ANALYSIS OF WOOD IN *HEVEA BRASILIENSIS*: ESTIMATION AND QUANTIFICATION OF LIGNIN BIO-POLYMER AND CELL WALL PHENOLICS

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ABSTRACT

Lignins are the second largest phenolic bio-polymers of the cell wall after cellulose and their deposition is associated with mechanical strength, improved sap conduction, defense mechanisms and imperviousness to biodegradation. As the biosynthesis of lignin and polyphenolic derivatives in living trees, especially in fast growing woody species like *Hevea*, facilitates the improvement of quality and durability of timber to a great extent, these two wood quality traits has been considered for the early selection of timber quality in *Hevea brasiliensis*. A study was conducted in stems of 19 wild *Hevea* germplasm accessions and six popular Wickham clones in the juvenile growth phase to estimate and quantify the insoluble lignin content and wall associated phenolics. The results revealed that the quantity of lignin produced was increased in association with the progress of secondary thickening. Variation in the percentage of lignin and cell wall phenolics between the wild accessions and Wickham clones was highly significant.

In wild *Hevea* germplasm the lignin percentage ranged from 19.28 – 24.75 % whereas 20.84 – 23.36 % in Wickham clones. Of the 19 wild accessions analysed, three showed significantly higher percentage of lignin over five Wickham clones. With regard to cell wall phenolics, twelve wild accessions had significantly higher percentage than that of Wickham clones.

The study revealed that the quantification of lignin bio-polymer and cell wall phenolics can be used as reliable tools for the early detection of wood quality in *Hevea brasiliensis*.

Key words: Cell wall phenolics, Hevea brasiliensis, lignification, rubberwood, timber quality, wild Hevea germplasm.

INTRODUCTION

Lignins are phenolic polymers of the cell wall and form the second most abundant group of biopolymers after cellulose (Roth *et al.*, 1997; Boudet, 2000). The deposition of lignin in plant cell walls is one of the mechanisms which allowed the development of upright plants adapted to terrestrial habit. In different species of woody plants, lignin content range between 15 and 36% of the dry weight of wood (Zobel and Buijtenen, 1989). The functional significance of lignin has been mainly associated with mechanical support, improved sap conduction, defense mechanisms, strengthening plant tissue and imperviousness to biodegradation. (Piquemal *et al.*, 1998; Gierlinger *et al.*, 2004).

Lignins are heterogenous tridimensional phenolic polymes formed by the oxidative polymerization of at least two of the three monolignols viz. p-coumeryl, coniferyl and sinapyl alcohols giving rise to hydroxyphenyl (H), Guaiacyl (G) and Syringal (S) lignin units, respectively. (Campbell *et al.*, 1996; Boudet *et al.*, 1996; Yahiaoui *et al.*,1998; Boudet,2000). The typical lignins of hardwoods are Guaicyl and Syringyl lignins, formed from the co-polymerization of coniferyl and sinapyl alcohols, respectively. (Higuchi, 1985). From the

biosynthetic point of view, lignification is a tightly regulated and dynamic process subject to modulation at different levels during normal development and response to different environmental stresses. Recent enzymatic data and various lignin genetic engineering studies revealed that there must be a specific route to the synthesis of lignin precursors in the cytoplasm, which in turn is translocated to the cell wall for polymerization (Boudet, 2000; Gierlinger *et al.*, 2004). Regulation of transport or polymerization could effect the quantity of lignin produced through specific enzymatic activity and metabolic chanelling of substrates and products.

The potentiality of rubber timber for various industrial applications has been well established. However, the major limitations preventing the wide utilization of rubber wood are (i) the formation of high proportion of unlignified or partially lignified tension wood fibers and low level of lignification in normal fibers and (ii) high susceptibility to biological deterioration due to the low level of phenolic conversion of reserve metabolites into extractives. As the biosynthesis of lignin and polyphenolic derivatives in living trees, especially in fast growing woody species like *Hevea*, facilitates the improvement of quality and durability of timber to a great extent, studies on the pattern of lignification and wall associated phenolics in *Hevea brasiliensis* assumes significance. Reghu *et al* (2007) studied the lignification pattern in developing stems of *Hevea brasiliensis* and reported positive correlation with CAD activity and lignification pattern .The authors further opined that CAD activity and lignification could be used as early selection parameters for wood quality in developing stems of *Hevea brasiliensis*.

In this context the present study was taken up to understand the variability in the insoluble lignin content and associated cell wall phenolics in selected wild *Hevea* germplasm and popular Wickham clones.

MATERIALS AND METHODS

Stem samples were collected from three inter-whorl positions (1st, 2nd and 3rd) of four year old plants (Fig.1)of 19 wild *Hevea* germplasm accessions representing the three provenances of Brazil *viz.* Acre, Rondonia and Matto Grosso from the timber evaluation trial at Regional Research Station, Padiyoor, Northern Kerala situated at 75° 36' E and 11° 58' N. The annual rainfall of the station was 3500-4000 mm. The statistical design adopted was Randomized complete block with three replications and a plot size of four plants at a spacing of 4.9X4.9 m. The popular six clones *viz.* RRII 105, RRII 33, RRII 118, PB 235, PB 260 and RRIM 600 were used as control.The characters studied were (i) insoluble lignin percentag represented as Klasson lignin (% wt. of EXR / mg) and (ii) percentage of cell wall phenolics (μ OD / mg. of dry wt. of EXR). The estimation and quantification of lignin and cell wall phenolics were carried out using the Extractive free Xylem Residue (EXR) prepared from the sampled wood tissue as follows.

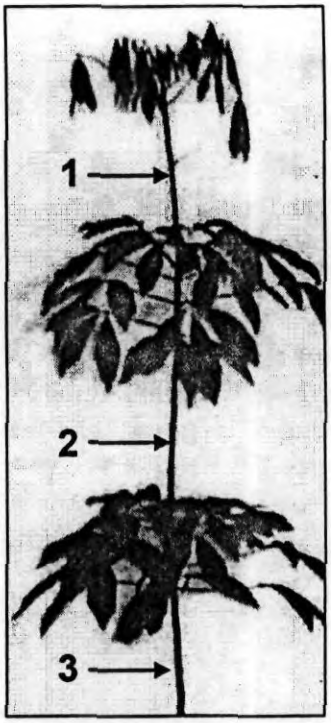

Fig.1 Four year old plant: 1)1st interwhorl 2)2nd iterwhorl 3) 3rd interwhorl positions

Estimation of insoluble lignin

The insoluble lignin content was estimated from the cell wall residue (CWR) obtained through solvent extraction of the powdered xylem tissue prepared from the same stem samples as per Dence (1992). The frozen stem samples in liquid nitrogen for 1h immediately after collection were freeze dried at – 80 ^{0} for 72 hrs. The frozen wood tissue were then fine powdered at 180 µm mesh size using the laboratory ball mill and sequentially extracted with water, ethyl alcohol and toluene in cellulose thimples using soxhlet apparatus. The resulted cell wall residue (CWR) obtained was rinsed with acetone, dried and prepared.the extractive free xylem residue (EXR) The EXR was used for the estimation and quantification of lignin content by weighing the residue left after saccharification of cellulose and other cell wall polysaccharides with sulphuric acid through vacuum filtration in glass microfibre filter adopting gravimetric Micro klasson technique (Whiting *et al.*, 1981). The GF filter along with the residue (Fig.2) obtained after vacuum filtration was dried in Pasteur oven at 37°C overnight were then re-weighed The percentage of lignin, represented as Klasson lignin (% wt. of EXR) was calculated as follows.

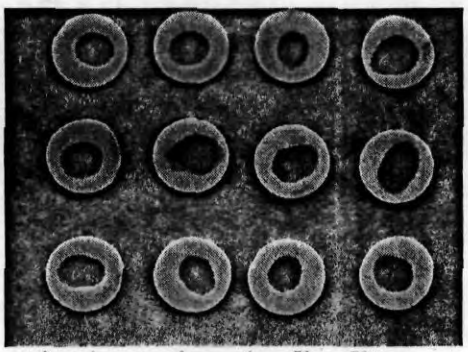

Figure 2 Insoluble lignin estimation on glass microfibre filter

$$\text{Lignin (\%)} = \frac{Z - Y}{X} \times 100$$

Where X - wt. of EXR (g)
Y - wt. of pre-weighed GF filter
Z - wt. GF filter + Residue

Estimation of Cell wall phenolics

The estimation of cell wall phenolics was carried out using EXR .The pre-weighed EXR hydrolysed with 1M Sodium hydroxide and agitated in the dark in a rotary shaker for 2 hrs. and extracted with ethyl acetate solution. The resultant extract was dried in speed vacuum concentrator till to get phenolics pellets. The phenolics pellets thus formed were re-dissolved in Methyl alcohol and measured the absorbance at A 280 to calculate the percentage Cell wall phenolics (U OD / mg. of dry wt. of EXR) as follows.

$$\mu \text{ OD / mg. of dry wt. of EXR} = \frac{A\ 280}{\text{Wt.of EXR}}$$

For comparison of OD between different samples, the absorbance of control samples were taken as 100%.

Analysis of variance was done and the significance was tested with reference to the standard F table

RESULTS AND DISCUSSION

Lignin Content

Lignin content was estimated from the wood samples of three inter-whorl positions from each plant and worked out the mean value as plant average. Within plants the percentage of lignin was minimum in the first inter-whorl and maximum in the 3rd inter-whorl indicating lignification gradually increased from top to bottom in association with the secondary thickening.

Table 1 represents the quantity of lignin estimated from the three inter-whorl positions of four year old plants of the wild accessions as well as popular clones. The test for significance revealed that the variation in the quantity of lignin produced in the three inter-whorl positions was significant. With regard to the plant average, 3 wild accessions *viz.* AC 685 (24.75%), RO 322 (23.99%) and RO 255 (23.90%) showed significantly higher lignin percentage over all the six control clones; 12 accessions over RRIM 600; 9 accessions over PB235; and 6 accessions over PB 260.. Among the Wickham clones, RRII 118 (23.36%) had comparatively high lignin percentage followed by RRII 105 (22.77 %),, RRII 33 (22.73 %),, PB 260(21.97 %), PB 235 (20.84%) and the least in RRIM 600 (20.08 %).

Table 1 Estimation of lignin content (%) in wild accessions and Wickham clones

Sl. Nos.	Wild Accns.	Lignin (%)
1	AC 685	24.75
2	RO 322	23.99
3	RO 255	23.92
4	MT 922	22.73
5	MT 941	22.73
6	AC 635	22.52
7	MT 1020	21.98
8	RO 879	21.50
9	MT 1021	21.43
10	MT 935	21.04
11	AC 650	21.03
12	MT 1032	20.77
13	AC 637	20.50
14	MT 919	20.34
15	AC 655	20.27
16	AC 707	20.22
17	MT 915	20.12
18	AC 651	19.32
19	MT 999	19.28
	Wickham clones	
1	RRII 118	23.36
2	RRII 105	22.77
3	RRII 33	22.73
4	PB 260	21.97
5	PB 235	20.84
6	RRIM 600	20.08
	Cd (P = 0.05)	**0.54**

Cell wall Phenolics

Table 2 Depicts the quantity of cell wall phenolics estimated from the three inter-whorl positions of four year old plants of the wild accessions as well as wickham clones. The result indicated that though the variation in the percentage of cell wall phenolics was not statistically significant among the wild and wickham clones, numerical variation was very evident. With regard the percentage of cell wall phenolics in comparison between wild accessions and wiskham clones, 4 accessions (MT 1021, MT 1020, MT935 & AC 650) had a higher percentage over all the six wickkham clones. Similarly, 7 accessions (MT 922, MT 919, RO 255, AC 685, AC 655, AC 651 and AC 707) had the CWP % five wickham clones except PB 260. One accession, MT 915 showed high percentage of CWP over 4 wickham clones and 2 accessions over 3 wickham clones Rest of the wild accessions evaluated showed a decreasing trend in the percentage of cell wall phenolics in comparison with that of the control clones.

Table 2 Percentage of Cell wall Phenolics in Wild accessions and Wickhan clones

Wild Accns.	Cell Wall Phenolics (μ OD/mg/dry wt. Of EXR)	% of Cell all Phenolics in comparison with Control clones					
		PB 235 (0.310)*	RRII 33 (0.295)*	RRII 105 (0.304)*	RRII 118 (0.307)*	RRIM 600 (0.319)*	PB 260 (0.359)*
MT1021	0.372	+20.8	+25.9	+22.3	+ 21.3	+16.6	+3.6
MT1020	0.368	+18.4	+24.4	+20.9	+ 19.0	+15.2	+2.4
MT 935	0.362	+16.5	+22.5	+19.0	+17.9	+13.4	+1.0
MT 922	0.321	+ 3.4	+ 8.7	+ 5.6	+ 4.6	+ 0.5	-11.0
MT 919	0.349	+12.6	+18.3	+14.8	+13.8	+ 9.4	- 2.7
MT 999	0.294	- 5.3	- 0.5	- 3.3	- 4.1	- 8.0	-18.0
MT 1032	0.279	- 10.0	- 5.4	- 8.0	- 8.9	- 12.4	-22.1
MT 915	0.318	+ 2.6	+ 7.8	+ 4.8	+ 3.8	- 0.2	-11.3
MT 941	0.276	-11.3	- 6.7	- 9.4	- 10.2	- 13.7	-23.3
AC 635	0.297	- 4.3	+ 0.6	- 2.3	- 3.1	- 6.9	-17.2
RO 322	0.252	-19.2	-15.0	- 17.5	-18.2	- 21.4	-30.4
RO 255	0.338	+ 8.8	+14.4	+11.2	-10.2	+ 5.9	- 8.0
AC 685	0.339	+ 9.2	+14.8	+11.6	+10.6	+ 6.3	- 5.6
RO 879	0.460	+48.1	+55.7	+51.3	-49.9	+ 44.1	+28.1
AC 637	0.289	- 6.7	- 1.9	- 4.7	- 5.6	- 9.2	-19.3
AC 650	0.374	+20.4	+26.6	+23.0	+21.9	+ 17.2	+ 4.2
AC 655	0.329	+ 5.9	+11.4	+ 8.2	+ 7.2	+ 3.1	- 8.4
AC 651	0.341	+ 9.8	+15.5	+12.2	+11.2	+ 6.9	- 5.0
AC 707	0.347	+11.7	+17.5	+14.1	+13.1	+ 8.7	- 3.4
Cd (P = 0.05)	NS						

* Values in parenthesis : CWP % of Wickham clones ; + = higher CWP % over Control
- = lower CWP% over control

Reghu *et al* (2007) reported the positive correlation of Cinnamyl Alcohol Dehydrogenase (CAD) activity and lignification pattern in young stems of *Hevea brasiliensis*. The authors further opined that CAD activity and lignification could be used as early selection parameters for wood quality. The present study revealed that the quantity of lignin produced significantly varied among wild *Hevea* germplasm as well as domesticated clones. Moreover, the wall associated phenolics, being the precursor of lignin bio-polymer,also showed considerable variation among *Hevea* clones indicating that the quantification of cell wall phenolics can also be used as an early selection parameter to ascertain wood quality in the juvenile growth phase Those wild accessions showed high content of lignin also had high content of cell wall phenolics.

In general, twelve wild *Hevea* accessions had high percentage of lignin than that of control clones and fourteen accessions with a corresponding increase in cell wall phenolics. These potential accessions can be used as parents for future breeding programmes for the improvement of timber quality of *Hevea brasiliensis*. This is the first report on the variation in cell wall phenolics in *Hevea brasiliensis*

The quality of lignin produced in hardwoods is assessed by the Syringyl / Guaiacyl lignin monomer units (Higuchi, 1985). Hence further investigation on the status and role of wall associated phenolics in the synthesis of Syringyl / Guaiacyl lignin monomer units leading to lignin biosynthesis in *Hevea brasiliensis*.

CONCLUSIONS

A study was carried out in four years old stems of 19 wild *Hevea* germplasm accessions and six popular Wickham clones A study was conducted in stems of 19 wild *Hevea* germplasm accessions and six popular Wickham clones to estimate and quantify the insoluble lignin content and wall associated phenolics in the juvenile growth phase. The quantity of lignin produced was increased in association with the progress of secondary thickening. Variation in the percentage of lignin and cell wall phenolics between the wild accessions and Wickham clones was highly significant. The estimation and quantification of lignin bio-polymer and cell wall phenolics can be used reliable parameters for the early selection of potential genotypes for wood quality. High percentage of lignin and cell wall phenolics was recorded in twelve and 14 wild accessions, respectively. These l accessions can be used as potential parents for future breeding programmes for the improvement of timber quality and development of latex-timber clones.

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REFERENCES

Boudet, A.M. 2000 . Lignin and lignification: Selected issues. *Plant Physiology and Biochemistry*, **38**(1&2), 81-96

Boudet, A.M., Goffner, D.P. and Pettenati, J.G. 1996. Lignins and lignification: recent biochemical and biotechnological developments. *Plant Biology and Pathology*, **319**, 317-31.

Campbell, M.M.,and Sederoff, R.R.1996. Variation in lignin content and composition. *Plant Physiol.* , **110**, 3-13.

Dence, C.W. 1992.The determination of lignin. *Methods in lignin chemistry*,(Lin S.Y. and Dence, C.W.) Springer-Verlag, Berlin pp.31-70.

Gierlinger,N., Jacques,D., Schwanninger,M., Wimmer,R., & Paques, L.E. 2004. Heartwood extractives and lignin content of different larch species (Larix sp.) and relationships to brown-rot decay-resistance. *Trees*, **18**, 230-236.

Higuchi, T. 1985.Biosynthesis of lignin. In: *Biosynthesis and biodegradation of wood components*.(ed. T. Higuchi). Academic Press, New York, pp.141-160.

Montis, B. 1984. Recent advances on lignin inhomogeneity. *Annual proceedings of the Phytochemical Society of Europe*, **25**, 161-81.

Piquemal, J., Lapierre, C., Myton, K., O'Connel. A., Sahuch, W., Grima-Pettenati, J., & Boudet, A.M. 1998. Down-regulation of Cinnamyl-CoA Reductase induces significant changes of lignin profiles in transgenic tobacco plants. *The Plant Journal*, **13**(0) ,101-113

Reghu, C.P., George, P., and Varghese, Y.A.2007. Screening of *Hevea brasiliensis* germplasm for wood quality using cinnamyl alcohol dehydrogenase (CAD) activity and lignification pattern. *Natural Rubber Research*, **20** (1&2), 1-8.

Roth, R., Boudet, A.M.,and Lezika, R.P. 1997. Lignification and Cinnamyl alcohol dehydrogenase activity in developing stems of tomoato and poplar: a spatial and kinetic study through printing. *Journal of Experimental Botany*, **48**(307), 247-254.

Whiting, P., Favis, B.D., St-Germain, FGT., & Goring, DAI. 1981. Fractional separation of middle lamella and secondary wall tissue from spruce wood. *J.Wood. Chem.Tech.*, **1**, 29-42.

Yahiaoui, N., Marque, C,. Myton, K.E., Negrel, J., and Boudet, A.M. 1998. Impact of different levels of cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco plants. Planta, **204**, 8-15.

Zobel B.J and Buijtenen, J.P. 1989. Wood variation. Its causes and control. *Springer Verlag*, Heidelberg, Germany.