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RESEARCH PAPER

Activity of Lignin Biosynthesis Enzymes During Firbre Ring and Shell Formation of Oil Palm (*Elaeis guineensis* Jacq.) Fruit - *Tenera* variety

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ABSTRACT

The functional compartmentation of lignifying enzymes Phenylalanine ammonia lyase (PAL), Cinnamyl alcohol- NADPH- dehydrogenase (CAD) and Peroxidase (POD) during the development of mesocarp, fibre ring and endocarp in *tenera* variety was established by biochemical analysis. Maximum lignin content was noticed at 8, 12 and 16 weeks after pollination (WAP) in endocarp, mesocarp and fibre ring respectively, suggesting the three different maturation phases of the tissues. The soluble phenols of mesocarp, endocarp and fibre ring at their developmental stages was analysed using reverse phase high- performance liquid chromatography (RP-HPLC). Scanning electron microscopic (SEM) observation demonstrated the differentiation of mesocarp, endocarp and fibre ring tissues and their lignified nature in *tenera* fruit. The waxing and waning of cinnamic acid content with respect to the lignin synthesis corroborates the biochemical data.

Key words: Oil palm fruit, *tenera*, mesocarp, endocarp, fibre ring, functional compartmentation, cinnamic acid, lignifying enzymes, lignin.

INTRODUCTION

Enzymic reaction of lignin biosynthesis and mechanism of its control and implication for the genetic improvement of the plant have been extensively studied by several workers (Grand *et al.*, 1985; Halpin *et al.*, 1992; Whetten and Sederoff, 1992; Grima Pettenati *et al.*, 1993; Morrison and Buxton, 1993; Morrison *et al.*, 1994; Hawkins and Boudet, 1994; Salini Bhasker, 2000). At different steps in the biosynthetic pathway, several enzymes have been identified with diversity of functions. Lignification during cell wall development indirectly involves the enzymes of the general phenyl propanoid pathway. Phenylalanine ammonia lyase (EC 4.3.1.5, PAL) is one of the most intensively studied enzymes in plant secondary metabolism because of its key role in phenyl propanoid pathway (Griesbach, 1981). This enzyme catalyzes deamination of phenylalanine to cinnamic acid. Cinnamic acid formed by deamination is then converted by a sequence of hydroxylation and methylation reactions to several substituted acids that can be converted as the corresponding esters of Co- enzymes. These activated enzymes can then enter different biosynthetic pathways leading to lignin, flavanoids, stillbenes, benzoic acids and other compounds. Cinnamyl alcohol - NADPH - dehydrogenase (EC 1.1.1.2,CAD) has been considered to be the indicator of lignin biosynthesis because of its specific role in the reduction of cinnamyl co-enzyme esters to cinnamyl alcohols . Hence it is a potential target enzyme for biotechnology directed towards modulating the quality and quantity of lignin in plants (O'Malley *et al.*, 1992). Polymerisation of cinnamyl alcohol to lignin, the last step of lignin synthesis has been attributed to two different classes of enzymes such as peroxidases (EC 1.11.1.7, POD) and laccases. The evidence for the involvement of peroxidase as the last enzyme in lignification has recently been reviewed (Abeles and Biles, 1991; Whetten and Sederoff, 1995).

Oil Palm (*Elaeis guineensis*) is the most productive tropical oleaginous crop in the world. Under this species there are three so called varieties - *dura*, *pisifera* and *tenera*. *Dura* is thick shelled, *tenera* is thin shelled and *pisifera* is shell less. However, confusion may arise in some cases of *dura* and *tenera*, where the fruits exhibit thin and thick shells respectively. The alternative method to identify *tenera*

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from such thin-shelled *dura* is the presence of a fibre ring in the mesocarp around the shell. Since the fibre ring along with thin endocarp ultimately decides the variety *tenera,* it would be appropriate to reveal the enzymic mechanism of lignin synthesis in these fruit forms in order to understand the functional diversity of major lignifying enzymes in the formation of fibre ring and shell. So the objective of this study was to follow more closely the activities of the lignifying enzymes PAL, CAD and POD during fibre ring and shell formation of the *tenera* fruit.

MATERIALS AND METHODS

Plant material: Healthy *tenera* palms were identified from the experimental plantations of National Research Centre for Oil Palm, Regional Station, Palode, Thiruvananthapuram. The fruit samples from different palms were collected separately at 4 and 8 weeks after pollination and pooled for the study. Assay and analysis were carried out in triplicate and the mean value was taken.

Scanning Electron Microscopy (SEM): Fruit samples were processed for SEM following the procedure of Salini Bhasker *et al.* (1997). Young fruits at 4 weeks after pollination (WAP) were fixed in 3% glutaraldehyde in phosphate buffer (pH 6.8) for 4 hours. The fixed sections were dehydrated with acetone series and dried by critical point drying (CPD). The dried sections were mounted and gold coated for SEM observation (8-2400 Hitachi).

Isolation and assay of enzymes: Frozen fruit samples were used for isolating the enzymes. PAL and CAD were isolated from the mesocarp and endocarp tissues of young fruits at the stage of 4 and 8 weeks after pollination following the method of Morrison *et al.* (1994). The homogenizing medium of PAL and CAD contained 0.1 M Tris-HCl buffer, 20mM 2- mercaptoethanol and 0.5% polyethylene glycol (pH 7.6). POD was isolated with 0.1 M phosphate buffer (pH 6.0). The reaction mixture of PAL assay contained 2.5ml of 12mM phenylalanine in 0.1 M Tris- HCl buffer (pH 8.5) and 0.5ml enzyme extract (Whetten and Sederoff, 1992). The reaction was allowed to proceed for 30 minutes at room temperature and stopped by the addition of 2N HCl. The reaction mixture was then extracted with 3ml of toluene by vortexing for 10s and centrifuged at 1000g for 5 minutes. The absorbance of cinnamic acid released was measured at 290nm against toluene as blank.

The activity of CAD was assayed using a reaction mixture containing 1150 µl, 0.1M KH_2PO_4/Na_2HPO_4 buffer; 50 µl, 20mM $ZnSO_4$ $7H_2O$; 600µl 1mM NADPH; 600 µl enzyme and 600 µl, 1mM cinnamaldehyde in ethanol. The reaction mixture without cinnamaldehyde was used as control. The oxidation of NADPH resulted in a gradual decrease in absorbance, which was measured at 340nm initially (0 min) and after 15 min of incubation at 30°C. POD was assayed in a mixture containing 0.1M phosphate buffer at pH 6.0; 20mM guaiacol solution; 10mM H_2O_2 and enzyme extract. Increase in absorbance at 470nm was recorded. As control, enzyme was not added in the medium. Activity of POD was defined as the quantity of enzyme required to oxidize 1mM of guaiacol/gram tissue in presence of H_2O_2 under test conditions. The assay values are the means of five determinations per sample expressed in units/g tissue.

Quantification of lignin and phenols: The amount of lignin content in fruit tissue was isolated by acetyl bromide method and estimated spectrophotometrically at 280nm (Liyama and Wallis, 1988). Dehydroxyconiferyl alcohol was taken as standard. Aqueous methanol was used to extract soluble phenols and quantified colorimetrically at 650nm. The standard graph of Catechol was used for the estimation of total phenolic content.

HPLC analysis of phenols: The different phenolic acids present in the mesocarp, fibre ring and endocarp at various stages of fruit development were separated by RP-HPLC. A HPLC system (Waters Associates) equipped with Rheodyne injector model 7725 was used for the determination of free phenolic acids (Wang and Lee, 1996). 4.6x250 mm id, C_8 model column with a Waters 510 pump and tunable absorbance detector of model 486. Sample (20 µ l) was injected and the detection of phenolic compounds was done at 280nm. Cinnamic acid was used as the standard.

RESULTS AND DISCUSSION

Structure of fruit tissue under SEM: Being a drupe, the differentiation of fruit tissue into mesocarp and endocarp was distinct at 4WAP. A clear and close association of vascular elements appeared beneath the mesocarp representing the fibre ring with a thickness of 1mm (Fig.1). In continuation of the fibre ring, the inner region showed a deep contrasting appearance representing the hard endocarp. The mesocarp tissue consists of polygonal parenchymatous cells for oil storage. The solid deposits in the peripheral cells closely associated with vascular elements (Fig. 2) indicating its hard nature other than ligniification. The crystalline nature of the thick deposits occupied in the inner lumen of the cells was observed (Fig. 3).

Activity of major lignifying enzymes: The biochemical relationship of lignifying enzymes PAL, CAD and POD during mesocarp development, fibre ring formation and shell synthesis was assessed by assay. Table 1 displays the activity of PAL, CAD and POD during fruit development of *tenera* fruit. The assay values expressed the functional diversity of the enzymes PAL, CAD, and POD in the development of mesocarp, fibre ring and endocarp during fruit development. From the peak values, two maturation phases were observed for the synthesis of fibre ring and endocarp at 16 and 8WAP respectively. It indicated the independent nature of both the tissues. During mesocarp

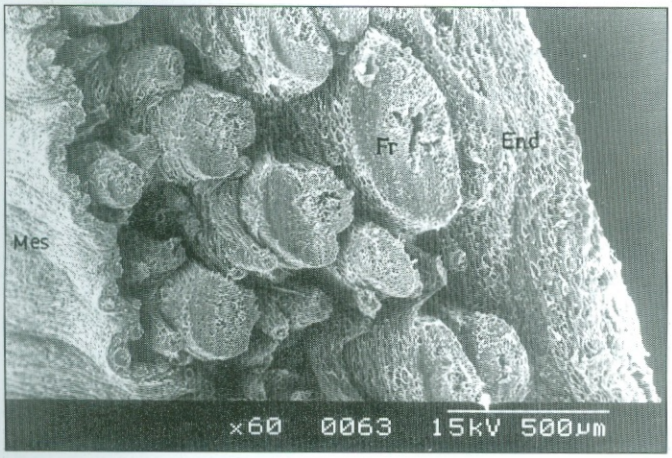

Fig. 1: Scanning electron micrograph of the pericarp of *tenera* fruit at 4WAP. Mes-mesocarp, Fr-Fibre ring, End - Endocarp

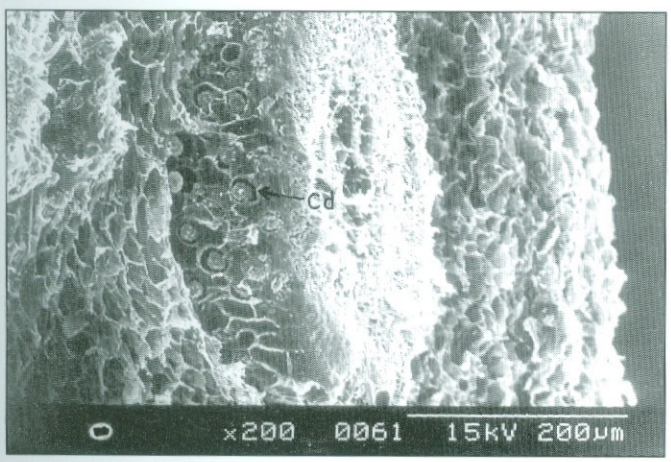

Fig. 2: Fibre ring of *tenera* fruit under SEM showing cellula deposits (Cd)

Fig. 3: Cellular deposits (Cd) on the fibre ring region, an enlarged view under SEM

development, the activity of these enzymes expressed a gradual decrease from 4 to 24WAP as they matured, (Table 1) suggesting the normal lignification process of the tissues. The occurrence of high activity of PAL in the mesocarp and endocarp at 4WAP indicates the formation of cinnamic

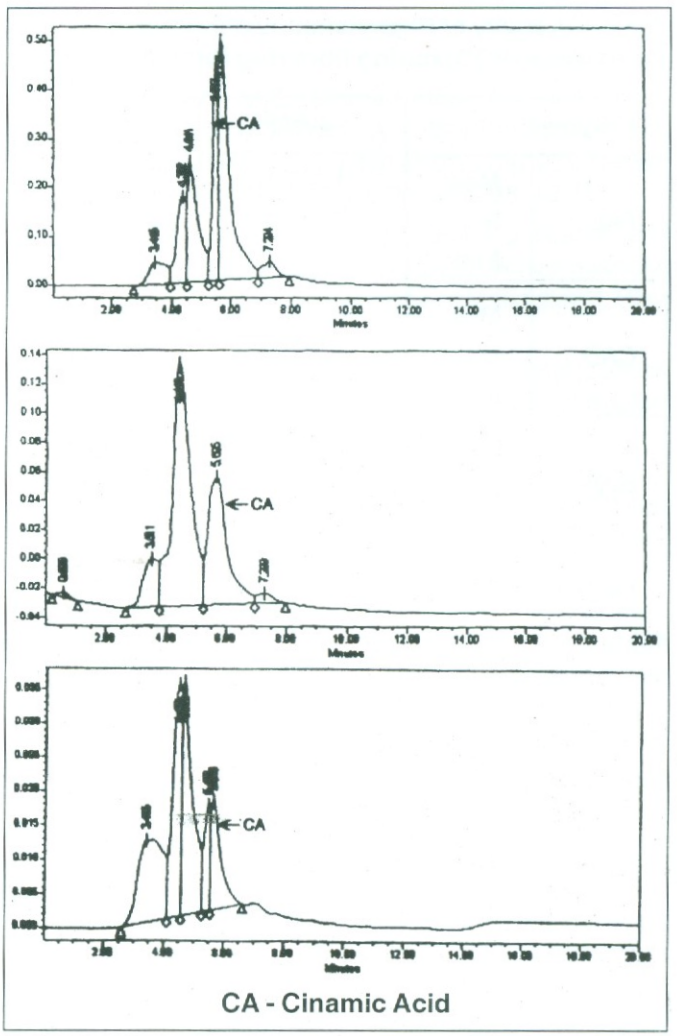

Fig. 4: Chromatogram representing peaks of cinnamic acid in mesocarp, endocarp and fibre ring tissues of *tenera* fruits

acids by de-amination of phenylalanine, the initial compound in lignin biosynthesis. Similarly, CAD and POD, the major enzymes of lignin biosynthesis exhibited higher activity in the endocarp tissue at 4WAP. At the next phase of endocarp development i.e., 8WAP the activity of these enzymes expressed a sound reduction suggesting the transformation of cinnamyl precursors to lignin by the action of CAD and POD. The moderate level of activity of these enzymes in the mesocarp without any prominent variation at any stages of fruit development indicated its normal process of lignin synthesis, whereas the significant reduction in activity from 4 to 8WAP in the case endocarp strongly suggested the role of these enzymes in shell synthesis. CAD and POD in lignin synthesis catalysed the reduction reaction of cinnamyl co-enzyme esters and polymerises cinnamyl alcohol to lignin. Because of the hard texture of tissue, it was found difficult to extract the enzyme from the endocarp. Hence no activity was observed in the subsequent stages of fruit development. In the case of fibre ring formation, the pattern of activity of these

Table 1: Activity of Phenylalanine ammonia lyase (PAL), Cinnamyl alcohol - NADPH- dehydrogenase (CAD) and Peroxidase (POD) during fibre ring formation, shell synthesis and mesocarp development (units/g tissue).

Enzymes		4WAP	8WAP	12WAP	16WAP	20WAP	24WAP
PAL	Mes	1.3±0.61	0.8±0.35	0.7±0.19	0.4±0.09	0.4±0.09	0.4±0.09
	Fr	0.5±0.1	0.72±0.2	0.98±0.2	0.26±0.09	0.12±0.03	-
	End	1.8±0.61	0.2±0.07	-	-	-	-
CAD	Mes	24.53±0.4	18.5±0.3	15.7±0.2	2.55±0.3	1.8±0.59	1.6±0.4
	Fr	10.1±0.13	13.0±0.2	32.0±0.5	3.4±0.11	0.4±0.12	-
	End	68.6±0.68	6.4±0.42	-	-	-	-
POD	Mes	4.2±0.32	3.5±0.4	1.6±0.4	1.46±0.31	1.0±0.2	0.6±0.2
	Fr	3.5±0.38	5.0±0.4	7.4±0.2	1.2±0.1	0.5±0.1	-
	End	6.0±0.5	0.6±0.01	-	-	-	-

Mes = Mesocarp Fr = Fibre ring End = Endocarp

Table 2: Distribution of Lignin (µg/g tissue) and Cinnamic acid content (µg/g tissue) during shell and fibre ring formation in *tenera* variety.

Concentration (µg/g tissue)	Fruit tissue	4WAP	SWAP	12WAP	16WAP	20WAP
Lignin	Mes	338.6±1.28	468.2±0.6	546.7±1.8	548.0±0.16	550.0±1.7
	Fr	-	404.0±0.5	407.0±0.05	590.0±0.92	591.0±0.24
	End	342.0±0.37	689.0±0.61	700.0±1.2	-	-
Cinnamic acid	Mes	9.74	8.43	5.51	4.8	-
	Fr		0.68	3.62	0.143	-
	End	7.35	0.63	-	-	-

Mes = Mesocarp Fr = Fibre ring End = Endocarp

enzymes showed greater variation than mesocarp and endocarp. At 4WAP the activity of PAL, CAD and POD was lower than that of mesocarp and endocarp. At the next two stages of development i.e., 8 and 12WAP the enzymes showed a significant increase in activity with a maximum peak at 12WAP, assuming the physiological activeness of the tissue during fibre ring formation. At 16WAP, the activity of these enzymes reduced to a negligible level. Thus the enzyme assay results during endocarp and fibre ring formation in *tenera* fruit clearly indicates the independent nature of these two tissues that developed separately. The enzyme data were substantiated by the lignin content of mesocarp, fibre ring and endocarp (Table 2). The lignin content of endocarp showed a steep increase from 4 to 8WAP coinciding with the activity of lignifying enzymes PAL, CAD and POD. Higher level of lignin concentration and the negligible level of enzyme activity in the endocarp and fibre ring at 8 and 16WAP respectively, confirm the differential nature of lignification process. i.e., in endocarp and fibre ring, bic synthesis of lignin took place between 4 and 8WAP and between 12 and 16WAP respectively. The activity of PAL, CAD and POD during lignification was studied earlier for confirming their role (Morrison *et al.*, 1994; Salini Bhasker *et al.*, 1997; Salini Bhasker, 2000). However, the biochemical evidence to substantiate the role of PAL, CAD and POD in endocarp and fibre ring formation in *tenera* variety has not been reported so far.

RP-HPLC analysis: The role of cinnamic acid as a precursor of lignin biosynthesis during fibre ring and shell formation was analysed under RP-HPLC using the methanolic concentrates dissolved in phosphate acetonitrile mixture at pH 2.54. The chromatogram of phenolic acids of mesocarp, endocarp and fibre ring tissues was presented in Fig. 4. The cinnamic acid peak and its amount was detected and estimated from the chromatogram using the standard peak of cinnamic acid (CA). It could be noticed that the concentration of CA and the formation of lignin during the development of the different regions of the pericarp exhibited a reciprocal relationship. The lignin concentration of the tissues was also quantified. Distribution

of cinnamic acid and the concentration of lignin during fibre ring and shell formation is presented in Table 2. The concentration of lignin showed a progressive level of increase from 4 to 20WAP. The level of cinnamic acid showed a progressive decrease indicating its role in lignin biosynthesis. It was significant to note that the increase in lignin content in the mesocarp from 12 to 20WAP was negligible. In other words the active phase of lignin synthesis in mesocarp tissue was from 4 to 12WAP. The enzymatic reaction leading to synthesis of monolignols from cinnamaldehyde revealed the role of cinnamic acid as a lignin precursor (Whetten and Sederoff, 1995). The low content of cinnamic acid in the endocarp region and the high level of lignin deposition at 8WAP compared to 4WAP supported the role of cinnamic acid for the synthesis of lignin. Unlike the high level of lignin content noticed in endocarp and mesocarp at 8 and 12WAP, the fibre ring showed its peak at 16WAP indicating the more delayed nature of lignin synthesis in fibre ring tissue than endocarp and mesocarp

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REFERENCES

Abeles, F.B. and Biles, C.L. 1991. Characterization of peroxidases in lignifying peach fruit endocarp. *Plant Physiol.*, **95**: 269-273.

Grand, C., Parmentier, P., Boudet, A. and Boudet, A.M. 1985.Comparison of Lignins and of enzymes involved in lignification in normal and brown midrib (bm3) mutant maize seedlings. *Physiol. Veg.* **23**: 905-911.

Griesbach, H. 1981.Lignin. In: *Biochemistry of plants.* (Ed. Conn, E.E.) Vol **7**: Academic press, New York. pp.457-478.

Grima Pettenatti, J., Feuillet, C., Goffner.D., Borderies, G. and Boudet, A.M., 1993.Molecular cloning and expression of Eucalyptus gunnii c DNA clone encoding Cinnamyl alcohol dehydrogenase. *Plant Mol. Bioi.*, **21**:1085-1095.

Halpin, C., Knight, M.E., Grima Pettenatti.J. Gofner, D., Boudet, A. and Schuch.W.1992. Purification and characterization of Cinnamyl alcohol Dehydrogenase from Tobacco stems. *Plant Physiol.*, **98**: 12-16.

Hawkins, S.W. and Boudet, A. M.1994. Purification and characterization of cinnamyl alcohol dehydrogenase Isoforms from the periderm of *Eucalyptus gunnii. Plant Physiol.*, **104**: 75-84.

Liyama,K, Wallis, AF.A. 1988.An improved acetyl bromide procedure for detecting lignin in wood and wood pulps. *Wood Sci. Technol.*, **22**: 271-280.

Morrison, T.A. and Buxton, D.R. 1993.Activity of phenylalanine ammonia lyase, tyrosine ammonia lyase and cinnamyl alcohol dehydrogenase in maize stalk. *Crop Sci.*, **33**: 1264-1268. -1f 1

Morrison, T.A., Kessler, J.R., Hatfield, R.D. and Buxton, D.R. 1994. Activity of two lignin biosynthesis enzymes during development of a maize internode. *J. Sci Food Agric Technol.*, **22**: 391-397.

O'Malley, D.M., Porter, S., Sederoff, R.R., 1992. Purification, characterization and cloning of cinnamyl alcohol -NADPH -dehydrogenase in Loblolly pine (*Pinus taeda*). *Plant Physiol.*, **98**: 1364-1371.

Salini Bhasker, Mohankumar. C. and Pillai.R.S.N. 1997.Early identification of Oil Palm (*Elaeis guineensis*) varieties *dura*, *tenera* and *pisifera*. Proceedings of the Ninth Kerala Science Congress, Thiruvananthapuram., pp. 121-124

Salini Bhasker 2000. Biochemical studies on lignification of Oil Palm (*Elaeis guineensis*) varieties Dura and Pisifera. PhD Thesis, Kerala University, Thiruvananthapuram.

Wang,C.K., Lee, W.H. 1996. Separation, characteristics and biological activities of phenolics in areca fruit. *J. Agric. Food Chem.*, **44**: 2014-2019.

Whetten, R.W. and Sederoff, R.R. 1992. Phnylalanine ammonia lyase from Loblolly pine. *Plant Physiol.*, **98**: 380-386.

Whetten, R.W. and Sederoff, R.R. 1995. Lignin biosynthesis. *The Plant Cell.*, **7**: 1001-1013.