# CHAPTER II

# MATERIALS AND METHODS

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Populations of different plant species included in the present work were collected from various localities of Gujarat state, Delhi, Jodhpur and adjoining areas. Routine observations regarding the habit, size, state in which observed, colour and/or smell of flowers and other striking morphological features, if any, were recorded for each population. Each collection was given a separate voucher number and specimens were collected for herbarium preparation. Tentative field identifications were later on confirmed by dissecting various parts and by referring to standard floras available. After confirming the identity herbarium sheets representing all the populations were prepared following Lawrence (1951) and the same are deposited in the departmental herbarium.

Mature seeds of the populations collected from different localities during field trips and those obtained from foreign countries, were raised in the botanical garden of the Dept. of Botany under uniform conditions. This helped in checking the nature and consistency of observed variations present in different populations of a species, thereby the presence of ecological races, ecads, or ecotypes, if any, could be detected.

Populations of various species studied, their sources and collection numbers are presented in the given table.

Table I. showing taxa, collection number, source and/or locality.

| Taxa | Collection Number | Source or locality |
|---|---|---|
| Nicandra physalodes (L.) Gaertn. | 16 | Saputara |
| | 08 | Kew gardens |
| | 21 | Copenhagen |
| Lycium barbarum L. | 15 | Kutch |
| Withania somnifera (L.) Dunal | 14 | Baroda |
| | 60 | Mandvi (Bhuj) |
| Physalis longifolia Nutt. | 38 | Baroda |
| | 39 | Dumas |
| | 41 | Ranoli |
| | 43 | Karodia |
| | 45 | University Campus Baroda |
| P. minima L. | 37 | " |
| | 40 | Raika |
| Solanum villosum Mill.subsp.villosum | 42 | Karodia |
| | 31 | Copenhagen |
| S. villosum Mill. subsp. puniceum (Kirschleger) Edmonds | 32 | " |
| S. chenopodioides Lam. | 33 | " |
| S. scabrum Mill. | 36 | " |
| S. americanum Mill. | 35 | " |

Contd....

Table I. Contd..

| Taxa | Collection Number | Source or locality |
|---|---|---|
| S. roxburghii Dunal | 03 | Rajpipla |
| | 06 | Ranoli |
| | 18 | University Campus Baroda |
| | 22 | Bajuwa |
| | 24 | Pratap Nagar |
| S. purpureilineatum Sabnis & Bhatt | 11 | University Campus Baroda |
| | 46 | Baroda |
| | 47 | Harni Road |
| | 50 | Pani Tank, Ajwa Road |
| S. nodiflorum Jacq. | 26 | Pratap Nagar |
| | 27 | Jawahar Nagar |
| | 28 | Delhi |
| S. nigrum L. | 02 | Bhuj |
| | 19 | Raika |
| | 30 | Mandvi - Kutch |
| S. nigrum L. (Red veined leaf) | 04 | Jawahar Nagar |
| S. viarum Dunal | 25 | Dehra Dun |
| S. trilobatum L. | 10 | Sunderpura |
| | 48 | Zhambuva |
| S. heterodoxum Dunal | 34 | Copenhagen |

Contd...

Table I. Contd..

| Taxa | Collection Number | Source or locality |
|---|---|---|
| Tephrosia strigosa Dalz. | 63 | L.V.Palace, Baroda |
| | 59 | Jawahar Nagar |
| T. jamnagarensis Santapau | 64 | Surat |
| T. uniflora Pers. subsp. petrosa Blatt. & Hall | 65 | Jodhpur |
| T. subtriflora Hochst | 66 | Jodhpur |
| T. villosa (L.) Pers. | 20 | Raika |
| | 52 | Delhi |
| | 09 | Bhimpura |
| T. falciformis Ramaswamy | 67 | Jodhpur |
| T. wallichii Grahm | 68 | Jodhpur |
| | 53 | " |
| T. candida DC. | 62 | " |
| T. purpurea Pers. | 56 | Baroda |
| | 05 | Bhimpura |
| | 61 | Raika |
| T. pumila (Lamk.) Pers. | 57 | Jawahar Nagar |
| | 01 | L.V.Palace, Baroda |
| T. hamiltonii Drumm. | 58 | Raika |
| Psoralea corylifolia L. | 07 | Zhambuva |
| | 51 | Sunderpura |

Mature seeds of different populations were germinated in Petri dishes on moist filter paper at room temperature. On some occasions, subjecting those Petri dishes to higher temperature (upto 35°) in the initial stages, considerably enhanced the germination. Seeds of Tephrosia species having hard seed coat, were first treated with conc. $H_2SO_4$ for 30-40 seconds and then thoroughly washed in running water for 2-3 hrs. before placing them for germination in Petri dishes (Rao, 1947).

Various pretreatment chemicals, such as : 8-Hydroxy-quinoline, p-Dichlorobenzene and Colchicine were tried. Of all these chemicals 8-Hydroxyquinoline gave most satisfactory results in showing exaggerated constrictions and suitable shortening of arms. For mitotic preparation Tjio and Levan's (1950) oxyquinoline aceto orcein method was adapted. Young healthy root tips collected between 9.10-9.35 a.m. were treated with 0.002 M 8-Hydroxyquinoline for 3 hrs. at 12-15°C. Pre-treated root tips were then fixed in mixture of acetic alcohol (1:1). Fixed root tips were hydrolysed in a mixture of 2% aceto orcein and 1N HCl (9:1) and were then squashed in 1% aceto orcein applying uniform pressure over the cover glass. Slides showing good metaphase plates were temporarily sealed with wax, which remained good for about a week or so. For ascertaining the chromosome number and to study the chromosome morphology, a number of preparation for each collection were made.

The flower buds of suitable size were selected for meiotic preparation. Both fresh as well as fixed flower buds were tried. However, in most cases the fixed material gave better results. Suitable sized buds were fixed in Carnoy's fixative, having 6, 3 & 1 parts of alcohol, chloroform and glacial acetic acid respectively, for 24 hours. For longer duration preservation, buds were transferred to fresh fixative. In case of Solanaceae members buds fixed between 11.15 to 11.45 a.m. and for Fabaceae members buds fixed between 8.30 to 9.15 a.m. gave good results. However, before smearing fixed buds were transferred to 45% acetic acid at least for 30 mins. Smear preparations were made in 1% acetocarmine.

Both, for mitosis and meiosis, best preparations were selected for camera lucida drawings and microphotographs.

Drawings were made at table level on Steindorff's Research microscope, using E Leitz-Wetzlar Camera-lucida apparatus, having removable filters. Eyepieces having X15 or X30 and objectives having X100 (oil immersion) or X120 (apochromatic oil immersion) magnifications were used for drawing. Microphotographs were taken on Amplival Carl Zeiss Research microscope using mf camera attachment (24X36), MF projective (K-3,2:1) and ORWO 35 mm black & white film.

Temporary slides were made permanent following Celariar's (1956) butylalcohol-acetic acid schedule and mounted in euparal.

Camera lucida drawings of mitotic metaphase plate were used for detailed karyomorphological studies. Chromosomes of the karyotype were numbered and measured. Pairing of the chromosome was made on the basis of length of long and short arms, presence of secondary constriction or satellite and total length. Mean values of length of long arm and short arm was calculated for each pair. The primary and secondary constriction gap were not taken into consideration while measuring chromosome length. The above mentioned values were then converted into microns by multiplying them with the calibration constant determined for the magnification at which drawing was made. Total length of the chromosome complement of a taxon was also calculated. The size of satellite and β-chromosome were not taken into consideration for the total chromatin length.

Precise determination of centromeric position was done following Adhikary (1974). The prescribed values for $R_1$ and $R_2$ for the centromeric position are presented in the table given below.

Table II. Arm ratios and position of centromere.

| $(R_1)$ $\frac{\text{Short arm}}{\text{Long arm}}$ | $(R_2)$ $\frac{\text{Long arm}}{\text{Short arm}}$ | Position of the Centromere | Notation |
|---|---|---|---|
| 1.00 | 1.00 | Median | M |
| 0.33 | 3.00 | Submedian | SM |
| 0.99 - 0.61 | 1.01 - 1.63 | Nearly median | nm |
| 0.60 - 0.34 and 0.32 - 0.23 | 1.64 - 2.99 and 3.01 - 4.26 | Nearly submedian | nsm |

Relative length of chromosomes in a complement were calculated to minimise the error of chromosomal length due to pretreatment and other factors. The relative length is expressed as percentage length of chromosome in consideration to the longest chromosome in the complement(Huziwara, 1958; Kapoor and Löve, 1970). Relative length values facilitate in judging the nature of gradation within the karyotype.

The total form percentage (T.F.) represents,

$$\frac{\text{Total sum of short arm}}{\text{Total sum of chromosome length}} \times 100,$$ while L/S ratio represents, $$\frac{\text{Length of the longest pair}}{\text{Length of the shortest pair}}$$ (Huziwara, 1958).

TF % and L/S ratio values were useful in deciding the symmetry of the karyotype. A T.F.% of 50 indicates that all the chromosomes have a median centromere and the karyotype is absolutely symmetrical, whereas T.F.% of zero indicates all chromosomes with terminal centromere and absolute asymmetry of karyotype. Higher value of L/S ratio is also indicative of apparent asymmetrical nature of the karyotype (Kapoor and Löve, 1970).

Long arm and short arm values were then transferred on a graph paper, in decreasing order from left to right, to construct an idiogram. The ends of long arms were directed downward lying on the same abscissa. The width of the chromosome, the gap of the primary and secondary constrictions

and the distance between the two chromosomes were kept constant and uniform throughout. This was then transferred to a drawing paper, by the side of which the provided scale represents the measurement in microns. Categorisation type, to which it belongs, is mentioned below each chromosome. Both in camera lucida drawings and idiograms satellited, secondarily constricted chromosome and isochromosome are drawn in outline only. Wherever noticed, B-chromosomes are also drawn in karyotype and are indicated by an arrow.

Histogram represents the total chromatin length of diploid set of chromosomes of a complement. Histograms of different taxa are compared and suitable scale is provided to facilitate the comparison.

The apparent pollen fertility was estimated on the basis of stainability of pollens with Muntzing solution (1:1 proportion of glycerol and 1% acetocarmine). Only well inflatened and uniformly stained pollens were scored as fertile ones.

Epidermal peels were taken from the fresh as well as fixed leaves/leaflets. Wherever herbarium specimens were used, leaves were first boiled in distilled water on a hot waterbath for 3-4 min. to facilitate peeling. These peels were cleared with soft brush dipped in water. The cleared peels were then stained in Delafield's haematoxyline (1%) and thoroughly washed in distilled water. Peels were mounted on slide in

glycerine jelly and covered with a cover glass. In some cases, where peeling was difficult imprints of the epidermis were taken using 'favicol' (Inamdar and Patel, 1969).

Epidermal cell shape, size, frequency and stomatal types, guard cell size and stomatal frequency were determined on the basis of the study of 10 fields under microscope for each species. Mean values of these observations were calculated to represent the average size and frequency of epidermal cells and stomata. Stomatal index (SI) for each species was calculated on basis of $\frac{S}{E + S} \times 100$ in which 'S' is the number of stomata and 'E' is the number of epidermal cells over a given area (Salisbury, 1927, 1932).

Drawings representing epidermal cells, trichomes and stomata were drawn from the epidermal peel slides prepared under uniform magnification using Carl Zeiss camera lucida apparatus, on Carl Zeiss Research microscope. Drawings were compiled, inked; from which prints of suitable size were made. In some cases, microphotographs of abnormal stomatal types were taken directly from the slides on Carl Zeiss photomicroscope using incandescent light, yellow filter and ORWO NP-15 film.

In order to determine the leaf area, leaves were first traced on a graph paper and the number of the squares covered were counted.

For venation pattern study, leaves were cleared in 2:1

solution of Trichloroacetic acid and Phenol (MohanRam and VijayaLaxmi, 1978). The cleared leaves were then stained with Kores stamp pad (violet) ink (Rao, Shenoy and Inamdar, 1980). Permanent slides were made following customary procedure.

Directly from the leaves, number of secondary veins on either side of the midrib, their angle of divergence, presence or absence of intersecondary, were determined. However, permanently prepared slides were used for determining size of areole, number of areoles per $mm^2$, number of veinlets entering areoles per $mm^2$, number of vein termination per $mm^2$ and highest degree of vein order for each species. Absolute vein islet number and absolute vein termination number in thousands were calculated following Gupta (1961).

Direct photographs of cleared leaves/leaflets were taken on an enlarger.

For description of above mentioned features of the leaf, terminologies of Uphof and Hummel (1962), Roe (1971) for trichomes, Metcalfe and Chalk (1950) for stomata and Hickey (1973) for venation pattern have been adopted.

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