2. MATERIALS AND METHODS

2.1 COLLECTION OF SAMPLES

To study the development of cambial variant and pattern of secondary growth, samples of the main stems or roots measuring about 3-50 mm in diameter and 40-60 mm in length were collected from ten plants of 28 genera and 34 species. To study the primary growth, samples starting form young shoot (apical tip) up to 25th inter node including mature stems (about 20 mm thick) were collected. Before collection due care was taken that all the samples were collected from the healthy plants free from the injuries or free from diseases to avoid unusual structures developed in response to injury. Plants studied for both primary and secondary unusual growth are listed in Table 1. All the samples were collected from different parts of India while some of the samples were procured from Atlantic rain forest, Rio de Janeiro, Brazil.

2.2 MICROTECHNIQUES

2.2.1 Killing and Fixation:

Collection of samples as well as proper fixation of material is very important. These preliminary stages become the one on which everything else depends. Improper fixation would never give satisfactory result. Killing and fixing are two distinct processes. However, both are usually obtained by means of a single fluid which in turn is commonly a mixture of various chemical reagents. The term killing means the sudden and permanent termination of the life processes. Killing invariably precedes fixation since the reagents which do the killing penetrate tissues faster than those upon which responsibility for fixation rests. Fixation is the process in which

preservation of all cellular and structural elements occurs nearly at the natural living conditions as possible. Collected samples were fixed in FAA (Formaldehyde-Acetic acid-Alcohol) solution immediately after collection of the samples (Berlyn and Miksche 1976). After coming back to the laboratory, all the collected samples were aspirated in the vacuum by using Desiccator. After 12 hours of fixation, these samples were transferred into 70% alcohol for further processing and preservation.

2.2.2 Softening of Material:

Some of the hard and woody samples were processed by ethylenediamine method as described by Carlquist (1982). Fixed samples of hard and woody materials were washed for two-days under running water prior to treating them with softening agent. Suitably trimmed small wood blocks measuring ($1x1cm^2$) were kept in 10% ethylenediamine solution for three days. Depending on hardness of the materials, some of the harder wood samples were kept in 4% of ethylenediamine solution for a week. After softening in, materials were washed thoroughly with water, and thereafter they were infiltrated, and embedded in paraffin. Paraffin blocks not treated with ethylenediamine were preliminary sectioned with a rotary microtome just to expose the surface of the material. Thereafter the paraffin blocks were soaked in water for overnight for softening purpose. Treatment of wood blocks with water considerably improved the quality of sections. However, treatment timing varies from samples to samples and needs standardisation depending on the hardness of the material. Ethylenediamine was used with reasonable caution, because it is much less hazardous than hydrofluoric acid and is more effective in softening plant material.

2.2.3 Microtomy:

Depending on the type of material and its hardness, sectioning of the samples was carried out with the help of sliding/sledge and rotary microtome. Samples, particularly apical shoots, softened material and samples having abundant parenchyma were sectioned with rotary microtome while thick stem samples with moderate hardness were sectioned with sliding/sledge microtome.

1) Rotary Microtome Sections:

To obtain thin sections, samples were processed with routine method of paraffin embedding method as described by Berlyn and Miksche (1976), which is described in following steps:

Dehydration: Fixed samples were washed thoroughly to remove the fixative and softening reagent from the material (if used). It is necessary to insure removal of every trace of fixative and softening reagent from the samples for better staining and good contrast in the prepared slides. After washing, samples were dehydrated in graded series of Tertiary Butyl Alcohol (TBA). Suitably trimmed samples (i.e. 2×2 mm^2) were transferred to glass vials for dehydration series. Samples were transferred through graded series of TBA *viz.*, 30%, 50%, 70%, 90% and 3 times in 100% TBA (Johansen 1940; Berlyn and Miksche 1976). In each step samples were placed in solution for 8-9 hours or sometimes more if material is hard.

Infiltration: Infiltration of the samples was carried out in with paraffin. Samples from the pure tertiary butyl alcohol were gradually brought into paraffin by adding small amount para-TBA after every four hours till it reached to saturation. Thereafter, lids of the vials were kept open to allow evaporation of TBA from the samples. After evaporation of entire TBA, samples were brought into pure paraffin wax and such three changes were given to remove even minute traces of the TBA from the samples. Timing for transferring the material from the para-TBA to pure paraffin was dependent on the hardness of the material.

Embedding: After completion of infiltration, three changes of pure paraffin wax (melting point 58°C - 60°C) were given prior to embedding. Melted paraffin wax was poured into suitable receptacles, and the infiltrated material was arranged in proper order/orientation to get sections in all three planes (transverse (TS), tangential longitudinal (TLS) and radial longitudinal (RLS). After arranging the material, paraffin wax was then cooled quickly.

Sectioning and Mounting: Embedded samples were trimmed further into suitable size and mounted on wooden block for sectioning. Sections of 12-15µm thickness were taken with the help of on Leica rotary microtome. Paraffin ribbons along with embedded materials were mounted on the slide. Prior to mounting, one drop of

Haupt's adhesive was applied and then the slides were rubbed till the slides became dry (Johansen 1940). Suitable length of paraffin ribbon was placed on the slide with the help of scalpel and slides were flooded with 3% formalin solution before keeping them on warm plate. Slides were kept on the warm plate till the ribbons became wrinkles free and completely flattened out. Another reason for warming of the slides is to generate fumes of formalin which will serve to coagulate the gelatine of the adhesive. After drying for 7-8 days, these slides were passed through the downgraded xylene-ethanol series to remove the wax (Berlyn and Miksche 1976).

2) Sliding Microtome Sections:

Thick and woody stem samples were directly sectioned with or without softening process with a Leitz sliding microtome to obtain the sections of 15-20 µm thickness. Thin sections were chosen and then tied on the slide with help of cotton string. These sections were directly used for staining and mounting.

Staining: Before staining the sections, slides were passed through the xylene to dissolve/remove the paraffin. Slides/sections were then rehydrated through routine method of down grade alcohol series and brought to aqueous condition to stain the sections with Safranin and Fast green combination (Johansen 1940) or Safranin and Astra blue combination (Srebotnik and Messner 1994; Vasquez-Cooz and Mayer 2002).

2.2.4 Staining Procedure:

Sliding microtome sections were directly processed for staining while paraffin embedded sections obtained by rotary microtome were dehydrated as mentioned earlier were used for staining. Sections were stained with aqueous solution of Safranin (1%) and Fast green (0.5%) combination (Johansen 1940) or Safranin-O (1%) and Astrablue (1%) combination (Srebotnik and Messner 1994; Vasquez-Cooz and Mayer 2002). The period of staining differed from the material to material. Sometimes sections were also stained with Tannic acid-Ferric Chloride-Lacmoid combination to study the phloem (Cheadle *et al.* 1953). Stained sections were washed thoroughly in water before passing them through upgraded series of ethanol-xylene for dehydration. Dehydrated sections were mounted in DPX (Dibutyl Phthalate Xylene) and allowed to dry.

2.2.5 Macerations:

To study the general morphology and size of vessel elements, fibriform vessels, tracheids and fibres suitably trimmed samples were macerated with Jeffrey's solution (Berlyn and Miksche 1976) at 56°C - 60°C for 24-36 hours. About 1 mm^2 thick portion of xylem adjacent to the cambial zone, from the middle portion and xylem portion adjacent to the pith was used for the maceration of wood sample. After maceration samples were thoroughly washed with water and then stained with Safranin before mounting in glycerine jelly.

2.2.6 Measurements:

Slides prepared from the macerated material were used for the measurement of length and width of vessel elements, fibriform vessels and fibres. On the other hand, length and width of fusiform cambial cells and sieve tube elements were measured directly from the tangential longitudinal sections. To obtain average length of fusiform cambial cells, sieve tube elements, vessel elements, fibriform vessel elements and xylem fibres, one hundred measurements were chosen randomly to obtain mean and standard deviation for each cell type. Vessel frequency per 0.5 mm^2 area of xylem, vessel diameter and vessel wall thickness was measured in transverse section while ray height and ray cell diameter was measured in tangential longitudinal sections by using Qwin-plus Image Analyser software (Leica Germany). Term ray cambium is adapted from Carlquist and Hanson (1991) for the ray cells that dedifferentiate into meristematic cells while wood descriptions follow the IAWA Committee (1989) and Carlquist (2001a).

2.3 PHOTOGRAPHY

Slides were observed under Leica trinocular photomicroscope DM 2000 model and important results were micro-photographed by using Canon camera of 7.1 mega pixel having 15x zoom of both optical and digital image.

Table 1: Different places of collection of plant material used for given study.

No.	Plants Name	Family	Place of Collection
1.	*Aristolochia indica* L.	Aristolochiaceae	Botanical Garden MSU Baroda
2.	*Leptadenia reticulata* (Retz.) W.& A.	Asclepiadaceae	Campus of MSU Baroda
3.	*Begnonia alliacea* L.	Bignoniceae	Vadodara
4.	*Spinacia oleracea* Linn.	Chenopodiaceae	Baroda, Rajkot
5.	*Calycopteris floribunda* (Roxb.) Lam.	Combretaceae	Goa, Kolhapur
6.	*Argyreia nervosa* Lour.	Convolvulaceae	MSU Baroda, Rajkot, Dediapada
7.	*Creesa cretica* L.	Convolvulaceae	Sayajipura Baroda
8.	*Ipomoea aquatica* Forsk.	Convolvulaceae	Parola (Jalgaon)
9.	*Ipomoea biloba* Forsk.	Convolvulaceae	Panchmahal forest, Dabhoi, MSU Baroda, Dediapada Forest, Rajkot
10.	*Ipomoea hederifolia* L.	Convolvulaceae	Panchmahal forest, Saurashtra, Rajkot, Dediapada Forest, Dabhoi
11.	*Ipomoea quamoclit* L.	Convolvulaceae	Munjaka, Parola (MS)
12.	*Ipomoea triloba* L.	Convolvulaceae	Panchmahal forest, Dabhoi, MSU Baroda, Dediapada Forest, Rajkot
13.	*Coccinia indica* W. & A.	Cucurbitaceae	MSU Campus Baroda
14.	*Camptosema isopetalum* (Lam.) Taub.	Fabaceae	Rio Bonito, a cerradão area at Botucatu, São Paulo State, Brazil.
15.	*Canavalia ensiformis* (L) DC.	Fabaceae	MSU Campus Baroda
16.	*Dolichos lablab* L.	Fabaceae	Borkheda (MS)
17.	*Macherium aculeatum* Raddi. Lantana aff. Fulcata Lindl.	Fabaceae	Rio Bonito, a cerradão area at Botucatu, São Paulo State, Brazil.
18.	*Mucuna pruriens* var. *pruriens* (Linn.) DC.	Fabaceae	MSU Campus Baroda
19.	*Phanera glabra* Jacq.	Leguminosae	Rio Bonito, a cerradão area at Botucatu, São Paulo State, Brazil.
20.	*Phanera outimouta* (Aubl.) Queiroz	Leguminosae	Rio Bonito, a cerradão area at Botucatu, São Paulo State, Brazil.

No.	Plants Name	Family	Place of Collection
21.	*Coroupita guianensis* Hook.	Lecythidaceae	Arboratum MSU Campus Baroda
22.	*Strychnos bicolor* Prog.	Loganiaceae	Rio Bonito, a cerradão area at Botucatu, São Paulo State, Brazil.
23.	*Cocculus hirsutus* (L.) Diels.	Menispermaceae	Botanical garden MSU Baroda, Bhorkheda
24.	*Diploclisia glaucanscens* (Bl.) Diels.	Menispermaceae	Goa
25.	*Tinospora cordifolia* (Thunb.) Miers.	Menispermaceae	Baroda
26.	*Boerhaavia diffusa* L.	Nyctaginaceae	Borkheda (MS), MSU Campus Baroda
27.	*Mirabilis jalapa* L.	Nyctaginaceae	Borkheda (MS), MSU Campus Baroda
28.	*Nyctanthes arbortristis* L.	Ollaceae	Hansa Mehta halls of residence MSU Baroda
29.	*Antigonon leptopus* Hk. & Arn.	Polygonaceae	Botanical Garden MSU Baroda
30.	*Securidaca rivinaefolia* A. St. -Hill & Moq.	Polygalaceae	Rio Bonito, a cerradão area at Botucatu, São Paulo State, Brazil.
31.	*Salvadora persica* L.	Salvadoraceae	Dangs-Navsari and Baroda
32.	*Serjania corrugata* Radlk.	Sapindaceae	Rio Bonito, a cerradão at Botucatu, São Paulo State, Brazil.
33.	*Serjania caracasana* (Jacq) Willd.	Sapindaceae	Rio Bonito, a cerradão area at Botucatu, São Paulo State, Brazil.
34.	*Solanum pseudocapsicum* L.	Solanaceae	Botanical Garden MSU Baroda