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Review

Methods used for parental selection in pre-breeding of plants

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ABSTRACT

A key step in the pre-breeding stage of plant breeding is the selection of cultivars with desirable characteristics for use as parents to develop new crop varieties. With the development of technology, numerous laboratory techniques have emerged as key tools for breeding programs to save time and resources. For these reasons, the aim of this review was to describe techniques based on cytological and cytogenetic studies that are feasible for the selection of parents during the pre-breeding stage. Among the cytological techniques addressed in this review are: pollen viability and quality, stigmatic receptivity to know how long the stigma is receptive, and pollen tube growth to study incompatibility events. Another key step in pre-breeding is to determine the chromosomal number of the parents, which is possible by different techniques of classical cytogenetics (studies during mitosis and meiosis) and molecular cytogenetics (flow cytometry, Fluorescent *in situ* Hybridization and Genomic *in situ* Hybridization) that are discussed in this review.

Key words: biotechnology, karyotyping, cytogenetics, incompatibility, pollen viability.

INTRODUCTION

Since the beginning of agricultural activities more than 10 000 years ago, man has manipulated the genetic structure of plants and animals through numerous cycles of selection of the best adapted individuals. As a result, most of the plants cultivated today are different from their wild ancestors [(1)].

Today, the objectives of breeders, compared to our farmers' ancestors, still have the same mission, i.e., to produce crops with higher yields, more resistant and better adapted to different locations.

One of the main requirements for starting a breeding program is to have the genetic variability needed to identify potential genotypes for use as parents. Numerous factors favor genetic diversity, such as: sexual reproduction; mutations; gene flow and, of course, human action through artificial selection and hybridization [(2,3)].

After generating genetic variability, the next step is to discriminate among the variability in order to identify and select individuals with desirable characteristics to develop new potential cultivars, which we call artificial selection. In nature, natural selection is responsible for favoring genotypes most adapted to the environment in the species. In breeding, the same thing happens, but in this case the breeder is in charge of selecting the genotypes that show the highest yield or benefits.

In early years of genetic breeding, selection consisted on the observation of plant behavior and the subsequent selection of those with the best attributes, supported by different elements of biology, mathematics and statistics. With the advance of technology, a large number of techniques have emerged to facilitate selection work.

Conventional plant breeders, in order to save resources and time, carefully plan the crosses to generate the greatest possible variability [(4)]. It is important to determine the direction of the crosses; that is, which varieties or lines will be used as father or mother. Varieties that are going to be used as pollen donors must have high quality pollen and viability, parameters that are evaluated with pollen viability studies [(5)]. Likewise, it is necessary to know the adequate moment to carry out the pollination and that the pollen can germinate in the stigma of the female parent, which makes necessary stigmatic receptivity studies [(6)].

Another factor of great importance in selection is the knowledge of the chromosomal number, because differences in ploidy can prevent sexual reproduction and, therefore, the obtaining of seeds. Although this is not always the case, in some cases fertilization can occur and genotypes of other ploidy levels can be obtained with differential behavior in terms of the trait being evaluated. Karyotype studies can be performed by the discipline known as cytogenetics, which is divided into classical cytogenetic when chromosomes are analyzed under the microscope by means of staining or molecular one, when different molecular biology methods are applied [(7)].

Due to the above, the aim of this review is to expose techniques based on cytological and cytogenetic studies that are feasible for the selection of parents, during the pre-breeding stage.

Aspects related to pollen quality and viability

One of the fundamental steps in a breeding program is to determine which varieties or species will be used as pollen donors, which makes a parameter of great importance, the measurement of the pollen quality of possible parents [(5)]. In order to save time and resources, it is not advisable to use pollen from individuals with low pollen viability [(4)].

Having said this, studies aimed at studying quality and viability are necessary to ensure the success of hybridizations and increase efficiency, especially in conditions of the hot and humid tropics, where viability can be strongly affected by environmental conditions [(8)]. In addition to the importance of these studies in genetic improvement, they can also be very useful to determine physiological parameters, such as pollen vigor during storage, germination capacity after exposure to certain conditions, study its interaction with the stigma and fertility, and determine dispersion and gene flow [(5)].

Pollen viability is a parameter strongly influenced by environmental conditions such as temperature, humidity, atmospheric composition and oxygen partial pressure, and by several internal factors such as: duration of

microsporogenesis; interspecific genetic variability or metabolism; number and functionality of the nuclei; protection and exposure within the anthers; relative humidity and temperature at the time of dispersal [5,9-11].

There are different techniques to measure the ovule viability, which are classified into *in vivo* and *in vitro* methods. *In vivo* methods are much more accurate and reliable, but *in vitro* ones are the most widely used because they are simpler and faster to perform [12]. The choice of one method or another will depend on species under study and the final objectives of the research. Due to the above, many studies aimed to distinguish the most appropriate methods to estimate pollen viability. Such is the case of a study performed on wheat pollen [13]. In this experiment, Alexander's staining could not discriminate between viable fresh pollen and non-viable pollen. *In vitro* pollen germination was lower compared to pollen viability assessed by FDA and flow cytometry. Therefore, it is advisable to opt for a combined approach of *in vitro* germination with FDA or flow cytometry to correctly analyze germination potential and pollen viability for wheat.

In another study, triphenyl tetrazolium chloride (TTC) and *in vitro* germination methods were compared to measure pollen viability of *Cistus creticus* L. and *C. monspeliensis* [14]. Significant differences were obtained between the two tests and it was concluded that the in vitro germination test is more appropriate for these species. The above results indicate that the first case to estimate the pollen viability in a species is to determine that the most appropriate method because it can be different in each investigation.

Techniques for estimating pollen viability

The most accurate method, so far, to determine pollen viability, is *in vivo* germination, through which the pollen tube elongation is evaluated to carry out its function as pollinator and the number of seeds produced, in relation to the pollen viability; however, it is necessary to wait for the formation of seeds, which slows down the test and can be a problem when a quick evaluation is required [15].

Among the most commonly used *in vitro* methods to evaluate pollen quality are staining techniques to observe the cytoplasmic content (acetic carmine, acetic orcein, Alexander drug), enzymatic tests (benzidine, tetrazolium salts) and combined methods that allow determining the integrity of the plasma membrane and the presence of enzymatic activity (fluorochromatic reaction using fluorescein diacetate, FDA) and finally germinability tests [12].

In vitro germinability tests involve inducing pollen tube formation in an artificial medium, which is an indication of viability because it reveals the state of membranes, nuclei and the conversion rate of reserves [5]. However, *in vitro* germination depends on the genotype, environmental conditions, pollen maturity, composition and pH of the medium. For the above reasons, it is necessary to determine the optimal conditions for pollen germination of each species [16]. Culture media in liquid and solid condition, enriched with sucrose, calcium or boron are important components for germination, therefore, tests at different concentrations of these nutrients are relevant for the execution of viability tests [17].

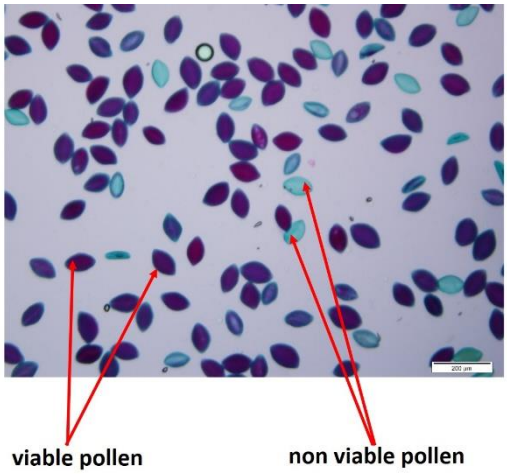
In order to facilitate researchers' choice of culture medium components, researchers at the University of Regensburg, Germany, compiled a compendium including 1572 recipes for culture media successfully used to germinate pollen grains or produce pollen tubes in 816 species representing 412 genera and 114 families (both monocotyledons and dicotyledons) [18]. Among the 110 components recorded from different recipes, the most common are: sucrose (89 % of species), H_3BO_3 (77 %), Ca^{2+} (59 %), Mg^{2+} (44 %) and K^+ (39 %).

Staining techniques to determine pollen viability

Staining methods detect the presence of cytoplasm, plasma membrane integrity or enzyme activity [5]. Most of them are based on the affinity of cells for certain dyes and generally overestimate viability or poorly determine the real germination power of pollen grains or the extension capacity of pollen tubes, but they are fast and feasible to develop when many crosses are made [19,20]. The following are the most commonly used staining techniques to estimate pollen viability:

Acetic carmine: this test measures the integrity of the cytoplasm; that is, pollen grains are colored red when the cytoplasmic membrane is intact and unstained pollen grains are considered non-viable [21,22]. Acetocharmine overestimates pollen viability, but gives additional information on nuclear morphology. In a study carried out on eight accessions of bell pepper at the Federal University of Piauí, Brazil, the method with 2 % reagent concentration proved to be effective [23].

Alexander staining: this method allows differentiation of non-viable pollen from viable one. Among the components of this solution, malachite green dye specifically stains the cell wall, while fuchsinic acid penetrates living cells and colors the cytoplasm red (Figure 1). Aborted pollen appears green, as it retains only its cell wall, while viable pollen grains also show a reddish coloration inside [24]. The method proved to be efficient in estimating pollen viability in 11 wild *Passiflora* species, with 2 % reagent concentration [25].



Viable pollen is observed with the cytoplasm stained, while in non-viable pollen grains, only the cell wall is stained (author's own creation)

Figure 1. Pollen viability test using Alexander stain on *Alstroemeria* (Cartagena)

Trypan blue: this test is based on the fact that when the plasma membrane of the cell is intact, the dye cannot enter the cytoplasm; therefore, the viable grains are not stained, while the non-viable ones are colored intense

blue-violet, because the dye does penetrate, and binding to the proteins present inside the cell. Generally this staining has been used in viability studies of cultured cells [26] and has recently been applied to pollen viability. This method was applied to estimate pollen viability in different olive cultivars, and the authors reported a significant correlation between germinability and this viability test, which indicates that it is adequate in the case of this species [12].

Techniques for testing enzyme activity in pollen

The following techniques allow detecting the existence of enzymatic activity in pollen, by means of color changes in cells:

Tetrazolium salt: this method is based on the use of the compound (2, 3, 5,-triphenyl-tetrazolium chloride), which, in the reduction processes of living cells, takes the hydrogen released by dehydrogenase enzymes and forms a red, stable and non-diffusible substance, triphenyl-formazan. Therefore, viable pollen that has dehydrogenase activity stains red, while non-viable pollen fails to stain [27]. This method was used to analyze the effects of low storage temperatures on the quality of pollen obtained from four cultivars of sweet cherry, showing satisfactory results with a concentration of the reagent of 1 % [28].

Para-phenylenediamine: this test uses para-phenylenediamine (p-phenylenediamine) as reagent and is based on the detection of the presence of peroxidases [5]. Pollen grains that stain dark brown are considered viable, while light brown grains are considered non-viable. It is recommended for future work, to make a primer where the color of the pollen grains is related to the germination percentage obtained in a given sample, since the coloration can vary with storage time, according to the results obtained in *Nothofagus nervosa* [4].

Combined methods for determining plasma membrane integrity and the presence of enzyme activity

Combined methods estimate pollen viability by checking membrane integrity and the presence of enzyme activity in the cytoplasm at the same time. The most commonly used techniques are described below:

Fluorochromatic reaction using DAF: this method is one of the most widely used in pollen viability studies. DAF is an apolar ester, which allows it to cross the cytoplasmic membrane. Once inside the cell, the ester is hydrolyzed by esterase enzymes found in the cytoplasm and releases a fluorochrome, which when excited at an appropriate wavelength (490 nm), emits bright green fluorescence. Since the fluorochrome is polar, it cannot exit through the cytoplasmic membrane and is retained in cells with an intact cytoplasmic membrane. As a consequence, only pollen grains with adequate levels of esterase activity and with an intact cytoplasmic membrane will show greenish fluorescence, indicating that these grains are viable [29]. This method was successfully applied to estimate pollen viability in *Flaveria bidentis* and *F. haumanii* in Santiago del Estero (Argentina) [30].

Fluorescein diacetate reaction (FDR): this test works much like the FDA. Pollen grains are mounted in fluorescein diacetate, which is an apolar compound that, like FDA, rapidly penetrates the pollen cytoplasm and is hydrolyzed by esterases, leaving the polar and fluorescent fluorescein free [31].

Aspects of floral biology of importance for plant breeding

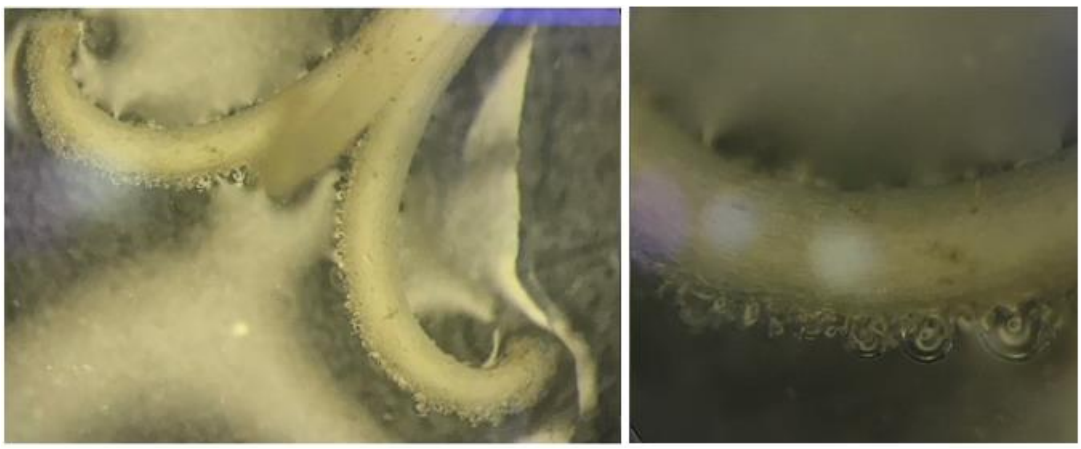
For classical genetic improvement, based on the selection of superior individuals and directed crosses, it is important to know aspects of the floral biology of plant species under study. In order to obtain successful fertilizations, it is not only necessary to estimate the quality pollen of the species used as male parent, but also at what moment the stigma of the female parent is receptive, for which stigmatic receptivity studies are carried out. Likewise, it is necessary to know if there is self-incompatibility in the species being worked on, in case self-pollinations are required to obtain pure lines. For this purpose, it is necessary to study the growth of the pollen tube, to know the place where self-incompatibility occurs and to find a way to avoid this barrier.

Stigmatic receptivity

Stigmatic receptivity reflects the ability of the stigma to receive pollen, allowing it to adhere, hydrate and finally germinate [6]. Pollination consists of the transfer of pollen from the male sexual organs to the female sexual organs, but for this process to occur, the transfer of pollen to the stigma must occur during the period when the stigma is receptive, otherwise the pollen cannot adhere and cannot germinate [32,33].

In nature; generally, once the flowers open, the stigma is receptive, but in the case of genetic improvement, many times the need to perform directed pollinations in the bud stage prevails. This is done in order to perform pollination at the same time of spaying, avoiding contamination with pollen grains foreign to the one used as parental [6].

At floral maturity, when the stigmas are ready for pollination, these are characterized by having high peroxidase enzyme activity; therefore, in order to determine the receptivity of the stigma, hydrogen peroxide is used to check for the presence of enzymes when bubbling occurs [34] (Figure 2). There is a scoring criterion to convert stigmatic receptivity into a quantitative variable and thus find the average receptivity per day evaluated, for which four levels of bubbling were proposed [35].



Bubbling can be observed on the surface of the stigma, indicating that it is receptive (author's own creation)

Figure 2. Stigmatic receptivity technique in *Alstroemeria*, using hydrogen peroxide

Stigmatic receptivity can be affected by environmental conditions, such as temperature, chemical application and plant nutrition, although generally, in studies on this subject, the male part has received the most attention. It has been shown that, under field conditions, temperature, through its effect on stigmatic receptivity, affects the effective pollination period and fruit set in several fruit species, such as apricot [36,37] or kiwifruit [38]. In peach, it was shown that under controlled conditions temperature had a great influence on the stigmatic receptivity duration which decreases with increasing temperature. The stigmatic receptivity loss was manifested in three consecutive processes: pollen adherence to the stigma, germination and penetration into the transmitting tissue. The loss of these capacities is gradual, first the capacity to penetrate the transmitting tissue is lost; secondly, germination and finally the adherence of pollen to the stigma [39].

Plant incompatibility systems

In traditional breeding, it is essential to have pure lines to make uniform hybrids; however, the development of pure lines requires self-pollination to induce a change in allele frequency and achieve homocigosis. According to the above, it is assumed that all plant species can be capable of self-pollination; however, the reality is not so, since self-incompatibility is present in more than half of the Angiosperm species. In the case of self-pollinated plants, obtaining pure lines is easy, because self-pollination occurs to a large extent; however, it is proposed that they have, with open pollination, 5 % of allogamy. In cross-pollinated plants, due to self-incompatibility mechanisms, self-pollination is often difficult [40].

Breeders resort to various techniques such as pollination between full sibs or half sibs, backcrossing or variability induction, making dialelic designs with various compatible species and, if this is not successful, biotechnological techniques are used to overcome pre- or postzygotic self-incompatibility barriers, haploid induction, among others.

Self-pollination is not possible in many plant species due to a mechanism known as self-incompatibility. This mechanism ensures genetic variability, avoiding inbreeding depression and promoting cross-pollination, so its effectiveness ensures the success of species evolution [41].

As part of evolution, to promote cross-pollination, several hermaphrodite species developed morphological adaptations such as, for example, the spatial separation of the pistil and stamens (hercogamy) and physiological adaptations such as differential maturation of the reproductive organs (dichogamy). These modifications largely prevent self-fertilization and promote cross-pollination, but there is still the possibility of gene flow through pollen to the parents or to other individuals in the progeny. To avoid such an event, a large number of species have developed a genetic-biochemical pollen recognition mechanism known as a sexual incompatibility (SI) system, which is defined as the inability of a fertile hermaphrodite plant to produce zygotes after self-pollination [40].

Classification of incompatibility systems

Incompatibility systems have been classified according to the genotype that determines it; if it is determined by the haploid genotype of the pollen, it is called gametophytic incompatibility; if on the other hand, it is determined by the diploid genotype of the plant that gives origin to the pollen, it is called sporophytic incompatibility [42].

Self-incompatibility can be expressed in the stigma, style or flower ovary, due to an accumulation of callose at the tip of pollen tubes and, therefore, the elongation of pollen tubes is prevented at some point in their trajectory to the oosphere. This accumulation of callose makes it possible to differentiate between compatible and incompatible pollen tubes [43,44]. In the case of gametophytic incompatibility, the interruption of pollen tube growth occurs in the style, whereas in sporophytic incompatibility it generally occurs in the stigma [41]. There is a third type of poorly studied self-incompatibility that occurs in the ovary, known as late-acting self-incompatibility, which is caused by inhibition of pollen tubes before they reach ovules, inhibition of fertilization, post-zygotic rejection, and inhibition in the ovule [45].

Performing self-incompatibility studies allows the breeder to save time and resources, since it can know which route to take at the moment of advancing the selection of the segregating generations.

Incompatibility studies were carried out in *Olea europea*, with the aim of explaining how self-fertilization occurs in some varieties, despite being a species with high degrees of self-incompatibility. The authors suggest that some determinants of self-incompatibility in the pollen tube and stigma are unstable and degrade, allowing the pollen tubes to reach the ovule and fertilize it. This explains that self-incompatibility may not be absolute and, therefore, some degree of self-fertilization may occur [46].

Apart from the intraspecific incompatibility described above, there is another mechanism known as interspecific incompatibility, where pollen is rejected because of the great genetic dissimilarity between the donor and recipient species [47]. This often occurs because the pollen cannot be nourished by the extracellular matrix of the maternal tissue [16] and nutrients of the maternal tissue are not sufficient to generate all the substances necessary for the tube growth to ovules [29]. This process often makes hybridization impossible, which makes it necessary to know at what moment the interruption of pollen tube growth is generated in order to be able to make decisions. This type of incompatibility has been studied in Solanaceae, where the cessation of pollen tube growth occurs along the style [44], as occurs in gametophytic self-incompatibility [48]; therefore, it is logical to think that both recognition processes could be similar [49].

A study was conducted in *Nierembergia* of interspecific crosses, in which different patterns of pre-zygotic incompatibility were detected. Studies of the stigma-pollen/style-pollen tube relationship showed that interspecific incompatibility is manifested by inhibition of pollen tube growth at different levels of the style. In no case were signs of incompatibility found at the stigma level [49].

To observe the pollen tube development, pistils are collected at intervals of one day after self-pollination or cross-pollination, until at least four days after pollination. The technique consists of staining the pistils with aniline blue, since this compound reacts with the callose in the pollen and, therefore, the path of the

pollen tube can be seen through the style under a fluorescence microscope. To analyze in which part of the pistil the interruption of the pollen tube occurs, the pistil is divided into four parts: stigma, first half of the style, second half of the style and ovary [41]. Figure 3 shows the growth of the pollen tube in *Alstroemeria* in the style and ovary.

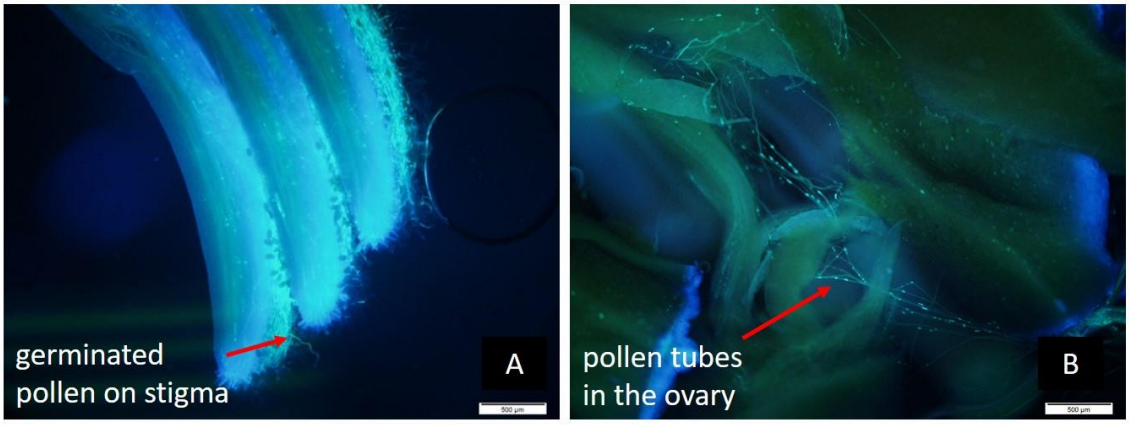


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Figure 3. A. Germination of *Alstroemeria* pollen on the stigma and progress of pollen tubes. B. Pollen tubes in the ovary of *Alstroemeria*

Cytogenetic studies for plant species karyotyping

Karyotyping consists of the study of genome structure including chromosome number and thus ploidy level, analysis of chromosome morphology (absolute and relative size, centromere position, location and number of satellites [50]. When selecting parents for crosses in a breeding program, it is important to have knowledge about chromosome number. If cultivars with differences in chromosome number are involved, sexual reproduction may be affected and, therefore, it may be impossible to obtain seed.

Chromosome number can be very variable, even within cultivars of the same species, as was found in a study on mulberry, where aneuploid and diploid varieties were found in *Morus indica* [51]. The phenomenon of polyploidy can occur and, therefore, new basic numbers can arise that have no direct relationship with the ancestral ones, due to new restructurings or hybridization between polyploids with different basic numbers. Furthermore, it is difficult to deduce differences in ploidy without cytogenetic analysis, because variations in karyotype can occur without noticeable changes in phenotype [52].

Karyotype studies can be performed by the discipline known as cytogenetics, which deals with the structure and behavior of chromosomes, as well as the genetic implications derived from their study [53]. In the last 20 years, cytogenetic studies have focused on the structure and evolution of the genome of different plant species, both wild and cultivated [7]. Cytogenetic analysis generates basic knowledge on the number and shape of chromosomes for subsequent studies focused on the use of biological diversity for breeding purposes [54]. This

type of studies was carried out in diverse crops such as *Triticum aestivum* [55], *Dipteryx alata* [56] and *Hibiscus rosa-sinensis* [57].

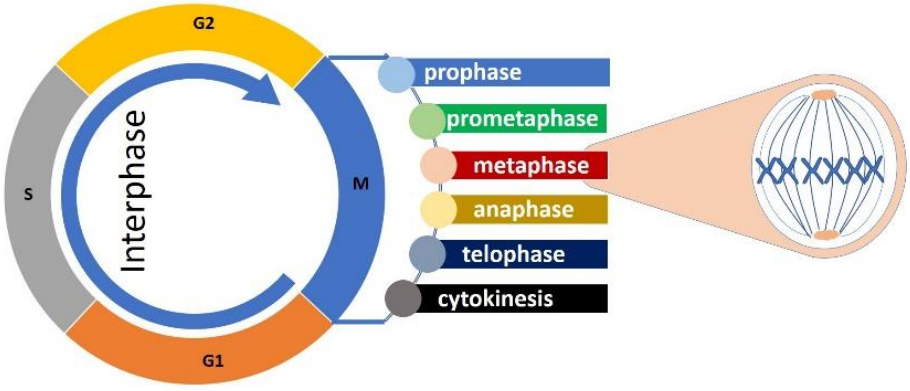
Cytogenetics is divided into classical and molecular cytogenetics. The former includes cytological studies in which chromosomes are analyzed under the microscope during the metaphase of mitosis or meiosis. Molecular cytogenetics could be considered the fusion between classical cytogenetics and molecular biology. This discipline groups a set of techniques that apply different methods of molecular biology directly on cytological preparations, such as tissues, cells, chromosomes and DNA fibers [7].

Classical cytogenetics

Classical cytogenetics consists of observing chromosomal characteristics in cells that are in the metaphase of mitosis or meiosis under a microscope. For this purpose, root cells are used in the case of mitosis or gametes in the case of meiosis.

Determination of chromosome number during mitosis

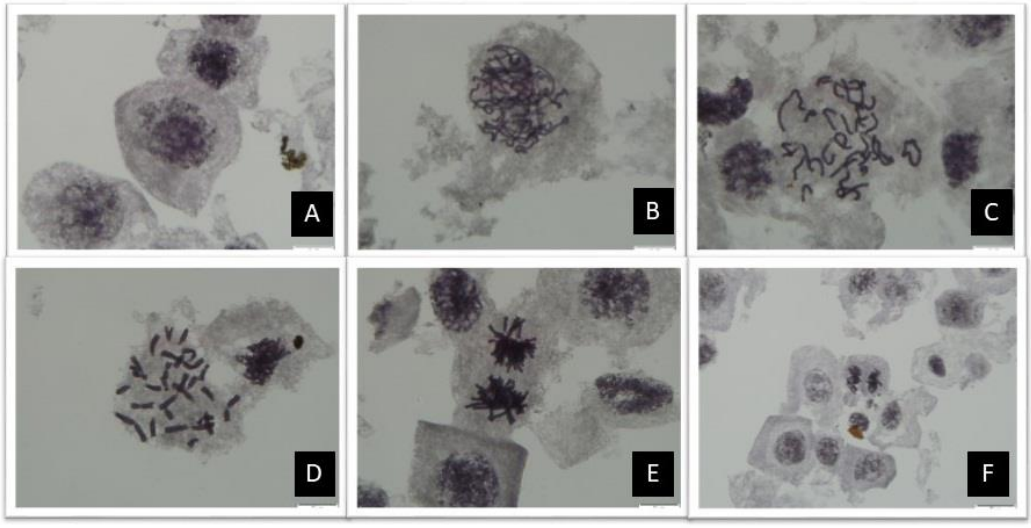
The cell cycle comprises four sequential ordered phases, which temporally distinguish the replication of genetic material from the segregation of duplicated chromosomes into two daughter cells. The G1 and G2 phases are referred to as "gaps", because no highly visible processes occur in the cell nucleus at these stages. However, the cells are actually very active, as they are growing and preparing for division. The S-phase refers to synthesis, in which DNA is copied or replicated, and the M-phase to mitosis [58]. The different phases of the cell cycle can be seen in Figure 4.



In M-phase mitosis occurs, which is divided into prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis.
In metaphase chromosomes are well defined and aligned in the equatorial plane of the cell; therefore, they can be easily observed under the microscope.
(author's own creation)

Figure 4. Cell cycle, composed of G1, S, G2 and M phase

Mitosis is divided into five phases: prophase, prometaphase, metaphase, anaphase and telophase. During metaphase chromosomes are arranged towards the equatorial plate and it is the appropriate moment for chromosome counting [59]. Figure 5 shows the different phases of mitosis in a meristematic tissue of *Altroemeria*, in an experiment developed by the authors at the Institute of Floriculture of Argentina, where in metaphase the chromosomes are perfectly visible.



A. Interphase or growth phase, B. Prophase (condensed chromosomes are observed, but the number cannot yet be defined)**, C.** Prometaphase (chromosomes begin to define themselves), **D.** Metaphase (chromosomes are well defined in the equatorial plane of the cell)**, E.** Anaphase (sister chromatids are migrating toward opposite poles), **F.** Telophase (the new nuclear envelope begins to form). (Author's own creation)

Figure 5. Phases of the cell cycle in *Alstroemeria* meristematic cells

In the first karyotyping studies, cytologists, in order to observe chromosomes under the light microscope, fixed the meristematic tissues in kerosene blocks and then made micrometer cuts. At the time, this technology provided good results, until the emergence of the root tip crushing technique known as "squash" [60].

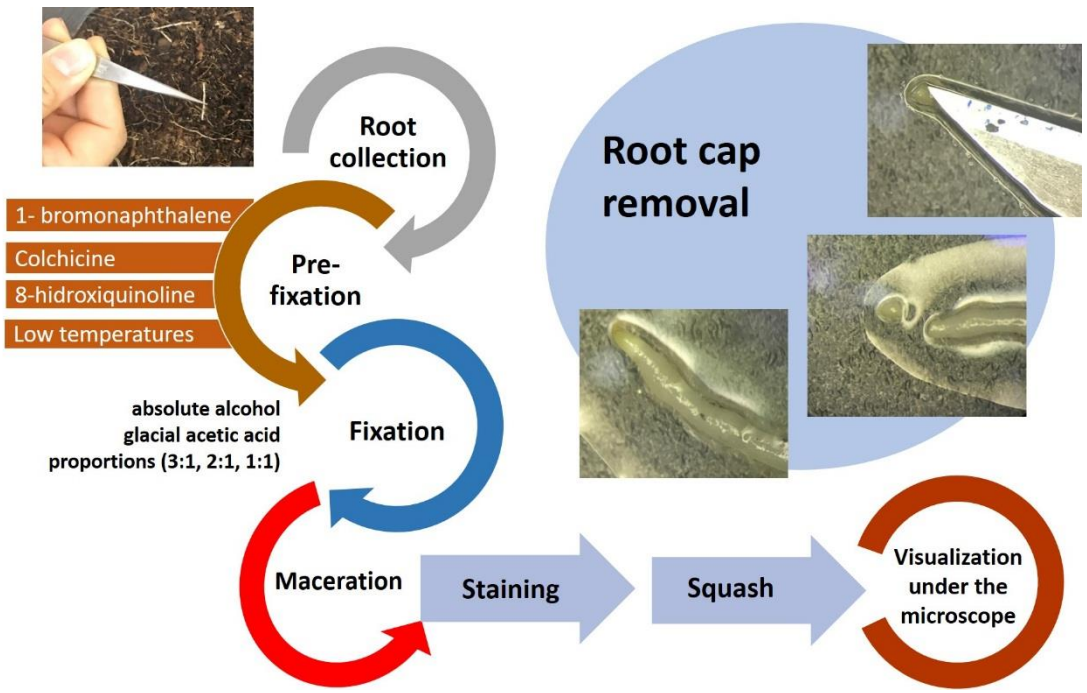
Chromosomes are found in cells of all organisms, but are visible when they are undergoing mitotic or meiotic division. If it is desired to analyze the chromosome number during mitosis, meristematic tissues must be used, which are characterized by constantly dividing cells with large nuclei [61]. The meristematic tissues most used by cytologists are those found in the apical zone of the root [60].

Firstly to take into account for the determination of the chromosomal number, through the observation of mitosis, is to determine the most appropriate time for the pretreatment of roots [62]. An experiment is carried out to find when a greater number of metaphase cells are found, which can be different in each locality, due to environmental conditions [60]. For this purpose, Valladolid, proposed to fix roots in the mixture absolute alcohol: glacial acetic acid (3:1) at different hours of the day, macerate and stain the samples to observe them under the optical microscope and later note the frequency of interphases, prophases, metaphases and telophases for each hour of collection [60].

Subsequently, the pretreatment that consists of putting the tip of the root in contact with substances called mitosis inhibitors was proceed (Colchicine, 8-hydroxyquinoline, 1-bromonaphthalene, low positive temperatures, etc.) to obtain a greater number of metaphase cells in the meristem. Metaphase is the mitosis stage, where chromosomes reach their maximum degree of condensation and they are individualized; therefore, it is very easy to count and characterize them. Mitosis inhibitors prevent the achromatic spindle formation and the cells do not pass to anaphase, the next stage of mitosis [62,63].

Mitosis inhibitors have also been used to induce polyploids with novel characteristics. In a study conducted in *Impatiens walleriana*, the application of colchicine at 0.05 % produced the highest tetraploid induction efficiency; therefore, this concentration would be a good starting point for inducing tetraploids in the future [64]. In another study, the effect of another mitosis inhibitor, oryzalin, was evaluated in *I. walleriana* and proved to be very effective for tetraploidy induction [65]. In both experiments, tetraploid plants exhibited greater leaf area, leaf thickness, ovary width, pollen length, and stomatal size and showed decreases in height, stomatal guard cell density, and flower number.

The next step consists of fixation in order to rapidly interrupt the vital processes of the sample to preserve cell structure [66]. Chemical agents or enzymatic treatments (cellulases and pectinases) are then used to remove the cell wall of cells and disperse them. The chromosomes are then stained to make them visible under the light microscope. Basic stains are used for this purpose, due to the acidic nature of chromatin. Once the cells have been stained, they are squashed, so that the chromosomes are dispersed and placed in the same plane. Finally, the sample is observed under an optical microscope [62]. Figure 6 shows different steps for the observation of chromosomes in metaphase.



The elimination process of the root cap to expose the meristematic tissue is observed (author's own creation)

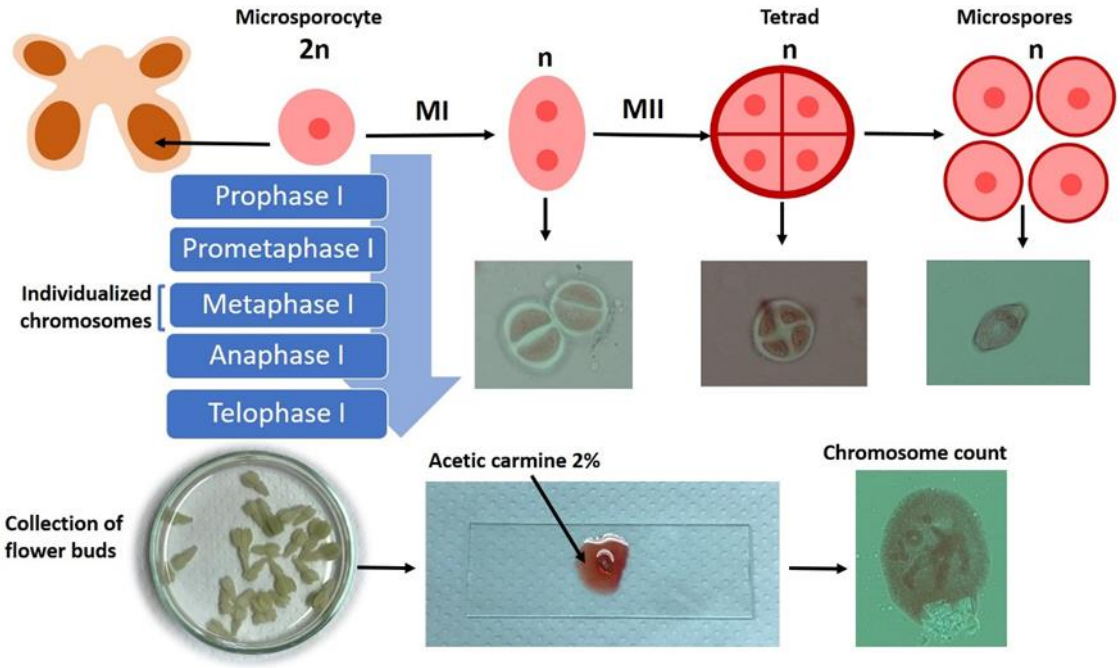
Figure 6. Different steps to observe chromosomes during metaphase mitosis

Despite the advancement of technology and the emergence of new techniques for karyotyping, chromosome counting during mitosis is still performed in many cultures. For example, the chromosome number of 14 species of *Iris* was determined by classical cytogenetics in Korea [67].

Determination of chromosome number in meiosis

In higher plants, meiosis takes place only in the mother cells of the pollen grains located in the microsporophyll (gymnosperms) or in the anther (angiosperms) and in the mother cells of megaspores located in the ovule (gymnosperms and angiosperms). As in mitosis, to analyze chromosome number during meiosis, it is performed during metaphase I or metaphase II. If it is performed during metaphase II, it is important to take into account that the chromosome number is reduced by half [68] (Figure 7).

Studies of chromosomal behavior during meiosis can detect chromosomal abnormalities that affect plant fertility. For example, a study was developed in three species of the genus *Capsicum*, where different meiotic abnormalities were observed, which can interrupt the process of cell division creating different chromosome numbers and decreasing pollen fertility. The following chromosome number variations were observed: 2n=2x=24, 2n=2x=26, 2n=4x=48 to 2n=6x=72 [69].



It was observed how meiosis occurs for the formation of pollen grains in plants. The first division occurs in the pollen sacs of immature anthers where the diploid microsporocyte results in two haploid nuclei. The second division results in the tetrad of microspores, also haploid. The ideal time to observe chromosomes is during Metaphase I, where they are individualized and easy to count. For this process, the buds are collected, the immature anthers are fixed in Farmer's solution and 2 % acetic carmine is applied to color the chromosomes. (author's own creation)

Figure 7. Chromosome counting during meiosis metaphase

Molecular cytogenetics for chromosomal analysis

As previously mentioned, for the development of a breeding program, a key step is the karyotypic characterization of cultivars to be used as parents. Currently, in well-equipped laboratories, techniques much more feasible than classical cytogenetics can be performed, such as: flow cytometry, fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) [(2)]. These techniques are grouped in a discipline known as molecular cytogenetics, which is defined as the fusion between classical cytogenetics and molecular biology [(7)].

Flow cytometry for chromosomal studies

Flow cytometry is a powerful tool for the analysis of nuclear DNA content (in its relative or absolute values) of plant cells [(70)]. It consists of analyzing particles flowing in a liquid suspension, due to optical properties (light scattering and fluorescence) [(71)]. Since the 1980s, application spectrum of flow cytometry for plant genome analysis has increased exponentially and it is now a routine technique used in many laboratories around the world [(72)].

Flow cytometry makes it possible to determine the number of nuclei, previously isolated and labeled with a fluorochrome, present in each phase of the cell cycle (G0/G1, S and G2/M). First, the fluorescence measurements of the nuclei are expressed on an arbitrary scale, then compared with the fluorescence of nuclei isolated from a reference standard with known genome size to estimate the nuclear DNA content of a given individual or tissue [(72)].

This technique was used to determine the ploidy of a CIAT (Colombia) germplasm collection of tropical grasses of the genera *Brachiaria*, *Megathyrus* and *Panicum*. In this way, the reproductive mode of the analyzed accessions was determined in order to select male and female parents in a crossbreeding program [(73)]. Moreover, the nuclear DNA content and ploidy level of 19 accessions of *Gagnepainia godefroyi* and *G. harmandii* in Thailand were estimated by flow cytometry [(74)].

FISH (fluorescence *in situ* hybridization) for chromosome characterization

FISH, unlike conventional cytogenetic techniques, is based on specific molecular reactions between chromosomal DNA and any other sequence called a 'sonda probe'. The principle of this technique consists of the hybridization of DNA probes, previously labeled with a specific fluorochrome, directly on the chromosomal DNA, taking advantage of the homology existing between them. For the detection of such probes, the chromosomal preparations are exposed to ultraviolet light, which causes the excitation of the electrons of the fluorescent molecule, which is characterized by an emission of rays, whose wavelength varies, depending on the type of fluorochrome used.

The adaptation of FISH protocols to an increasing number of plant species has opened up new possibilities for the study of plant genomes [(7)]. For example, using this technique, cytogenomic characterization of twenty

accessions covering eight species of the genus *Hedysarum* was performed for the first time to assess genetic diversity and karyotyping [75].

One of FISH modifications is called genomic *in situ* hybridization (GISH), which allows differential coloring of chromosomes from different ancestors (in the case of a species) or parental genomes (in the case of a hybrid) [7]. Recently, GISH in combination with FISH was applied for the study of chromosome structure in *Medicago sativa*, due to the fact that this species has very small chromosomes, which makes it difficult to study by means of classical cytogenetics [76].

Another variant of the technique is called Comparative Genome Hybridization or CGH (for *comparative genome hybridization*), in which the genomic DNA of a standard species (for example, Arabidopsis) is used as a probe to hybridize it on chromosomes of another species, which allows analyzing the karyotype of a given species with a molecular approach, without the need to use specific sequences that are difficult to obtain. This cross-hybridization generates a series of bands on chromosomes of the species studied, which correspond to conserved genomic regions (usually repetitive sequences) between the two species [77,78]. For example, this technique was recently used to study the chromosomal structure of two species of the genus *Carex*, and to test the taxonomic hypothesis of hybrid origins in *Carex salina* and *C. ramenskii* [79].

CONCLUSIONS

- The most commonly used method to estimate pollen viability in the case of male parent selection in a breeding program is *in vitro* germination. However, in the case of having many genotypes to evaluate, staining methods are more recommended because of their speed.
- The most effective method for determining stigmatic receptivity for the choice of the female parent is the use of hydrogen peroxide.
- Performing self-incompatibility studies, by observing pollen tube growth, allows the breeder to save time and resources in a breeding program. It is also advisable to carry out self-pollination and flower isolation trials, if the necessary equipment is not available.
- Knowledge of chromosome number is important when selecting parents for crosses; the most commonly used method for this purpose due to its versatility and speed is flow cytometry.

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