Chapter 3

Materials and Methods

3.1. Materials

Ten Wickham clones of *Hevea brasiliensis* (Willd. ex Adr. de Juss.) Muell. Arg., were selected from the Germplasm gardens I, II and III, and were planted during 1977, 1979 and 1981, respectively, at the Central Experimental Station of Rubber Research Institute of India, Chethekal, Ranni, Kerala. The experimental station is situated at 9° 22' N latitude and 76° 50' E longitude with an altitude of 80m above the MSL. These germplasm gardens comprised of 102 Wickham clones, planted in Randomised Block Design (RBD) with three replicates and three trees per plot. The trees were under regular tapping and had an age of 17-21 years.

In addition to this, seedling trees from two cross combinations of Wickham clones Vs Wild Brazilian germplasm accessions and budded clones of RRII 105 and RRIM 600 were also selected from the progeny evaluation trial, of age 4 years established at the Rubber Research Institute of India, Kottayam. The details of the materials selected for the present study are described in Table-2.

Table 1: Details of materials selected

SI. No	Wickham clones	Age (in years)	Origin/Parentage
1	Tjir 1	21	Primary clone evolved by Tjirandji Estate, Indonesia
2	Gl 1	21	Primary clone evolved by Glenshiel Estate, Malaysia
3	PB 86	21	Primary clone evolved by Prang Besar Estate, Malaysia
4	GT 1	21	Primary clone evolved by Gondang Tapen Estate, Indonesia
5	PB 28/59	21	Primary clone evolved by Prang Besar Estate, Malaysia
6	RRII 105	19	Hybrid clone (Tjir 1 x Gl 1) evolved by Rubber Research Institute of India
7	RRIM 600	19	Hybrid clone (Tjir 1 x PB 86) evolved by Rubber Research Institute of Malaysia
8	RRIM 703	19	Hybrid clone (RRIM 600xRRIM 500) evolved by Rubber Research Institute of Malaysia
9	PB 235	21	Hybrid clone (PB 5/51x PB 5/78) evolved by Prang Besar Estate
10	RRII 300	17	Hybrid clone (Tjir I x PR 107) evolved by Rubber Research Institute of India
Seedling plants (Wickham x Brazilian germplasm)			
1	Seedling Progeny	4	Hybrid progeny, (RRII-105 x MT 1005)
2	Seedling Progeny	4	Hybrid progeny, (RRIM –600 x AC 495)
Budded plants (Wickham clones)			
1	RRII 105	4	Hybrid clone (Tjir 1 x Gl 1) evolved by Rubber Research Institute of India
2	RRIM 600	4	Hybrid clone (Tjir 1 x PB 86) evolved by Rubber Research Institute of Malaysia

3.2. Methodology

3.2.1 Selection of trees

Nine mature trees from each clone (three trees per replication) and eight plants from seedling progenies and budded plants (four plants from each progenies) in the juvenile phase were selected, to study the structure of bark.

Three mature trees from each clone (one tree per replicate) were selected to study histochemical parameters.

3.2.2 Collection and processing of bark samples

To study the orientation and inclination of laticifers / phloic elements, virgin bark samples were collected from the selected trees at 150 cm height (for mature trees) and 20-30 cm height (for seedling plants) from the ground. The sampling method reported by Gomez (1967) with certain modifications was adopted as described in Fig.1. A vertical line was drawn on the tree trunk along the longitudinal axis of the tree (Fig. 1 a). One of the cutting edges of the bark sampler was placed parallel along the vertical line (Fig 1 b) and the bark samples (Fig. 1 c) of the size 2 x 2 cm and 2 x 3 cm were collected. Immediately after sampling, a marking was made on the sampled bark by cutting on the right top corner (Fig. 1 d) to maintain the orientation of the bark sample on the tree. The samples collected were fixed in formalin-acetic -alcohol (FAA) and were sectioned at 30 – 60 µm thickness at different planes *viz.* cross sectional (CS), tangential longitudinal (TLS) and radial longitudinal (RLS) plane, using Reichert Jung sledge microtome. Sections were stained with Oil Red O (Omman and Reghu, 2003) and mounted in 50% glycerine and the micro slides (Fig. 1 e) were prepared by maintaining the orientation of the tissues as in the tree.

3.2.3 Method of observation

The bark sections were observed under Leitz Aristoplan Research microscope attached to Leica Q 500Q I W Image Analysis System. The images of the bark sections documented in the Image Analysis System were used to measure the inclination of laticifers / phloic rays and other anatomical traits by means of Leica Q Win V.2.1 Image analysis software.

The TLS of the bark were used to measure the inclination, density and diameter of laticifers and frequency of interconnections. Cross section and RLS were used to count

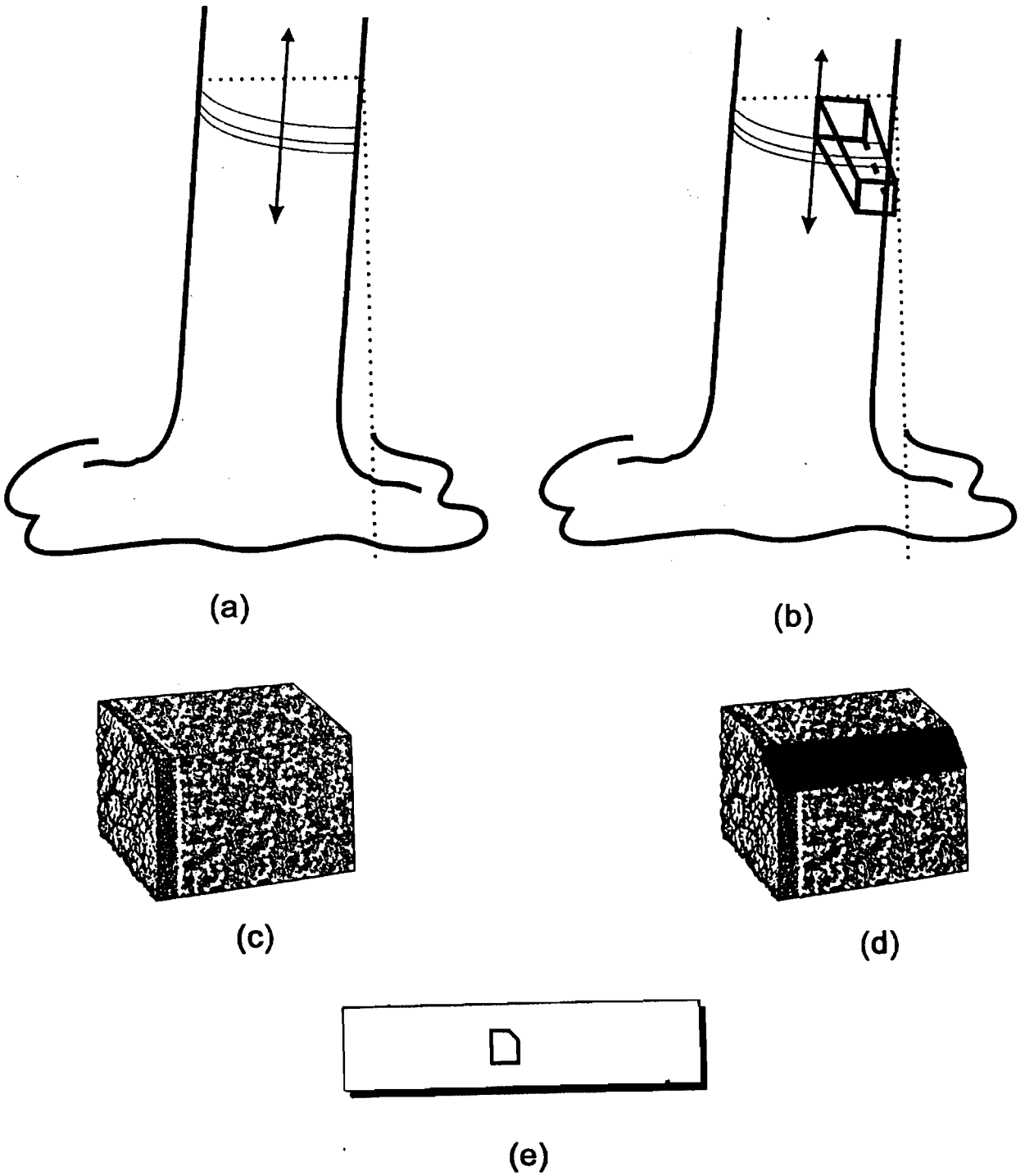

Figure 1. Method of bark sampling and mounting of sections. a. vertical line drawn on tree trunk along the longitudinal axis. b. bark sampler placed parellel along the vertical line. c. collected bark sample d. a cutting made on the corner of the bark sample. e. Mounting of sections on the slides maintaining the orientation of the tissue.

the number of laticifer rows, inter row distances, area occupied by stone cells and thickness of soft / hard bark. For each anatomical parameter, observations from ten microscopic fields were taken per plant.

3.3. Characters studied

3.3.1 Leaning angle of trees (in degrees)

3.3.2 Tree girth : Measured at 150 cm height from the ground.

3.3.3 Total bark thickness (mm): The sum of soft bark and hard bark thickness measured at 150 cm height. The thickness of the hard bark was further sub divided into the inner hard bark and outer hard bark thickness:

3.3.3.1 Soft bark (SB) thickness: The distance from the cambial zone outward upto the zone of initiation of stone cells

3.3.3.2 Inner hard bark (IHB) thickness: The distance from the inner most layer of stone cells to the inner most row of functional latex vessel.

3.3.3.3 Outer hard bark (OHB) thickness: The distance from the innermost functional laticifers to the remaining outermost hard bark zone.

3.3.4 Number of latex vessel rows in the soft bark and hard bark

3.3.5 Average distance between adjacent laticifers rows in SB and IHB (mm)

3.3.6 Average distance between the cambium to the 1st row of latex vessel (mm)

3.3.7 Total density of latex vessels per row per 1 mm distance

3.3.7.1 Density of latex vessel contiguous to rays

3.3.7.2 Density of latex vessels non-contiguous to rays

3.3.8 Frequency of interconnections between laticifers ($5x10^{-2}$ mm^2 area)

3.3.9 Diameter of latex vessels (μm)

3.3.10 Total cross sectional area of latex vessels (Laticifer area index): The total cross sectional area of the latex vessels at a given CS of the bark (Laticifer Area Index) was computed as per the following formula (Gomez *et al.*, 1972).

Total cross sectional area of latex vessels = nfG (μr^2)

Where *n* is the total number of latex vessel rows

f = density of latex vessels per row per 1mm circumference of the tree

G = girth of the tree (cm)

r = radius of latex vessel

3.3.11.1 Angle of inclination of laticifers in SB

3.3.11.2 Angle of inclination of laticifers in IHB

3.3.12.1 Angle of inclination of phloic rays in SB

3.3.12.2 Angle of inclination of phloic rays in IHB

3.3.13.1 Frequency of phloic rays contiguous to latex vessels per unit distance (765 µm) in TLS of SB and IHB

3.3.13.2 Frequency of uni-, bi- and multiseriate rays contiguous to latex vessels per unit distance (765 µm) in TLS of SB and IHB

3.3.14.1 Frequency of phloic rays in latex vessel free zone per unit distance (765 µm) in TLS of SB and IHB

3.3.14.2 Frequency of uni-, bi- and multiseriate rays in latex vessel free zone per unit distance (765 µm) in TLS of SB and IHB

3.3.15.1 Height and width of phloic rays (µm) contiguous to latex vessels in TLS of SB and IHB.

3.3.15.2 Height and width of phloic rays (µm) in latex vessel free zone in TLS of SB and IHB.

3.3.16.1 Height / width ratio of phloic rays contiguous to latex vessels in TLS of SB and IHB.

3.3.16.2 Height / width ratio of phloic rays in TLS of SB and IHB

3.3.17 Length and diameter of sieve tubes (µm)

3.3.18 Number of stone cell rows in IHB

3.3.19 Area occupied by stone cells per 255 x 10^{-3}mm^2 CS area in IHB and OHB..

3.4 Histochemical studies

The following staining methods and histochemical tests were employed using sledge microtome sections of the bark at 30 – 60 µm thickness.

3.4.1 **Starch**: Iodine-Potassium iodide (Johansen, 1940)

3.4.2 **Total polysaccharides**: Periodic acid – Schiff's (PAS) reagent (Ruzin, 1999).

3.4.3 **Lipids**: Sudan Black B (Ruzin, 1999).

3.4.4 **Total protein**: Mercuric-Bromophenol (Mazia *et al.*, 1953)

3.4.5 **Phenols**: Tannin acid-ferric chloride (Johansen, 1940)

3.4.6 **Tannin**: Ferric sulphate (Rawlins and Takahashi, 1952).

3.4.7 **Lignin**: Phloroglucin -HCl. (Purvis *et al.*, 1964; Ruzin, 1999)

3.5 Statistical analysis

The following statistical analysis were carried out (Gomez and Gomez, 1983; Panse and Sukhatme, 1985):

3.5.1 Coefficient of variation (CV) was calculated to ascertain the tree-to-tree variation within clones. Mean values were pooled to find out the CV values. The CV was not calculated whereever the data was absent / insignificant. The variation within trees was taken as low, medium and high with respect to the CV values. For example 0 to 30 was taken as low, 31 to 50 as medium and 51 and above as high.

3.5.2 Correlation : Simple correlation was worked out to findout the relationship among themselves and also between different characters.

3.5.3 Analysis of variation (ANOVA) was estimated to measure the extend of clonal variation between different clones.

3.5.4 Regression analysis was done to find out the effect of various independent variables and their associated influence on a dependent variable (the latex vessels inclination)

For statistical analysis of data ,softwares of excel (MS office) and SPSS 10 were used.

3.6 Photomicrography

Photomicrographs were taken in Leitz Aristoplan Research microscope attached to Wild MPS 46 Photo Automat using Kodak Gold 35mm colour film.

3.7 Image analysis: Quantitative image analysis was done using Leica Q Win V.2.1 Image analysis software.