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## Some Observations on Plant Karyology and Investigation Methods

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### 1. Introduction

Karyology deals with the structure of cell nuclei, especially chromosomes. Cytology dealing with the study of cells in terms of structure also, function is known nowadays to mean only the study of chromosomes or nucleus and made synonymous with karyology wrongly. Cytotaxonomy means the application of cytological data to taxonomy. Cytotaxonomy studies the morphological and cytological characteristics of the organism along with their chromosome numbers and structures (karyotype)[1, 2]. It is a secondary discipline that reinforces the principles of plant and animal taxonomy by abiding to the phylogenetic kinships. In classical taxonomy, the plants are categorized through determining their natural kinships in accordance with their morphological characteristics. Especially, the taxonomists are advisable in connection with chromosomes. So, often chromosome number is assumed to be the all important, if not the only, chromosome character of interest to taxonomists, but size, shape, and behavior of chromosomes may throw more light on a taxonomic problem than their number alone.

F. Ehrendorfer, in an erudite essay on Cytologie, Taxonomie und Evolution bei Samenpflanzen [Cytology, taxonomy and evolution in seed plants], gives a detailed outline of these developments in cytogenetics since 1900 that have had a bearing on problems of taxonomy. Examples of taxonomic corrections based on chromosome studies include the removal of *Yucca* and *Agave* from the Amaryllidaceae to the Agavaceae and a number of rearrangements of species and genera in the Gramineae, Liliaceae, Compositae and other families; warnings are sounded against an uncritical use of chromosome pairing as a criterion of affinity, secondary pairing being dismissed altogether. A concrete example of the use of morphological and cytological considerations in deciding questions of relationships and evolution is given for the Dipsacaceae, where two distinct lines of phylogenetic development are traced: one of bushy species with clear polyploid series and the other of

annuals with diploid series and a strong tendency towards structural chromosome differentiation [1].

In addition, findings about the status of the karyotypes, the chromosome numbers, the chromosome structures, sizes and enlightening data about the controversial situations of the members of the genus such as *Pandanus*, *Typha*, *Sparganium*, *Funcia*, *Polyantes* [2]. As it can be seen, karyological studies were helpful in classification considering the cytological characteristics. Today, palinological and micromorphological characteristics detected with a scanning electron microscope (SEM) and the DNA sequence analysis are helpful in classification along with the karyological studies. However, in plant taxonomy, the emergence and geographical diffusion of cytotaxonomic new karyotypes continues to be an important problem. The study of karyotypes is important for cell biology and genetics, and the results may be used in evolutionary biology and medicine [2, 3]. Karyotypes can be used for many purposes; such as to study chromosomal aberrations, cellular function, taxonomic relationships, and to gather information about past evolutionary events.

In recent years numerous plants have been published in the field of cytotaxonomy which have been concerned with the cytological aspects of many species. For example, the morphological, anatomical and molecular biological studies and the cytological examinations carried out have caused modifications to be made on the classification of algae [4].

There are two types of cell division for high plants; mitosis and meiosis. Mitosis is the type of division required for the plants to grow, develop and for the plant parts to be ingenerated. Mitosis is observed at the cambium, root tips and the somatic cells of other growth points. Mitosis allows for the genetic content of a cell to be transferred to the new generations without being distorted. Because, mitosis process of the haploid and diploid cells takes place after the chromosome duplication process.

Plant cells contain 3 genomes; in the nucleus, in the mitochondria and in plastids. During mitosis, the main cell doubles the chromosomes and its organelles such as mitochondria and chloroplast which proceed from the prokaryotic cells and membranes. Prokaryotes are multiplied with the method of binary fission. Mitochondria and chloroplast are organelles which are multiplied by division, as their ancestors. First the DNAs of these organelles are doubled. The small chromosomes produced, hold on to the inner membrane of the organelles. As the organelles grow in length, these two chromosomes move away from each other. At this stage, the membrane collapses inwards reciprocally at one point and it becomes narrower. As a result, two small organelles are produced.

In Eukaryotic cells, the cell cycle is consisted of the constant repetition of consecutive processes. The cell cycle includes the time period between the beginning of one cell division and the beginning of the following cell division. The cell division is divided into two stages; one long interphase and a short division stage. The division stage, or the M stage, includes the nucleus division (mitosis) and the cytokinesis (the division of the cytoplasm). The interphase takes place before the mitosis and cytokinesis. Interphase is the stage where the cell members are synthesized and the active growth occurs. Replication (DNA replication) and the duplication of chromosomes (doubling) occur in the interphase stage. The interphase is consisted of G1, S

and G2 stages. G is the abbreviation for the word 'gap' and S is the abbreviation for the word 'synthesis'. G1 is the stage where the molecules and the intracellular elements are intensively synthesized. In the S stage, replication of DNA takes place. G2 stage is where the required preparations for cell division are completed [3].

As the prophase progresses, the length of the chromosomes are contracted. As the contraction increases, it can be observed that the chromosomes are consisted of two chromatids and that they are connected to each other at the centromere. Although in some sources, centromere and kinetochore are used as synonyms, they have differences. Centromere has a special DNA sequence which is present in every chromosome and which connects the chromosome to mitotic fibers. Kinetochore, on the other hand, is a special protein complex formed by each chromatid in the centromere. The mitotic fibers consisted of microtubules emerge at the end of the prophase stage. The nucleus and the nuclear membrane are dissolved at the end of the prophase [3, 5].

During metaphase, the chromosomes are lined through the equator of the mitotic fibers. The kinetochores are connected at the equator level to the ends of the cell which is thought to be the + end of the microtubules. The - ends of the microtubules are on the polar side. When a kinetochore is connected to the microtubules, the chromosome begins to move towards the polar side to which the microtubule is extended. As all the chromosomes are aligned reciprocally at the equator level, the anaphase ends. The sister chromatids are separated from each other at the anaphase stage [3].

The nucleuses of the plant cells which do not divide are generally close to the cell walls. The nucleus takes place at the center of the cell before the division. Cytokinesis begins in the telophase stage. Phragmosome is produced firstly in the section where the cell is to be divided into two. Later, after the division of nucleus, the cell wall and fragmoplast allows for the cytoplasm to be portioned in two small cells. During the telophase, fragmoplast and the cell wall are visible.

As it is seen, if the division of the full-length of the chromosomes in mitosis did not occur equally, the new cells coming into existence as a result of the cell division would be very different from each other. If the number of chromosomes was not divided equally to both cells, large, immoderate and excessive cells would originate. In this case, the cells originated would not be able to perform fully.

Sometimes the chromosome number can be doubled without cell or nucleus divisions. The increase in the chromosome numbers of antipode nucleuses provides a good example for this situation [6, 7]. If the chromatids produced with endomitosis are separated from each other to form independent chromosomes, this is called endopolyploidy.

As it is in other organisms, the number and the morphology of chromosomes vary in plants. The number of chromosomes in a plant does not provide information with regard to its development level. For example, *Ophioglossum petiolatum*, which belongs to Pteridophyta, has  $2n=2000$  chromosomes, whereas *Allium cepa* (onion), which is a monocotyle has  $2n=16$  chromosomes. Also, one of the two plants which have equal chromosomes may be more developed than the other. For example, the number of chromosomes of *Acetabularia mediterranea*, which

is a green algae is equal to the number of chromosomes *Zea mays* (corn) has  $2n=20$ . As it can be seen, the important point is the data included in the chromosomes.

Some studies have shown that the changes in the number of chromosomes of the same species affect the flower sizes [8]. Also the idea that the chromosome number variety observed in the same species is related to the changes in the morphological characteristics of the plant was proposed [9, 10]. In reference [10], the differences seen in the number of chromosomes among the problematic genus such as the *Crocus* which is a monocotyle or among the problematic species such as the *Crocus chrysanthus* can be related to the differences in the morphological characteristics of the plant, such as the color of the anther, tepal or throat of the flower. This also constitutes a good example regarding the reflection of the karyotype differences on the phenotype (Figure 1). In references [10-15], the researcher even claims that these differences are indications of new taxa.



**Figure 1.** Three different anther types (wholly yellow, blackish lobed, blackish lined) of *Crocus chrysanthus* with different chromosome numbers [10]

Each chromosome includes a single DNA molecule in the form of a chain and in the length of thousands of nucleotides. DNA includes one nucleotide chain. Adenine (a), Guanine (g), Thymine (t) and Cytosine (c), which are the bases in the nucleotides which contain nitrogen, only connect to deoxyribose. The spine of the nucleotide chain is held together with the chemical bonds of sugar deoxyribose and phosphate groups. Chromosomes carry the genes coded for the synthesis of genetic proteins. Genes are units of genetic data.

Prokaryotes contain the smallest amount of DNA. Mycoplasma, which is a bacterium, is one of the organisms known that consist the minimal amount of DNA. Eukaryotes, as opposed to prokaryotes, contain greater amounts of DNA. However, approximately 90% of the DNA of eukaryotes do not code any proteins. 20-40% of the DNA in eukaryotes are consisted of unnecessary repetition series and their functions are unknown. Introns, which interrupt the protein coding series are an another example for DNAs which do not carry any coded data. Prokaryotes do not have any introns. Exons are regions which code a certain protein. The data



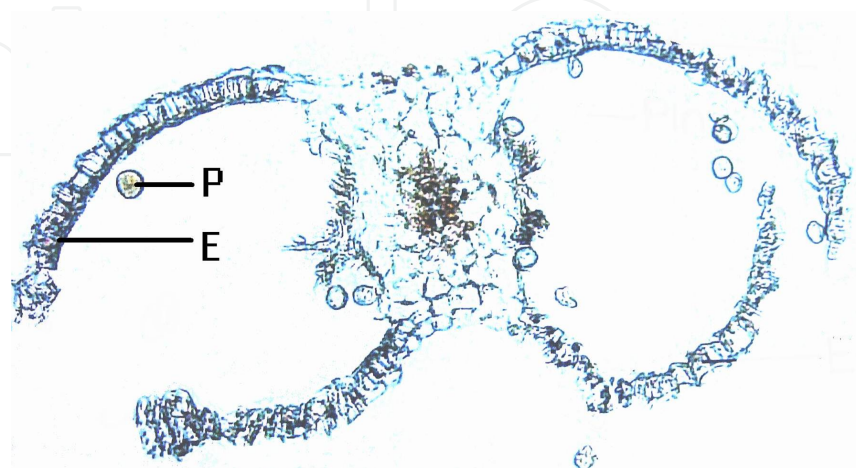
coded in mRNA are transformed into proteins in the ribosome. A protein includes 300-400 amino acids in average. In order to code this amount of amino acids, 1,000 base pairs are required unknown [3]. According to this view, it can be said that the *Zea mays* with 4,500,000 base pairs contain 4,500-5,000 coded proteins.

*Arabidopsisthaliana* species have a small amount of DNA. Its small size, its short reproduction period, the scarcity of the number of the chromosomes it contains, its availability for cross breeding experiments and the high production of seeds are required for an experimental organism and because of these characteristics, this plant is preferred by plant geneticists. The studies conducted have revealed that the DNA of the plant *Arabidopsis thaliana* is similar to many plants used by humans [3].

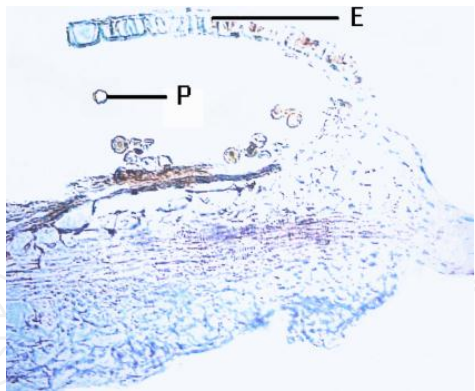
*Trillium* sp., which is a flower that blossoms in spring, is the plant with the greatest genome known. The DNA of this plant contains 100 billion base pairs. The reason this plant needs that many DNA is unknown [3].

Meiosis is the cell division that allows for the male and female gametes to originate in order to ensure the continuity of developed plants. During meiosis, one main cell produces 4 daughter cells with halved chromosome numbers after two consecutive divisions. In meiosis, there are two stages; reduction, which reduces the chromosome number in half and mitosis, which preserved the halved number. If mitosis takes place first and is followed with reduction division, it is called postreduction. If reduction takes place first and is followed with mitosis, it is called prereduction [6, 7].

Cytokinesis is the division of cytoplasm into two young cells after the division of nucleus. In plants, following the production of the young nucleuses, with cytokinesis, 4 cells (gons) are produced [6-8]. The microspore main cell produces 4 microspores. The group formed by these 4 microspores is called a tetrad. Later, these microspores are separated from each other and each form a pollen as they develop. The pollens are produce in the anthers. *Anemone coronaria* var. *coccinea* anthers show this structure clearly (Figure2, 3) [16].

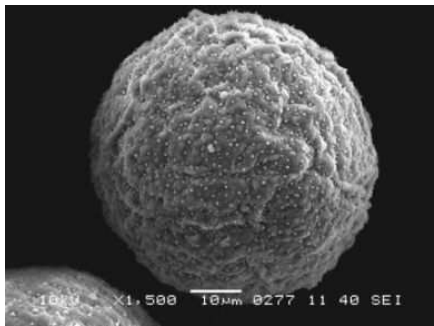


**Figure 2.** Anther cross section of *Anemone coronaria* var. *coccinea* (P:pollen, E:endotesium) [16]

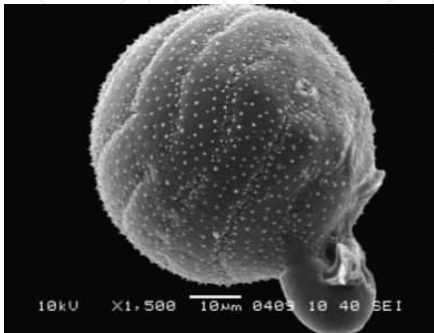


**Figure 3.** Anther transversal section of *Anemone coronaria* var. *coccinea* (P:pollen, E:endotesium) [16].

Proteins exist on the intine and exine layers of the pollen wall (Figure 4). These proteins are especially concentrated around the germination pores and the exine dents. The proteins in the intine have a gametophytic origin, whereas the proteins in the exine have a saprophytic origin. Following the contact of the pollens to the humid surface of the stigma, the proteins in the intine and exine are rapidly released and are diffused on the contact surface [17, 18]. The first significant stage of this interaction between the stigma and pollen is the pollen tube produced as a result of the intine stemming out of the germination pore (Figure 5).



**Figure 4.** *Crocussieheanus* pollen exine layer (SEM photograph) [10]



**Figure 5.** *Crocus ancyrensis* pollen with pollen tube (SEM photograph) [10]

Different fertility values may be observed as a result of the studies carried out on the pollen grains obtained from the stamens of male and female flowers of the same plant. The fertility values obtained from the male flowers are generally higher. If the fertility value of the female flower is also high, the plant will be able to self-fertilize, because the stamens are rich in pollens and that they almost cover the stigma. Thus, the main typical characteristics are often preserved. For example, Turkey constitutes a great variation center for the species, *Cucumis melo*. It is known that especially in the Eastern Anatolia region, gene exchanges are made and as a result of natural cross-breeding of the cultural and wide forms of this species [19].

Tissue culture studies generally use mitosis and meiosis divisions basically. The plant tissue culture, is the production of new tissue, plant or herbal products (such as metabolites) from a complete plant or plant parts such as cells (meristematic cells, suspension or callus cells), tissues (various plant parts=explants) or organs (apical meristem, root, etc.). Explant is the plant parts which can be collected from various sections of the plant and which can be used for culture. Creating new species and causing variability in the existing species can be regarded as the main purposes of tissue culture. For this reason, plant tissue cultures are important with regard to genetic optimization studies. Also various tissue culture methods are used for the preservation of endangered species and the reproduction of the species which are not easily reproduced [20].

The main method used in plant tissue culture processes and genetic optimizations is the regeneration capability of plants. Plant regeneration can be assessed in three parts with regard to the characteristics of the cultured cells: 1) regeneration from the somatic tissue consisting of organized meristematic cells, 2) regeneration from the somatic tissue consisting of non-meristematic cells, 3) regeneration from the gametic cells divided with meiosis. The first kind of regeneration consists of the reproduction of plants from the apical and lateral meristems. This is called clone reproduction with meristem culture method. The cells obtained look exactly like the donor plant. The second kind of regeneration is the formation of an embryo or a complete plant by the constant division of a somatic cell (direct somatic embryogenesis) or the formation of organs and then a complete plant by the division of some of the certain somatic cells on the cut surfaces of a plant explants, generally caused by plant growth regulators (especially auxins and cytokinins) and the organization of these divided cells (direct organogenesis). Also, both situations can occur following a certain callus, proto-callus or cell suspension generation phase (indirect regeneration). Some genetic or temporary variations can occur in the plants produced. Lastly, plants can regenerate directly or indirectly from the cells which include half the number of chromosomes it should normally have. With this method it is possible to reproduce haploid plants which are generally sterile and have half of the chromosomes the donor plant has [20, 21].

Explant should be chosen carefully with regard to tissue culture studies. Younger tissue is more easily dividable and has a higher capacity to form a callus. Cells should be actively dividable and they should not have the tendency to get in a dormancy period [22].

Comparative genomics, the study of the similarities and differences in structure and function of hereditary information across taxa, uses molecular tools to investigate many

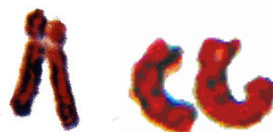


notions that long preceded identification of DNA as the hereditary molecule. Over the past two decades, multiple investigations of many additional taxa have delivered two broad messages: (1) In most plants, the evolution of the small but essential portion of the genome that actually encodes the organism's genes has proceeded relatively slowly; as a result, taxa that have been reproductively isolated for millions of years have retained recognizable intragenic DNA sequences as well as similar arrangements of genes along the chromosomes. (2) A wide range of factors, such as ancient chromosomal or segmental duplications, mobility of DNA sequences, gene deletion, and localized rearrangements, has been superimposed on the relatively slow tempo of chromosomal evolution and causes many deviations from colinearity [23].

## 2. Chromosome morphology

Prokaryotes have one chromosome and haploid genomes. The chromosomes of prokaryotes have an annular structure. The DNA of eukaryotes is consisted of long and linear shaped molecules to form distinctive and different chromosomes, as opposed to the annular chromosomes mentioned.

Every chromosome of eukaryotic cells is found in pairs and they have a diploid ( $2n$ ) structure. Thus, diploid organisms carry two copies of each gene. Every member of the chromosome pairs are the homologue chromosomes of each other (Figure 6). The chromosomes are found metacentrically, submetacentrically, acrocentrically and telocentrically in the cell. Haploid cells ( $n$ ) have one complete set of chromosomes. Chromatin is a mass of uncoiled DNA and associated proteins called histones. A small segment of DNA that contains the information necessary to construct a protein or part of a protein (polypeptide) is called a gene. Genes are the unit of inheritance.



**Figure 6.** Two chromosome pairs of submetacentric homologue chromosomes [10]

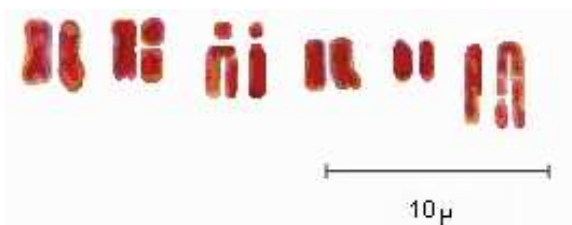
**Karyogram:** Chromosomes are cut or taken from the photos as regards metaphase stages of an individual, in which chromosomes can be observed clearly. Chromosomes which are morphologically, which similar have the similar or the same length are placed in juxtaposition in the horizontal axis. The karyograms allow for the chromosome characteristics of the individual to be in comparison in themselves, and it helps to present the relation between different individuals with regard to different chromosome properties. According to references [9, 10, 24, 25], performed karyograms for various *Crocus* taxa and compared them to each other. In reference [10], samples of the problematic species *Crocus chrysanthus* have been grouped up, which have different morphological structures and examined

them with respect to their cytological, palinological and micromorphological properties during and after her doctorate thesis. Consequently, she compared the karyograms of the samples (Figure 7, 8) she has examined and stated that their cytological properties differ as well as their other characteristics [10]. Then, she suggested a new identification key for 7 new taxa of *Crocus chrysanthus* [11-15].

**Ideogram:** Some cells can be observed in the right metaphase stage; however, since the chromosomes are small, these are not in the size to be made a karyogram with. In these cases, ideograms are prepared with chromosomes. The chromosomes are examined with a microscope and the drawings of chromosomes are made with camera lucida or another photographic computer program. Later, drawings of the chromosomes are made by beginning from the longest chromosome as straight lines which determine the average branch lengths. First the lower branch is drawn, than 1 mm space is left for the centromere and the short branch is drawn on it. Later, other chromosomes are drawn on the same axis by leaving 4-5 mm space in between. Thus the ideogram is prepared.

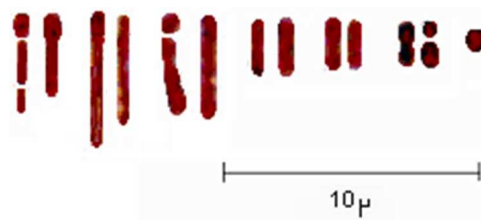


**Figure 7.** Karyogram of the *Crocus chrysanthus* sample which is suggested as a new taxon,  $2n=8$  [10, 15]



**Figure 8.** Karyogram of the *Crocus chrysanthus* sample which is suggested as a new taxon,  $2n=12$  [10, 15]

Sometimes the karyotype analysis are made by measuring the lengths of the chromosomes of the species similar to each other and the letters J and V are used in order to facilitate the comparison. The capital and lower case letters are used to determine the sizes of the chromosomes and each chromosome is classified in two types depending on the position of the centromere. If the centromere of the chromosome is close by 1/3 of the total length, this chromosome is represented with the letter J or j in the karyotype formula. According to this representation, the centromere is called subterminal or terminal. If the centromere of the chromosomes is near to the center or at the center, the chromosome is represented with the letter V or v. The centromere is called metacentric or submetacentric. In V type chromosomes the ratio between the length of the short branch and the total



**Figure 9.** Karyogram of the *Crocus ancyrensis*,  $2n=12+1B$  [10]

chromosome length is more than 33.3%, and in J type chromosomes this ratio is less than 33.3%. On the other hand, if the length of the chromosome is more than half the length of the longest chromosome, it is represented with V or J and if it is less than half the length of the longest chromosome, it is shown with v or j [26, 27].

### 2.1. B Chromosomes

B chromosomes can be observed in plants and animals. The chromosomes which are part of the genome and carry the essential genetic data are known as the 'A' chromosomes. The organism does not need the 'B' chromosomes for survival. For this reason, they are called as 'B' chromosomes. If an organism has many B chromosomes, its development (phenotypic defects), fertilization and its capacity to produce effective seeds (differences in pollen sizes, sterile seeds or seeds which include different genetic data) are negatively affected. B chromosomes are not observed in polyploid plants. It can be observed in monocotyledons, particularly in herbaceous dicotyledons and in primitive plants such as bryophytes. B chromosomes may exist on the individuals of a species. It may also be observed in the pollen mother cells of an individual, even if its root cells do not consist of B chromosomes.

B chromosomes may be observed in monocotyledons and dicotyledons in the populations of the same species, which have been subject to differentiation, and to ensure the adaptation to the environmental conditions. For example, some samples of *Crocus ancyrensis*, which is an endemic species with  $2n=12$  chromosomes [28], were observed to have  $2n=12+1B$  chromosomes (Figure 9). The fact that there were some individuals of *Crocus ancyrensis* species with B chromosomes and that no individuals of the subspecies *Crocus flavus* subsp. *flavus* and *Crocus flavus* subsp. *dissectus* with B chromosomes were observed, has led us to believe that the environmental adaptation capability of *Crocus ancyrensis* is stronger, when compared to the species *Crocus flavus* [10].

B chromosomes were observed in the samples collected from different populations, as seen in the samples of the species *Crocus chrysanthus* with  $2n=20+2B$  chromosomes (Figure 10). In reference [15], the researchers have observed during their field studies and herbarium examinations that these samples, which also have a different morphology and blackish lined anthers (Figure 1), are differentiated within the species and they proposed for them to be accepted as a subspecies.

B chromosomes are not observed in all of the cells of a certain plant. It only can be observed in some of its cells. Smaller numbers of B chromosomes in a plant (1-3, sometimes 4) may be



**Figure 10.** Karyogram of the *Crocus chrysanthus* samples with balckish lined anthers suggested as a new subspecies,  $2n=20+2B$  [10]

accepted as an indicator of a good adaptive capability. For a study conducted, which was related to B chromosomes, the samples of the *Secale cereale* with  $2n=14$ ,  $2n=14+1B$ ,  $2n=14+2B$ ,  $2n=14+3B$  and  $2n=14+4B$  chromosomes were compared with regard to the growth rate of the pollen tube. The results revealed that the pollen percentage and the pollen tube length of the sample with  $2n=14+2B$  chromosomes was higher than the other samples. The second in ranking was the sample with  $2n=14$  chromosomes [29, 30].

The studies performed revealed that when there are more than two B chromosomes, multivalents produces. The B chromosomes in the mother pollen cells sometimes replicate themselves and they are observed in all the cells produced with the division. Sometimes, in meiosis, it is observed that when there is one B chromosome in the cell, this chromosome remains out of the equatorial level. In this case, the B chromosome is an underdeveloped chromosome. It is not observed in the other nucleuses. However, this chromosome may exist as a micronuclei in 80-85% of the mother pollen cells at the end of the meiosis stage [30, 31].

## 2.2. Chromosome enormity

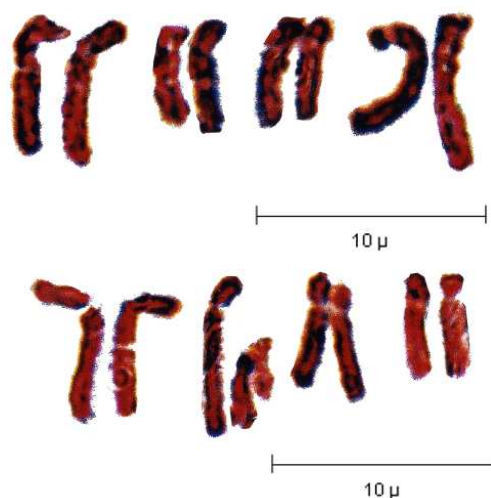
The chromosome enormity is regarded as an indicator of primitiveness. Small chromosomes have been differentiated throughout evolution as a result of chromosome disassociations. If all the chromosomes are metacentric, the karyotype will be symmetrical. The differences in sizes of the chromosomes and the existence of acrocentric chromosomes show that the karyotype is asymmetrical. Excess asymmetry of the karyotype may point out that the species is at a more advanced level with regard to evolution. In other words, between the similar species, the one with metacentric chromosomes is regarded more primitive than the species which include submetacentric chromosomes in majority. Thus, the species with submetacentric chromosomes in majority is a more recent flora element than the other. The chromosome enormity may be regarded as a reliable characteristic in the phylogenetic aspect, if similar species are being compared. During evolution, the karyotype may transform from being asymmetrical when it is symmetrical and chromosomes may have disassociate. With an advanced specialization, even polyploidy may emerge. In similar taxa it can be said that the ones with smaller chromosomes are more specialized than the ones with larger chromosomes [32, 33].

Different environment factors cause ecotypes to emerge. Ecotypes can be examined under 5 categories; climatic ecotypes, edaphic ecotypes, culture ecotypes, physiologic ecotypes and chemotypes [33]. It would be imperfect to state that the chromosome number, size and form of a species will be constant. Because of ecological properties and edaphic factors, the total size

of the chromosomes of the members of the same species grown in different places. For this reason, it is natural for a plant with edaphic ecotypes such as *Lythrum salicaria*, which is grown in clayed, sandy and salty soil, to have different chromosome lengths in different soils.

For example, it was determined that the chromosomes of *Allium cepa* grown in an environment rich in phosphate, are twice the size of the ones grown in environments with less phosphate [30]. In reference [10], the researcher has determined the chromosome sizes on the karyograms as regards the samples collected from two different populations of *Crocus flavus* subsp. *flavus* and has resulted that the chromosome lengths of these two populations are different from each other (Figure 11). It is stated that this difference is caused by the differences in the chemical content of the soil [10, 34].

Enormous chromosomes are most generally found in animal cells. Also, if the chromatids emerging with endomitosis stay stuck to each other, they may form enormous chromosomes which consist of chromatide packs. This is called polyteny.



**Figure 11.** Karyograms of *Crocus flavus* subsp. *flavus* collected from different localities [10]

### 3. Procurement of the material, the first treatment and fixation

Root tips obtained from the plant grown in land or in a pot, root tips from the seed germinated in petri dish, tips of very young leaves, flower primordiums, growth points of the buds of lateral branches, very young petals, glumes of some monocotyle plants can be used for the examination of somatic chromosomes. Root tips are the most commonly used part among all these, when conducting a mitosis examination.

In order for the chromosomes to be examined quantitatively and structurally in the somatic sense in detail, first, the material needs to be pretreated. The pretreatment allows for the chromosomes to remain at the metaphase level, to decrease in length by increasing the number



of spirals and to be observed clearly. The pretreatment solutions alter the viscosity of the plasma in the cell, the chromosomes diverge from each other and this allows for them to be observed separately. In addition to this, all pretreatment solutions insure the coagulation of proteins in organelles of the cell and perform the fixation process at a certain level. The cleanness and the pH and oxygen levels of the pretreatment solution are extremely important. If the root tips are collected from the land or a field, they should be decontaminated of soil particles and dirt before being rested in the pretreatment solution. The most practical method when cleaning root tips is to use water. However, they should be wiped with a blotting paper before being put in the pretreatment solution. If the root tips are placed in the pretreatment solution without being cleaned or with water drops on them, the concentration of the solution may change and it may not perform well. Also, the bottle which holds the pretreatment solution should be left in an airy environment with its lid open at least one day before the treatment, so that it would aerate. There are several pretreatment solutions. It may be necessary to use different kinds of pretreatment solutions for different kinds of plants. Also, the periods of the samples are to be rested in the solutions differ according to each plant. Thus, it would be beneficial to collect many samples and perform many trials.

Generally, the most commonly used pretreatment solutions are iced water, monobromonaphthalene ( $\alpha$ -bromonaphthalene), colchicine, 8-hydroxyquinoline, coumarin, paradichlorobenzene, acenaphthene. Samples should not be rested for a long time in pretreatment solutions such as 8-hydroxyquinoline or  $\alpha$ -bromonaphthalene. Each sample should be tested for the resting duration and the duration which gives good results should be determined. For example, it was observed that if Fabaceae and Poaceae family members are rested in  $\alpha$ -bromonaphthalene, at +4 °C for 16 hours or at room temperature for 1 hour, the results would be satisfactory [30]. It would be sensible to use this solution with plants which have large chromosomes. Also, it works when 0.01% colchicine is applied on the root tips of the plants for 3 hours [33, 35]. Applying 2% colchicine on the young leaf tips is also a good solution. The root tips are rested in 8-hydroxyquinoline at 10-18 °C for 3-6 hours. In references [10, 15, 24, 25], the root tips are rested in 8-hydroxyquinoline at room temperature for 4 hours during the cytological studies conducted on the *Crocus* species and the results were satisfactory. 8-hydroxyquinoline pretreatment can also be applied to the root tips on the plant. It can be prepared with the slide taken from the root tip in 8-hydroxyquinoline. However, it would be more convenient if the slide is prepared after being dyed with aceto-orcein and after maceration is employed. When the root tips are being rested in this liquid, the excess temperature of the room may cause the root tips to get stuck to each other. The coumarin application suggests for the root tips to be rested in solution at 16 °C for 2-3 hours and this application facilitates the examination. The water saturated solution of coumarin (2%) should be used. If the coumarin is used with chloral hydrate, paradichlorobenzene sulphanilamide and bromonaphthalene combinations, better results will be obtained. If paradichlorobenzene will be used for the examination of somatic chromosomes, the root tips are rested in the saturated solution of paradichlorobenzene in pure water (1-2%) for 1-4 hours. For example, if the root tips as regards members of Poaceae and Cyperaceae are heated in the acid solution and dyed with aceto-orcein, after being rested in this solution at 12-16 °C for 3 hours, satisfactory results will be obtained [33]. With the

application of paradichlorobenzene the images of many plants with 120 or more chromosomes were obtained in the metaphase where the chromosome length is reduced. Acenaphthene was used in the karyotype studies of pollen tube chromosomes. This liquid was not regarded as the appropriate solution for the karyotype examinations of root tips [36].

After the pretreatment, the sample should be put to fixation. There are many kinds of fixatives. The most important ones among all are; alcohol, acetic alcohol (1 measure of glacial acetic acid: 3 measures of absolute alcohol), Carnoy's fixative (1 measure of glacial acetic acid: 3 measures of chloroform: 6 measures of absolute alcohol or 1 measure of chloroform: 3 measures of acetic acid: 6 measures of absolute alcohol), Helly's fixative and Navaschin's fixative. In reference [10], the root tips have been collected from the plants in pots in different hours of the day based on the weather being sunny or rainy and rested them in 8-hydroxyquinoline pretreatment solution for 4 hours and fixated them in acetic alcohol during the studies on the genus *Crocus*.

In reference [19],  $\alpha$ -bromonaphthalene is used as the pretreatment solution on the root tips the researcher has collected for the examination of mitosis during the studies on the species *Cucumis melo*. In references [19, 37], researchers have rested the flower sprouts directly in the Carnoy's fixative without using a pretreatment solution during their meiosis examinations they have conducted using pollen mother cells.

In reference [27], cytotaxonomical studies were made on several *Musci* species deployed among the Aegean region of Turkey. The researcher cultured the samples she has brought in the laboratory by ensuring the humidity of the material at 10-15 °C in petri dishes. As the new suckers develop, they were cut from the plant at an hour close to midnight and were fixated in the Carnoy's fixative at 18-20 °C for 3 hours.

If acetic alcohol is used as a fixative and the samples are put in this solution after being taken from the pretreatment solution, there is no need to place the samples in another solution or in ethyl alcohol to be able to preserve them for a long period, because, acetic alcohol esterifies and the acid loses its effect in time. Also, since the glacial acetic acid and alcohol mix esterifies in time and since they lose their effect as a fixative in time, this mixture should be prepared right after the root tips are removed from the pretreatment solution. Also, the small bottles in which the samples are to be preserved should be chosen carefully. Bottles with a capacity of 5 cc would be adequate to hold the samples. These small bottles may be used during the period when the root tips collected from the plant for the pretreatment. The time and whether conditions when the root tips are collected for the pretreatment should definitely be noted on the bottle, because this data will provide information about when mitosis divisions occur in general. This information will be helpful in determining on what time and in which weather conditions root tips should be collected in order to observe the chromosomes in the metaphase stage. It would be practical to take the pretreatment solution from the bottle with an injector carefully and this way, it will be ensured that there are not any liquids left in the bottle. Also, before the root tips are taken from the pretreatment solution and put in the fixative, the pretreatment solution drops remaining on the samples should be removed with a blotting paper. If the samples are put in the fixative without paying attention to this step, the purity of the fixative

may change and the required effects may not be realized on the chromosomes. If the bottle caps are made of plastic or if the structure of the caps is similar to plastic, it may get fractured in time. Because the bottle includes acid and the corrosive effects of acid are well known. If the caps are fractured, the acetic alcohol inside the bottle will evaporate and the samples will dry. On the other hand, even if the cap is not fractured, the acetic alcohol in the bottle will vaporize, if the sides of the cap allow air to get in; thus the samples will dry and they will become unusable. For this reason, the cap should be covered with a cloth plaster (1 cm wide depending on the size of the bottle) carefully, without leaving any space for air between. If the samples will be preserved for a long time, they should be checked regularly to see if they have dried. If the acetic alcohol level of the bottle has decreased, adding ethyl alcohol on the sample would be sufficient. If the samples are kept in the refrigerator (+4°C), their storage time would increase and it would be ensured that the samples are maintained without decaying. The samples stored in this manner could be examined for years after they were prepared. In order to examine the chromosomes during meiosis, the formation of pollens (microspore) and the ovum (megaspore) of the phanerogams should be assessed. Pollen mother cells (microspore mother cells) produce pollens as a result of meiosis. Ovule mother cells (megaspore mother cells) produce ovums as a result of meiosis. In short, in order to examine the meiosis of the flower, the pollen mother cells, mother cells of the embryo sac and the embryo cells (ovaries) can be used.

In order to examine pollen mother cells, pretreatment should be applied to very young flower blossoms. After the proper procedure is carried out on the fixated samples, the blossom is opened, the pollen mother cells without a fractured callus wall are chosen by disjoining the anthers and meiosis is observed.

### 3.1. Dying the chromosomes

For the chromosomes of the collected root tips, at different stages of mitosis to be examined clearly, the samples should be dyed. Also, in order to examine the meiosis of the fixated flower primordium pollens, the samples should be dyed thoroughly. Some researchers have examined the root tips by dying them with Feulgen dye [30, 38, 39]. Crystallized basic fuchsin should be used when preparing the Feulgen dye. If basic fuchsin is in powdered form, the Feulgen dye prepared with this will not dye the chromosomes adequately.

Bands are formed when the chromomeres in polytene chromosomes are in juxtaposition. The part between two bands is called an interband. The bands can be dyed with Feulgen dye or with basic dyes, but interbands cannot be dyed.

If mitosis will be examined by using fixated root tip samples, the most convenient material to dye the chromosomes with would be aceto-orcein. If the pollens in the anthers of flower primordia will be used as samples to examine meiosis, chromosomes should be dyed with aceto-carmin.

In reference [10, 24, 25], aceto-orcein is used to dye the root tips taken from the fixative when working with some *Crocus* taxa. This way, the photos of the available metaphase stages were taken and the karyograms were prepared. In reference [40], they used the same method to

examine the karyotypes in their study related to the seed development and DNA structure of *Cynara scolymus* and *Phaseolus coccineus* seeds with Pb and Cu heavy metal stress.

In reference [27], the researcher rested the *Musci* samples at 15-20 °C for 10 hours in 2% aceto-orcein after fixation. After the dying process, the samples were examined with Feulgen preparation technique, performed the karyotype analysis and prepared ideograms. The researcher has also stated that the fixation period and the dying characteristics of each species they have worked with was different and for this reason, they had to develop different methods.

The dye acceptance of pollens and consequently the adequate dying of the chromosomes is in direct proportion with their vitality. For that matter, it would be appropriate to carry out a pollen vitality test before the karyological study. The dye acceptability of pollens can be tested on alive samples, samples which are fixated with Carnoy's fixative or 80% alcohol and herbarium samples. In cytological studies, pollen infertility is generally regarded as a measure for meiosis irregularity.

In reference [19], the meiosis of the pollen mother cells examined were obtained from the flower buds along with the root tips when conducting a cytological study on some *Cucumis melo* taxa. The researcher dyed the root tips with nigrosin and examined the chromosomes. He also examined some of the root tip samples with pectinase enzyme and aceto-orcein dye. The researcher examined the meiosis divisions with Feulgen method. He has dyed the pollen mother cells he had obtained from the anthers of flower buds with 2% aceto-carmin.

There are several ways to prepare aceto-orcein and aceto-carmin and the procedures given below have been tested many times and satisfactory results were obtained [10, 19, 37, 41].

### 3.2. Preparation of aceto-orcein

5 grams of powdered orcein and 250 cc 45% acetic acid are mixed and shaken. They are boiled for 30 minutes by using a Soxhlet apparatus. The apparatus should certainly be held by a stative. The Soxhlet apparatus looks like a glass tube with a spiral back cooler connected to the volumetric flask, it has a tap for water intake below and a place for water outlet. The volumetric flask under the Soxhlet apparatus is placed on the amiant wire on the heater. A boiling chip should be placed inside the volumetric flask. If there are not any boiling chips available, 1-2 cm long glass bars can be used for the same function. Acetic acid dye mix is poured in the volumetric flask and the heating process is started. The aceto-orcein prepared is filtered after it has been cooled down. Aceto-orcein prepared in this manner shall be ready for use.

### 3.3. Preparation of aceto-carmin

1 gram of powdered carmin and 200 cc 45% acetic acid are mixed and shaken. They are boiled for 5 minutes by using a Soxhlet apparatus. This duration is ideal for Turkey. For example, if the boiling process for dye preparation would take place at a location in the north of Turkey, a country with colder weather, the boiling duration may be 30 minutes. After the boiling process, 1-2 drops of Fe acetate solution saturated with 45% acetic acid is added to the dye.





**Figure 12.** Radicula and primary roots on the germinated *Phaseolus coccineus* seed [40]

Excess Fe acetate settles the carmine. In this case, the dye cannot be used expediently. The aceto-carmine is filtered after being cooled down. Aceto-carmine prepared in this manner shall be ready for use.

3-4 cc of aceto-carmine should be spared before every use. If Fe acetate is not used when preparing aceto-carmine, a nail is placed in the aceto-carmine spared. The iron nail should not remain in the dye for a long time. Because, aceto-carmine with a heightened Fe proportion dyes the cytoplasm in very dark color as well as the chromosomes and it gets extremely difficult to examine the slide. However, if the cytoplasm of the cells are dyed in a dark color, 45% acetic acid can be dropped on the side of the cover slip with the help of a thin pipette. When the acetic acid reaches below the cover slip, it may lighten the color of the cytoplasm. The reason a lighter cytoplasm is required is that, it is preferred when there is a contrast between the cytoplasm and the dyed chromosomes. When there is a contrast between the cytoplasm and the chromosomes, it is easier to examine the chromosomes. Also, the photos of the meiosis phases taken are clearer when there is contrast.

#### **4. The points to take into consideration when taking examination samples**

The chromosomes of dicotyle plants are generally examined by using the root tips obtained with the germination of their seeds. The seed embryo is a fertilized mature ovule, which consists of endosperm and testa. When the germination begins, deterioration first takes place in the micropyle area. The radicula starts to grow towards the micropyle. Root hairs emerge on the radicula and primary roots become apparent (Figure 12). Germination varies in accordance with the species.

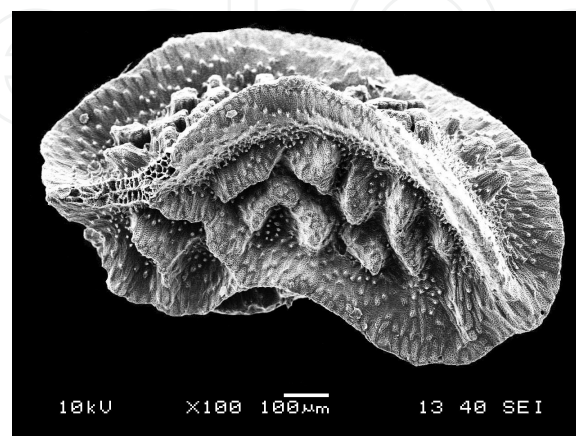
The testa is a diploid tissue of the sporophyte. The seed coats of beans and groundnuts are thin. It can be thick and rigid as the seed coat of nuts. The variety in the thickness of seed coats may affect the germination of a seed. In addition to this, the seed sizes also differ. For example, the fresh weight of one orchid seed is 0.000002 grams. The seed of *Mora oleifera*, which is 1000



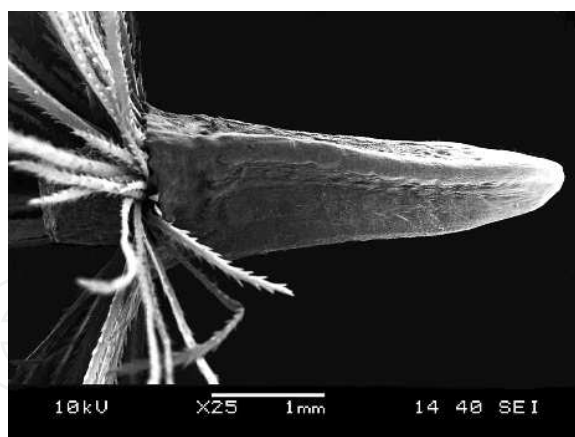
gr, is one of the heaviest seeds known [18]. As it can be seen, if the chromosome examination will be conducted by using the root tips obtained with seed germination, the characteristics of the seed need to be determined clearly and if necessary some pretreatment procedures should be applied before the germination. For example, if the seed coat is thick, it should be sanded or perforated to facilitate the water intake.

The impact of environmental conditions is also important with regard to seed germination. For a successful germination process; the temperature, water and oxygen levels should be adequate. Water is required for the development of the embryo and the enzymatic reactions to start by secreting hormones. Temperature is significant with respect to the functioning of the enzyme. Oxygen is required for the respiration need of the developing seedlings. Although the seeds of some plants germinate in dark, seeds of several plants can only start the germination process after being left under light for a certain amount of time. This pretreatment performed with light can be significant when the seeds absorb water [18, 42].

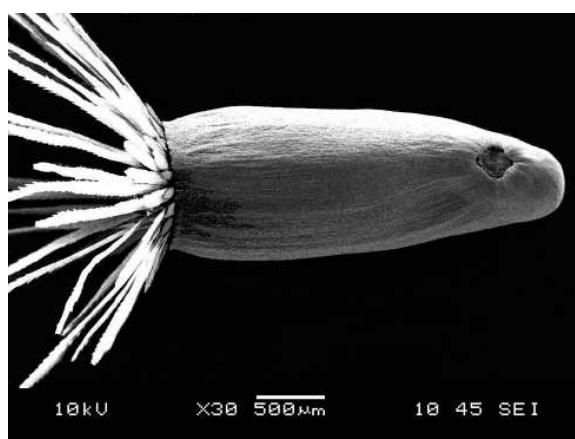
Therefore, the ecological factors of the environment plant lives in should be determined and a seed germination environment should be prepared accordingly. For this, generally climate cabinets are used. In some instances, even though the conditions required for the seed to be germinated are present, germination does not take place. For example, for the orchid seeds to begin germinating, they should form a mycorrhiza with some fungi types. As it can be seen from the examples, the germination factors of seeds may vary significantly. Thus, more detailed preliminary examinations may be due for the germination of some seeds. In addition to this, if the dicotyle plant examined is endemic, its seeds may be small in quantity or the fertile seeds may be scarce. Also, it is possible for the mature seeds obtained from endemic plants to require more care and time for germination. If the seed of the dicotyle plant is spread around the land as the fruit dries, such as endemic *Linaria corifolia* of the Scrophulariaceae family (Figure 13), it would be appropriate to take the root tips by collecting and germinating these seeds [42]. However, if achene fruits, such as the members of the family Asteraceae; for example endemic *Centaurea zeybekii* or endemic *Jurinea pontica*, are examined (Figure 14, 15), they should directly be subject to germination without attempting to remove the seeds [43-45]. Because, pericarp is fused to the thin seed coat in the grain.



**Figure 13.** Seed of *Linaria corifolia* (SEM photograph) [43]



**Figure 14.** Seed of *Jurinea pontica* (SEM photograph) [43]



**Figure 15.** Seed of *Centaurea zeybekii* (SEM photograph) [44, 45]

The germination durations of seeds may vary significantly among plants. Also, the optimum temperature required for the germination of the seed may also vary. Thus, many tests and observations in different places and periods could be needed for the determination of the right germination duration and the optimum temperature for the plant.

Also, sometimes, in order to end the dormancy of the genetic material inside the seeds, germination tests can be made after it is rested in the refrigerator (+4°C) for hours or days. The germination test can be carried out inside a petri dish with a humid blotting paper inside, covered with another petri dish. The point to take into consideration here is this; before placing the blotting paper inside, first a piece of glass in the shape of a square or a rectangle should be put in the petri dish. Later the blotting paper cut in the shape of a circle is placed on the piece of glass. The size of the blotting paper should be smaller than the petri dish. Later, water is poured on the blotting paper, whose center is heightened with the piece of glass. The water should be pure. The water poured on the blotting paper may be 1-2 mm above the surface level. Later, the seeds are placed on the center of the blotting paper which is heightened with the piece of glass. A circular and humid blotting paper of an appropriate size may be placed

on the seed as well. This blotting paper can also be placed in the second petri dish after being humidified. This method can work in some instances. For example, if the radicle and the primary roots grow upwards, this humid blotting paper prevents the root tips from getting dry. Because, it is impossible to examine mitosis in dry root tips.

The number of seeds to be placed in the petri dish varies depending on the size and the water absorption capacity of the seed. For example, the swelling capacity of the seeds which belong to Fabaceae family, is high. If a seed with these characteristics is being used, the seeds to be put in the petri dish should be small in number and the water to be poured needs to be plenty. It is important to adjust the amount of water to be poured in the petri dish. If one pours excessive amount of water, it may spill from the sides of the petri dish. If the seeds are observed well during the germination period, water may be added after the level of water in petri dish has decreased and the beginning stages of dryness are observed.

Collecting root tips from a germinated seed in the petri dish relatively easier than collecting the root tips of plants which are grown in fields or on land. The root tip collected from a germinated seed in petri dish is cleaner and there is no risk of tearing the part above the calyptra, where mitosis takes place. Also, the most important point to take into consideration when applying this method is the length of the primary roots shooting from the seed. If the root tip is too long, the part where mitosis takes place can dry. In addition to this, if the root tip is too small, it may get damaged during the dying process and it cannot be examined. It is sufficient for the primary roots to be 1-2 cm long (Figure 16).

Sometimes the seeds placed in a petri dish for germination can be contaminated, they can be molded for example. In this case, they do not shoot root tips. Also, in some instances the seed gets infected after germination. There is a root tip; however, it is impossible to carry out the appropriate chromosome examinations on this root tip, because it is infected. Therefore, the seeds placed in the petri dish should be observed frequently (1-2 times a day).



**Figure 16.** Primary root of the *Phaseolus coccineus* on the left is suitable to collect [40]

It is difficult to collect root tips from dicotyle plants on the land. Especially, if the soil the plant is located on is clayed or stoned, if the plant is on a cliff or on a sloped environment, it is difficult to collect root tips, even though the roots and the lateral roots of the plant do not grow too deep. Also, even if the place the plant is on is flat, if the roots grow deep or if the soil type is not suitable for collecting the root tips, the process of taking root tips will require effort. For

the chromosome examinations of these plants, germinating their seeds would be an easier method which gives useful results.

Perlite can be used for the seeds which are to be germinated. Perlite also is used in greenhouses to grow seedlings from seeds. Torf can also be used for the tests of growing plants in pots. The pots are turned upside down, the torf surrounding the plant is collected carefully and the lateral roots are reached. One should be very careful in order not to damage the root tips.

If the chromosomes to be examined belong to a monocotyle plant and it reproduces with stems or corms like the genus *Crocus* (Figure 17, 18), it should be known that the number of the seeds may be less for these plants. Even if there are plenty of seeds, there is a great probability that many of them are sterile (Figure 19, 20) [10].



**Figure 17.** Young corms of *Crocus chrysanthus* [10]



**Figure 18.** Corms of *Crocus chrysanthus* [10]





**Figure 19.** Ripped fruit and scattered seeds of *Crocus chrysanthus* [10]



**Figure 20.** Ripped seed of *Crocus flavus* subsp. *flavus* [10]

If the chromosomes of monocotyles (for example Iridaceae or Liliaceae) are to be examined, root tips may be collected on the land. However, the most appropriate and easy method would be to obtain them on the land, sew them on the pot and to try and collect the root tips. If the plants are on a mountain or on highlands, the environment in your house or your garden may not be suitable for the plant to grow. In this case, the plants sown will die. However; if the corms and bulbs are preserved under convenient conditions (in a dry and dark environment), they will give roots the next year. The corms of *Crocus* (Iridaceae) and bulbs of *Colchicum* (Liliaceae) species shoot primary or contractile root tips the following year even if they are not placed under soil or torf.

If root tips are to be collected from monocotyles on land, it should be noted that this process requires extreme attention and patience. The root depth of the plant is examined and a proper garden tool (such as digger, shovel and hoe) needs to be taken to the land. When removing the plant from the land, the distance between the tool and the plant should be maintained. Otherwise, there is a risk of tearing the root or the stem of the plant and rupturing the lateral



roots. The plant should be removed with the soil surrounding it and later, the soil and the weed around the plant should be sorted out for the unruptured root tips to be collected. If the soil is clayed, it is difficult to collect root tips since, the soil is sticky. In this case, the corm or the bulb should be taken, the plant should be sown in a pot and the root tips shoot in that environment should be collected. For example, the chromosomes you wish to examine could be of a plant which grows in a mountain, far away from where you live. The region and the climate the plant grows in may not be suitable for you to visit the area often to collect root tips. It is difficult for the researcher to grow this plant, which is adapted to the mountain climate and cold weather, and to collect its root tips. In this case, it may be convenient to sew the samples collected from the land in pots and to take the pot to a mountain near (or another place similar to the original environment of the plant). An area on the mountain or the natural site is surrounded with a wire or a fence, after obtaining the necessary permits from the authorities. The pots are placed in this area. Thus, it may be possible to prevent the pots from getting harmed by some animals.

The root tips of the plants taken from the soil may continue performing mitosis divisions. For this reason, it would be appropriate to use the natural water of the environment in order to prevent the root tips from drying out until they are collected for examinations. For example, if a field study is being conducted on a highland, it would be convenient to use water from the mountain. If the land is snow-covered, it would be best to use melted snow. If the water reserves of the land are scarce, drinking water or pure water may be used to prevent the root tips from drying. However, if the environment is cold, the water used should also be cold. As it is known, temperature affects mitosis.

If the fixated pollens in the anthers of flower primordiums will be used to examine meiosis, samples should be taken during the periods when there are plenty of flower buds. The flower buds should be collected at different times of the day and they should be in different sizes. Because it is extremely difficult to estimate at what time and in which size (necessary maturity) the pollens will be in the appropriate phase to be examined. Thus, it would be beneficial for the study to take many samples while changing the variables.

## **5. The points to take into consideration when preparing the mitosis or meiosis slides and when performing the examination**

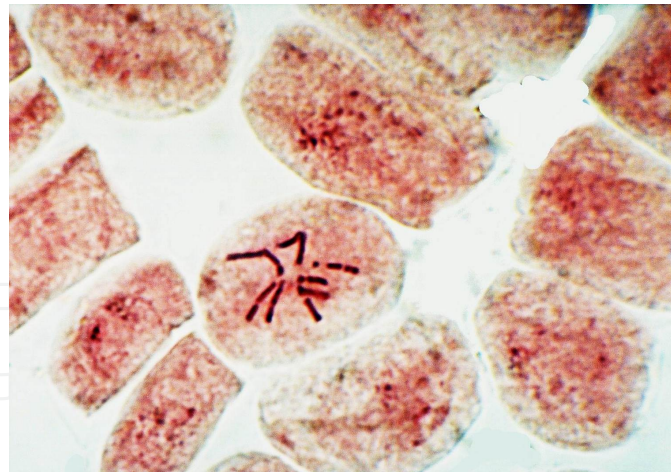
If slides are to be prepared by using root tip squash samples, it would be convenient to place the pretreated and fixated root tips 1-2 cm length, collected from the seed or the root in a clean petri dish which includes 70% clean ethyl alcohol. The samples placed in the petri dish should be pre-examined with a stereo microscope. Because, the part of the root to be examined, which is 1-2 mm long and is above the calyptra, may be torn in the sampling process. Also, the samples may be contaminated. These cases are most commonly observed in the root tip collection processes carried out in the fields. If the soil conditions are favorable, the hairy roots may grow and spread in the soil. Sometimes, for example, when the soil is clayed, it will be difficult to collect the root tips. Since the pieces of soil will stick

to the roots, the sensitive root tips will be ruptured. Even when the root tip is collected properly from such an environment or a different environment, the soil pieces stuck on the roots should be cleaned before the slides are prepared. There is also a possibility for particles and dirt to remain on the root tips collected from torf or perlite. Thus, before starting the slide preparation process, the samples should be examined in alcohol with a stereo microscope. If the root tips are very dirty, the alcohol in petri dish should be replaced for a couple of times. The cleaning of root tips can be carried out by shaking the samples in alcohol and removing the dirt by using a forceps or a needle.

It would be sufficient for aceto-orcein or aceto-carmin dyes to be filtered for a short time during the preparation process, because, there may be sedimentations in the dyes which are rested for a long period. It may be convenient to transfer small amounts of dye from the total amount stored in dark glass bottles in a dark place to small bottles which can contain 5 cc of liquid. Because, the amount of dye required for an examination is 3-5 cc. The required amount of dye is filtered with a diameter of 2-3 cm prepared blotting or filtering paper. The filtered dye is poured in a middle sized watch-glass. The fixated root tips are placed in here. The watch-glass which contains dye and root tips heated on the burner until it boils. Since there is a small amount of liquid, the boiling process should not take a lot of time. Also, the acetic acid in the dye will evaporate. The samples may dry if the heating process lasts too long, since the acid will be vaporized. In this case, the root tips in the watch-glass will be unusable. The heating process made in a careful way should be repeated three times. After each heating process, the watch-glass is taken aside and another watch-glass larger than the one used is covered on top of it. This should be done very quickly. The purpose here is to prevent the acetic acid in the dye to vaporize. Also, the point to take into consideration is the size of the watch-glass which is used to cover the watch-glass used. If a significantly larger watch-glass is used to cover the heated watch glass, the acetic acid would vaporize and the dye would dry. If the heating process is carried out carefully, it will enable the maceration of the roots as well as allowing for the dye to perforate in the cells and ensuring the dying of the chromosomes; therefore, there will be no need for hydrolysis.

The slide prepared for mitosis examination does not only reflect one phase of the mitosis. When some cells are in interphase, some may be in anaphase, some in telophase and some may be in metaphase (Figure 21). If lengthened cells are observed in the slide, this shows that not only the necessary part of the root tip required to examine mitosis is squashed. The excessive parts squashed are the cells in the stage of elongation. Also, vascular tissue can be seen in the slide. The reason of this observation is similar to the reason for seeing the elongated cells. Even if there are proper metaphase cells in the slide, the existence of elongated cells and vascular tissue could make it difficult for the chromosomes to be examined. For this reason, one should decide very carefully when determining how much of the root piece will be left in the slide when examining the length of the calyptra shot from the root tip.

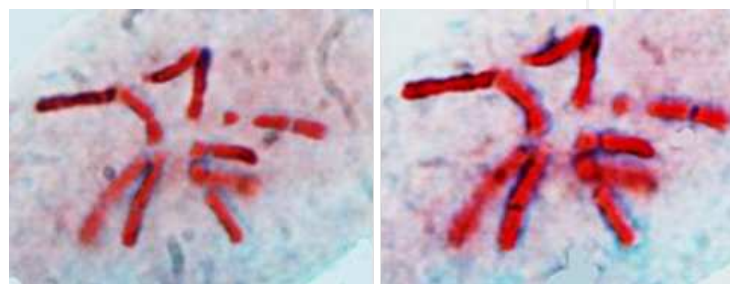
The root piece on the slide which is 1-2 mm long has become very soft and is ready to be squashed. The dyed root tip is placed on the slide. After the calyptra is cut with a sharp razor blade (a piece almost in the size of a pinhead), 1-2 mm of the remaining piece is cut and this



**Figure 21.** Different phases in a squashed root tip of *Crocus chrysanthus* [15]

piece is placed on the slide. The remaining root piece is thrown away. 1 drop of the aceto-orcein filtered for the study shall be poured on the material. The cover slip is closed on the sample without allowing any air to get in (with an angle equal to  $45^{\circ}\text{C}$ ). A piece of blotting paper (a little larger than the cover slip) is placed on the cover slip carefully. The blotting paper absorbs the aceto-orcein spilling from the sides of the cover slip. The thumb is placed on the cover slip carefully, without moving the cover slip on the slide. The thumb is not removed, but is moved from side to side for the mitosis cells of the root tip squashed to spread under the cover slip. Thus, the cells which have overlapped and the chromosomes of the cells will be separated from each other and are spread on one plane. As a result, the cells and the chromosomes will be easily examinable.

However, there may be cells in the slide which are not spread on the plane as well as the cells which reflect the chromosomes clearly. In this case, various images of the same cell should be taken using the microscREW (Figure 22). Later, the images are put together and examined. For example, a chromosome observed in one image may not be present in the other. As a result, a karyogram can be prepared with these images.



**Figure 22.** Various images of the same cell of *Crocuschrysanthus* using the microscREW [15]

Sometimes the photographs taken by replacing the microscREW may not be sufficient in providing the chromosome data. In this case, the microscope coordinates of the cell examined are noted in accordance with the objective used. Later, the slide is taken from the microscope flange and is put on a hard surface. A blotting paper is placed on the cover slip once more and the cover slip is pressed with the thumb carefully. This pressure may allow for the chromosomes of the cell, whose coordinates were noted, to be observed individually. If this operation is carried out carefully, there is a great chance that it will be useful. However, even the slightest gliding of the slide may cause the cells to overlap or it may result in the emergence of new cells which have been flattened and cannot be observed clearly. This second squashing process can only work after many practices. As it can be seen, a minor error in the second squashing process may cause the slide to be unusable. Thus, before the second squash and after the coordinates of the examined cell is noted, other photographs of the cells should be taken. So that, when later, the slide is taken from the flange to be squashed once more and the slide ends up being unusable, the negative impact will partially be eliminated.

In some instances, the researcher may need to take a break when observing the phases of mitosis or meiosis. When the slide is being examined as described above, it may only be preserved for 15 minutes, depending on the temperature, because the acetic acid in the aceto-orcein used to dye the chromosomes and the acetic acid in the aceto-carmin used in meiosis examinations will vaporize. Thus, a dried slide is not functional and it cannot be used for examination. In this case, it would be convenient to cover the all sides of the cover slip with paraffin in order to be able to examine it in 1–2 days. For this appliance, the back of the metal spatula should be heated on the burner and it is contacted with the paraffin. The liquid paraffin poured on the metal is applied thinly around the cover slip. The slide prepared in this manner can be maintained for 1-2 days without being dried and it can be used. If the slide is preserved in the refrigerator (+4°C) in a petri dish covered with another petri dish, the life cycle of the slide will increase. Covering of the sides of the cover slip with rubber solution, has the same effect.

It may take a long time to make a careful examination on all the root tip squash under the cover slip with microscope. If the slide is prepared with aceto-orcein (for the examination of mitosis) or with aceto-carmin (for the examination of meiosis), under the sides of the cover slip will begin drying circularly. Since the drying of the slide will prevent us from completing the examination, a small amount of dye shall be added on the side of the cover slip with the help of a needle. It will be observed that the dye steals into the dried parts under the cover slip after a while. The excess dye on the side of the cover slip can be cleaned with a blotting paper. This process will delay the drying process and increase the time allowed for examination.

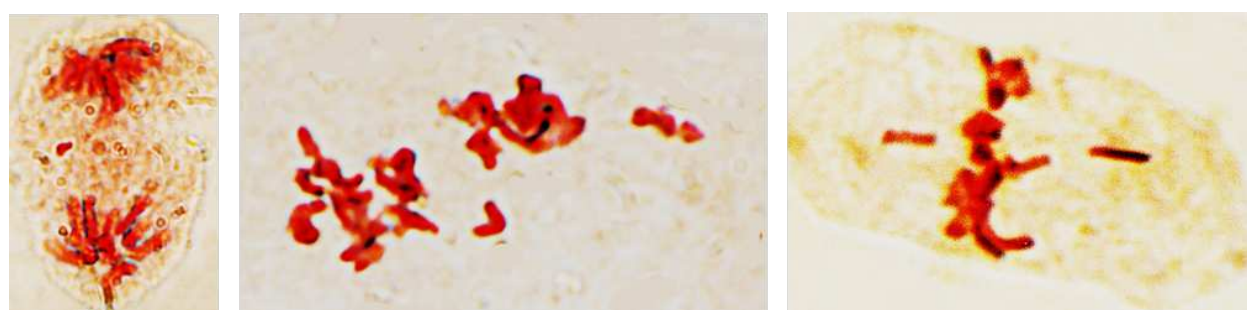
In some instances Feulgen's dye is used for the chromosomes dying. In this case, 45% acetic acid is dropped on the sample and the slide is covered with the cover slip. The squashing process is carried out carefully. The cover slip may start to dry from the sides when examining the slide, as it does with other dying methods. In order to prevent the drying of the slide, the researcher should drop 45% acetic acid on the side of the cover slip with a needle and should



wait for it to steal into the dried parts of the squashed root tip under the cover slip. The excess acetic acid on the side of the cover slip is dried with a blotting paper.

## 6. Chromosomal abnormalities

Chromosomal abnormalities are observed in plants for various reasons. The existence of a chromosomal abnormality in the cell can also be understood from the morphology of the root or another part of the plant. The chromosomal abnormalities which reflect themselves in the morphological properties can occur with relation to the differentiation and adaptation processes. For example, in reference [10], when examining the problematic species *Crocus chrysanthus*, observed lagging chromosomes (Figure 23) and determined  $2n=20+2B$  as the chromosome number for the plant samples with blackish anthers, which are differentiated and morphologically different suggested as a subspecies in reference 15.



**Figure 23.** Lagging chromosomes of *Crocus chrysanthus* in different cells [10]

Rapidly increasing world population in recent years brings about an increasing demand for nutrition, which is one of the most important problems for mankind. Various methods have been discussed in order to solve this problem. One of these methods is using chemicals against harmful organisms in plants. The use of pesticides in agricultural areas increases the plant yield; however, some chemical substances may result in pollution in nature and health problems [46].

Modern agriculture and industry depend on a wide variety of synthetically produced chemicals, including insecticides, fungicides, herbicides, and other pesticides. Continual widespread use and release of such synthetics has become an everyday occurrence, resulting in environmental pollution [47]. It was indicated that many cytogenetic studies have been carried out to detect the harmful effect of different pesticides on different plants [48]. These chemicals used at recommended dosage and double the recommended can give rise to abnormal chromosomes and degeneration in meiosis cycle, such as ring shaped chromosomes, linear chromosomes and binding chromosomes [37]. Benomyl, a systemic fungicide, affects germination, mitotic and meiotic activity, and pollen fertility in barley [49]. Besides, the

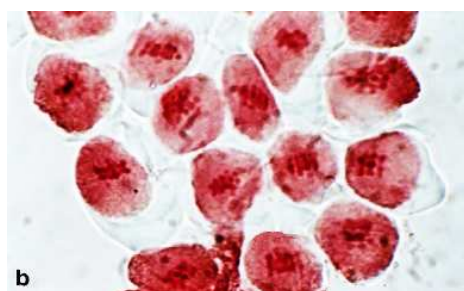


fungicide phosphite reduced pollen fertility in *Petunia hybrida*, *Tradescantia virginiana* and *Vicia faba*, while phosphite, application increased the number of abnormal meiotic cells at all stages in *Tradescantia virginiana* microspores [50]. Furthermore, some common pesticides (Thiodan, Folithion, Lebaycid, and Kitazin) caused a spectrum of cytogenetic abnormalities such as chromosome fragmentation, lagging of chromosomes, anaphase bridge formation as well as tripolar and tetrapolar spindle formation in barley [51].

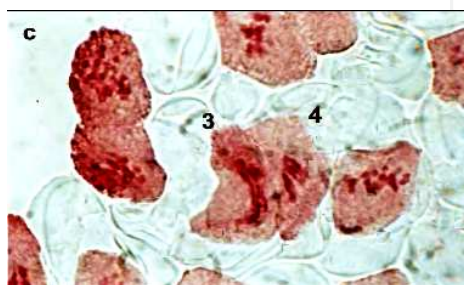
The abnormalities occurring in meiosis are very important because they cause sterility in pollens and genetic damage can be transmitted to the offspring via male gametes, leading to congenital abnormalities. It was studied on *Lycopersicon esculentum* that all dosages of the fungicides Agri Fos 400 [80% fosetyl-Al (400 g/l mono- and di-potassium phosphonate)] caused various abnormalities in meiosis when compared with the control (Figure 24-27) [37].



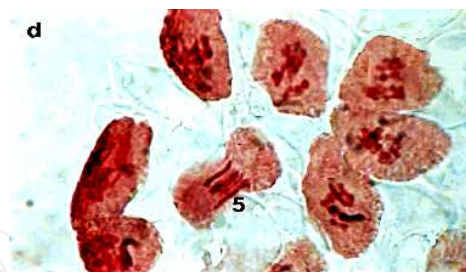
**Figure 24.** Ring-shaped chromosomes in 400 ml/100 l group a (1, 2) [37]



**Figure 25.** Binding chromosomes in 800 ml/100 l group b [37]

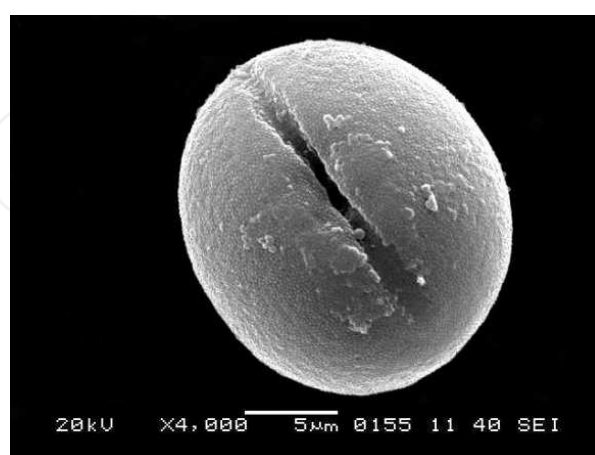


**Figure 26.** Binding chromosomes and abnormal shape in 800 ml/100 l group c (3, 4) [37]

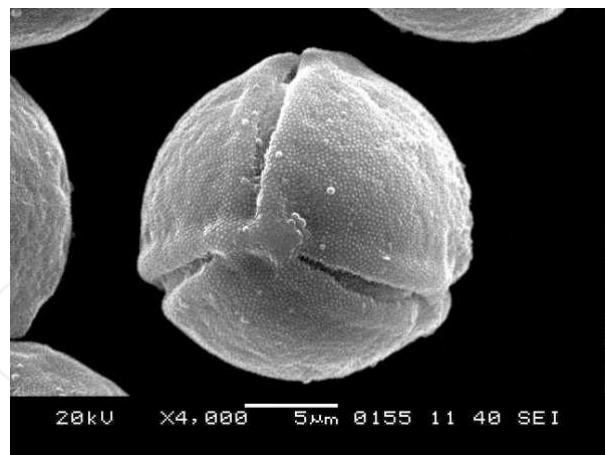


**Figure 27.** Abnormal shape and linear chromosomes in 800 ml/100 l group d (5) [37]

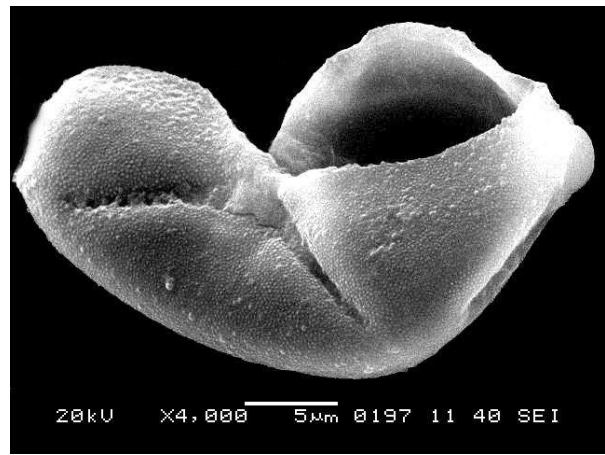
In another studies, the fungicides Fosetyl-Al (80% Aliette WG 800) and Equation Pro (22.5 % Famoxadone + 30 % Cymoxanil) widely applied on tomato plants (*Lycopersicon esculentum*) grown in greenhouse in Turkey caused various anomalies in pollen meiosis such as thread-like, ring shaped, linear and binding chromosomes (Figure 24, 25). This situation could lead to a decreased in the productivity of fruits [52, 53]. In references [54- 56], the effects of fungicide and applicator application on pollen structure in tomato (*Lycopersicon esculentum*) were investigated. SEM photographs of *Lycopersicon esculentum* as regards control group and non-viable pollen grains in the ACT-2 groups are given below (Figure 28-33).



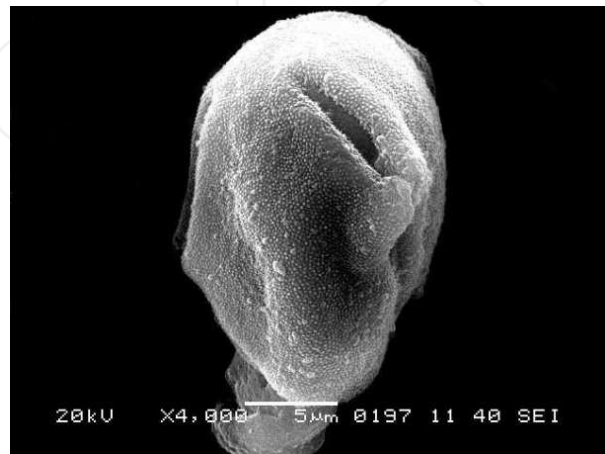
**Figure 28.** Pollen grain of control group (SEM photograph) [56]



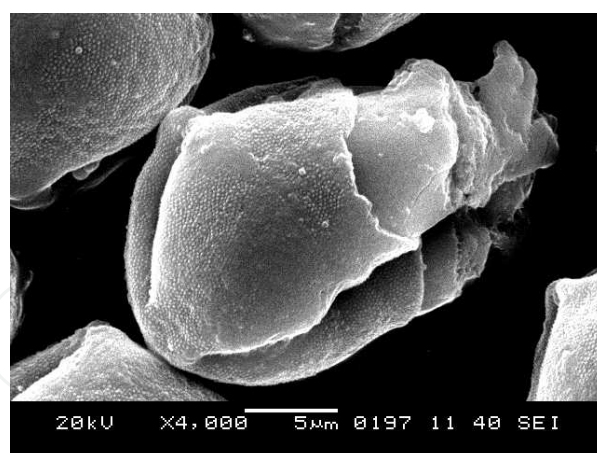
**Figure 29.** Pollen grain of control group, polar view (SEM photograph) [56]



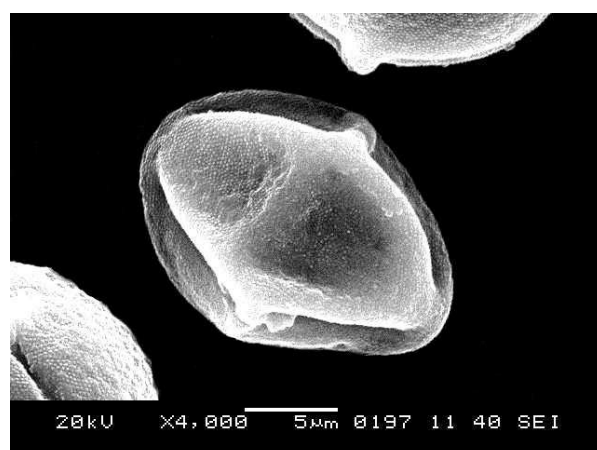
**Figure 30.** Abnormally shaped pollen grain in 150 cc/100 L. (SEM photograph) [56]



**Figure 31.** Abnormally shaped pollen grain in 150 cc/100 L. (SEM photograph) [56]



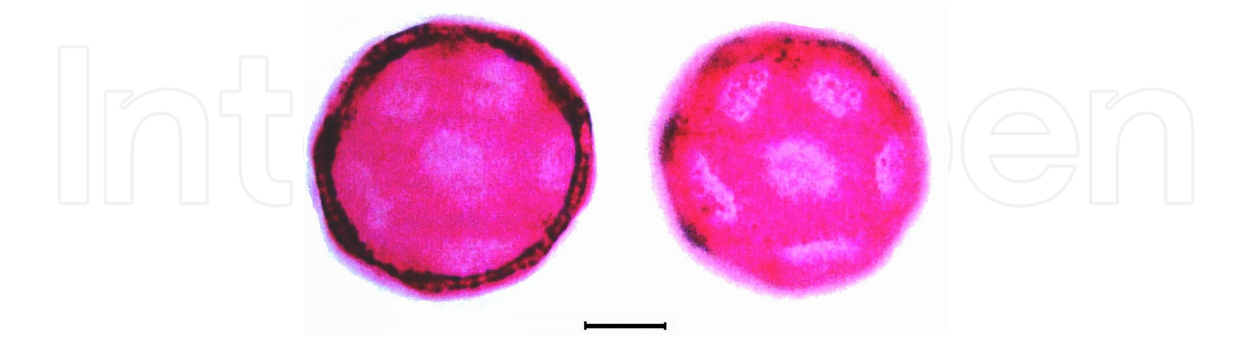
**Figure 32.** Abnormally shaped pollen grain in 300 cc/100 L. (SEM photograph) [56]



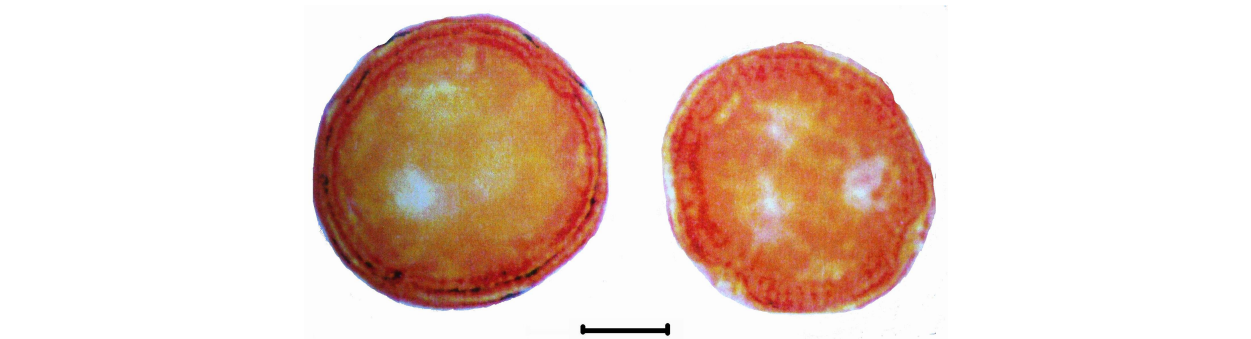
**Figure 33.** Wrinkled pollen grain in 300 cc/100 L. (SEM photograph) [56]

Agricultural pesticides or hormones commercialized may cause the flowers and pollens of the flowers to have formal differences and abnormalities and it may reduce productivity. The species, *Anemone coronaria* is a flower with corm, which can be grown in greenhouses with no heating between October and May and is very popular in domestic and foreign markets, also, its origin is Mediterranean and it is ecologically advantageous; because of these reasons it has a significant status in export with regard to differentiation. In references [16, 57], it has been pointed out that while there is homogeneity in the pollen sizes of the natural forms of the variety within the species, the pollen sizes, pollen deformations, pollen failure to thrive and vitality of the pollens taken from the members to be traded are significantly different (Figure 34-40). The agricultural pesticides used to enhance and accelerate the growth and blooming can be the reason behind this differentiation. The pollen vitality percentages were determined as a result of the test made with TTC (triphenyltetrazolium chloride) in order to observe the pollen vitality of natural and commercial forms of *Anemone coronaria* taxa. In reference [57],

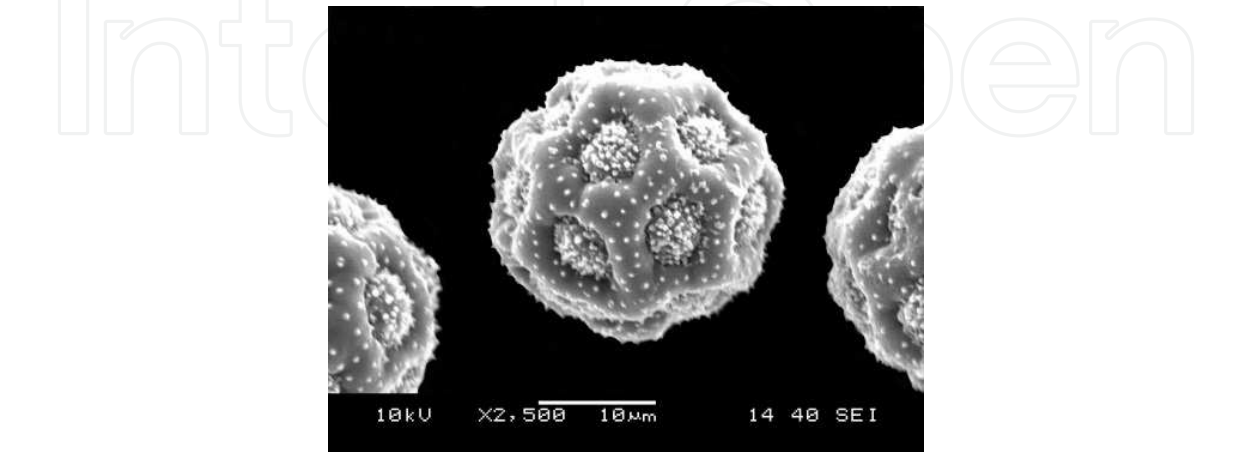
viable pollen ratio of natural *Anemone coronaria* var. *coccinea* is 94%, viable pollen ratio of commercial forms of that taxon is 69%.



**Figure 34.** Pollen grains of *Anemone coronaria* var. *coccinea* as regards control group, scale bar:10  $\mu$ m (Wodehouse Method used) [16]

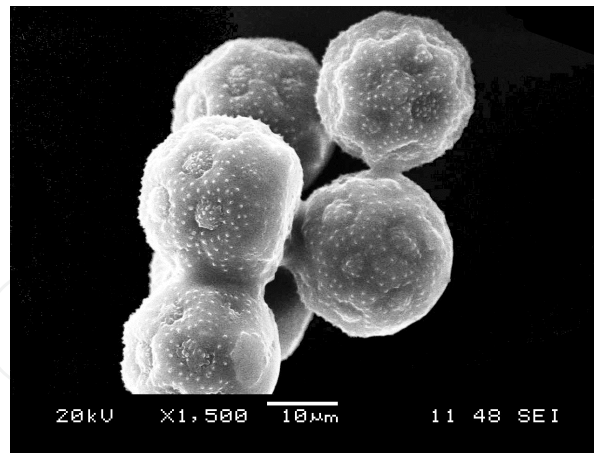


**Figure 35.** Pollen grains of *Anemone coronaria* var. *coccinea* as regards control group, scale bar:10  $\mu$ m (Erdtman Method used) [16]

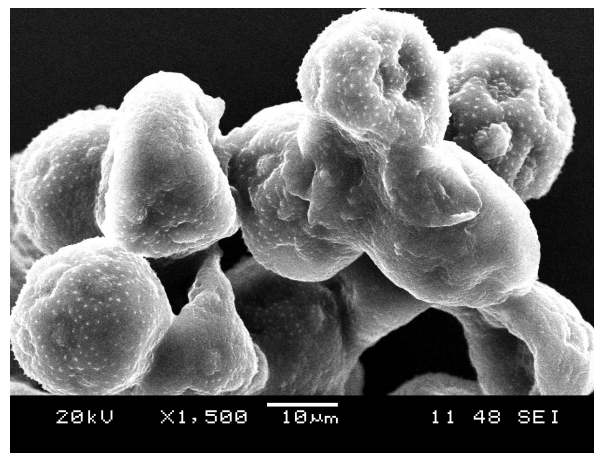


**Figure 36.** Pollen grain of *Anemone coronaria* subsp. *coccinea* as regards control group (SEM photograph) [57]

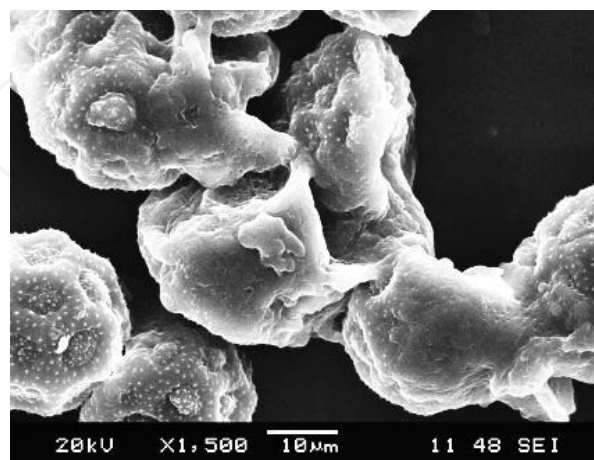




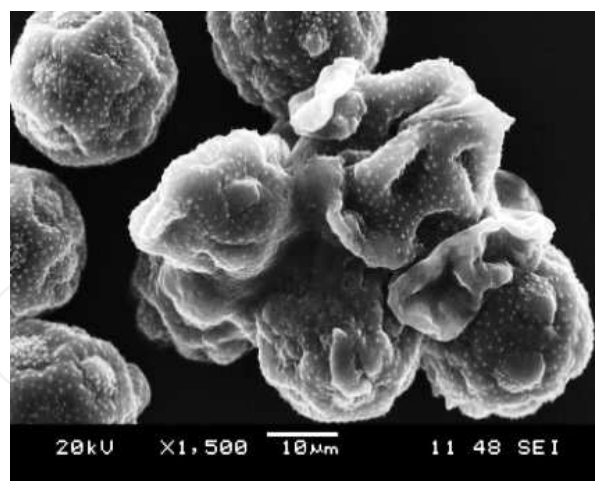
**Figure 37.** Abnormal shaped pollen grains of *Anemone coronaria* var. *coccinea* as regards commercial group (SEM photograph) [57]



**Figure 38.** Abnormal shaped pollen grains of *Anemone coronaria* var. *rosea* as regards commercial group (SEM photograph) [57]



**Figure 39.** Abnormal shaped pollen grains of *Anemone coronaria* var. *cyanea* as regards commercial group (SEM photograph) [57]



**Figure 40.** Abnormal shaped pollen grains of *Anemone coronaria* var. *alba* as regards commercial group (SEM photograph) [57]

Also, environmental conditions which are stem from pollution, such as heavy metal stress may cause development disorders and chromosomal abnormalities in plants. For example, in reference [40], they applied different values of Pb stress to artichokes for 5 days and have observed their development. 40, 80, 160, 320, 1280 ppm Pb acetate solution was used. The researchers have also germinated control groups in Hoagland solution. The seeds to which stress are applied, delays in germination were observed and some did not even germinate depending on the stress level (Figure 41). Also, the root tips collected from the seeds have revealed chromosomal abnormalities and deviations.



**Figure 41.** Photographs of germination related *Cynarascolymus* seeds in a row of control group and 40, 80, 160, 320 ppm Pb application [40]

## 7. Uncontrolled and abnormal cell reproduction of plants

Galls are abnormal growths that occur on leaves, twigs, roots, or flowers of many plants. Most galls are caused by irritation and/or stimulation of plant cells due to feeding or egg-laying by insects such as aphids, midges, wasps, or mites. Some galls are the result of infections by

bacteria, fungi, or nematodes and are difficult to tell apart from insect-caused galls. Seeing the insect or its eggs may help you tell an insect gall from a gall caused by other organisms. In general, galls provide a home for the insect, where it can feed, lay eggs, and develop. Each type of gall-producer is specific to a particular kind of plant [58].

The gall structure observed in oak, apple, cherry, nut, walnut and maple trees and grapefruit contain a liquid similar to the gall of the mammals with its green tone and bitter taste. Although many galls do not critically harm the plant, each one of them is a parasite. The parasites causing the gall generation accelerate the uncontrolled cell division of the organism on which they live and as a result cause the number of the cells to increase.

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