MATERIALS AND METHODS

2.0 Selection of cultivars :

Five cultivars of *M. indica* namely Rajapuri, Amrapali, Langra, Dadamio and Kesar were selected. Among these varieties Rajapuri was studied in detail as it exhibited high disease incidence. Besides these, male and bisexual flowers of other four varieties were also studied. All the cultivars are grown in Central fruit Nursery, Baroda and they fall under the age group of 10-15 years.

2.1 Collection of samples :

Malformed and healthy inflorescences and shoots of the selected cultivars were excised from the trees. Samples of terminal buds were excised at monthly intervals from the trees (var Rajapun) from January to December 1995. Soon after the collection, the samples were trimmed and immediately fixed in formalin-propanionic acid - alcohol, FPA (Dring, 1971). After reaching the laboratory the fixed samples were aspirated. Twenty four hours after fixation, the samples were cut into small pieces and processed following the conventional paraffin embedding method (Berlyn and Miksche, 1976).

2.2 Microtomy and Staining :

Samples of peduncle, pedicel, ovary with gland, anthers, shoots and leaves of malformed and healthy panicles and twigs were sectioned at 8-10 μm thickness in transverse and tangential planes using Leica rotary microtome. Terminal and axillary buds were sectioned longitudinally Paraffin ribbons containing serially sectioned samples were arranged in rows on glass slide after applying Haupt's adhesive. After spreading the ribbons with 4% formalin on a hot plate the slides were kept for drying.

Following dewaxing in xylene, the sections were stained either with periodic acid - Schiff's reagent (PAS - reagent) in combination with chlorozol black 'E' or PAS - reagent alone (Dring, 1971) Some slides were stained with toluidine blue 'O'.

2.3 Histochemical techniques :

Paraffin embedded and hand sectioned samples were used for histochemical studies

2 3 1 Phenolic catechins: Dewaxed or hand-cut sections were kept in equal volumes in succession. (i) 10% sodium nitrate, (ii) 10% Urea and (iii) 10% acetic acid. After few minutes two volumes of 2N sodium hydroxide was added. The resultant reaction gives cherry-red colour indicating the presence of phenolics (most probably catechol tannins) (Reeve, 1951) or the sections were stained with vanillin reagent (equal volumes of Hcl and a mixture of vanillin 2% w/v and acetaldehyde 1% v/v in 95% ethanol (Blunden *et al.*, 1966).

2.3.2 Total proteins · The dewaxed sections were washed and kept in 0.25% Coomassie brilliant blue in 7% aqueous acetic acid solution. Following a rinse in 7% aqueous acetic acid and distilled water the sections were dehydrated and finally mounted in DPX (Ekalavya, 1979).

2.3.3 Starch : After dewaxing, sections were washed in distilled water and mounted in iodine potassium-iodide solution (Johansen, 1940).

2.3.4 Total Lipids: The sections were treated with ethanolic sudan black 'B' for 1 hr at 60°C Then these were washed in 70% alcohol, rinsed in distilled water and mounted in glycerine jelly (Bronner, 1975)

2.4 Enzyme Localization:

Hand cut sections were used for histochemical localization of peroxidase (E C 1 11 1 7) and succinic dehydrogenase (E.C 1 3.99.1) enzymes. Peroxidase was localised following DAB - technique (Graham and Karnowsky, 1966) and succinic dehydrogenase by NBT - technique (Gahan, 1984).

2 4 1 Peroxidase (DAB - technique) : Fresh tissues were fixed in 6% glutaraldehyde and formaldehyde in phosphate buffer Sections from fixed tissues were placed in incubation medium (for 5 min) which consisted of 3-3-diamino benzidine tetrahydrochloride 5 mgs, in Tris-HCl buffer pH 7 6 (10 ml) and 1% H_2O_2 (1.2 ml). Incubated sections were washed well in distilled water and mounted in glycerine jelly. The site of enzyme activity stained dark brown in colour

2 4 2 Succinic dehydrogenase (NBT - technique) : Tissues were placed in freshly prepared incubation medium for 5-6 hrs at 37°C which consisted of phosphate buffer 0 1M pH 7 2 (10 ml), EDTA (5 mg), sodium succinate (81 mg), Potassium cyanide (4 mg), Nitroblue tetrazolium salt (1.3 mg), Phenazine methosulfate (PMS) (15 mg/ml). After 5-6 hours in incubation medium sections were washed well in distilled water and mounted in glycerine jelly. The site of enzyme reaction was identified by the appearance of black precipitate in the tissues.

For controls the sections were heat treated and kept in incubation medium. These sections were observed and photographed under Zeiss research microscope.

2.5 Maceration :

To measure the dimensions of fibres and vessel elements xylem tissue was macerated using Jeffrey's fluid (Berlyn and Miksche, 1976) or combination of hydrogen peroxide and acetic acid (1:1).After thorough washing with 1-2% sodium bicarbonate and distilled water, the macerated tissue was stained with Saffranin or toluidine blue

2.6 Measurements :

Vessel lumen diameter, length and width, gum-resin duct lumen diameter were measured with an occular micrometer scale mounted in Carl Zeiss Amplwal research microscope. The number of vessels, gum-resin ducts per 0.5 or 1 square mm in transverse sections of peduncle, shoots or longisections of terminal buds was counted on Metzer projection microscope. For each parameter one hundred random measurements were recorded from healthy and malformed tissues. The readings were

statistically analysed to determine the mean and standard deviation. The dimensional details of elements and meteorological data were represented either in histograms, tables or graphs.

2.7.0 Electronmicroscopy :

2 7 1 Transmission Electron Microscopy: Trimmed samples of stamens and ovary measuring 0.5 to 1 mm in size were fixed in 6% glutaraldehyde in 0.1M phosphate buffer at pH 7.2 for 24-48 hrs. Post fixation was done in 2% Osmium tetroxide for overnight. The samples were then en-block stained in 2% acqueous Uranyl acetate for 30 mins, dehydrated in graded acetone series and embedded in Araldite medium (Glauert, 1975).

Semithin and Ultrathin sections from polymerized blocks were cut with Joel ultramicrotome using glass and diamond knives respectively. Semithin sections were stained with Toulidine blue O in borax on a glass slide while ultrathin sections collected on 200 mesh copper grids were stained in Uranyl acetate and lead citrate (Reynolds, 1963) Ultrathin sections were observed under Philips CM 10 Transmission Electron microscope at 80 KV and photographed at different magnifications.

2.7.2 Scanning Electron Microscopy : Samples of sepals, petals, entire anthers, and longitudinally cut ovary were fixed in 6% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 24-48 hrs. After fixation, samples were dehydrated in graded ethanol series, coated with gold and observed under Hitachi S-520 Scanning electron microscope at 20 KV.

2.8 Photography :

Photomicrographs were taken from stained sections on zeiss research microscope using ORWO NP55 negative film. Black and white positive prints were taken using sterling special grade bromide paper.