Chapter 16
Plant Chromosome Preparations and Staining for Light Microscopic Studies

Subhash C. Hiremath and C. C. Chinnappa

16.1   Introduction

Swiss botanist Wilhelm Von Nägeli in 1842 discovered thread-like structures in plant nuclei and called them in German stäbchen or little sticks in English, which are now known as chromosomes [1]. Waldeyer [2], a German anatomist, coined the word chromosome in 1888 for these structures. In 1886, Mendel [3] formulated the laws of genetics without any knowledge of chromosomes and his work remained unknown. Three botanists Carl Correns (Germany), Hugo de Vries (The Netherlands) and Erich Von Taschermark (Austria) in 1900, independently rediscovered the Mendelian principle of heredity [4]. In 1902, Sutton and Boveri proposed the chromosome theory of inheritance and linked the behaviour of chromosomes with Mendelian principles [5]. Thus, arose the discipline of cytogenetics. Pioneering work of Morgan and his students with fruitfly and McClintock's contribution with maize laid the firm foundation of cytogenetics. It is a hybrid discipline nourished by contributions from cytology, genetics and lately molecular biology. Cytogenetics involves handling of chromosomes, structure, movement, function and behaviour of chromosomes including recombination, transmission and expression of genes [6].

Eukaryotic nuclear genome is constituted of discrete linear chromosomes and apparently, a segregational device for cell division. Each chromosome is made up of a single long linear DNA molecule associated with different protein molecules. It provides a framework structure for linkage groups, allows replication, transcription and transmission of genetic information. Each species is characterized by a precise number of chromosome in the nucleus. Plants show great diversity in chromosome number. In a well-documented work, lowest chromosome number has been reported as $2n=4$ in *Haplopappus gracilis* [7] and in several other species [8]. Highest chro-

S. C. Hiremath (✉)
Department of Applied Genetics, Karnataka University, Dharwad 580003, Karnataka, India
e-mail: subhashhiremath@yahoo.com

C. C. Chinnappa
Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada

mosome count is known from a fern *Ophioglossum reticulatum* as $n=720$ [9]. There are a few exceptions to the constancy of chromosome number within a species that shows different chromosome numbers, for example *Eleusine floccifolia* shows diploid ($2n=2x=18$) and tetraploid ($2n=4x=36$) races [10]. Several such examples are available in literature. Another source of variation in chromosome number is due to the presence of B chromosomes in addition to the normal chromosomal compliment in some plants [11]. Knowledge of chromosome number is essential in taxonomy, systematics, phylogeny, plant breeding, genetics, physical mapping and DNA-sequencing projects [12].

Light microscopic observation of metaphase chromosomes reveals three distinct features: centromere (primary constriction), nucleolar organizing region (secondary constriction) and telomere at the two free ends of linear chromosomes. Position of centromere determines the shape of the chromosome. Levan et al.'s system of classification and nomenclature of chromosome is widely used by cytogeneticists [13]. Chromosome shape can be metacentric, submetacentric, acrocentric and telocentric depending upon the position of centromere along its length (Fig. 16.1).

Development of staining procedures of root tip squash method and observing condensed chromosomes at metaphase stages have led to the popularity of analysing chromosomes for various cytogenetic studies. At this stage, individual chromosomes are amenable for identification in a genome. In this direction, Levitsky [14] proposed and developed the concept of karyotype, and defined it as phenotypic appearance of somatic metaphase chromosomes complimenting their genic content. Diagrammatic representation of karyotype is often termed as ideogram or karyogram. Karyotype analysis has helped in the identification of individual chromosomes within a genome. Such studies have been undertaken in diverse group of plants over past several decades. These data have been useful in evolutionary and phylogenetic studies at taxonomic level between species and family [12].

With conventional staining methods, it has not been possible to differentiate all the chromosomes in a karyotype of several groups of plants and animals. Various

Fig. 16.1 Diagrams showing somatic chromosome morphology (modified [37])
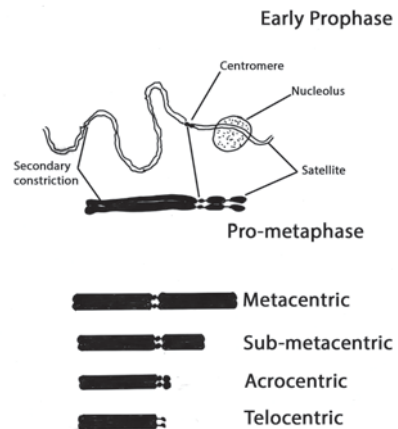
chromosome banding methods were developed after 1960s, which produced transverse dark and light bands along the length of chromosomes. These banding methods are widely used for the identification of individual chromosomes in the karyotype, detection of structural changes in chromosomes, aneuploid identification, chromosome polymorphism and genome analysis of polyploids. Various chromosome banding methods that are reported in usage in plants and animal species are Q-, G-, C-, N-, F-, Hy-, R- and Ag-NOR-banding [15–17]. Among these banding methods, Q- and C-banding are widely used in plants.

Development of in situ hybridization (ISH) technique [18, 19] heralded the dawn of molecular cytogenetics. In this procedure, radioactively labelled nucleic acid sequence or probe was hybridized to chromosomal preparations on the slide. Probe-targeted hybrid sequence along the chromosome was detected by autoradiography. In later techniques, radiolabelling was replaced by fluorochromes or fluorescent stains and DNA: DNA hybridization (probe-target sequence) on chromosomal location was visualized by fluorescent microscopy. This modified ISH is now known as fluorescent in situ hybridization or FISH technique, and various aspects of this procedure have been reviewed [17, 20–22]. FISH technology has enabled cytogeneticists to recognize individual chromosomes for karyotype analysis, and phylogenetic studies to prepare physical maps, to study the role of chromatin at cytological level that regulates gene expression [20, 21]. Modification of FISH is known as genomic in situ hybridization (GISH) in which whole genomic DNA is used as probe instead of specific DNA sequences like 45S or 5S r-RNA genes, etc. GISH allows the identification of whole genome in a polyploidy species or a hybrid. FISH procedures and allied aspects are detailed in Chap. 17 of this book.

Chromosome preparation is a basic indispensable tool in cytogenetics. It is required for recording chromosome numbers and ploidy level, karyopype analysis, chromosome pairing data in species and hybrids, chromosome banding, FISH, GISH and hosts of situations in genetic studies. In modern genomic projects, chromosome preparations provide a framework for physical maps, number of linkage groups and guide in genome sequencing projects [12, 17].

16.2   Materials

16.2.1   General Laboratory Equipment and Supplies

Microscope with digital camera attached, stereomicroscope, slide warmer, water bath, forceps, camel hair brush, slides and coverslips, dissecting needles, Lancet needle, blade, spirit lamp, beakers, vials, cavity block or Syracuse dish, watch glass, and Coplin jars.

16.2.2   Chemicals for Plant Treatment

1. Colchicine (*see Note 1*), 8-hydroxyquinoline (*see Note 2*), p-dichlorobenzene (*see Note 3*), monobromonaphthalene (*see Note 4*)
2. Stains: carmine (*see Note 5*), orcein (*see Note 6*), Basic fuchsin (*see Note 7*), Giemsa (*see Note 8*)
3. Chemicals for fixative preparations: chloroform (*see Note 9*), ethanol (*see Note 10*), propionic acid (*see Note 11*), glacial acetic acid (*see Note 12*)
4. Mounting media: Canada balsam (*see Note 13*), Euparal (*see Note 14*), DPX (*see Note 15*)
5. Other chemicals: hydrochloric acid, barium hydroxide

16.2.3   Preparation of Reagents and Solutions

1. 0.1% Colchicine solution: dissolve 1 g of colchicine in 100 mL dH_2O and use it as stock solution. From this prepare 0.1% solution by dilution. Store in refrigerator.
2. 0.002 M 8-hydroxyquinoline: dissolve 0.3 g of this chemical in 1 L dH_2O. Keep at 60 °C overnight. Store in refrigerator.
3. Paradichlorobenzene-saturated solution: weigh and dissolve 3 g of this chemical in 200 mL dH_2O by keeping the bottle at 60 °C overnight in a stoppered bottle. Store in refrigerator [23].
4. Monobromonaphthalene-saturated solution: prepare saturated solution by adding 1 mL of monobromonaphthalene to 98 mL of water by constant shaking. Later add more of this chemical drop by drop with constant shaking. Let it settle down. Undissolved monobromonaphthalene settles down and can be reused.
5. Preparation of acetocarmine [24]: in a large Erlenmeyer flask, provide heat to boil 100 mL of 45% glacial acetic acid. Add 1 g of carmine powder to the boiling solution and let the solution simmer for 5 min. Allow it to cool and settle. Filter and store in coloured bottle. Keep it in a refrigerator (*see Note 16*).
6. Preparation of acetoorcein: 2% and 1% acetoorcein is prepared in a manner acetocarmine is prepared. Carmine is replaced by orcein. No iron mordanting is needed for acetoorcein staining. Store in refrigerator and use within 2–3 months.
7. Preparation of propino-carmine: propino-carmine is prepared in the same manner as acetocarmine. Instead of 45% acetic acid, 45% propionic acid is used. Addition of ferric salt is recommended.
8. Preparation of Lillie's Feulgen stain [25]: dissolve 1 g of basic fuchsin in 100 mL of 0.15 N HCl (15 mL N HCl to this add 85 mL dH_2O) in a tightly stoppered bottle. To this dye solution, add 2.2 g of sodium metabisulphite. Shake frequently or place on a shaker for 2–4 h. Solution may be clear to pale yellow in colour. Add activated charcoal or carbon and shake for few minutes and filter quickly using coarse filter paper. Good Feulgen stain will be clear with strong sulphur dioxide odour. Store in amber-coloured bottle in refrigerator; it can be stored for a few months.

9. Preparation of de Tomasi's Feulgen stain [26]: pour 200 mL of boiling dH_2O into 500 mL Erlenmeyer flask containing 1 g basic fuchsin powder. Shake well and allow it to cool to 50 °C. Filter into a bottle. Add 30 mL 1 N HCl and 3 g of potassium metabisulphite, stopper the bottle tightly. Shake and leave in dark for 24 h. Solution will be clear. In case it is pale yellow or straw coloured, add 0.5 g carbon and shake for few minutes. Filter quickly using coarse filter paper into a new dry coloured bottle with tight stopper. Store in a refrigerator; it remains in a good condition for 2–3 months. One can get excellent Feulgen reagent using pure pararosaniline instead of basic fuchsin.

10. Preparation of Giemsa stain [27]: dissolve 1 g of Giemsa dye in 66 mL of glycerine at 60 °C with constant stirring for an hour. Add 66 mL methanol and continue stirring at 60 °C for 24 hrs. Filter and keep in refrigerator. The stain solution is useful for up to 2 months. Giemsa stain is available in premade and ready-to-use stock solution of different strength from various venders. From this stock solution, the required per cent of Giemsa stain is prepared by mixing with phosphate buffer (pH 7).

11. Carnoy's Fixative I (*see Note 17*): 3 parts ethanol 95–100 % and 1 part glacial acetic acid.

12. Carnoy's Fixative II (see *Note 18*): ethanol 95–100 %: glacial acetic acid: Chloroform (6: 3: 1; v:v)

13. Preparation of Sorenson phosphate buffer: Part-A, dissolve 9.47 g sodium phosphate dibasic (Na_2HPO_4) in 300 mL dH_2O and make to 1 L. Part-B, dissolve 9.07 g potassium dihydrogen phosphate (KH_2PO_4) in 300 mL dH_2O and make to 1 L. Label and store A and B in separate bottles and leave them at room temperature. Mix 58 mL of part-A and 42 mL of part-B to get 100 mL of buffer at pH 7.0.

14. Preparation of 2x SSC solution: prepare 20X SSC (Saline Citrate Solution) stock solution by dissolving 88.2 gm of trisodium citrate ($Na_3C_6H_5O_7.2H_2O$) and 175.3 g of sodium chloride (NaCl) per liter dH_2O. This solution can be stored for several months. Dilute 1 part of 20x SSC with 9 part of dH_2O to get 2x SSC.

16.3   Methods of Studying Chromosomes at Mitosis

16.3.1   Specimen Collection

Microscopic examination of any biological specimen for chromosomal analysis involves several steps like sample collection, pretreatment, fixation, staining and mounting. Important aspects of this exercise are to obtain a good chromosomal preparation having large number of cells with well-spread brightly stained chromosomes.

Actively growing meristematic tissues like root tips, shoot tips and leaf tips are favourable material for chromosome preparation. Among these, root tips are chosen as specimen because of the ease of obtaining them at short notice. Root apices can be easily obtained from seeds. They can be easily germinated on moist filter paper at 20–25 °C. Root tips of about 1–3 cm long touching the filter paper are collected in a vial with cold water.

Healthy potted plants at early developmental stage are also good source of actively growing root tips. Take the pot, carefully turn it upside down and remove the pot. The healthy growing roots would be seen on sides of soil mould holding root system. Carefully collect 2–3 cm long root apices with forceps and put them in vial containing cold water. Using a camel hair brush, clean the root tips in running water for any soil particles adhering to them.

16.3.2   Pretreatment

Various pretreating agents have been used to obtain well-condensed chromosomes with clear constriction regions. Further, these agents inhibit spindle formation thereby arrest the chromosomes at metaphase stage with increased number of metaphase cells. All these changes are brought about due to the physical changes in cytoplasm by pretreating agents [28].

Low temperature (2–8 °C) has been widely used as a physical pretreating agent for cereal chromosome analysis. Root apices in cold water are pretreated at low temperature in a fridge for 12–24 h. This causes chromosome condensation with high mitotic index.

Large number of chemicals have been evaluated for the pretreatment of chromosomes. Some chemicals are sparingly soluble in water and these are used as saturated solutions. Only few of the chemicals have been found to be useful as pretreatment chemicals for chromosomes.

16.3.3   Study of Chromosomes at Mitosis

Ability to reproduce is a fundamental property of living organisms. Eukaryotes reproduce by two types of cellular divisions, Mitosis and Meiosis. Mitosis is characterized by perseverance of constant chromosome number. This type of cell division is the property of body cells or somatic tissue. In contrast, meiosis occurs in sporogenous or germinal cells and chromosome number is reduced from diploid to haploid or halved. Both types of cell divisions involve the cell cycle comprising growth (G1), DNA synthesis (S), growth (G2) mitosis or meiosis (M) and cytokinesis (C) phases [29]. Mitotic division involves division of nucleus to form two identical daughter nuclei and this process is referred as karyokinesis or mitosis followed by division of cytoplasm or cytokinesis. Net result of this is the formation of two daughter cells each having one nucleus. In plants, cytokinesis is by cell plate formation.

Fig. 16.2 Feulgen stained mitotic chromosomes and karyotype diagrams of three species of *Krigia*. **a** Mitotic metaphase chromosomes of *K. cespitosa* (2*n*=8). *Arrows* show satellite chromosomes. **b** Mitotic metaphase chromosomes of *K. biflora* (2*n*=10). *Arrows* show satellite chromosomes. **c** Mitotic metaphase chromosomes of *K. montana* (2*n*=20). **d**, **e**, **f** Idiograms of haploid sets of karyotype chromosomes of *K. cespitosa, K. biflora* and *K. montana* respectively Modified from Chinnappa, 1981 Cytological studies in *Krigia* (Asteraceae), Can. J. Genet. Cytol. 23. 671–678. [63]
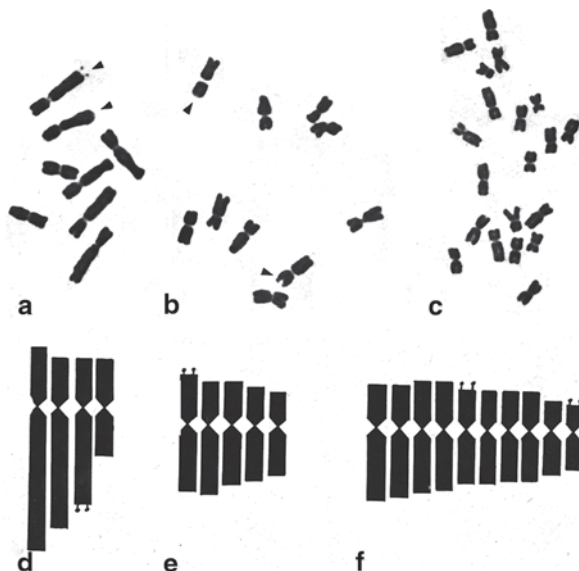
16.3.3.1   Stages in Mitosis

Somatic cell division or mitosis consists of two major phases: karyokinesis or nuclear division or mitosis and cytokinesis. Six stages have been recognized in the mitosis and they are interphase, prophase, metaphase (chromosome morphology for karyotype analysis is normally studied at this stage using various staining procedures), anaphase, telophase and cytokinesis.

16.3.4   Protocols for Root Tip Squash Preparation

Main aim of somatic chromosome preparation is to determine the chromosome number and study chromosome morphology. For this purpose large number of stains have been used to device the staining methods. Among these, most widely used schedules for chromosome preparations are given here. To get good, consistent, high-quality reproducible chromosome preparations, one must use stains certified by the Biological Stains Commission.

16.3.4.1   Acetocarmine Staining Schedule

Material

Collect fresh root tips or leaf tips or any other meristematic tissue. Pretreat them with appropriate pretreating agent.
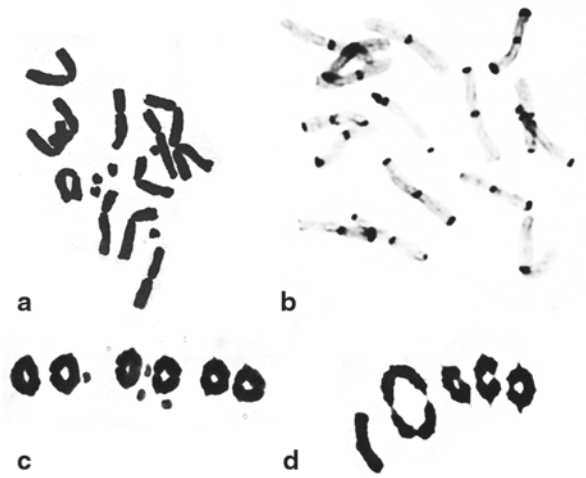
Fig. 16.3 Mitotic and meiotic chromosomes. **a** Mitotic metaphase chromosomes of *Tradescantia hirsuticaulis* 2*n*=12 and B chromosomes. **b** Geimsa C-banding chromosomes of *Allium schoenoprassum* 2*n*=16. **c** Meiotic metaphase chromosomes of *Tradescantia hirsuticaulis*. Showing 6 ring bivalents and 4 B chromosomes. **d** Meiotic metaphase chromosomes of *Tradescantia hirsuticaulis*, showing 1 rod bivalent, 1 quadivalent and 3 ring bivalents. Fig. 16.3**a**, **c**, **d** Modified from [64] Structural hybridity and supernumerary chromosomes in a diploid *Tradescantia hirsuticaulis* Can. J. Bot. 53: 456–465. Fig. 16.3b. Modified from [45] Giemsa C-banded kazyotypes of seven North American species of *Allium*. Amer. J. Bot. 74: 1087–1092.

Fixation

Wash and fix the material in Carnoy's fixative for 12–24 h and store at −20 °C or transfer it to 70 % alcohol.

Staining Procedures:

1. Take two root tips in a watch glass. Add several drops of 1 N HCl and leave them for few minutes. Root tips are now soft and supple.
2. Transfer one root tip on a slide with forceps and put a drop of 1 % acetocarmine over it. Examine under a dissecting microscope, identify the conical part of a root apex with attached root cap and the zone of elongation at the opposite end. Using a lancet needle, cut and remove root cap and zone of elongation. One must remove the root cap as these cells are tough and prevent uniform spreading of dividing cells. Only 1–2 mm of meristematic tissue is left in carmine solution on the slide.
3. Add a drop of carmine and warm over a gentle spirit lamp flame. Using an iron needle macerate the tissue. Carmine will now have bluish tinge. With a needle evenly spread the tissue in carmine solution (*see Note 19*).

4. Now keep the slide on a flat surface having thick blotters. Pick up a coverslip with forceps and gently place it on the carmine tissue preparation. Gently warm to remove any air bubbles. Do not boil.
5. Place a folded blotting paper over the coverslip on the slide and press hard and firmly on the coverslip with your thumb. This squash process flattens the cells and scatters the condensed chromosomes in the cell. Often the squashing breaks the thin walls of meristematic cells and chromosomes are scattered evenly without any overlap. While squashing, care must be taken not to cause lateral movement of coverslip as this will destroy the well-spread chromosome preparation (Fig. 16.3a).
6. Seal the edges of cover glass with rubber solution or gum mastic.
7. Observe the preparation under microscope. The chromosomes would appear dark translucent red and cytoplasm faintly red.

16.3.4.2   Feulgen Staining Schedule

Feulgen staining has many advantages. In this staining method only chromosomes and nuclei are stained. With this schedule several samples can be processed simultaneously. Stained tissue can be stored. Only disadvantage is that it is long and a little complicated process. Slides can be made permanent using Conger and Fairchild's dry ice method [30] or Celarier's t-butyl alcohol series [31] (*see* Sect. 16.6).

Material and Fixation Protocol

As in subheading "Material" in Sect. 16.3.4.1.

Staining Procedure

1. Transfer root tips from 70 % alcohol to water. Give two washes in water for 2–3 min each.
2. Dip the root apices in a vial containing 1 N HCl. Keep the vial in a water bath at 60 °C and hydrolyze the tissue for 8–15 min. The correct time of hydrolysis at which optimum staining occurs must be empirically determined.
3. Remove the vial from the water bath and drain off the hot 1 N HCl and replace with fresh 1N HCl maintained at room temperature. Allow it to stand for 2–3 min.
4. Replace the 1 N HCl in the vial by water. Give two rinses of water for 2–3 min each.
5. Gently transfer the material into vial containing the Feulgen stain. Care should be taken to blot out any carry over water by blotting paper. Keep the vial in dark for 1–1½ h for staining (*see Note 20*).
6. Take a stained root tip on a slide and place a drop of 45 % acetic acid. With a lancet needle head, cut and remove the root cap and zone of elongating cells to

expose meristamatic tissue. On the 1–2 mm meristematic tissue, place the cover glass and warm. Squash the cells as in Sect. 16.3.4.1.

7. Seal the edges of coverslip with rubber solution or gum mastic. Observe under microscope (Fig. 16.2a, b, c). Chromosomes and nuclei would appear magenta red and cytoplasm colourless.

16.3.4.3   Acetoorcein Staining Schedule

Acetoorcein schedule is simple and the most widely used method for somatic chromosome preparation. This procedure does not require iron or any mordant. Slides can be made permanent (*see* Sect. 16.6).

Material and fixation protocol

As in subheading "Material" in Sect. 16.3.4.1.

Staining Method According to La Cour [15]

1. Take root tips in vial containing 1N HCl and hydrolyze for few minutes at 60 °C in a water bath. Time of hydrolysis should be empirically determined as it has bearing on separation of cells and staining of chromosomes.

2. Transfer the root tips to a watch glass and put 9–10 drops of 1% acetoorcein solution. Heat over a spirit lamp until fumes start appearing. Heat for 2–3 min. Do not boil. Allow it to cool.

3. Take a clean slide with a drop of 1% acetoorcein and put a stained root tip. Examine under dissecting microscope to locate conical root apex. With a lancet needle remove the root cap and the zone of elongation.

4. Macerate the 1–2 mm meristematic tissue with a needle and apply a clean coverslip. Warm and allow it to cool.

5. Squash the cells and seal the edges of coverslip as in Sect. 16.3.4.1.

16.3.4.4   Giemsa C-banding Schedule

Vosa and Marchi [32] developed this banding procedure. It has undergone several modifications. Improved techniques enable the recognition of individual bands in the chromosomes of plant genome using light microscopy.

Material

As in subheading "Material" in Sect. 16.3.4.1.

Fixation

Fix root tips in 45 % acetic acid for few minutes at room temperature or for 20 min at 60 °C [33]. Enzymes like pectinase, cellulase, etc. can be used for softening purpose [6].

Processing and Staining Procedures

1. Squash the root apices on a clean slide in 45 % acetic acid.
2. Remove the cover glass by dry ice method [30]. After freezing the coverslip is prised off with a blade.
3. We describe here the standard protocol of Giraldez et al. [34]. Variants from their method are indicated at each step. For dehydration, slides are dipped in 95–100 % ethanol overnight. Most workers keep the slides in above alcohol for 1–2 h for good bands in rye, barley, maize and other plants [35].
4. Air dry the slide for few minutes at room temperature. Some investigators air dry the slides overnight [36] to a few weeks [37].
5. Incubate the slides for 2–5 min in 0.2N HCl at 60 °C in water bath. The time and temperature are important for resolution of good bands.
6. For the Barium hydroxide/Saline/Giemsa stain (BSG) procedure, wash the slides briefly in dH$_2$O.
7. The slides are denatured by incubating the slides in Coplin jar in freshly prepared saturated solution of Ba(OH)$_2$ for 7 min at room temperature or at 50–55 °C in a water bath [34]. Wash carefully in distilled water and ensure all Ba(OH)$_2$ has been removed. Alkali treatment denatures the DNA helix and results into ssDNA. Ba(OH)$_2$ must be prepared fresh for each denaturation reaction.
8. Renaturation of slides is carried out by transferring the slides to a Coplin jar with 2x SSC and incubate it at 60 °C in water bath for 1 h.
9. Remove the slide from SSC and directly place them in 3 % Giemsa stain in Sorenson phosphate buffer pH 7.0 (*see Note 21*).
10. Monitor the staining under microscope. Increase the stain concentration if required. Staining time of 10–45 min would give good results. About 30 min would be optional. In case stain is too blue, increase treatment time of HCl or decrease the time of Ba(OH)$_2$ treatment. In case of deep red staining of cytoplasm, the HCl treatment time is reduced. Giemsa stain should be from good source with stain commission certification for chromosomal staining purpose.
11. Dip the slides in distilled water and air-dry them.
12. Keep the slides in xylene overnight and mount in Euparal or Canada balsam (Fig. 16.3b). Slides can be kept for years at room temperature without any loss of contrast.

16.4   Methods for Karyotype Analysis

The number of chromosomes found in the somatic cells is termed as somatic chromosome number and is referred as $2n$. Whereas, the number of chromosomes present in gametic cells is half of the somatic number and is termed as gametic chromosome number or n. Every eukaryotic species is normally characterized by a definite number of chromosomes in their cells and it is a species-specific trait.

Levitsky [14] proposed the concept of karyotype. He defined the karyotype as phenotypic appearance of somatic metaphase chromosome in contrast to their genic content. In a simpler term, karyotype is the number and appearance of chromosomes at somatic metaphase stage. Diagrammatic representation of a karyotype is referred to as ideogram or karyogram (Fig. 16.2d, e, f).

Six features of karyotype are recognized and compared [37]. They are differences in (i) absolute size of chromosomes, (ii) position of centromere, (iii) relative size of chromosome, (iv) basic chromosome number, (v) number and position of satellite, and (vi) degree and distribution of heterochromatin. Analysis of karyotype helps us in identifying each chromosome pair in the chromosome complement. This has several theoretical and practical applications. Karyotype and its significance in genetics, evolution and systematics has been discussed by eminent geneticists [37–42].

16.4.1   Chromosome Morphology and Classification

Position of centromere along the length of chromosome is the most important criterion for identification and classification of chromosome in the karyotype. Several systems of classification and nomenclature have been proposed [13, 41, 43, 44]. Levan et al.'s [13] system of nomenclature and classification of chromosomes is widely used in karyotype analysis. This system of classification is given in Table 16.1. Position of centromere is correlated to the resulting type of chromosome. Arm ratio (R) that is long arm/short arm is an important parameter in determining the position of centromere on the chromosome. In a karyotype, chromosomes can be identified as metacentric (M or m), submetacentric (Sm), subtelocentric (St), acrocentric (T) and telocentric depending upon the arm ratio (Table 16.1, Fig. 16.1).

Satellite is a spherical body separated from the main body of chromosome by a secondary constriction. It is connected to the main body of chromosome by thin

Table 16.1 Classification of chromosomes. (Based on data from [13])

Chromosome type	R	Symbol	Position of centromere
Metacentric	1.0	M	Median point
Metacentric	1.0–1.7	M	Median
Submetacentric	1.7–3.0	Sm	Submedian
Subtelocentric	3.0–7.0	St	Subterminal
Acrocentric	7.0	T	Terminal
Telocentric	∞	T	Terminal point

chromatin fibre. Nucleolus is formed at secondary constriction [41]. Each chromosome complement or karyotype contains two or more satellites. Secondary constriction is constant in position on a chromosome. This feature is used in identifying particular chromosome. A chromosome possessing satellite is termed as satellite chromosome or SAT-chromosome.

Battaglia [41] classified the satellite based on position and size. Based on position, terminal and intercalary satellites are recognized. In the former, satellite is on the extreme end of secondary constriction and in the latter, the satellite is between two nuclear constrictions. Satellites have been further classified based on size: (a) microsatellite—small spherical satellite, that is having diameter equal or less than one half of the chromosomal diameter; (b) macrosatellite—large spheroidal satellite, that is having diameter greater than one half of the chromosome diameter; and (c) linear satellite—a satellite having the shape of a long chromosome segment.

16.4.2 Construction of Karyotype and Idiogram

About 15–20 complete metaphase plates with well-spread clear chromosomes from three to eight individuals from each population are analyzed. Obtain photo prints of 10 metaphase plates and select 5 plates for measurements of chromosomes. Classification of chromosome types is based on Levan et al.'s [13] system. Karyogram is prepared by arranging chromosomes in decreasing order.

16.5 Study of Chromosomes at Meiosis

Meiosis is a characteristic feature of sexually reproducing organisms. In animals, meiosis normally takes place in germinal cell leading to formation of haploid gametes. In contrast, in plants, meiosis occurs in sporogenous tissue of anther and ovule to form haploid microspores or megaspores. These divide mitotically to form male and female gametophytes. Two haploid gametes fuse to form a diploid zygote that develops into a diploid sporophyte.

16.5.1 Outline of Meiotic Process

It consists of two cell divisions, meiosis-I and meiosis-II. DNA synthesis or chromosome duplication occurs prior to meiosis-I. In meiosis-I, the chromosome number is halved and sometimes this division is referred to as reduction division. Second meiotic division is typically a mitotic division. The net result of meiosis is from one diploid sporogenous cell four haploid cells are produced. From these, later by mitotic divisions, gametes are formed. Thus meiosis plays a crucial role in reproduction of eukaryotic organisms.

16.5.1.1   Meiotic Division I

The first meiotic division is basically a reduction division. The sporogenous cells contain diploid or 2*n* chromosome number. These chromosomes pair during prophase of this division. The paired chromosomes are known as homologous chromosomes and they are normally identical in size, shape and gene sequence. This pairing of homologous chromosomes is the basis for orderly process that leads to reduction of chromosome number to half or haploid condition. The reduction of chromosome number takes place in such a way that two haploid cells of Division I receive one chromosome from each chromosome pair.

Stages in Meiotic division I: prophase I—leptotene, zygotene, pachytene, diplotene, diakinesis; metaphase-I; anaphase-I and telophase-I. Exchange of chromosome segments by a process called crossing over is visualized at diplotene, diakinesis and metaphase stages. This results in segregation of genes.

16.5.1.2   Meiotic Division II

Mechanistically, meiotic division II is similar to somatic mitotic process. It follows prophase-II, metaphase-II, anaphase-II and telophase-II as in typical somatic mitosis (prophase, metaphase, anaphase and telophase). Anther pollen mother cells (PMCs) and megaspore mother cells (MMCs) in ovule are both diploids. They undergo meiosis and produce four haploid uninucleate microspores and megaspores. These haploid cells develop into male and female gametes. The result of meiotic division of a single diploid cell leads to formation of four haploid cells. Unlike mitotic products, these four cells are not genetically identical.

16.5.2   Protocol for Meiotic Chromosome Preparation and Staining

Meiotic investigations provide cytogenetic data useful in genetic analysis, plant breeding and evolutionary studies. Generally, all protocols for meiotic chromosome preparations utilize classic anther squash technique using variety of stains. Commonly used stains are carmine, Feulgen and Giemsa. These protocols are described here.

16.5.2.1   Acetocarmine Staining Schedule

This staining schedule is widely used in meiotic chromosome preparation in plants. Its popularity is due to simple staining schedule and intense staining of chromosomes with bright contrast between chromosomes and cytoplasm.

Material and Fixation

Collect inflorescence or young flower buds and fix them in freshly prepared Carnoy's fluid for 12–24 h. Store them at −20 °C or transfer them to 70 % alcohol at 0–4 °C. Volume of fixative should be 10–20 times more than the volume of the material.

Staining Procedure

1. Take out the inflorescence or bud from storage vial and place it on a watch glass containing few drops of 70 % alcohol. Work from young bud to older ones in a graded manner.
2. Place the bud on the slide and dissect out the anthers and discard the remaining floral parts. Keep all the anthers in a watch glass with few drops of 70 % alcohol.
3. Put an anther on the slide in a drop of 1 % acetocarmine. Crush it with a lancet needle head. Warm over a spirit lamp. Check the presence of PMC with meiotic stages under a microscope. In case PMC do not show the meiotic stage, discard the slide. Repeat the above process with next older bud until you find a bud having anther with meiotic stages.
4. Now take the anther with desired meiotic stage on a clean slide and put a drop of 1 % acetocarmine over it. With a needle, tease and macerate the anther so as to free the PMC from the anther wall. Remove all the debris with a needle and put a cover glass, warm over the spirit lamp (*see Note 22*).
5. Put a folded blotting paper over this slide and press down over the cover slip with your thumb. This pressure will flatten the meiocytes and chromosomes will appear in one plane. Generally, all the meiocytes in a given bud tend to be in the same stage of meiosis. They follow synchronous division. Anthers from different flower buds may show different stages.
6. Examine the slide under compound microscope first under low power and then in high power. Instead of acetocarmine one can use propinocarmine or acetoorcein and follow the above schedule.
7. Slides can be made permanent using Celeriar's method [31] or McClintock's method [42] or Conger and Fairchild's dry ice method [30].

16.5.2.2   Feulgen Staining Schedule

Material and Fixation

Collect inflorescence or flower buds. Fix them in freshly prepared Carnoy's fluid (3:1) for 12–24 h and store them at −20 °C or transfer to 70 % alcohol.

Staining Procedures

1. Take out the inflorescence or flower bud from the storage vial and keep in a watch glass with few drops of 70 % alcohol. Work from the young to mature buds.
2. Steps 2 and 3 as in acetocarmine meiotic staining schedule, *see* subheading "Staining Procedure" in Sect. 16.5.2.1.
3. Collect large number of anthers at different meiotic stages. Give them two washes of dH$_2$O for 2–3 min.
4. Transfer all the anthers to a vial containing 1 N HCl. Keep the vial in a water bath at 60 °C and hydrolyze them for 8–15 min. Correct time of hydrolysis at which optimum staining occurs must be empirically determined.
5. Take out the vial from water bath and drain off the hot 1 N HCl. Pour 1 N HCl maintained at room temperature and allow it to stand for 2–3 min.
6. Replace the 1 N HCl in vial by dH$_2$O. Give two changes of dH$_2$O for 2–3 min each.
7. Transfer all the anthers into vial containing Schiff's reagent or Feulgen stain and put a stopper (*see Note 23*). Keep the vial in dark for 1–1½ h. Stained anthers can be stored. Give a wash in dH$_2$O and transfer the stained anthers to 70 % alcohol at −20 °C. They can be stored up to 3 months.
8. Take a right anther on a clean slide and put a drop of 45 % acetic acid over it. With a needle, tease and macerate the anther so as to free PMC's from anther wall. Remove all the debris with a needle and put a cover glass. Warm over spirit lamp.
9. Squash the preparation as in subheading "Staining Procedure" in Sect. 16.3.4.1. Seal the edges of cover glass with rubber solution or gum mastic. Normally all the PMC in a given bud tend to be in same stage of meiosis. They follow synchronous division. Anther from different flower buds may show different stages.
10. Slides can be made permanent as in Sect. 16.6 (Fig. 16.3c, d).

16.5.2.3   Giemsa C-banding Schedule

Material and Fixation

As in acetocarmine schedule for meiotic chromosome preparation.

Staining Procedures

1. Make an anther squash preparation as in acetocarmine meiotic staining schedule (Steps 1–5). Instead of acetocarmine, use 45 % acetic acid for squash preparation.
2. Remove the cover glass by dry ice method. After freezing the slide prise off the cover glass with a blade.

3. Dip the slides in ethanol for 1–2 h and air-dry them. The procedure followed here is from Cai and Chinnappa [45].
4. Place the air-dried slides in a Coplin jar with 0.2N HCl for 2 min at room temperature or at 60 °C [34].
5. BSG Procedure (Ba(OH)$_2$/saline/Giemsa stain)—denaturation**:** Transfer the slides to freshly prepared saturated solution of Ba(OH)$_2$ for 7 min at room temperature or at 50–55 °C in water bath [34]. Wash all the Ba(OH)$_2$ with dH$_2$O or deionized water.
6. Renaturation: Keep the slides in a Coplin jar with 2x SSC and incubate at 60 °C in a water bath for 1 h.
7. Remove the slides from SSC solution and place them in 3 % Giemsa solution with pH 7.0. Some investigators wash the slides from SSC in distilled water three times and air dry them. Then place them in 3 % Giemsa stain [34]. Staining time of about 10–45 min would give good results. Dip the slides in distilled water and air dry them.
8. Keep the slides in xylene overnight and mount them in Euparal or Canada balsam.

16.6   Permanent Slide Preparation

Permanent preparations of cytological slides are much desired. These are required for later observation and record keeping. We describe here the widely used methods for making the slides permanent.

16.6.1   McClintock's Method [42]

1. Scrape the sealing and dip the slide in a Coplin jar containing 10 % acetic acid. Wait for 10–15 min for cover glass to separate from the slide. In case it does not separate flick off the cover glass with a blade.
2. Transfer the slide and cover glass to a next Coplin jar having a mixture of 1:1 glacial acetic acid and absolute alcohol. Leave them in this fluid for 15 min.
3. Pass through acetic acid—absolute alcohol 3:1 and 1:9 mixture for 5 min at each step.
4. Keep the slides and cover glass in absolute alcohol for 5 min. Give one more change of absolute alcohol.
5. Next give two changes in absolute alcohol for 5 min each.
6. Mount the cover glass on a new slide and use new cover glass to mount on old slide using Euparal (*see Note 24*).

16.6.2   Dry Ice Method (Conger and Fairchild [30])

1. Keep the squashed slides on the flat bed of dry ice for about 2–3 min. Freezing for longer duration is harmless.
2. Pry off the cover glass with a blade while the slide is still on the dry ice bed. Most of the material will be sticking to slide only.
3. Quickly dip the frozen slide into a Coplin jar containing absolute or 95 % alcohol for 5 min.
4. Transfer the slide to a next Coplin jar with absolute alcohol or 95 % alcohol. Allow it to remain in this fluid for 10 min (*see Note 25*).
5. Mount the preparation in Euparal or Canada balsam.

16.6.3   Celarier's Method [31]

1. Keep the squashed or smeared slides in a rectangular horizontal staining jar containing mixture of 1:1 glacial acetic acid and t-butanol. Cover glass will loosen after 5–10 min and with a needle flick off the cover glass. Allow them in this fluid for another 10–20 min.
2. Pick up the slide and cover glass with forceps and blot the fluid on a blotting paper. Place them in the next staining jar having mixture of 1 part glacial acetic acid and 3 parts butanol. Retain them in this dehydration grade for 15–30 min.
3. Transfer the slide and cover glass after blotting away the excess dehydration fluid to a next staining jar containing pure butanol. Leave them for 10 min.
4. Remove the slide from butanol and mount in a drop of Euparal. Mount the cover glass in a drop of Euparal on a new slide.

16.7   Notes

1. Colchicine ($C_{22}H_{25}NO_6$, MW-399.4) is an alkaloid obtained from autumn crocus *Colchicum autumnale* used in the treatment of gout. Colchicine is a pale yellow crystal or powder soluble in water. Low concentration from 0.05 to 0.5 % in water for 1–3 h at room temperature brings about the shortening of chromosome arms and constriction regions become highly conspicuous. Higher concentrations of this chemical induces polyploidy. Roots should be washed properly after pretreatment and fixed in a fixative. This pretreatment appears to allow the easy penetration of fixative at later stages. Handling and disposal of colchicine should be done with great care as it is a toxic compound. Washing of root tips after pretreatment should not be prolonged as the dividing cells may enter into interphase.
2. 8-hydroxyquinoline (C_9H_7NO, MW-145.6) is a light-yellow crystalline solid organic compound and sparingly soluble in water with melting point of 72–74 ºC.

It is a most popular pretreating agent for medium to long chromosomes. Excised root tips are pretreated with 0.002 M 8-hydroxyquinoline at 12–16 ºC for 3–5 h results into same shortening of chromosomes as after colchicine [46]. Pretreatment is followed by washing and fixation. Pretreatment above 18 ºC causes clumping of chromosomes. Unlike colchicine, hydroxyquinoline allows the metaphase chromosomes to maintain their relative arrangement at equatorial plane.

3. p-dichlorobenzene ($C_6H_4Cl_2$, MW-147) is a chlorinated aromatic hydrocarbon compound used as a fumigant, insecticide and repellent. The compound is a white solid and sublimes into gas at room temperature with poor solubility in water. Plants with small chromosomes are pretreated with this chemical agent. A saturated aqueous solution is used to pretreat the somatic tissue for 3–5 h at 12–16 ºC or at room temperature [47].

4. Monobromonaphthalene ($C_{10}H_7Br$, MW-207.07) is a clear-yellow liquid with high density (1.4 g/mL at 20 ºC) and it is sparingly soluble in water but miscible in alcohol, ether and benzene. Somatic tissue is pretreated with saturated solution of this chemical for 2–4 h at room temperature [38]. This pretreatment has been found to be effective for *Lolium* spp., tomato, egg-plant, pepper, wheat and barley chromosomes [48].

5. Caramine is a most frequently used dye to stain chromosomes in plants, animal and human tissue. Carmine is obtained from female cochineal insects *Coccus cacti* growing on cactus plants *Opuntia coccinellifera* in South America. Large quantity of carmine is produced in South America for food, cosmetic, drug, art and textile industries. Only a fraction of it has usage in science. Cochineal is a crude dried material containing dead dry female insects and plant remains. Carmine of commerce is obtained from this by precipitation with aluminium or other metal ions. Dye available in market is inconsistent in quality. It is recommended to use carmine certified by stain commission. Recently, Dapson [49] has reviewed various aspects of this dye. Dye is a mixture of carminic acid and carmine. Carminic acid has anthroquinone nucleus having methyl, carboxylic acid and hydroxyl groups with attached sugar moiety [50]. It could be in free acid form or salt of sodium and potassium. In contrast, carmine appears to be made up of two molecules of carminic acid co-ordinately bound to single atom of aluminium at carboxyl-hydroxyl pairs [51]. Some dye samples probably contain amino-carmine. As occasional extraction of dye from cochineal by ammonium hydroxide produce aminated carmine, it is used in food industry. Dapson [49] proposed that in acetocarmine staining, aluminium ions of carmine are replaced by ferric ions and carmine forms a tightly bound coordination complex with DNA. Carmine has no potential for covalent bonding to tissue or DNA. Chromosome appears red with bluish tinge. Low pH of acetocarmine solution prevents the staining of cytoplasm. Carmine is soluble in water and alcohol. It is the most popular dye for staining meiotic chromosomes and its use in staining somatic chromosomes is less prevalent in plants.

6. Orcein is a deep purple dye obtained from two species of lichens *Rocella tinctoria* and *Lacanora parella*. Lichen extract, which contains colourless parent substance orcinol, is treated with ammonia in the presence of air resulting in the

formation of orcein dye. Orcein is a variable mixture of 14 different compounds and are phenoxazone derivates. Among these eight compounds α, β, γ amino orcein, α, β, γ hydroxy orcein and β amino orceimine, γ amino-orceimine are the major components constituting 98–99% of orcein dye. Remaining 1–2% are secondary components [52]. Orcein is soluble in acetic acid, ethanol, acetone and aqueous alkali and not soluble in water, benzene, chloroform and ether. One or two per cent of orcein in 45% acetic acid is used for staining chromosomes of plants and animals. Orcein is available in synthetic form but natural orcein appears to give better results. Mechanism of orcein staining is not clearly known, as the orcein itself is a mixture of several related compounds. The stain may interact at acid pH with negative-charged group or possibly interact hydrophobically with chromatin [53]. This is a widely used stain for somatic chromosomes and infrequently used these days for staining meiotic chromosomes in plants.

7. Feulgen and Rossenbeck [54] for the first time demonstrated that DNA can be localized in the cell using Schiff's reaction for aldehyde. With Schiff's reagent, only the chromosomes are stained magenta red and rest of the part of cell appears colourless. Basic fuchsin used in cytology is a variable mixture of triphenyl methane analogoue. Pararosaniline (= magenta = 0), rosaniline (Magenta I), Magenta-II (Basic fuchsin) and new Magenta-III (new fuchsin). Rosaniline, magenta-II and magenta-III have 1,2,3 methyl groups. Pararosaniline is unmethylated [55]. It is easily soluble in water and alcohol. Preparation of Feulgen reagent involves dissolution of basic fuchsin dye in water. Addition of HCl and sodium metabisulphite to above solution releases sulphur dioxide in the reaction medium and it reacts with water to produce sulphurous acid. This reacts with basic fuchsin to yield colourless fuchsin sulphurous acid or Leuco-basic fuchsin or Schiff's reagent. When the tissue is hydrolyzed with 1 N HCl, the purine containing fraction of DNA in chromosome is separated from sugar and aldehyde group is left free on sugar moiety. Fuchsin sulphurous acid of Feulgen reagent reacts with free aldehyde group of DNA to give a magenta-red colour [28]. Using this staining method, one can quantify the DNA amount in a nucleus using microdensitometer.

8. Giemsa dye is a mixture of methylene blue and its oxidation products Azures especially Azure B and eosin Y. It is generally prepared by dissolving the Giemsa powder in glycerine and methanol. Dye is also available as premade stock solution from various companies. Required percentage of stain solution is prepared from this by dilution with buffer. This dye stains the chromatin red and cytoplasm blue. Giemsa is not a general purpose stain for chromosomes. It is used in producing unique C-banding pattern in plants and animal including human chromosomes. This stain is also highly acclaimed in revealing characteristic G-bands in animal chromosomes. In plants G-bands appear to be of doubtful origin and are not popular [56, 57].

9. Chloroform is a sweet smelling colourless liquid and is sparingly soluble in water. It is miscible in all proportions with alcohol and acetic acid and acetone. Chloroform is a good solvent for lipids and this characteristic is useful in formulating fixatives. It makes tissue highly brittle and is seldom used in smear preparations.

10. Ethanol is a colourless fluid and soluble in water in all proportions. Ethanol is a component of most fixatives. This alcohol has great penetration power and dehydrating property. This causes tissue to shrink, harden and makes it brittle. Alcohol replaces the water molecules in the tissue. Alcohol denatures proteins and precipitates nucleic acids [58]. Ethanol as a single fluid is not used as a fixative. Ethanol is available as 95 % or absolute alcohol (100 %). Denatured spirit refers ethanol to which methanol is added to prevent its use for drinking.

11. Propionic acid is a colourless liquid with acrid odour. Propionic acid is miscible in all proportions with water, alcohol and many other organic solvents. It is used sometimes as a substitute for acetic acid. This causes less swelling of cells than the acetic acid and also a good solvent for aniline dyes.

12. Glacial acetic acid is a colourless liquid having pungent odour and miscible with water and alcohol in all proportions. It does not denature protein and has greater penetrating power than alcohol [59]. The most striking feature of acetic acid is its swelling effect on cellular structure and tissues fixed in it are soft unlike fixed in alcohol. When combined with alcohol it offsets the shrinkage caused by alcohol [58]. This is a good solvent for aniline dyes and one can easily prepare aceto-carmine or acetoorcein.

13. Canada balsam is collected as bark exudates from balsam fir tree *Abies balsamea* which naturally grows in North America. Canada balsam is a thick lightly yellow, transparent liquid and composed of resins solubilised in the essential oil forming oleoresin. On evaporation of essential oil hard resin is left which is soluble in xylene, but not in alcohol. Canada balsam is an ideal mountant and its refractive index is same as that of a glass. Main drawback of this mountant is it dries slowly and basic dyes fade due to its acidic nature during long storage [40]. It is desirable to keep a marble piece in the Canada balsam bottle [59]. It is useful to mount in Canada balsam from after xylene not after dehydration from alcohol series. Neutral balsams are also available [40, 58, 59].

14. Euparal is a mounting medium widely used in cytological studies. It is a synthetic resin with refractive index of 1.483 and is soluble in xylene, butanol and alcohol. Euparal is a mixture of eucalyptol, sandarac (a resin from the tree *Tetraclinin articulata* grown in North-West Africa), paraldehyde and camsal (camphor and phenyl salicytate). Slides can be directly mounted in euparal from 95 % alcohol [60].

15. DPX is a neutral, colourless mounting medium with refractive index of 1.522 and most stains are well preserved. It is a mixture of plastic, polysterene dissolved in xylene [61, 62]. The disadvantage of this mountant is it sets quickly and retracts from the edge of coverslip. This has been overcome by adding a plasticizer [58].

16. Belling [24] originally recommended the addition of trace amount of ferric hydrate dissolved in 45 % of acetic acid until the acetocarmine solution turns bluish red and avoids precipitation. Recent modification is adding few drops of ferric chloride or ferric acetate in 45 % acetic acid.

17. Carnoy's Fixative I is prepared fresh whenever required. Root apices, flower bud, leaf tips and animal tissue are fixed in this fixative from 30 min to 24 h at room temperature. Finally they are stored in the same fluid at $-20\,°C$ until use. In case deep freezer is not available, transfer the material to 70 % alcohol and leave it to $0–4\,°C$ or room temperature for 1–2 months. Leaving the material in 70 % alcohol longer than 2 months would lead to over staining of cytoplasm and contrast would be lost.

18. Carnoy's Fixative II is popular with animal cytologists and fixing of flower buds. Duration of fixation and other conditions are similar to Carnoy's I fixative. This fixative makes the plant tissue more brittle [28, 58].

19. The iron in the needle will react with carmine solution and iron acts as a mordant. This helps in deep staining of chromosomes. In case iron is already added in the carmine there is no need to use iron needle.

20. If long term storage is required, wash the Feulgen stained root apices in water and transfer them to 70 % alcohol. The material can be stored up to 3 months.

21. Some investigators wash the slides form SSC in distilled water three times, air dry them, and finally place them in Giemsa stain solution [6].

22. In case the anther is large, then cut one end of it with a sharp blade. Squeeze the anther from the other end to facilitate the release of PMC into a pool of acetocarmine stain. Discard the anther wall.

23. In case anthers are very small, then whole bud can be hydrolyzed and stained in Feulgen reagent. Dissect out the stained anthers from flower bud and check for meiotic stages. Squash anthers having meiotic stages.

24. One must keep track of which side of cover glass and slide contains the tissue. One disadvantage of this method is lot of tissue is lost during transfer through dehydration grades.

25. At final stage it is possible to dehydrate the tissue with two changes of xylene for 5 min each instead of alcohol and mount in Canada balsam. This method is widely used due to its simple process.

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