Flavonoid Supplementation Reduces the Extractive Content and Increases the Syringyl/Guaiacyl Ratio in *Eucalyptus grandis x Eucalyptus urophylla* Hybrid Trees

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The *Eucalyptus* genus plays an important role in the worldwide forest industry, with highly productive plantations supplying high-quality raw material for pulp and paper, wood, and biomass that would otherwise come from native forests. Lignin and extractives are important components for wood structure and protection but they are disruptive elements with respect to some industrial processes involving paper, pulp, and biomass production. This work evaluated effects of supplementation of flavonoids on the wood composition of *Eucalyptus grandis* x *Eucalyptus urophylla* (*E. urograndis*), a commercial hybrid. The wood samples were analyzed for extractives and lignin contents by wet chemical analysis, and the composition of lignin monomers and the carbohydrate hexosan/pentosan ratio were determined by analytical pyrolysis. The results showed that supplementation with the flavonoids naringenin and naringenin-chalcone led to an overall reduction of the extractive content and altered the monomeric composition of lignins towards a higher syringyl content. Thus, the treatment of *Eucalyptus* with flavonoids results in the improvement of wood quality for technological purposes.

Keywords: Eucalyptus; Extractives; Lignin; Flavonoids

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INTRODUCTION

Wood is a natural resource that forms the basis of a global industry producing fiber, timber, and energy, and it is the fifth most important world trade product (Foucart *et al.* 2009). Wood represents one of the most important sources of energy and biomass on Earth and constitutes an environmentally friendly and renewable alternative to fossil resources. Moreover, wood is an important natural sink for carbon dioxide, one of the major causes of global warming due to the greenhouse effect (Demura and Fukuda 2007).

Eucalyptus is the most widely planted hardwood crop in the tropical and subtropical world due to its superior growth, broad adaptability, and multipurpose wood properties (Hu *et al.* 1999). Plantation-grown *Eucalyptus* supplies high-quality woody biomass for several industrial applications while reducing the pressure on tropical forests and their associated biodiversity (Hu *et al.* 1999).

The chemical composition of wood plays an important role in wood applications, especially in chemical conversion. Wood extractives, the non-cell wall components that can be removed by solvents, are necessary for protecting the living tree and derived wood products against disease; however, extractives can be detrimental to pulp and paper, paint, and varnish films and adhesives (Alves *et al.* 2012). Pulpwood from tropically-grown eucalyptus trees, including those grown in Brazil, contains a higher content of extractives than those grown in Europe, accounting for a decrease of up to 4% in the pulping yield (Gomide *et al.* 2005). The extractive content is lower at a young harvesting age, and the content increases rapidly with aging (Gomide *et al.* 2005). Lignin composition is also a very important parameter. *Eucalyptus* lignins are composed of syringyl (S) and guaiacyl (G) units in varying proportions. High syringyl/guaiacyl (S/G) ratios are advantageous for pulp production due to higher delignification rates, reduced chemical consumption, and higher pulp yields (Rodrigues *et al.* 1999). Because of high tree-to-tree variation (Rodrigues *et al.* 1999) and low variation within a tree, lignin composition is a wood trait under strong genetic control (Stackpole *et al.* 2010) even when tension wood is present (Rodrigues *et al.* 2001). Therefore, extensive research efforts are focused on comprehending the lignification process in an effort to design trees through genetic engineering that either have reduced lignin content, produce lignins that are more susceptible to chemical degradation (Weng *et al.* 2008; Mansfield *et al.* 2009), or have altered lignin content (Valerio *et al.* 2003).

Lignin synthesis is a relatively well-established process that starts with the assemblage of radicals produced during the single-electron oxidation of monolignols (Baucher *et al.* 2003). The units resulting from the monolignols, when incorporated into the lignin polymer, are called guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) (Vanholme *et al.* 2010).

The monolignols are synthesized from the aminoacid phenylalanine through the phenylpropanoid and monolignol-specific pathways. Phenylalanine is derived from the shikimate biosynthetic pathway in the plastid (Boudet *et al.* 2003; Rippert *et al.* 2009), which is responsible for the synthesis of a wide variety of secondary metabolic compounds, including lignin and phenolic extractives (Dixon *et al.* 1996; Vogt *et al.* 2010).

The catalytic step performed by the enzyme 4-coumaroyl:CoA-ligase (4CL) likely represents the most important branch point within the central phenylpropanoid biosynthesis pathway in plants (Campbell and Sederoff 1996; Voo *et al.* 1995). Through 4CL activity, cells can produce the precursors for either flavonoid biosynthesis or guaiacyl (G) and syringyl (S) lignin units (Vogt, 2010). The product of 4CL, p-coumaroyl-CoA, is the substrate of the enzyme chalcone synthase(CHS) (Besseau *et al.* 2007), the committing step in flavonoid biosynthesis. This pathway is reviewed in detail elsewhere (Besseau *et al.* 2007; Vanholme *et al.* 2010).

The flavonoids naringenin-chalcone and naringenin, synthesized by the enzymes chalcone synthase (CHS) and chalcone isomerase (CHI), respectively, constitute the primary C15 intermediates in flavonoid biosynthesis (Moustafa 1967;Chen *et al.* 2011). Naringenin was reported to inhibit the activity of 4CL (Voo *et al.* 1995), and this inhibition is positively associated with naringenin sensitivity in several plant species (Deng *et al.* 2004; Yun *et al.* 2009).

The objective of the present work was to evaluate the effects of naringenin-chalcone and naringenin supplementation on the wood composition of *E. urophylla x E. grandis*, a commercial hybrid referred to hereafter as *E. urograndis*.

EXPERIMENTAL

Materials

Plantlets of a 6-month-old commercial clone of *Eucalyptus urograndis* were provided by International Paper (Mogi-Guaçu, Brazil) and grown in a greenhouse. The plantlets were divided into 5 groups, according to supplementation conditions (apart from the standard nutritional solution supplied to all groups) as follows: control group (CT); experimental group 1 (CH), supplemented with 0.1 mmol of naringenin-chalcone for 5 months; experimental group 2 (NAR), supplemented with 0.1 mmol of naringenin for 5 months; experimental group 3 (CHSTOP), supplemented with 0,1 mmol of naringenin-chalcone for only the first month; and experimental group 4 (NARSTOP), supplemented with 0.1 mmol naringenin for only the first month. All solutions were administered by root application at approximately 100 to 150 mL daily. The treatments lasted 5 months. The composition of the standard nutritional solution has been described previously (Sarruje 1975). At the end of the experiment, all 5 groups of plantlets were cut, and the stems were kept for analysis; no growth differences were observed between the control and the treatment groups. A total of 30 plantlets were individually analyzed; 9 were only used for histology and 21 for chemical analyses. The same extracted samples were used for pyrolysis. All samples were analyzed 5 months after the start of the experiment regardless of their supplementation. The main wood stems were debarked and ground in a Thomas–Wiley mill model ED-5 to pass a 1 mm sieve and screened in a vibratory sieving apparatus, and the 40 to 60 mesh wood meal fraction was retained for analysis.

Naringenin (4'-,5-,7-trihydroxyflavanone, 95%) and naringenin-chalcone (1,3-diphenyl-2-propen-1-one, 97%) were purchased from Sigma-Aldrich Co. (Tokyo, Japan) and AcrosOrganics Co. (Tokyo, Japan), respectively.

Histology

After harvesting, three stem samples per group were fixed in FAA (formalin: acetic acid: 50% ethanol, 1:1:18 v/v) for at least 24 h (Johansen 1940). All materials were dehydrated using the tertiary butyl alcohol series (Johansen 1940), embedded in paraffin (Paraplast Plus® - Fischer), and sectioned into 12 to 14 µm thick sections with a rotary microtome (Model and brand). Deparaffinized sections were double stained with a 1% alcoholic solution of safranin-O and1% aqueous astral blue (Gerlach 1969). The sections were observed using an Olympus BX51 microscope under white light, and the images were obtained using a DP-72 digital camera and Image Pro Plus 6.3 software. Due to group size limitation, only the prolonged treatment groups (CT, CH, and NAR) were analyzed.

Extractives Content

Between two and eight samples per group (0.3 to 1.5 g) were kept individually in Ankon filter bags (Ankon Technology, New York, USA) and sequentially extracted for 16 h with 95% ethanol followed by another 16 h in distilled water in a 125 mL Soxhlet apparatus. Afterwards, the extracted samples were allowed to cool and dry under room conditions overnight and then dried at 60 ºC overnight, followed by 2 h at 102 ± 2ºC. Extractive content was assessed according to weight loss after each step (Alves *et al.* 2012). Only one determination per sample was possible due to the low amount of sample available.

Lignin Content

For lignin analysis, a pool of five randomly selected individuals was prepared per group treatment, and Klason (acid-insoluble lignin) and total lignin content (acid-insoluble lignin plus acid-soluble lignin) were assessed using the averaged values based on the oven-dried, extractive-free weight of each pooled sample as determined using wet chemistry methods. Klason (acid-insoluble) lignin content was determined according to TAPPI T 222 om-02 following the modifications by Schwanninger and Hinterstoisser (2002), and the acid-soluble lignin content was determined according to TAPPI UM 250.

Analytical Pyrolysis

A 30 mg aliquot of each extracted sample was further milled in a vibratory ball mill (Mixer Mill MM, Retsch) for 5 min and kept in desiccators prior to analysis by analytical pyrolysis. Analytical pyrolysis was performed using a CDS Pyroprobe 1000 with a coil filament probe connected to a gas chromatography (GC) unit (Agilent 6890) with a flame ionization detector (FID) using a heated interface (270 ºC). The pyrolysis was carried out at 600 ºC for 5 s using 75 to 77 µg of the extractive-free milled samples. Capillary column: DB1701 (60 m x 0.25 mm, 0.25 µm film, J&W Scientific). GC conditions: injector, 270 ºC; detector, 270 ºC; temperature program, 45 ºC, 4 min isothermal, then heating rate 4 ºCmin^{-1} to 250 ºC and 6 ºC min^{-1} to 270 ºC, hold for 8 min (Rodrigues *et al*. 1999, 2001; Alves *et al*. 2011).

From the pyrolysis product peaks, the following ratios were computed using Chemstation Software (Agilent Technologies, Palo Alto, USA). S/G and H/G ratios were calculated from the sum of the peak areas of the pyrolysis products assigned to syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) type phenols. The cP/cH ratio was calculated from the sum of the peak areas of the characteristic pyrolysis products of pentosans (cP) and hexosans (cH) and represents a rough estimation of the relative proportion of hemi-celluloses and cellulose. Py-lignin was calculated as the ratio of the sum of the areas of the peaks from the lignin products divided by the sum of the area of all peaks used (lignin and polysaccharides, *ca*. 75% of the total area) multiplied by 100% (Alves *et al*. 2006a).

Statistical analysis

To verify significant differences between the controls and the flavonoid-supplemented groups, a one way ANOVA test was performed between the control and each supplemented group. The results were considered significant if $p < 0.05$ and were classified as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. The correlation between ethanol and water extractives was also assessed. All statistics were performed using Analysis ToolPak for Excel 2007.

RESULTS AND DISCUSSION

Histochemical Analysis

The photomicrographs of the stained (astral blue-safranin) transverse section cuttings show that the treatment groups had a more pronounced blue coloration than the control group, especially evident in the NAR group (Fig. 1). These results suggest a relative decrease in the lignin content with a consequent increase in the polysaccharide fraction of the flavonoid-treated wood plantlets because astral blue-safranin dye confers a distinct coloration for carbohydrates and colors cellulose in the absence of lignin blue and

lignin-rich regions red. As shown later using wet chemical analysis, no significant changes in lignin content were found, whereas a clear reduction in the content of ethanol extractives seemed to indicate that the difference in the coloration could result from different amounts of ethanol extractives. Another possibility is the mobilization of the stained compounds during the preparation stages of the material. In fact, mobilization alone could account for the differences between the stain results and the analytical results.

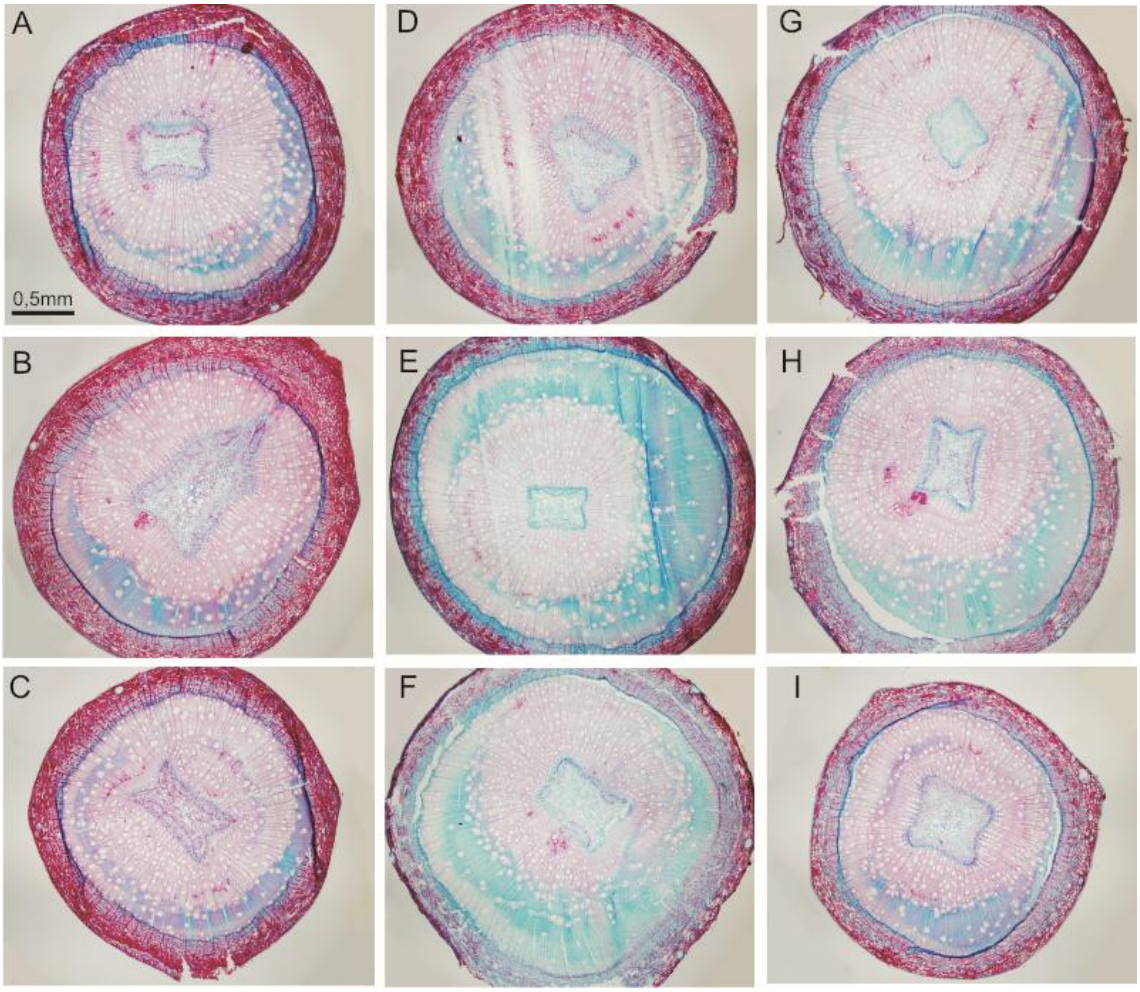

Fig. 1. Photomicrographs of the stained (astral blue-safranin) transverse section cuttings from the control (A-C) and the naringenin (D-F) and naringenin-chalcone (G-I) treatment groups

Extractives and Lignin Content

A summary of results for the extractive and lignin content of the samples is given in Table 1.

Across groups and plants, the ethanol extractives content varied between 4.4% and 13.7% of the dry weight of the wood meal, representing between 51% and 83% of the total extractives content. After ethanol extraction, the water extractive content varied between 2.0% and 6.6% and represented between 26% and 41% of the total extractives content. The ethanol and water extractives contents were positively correlated ($r = 0.56$), and the correlation was highly significant ($p < 0.004$).

Table 1. Summary of the Results of the Extractive and Lignin (Klason and total) Contents

	Extractives (%)			Lignin (%)	
	Ethanol	Water	Total	Klason	Total
CT	10.3(0.34)	3.8(1.09)	14.2(1.25)	22.6(0.03)	26.7(0.01)
CH	6.1(1.01) ***	2.9(0.47)	9.0(1.82) ***	21.5(0.03)	25.5(0.01)
CHSTOP	7.1(0.23) ***	3.4(0.44)	10.5(1.31) **	22.6(0.14)	26.6(0.03)
NAR	7.6(1.57) ***	4.2(0.14)	11.8(0.90) *	22.3(0.01)	26.3(0.17)
NARSTOP	6.9(0.99) **	2.5(0.28)	9.4(0.19) **	22.5(0.02)	26.2(0.40)

Extractive data refer to the mean values of the individual samples from each group. Lignin data refer to the replicate analysis of a 5-sample pool per treatment. CT – control group; CH – 5 months naringenin-chalcone; CHSTOP – 1 month naringenin-chalcone; NAR – 5 months naringenin; NARSTOP- 1 month naringenin. Values in brackets refer to variance.

A decrease in the ethanol extractives content was observed for the flavonoid-treated plants. The average ethanol extractives content decreased from 10.3% (control group) to between 6.1% (CH) and 7.6% (NAR), representing a decrease between 41% (CH) and 26% (NAR) compared with the control group. The water extractive content was also reduced with flavonoid treatment from 14% (CH) to 34% (NARSTOP), with the exception of the NAR-treated group, which showed an 11% increase compared with the control group. The total extractive content followed the same pattern as the ethanol extractives.

The extractive content of these plants was higher than the extractive content of *Eucalyptus urograndis* at the age of commercial pulpwood. The ethanol extractive content alone was above or close to the total extractive content of the heartwood (7.6%) and at least twice that of the total extractive content of the sapwood (3.7%) of 5.6-year-old trees from an *E. urograndis* clone in a commercial pulpwood plantation in Brazil (Gominho *et al.* 2001). These differences could be explained, at least in part, by the fact that living cells (developing xylem) filled with easily extractable metabolites (Paiva *et al.* 2008) represent a larger percentage of the stem at this age than at older ages.

The reduced extractive content due to flavonoid treatment will increase the productivity of pulping because a higher extractives content accounts for up to 4% of the losses to the kraft pulping yield of clonal eucalyptus in Brazil (Gomide *et al.* 2005).

The Klason and total lignin contents of these plantlets were lower than the reported values for *E. urograndis* at commercial age in Brazil (6 to 7 years old), ranging from 24.2% to 27.1% (Klason) and 27.5% to 30.6% (total) lignin content (Gomide *et al.* 2005). However, these results were obtained on a limited number of samples (7 clones, each a composite sample of three individuals).

The lignin content (Klason and total) was not affected by the flavonoid treatment (Table 1), which was unexpected in light of the histological results (Fig. 1). This result seems to indicate that, in addition to changing the extractive content, the flavonoid treatment could also have changed the extractive composition, accounting for the differences in coloration between the treated and control samples.

Alternatively, the flavonoid treatment could have altered the lignin composition results by making cellulose and other carbohydrates more accessible to the dye astral blue (Fig. 1).

Analytical Pyrolysis

The summary of the analytical pyrolysis results is shown in Table 2. According to these results, flavonoid-treated plants showed a statistically significant increase in the S/G ratio (between 8 and 10% compared with the control) and a decrease in the H/G ratio (between 11 and 16%). Both changes have a favorable impact on pulping because of their effects on delignification rates, chemical consumption, and pulp yields (Rodrigues *et al*. 1999).

Table 2. Summary of the Analytical Pyrolysis Results

		cP/cH	H/G	S/G	Py-lignin%
CT	5	26.8(1.6)	0.092(0.008)	1.41(0.05)	23.2(0.6)
CH	8	28.8(3.3)	0.078(0.007) **	1.55(0.03)**	23.7(0.8)
CHSTOP	3	29.5(2.5)	0.078(0.006) *	1.55(0.03) **	24.2(0.3)
NAR	3	28.2(1.2)	0.077(0.004) *	1.52(0.02) *	24.1(0.7)
NARSTOP	2	29.6(4.2)	0.082(0.009)	1.52(0.01) *	23.0(0.1)

The mean values and standard deviation (brackets) results of the analytical pyrolysis. The first column refers to the number of individual samples analyzed.

The syringyl/guaiacyl (S/G) ratio ranged from 1.41 (CT) to 1.52 (CH and NAR), representing an approximate 7.8% increase in syringyl lignins in the flavonoid treatment. The same result was observed in the short-term flavonoid supplementation groups: the S/G ratio increased to 1.52 and 1.53 in the CHSTOP and NARSTOP groups, respectively, which represents an approximately 7.8% (CHSTOP) and 8.5% (NARSTOP) increase. These values are lower than the values reported for this species (S/G ratio above 2) using analytical pyrolysis and nitrobenzene oxidation (Barbosa *et al*. 2008; Lima *et al*. 2008). However, the analytical pyrolysis results cannot be directly compared because differences in the pyrolysis instruments and columns will inevitably lead to different results.

The H/G ratio decreased from 0.092 (CT) to 0.078 for both CH and NAR groups and to 0.078 and 0.081 in the CHSTOP and NARSTOP groups, respectively, representing an approximate 15% decrease in the H/G ratio in the prolonged treatments (CH and NAR) and 15% (CHSTOP) and 12% (NARSTOP) in the short-term treatments. Interestingly, these H/G values are higher than the average H/G ratios for *Pinus pinaster* (0.64) (Alves *et al*. 2006b), *Pinus caribaea* (0.50) (Godoy *et al*. 2007), and *Picea abies* (0.50), all determined by analytical pyrolysis using the same methodology (Alves *et al*. 2009).

Because no changes in the total lignin content were observed and the S/G ratio increased while the H/G ratio decreased, a direct substitution of p-hydroxyphenyl and guaiacyl units by syringyl units in the composition of lignin is likely. This substitution is one of the key desired aspects of *Eucalyptus* plants (Baucher *et al.* 2003; Grattapaglia and Kirst 2008; Jung *et al.* 2011), increasing lignin solubility and cellulose accessibility by promoting better delignification (Baucher *et al.* 2003; Huntley *et al.* 2003; Stewart *et al.* 2006). To explain this result, a change in the phenylpropanoid pathway is necessary, with

a shift towards S lignin synthesis; expression analyses of supplemented *Eucalyptus* might help elucidate this change.

Both the cP/cH ratio and the Py-lignin content showed no statistically significant differences between the control and treatment groups, although the cP/cH ratio was, on average, higher for all treated groups compared with the control.

In conclusion, it was demonstrated that flavonoid supplementation of *Eucalyptus urograndis* plantlets has a strong influence on its wood composition. The present results are preliminary but have great potential for the improvement of this species towards increased productivity. The addition of flavonoids to a common nutrient medium is inexpensive and easy to accomplish in a *Eucalyptus* nursery. The fact that short-term supplementation (only 1 month) was enough to produce changes in wood composition four months later was encouraging, and longer experiments should be performed to determine the impact of supplementation on full-grown *Eucalyptus*.

CONCLUSIONS

1. Root-applied flavonoid supplementation promotes a significant reduction in extractive content and increases the syringyl monomeric composition (S/G ratio) of young *E. urograndis* trees.
2. These preliminary results indicate that flavonoid supplementation can potentially be used as a nutritional complement being a new, viable, and interesting method for improving *Eucalyptus* wood quality for its utilization by the paper and pulp industry and for biomass exploitation.

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