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Early detection of wood quality in *Hevea brasiliensis* (Rubber wood) using cinnamyl alcohol dehydrogenase (CAD) activity and lignification

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Abstract Lignins are phenolic polymers of the cell wall and cinnamyl alcohol dehydrogenase (CAD) is the key enzyme involved in the synthesis of lignin monomers. A study was conducted in stems of 18 wild *Hevea* germplasm accessions and five popular clones to localize and correlate CAD activity and lignification at various stages of xylogenesis through tissue printing and in situ localization as well as quantitative image analysis techniques. Simultaneously, quantification of lignin produced was also done by classical histochemistry and gravimetric estimation. CAD activity was maximum during the early stages of stem development and minimum during the mature stage of xylogenesis whereas the pattern of lignification showed a reverse trend indicating that as xylogenesis increased, CAD activity decreased. The quantity of lignin produced was also increased in association with progress of secondary thickening. The percentage of lignin at different developmental stages of the stem was highly significant. The percentage of lignin ranged from 21.04–27.4 % in wild *Hevea* germplasm and 20.01–23.00 % in popular clones. Of the 18 wild accessions screened, nine showed significantly higher percentage of lignin over five popular clones. The present study revealed that CAD activity is involved in the lignification process in *Hevea* stem and its activity is restricted in the mature xylem. The CAD activity and lignification pattern in the juvenile growth phase can be used as reliable tools for the early detection of wood quality in *Hevea brasiliensis*.

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Introduction

Lignins are phenolic polymers of the cell wall and form the second most abundant group of biopolymers after cellulose (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. (Gierlinger et al. 2004).

Lignins are heterogenous tridimensional phenolic polymers 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 Syringyl (S) lignin units, respectively. (Campbell and Sederoff 1996; Boudet et al. 1996; Yahiaoui et al. 1998; Boudet 2000). The typical lignins of hardwoods are Guaiacyl and Syringyl lignins, formed from the co-polymerization of coniferyl and sinapyl alcohols, respectively (Higuchi 1985). Lignification is a tightly regulated and dynamic process subject to modulation at different levels during normal development and response to different environmental stresses. 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 (Gierlinger et al. 2004). Regulation of transport or polymerization could affect the quantity of lignin produced

through specific enzymatic activity and metabolic channeling of substrates and products.

The biosynthesis of lignin follows a long sequence of reactions involving (i) shikimate pathway which provide phenylalanine and tyrosine; (ii) the common phenylpropanoid pathway leading to the synthesis of cinnamoyl CoAs, which are the general precursors of a wide range of phenolic compounds, and (iii) the lignin specific pathway which channelises the cinnamoyl CoAs towards the synthesis of monolignols or lignin precursors, (Higuchi 1990). The targeting enzymes involved in the synthesis of monolignols are cinnamyl co-enzyme reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). The former enzyme (CCR) catalyse the conversion of cinnamyl CoAs to cinnamyl aldehydes whereas the latter (CAD) is an $NADPH^{+}$ specific oxidoreductase catalyzing the reversible conversion of cinnamyl aldehydes to the corresponding alcohols. Hence the enzyme CAD is closely related to lignification and the inhibition of its activity reduces the synthesis of lignin (Munch-Mani and Slusarenko 1996).

Several studies have shown that CAD may be polymorphic with isoforms that differ not only on substrate affinity, but also on molecular mass (Mansell et al. 1974; Goffner et al. 1992). Extensive works have also been done to down regulate CAD activity to overcome the negative impact of lignin during the delignification process in pulp and paper industry (Munch-Mani and Slusarenko 1996). Variation in the quantity and quality of lignin must be based on the control of metabolic flux into the pathway influenced by the levels of this enzyme activity.

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 the activity of lignin precursor enzymes in *Hevea brasiliensis* assumes significance. So far no such studies are known to have been conducted in *Hevea.*

Hence the present investigation is a pioneering attempt to understand the lignification pattern in developing stems of *Hevea brasiliensis* through identification and localization of CAD activity by tissue printing and in situ techniques and correlation of CAD activity with lignification by quantitative image analysis techniques. This study also aimed at early selection of wild *Hevea* accessions and Wickham clones for wood quality through lignin biosynthesis studies.

Materials and methods

Plant material

Stem samples were collected from three interwhorl positions (1st, 2nd and 3rd) of 1 year old plants of four popular rubber clones RRII 105, IRCA 111, IRCA 652 and PB 5/51 raised in polybags. Similarly stem samples of 4 year old plants of 18 wild *Hevea* germplasm accessions belonging to the three provenances of Acre, Rondonia and Matto Grosso in Brazil and two clones viz. RRII 105 and RRIM 600 were also collected from the source bush nursery established at Rubber Research Institute of India, Kottayam, Kerala as shown in Fig. 1.

Localization of CAD activity on tissue print/in situ localization

Cross sections were made from the fresh stem samples with disposable blades attached to Rechert Jung sledge microtome at 30 μm thickness. Four tissue prints were made of each interwhorl zone on nitrocellulose membrane (0.45 pore size, Schleicher and Shell). Fresh tissue prints were washed in cold 20 mM TRIS–HCl (pH 8.8) for 15 min to eliminate all hydrophilic metabolites that do not bind the membrane and to preserve the stability of the enzyme. In the case of in situ localization fresh sections were directly washed in TRIS–HCl prior to incubation.

Fig. 1 Interwhorl position in 1 year old plant

Cinnamyl alcohol dehydrogenase activity was detected histochemically on prints and fresh sections as described by Roth et al. (1997) with some modifications. Activity of the enzyme was tested by incubating the printed membrane/in situ section for 1 h at 37 °C with the following reaction medium: 1 mg ml^{-1} $NADP^{+}$; 3.6 mg ml^{-1} coniferyl alcohol (105778 CH: Chemos, Germany); 0.35 mg ml^{-1} nitro blue tetrazolium (NBT) in 20 mm TRIS–HCl (pH 8.8). The reaction was stopped by washing the membranes/sections in H_2O for 10 min and air-drying the samples. In the control, either coniferyl alcohol, or NADP or both were not added.

$$\text{Lignin } (\%) = \frac{\text{Wt. ofG. M.F. filter} + \text{residue} - \text{Wt. ofpre} - \text{weighedG.M.F. filter}}{\text{Wt. of EXR}} \times 100$$

Lignin Staining

Lignins were detected in stem sections by the Weisner reaction (Montis 1984). Stem sections prepared from the same interwhorl and the tissue prints were incubated for 3 min in the reaction medium. The images of the prints as well as lignin stained sections were documented in the image analysis system for quantification.

Image analysis of CAD activity and lignification

In order to correlate CAD activity with lignification on different stem interwhorl, image analysis was done. The tissue prints and the sections stained for lignin were transferred in a video image using CCD camera attached to Leitz Dm IRBE microscope and stored as a 512 × 512 pixel image into a synapse digital frame store and analysis was done with Image pro-plus software (Media Cybernetics, USA). The area of CAD activity and lignification were quantified in pixels. The number of pixels for each section was added to obtain the total number of pixels. An integrated value taking the surface of the stain and the optical density was used in the case of enzyme activity. This provides an estimation of CAD activity and lignified areas in each section.

Lignin quantification

The lignin content was estimated from the cell wall residue (CWR) obtained by solvent extraction of the powdered xylem tissue prepared from the same stem samples used for CAD activity as per the method suggested by Dence (1992). Stem samples collected were immediately frozen in liquid nitrogen for 1 h and then freeze dried at –80 °C for 72 h. The wood tissue was manually removed from the pith and bark, ball milled to a fine powder (180 μm mesh size) and sequentially extracted with water, ethyl alcohol and toluene: (1:1 v/v) using a soxhlet apparatus. The resulting CWR was rinsed in acetone, dried and the extractive free xylem residue (EXR) was prepared. 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 percentage of lignin was calculated as % weight of EXR as follows..

Results and discussion

CAD activity on tissue prints/in situ sections

The histochemical assays started within 1 h in tissue prints and 45 min in the case of in situ localization. The activity of CAD could be localized by observing the formation of the blue, insoluble formazan product (Figs 2, 3), whereas in the control no such product was observed revealing the absence of CAD activity.

Specificity of CAD reaction

Cinnamyl alcohol dehydrogenase activity on tissue prints as well as in stem sections was tested by coupling the reverse reaction of CAD (in vivo CAD reduces the coniferyl aldehyde to coniferyl alcohol) to reduction of NBT into formazan.

$$\text{Coniferyl alcohol} + \text{NADP}^{+} \rightleftarrows \text{CAD coniferyl aldehyde} + \text{NADPH} + \text{H}^{+}$$
$$\rightarrow \text{NADPH} + \text{H}^{+} + \text{NBT} \rightarrow \text{formazan} + \text{NADP}^{+}.$$

In the controls without substrate (coniferyl alcohol) no staining was observed, confirming the absence of CAD activity.

Correlation of CAD activity with lignification

The localization of CAD activity was the maximum in the first interwhorl which gradually decreased towards the 2nd and 3rd interwhorl. As xylogenesis increased, the activity

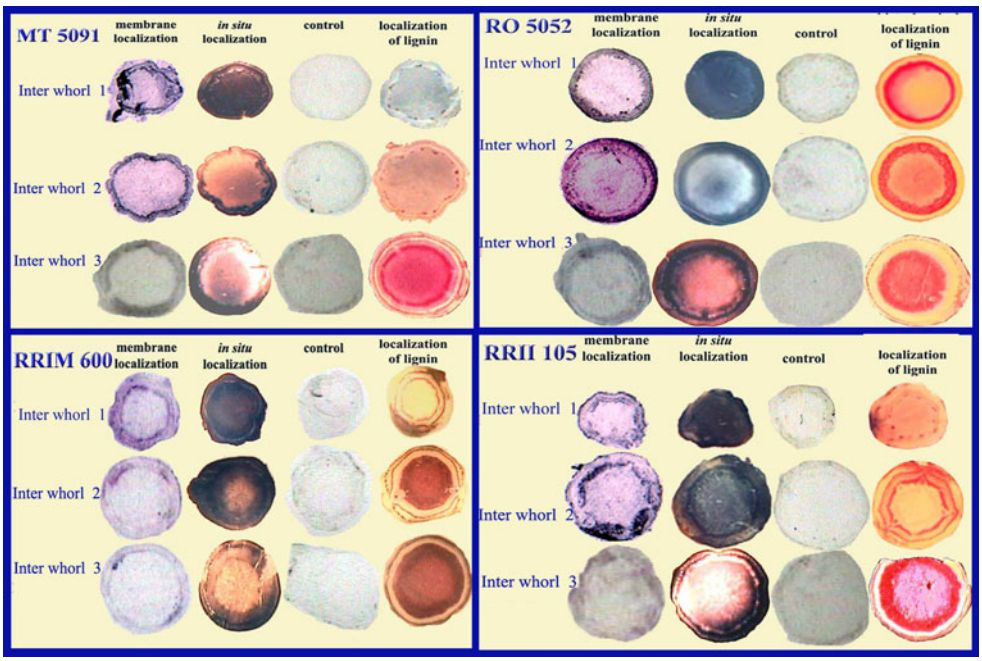

Fig. 2 Nitrocellulose membrane localization of CAD activity and lignification in Wild *Hevea* accessions and popular clones

of CAD decreased in the mature xylem (Fig. 3). In general, the CAD activity was observed on the periphery of the vascular tissue where new xylem and phloem differentiate. Observation of the lignified sections stained purplish red with Weisner reagent showed that CAD activity was found only in the vicinity of the cambial zone. Microscopic examination revealed that the intensity of CAD activity was more in nine wild accessions than the popular clones, RRII 105 and RRIM 600 (Fig. 2). Among the 18 wild accessions the intensity of CAD activity was minimum for MT 5091 and maximum for RO 5022 (Figs. 2, 4).

Lignin Content

Lignin content was estimated from the three interwhorl positions in four 1 year old clones is presented in Table 1. Within plants the percentage of lignin was minimum in the first interwhorl and maximum in the 3rd interwhorl indicating lignification gradually increased from top to bottom in association with the secondary thickening. With regard to the plant average, the percentage of lignin was more or less same in all the clones ranged from 20.64 (IRCA 652) to 21.33 (PB 5/51).

Table 2 represents the quantity of lignin estimated from the three interwhorl positions of 4 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 interwhorl positions was highly significant. With regard to the plant average, nine wild accessions comprising three from Acre (AC 4830, AC 4654 and AC 4638), two from Rondonia (RO 5022 and RO 4911) and four from Matto Grosso (MT 6180, MT 4697, MT 5085 and MT 4859) had significantly higher lignin percentage over the popular clones, RRIM 600 and RRII 105. Among the popular clones, RRII 105 had comparatively less lignin percentage (21 %) compared to RRIM 600 (23 %). Similar results also recorded for RRII 105 in the 1 year old stem samples of four popular clones. Those nine accessions which showed higher CAD activity also had higher percentage of lignin content.

The result obtained with tissue prints and in situ localization in *Hevea* stems indicated that the formazan precipitate obtained in the experimental conditions was due to CAD activity. This is evident from the lack of precipitate on the control (with out coniferyl alcohol) compared to those that included the substrate that produced the formazan as reported by Roth et al. (1997) in poplar and tomato. CAD activity was found in the younger internodal region and on restricted areas of developing xylem in older internodes. The site of CAD activity was limited to the differentiating xylem on the periphery of the lignified region in *Hevea.* CAD activity was expressed in living cells where $NADP^{+}$ and substrate were available (Goffner et al. 1992) and the monolignols are exported from their site of synthesis in the parenchyma cells towards the site of assembly in the vessel and fibers (Feuillet et al. 1995). The present study revealed that in *Hevea* the CAD activity drops after the third internode whereas the lignin deposition increased as reported in tomato stem (Roth et al. 1997). The result indicated that the flux of monolignol synthesis is limited in the mature xylem of *Hevea.*

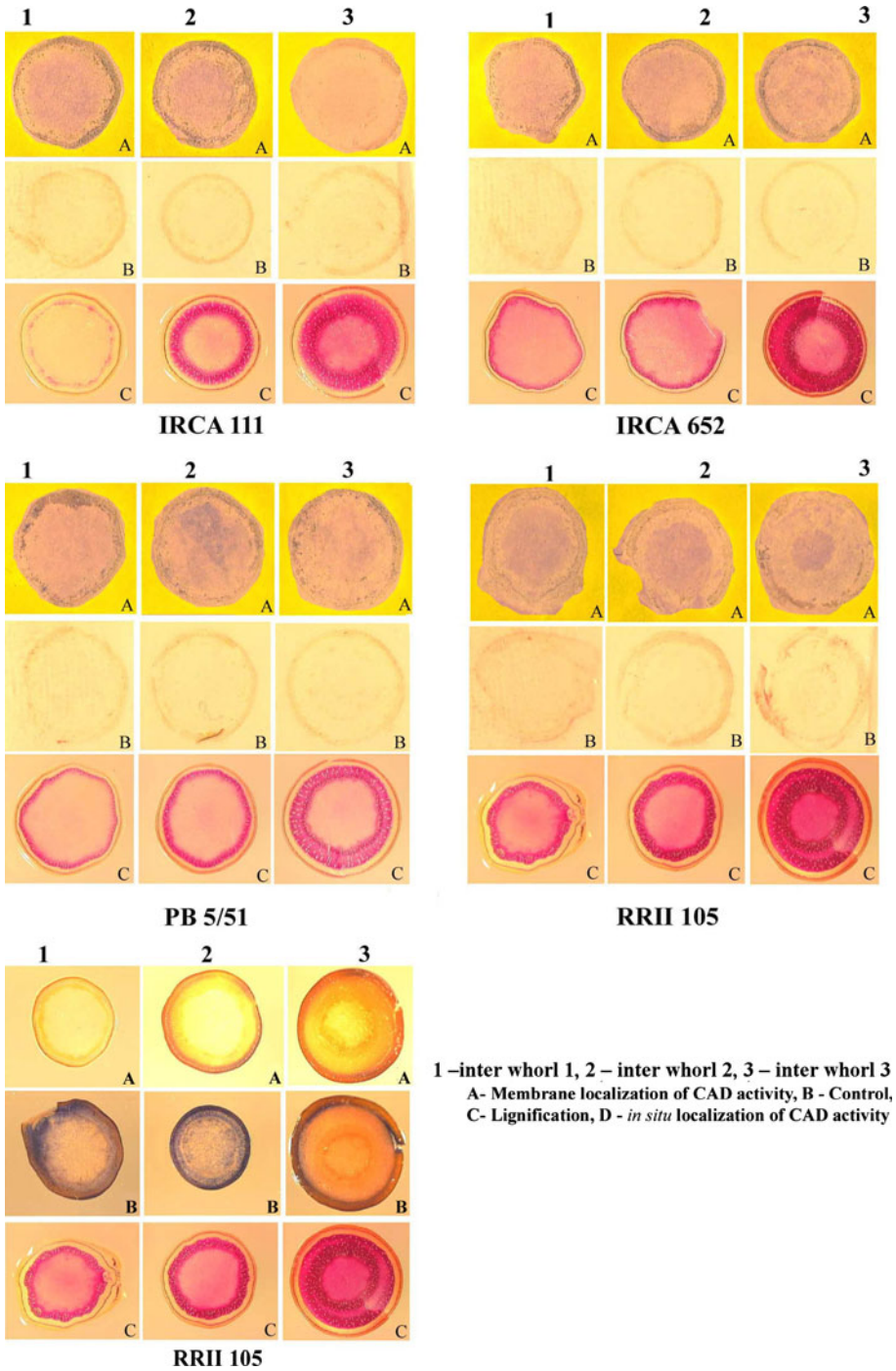

Fig. 3 Membrane localization (*top*) and in situ localization of CAD (*bottom*) in popular *Hevea* clones

Secondary xylem formation was initiated early in the developing stem and as more cells were laid down, CAD activity decreased in intensity which means that the enzyme CAD might have been utilized in the lignification process during secondary thickening.

The percentage of lignin in *Hevea* stems ranged from 20.81–23.00 % in the case of Wickham clones and 21.04–27.43 % (Table 2) in wild accessions. Out of the 18 wild accessions screened, nine showed high CAD activity and lignification than the five popular clones. It is interesting to note that the quantity of lignin produced in stems collected from 1 year old polybag plants and in twigs of 4 year old plants were comparable (Table 1).

The present studies indicate that the localization of CAD activity and quantification of lignin in the juvenile growth phase can be used as an early selection parameter for wood quality traits. Moreover, since the quality of lignin deposited in hardwoods is assessed by the Syringyl/Guaiacyl

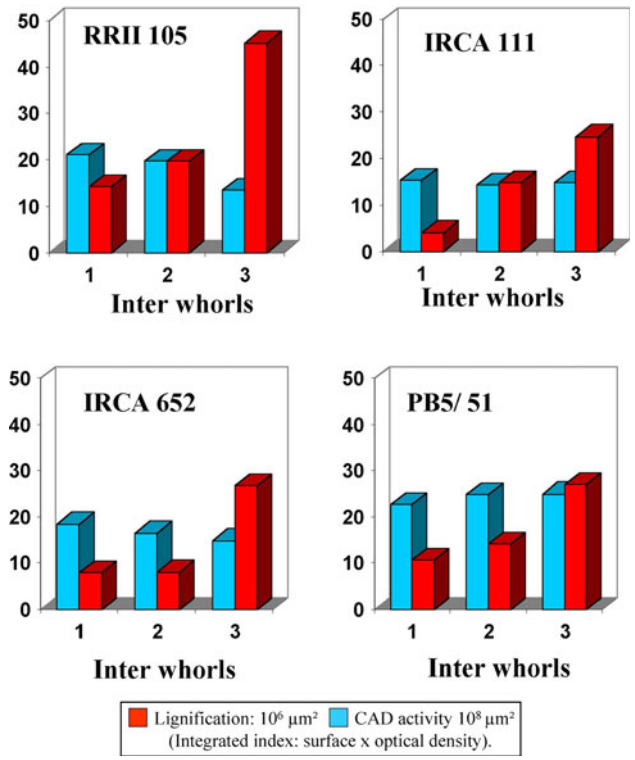

Fig. 4 Quantification of CAD activity and lignification

Table 1 Gravimetric estimation of lignin content in wild *Hevea* germplasm and popular clones at the age of 4 years

Accessions	Lignin (% wt. of EXR)			
	Ist Internode	2nd Internode	3rd Internode	Accn. mean
RO 5022	26.35	27.58	28.36	**27.43**
AC 4830	25.49	27.13	29.16	**27.26**
AC 4654	24.96	27.23	28.98	**27.06**
MT 6180	22.56	26.34	28.40	**25.77**
MT 4697	23.18	25.34	28.37	**25.63**
MT 5085	23.24	25.38	28.26	**25.63**
RO 4911	21.18	26.12	29.42	**25.57**
AC 4638	22.04	24.97	27.29	**24.77**
MT 4859	21.65	24.79	26.90	**24.45**
MT 4804	21.52	25.14	26.43	24.36
RO 4617	22.04	24.33	26.28	24.22
AC 4937	19.53	23.03	28.58	23.71
RO 4574	19.77	22.38	25.01	22.39
AC 4833	19.74	23.36	24.19	22.43
RO 4605	18.29	20.03	28.33	22.22
AC 4677	19.72	21.61	23.96	21.76
RO 4942	19.11	21.22	23.12	21.15
MT 5091	18.51	20.32	24.34	21.06
RRIM 600	20.35	22.50	26.15	**23.00**
RRII 105	18.31	20.20	24.51	**21.00**
1–2 = 5.56**	**2–3 = 6.75****	**1–3 = 13.10****	**CD (*P* = 0.05)**	**1.42**

Table 2 Gravimetric estimation of lignin content in four *Hevea* clones at the age of 1 year

Accessions	Lignin (% wt. of EXR)			
	Ist Internode	2nd Internode	3rd Internode	Accn. mean
RRII 105	17.89	20.63	23.82	**20.81**
IRCA 111	18.13	21.41	24.36	**21.23**
IRCA 652	17.14	20.66	24.08	**20.64**
PB 5/51	18.36	21.04	24.60	**21.33**

lignin ratio (Higuchi 1985), further investigations are essential to identify the status of in vivo CAD activity on sinapyl alcohol leading to the synthesis of Syringyl lignin monomers in *Hevea*.

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