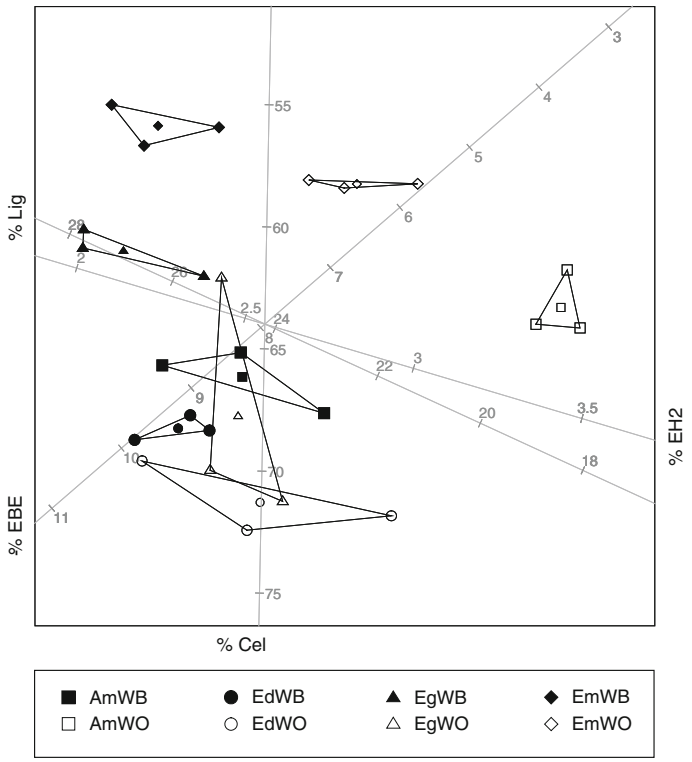

Fig. 5 AOD biplot displaying differences between the culture combinations. *Solid symbols* = Fungal-treated wood chips; *Open symbols* = Untreated wood chips; % Cel = % cellulose in wood after treatments; % Lig = % lignin in wood after treatments; % EBE = % alcohol-benzene extractive content in wood after treatments; % EH2 = % water-soluble extractive content in wood after treatments

the chemical composition of wood from different tree species, a canonical variate analysis (CVA) biplot was constructed (Fig. 4). The construction of a CVA biplot, however, makes use of equality of the within classes covariance matrices. Inspection of the boxplots in Fig. 1 as well as the PCA biplot in Fig. 3 may cast a shadow of doubt on the truth of this assumption. Consequently, an analysis of distance (AOD) biplot that does not require this assumption was constructed (Fig. 5). From the resulting biplot, it was obvious that the fungal co-culture impacted differently on the chemical components measured in wood chips from the various tree species. For example, despite the relative large variation observed within some treatments, such as *A. mearnsii* inoculated with the co-culture and hot water-washed *E. dunnii* wood chips, it was clear that the co-culture notably increased the perceived Klason lignin content of the *A. mearnsii* wood chips, while such an obvious fungal-induced increase was not observed for the *E. dunnii* chips. In the latter case, the most prominent alteration was a decrease in the cellulose content of the wood chips during fungal growth. In contrast, growth of the fungal co-culture increased the relative cellulose content of the *A. mearnsii* chips. *E. grandis* chips were affected similarly but with more variation in the cellulose

content of the untreated samples. *E. macarthurii* chips show low % cellulose content for both untreated and fungal-treated samples.

The investigation was primarily focused to determine the co-culture and hot water washing effects on the relative concentrations of the chemical components in selected wood species. Further investigation into the mechanisms of extraction and fungal degradation as influenced by anatomical and macromolecular characteristics, as well as the types and amounts of extractives, would hopefully provide answers to why the fungal co-culture and timber species combinations exhibit different degradation patterns. Macromolecular characteristics that need to be considered in such experiments include types and amounts of hemicelluloses, degree of polymerization, polydispersity, type of lignin-polysaccharide complexes, as well as amount and type of lignin (ratio of p-hydroxy phenyl, guaiacyl and syringyl). The latter is important since it is known that the lignin content of *Eucalyptus* species is generally higher than that of *Acacia* (Kumar and Gupta 1992) and differs in composition with respect to the guaiacyl and syringyl units. Since white-rot fungi degrade syringyl preferentially to guaiacyl, and lignin serves as the barrier to cellulose, the ratio of these units in wood may play a pivotal role in determining the degradation rate of different wood species (Yu et al. 2008).

Conclusion

The authors' results indicated that the *P. sanguineus/A. flavipes* co-culture altered the chemical composition of each tree species in a different manner. This agrees with the highly significant interaction found in the MANOVA. This phenomenon may have implications in the ecology of lignocellulosic degradation in the natural environment. Since lignocellulosic degradation is important in the bio-pulping industry, the results also indicate that optimization of the bio-pulping process may depend both on the fungi used and the tree species involved.

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