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SCREENING OF *HEVEA BRASILIENSIS* GERMPLASM FOR WOOD QUALITY USING CINNAMYL ALCOHOL DEHYDROGENASE (CAD) ACTIVITY AND LIGNIFICATION PATTERN

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Lignins are phenolic polymers of the plant cell wall associated with mechanical strength, sap conduction, defense mechanisms and imperviousness to biodegradation. Cinnamyl alcohol dehydrogenase (CAD) is the key enzyme involved in the synthesis of lignin monomers. A study was conducted in stems of 18 wild germplasm accessions and five Wickham clones of *Hevea brasiliensis* to localize and correlate CAD activity and lignification at various stages of xylogenesis. 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. The quantity of lignin also increased in association with the progress of secondary thickening. The lignin percentage in the wild accessions ranged from 21.0 - 27.4 per cent and it was 20.0 – 23.0 per cent in the Wickham clones. Nine wild accessions showed significantly higher percentage of lignin over the Wickham clones. The localization of CAD activity and quantification of lignin in the juvenile growth phase can be used as early selection parameters for wood quality in *Hevea brasiliensis.*

Keywords: CAD activity, *Hevea brasiliensis,* Lignification, Wild germplasm, Wood quality.

INTRODUCTION

Lignins, the phenolic polymers of the plant cell wall, form the second most abundant group of biopolymers after cellulose (Roth *et al.,* 1997; Boudet, 2000). Functionally, lignin is associated with mechanical support, sap conduction, defense mechanisms, strengthening of plant tissue and its imperviousness to biodegradation (Piquemal *et al.,* 1998; Gierlinger *et al.,* 2004).

Lignification is a tightly regulated and dynamic process subject to modulations during normal development and response to different environmental stresses. Recent enzymatic and genetic engineering studies on lignins revealed a specific route to the synthesis of lignin precursors in the cytoplasm, which is translocated to the cell wall for polymerization (Boudet, 2000; Gierlinger *et al.,* 2004). Regulation of transport or polymerization affects the quantity of lignin produced.

The lignin biosynthesis involves the shikimate, phenyl propanoid and lignin specific pathways (Higuchi, 1990). The cinnamyl alcohol dehydrogenase (CAD) is an $NADPH^+$ specific oxidoreductase enzyme catalyzing the reversible conversion of cinnamyl aldehydes to the corresponding alcohols in the lignin specific pathway. As

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CAD is closely related to lignification, inhibition in its activity to reduces the synthesis of lignin (Moesbacher *et al.,* 1990; Mauch-Mani and Slusarenko, 1996).

CAD has been purified and characterized from several herbaceous and woody species (Boudet *et al.,* 1996). CAD is reported to be polymorphic with isoforms that differ not only on substrate affinity, but also on molecular mass (Mansell *et al.,* 1974; Goffner *et al.,* 1992; Boudet *et al.,* 1996). Variation in the quantity and quality of lignin seems to be based on the control of metabolic flux into the pathway influenced by the levels of this enzyme activity.

The potential of rubber timber for various industrial applications has been well established. However, the major limitations preventing the wide utilization of rubberwood are (i) the presence of high proportion of unlignified or partially lignified tension wood fibers along with 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 polyphenol derivatives in living trees, especially in fast growing woody species is known to improve quality and durability of timber, an attempt was made to understand the lignification pattern in developing stems of *Hevea brasiliensis* through identification and localization of CAD activity. This study also aimed at the early selection of *H. brasiliensis* clones for wood quality through lignin biosynthesis studies.

MATERIALS AND METHODS

Stem samples of (i) one year *H. brasiliensis* polybag plants of four Wickam clones *viz.,* RRII 105, IRCA 111, IRCA 652 and PB 5/51 from CIRAD, Montpellier, France and (ii) four years old plants of 18 *H. brasiliensis* germplasm accessions (from the provenances of Acre, Rondonia and Matto Grosso in Brazil) and two Wickham clones (RRII 105 & RRIM 600) of the same age from the source bush nursery of Rubber Research Institute of India (RRII), Kottayam were selected for the study. Fresh stem samples from three inter whorl regions such as inter whorl 1, 2 and 3 (Fig.1) were used for the histochemical localization of CAD activity and lignin and also for the gravimetric estimation of lignin.

Localization of CAD activity on tissue print and *in situ* localization

Cross sections of fresh stem samples (30µm) were prepared using as Rechert Jung sledge microtome. Four tissue prints were made for each inter whorl region on nitro-

Fig. 1. Stem sampling points

cellulose membrane (pore size: 0.45 µm). The prints were washed thoroughly in 20 mM TRIS HCl (pH 8.8) for 15 minutes to eliminate all free hydrophilic metabolites and to preserve the stability of the enzyme. Fresh stem sections were directly washed in 20 mM TRIS HCl for 15 minutes prior to incubation for *in situ* localization.

CAD activity was observed on tissue prints and fresh sections as per Roth *et al.* (1997) by incubating the printed membrane and *in situ* sections for 1 h. at 37°C with the following reaction medium containing 1 mg/ml $NADP^+$, 3.6 mg/ml coniferyl alcohol (Chemos, Germany), 0.35 mg/ml nitro blue tetrazolium (NBT) in 20 ml TRIS HCl (pH 8.8). CAD activity on tissue prints and 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, blue precipitate.

$$\text{Coniferyl alcohol} + NADP^+ \overset{\text{CAD}}{\rightleftharpoons} \text{Coniferyl aldehyde} + NADPH + H^+$$

$$NADPH + H^+ + NBT \longrightarrow \text{Formazan} + NADP^+$$

The reaction was stopped by washing the tissue prints and sections in distilled water for 10 minutes followed by air-drying. Control prints were prepared without adding either coniferyl alcohol or NADP or both in the reaction medium.

Histochemical localization of lignin

Lignin was localized histochemically by using Weisner reaction medium as per Monties (1984). Stem sections prepared from the same inter whorl zones were incubated in the reaction medium for three minutes.

Image analysis of CAD activity and lignification

The images of the CAD activity on tissue prints as well as the lignified areas in the sections were quantified with an image analysis system (Leica Q500W). The tissue prints and the lignin localized sections were transferred in a video image using CCD camera attached to Leitz DM IRBE microscope and stored as a 512 x 512 pixel image into a synapse digital frame store and analyzed with the 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 to provide an estimation of CAD activity and lignification pattern 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 Dence (1992). Fresh stem samples were collected and incubated for 1h in liquid nitrogen followed by freeze drying for 72 hrs. at -80°C. The wood tissue was separated from the frozen sample after removing the pith and bark tissue and was powdered to 80µm size in a ball mill. The powdered wood was then sequentially extracted with distilled water, ethyl alcohol,

toluene (1:1 v/v) and acetone using a soxhlet apparatus and dried to get the extractive free xylem residue (EXR).

The estimation and quantification of lignin was done using the EXR by weighing the residue left after saccharification of cellulose and other cell wall polysaccharides with sulphuric acid through vacuum filtration on glass microfibre filter (GMF) adopting gravimetric Micro Klasson technique (Whiting *et al.,* 1981). The percentage of lignin was calculated as percentage weight of EXR as follows.

$$\text{Lignin (\%)} = \frac{\text{(Weight of G.M.F + residue) – Weight of pre-weighed GMF}}{\text{Weight of EXR}} \times 100$$

RESULTS AND DISCUSSION

The histochemical assays were initiated within one hour for tissue prints and 45 minutes for the *in situ* localization. The activity of CAD was localized by the formation of the blue, insoluble formazan product in tissue prints (Fig. 2) as well as in *in situ* sections (Fig. 3), whereas in the controls no stained product was observed revealing the absence of CAD activity. Similar observations on indirect evidence for CAD activity through formazan precipitation has been reported in poplar and tomato samples (Roth *et al.,* 1997).

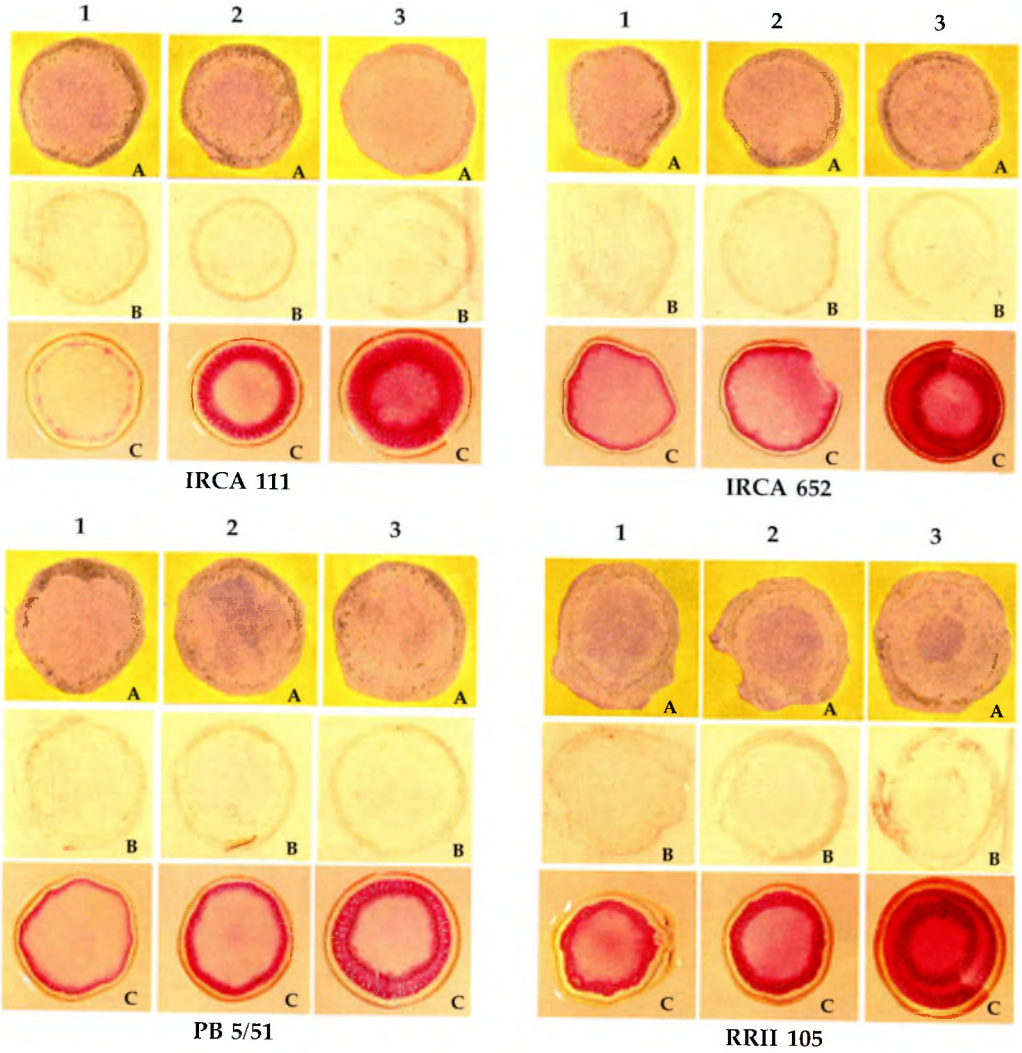
The CAD activity was the maximum in the first inter whorl region which gradually decreased towards the second and third inter whorls (Fig. 2, 3 & 4). As xylogenesis increased, the activity of CAD decreased in the mature xylem. In general, the CAD activity was observed on the periphery of the vascular tissue where new xylem and phloem differentiate. Observation on the lignified areas, 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 *H. brasiliensis* accessions than the Wickham clones (RRII 105 & RRIM 600). Among the 18 wild accessions the intensity of CAD activity was minimum for MT 5091 and maximum for RO 5052 (Fig. 4).

Lignin content estimated from the three inter whorl positions in four one year old Wickham clones is presented in Table 1. The percentage of lignin was minimum in the first inter whorl and maximum in the third inter whorl indicating that lignification gradually increased from top to bottom in association with xylogenesis and secondary thickening. The content of lignin on plant average basis was more or less same in all the four clones (20.6 to 21.3 %).

The quantity of lignin produced in the three inter whorl positions of the four year old wild accessions and Wickham clones

Table 1. **Lignin content in Wickham clones at the age of one year**

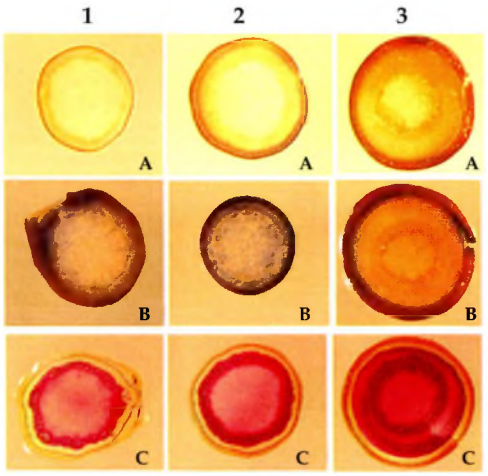
Accession	Lignin (% wt. of EXR)			
	Inter whorl - 1	Inter whorl - 2	Inter whorl - 3	Mean
RRII 105	17.9	20.6	23.8	20.8
IRCA 111	18.1	21.4	24.4	21.2
IRCA 652	17.1	20.7	24.1	20.6
PB 5/51	18.4	21.0	24.6	21.3



1 - First inter whorl, 2 - Second inter whorl, 3 - Third inter whorl
A - Membrane localization of CAD activity; B - Control; C - Lignification

Fig. 2. Membrane localization of CAD activity and lignification in four year old *H. brasiliensis* clones

viz., RRII 105 and RRIM 600 was highly significant (Table 2). The percentage of lignin in *H. brasiliensis* stems ranged from 20.8 – 23.0 per cent in the case of Wickham clones and 21.0 – 27.4 per cent in wild accessions. Out of the 18 wild accessions screened, nine showed higher CAD activity and lignification than the five Wickham clones. Nine wild accessions comprising three from Acre (AC 4830, AC 4654 & AC 4638), two from Rondonia (RO 5052 & RO 4911) and four from Matto Grosso (MT 6180, MT 4697, MT 5085 & MT 4859) had significantly higher lignin content over



RRII 105

1 - First inter whorl, 2 - Second inter whorl, 3 - Third inter whorl
A - Membrane localization of CAD activity; B - Control; C - Lignification

Fig. 3. *In situ* localization of CAD activity and lignification at the age of one year

the Wickham clones. RRII 105 had lesser lignin content (21.0 %) compared to RRIM 600 (23.0 %). It is interesting to note that the quantity of lignin produced in stems collected from one year old polybag plants and in twigs of four year old plants were comparable (Table 1 & 2). The nine wild accessions which showed higher CAD activity also had higher percentage of lignin.

CAD activity was found at the younger inter whorl zone and restricted areas of developing xylem in older inter whorl. The site of activity was limited to the differentiating xylem on the periphery of the lignified region in *H. brasiliensis*. 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

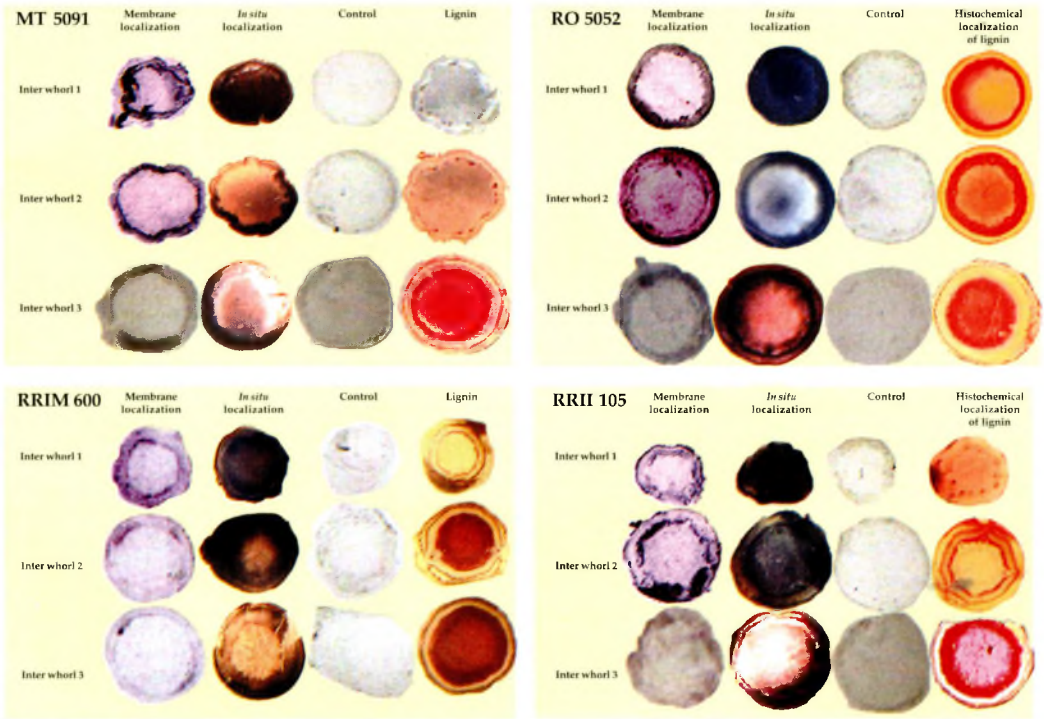


Fig. 4. Pattern of CAD activity and lignification in four years old wild *H. brasiliensis* accessions and Wickham clones

Table 2. **Lignin content in wild accessions and Wickham clones at the age of four years**

Accession	Lignin (% wt. of EXR)			
	Inter whorl - 1	Inter whorl - 2	Inter whorl - 3	Mean
RO 5052	26.4	27.6	28.4	27.4
AC 4830	25.5	27.1	29.2	27.3
AC 4654	25.0	27.2	29.0	27.1
MT 6180	22.7	26.3	28.4	25.8
MT 4697	23.2	25.3	28.4	25.6
MT 5085	23.2	25.4	28.3	25.6
RO 4911	21.2	26.1	29.4	25.6
AC 4638	22.0	25.0	27.3	24.8
MT 4859	21.7	24.8	26.9	24.5
MT 4804	21.5	25.1	26.4	24.4
RO 4617	22.0	24.3	26.3	24.2
AC 4937	19.5	23.0	28.6	23.7
RO 4574	19.8	22.4	25.0	22.4
AC 4833	19.7	23.4	24.2	22.4
RO 4605	18.3	20.0	28.3	22.2
AC 4677	19.7	21.6	24.0	21.8
RO 4942	19.1	21.2	23.1	21.2
MT 5091	18.5	20.3	24.3	21.1
RRIM 600	20.4	22.5	26.2	23.0
RRII 105	18.3	20.2	24.5	21.0
CD (P≤0.05)				**1.4**

t : inter whorl 1 & 2 = 5.6**, inter whorl 2 & 3 = 6.8**, inter whorl 1 -3 = 3.1

parenchyma cells towards the site of assembly in the vessel and fibers (Feuillet *et al.*, 1995).

In *H. brasiliensis* as the CAD activity drops from the first to the third inter whorl the lignin deposition increased (Fig. 5) as reported for tomato stem (Roth *et al.*, 1997) indicating that the flux of monolignol synthesis is limited in the mature xylem of *H. brasiliensis*. Secondary xylem formation was initiated early in the developing stem and as more cells were laid down, CAD activity decreased in intensity. This means that the CAD might have been utilized in the lignification process during secondary thickening.

The present study revealed that the localization of CAD activity and quantification of lignin in the juvenile growth phase can be used as a tool for the early selection

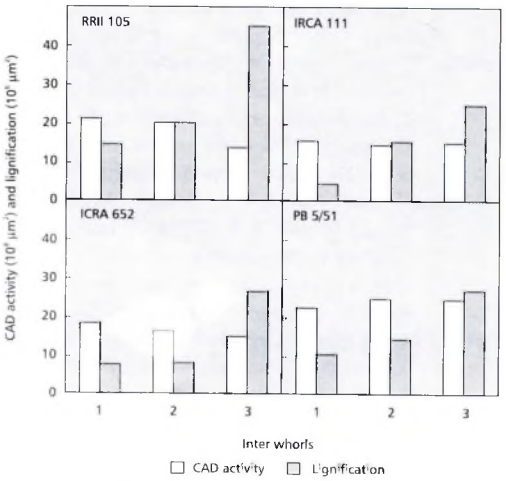


Fig. 5. Correlation of CAD activity with lignification at the age of one year

of *H. brasiliensis* clones for wood quality traits. Since the quality of lignin deposited in hardwoods is assessed by the Syringyl / Guaiacyl lignin ratio (Higuchi, 1985), fur-

ther investigations are essential to identify the status of *in vivo* CAD activity on sinapyl alcohol leading to the synthesis of Syringyl lignin monomers in *H. brasiliensis*.

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