CHAPTER 3

MATERIALS AND METHODS
The present studies were undertaken on the cytomorphological diversity of the dicotyledonous plants of Kinnaur district. The work encompasses extensive surveys for the collection of plant material, identification and submission of plant specimens, morphological and cytological studies followed by ethnobotanical study about the knowledge on the utilization of plants by local inhabitants.

3.1 SURVEYS AND SAMPLE COLLECTION

For exploration of cytomorphological diversity, study materials were collected by surveying the different localities of Kinnaur district during the months of April to September for four years (2007-2010). All the accessible localities were visited at different times when the area is open for traffic (see: Map 2). A total of 200 species falling under 119 genera and 33 families of Dicot plants have been cytologically investigated. Data under each species regarding the locality/localities with altitude from where the material was collected, accession number/s (PUN*), meiotic chromosome number, photomicrograph/s, ploidy level, pollen fertility %age, and previous chromosome reports are provided in Table 2. The arrangement of families in Tables 1, 2 and in the text is after Bentham and Hooker (1862-1883), whereas the name of genera, species and varieties within each family is arranged alphabetically. Some modification in the treatment of certain families is followed as per recent trends.

3.2 IDENTIFICATION AND SUBMISSION OF PLANT SPECIMENS

Plant specimens were identified by consulting the Herbaria of Botanical Survey of India (BSI, Northern circle), Dehra Dun and Herbarium, Department of Botany, Punjabi University, Patiala, India. Voucher specimens of the cytologically worked out species were deposited in the Herbarium (PUN*), Department of Botany, Punjabi University, Patiala, India.

3.3 CYTOLOGICAL STUDIES

3.3.1 Meiotic Preparations

The chromosome counts in each case were made through male meiotic studies for which young and developing floral buds were fixed in freshly prepared Carnoy’s fixative (6 Ethanol: 3 Chloroform: 1 Acetic acid v/v) for 24 hours.

* Code of Herbarium maintained by Department of Botany, Punjabi University, Patiala, India as per “Index Herbariorum” by Holmgren and Holmgren (1998).
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Materials were then transferred to 70% ethanol and stored in a refrigerator. Meiotic preparations were made from the developing anthers through standard squash technique in 1% acetocarmine. A number of freshly prepared slides were carefully examined from each collection to determine the exact chromosome number at different stages of meiosis which were confirmed by observing a number of well spread Pollen Mother Cells (PMCs)/meiocytes. Chromosome behaviour and various meiotic irregularities wherever exist, were analyzed at different stages of meiosis from prophase-I to telophase-II by examining a number of PMCs/meiocytes.

3.3.2 Meiotic Products (Sporads and Pollen Grains) Analysis

For sporad analysis, floral buds were squashed in 1% acetocarmine and PMCs/meiocytes were analyzed at late telophase-II. Sporads were analyzed and categorized on the basis of number of microspores units present in the meiocytes as monads, dyads, triads, tetrads and polyads. Pollen fertility in all the cytologically investigated species was estimated through stainability tests. In each case mature anthers from different flowers and also from the different individuals were smeared in glycerol: acetocarmine (1:1) mixture and 1% aniline blue dye. Well filled pollen grains with stained nuclei were taken as apparently fertile while shriveled and with unstained or poorly stained cytoplasm were counted as sterile. Size of pollen grain was measured by using Occulomicrometre in meiotically abnormal species or in different cytotypes and morphotypes.

3.3.3 Photomicrographs

Photomicrographs of chromosome counts, meiotic abnormalities, sporads, pollen grains, and stomata were made from the freshly prepared slides using Leica Qwin Digital Imaging System (X290, X900 and X2180) and Nikon Eclipse 80i microscope (X330, X1340 and X3400). Presently, all the photomicrographs of chromosome counts and some of the meiotic abnormalities are at X2180 of Leica Qwin Digital Imaging System and at X3400 of Nikon Eclipse 80i microscope. The photomicrographs of meiotic abnormalities, sporads, pollen grains, and stomata are made at X900 and X2180 of Leica Qwin Digital Imaging System, and X330, X1340 and X3400 of Nikon Eclipse 80i microscope.
Important points of cytological interest regarding various meiotic irregularities such as interbivalent/chromosomal connections, chromatin stickiness, pycnotic chromatin, chromatin transfer, extra chromatin masses, out of plate bivalents, laggards and chromatin bridges, early and late disjunction, irregular segregation of chromosomes, fusion of pollen mother cells (PMCs), fusion of pollen grains, hypo-, hyperploid and enucleated pollen mother cells (PMCs), multivalent/s, univalent/s, B-chromosomes and micronucleus/micronuclei are recorded and indicated by arrow/s in photomicrographs.

3.4 MORPHOMETRIC ANALYSIS

The morphological analysis is evaluated by field observations to mark out the different morphovariants by focusing on vegetative (plant height; number of branches per plant; number of leaves per branch; number of leaflets per leaf; shape and size of leaf and leaflets; leaf margin, surface, colour, fleshiness, thickness) and reproductive (length of inflorescence; number of flowers per inflorescence; size and colour of flower; size of capsule) parameters.

Characters like size and frequency of stomata, and pollen grains were studied in the laboratory by slide preparations and observations were made under microscope. Stomatal studies, wherever made, were from the abaxial epidermal peels obtained from the middle portion of the mature leaves through KOH treatment. Thoroughly washed peels were mounted in glycerol. Stomatal index is calculated by counting the stomata using a 40X objective and a 10X ocular. Following expression was used to calculate the stomatal index:

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SI = \frac{S}{E+S} \times 100
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Where, SI = stomatal index,

S = number of stomata per field,

E = number of epidermal cells per field.
3.5 ETHNOBOTANICAL STUDIES

While exploring the cytomorphological diversity of plants, documentation of traditional knowledge on the utilization of plants has also been made from different parts of Kinnaur district. The information was documented through interviews with the tribal people, elders and other local inhabitants residing in the study area. The ethnobotanical information about each plant species include vernacular/local names, plant part used, and mode of uses, and is provided in Table 19.