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Presence of Lignin and Cellulose Intensity Signals in Knotted and Knotless Wood of *Pinus elliottii* var. *elliottii* Engelm

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Abstract The fundamental chemical components of wood (cellulose, hemicellulose and lignin) are anatomically inherent and vary along the trunks of each individual tree. The aim of this study was to analyse the formation and concentration gradient of lignin and cellulose intensity signals between knotless wood, the knot-to-wood transition zone and knots in the species *Pinus elliottii* var. *elliottii* Engelm. Wood samples in knotted and knotless regions of *Pinus elliottii* var. *elliotti* trees from a plant shop in Agudos-SP, were analysed in the laboratory of the Federal University of Rio de Janeiro. Based on the degradative methods of Klason Lignin, 13C spectroscopy and nuclear magnetic resonance and Fourier transforminfrared and histochemical tests in histological sections, differentiated patterns in the development dynamics between the anatomical elements of knotless wood and knotted wood were verified. The structure of lignin in knotted and knotless wood was different, despite the proximity of these regions to one another. Crystalline cellulose signals were more intense in the middle knot and top knot regions. It is possible to conjecture that these characteristics interfere with wood resistance in these regions along the trunk.

Keywords Trunk Regions, Anatomical Elements, Spectroscopic Methods, Resistance

1. Introduction

Cellulose, hemicellulose, lignin, and extractives are considered fundamental chemical components of wood, and can vary as a result of silvicultural treatments, the anatomical characteristics inherent to each individual and the position along the bole of a tree (Oliveira, 1997; Silva, 2002; Gatto *et al*., 2008; Deus *et al*., 2022). However, the quantities of these components do not differ significantly between species (Oliveira, 1997; Silva, 2002).

The chemical heterogeneity of wood, while increasing its potential uses, represents an inconvenience to the transformation and processing industry, since it pejoratively interfereswith the technological properties required for the use of wood [Oliveira, 1997; Silva, 2002; Gatto *et al*., 2008). For example, variation in lignin influences hygroscopicity, the behaviour of dimensional variations and consequently, wood quality.

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While hemicellulose has a greater water absorption capacity and increases dimensional variation, lignin, which is essentially hydrophobic, promotes the opposite action. The higher the lignin content in the wood, the greater its resistance to atmospheric water absorption and the lower its dimensional variation (Oliveira, 1997; Silva, 2002; Gatto *et al*., 2008; Deus *et al*., 2022).

Furthermore, many physical and mechanical properties of wood depend on the presence of lignin (Britez and Nogueira, 2996; Deus *et al*., 2022). For example, basic density is one of the most studied properties and is used as a factor for assessing wood quality, directly influencing its use. The greater the lignin deposition in the cell wall, the better the density value, a characteristic which is required for the structural use of wood (Britez and Nogueira, 2996).

Understanding the mechanism of lignin formation still requires further theoretical and experimental research, especially in terms of relating lignification to the quality of the final product in the various areas of the forestry sector. Content control and the modulation of lignin biosynthesis, for example, offers a technological advance for the partial removal of lignin from woody tissues, a process which

requires high investment, especially due to its importance in the cellulose pulp industry (Deus *et al*., 2022 (Abreu *et al*., 2004; Deus *et al*., 2023).

The reduction in lignin content, which has already been associated with a reduction in the resistance capacity of trees, can be studied from other perspectives. Studies have shown that young branches are able to develop compensatory mechanisms for maintaining the structural, biophysical and biomechanical properties of tissues, despite a 64% reduction in lignin content ((Patten *et al.*, 2007; Deus *et al*., 2022).

However, it is worth noting that the major component of wood is cellulose, comprising approximately 50% of wood, in both conifers and hardwoods (Fengel and Wegener, 2003), with chemical and physical properties and a supramolecular structure fulfilling its function as the main component of cell walls (Klock *et al.*, 2005).

The structural organisation of cellulose is found both in both its amorphous and crystalline form. Crystalline cellulose corresponds to two thirds of the cellulose present in wood and is characterised as being more robust and resistant to heat, while the amorphous form is more sensitive, and assumes a similar characteristic to hemicellulose, in this regard, and is responsible for the absorption of water molecules, due to the empty spaces in its structure (Wilkie, 1961; Nishiyama *et al*., 2002; Oliveira, 2009).

Furthermore, there are no well-defined boundaries between the amorphous and crystalline regions, but there is a transition from the arrangement of cellulose chains to the ordered or amorphous state (Mokfienski, 2004). Even a combination of heat + water, allows for the conversion of the amorphous to the crystalline region, where an increase in the crystalline region is associated with the reorganisation of amorphous cellulose combined with its partial degradation (Stam, 1964; Fengel and Wegener, 2003).

The more crystalline the structure of cellulose, the more limited the access of compounds to functional groups and chemical bonds (Thygesen *et al*., 2005). The crystallinity of cellulose is an important factor impacting wood properties, changing the direction of production and even defining the quality of the final product (Dufresne and Belgacem, 2013).

Like lignin, cellulose is closely related to the technological properties of wood products. The tensile strength of fibres and the elasticity modulus of wood parts, depend on and vary according to the cellulose content present in cells (Klinke *et al*., 2000; Lahr *et* al., 2018; Marvila *et al.*, 2021).

The hypothesis of the influence of lignin on the formation of cracks in wood (Abreu *et al.*, 2004), demonstrates the need for further studies on cell wall lignification. As knots and their surroundings are largely affected by cracks, the study of lignin in these regions in justified by the need to increase wood quality. Thus, the aim of this study is to analyse the formation and concentration gradient of lignin and cellulose intensity signals between knotless wood, the knot-to-wood transition zone and knots in the species *Pinus elliottii* var. *elliottii* Engelm.

2. Materials and Methods

Five trees of the species *Pinus elliottii* var. *elliottii* Engelm from a plant shop located in the municipality of Agudos, in the State of São Paulo, at the coordinates Lat: 22°53'20" S and Long: 47°04’39” W, were studied. The wood of the species was identified, felled and deposited in the Xylotheque of the Rio de Janeiro Botanical Gardens, with the records 10191, 10192, 10193, 10194 and 10195. Three disks were extracted from each tree, containing at least one knot/branch from the bottom (25% of bole height), middle (50% of bole height) and top (100% of commercial height) positions.

Samples of knotted and knotless regions were taken from the disks, from which small wood chips were obtained using a machete. The samples were taken to the Chemistry Laboratory of the Institute of Exact Sciences at the Federal Rural University of Rio de Janeiro (UFRRJ).

Using a Willey 340 (TE 040 model) knife mill, the chips were converted into sawdust for subsequent granulometry reduction in a rotating ball mill to obtain the material for the analyses.

For the material extraction process, a Soxhlet device was used, using 25 grams of ground wood and homogenous wood separately, from the parts of the disk with and without knots. The material was packaged in a cartridge made with filter paper and placed inside the extraction tube. The solvents were placed in a 500mL flask, following the eluotropic scale in increasing order of polarity (cyclohexane; ethyl acetate; methanol). The extraction time for each solvent was 24 uninterrupted hours (Browning, 1967). Following this period, the extract was concentrated in a rotavapor, and the concentrates were transferred to a container until the completion of solvent evaporation at room temperature.

The characterisation of lignin in the knot samples was performed using the Klason Lignin degradative method and spectroscopic methods: 13C Nuclear Magnetic Resonance and Fourier transform infrared. Additionally, to corroborate or compare the results obtained in the tests mentioned above, Wiesner, Mäule and Fluorescence histochemical tests were used.

Klason Lignin (acid-insoluble lignin) was determined using approximately 300mg of extractive-free material, transferred to a test tube with the slow addition of 3mL of H_2SO_4 – 72%. The material was stirred for 1 hour at a temperature of between 25 and 30 ℃, transferred to a 250mL flask and diluted in a 15% H_2SO_4 solution, in addition to 84mL of distilled water. This was later left at reflux for 4 hours, and then left to rest. After resting, the residue was washed with 500mL of distilled hot water in a synthesised plate funnel, with the aid of a vacuum. The material retained on the filter was dried in an oven at 105℃ until the weight remained constant (Abreu *et al.*, 2006). The insoluble lignin sample was represented as a percentage, according to Equation 1.

$$Li\text{-}\% = m_2/m_1 * 100 \quad (1)$$

Where: Li = insoluble lignin in the sample (%); m_1 = residue mass, dry weight (mg); and m_2 = sample mass, dry weight (mg).

The Carbon-13 Nuclear Magnetic Resonance (13C NMR) analysis of the extractive-free material was performed using a spectrometer model DRX-500 operating at 125 MHz for the 13C nucleus. 13C NMR spectra were obtained from the knotted regions at 25%, 50% and 100% (bottom, middle and top, respectively) of the commercial height, as well as for the evaluation of molecule crystallinity levels (Park *et al.*, 2010).

For comparison purposes, 13C NMR spectra of knotless wood from regions at the same positions were obtained.

For infrared spectroscopy analyses, 1mg of the dry material was mixed and ground with 100mg of KBr (Morais *et al*., 2005) and pressed into a tablet and introduced to the spectrometer compartment, model VARIAN 640-IRFT-IR spectrometer. The spectra were recorded in transmittance mode.

For the histochemical tests, histological sections were obtained using a Leica SM 2000R microtome at the Structural Botany Laboratory of the Rio de Janeiro Botanical Gardens and were clarified with 50% sodium hypochlorite, neutralised with a 1% acetic acid solution and washed in distilled water (Vazquez-Cooz and Meyer, 2002), before performing the Mäule, Wiesner and fluorescence tests. Photomicrographs were obtained using an Olympus BX 51 microscope coupledwith a computer, equipped with a Cell Imaging Software image processing system.

To detect lignin, sections from each position (bottom, middle, top) were placed in an alcoholic solution of phloroglucinol for reaction, at a low temperature (+/-10°C), with HCl (Lin and Dence, 1992).

To perform the Mäule's test, fresh cuts were placed in a $KMnO_4$ solution for 5 minutes. They were then washed with distilled water and placed in a 3% HCl solution until the colour changed and stabilised. They were then washed again in distilled water and were finally placed in a NH_4OH solution to confirm the type of lignin (Lin and Dence, 1992).

Sections were observed in their natural state under an optical microscope (Olympus BX 51), equipped with a digital analysis system (Cell^F Imaging Software), with an excitation filter at 470-490nm, emission at 515-565nm and a FITC filter (U-MWB2).

3. Results

The percentage data of Klason lignin obtained from the wood of *Pinus elliottii* var. *elliottii* Engelm, present a normal or relatively normal distribution, with the exception of the "middle" section of the trunk, bothin the "knot-to-wood transition zone" and "knot". The variation coefficients presented low percentages, suggesting a relative numerical consistency of the obtained averages (29-37%), as shown in Table 1.

For the knotless wood, a higher average percentage of Klason lignin, although discrete, was observed in the middle region of the trunk, while in the knot-to-wood transition region, as well as for the samples obtained exclusively from knots, the higher average percentages were observed at the bottom, transition zone and knot, respectively. The two-way analysis of variance showed significant differences between the Klason lignin percentages in the conditions of "knotless wood", "knot-to-wood transition" and "knot", with 49% of data variation justified by this condition (Table 2).

Considering the position factor (bottom, middle and top) of the trunk, the test shows a trend of similarity between the lignin percentages; thereby, demanding further analyses, since less than 5% of the data variation can be explained by position. The interaction between the two factors (condition and position) accounted for approximately 8% of the total data variation. Since the total percentages associated with the analysed factors reached approximately 62%, it is likely that other factors, in addition to the conditions and positions analysed, also influence variation in Klason lignin percentagesin this species (Figure 1).

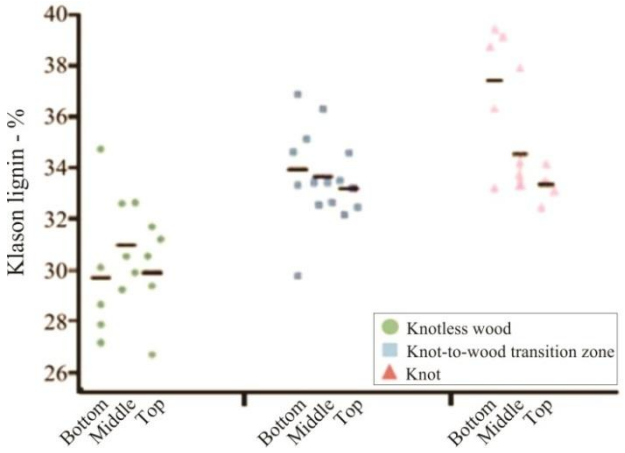

Figure 1. Percentage of Klason lignin from *Pinus elliottii*var. *elliottii*, considering the conditions of "knotless wood", "knot-to-wood transition" and "knot" and the position on the trunk (bottom, middle and top). Black lines represent the averages in each situation

The spectra resulting from the 13C magnetic resonance of the wood from "bottom knot", "middle knot" and "top knot", were highly similar. The spectra of the "knot" regions are illustrated in Figures 2a, 2b, 2c. The chemical shifts represented by the peaks are detailed in Table 3.

Table 1. Percentage of Klason lignin in *Pinus elliottii* var. *elliottii* Engelm, considering the conditions of "knotless wood", "knot-to-wood transition" and "knot" and the position on the trunk (bottom, middle, top)

Descriptive statistic	Knotless wood		
	Bottom	Middle	Top
Average (± standard deviation)	29.71 (±3.02)	31.00 (±1.56)	29.92 (±1.99)
Minimum	27.17	29.26	26.71
Maximum	34.75	32.65	31.70
Coefficient of variation	10.16%	5.03%	6.66%
K-S	0,2465	0,2496	0,2274
P value	> 0.1000	> 0.1000	> 0.1000
Summary K-S	ns	ns	ns
Asymmetry	1.57	0.23	-1.32
Kurtosis	2.48	-2.76	1.49
Descriptive statistic	Knot-to-wood transition zone		
	Bottom	Middle	Top
Average (± standard deviation)	33.95 (±2.66)	33,68 (±1.52)	33,20 (±0.95)
Minimum	29.78	32.57	32.17
Maximum	36.89	36.31	34.59
Coefficient of variation	7.84%	4.52%	2.87%
K-S	0.2077	0.3655	0.1791
P value	> 0.1000	0.0277	> 0.1000
Summary K-S	ns	*	ns
Asymmetry	-1.02	1.85	0.61
Kurtosis	1.50	3.65	-0.17
Descriptive statistic	Knot		
	Bottom	Middle	Top
Average (± standard deviation)	37.41 (±2.63)	34.55 (±1.93)	33.37 (±0.61)
Minimum	33.24	33.33	32.49
Maximum	39.48	37.94	34.17
Coefficient of variation	7.03%	5.59%	1.84%
K-S	0.2993	0.3633	0.2033
P value	> 0.1000	0.0297	> 0.1000
Summary K-S	ns	*	ns
Asymmetry	-1.30	2.05	-0,.29
Kurtosis	0.74	4.31	0.87

Table 2. The two-way analysis of variance of Klason lignin percentages from *Pinus elliottii* var. *elliottii* Engelm considering the conditions of the "knotless wood", "knot-to-wood transition" and "knot" and the position on the trunk (bottom, middle and top)

Bidirectional ANOVA (Alpha = 0.05)				
Varying source	% total variation	P value	Sumary P	Meaningfulness
Interaction	8.2	0.1261	ns	No
Fator "position on the trunk"	4.6	0.1279	ns	No
Fator "knot ou knotless wood"	49.0	< 0.0001	****	Yes

ANOVA					
Varying source	SS	DF	MS	F (DFn; DFd)	P value
Interaction	31.64	4	7.91	F (4; 36) = 1.932	P = 0.1261
Fator "position on the trunk"	17.83	2	8.92	F (2; 36) = 2.178	P = 0.1279
Fator "knot ou knotless wood"	189.10	2	94.55	F (2; 36) = 23.100	P < 0.0001
Residue	147.40	36	4.09		

Table 3. Chemical shifts (ppm) of carbon atoms in the 13C NMR spectra of the knots in *Pinus elliottii* var. *elliottii* Engelm

Signal	Chemical displacement (ppm) in the knots			Assignments (adapted from Baptista, 2006; Souza et al., 2011)
	Bottom	Middle	Top	
1	171.71	171.70	171.60	Hemicellulose ester carboxyl
2	149.80	149.70	149.80	C3 of the guaiacyl lignin
3	147.74	147.74	147.60	C1 and C4 guaiacyl lignin
4	146.40	146.40	-	C4 guaiacyl lignin
5	133.10	133.00	-	C1 of the guaiacyl lignin
6	132.57	132.56	132.57	C2 of the guaiacyl lignin
7	119.20	119.20	-	C6 of guaiacyl lignin
8	113.57	113.53	113.57	C5 and C6 of the guaiacyl lignin
9	104.47	104.46	104.47	-OCβH2 of cellulose and hemicellulose
10	88.51	88.49	88.50	C4 crystalline cellulose
11	81.80	81,80	81.90	C4 of non-crystalline cellulose and hemicellulose and -OCβH2 of the lignin
12	74.29	74.30	74.28	C2, C3 and C5 of cellulose and hemicellulose
13	71.76	71.70	71.80	C2, C3 and C5 of cellulose and Cα H2 of the lignin
14	64.46	64.46	64.39	C6 crystalline cellulose
15	61.79	61.79	61.77	C6 of non-crystalline cellulose; C6 of hemicellulose; -OCγH2 of the lignin
16	55.44	55.44	55.43	Lignin metoxyl
17	20.91	20.92	20-38	-CH3 and –CH2 in saturated aliphatic chains

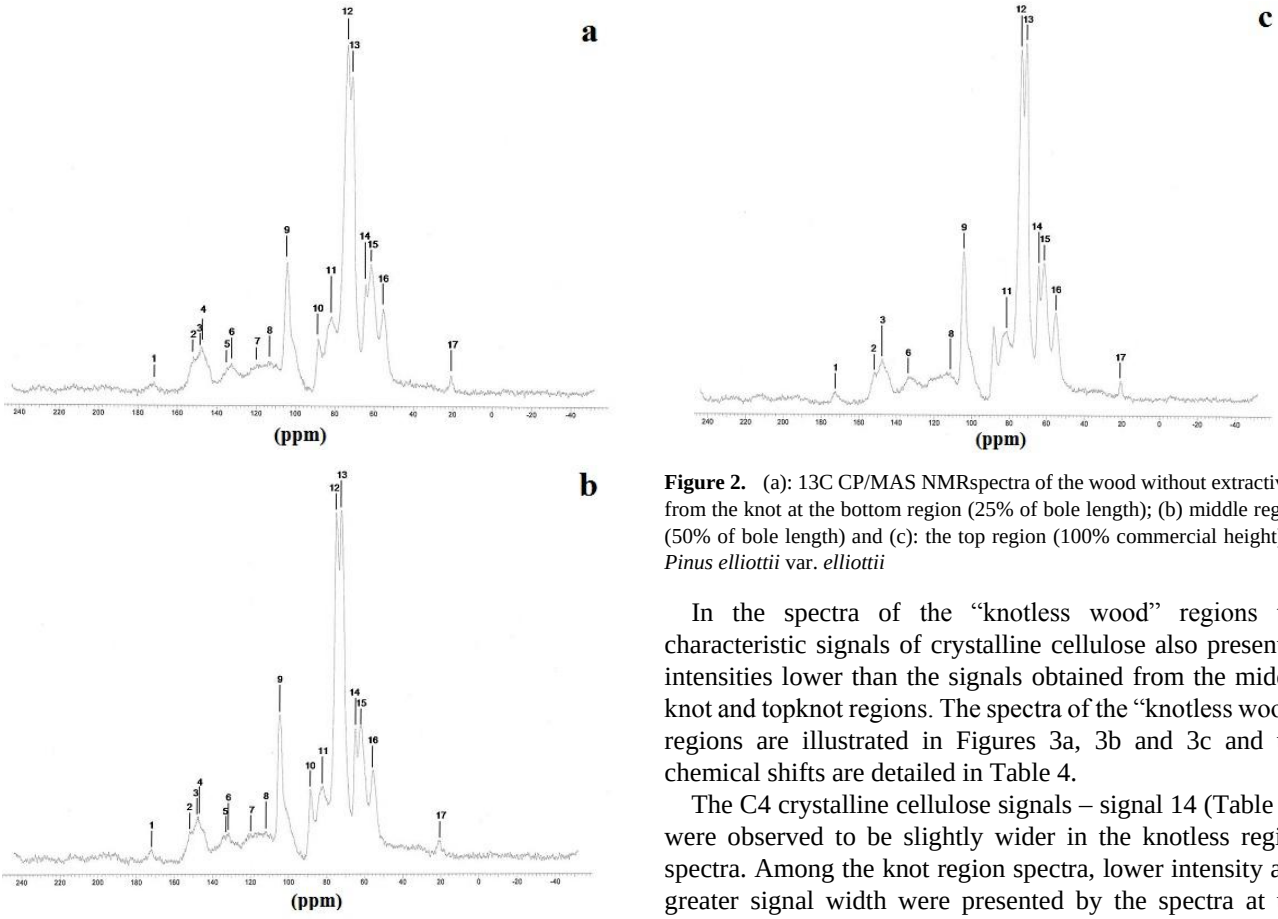

Figure 2. (a): 13C CP/MAS NMRspectra of the wood without extractives, from the knot at the bottom region (25% of bole length); (b) middle region (50% of bole length) and (c): the top region (100% commercial height) of *Pinus elliottii* var. *elliottii*

In the spectra of the "knotless wood" regions the characteristic signals of crystalline cellulose also presented intensities lower than the signals obtained from the middle knot and topknot regions. The spectra of the "knotless wood" regions are illustrated in Figures 3a, 3b and 3c and the chemical shifts are detailed in Table 4.

The C4 crystalline cellulose signals – signal 14 (Table 4), were observed to be slightly wider in the knotless region spectra. Among the knot region spectra, lower intensity and greater signal width were presented by the spectra at the bottom knot region.

In general, the bottom knot region (Figure 2a) presented spectra with signal intensities that were similar to the three knotless wood regions (Figures 3a, 3b and 3c), while themiddle and top regions of the knotted wood presented signal intensities that were similar to each other. However, there were differences between these signals and the signals obtained from the bottom knot and regions of knotless wood.

In addition to the variation in signal intensity observed between the obtained spectra, a greater number of characteristic carbon signals in guaiacyl units was observed in the spectra of knotless wood regions – signals 2-12 and signal 15 (Table 4).

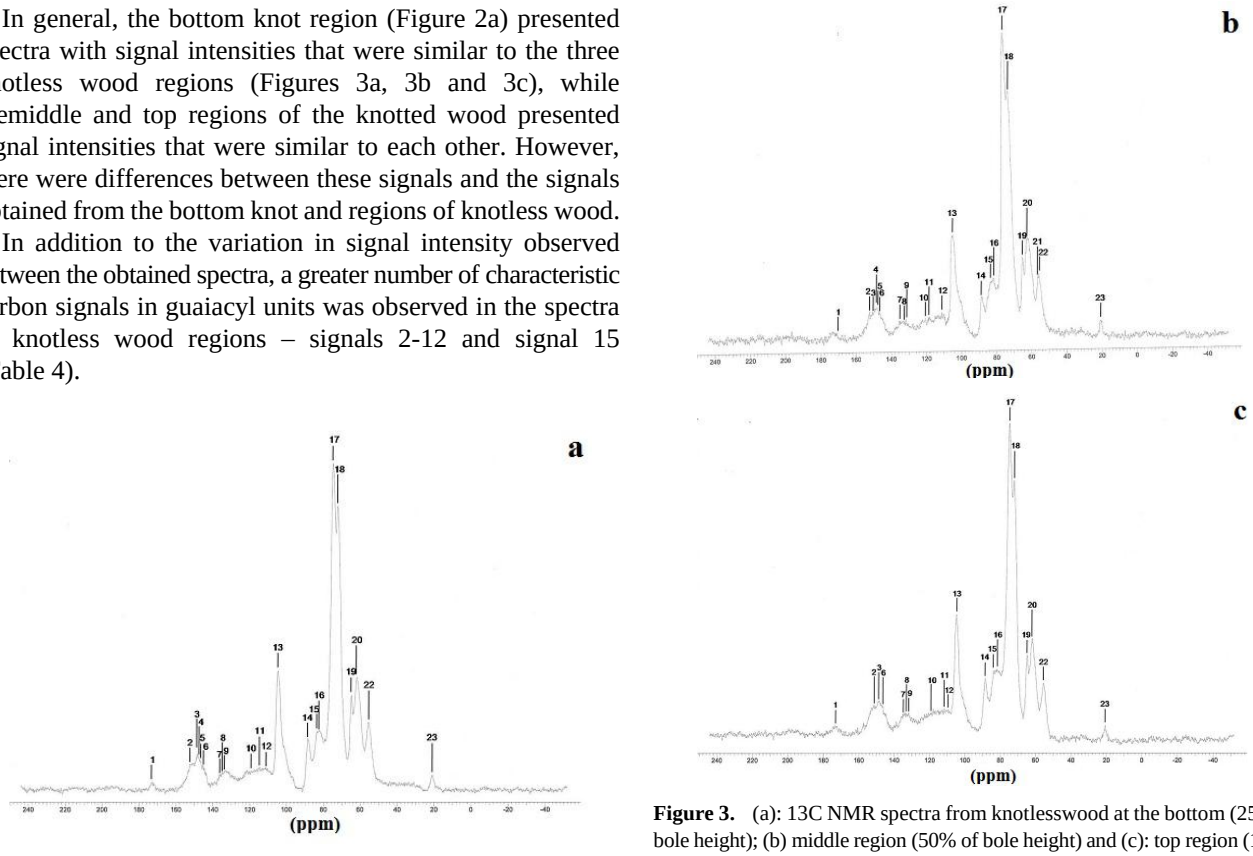

Figure 3. (a): 13C NMR spectra from knotlesswood at the bottom (25% of bole height); (b) middle region (50% of bole height) and (c): top region (100% of bole height) of *Pinus elliottii*, var. *elliottii* Engelm

Table 4. Assignments of carbon atom chemical shifts (ppm) in the 13C NMR spectra of knotless wood samples of *Pinus elliottii*, var. *elliottii* Engelm

Signal	Chemical displacement (ppm) (knotless wood)			Assignments (adapted from Baptista, 2006; Souza et al., 2011)
	Bottom	Middle	Top	
1	171.90	171.60	171.70	Hemicellulose ester carboxyl
2	149.70	149.70	149.80	C3 of the guaiacyl lignin
3	149.40	149.70	149.40	C3 guaiacyl lignin
4	147.72	147.60	-	C1 and C4 guaiacyl lignin
5	146.40	146.40	-	C4 of the guaiacyl lignin
6	146.40	146.30	146.30	C4 guaiacyl lignin
7	135.10	135.30	135.00	C1 in guaiacyl lignin
8	133.10	133.00	133.00	C1 in guaiacyl lignin
9	132.55	132.50	132.49	C2 of the guaiacyl lignin
10	119.15	119.20	119.20	C6 of the guaiacyl lignin
11	113.57	113.57	113.50	C5 e C6 of the guaiacyl lignin
12	111.80	111.90	111.90	C2 guaiacyl lignin
13	104.47	104.39	104.47	-OCβH2 of the lignin
14	88.50	88.51	88.50	C4 of the crystal cellulose
15	84.10	84.00	84.00	Cβ in guaiacyl unit
16	81.83	81.83	81.90	C4 of non-crystalline cellulose and hemicellulose and -lignin -OCβH2
17	74.29	74.29	74.31	C2, C3 and C5 of cellulose and hemicellulose
18	71.74	71.76	71.70	C2, C3 and C5 of cellulose and Cα H2 of the lignin
19	64.46	64.46	64.39	C6 of the crystal cellulose
20	61.79	61.79	61.73	C6 of non-crystal cellulose; C6 of hemicellulose; -OCγH2 and lignin
21	-	55.80	-	O-CH3
22	55.44	55.44	55.41	Lignin metoxyl
23	20-38	20-38	20-38	-CH3 and –CH2 in saturated aliphatic chains

The evaluated crystalline cellulose showed differences in molecule behaviour between the three positions (bottom, middle and top), as shown in Figure 4.

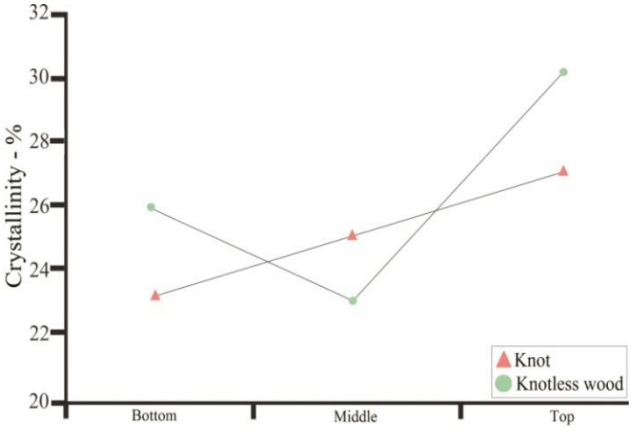

Figure 4. Percentage of cellulose crystallinity in knotted wood region and knotless wood, along the trunk of *Pinus elliottii*, var. *elliottii* Engelm

Figure 5 shows the variation trend of cellulose crystallinity in the knotted wood (a, c, e) and knotless wood (b, d, f) regions estimated by 13C NMR at different commercial heights.

The FT-IR spectra from the infrared spectroscopyof the knotted wood and the knotless samples obtained in the region of 4,000-800cm^{-1} and their respective attributes, are shown in Table 5. These values confirm the results obtained from the 13C NMR spectra of samples taken along the trunk of *Pinus elliottii*, var. *elliottii* individuals, showing the adequacy of the method used to extract and purify the lignin from the wood of the study species.

Signals in the region of 1,200-800 cm^{-1}, which is dominated by stretching vibrations of C-O, C-C ring structures and strain vibrations of CH_2 groups, were found in all samples. Characteristic signals of microcrystalline cellulose, 1,033 cm^{-1}, 1,060 cm^{-1} and 1,160 cm^{-1}. (Hori and Sugiyama, 2003), were observed to be more similar in the knotted wood samples in the middle and top regions of the trunk (Figures 6a, 6b and 6c).

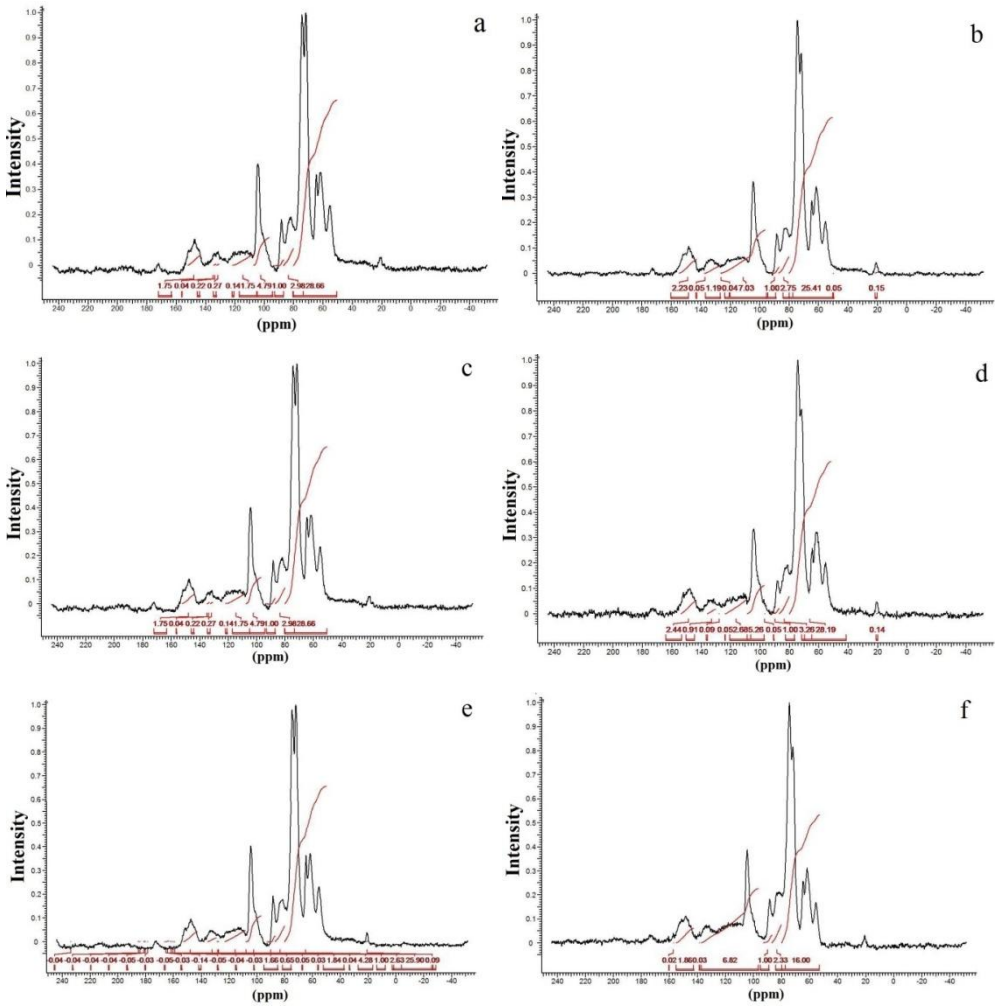

Figure 5. 13C NMR CP/MAS spectra of extractive-free wood, integrated in the region between (80-97 ppm) from the bottom region (25% of bole height) of the knot (a) and normal (b), from the middle region of the trunk (50% commercial height) for the knot (c) and normal (d) and from the top region (100% commercial height) of the knot (e) and normal (f) in *Pinus elliottii*, var. *elliottii* Engelm

Table 5. Fourier transforminfrared spectroscopy signals from knotted and knotless wood samples of *Pinus elliottii*, var. *elliottii* Engelm

Interval (cm^{-1})	Attributions	Signals					
		Knotless wood			Knot		
		Bottom	Middle	Top	Bottom	Middle	Top
3412-3460	O-H stretching						
2842-3000	C-H stretching of methyl and methylênic groups	2929	2923	2923	2930	2905	2903
1709-1738	C=O stretching in unjugated ketone, ester group (often carbohydrate) and carboxylic acids		1738	1738	1738	1738	1738
1505-1515	Vibration of the aromatic skeleton	1513	1513	1512	1512	1512	1513
1460-1470	Asymmetrical deformation in -CH3 and -CH2 -CH2-	1462	1462	1462	1462	1461	
1422-1430	Vibration of the aromatic skeleton combined with deformation in the C-H plan influenced by the replacement of the ring	1425	1425	1425	1425	1425	1425
1266-1270	Breathing of the guaiacyl ring with stretching C=O	1268	1268	1268	1268	1268	1268
1221-1230	C=O stretching, -C with stretching of C=O sensitive to the replacement of the aromatic ring G				1230		
1086-1125	Secondary alcohol C-O deformation and aliphatic ether						1159
1030-1095	(In the plane) deformation of C-H of the guaiacyl ring more C-O deformation in primary alcohol and ether with non-conjugated C=O stretch contribution	1057	1057	1057	1031	1056/1032	1056

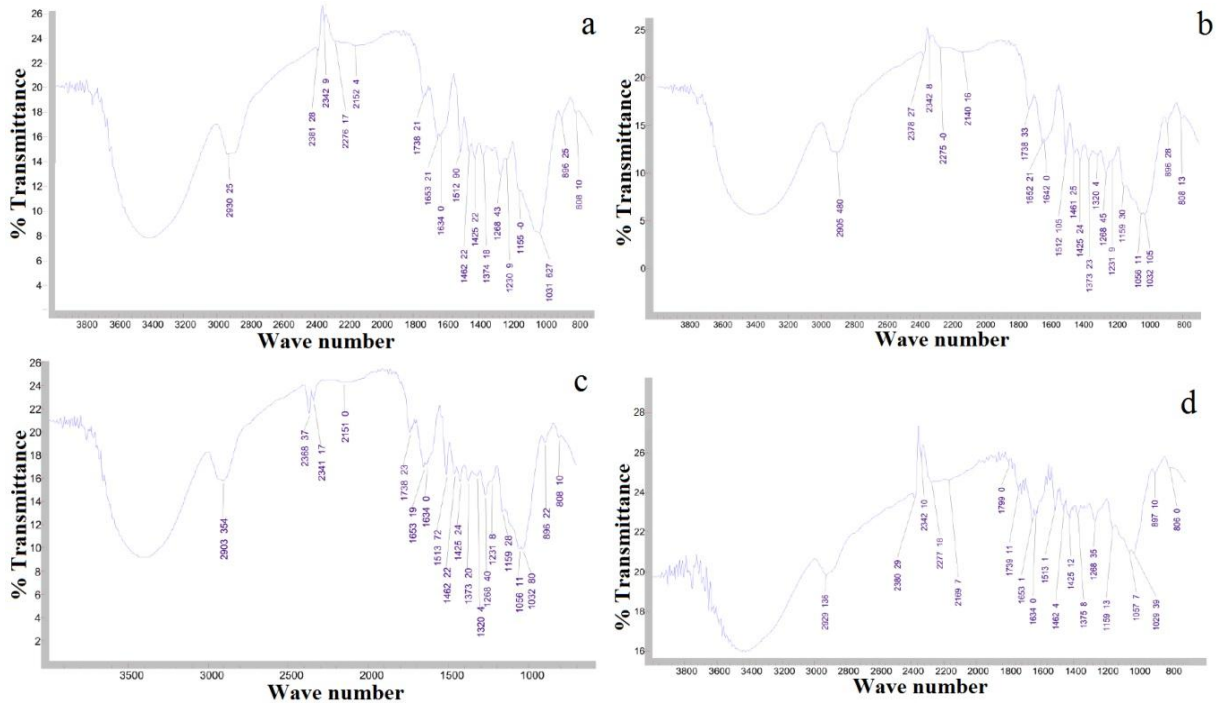

Figure 6. (a): Fourier transform infrared spectrum in the bottom region of extractive-free knotted wood; (b) in the middle region of extractive-free knotted wood; (c): in the top region of extractive-free knotted wood; (d): in the bottom region of extractive-free knotless wood; (e): in the middle region of extractive-free knotless wood and (f) in the top region of extractive-free knotless wood of *Pinus elliottii* var. *elliottii* Engelm

As observed in the 13C NMR analyses in FT-IR, a greater similarity was observed between the bottom region of knottedwood and the knotlesswood regions. In these spectra, the characteristic signals of crystalline cellulose were 1155 cm^{-1} and 1030 cm^{-1}. Signals between 1030-1095 cm^{-1} and 1266-1270 cm^{-1}, characteristics of C-H deformation of the guaiacyl ring and respiration of the guaiacyl ring with the contribution of C=O stretching, respectively, were observed in all spectra (Figures 6d, 6e and 6f).

Regarding the histochemical tests, the lignification of the cell wall, shown by the Wiesner test was divergent between knotted and knotless wood (Figures 7 and 8).

More intense staining expression was observed at the intersection points of cells of the knotless samples (Figures 7A and 7B), indicating the presence of more aldehydic lignin in these samples. In Figures 7C and 7D, in the cross-section, it is possible to observe highlighted points at the connecting edges of the cells from the knotless wood samples in the middle bole region.

In the fluorescence test, where the presence and location of lignin are revealed, it was possible to observe lignin surrounding the cells of the knotless wood cells (Figures 7E and 7F), Despite the histochemical, 13C NMR, infrared and % of Klason lignin tests showing the presence of lignin in all the analysed samples, lignin auto fluorescence was less intense in the knotted wood samples (Figures 8E and F) than in knotless wood.

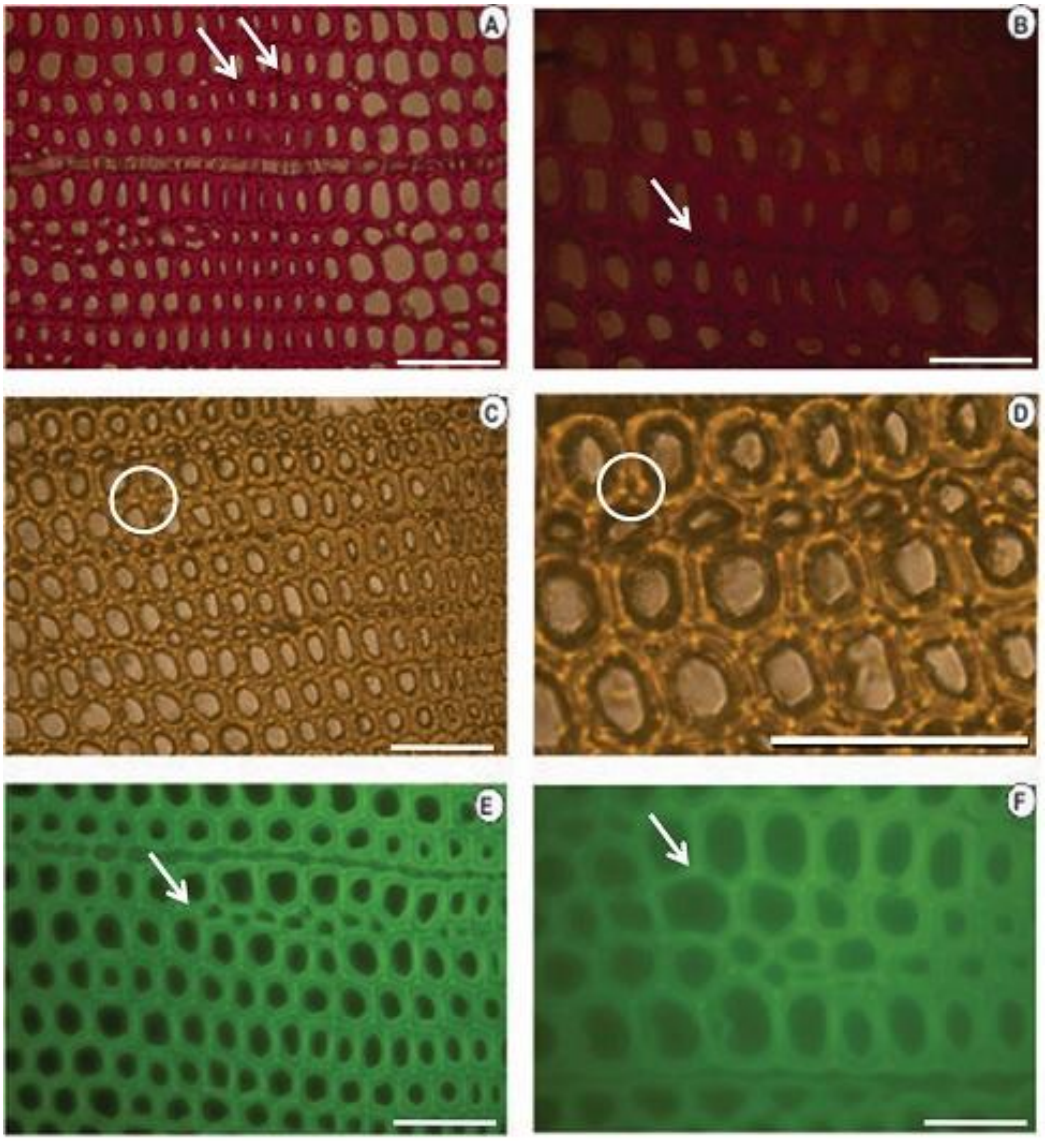

Figure 7. Histochemical tests applied to a cross-section of knotless wood from *Pinus elliottii* var. *elliottii* Engelm (A, B): Wiesener test showing the presence of more aldehyde lignin (darker colour) at the edges at the start of the lignification process. (C, D): Maüle test showing the presence of type G lignin. (E, F): Lignin autofluorescence showing the start of the lignification process at the edges (SETAS) of the cells. Bars (A, C, D, E) = 20μm; (B, F) = 100 μm

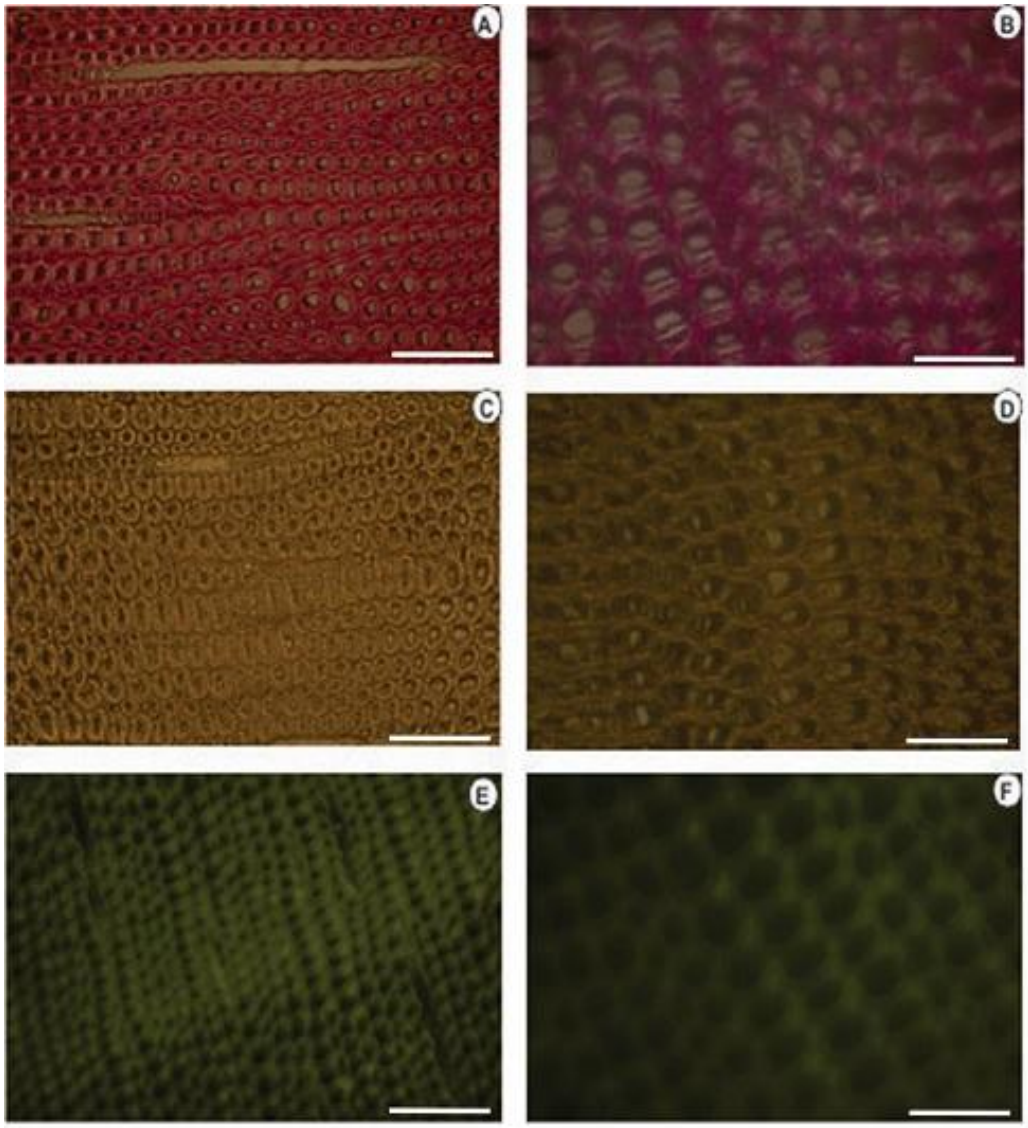

Figure 8. Histochemical tests applied to the cross-section of knotted *Pinus elliottii* var. *elliottii* Engelm wood (A, B): Wiesener test showing less aldehyde lignin (lighter colour compared to the colour of knotless wood). (C, D): Maüle test (E, F): Lignin autofluorescence. Bars: (A, C, E) = 20μm; (B, D and F) = 100

4. Discussion

For some authors, the lignin content reported for coniferous wood ranged from 25-35% (Sansígolo and Barreiros, 1998; Carvalho *et al.*, 2009). Analysing the percentages found in *Pinus elliottii* var. *elliottii* Engelm in this study, both for knotless wood and transition wood, the values for almost all individuals correspond to the expected range for coniferous wood. Studies have found values of 28% of insoluble lignin for the same species (Balloni, 2009). In knotted wood, the maximum percentage values of Klason lignin represent 39% and 37%. In studies with samples of 30 Norway Spruce knots (Willför *et al*., 2003c), variations between 6-24% were found for lignin content.

In this study, considering the radial orientation, the samples were taken horizontally more externally. Authors claim that there is a gradient of lignin concentration in knots, with a higher percentage occurring in the knotted portions inserted inside the trunk, progressively decreasing to a level below 1% as the knot is projected between 10-20 cm out of the trunk (Willför *et al*., 2003c). This suggests that, for knotted *Pinus elliottii* var. *elliottii* Engelm wood, the percentage of Klason lignin could be even higher than found here.

Compared to *Araucaria angustifolia* (Bertol.) Kuntze and *Pinus silvestre* L., the percentage of lignin for *Pinus elliottii* var. *elliottii* was lower, but confirms the percentage increase of lignin in the knot (Hillis and Inoue, 1968; Anderegg and Rowe, 1974), compared to the tissue adjacent to the knot and the tissue of the knotless wood. According to the authors, in *A. angustifolia* the percentage of lignin in knots is up to 20% higher than in the wood adjacent to the knots. This may be related, according to Willför *et al.* (2003a), to the oligolignans formed at an early age of the tree or knot and mainly to the lignans produced as the knot ages.

Other studies (Willför *et al*., 2003a; (Willför *et al*., 2003b) found that *P. silvestris* knotted wood (predominant species in Europe) contains from 0.4-2.9% more lignin than the wood adjacent to the knot. Other authors (Phelan *et al*., 2009)

stated that the content of phenolic substances varies significantly, not only between species, but between the knots of the same tree. The scarcity of literature that justifies the variation of lignin content exclusively in knots, limits the comparison of the results obtained in this study with the variation of lignin observed in compression wood.

The similarity of the lignin concentration found in *Pinus elliottii* var. *elliottii* for the three regions (bottom, middle and top), can be explained by the reduced number of individual samples (five), since for the series constituted by the set of average samples converging to the population average, the sample size (n) must be significantly large, based on the Central Limit Theorem (Gotelli and Ellison, 2011; Rodrigues, 2011). In a study of more than 30 samples of knots from seven Norwegian spruce trees, the authors demonstrated a percentage of 6 to 24% more lignin (Willför *et al*., 2003c), while the other authors (Holmbom *et al*., 2003), studying more than 50 individuals and showed that the knots could contain higher quantities of polyphenols than the wood next to the knot, with these amounts reaching up to 100 times more for some species. In an evaluation of knot extracts from *Pinus sitchensis* (Bong.) Carrière and *Pinus banksiana* Lamb. (Phelan *et al*., 2009), the authors verified that there was no similarity in the lignin values between species, with the *P. sitchensis* knot extract presenting content that was three times higher. Other authors did not evaluate the variation in the lignin content of knots along the trunk.

Regarding the 13C magnetic resonance spectra of wood from the "bottom knot", "middle knot" and "top knot" regions, the comparison of values obtained with the results of other authors (Landucci *et al*., 1998; Souza *et al*., 2011) showed that spectra signals of 13C NMR from *P. elliottii*, var. *elliottii* can be divided into three main types: acetyl groups (168-171 ppm); aromatic carbons, which can be divided into quaternary (125-160 ppm) and methylene carbon atoms (110-125 ppm) and signals from side chain carbon atoms (50-90 ppm).

Despite the similarity of the 13C spectrum signals for the three regions, differences in absorption intensity relativeto each signal were observed when comparing signals obtained from the bottom knot region with the carbon signals from the middle knot and top knot regions. Although the absorptions may reveal errors due to contributions from the 1H nucleus, they are minimised in their amplitude due to the combination of radiation between the 13C and 1H nuclei.

As such, the crystallinity indices of cellulose from different regions of wood were estimated. This estimate was based on dividing the areas of crystalline and non-crystalline C4 signals between 80-93 ppm. The determination was calculated based on dividing the area of the crystalline cellulose C4 signal (87-90 ppm) by the sum of the C4 signals between in 80-93ppm (Park *et al.*, 2010).

Signals characteristic of C2, C3 and C5 carbons and of cellulose and hemicellulose – signal 12 (Table 3) with the same intensity, were obtained from the bottom knot, middle knot and top knot regions. Signals characteristic of C- α in β-O-4 G units (guaiacyl lignin) were similar between the spectra obtained from the middle and top regions of knotted wood – signal 13 (Table 3) and Figures 2b and 2c, but different from signals observed in the spectra obtained from the bottom knot wood (Figure 2a).

These signals (C-α in units β-O-4-G) were similar to the signals obtained from the knotless wood from the three analysed regions (bottom, middle, top). The C2, C3 and C5 regions of cellulose and C-α H2 lignin presented higher intensities in the middle and top samples compared to bottom samples. C6 signals, characteristic of crystalline cellulose, were observed in all the knot region spectra – signal 19 (Table 3), but lower signal intensity and greater signal width were observed in the spectra of the bottom knot region (Figure 2a).

While the percentage of Klason lignin did not differ when compared longitudinally, the percentage of cellulose crystallinity showed significant variation. In the knot region there was an increasing linear variation in cellulose crystallinity, with the bottom knot wood presenting the lowest percentages (23%) followed by the middle knot wood (27%) and the top knot wood presenting the highest cellulose crystallinity (30%).

Comparing the "bottom knot" and "knotless wood bottom" conditions separately, the percentage of cellulose crystallinity washigher in "knotless wood bottom". The isolated comparison between the "middle knot" and "knotless middle" revealed the inversion of these values, where the "middle knot" presented more molecular crystallinity. In the evaluation of the top regions, the condition "knotless top" presented the highest percentage of molecular cellulose crystallinity. Compared to each other, it is possible to identify the same percentage of cellulose crystallinity between the "bottom knot" and "knotless middle" conditions.

In the analysis of 13C NMR CP/MAS spectra, the similarity of signals found between knotted and knotless wood samples and the comparison of the results with values found in the literature, suggest the same chemical composition between regions. This presupposes that in all the samples, the physiological process quantitatively maintained the same ratio (Souza *et al*., 2011). Broad signals, which indicate high heterogeneity and altered chemical structure (Froass *et al.*, 1996), were discretely observed in knotless wood spectra.

The comparison of 13C NMR data with values described in the literature (Lago and Roque, 2009), suggest that the lower quantity of guaiacyl lignin signals observed in the knot sample spectra is related to the interference of other substances, observed in the knot samples spectra is related to the interference of other substances, where the functional groups are obscured by the complexity of natural polymers.

Lignin isolated from most hard woods is essentially pure guaiacyl lignin (Obst and Landucci, 1986) but the difficult removalof traces of carbohydrates and foreign components, especially if they are covalently connected to the lignin polymer, interferes with the visualisation of the corresponding signal (Landucci *et al*., 1998). The heterogeneity of signal intensity, especially for signals characteristic of cellulose, hemicellulose and cellulose molecule crystallinity, although subtle, also indicate the interference of other chemical elements.

Comparing the Klason lignin percentage graphs with thecellulose crystallinity percentage graph, it is possible to infer the existence of a correlation between the lignin content and cellulose molecule crystallinity. Other authors (Donaldson and Knox, 2012) claim thatthere are consistent correlations between hemicellulose components (polysaccharides containing galactose, mannose and xylose) and cell wall lignification, and although less than 5% of data variation in the percentage of lignin in the bottom, middle and top regions of *P. elliotti* var *elliotti* was explained by the variable position on the trunk, the isolated values of these percentages suggest increasing variation along the trunk.

The lack of similarity presented for the percentages of lignin observed between knot regions and the adjacent regions, corroborates the thesis of the existence of differentiated patterns in the process of formation, differentiation, and maturation of knotted wood cells. The variation in cellulose crystallinity showed an increasing percentage as trunk height increased. This variation can be attributed to the influence of hormonal factors. The closer to the apex, the greater the influence of AIA (auxin) on the formation of tracheal elements and the greater the number of cells in the process of cellular division, the lower the increments of lignin disposition or secondary chemical components (Aloni, 2023).

For cell formation, several cellulose chains unite through hydrogenic connections to form microfibrils, which can be associated with other macromolecules through their -OH groupings. At the start of microfibril formation, cellulose is presented in its crystalline form. As the microfibril increases in size, with the deposition of more cellulose chains, it starts to suffer from the interference of localised defects, contributing to the formation of a greater amorphous region of cellulose (Oliveira, 2009). Since the cells of the knot top region are experiencing a greater rate of division, the cellulose chains responsible for the formation of microfibrils do not associate with other macromolecules, remaining mainly crystalline.

The decreasing polar flow of auxin, from the cauline apex, induces the differentiation of tracheal elements, promoting more cell division and an increase in the diameter of elements (Aloni, 1987; Aloni and Peterson, 1997; Evert, 2013). However, this gradient is also responsible for the decrease in element density, in the direction of leaves to roots (Aloni and Zimmermann, 1983). As they are closer to the physiologically more active zones of AIA production (young leaves), the branch/trunk intercession points (knots) are more susceptible to the action of this hormone.

Regarding the induction of tracheal differentiation by decreasing auxin, this may be associated with hormonal balance, with an increase in cytokinin regulating cell division. As well as the reduced concentration of the inhibitory hormone abscisic acid and the increase in day length, inducing greater exchange activity (Galston and Davies, 1972; Taiz *et al*., 2021). According to the authors, the plant's response to auxin depends both on the nature of the tissue and on the concentration of the substance present.

The difference in crystallinity behaviour observed in the knotless wood region, where the wood region at the bottom presented more crystallinity than wood in the middle of the trunk, can be explained by the heterogeneity of growth, thickening of anatomical elements and hormonal action as described in the literature.

In the histochemical tests, the lignification of the cell wall was divergent between knotted and knotless wood. Studies have shown that aldehyde structures are constructed during the initial stages of xylem cell wall lignification and that the Wiesener staining test can be used for the specific detection of these structures contained in lignin (Pomar *et al.*, 2002). In a study using the stem sections of *Zinnia elegans* L. treated with the Wiesener and Maüle test (Barceló *et al*., 2000), the authors observed that the colour of sections stained with phloroglucinol-HCL (Wiesener) extended beyond young xylem cell differentiation, while staining with Maüle reaction was restricted to already differentiated xylem cells.

Compared to the results in the literature (Fukushima and Terashima, 1991) and supported by the results of the chemical lignin analyses, where lower lignin contents were obtained for knotless wood, it is possible to infer that the knotless wood and knotted wood tissues, although close, present different phases of lignification. Since the process is mirrored through the secondary wall towards the lumen (Donaldson, 2001) and is controlled to a significant extent for individual tracheids (Donaldson, 1994), the brown-gold colour observed in the cells of knotless wood using the Maüle test suggests a lower level of lignification maturation and a lower lignin content in the cell wall.

Studying reaction tissue formation in *Medicago sativa* L. (Patten *et al*., 2007), the authors found that the percentage of lignin can increase tenfold between the stages of cell wall lignification. These authors observed that the greater the proportion of guaiacyl lignin in the tissue, the more potentialized (dark brown) the Maüle test reaction.

The results obtained from the histochemical tests corroborate the results obtained from the 13C NMR, where greater quantities of carbon signals characteristic of guaiacyl lignin were attained from the spectra of the knotless wood regions. In the fluorescence test, it was possible to observe lignin surrounding the cells of knotless wood samples.

Based on Emonet and Hay (2022), the local lignin deposition surrounding the cells of the knotless wood cells is a common mechanism that follows precise patterns, although with distinct genetic basis in different cell types with functions in different tissue contexts. Mainly for tracheal elements, as they depend on a lignified secondary cell wall to provide mechanical strength, rigidity and hydrophobicity.

The vast majority of scientific research on macromolecular lignin, its initiation sites and cell wall lignification are limited to the investigation of these processes in wood cells whose growth is normal or at best, compression and rection cells (Whetten and Sederoff, 1995; Donaldson, 2001; Grabber, 2005; Donaldson and Knox, 2012). Given the high variety found in the wood, either longitudinally or radially, between the anatomical elements and even between the chemical elements (Zimmermann and Potter, 1982; Muniz, 1993; Ballarin and Palma, 2003; Silva *et al.*, 2005) it can be

assumed that the results obtained in such research express, with a certain approximation, but not certainty, the dynamics of anatomical formation and chemical composition of cells that compose knotted wood.

The differentiated lignification between normal wood and compression wood, is explained by the chemical variation of the cell wall (Donaldson, 2001), where the middle lamella of normally growing wood cells is more lignified than the secondary wall. In compression wood, this pattern is significantly modified, with a reduction in average lignification in the middle lamella and an increase in the lignification of the outer secondary wall (Donaldson, 2001).

The auxin concentration gradient, proposed by some authors (Aloni and Zimmermann, 1983), also allows lower lignin deposition and secondary chemical elements in the cells of knotless samples, both at the bottom and middle bole, since the low auxin concentrations induce slow cell differentiation and that the cells of these regions are more distant from the auxin producing regions, which limits the action of the hormone (Shimoyama, 2005; Evert, 2013).

5. Conclusions

There is anincreasing gradient of lignin percentage between knotless wood, transition wood and knotted wood.

In knotted wood tracheids, lignification is at a more advanced stage of maturation than the knotless wood trachids and the lignin present in the knotless wood tracheids are more aldehydic than lignin in knotted tracheids.

The development dynamics between the anatomical elements of knotless wood and knotted wood showed different patterns. It is possible to conjecture that such characteristics interfere with the resistance of wood in these regions along the trunk.

Crystalline cellulose signalin “knotless wood” were less intense compared to signals in the middle knot and topknot regions. The bottom region of wood showed higher crystallinity than the wood in the middle trunk and lower than the top region.

The lignin structure in the regions of knotless and knotted wood was different, despite the proximity of these regions to each other.

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