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Technical Manual

Potato reproductive and cytological biology

Benny Ordoñez, Matilde Orrillo and Merideth Bonierbale

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Foreword

The techniques and protocols described in this manual are those currently used in the cytogenetics laboratory of the International Potato Center (CIP, in its Spanish acronym).

They refer mainly to the determination of ploidy in somatic cells, chromosomal behavior in sexual cells, determination of pollen fertility, and *in vitro* culture of immature embryos in *Solanum spp*.

These techniques are valuable tools when studying mating and cross-over between homologous chromosomes, the pollen-pistil relationship, identification of barriers of incompatibility, meiotic irregularities in interspecific hybrids, and the formation-production of gametes. Therefore, these techniques have applications not only in the genetic improvement of potato, but also in taxonomic and evolutionary studies.

Part One of the Manual includes theoretical concepts that make it easier to understand the different techniques and the interpretation of results: a great help for designing a good breeding plan. Part Two presents the basic cytogenetic techniques used in potato breeding. The potential applications are mentioned for each technique, and the procedures used are described in detail in a separate table. Finally, the Annex describes the reagents and solutions required, and how to prepare them, as well as the pollination techniques and obtaining of botanical seed used in the breeding work carried out in CIP greenhouses.

We are sure of the importance of this publication for the field of cytology as applied to potato breeding, not only for its structure, but also for the richness of its contents, which make for easy and useful application for those who need a rigorous scientific approach to basic cytology.

The authors

Introduction

The *Solanaceae* family comprises the largest group of angiosperms in the world. Within this family, the most economically important species is the cultivated potato, *Solanum tuberosum*, which is autotetraploid (2n=4x=48). It has 4 sets of similar chromosomes (where "n" is the gametic number and "x" is the basic number). The potato forms a polyploid series consisting of diploid species (2n=2x=24), triploids (2n=3x=36), tetraploids (2n=4x=48), pentaploids (2n=5x=60) and hexaploids (2n=6x=72), the diploids accounting for 70% of the species. *Hawkes* (1990) recognized 232 species divided into 21 series. The tetraploid species cover a wide range of distribution, from the southern part of the United States to the southernmost region of Chile, and are the most economically important. However, the diploid level has greater genetic diversity associated with resistance to various biotic and/or abiotic factors and consequently, it is of great use in the genetic breeding of the cultivated species and has a greater concentration of diversity in the Andes.

Through the use of cytological techniques, ploidy, pollen viability and fertility can be determined, as well as the presence of non-reduced gametes, among other important characteristics in the different potato species. The results obtained provide the knowledge required for an efficient and effective manipulation of the wild germplasm and potato cultivation, thus contributing to the development of more efficient strategies for the transfer of desirable characteristics such as resistance to biotic and/or abiotic factors.

Part One: Potato reproductive biology

Theoretical Basis

1. The life cycle of potato

As occurs in angiosperms, in the life cycle of the potato there is a diploid phase (2n), which comprises a series of divisions by mitosis (leading to an equitable distribution of hereditary material), followed by a haploid phase (n); it begins by meiosis division and ends with the fusion of two haploid nuclei (gametes) forming the diploid zygote (2n). Conventionally, the haploid phase is called gametophyte and the diploid phase, sporophyte.

1.1 Asexual Reproduction

The vegetative or asexual reproduction of the potato ensures the clonal conservation of the genotype. Generally speaking, a new plant is formed from vegetative parts (tubers, sprouts or buds) giving rise to clones; that is, plants genetically identical to the original plant. This type of reproduction occurs through mitosis.

In potato breeding, asexual propagation facilitates the fixation, selection and multiplication of genotypes. Tissue cultures and meristem cultures for the eradication of pathogens are other practical applications of asexual reproduction.

1.2 Sexual Reproduction

Sexual reproduction in potatoes enables the genetic material of two individuals to be exchanged to form new allelic combinations giving rise to new genotypes. This requires the participation of the male (anther) and female (pistil) reproductive organs, followed by a process of pollination and fertilization to form berries with seeds, each constituting a new individual.

1.2.1 Floral Morphology

The flowers of the potato are assembled in aggregates called inflorescences. The inflorescence is supported by the peduncle, and its branches (pedicels) end in flowers.

The flower contains two sets of floral envelopes: the calyx (five sepals) and the corolla (five petals), which form the floral receptacle located beneath the flower's reproductive organs, stamens and pistil (Fig. 1).
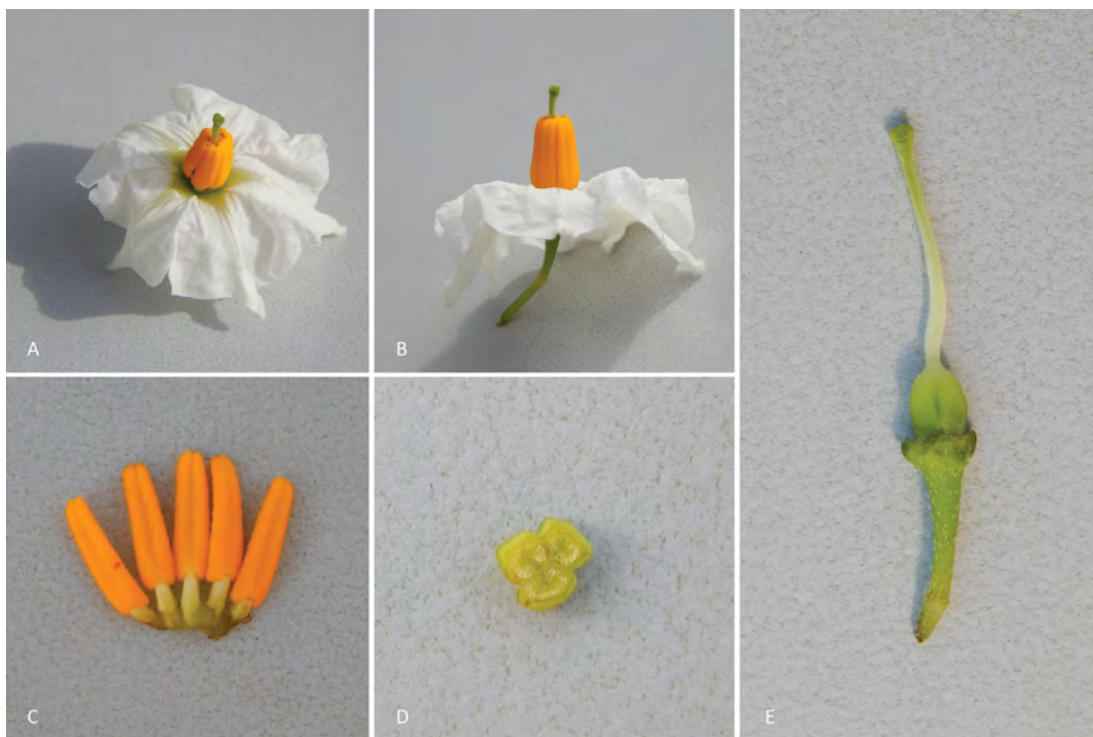
Fig. 1. Floral morphology of a potato flower:
 A) Corolla (frontal view), B) Corolla (side view), C) Stamens, D) Ovary and E) Pistil.

The stamen consists of a short filament, which holds the anther, with two lobes containing the pollen. The five stamens are separated from the corolla. The pistil comprises the ovary, which contains 300-600 ovules, the stigma - which receives the pollen - and the style, which connects both parts. Each ovule contains a mother cell of the megaspores, surrounded by a tissue called nucellus and an integument.

1.2.2. Megasporogenesis

Megasporogenesis is the process of forming reproductive cells called embryonic sacs, which contain the female gamete or oosphere. Inside the ovum, a stem cell (2n) is divided by meiosis, forming in its first division a pair of haploid cells. The second meiotic division produces four haploid megaspores.

Three of the megaspores will degenerate and the fourth (functional megaspore) undergoes three mitotic divisions without cytokinesis, giving rise to eight haploid nuclei, and when these align they form the embryonic sac. Three nuclei, located toward the sac's micropyle (opening in the integuments through which the pollen tube will penetrate) constitute the two synergids and the oosphere (female gamete). These three are located at the opposite end toward the chalaza, and are called antipodes. The two located in the center of the embryonic sac are the polar nuclei which then fuse to give rise to a diploid nucleus.

1.2.3. Microsporogenesis

Microsporogenesis is the process through which pollen grains are formed. This process takes place in the sporagenous tissue of the anthers. A pollen stem cell (microsporocyte, 2n) is divided by meiosis. During the first division a pair of haploid cells (dyads) are formed. The second meiotic division produces a tetrad of haploid microspores. Each microspore or pollen grain undergoes two mitotic divisions without a cytoplasmic division forming the male gametophyte. The first mitotic division produces a cell with two haploid nuclei: a vegetative nucleus (that will form the pollen tube on germinating in the stigma) and a generative one. The second mitotic division (which occurs after the development of the pollen tube) occurs only in the generative nucleus, and forms two spermatic nuclei.

1.2.4. Fertilization

When the anthers become dehiscent, pollen grains are transferred to the stigma by insects, or by human action. This process is known as pollination. Once in contact with the stigma, the pollen grains germinate and form the pollen tube that grows through the stigma and style, toward the ovules in the ovary. The generative nucleus of the pollen grain divides and forms into the two male gametes (spermatic nuclei) that move downward through the pollen tube. The tube penetrates the ovule through the micropyle and grows into the embryo sac through one of the synergids flanking the oosphere, releasing the spermatic nuclei.

A double fertilization occurs inside the embryo sac. One of the sperm nuclei (male gamete, n) fuses with the oosphere nucleus (female gamete, n) giving rise to the zygote (diploid, 2n), which then develops to become the embryo. The other spermatic nucleus joins together with the two polar nuclei forming the

nutritional reserve tissue called endosperm (triploid, 3n), important for the embryo's development and growth.

The embryo then goes through its early stages of development as it lies within the ovary of the flower, the integuments develop on the seed cover, and the ovary matures to become the fruit.

2. Cell Division

This is an ongoing process that occurs in all living organisms. The stages through which a cell passes, from one cell division to the next, constitute the cell cycle. The cell cycle is divided into two main phases:

The period of division or Phase M (mitosis or meiosis) and the interphase, so named because it was previously thought to be a cellular resting stage between divisions. Actually, in this phase a cellular division checkpoint is carried out, where the regulatory systems (blockers or inducers) act; this is the cell's period of maximum metabolic activity.

The Interphase comprises three stages:

• G1 (active cell growth phase, synthesis of proteins and RNA),

• S (DNA synthesis phase, the genetic material is replicated giving rise to two new chains), and

• G2 (another stage of growth, briefer than G1, in which the products necessary for cell division accumulate).

2.1 Mitosis

This is the process whereby genetic material, previously duplicated, is evenly distributed giving rise to two genetically identical cells. The number of chromosomes remains constant through successive cell divisions, with an exact chromosomal distribution in the newly formed cells, maintaining the original ploidy. Mitosis takes place mainly in meristems, the tissues at the ends of stems and in roots where plant growth can take place. These structures have the ability to divide throughout the entire life cycle of the organism.

Mitotic cell division consists of two sequential processes: nuclear division (karyokinesis) and cytoplasmic division (cytokinesis).

There are four stages in mitosis: prophase, metaphase, anaphase and telophase. Each stage can be recognized by the arrangement of chromosomes in the cytoplasm.

2.1.1. Prophase

Chromosomes appear and can be observed clearly; they are sufficiently condensed and are visible under an optical microscope. Each chromosome consists of two longitudinal copies, known as sister chromatids, which are clearly seen joined by centromeres. During the prophase the nucleolus loses visibility, the nuclear membrane disappears, and the achromatic spindle begins to establish itself. The latter consists of polar fibers, which extend from each pole of the spindle toward the central region, and of kinetochore fibers that insert themselves into the kinetochores (protein structures located on each side of the centromere) of duplicated chromosomes. The spindle fibers make it possible for the sister chromatids to separate during mitosis, while the chromosomes begin a process of shortening and thickening. By the end of the prophase, the chromosomes are completely condensed and, with the disappearance of their nuclear envelope, they are now in direct contact with the cytoplasm.

2.1.2. Metaphase

At the end of the prophase, the sister chromatids are joined to each other and also to the kinetochore fibers. During metaphase, these fibers are lengthened through polymerization and proceed to displace the chromosomes toward the center of the spindle where they form the equatorial or metaphase plate. The chromosomes reach their highest degree of shortening and condensation, appearing perfectly individualized, and thus visible under an optical microscope.

2.1.3. Anaphase.

Centromeres become doubled and divided. The sister chromatids separate and each chromatid becomes a new chromosome. As the anaphase progresses, the two identical sets of chromosomes are directed toward the cell's opposite poles through the fibers of the mitotic or achromatic spindle.

2.1.4. Telophase.

This begins when the offspring chromosomes reach the cell's poles. The chromosomes lose their state of condensation and begin to elongate, the mitotic spindle disappears, and the nuclear envelope and nucleolus form.

Simultaneously, cytokinesis is under way and the formation of the cell plate is completed, giving rise to the new cellular wall between all newly formed cells.

2.2. Meiosis

This is the process whereby specialized cells are obtained that intervene in sexual reproduction. In meiosis two cell divisions occur, resulting in four cells with the number of chromosomes reduced to half. These two successive divisions, known as meiosis I (first meiotic division) and meiosis II (second meiotic division), are preceded by a single DNA duplication.

In every diploid cell, one of the chromosomes comes from the gamete of one of the progenitors and its pair from the gamete of the other progenitor. These pairs of chromosomes, which resemble each other in shape, size, and type of hereditary information they contain, are known as homologous pairs. In Meiosis I, a series of genetic material exchanges occur between homologous chromosomes. This process is dynamic; it encompasses attraction, mating, exchange and subsequent separation of homologous paternal and maternal chromosomes. The distribution of chromosomes of each homologous pair occurs at random, which contributes to the genetic variability of the gametes.

In the second meiotic division (Meiosis II) the distribution of sister chromatids of each chromosome occurs between the nuclei of the daughter cells.

Each meiotic division is divided into prophase, metaphase, anaphase and telophase.

2.2.1. Meiosis I

Prophase I. In this phase, chromosomal cross-over occurs (homologous chromosomes mate and exchange fragments of genetic material). This meiotic phase, the longest and most complex, comprises five sub-phases:

Leptotene. In this phase the nucleus increases in size. Chromosomes begin to condense into filaments within the nucleus and become visible, resembling long thin strands. Further examination reveals that the chromosomes are clearly double, in other words, they have divided longitudinally into two chromatids. The nuclear membrane and nucleolus are present. In addition, the development of small areas of thickening along the length of the chromosome can be observed: these are called chromomers, and they give it the appearance of a pearl necklace, (Fig. 2A).

Zygotene. The mating and pairing of homologous chromosomes (paternal and maternal) begins, a process called synapses (Fig. 2B). Homologous chromosomes draw near and join along their entire length through a synaptonemal complex of protein nature, forming a bivalent. Each homolog consists of two sister chromatids and the homologous pair consists of four chromatids. The mating between homologs also involves the gene sequence of each chromosome, which prevents the mating of non-homologous chromosomes.

Pachytene. Once the bivalents are formed, the phenomenon of cross-over occurs, in which the homologous chromatids break to an identical level and exchange genetic material (Fig. 2C). The resulting genetic recombination greatly increases the genetic variation among the progenitors' offspring.

Diplotene. The homologous chromosomes begin to separate from each other, except at the sites where recombination exchanges called chiasmas took place. The nucleolus and nuclear membrane begin to disappear and cross-over becomes visible (Fig. 2D). The result is the formation of new genetic combinations, with altered genetic information localized in the chromosomes involved.

Diakinesis. At this stage, the bivalents are now more condensed. The achromatic spindle begins to appear, and the bivalents end up bound to the spindle via their centromeres. The nuclear membrane disappears (Fig. 2E).

Metaphase I. The bivalents place themselves on each side of the equatorial plate (Fig. 2F). The spindle has formed completely and each homolog has its centromere. Thus, the entire chromosome is directed to one of the poles. The chromosomes are now seen to be more condensed.
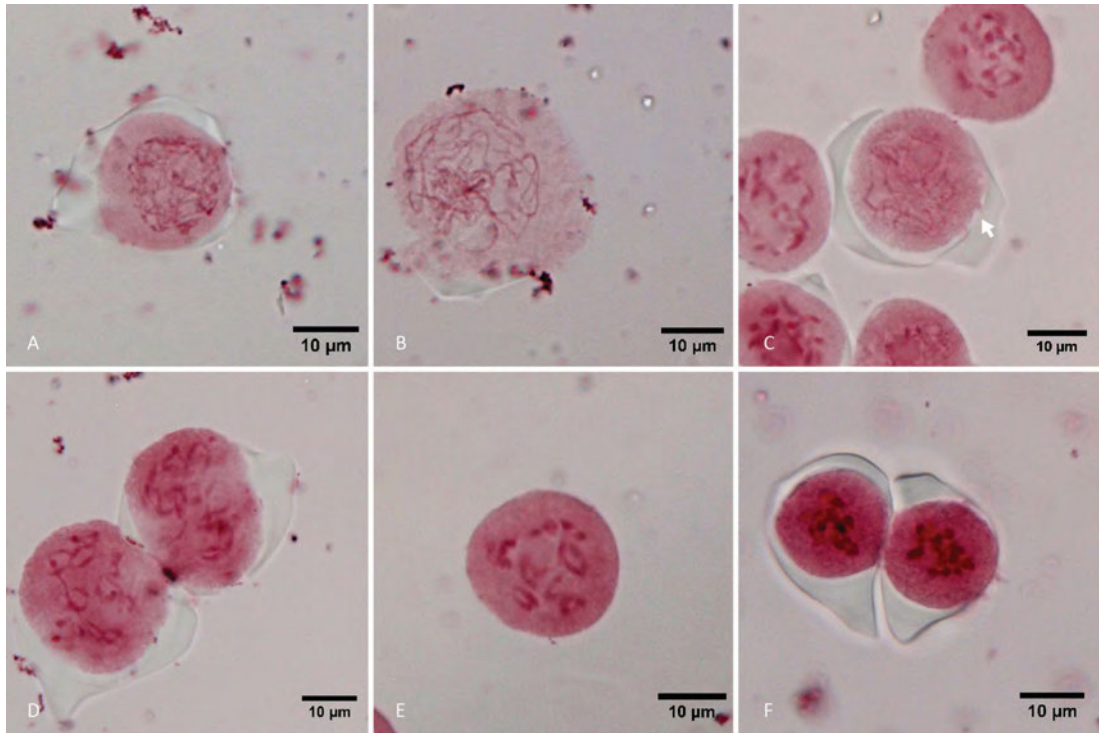
Fig.2. Early stages of meiosis
A) Leptotene, B) Zygotene, C) Pachytene (arrow), D) Diplotene, E) Diakinesis and F) Metaphase I. 600X Magnification, Scale bar 10 μm.

Anaphase I. Homologous chromosomes separate and start moving toward their respective or opposite cell poles (Fig. 3A) as a result of the action of microtubules in the achromatic spindle. However, because of the cross-overs that have taken place, the chromatids are not identical as they were at the beginning of meiosis. Centromeres do not divide, and sister chromatids remain attached at their centromeres, but homologs do separate; as a result, one of the homologous pairs receives a chromosome of paternal origin and the other, in the opposite pole, a chromosome of maternal origin.

Telophase I. Pairs of homologous chromosomes reach the cell poles and the nuclear envelope is formed. Cytokinesis produces two daughter cells (Fig. 3B), each containing half the number of chromosomes present in the parent cell.

2.2.2. Meiosis II

The second meiotic division resembles mitosis, except that it is not preceded by the replication of the chromosomal material.

Prophase II. Each cell is haploid, and the nuclear envelope begins to disintegrate. The nucleolus disappears toward the end of this stage and the achromatic spindle is formed (Fig. 3C).

Metaphase II. The chromosomes that remained at the poles align along the cell's equatorial plate and adhere themselves to the spindle fibers (Fig. 3D).

Anaphase II. The centromeres divide, and the sister chromatids separate and migrate toward the opposite poles (Fig. 3E).

Telophase II. A nuclear envelope forms around each set of chromosomes and cytokinesis occurs, producing four daughter cells, each one with a haploid number of chromosomes (Fig. 3F). Due to cross-overs, some chromosomes end up with recombinant segments of the original progenitor chromosomes.
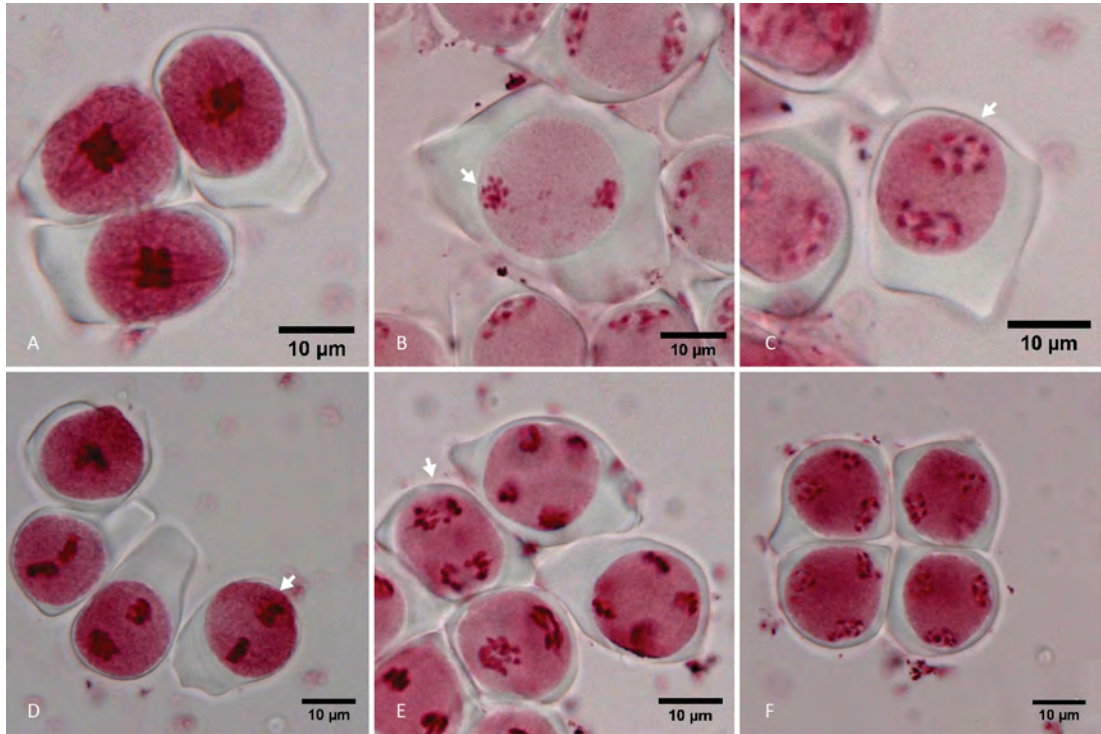
Fig.3. A) Anaphase I, B) Telophase I, C) Prophase II, D) Metaphase II (arrow), E) Anaphase II (arrow) y F) Telophase II. 600X Magnification. Scale bar 10 μm.

3. Unreduced gametes (2n)

The occurrence of 2n gametes seems to happen under genetic control, but the expression of these genes is influenced by the environment (Ramana & Jacobsen, 2003). Thus, some authors presume that one major gene is responsible for the formation of 2n gametes while several genes control their frequency (Dewitte *et al.*, 2012).

Depending on the stage at which a gene acts, it is classified as being pre-meiotic, meiotic, or post-meiotic. The mutation of these genes significantly alters meiosis, thereby affecting gametic fertility which can lead to the formation of gametes with the somatic chromosome number or unreduced gametes. (Bretagnolle & Thompson, 1995).

The origin of unreduced gametes is usually the result of the deviation of two meiotic processes. Remember that during a normal meiosis, meiotic cells undergo two successive divisions; the first division leads to the separation of the paired homologous chromosomes and the second division leads to the separation of sister chromatids within each chromosome.

During the first meiotic division, at the formation of the *first division restitution* (*FDR*) the mating and/or separation of homologous chromosomes does not occur at Anaphase I; thus, the first division occurs as a mitotic division and the second meiotic division occurs as it normally would, with the two sister chromatids moving toward opposite poles.

During the second meiotic division, at the formation of the *second division restitution* (*SDR*), the mating and separation of homologous chromosomes occurs as it normally would during the first meiotic division, but the sister chromatids do not separate during the second meiotic division.

An unreduced gamete is considered to be one resulting from an FDR if it has two non-sister chromatids, and it is regarded as a gamete resulting from an SDR if it has two sister chromatids.

The frequency of unreduced gametes is common in the plant kingdom and its occurrence has been reported in many species of families such as *Solanaceae*, *Brassicaceae*, *Leguminosae*, *Rosaceae* and *Vitaceae* (Veilleux, 1985; Camadro, 1986; Barba-González *et al.*, 2005).

Unreduced gametes are of great importance in genetic breeding, due to the fact that they facilitate sexual polyploidization and, at the same time, provide a very effective method for transmitting the genetic diversity of diploid species (2x) to a tetraploid level (4x). They can be detected by the occurrence of a tetraploid progeny when crosses 4x × 2x (pollen 2n) or 2x × 4x (eggs 2n) are made, by a bimodal distribution of pollen diameter, as well as by the presence of dyads (Fig 4A), triads (Fig. 4B), and/or tetrads (Fig. 4C), at the microspore formation stage (Fig. 4D).

In addition, there are mechanisms that regulate the crossability, which are mentioned in the following section.

Fig. 4.
A) Dyad. 400X Magnification
B) Triad, 200X Magnification
C) Tetrad, 400X Magnification
D) Microspore, 600X
Magnification Scale bar 10 μm.
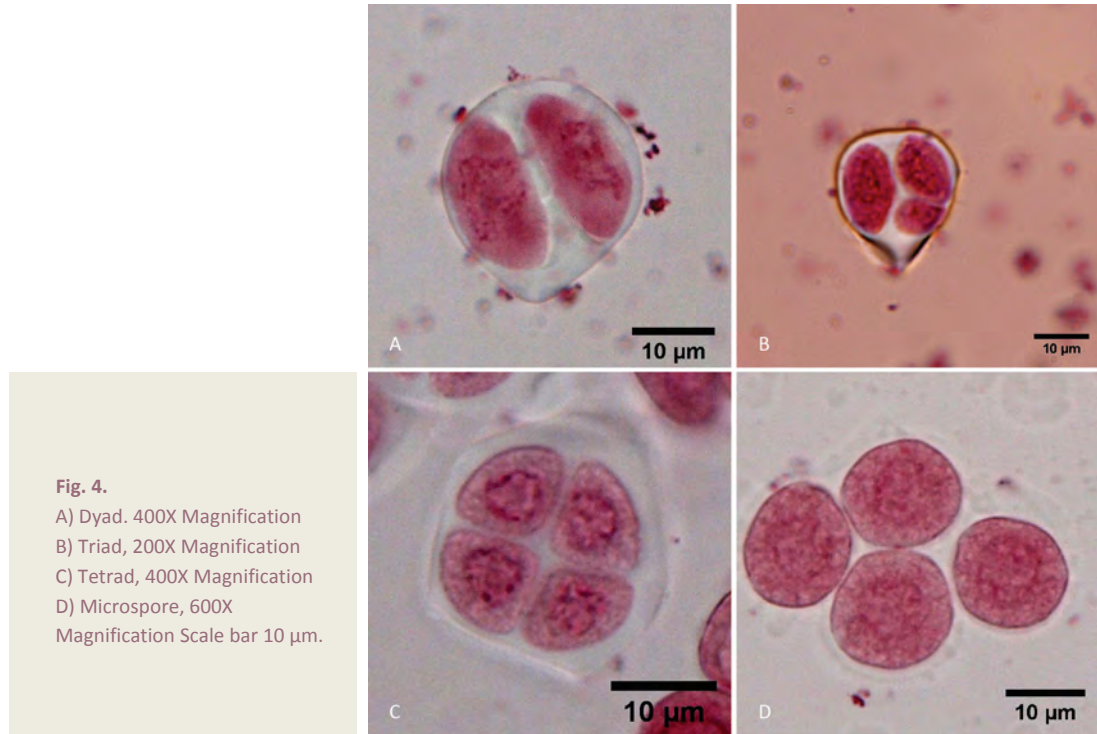
4. Barriers of incompatibility

Sometimes a grain of pollen (in spite of being fertile), does not germinate, or it germinates but the pollen tube stops its growth when it finds itself over a certain style; therefore fertilization is not achieved.

This mechanism aims to avoid cross-fertilization between related genotypes, favoring heterozygosity in populations, thus maintaining hybrid vigor and preventing the consequences of inbreeding depression.

It is estimated that 39% of angiosperm species present mechanisms of incompatibility.

Incompatibility has been determined in more than 3000 species belonging to the phanerogams. Within one family, the type of incompatibility is constant (although not all species within such a family necessarily present incompatibility).

Incompatibility is determined genetically and there are different genetic mechanisms that vary in their degree of efficiency.

4.1. Gametophytic Incompatibility System (GSI)

In this system, pollen recognition specificity is determined by the haploid genotype of the polymorphic S locus: the pollen tube is stopped when this S haplotype is the same as one or some of the diploid pistil haplotypes. That is, pollination occurs when the pollen genotype is different from the female genotype (Fig. 5A).

This system is the most widespread and has evolved to prevent homozygosity, consequently favoring heterozygosity.

The families that possess this type of incompatibility are the *Solanaceae*, *Plantaginaceae* and *Papaveraceae*.

4.2. Sporophytic Incompatibility System (SSI)

In this system, the specificity of pollen recognition is determined by the diploid genotype of the parent (sporophyte). The pollen tube is stopped when one of the S haplotypes coincides with any of the diploid pistil S haplotypes, but it is compatible when the pollen carries an S haplotype not present in the pistil. The sporophytic system differs from the gametophytic system in that the S allele exhibits dominance; furthermore, it can have individual action in both pollen and style, making the system more complex (Fig. 5B).

This type of incompatibility occurs in *Brassicaceae* and *Asteraceae*.

Occasionally, incompatibility is weakened by the mutation to alleles of low efficacy (partial incompatibility) or by environmental action (pseudo-compatibility), producing systems that are not very effective and which permit self-fertilization to a greater or lesser degree.
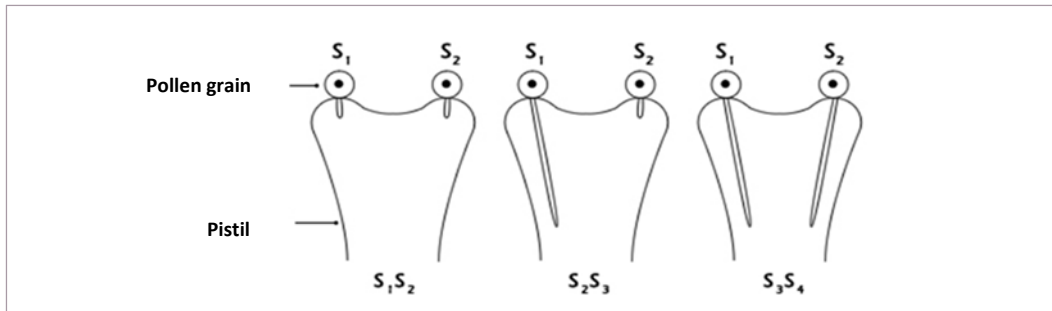
Fig. 5A. Gametophytic incompatibility system and compatibility by cross type.

Compatibility by cross type

$S_1 S_2 \times S_3 S_4$: Total Compatibility
$S_1 S_2 \times S_1 S_3$: Partial Compatibility
$S_1 S_2 \times S_1 S_2$: Total Incompatibility
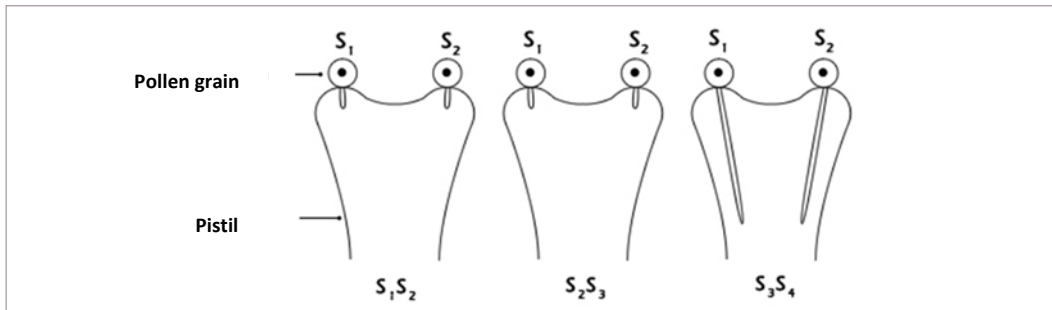
Fig 5B. Sporophytic incompatibility system.

Pollen does not germinate in the stigma of a flower containing either of the two alleles that are present in the sporophyte that produced the pollen.

The same condition applies even though each grain of pollen (haploid) contains only one of the alleles. Therefore, the pollen grains (S_1 o S_2) produced by plant $S_1 S_2$ will germinate only in plant $S_3 S_4$ and not in plant $S_1 S_2$ or plant $S_1 S_3$ and so on.

The order of dominance is $S_1 > S_2 > S_3 > S_4$

5. Androsterility

It refers to a plant's inability to produce fertile pollen. It is classified according to the genetic causes of androsterility (male sterility).

5.1. Genetic male sterility

This is usually determined by a locus with two alleles: Ms and ms ("male sterility"), the recessive homozygote being the one that determines male sterility; the other genotypes produce androfertile or male-fertile plants. To be useful for purposes of genetic breeding, this type of sterility must be stable in a wide range of environments and inhibit all seed production.

5.2. Cytoplasmic Cause

This is determined by cytoplasmic factors. The genes responsible are located in the mitochondria. The transmission of this sterility trait comes from the maternal side.

5.3. Genetic-cytoplasmic interaction

This type of sterility is determined by the interaction between nuclear (R and r) and cytoplasmic genes (carriers of plasma genes that produce – or not – male sterility).

Part Two: Cytological Techniques

1. Determination of ploidy in somatic cells

Knowledge of a species' ploidy is of great assistance in determining taxonomy and of great importance in breeding programs.

The three most commonly used methods of ploidy determination are:

Counting of chloroplasts contained in stomata guard cells, chromosome count at root tips, and flow cytometry (Ochat *et al.*, 2011).

1.1 Chloroplast count in the stomata guard cells

Although it is not an exact technique for the determination of ploidy of a genotype, a chloroplast count of the stomata guard cells allows for the distinction of the diploid group from the other groups (Huamán, 1995).

It is recommended that this technique be used only as a preliminary evaluation for the selection of diploids and not for larger ploidies. The average number of chloroplasts gives us an indication of the level of ploidy (Table 1). For a 2x ploidy, the range is 6 to 8 chloroplasts per guard cell, while 9 to 14 chloroplasts indicate a higher level of ploidy.

Table 1. Scale to determine the ploidy of a genotype according to the number of chloroplasts.

Number of chloroplasts in stomata guard cells	Ploidy
6 - 8	Diploid (2n=2x=24)
9 - 11	Triploid (2n=3x=36)
12 - 14	Tetraploid (2n=4x=48)

Taking the sample

Collect 3 to 5 leaflets from the upper third of each genotype to be evaluated.

Procedure

Preparation

1. Place the leaflets in a Petri dish containing paper towel in the bottom, moistened with distilled water (Fig. 6A).

2. Place 1 or 2 drops of Iodine-potassium iodide solution (I-KI) (Annex 1e) in the center of a slide.

3. With the help of fine-point tweezers, remove the epidermal tissue from the underside of the leaf, from an area close to the veins, and immediately place it gently on the slide (Fig. 6B).

4. Add another 2 drops of the Iodine-potassium iodide solution and cover with a coverslip (Fig. 6C).

Observation

5. Examine under an optical microscope at a magnification of 100, 200 or 400X. The chloroplast count is performed on one of the two stomata guard cells, in 10 different stomata. (Fig. 7A, B and C).
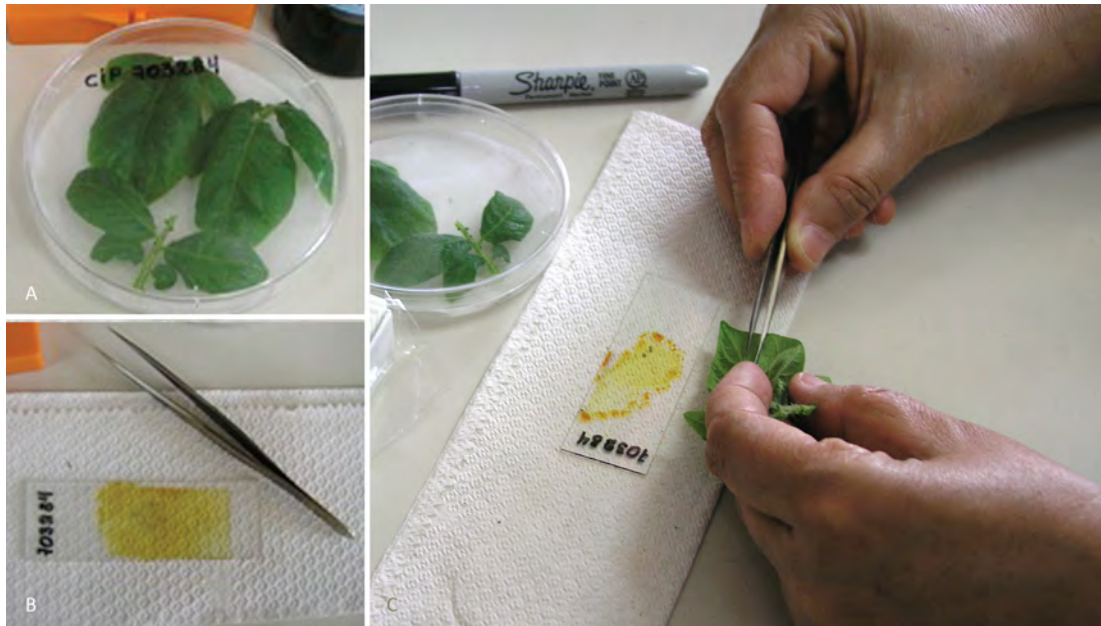
Fig. 6. A) Collection of potato leaflets, B) Epidermal tissue removal from the underside of the leaf; and C) Slide ready to observe under a microscope.

Fig. 7. A) Parts of a stomata: Nucleus, Guard cell, Chloroplast. B) Tetraploid genotype (arrow, 12 chloroplasts in guard cell, C) diploid genotype (arrow, 7 chloroplasts in guard cell). Magnification 400X. Scale bar10 µm.

1.2. Chromosome count in root tips

The potato has very small chromosomes, which makes it difficult to observe and count them. For an accurate determination of the number of chromosomes it is necessary to use cytological techniques that show reliable results, and to have well separated and colored chromosomes; so the samples must be collected in the metaphase stage.

Determination of the chromosomal number in the potato's somatic cells is carried out at the root tips.

Initially, the roots are subjected to a pretreatment or prefixation that allows:

• the accumulation of a large number of metaphase cells in the meristem,

• separation and shortening of chromosomes, and;

• clarification of centromeres, thus facilitating the chromosome count.

This pretreatment is performed with substances called mitotic inhibitors, such as cold water, 8-hydroxyquinoline, colchicine, paradichlorobenzene and pyrethroids (Tjio and Levan, 1950; Hermsen, 1971; Klein, 1990; Watanabe and Orrillo, 1993; Singh, 1993; Chen & Li, 2005).

These mitotic inhibitors block the formation of the microtubules that make up the mitotic spindle fibers, thus preventing the separation and migration of chromatids toward the poles, and stopping cell division in the metaphase stage.

In the cytogenetics laboratory, a pretreatment with permethrin is carried out. This is a pyrethroid insecticide, and its chemical composition is 3-phenoxyphenyl methyl () cis-trans 3- (2,2-dichloroethenyl) 2,2-dimethylcyclopropanecarboxylate.

In fact, Watanabe and Orrillo (1993) report that a permethrin pretreatment produces better chromosomal visibility than cold water or 8-Hydroxyquinoline.

The prefixed roots are then fixed to preserve the cell structure unvaried. Alcohol-acid mixtures are normally used as fixatives.

When observing the chromosomes under an optical microscope it is important to ensure that the cells are dispersed to form a single layer, avoiding any overlap. To this end, chemical agents or enzymatic treatments (cellulases and pectinases) can be used to destroy the cell wall as well as pectin from the intercellular junctions. The most commonly used treatment is with hydrochloric acid. This produces a hydrolysis of the pectic substances in the middle lamella, facilitating the separation of cells.

Finally, the roots are stained to view the chromosomes using dyes such as lacto-propionic orcein or acetic orcein. Orcein squashes complete the dissociation of the tissue, thereby facilitating observation of the chromosomes.

Taking the sample

Procedure

1. Plant individually in 4" pots (containing a substrate composed of soil and moss in a 2 to 1 ratio) tubers with a diameter of 3 to 5 cm already showing good-sized sprouts. Also, seeds can be germinated, cuttings rooted, or plants grown *in vitro*.

2. Let the plants grow to a height of 5 cm. The plants should be in a perfect state of development and have fine roots.

3. Perform the collection of roots considering the mitotic index (lm). In the case of Lima, Peru, carry out the collection between 10 and 11 in the morning (Talledo *et al.*, 1993).

4. Remove the plants by flipping the pots and applying a light blow to the base of the pot.

5. With the aid of fine-point tweezers, cut approximately 10 mm from the terminal part or root tip, which has a hyaline white color (Fig. 8A).

Alternative Pretreatments:

- Cold water: place the roots in jars with distilled water and store the jars in a recipient containing water and ice, for 48 hours, under refrigeration at 4°C.

- 8-hydroxyquinoline: place the roots in jars containing an 8-hydroxyquinoline solution (Annex 1a), for a minimum of 3 hours (the optimum time is 5 hours), at 15°C.

- Colchicine: leave the roots in a colchicine solution 0.05 to 0.5%, (Annex 1b) for 4 to 6 hours, at 10-15°C.

Prefixation

6. Put the collected roots into jars or small flasks containing distilled water at room temperature (Fig. 8B). The end purpose is cell expansion through turgor.

7. After one hour, transfer the roots to a prefixation solution containing the pyrethroid (15 µL of permethrin dissolved in 100 mL of ice water at a pH of 5 to 5.8), leaving them for 24 hours under refrigeration at 4 °C (Fig. 8C).

Fixation*

8. Transfer the roots to Farmer's solution (Annex 1c), and keep at room temperature for 24 hours.

* This step can be skipped if samples are processed immediately.

Hydrolysis

9. Add 1N hydrochloric acid (HCl) to the roots, previously heated to 60 °C (Fig. 8D).

10. Heat the hydrochloric acid (HCl) solution containing the roots in an incubator at 60 °C for 8 to 10 minutes (Fig. 8E).

11. Remove the hydrochloric acid (HCl) and carefully wash the roots with distilled water.

Staining

12. Place the roots in a lacto-propionic orcein solution (Annex 1d) in small containers or watch glasses for 5 minutes (Fig. 8F).

Squash

13. Cut 1-2 mm from the root tip and place it in the center of a slide (Fig. 8G).

14. Add a drop of lacto-propionic orcein and place a coverslip over it.

15. Securing one end of the slide, gently press the other end with a pencil eraser, or tap it with repeated firm blows.

16. Place the slide between sheets of filter paper and press strongly with your thumb; this step is called "squash". Avoid any lateral movement of the coverslip sheet (Fig. 8H).

Observation

17. Place the slide holding the sample under an optical microscope at a magnification of 100 or 200X.

18. For chromosome count, select the fields with the best metaphases and observe with a higher magnification lens of 400X or 1000X (Fig. 9A-H).

Only intact and non-overlapping cells will be considered valid so as not to distort the exact count of chromosomes.

Fig. 8.
A) Root collection
B) Prefixation with distilled water
C) Fixation with Permethrin
D, E) Hydrolysis and heating with HCl (1N)
F) Lacto-propionic orcein staining
G) Sample slide preparation
H) Squash
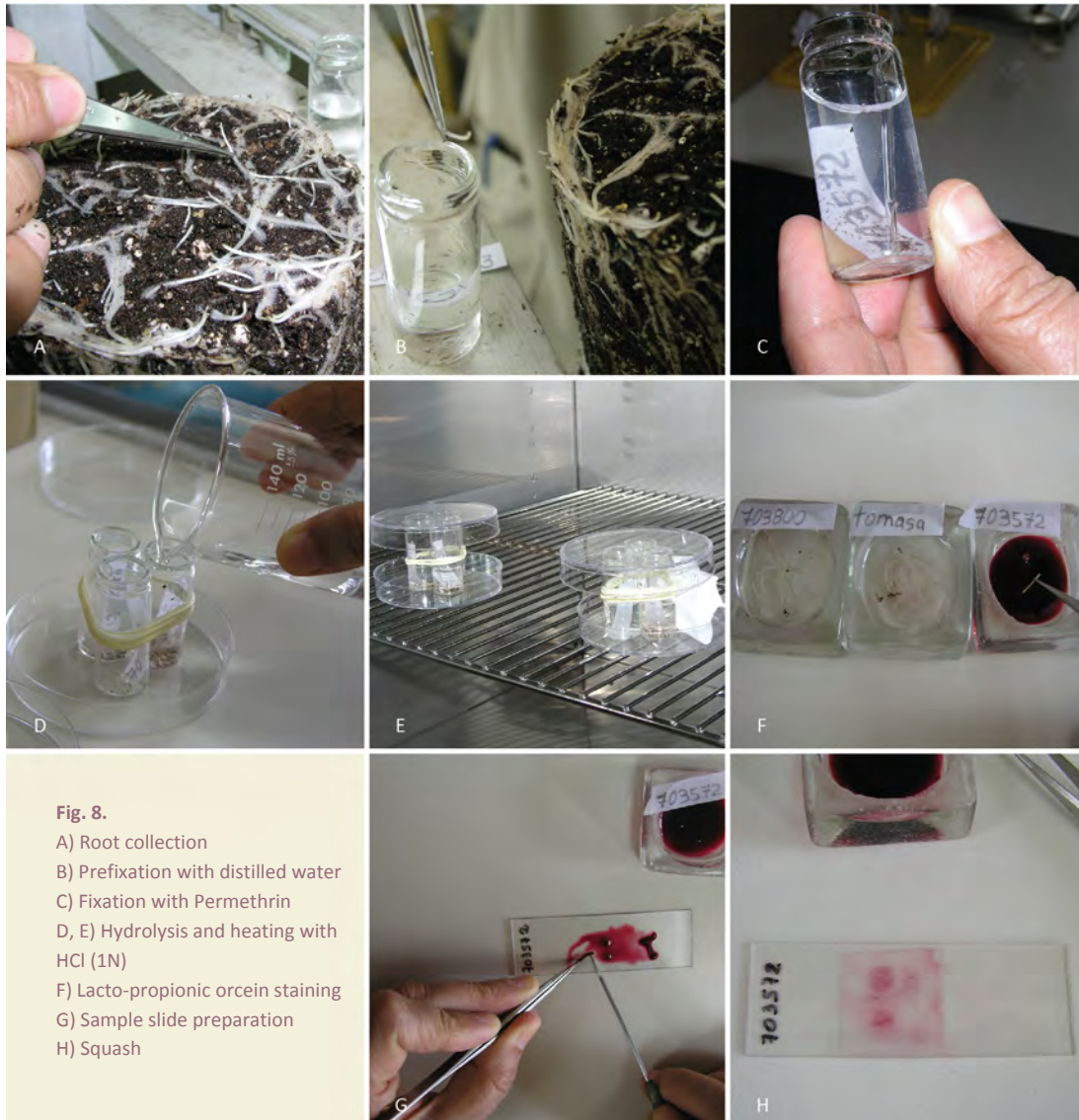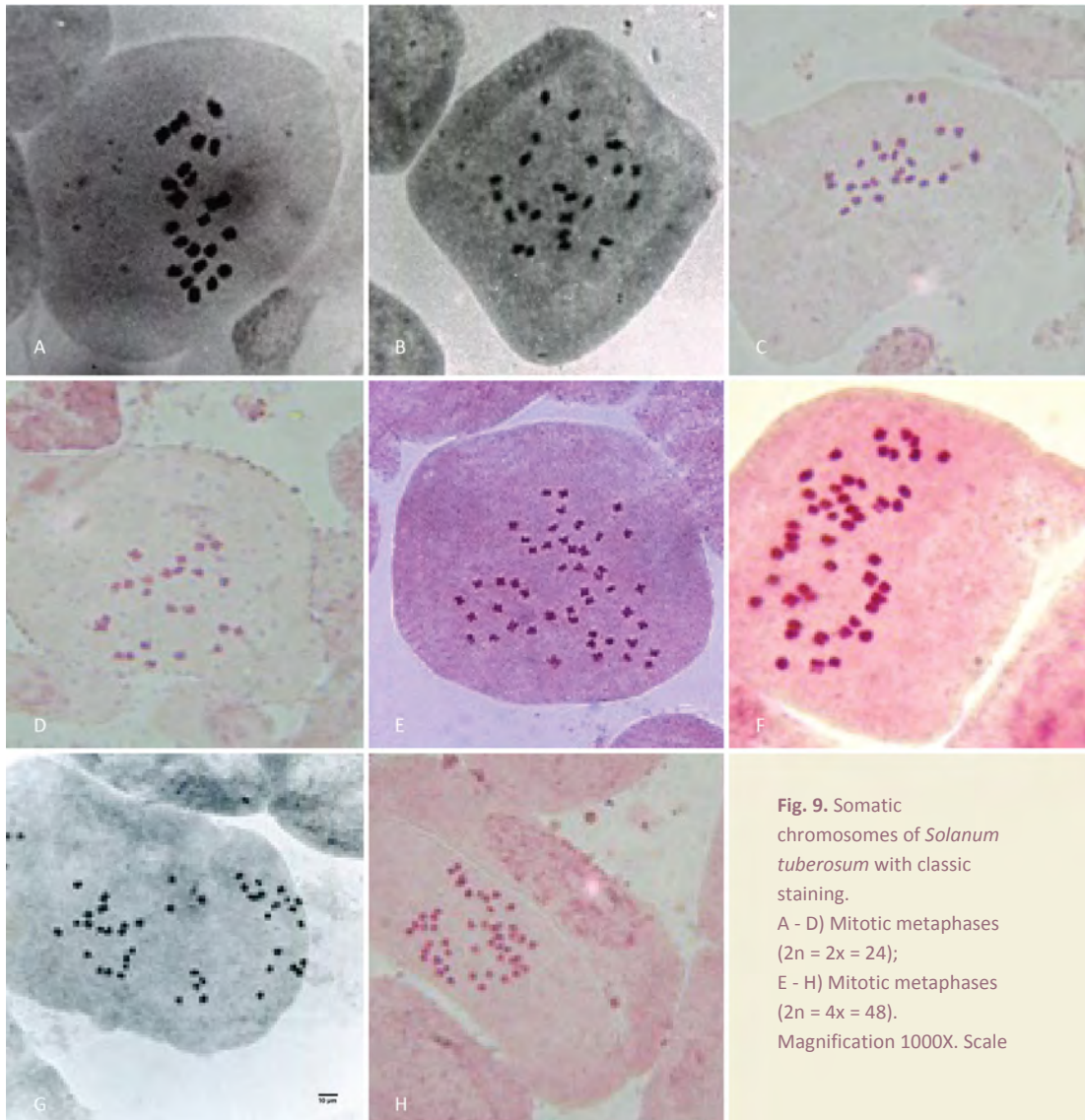
Fig. 9. Somatic chromosomes of *Solanum tuberosum* with classic staining.
A - D) Mitotic metaphases (2n = 2x = 24);
E - H) Mitotic metaphases (2n = 4x = 48).
Magnification 1000X. Scale

1.3. Determination of ploidy by flow cytometry

Flow cytometry is a rapid method which helps to infer the ploidy of a plant, based on measurement of the amount of DNA in the nuclei of its cells.

This method enables a large number of samples to be evaluated per day, as their preparation takes only a few minutes and expensive reagents are not required. There are more than 800 publications, as well as a database, that are dedicated to studies of flow cytometry in plants; such as FLOWER (https://botany.natur.cuni.cz/flower/search.php) with some 6000 species of angiosperms evaluated (Bennett & Leitch, 2011).

The technique is based on the optical properties (light scattering and fluorescence) of the flowing particles in a liquid suspension. This technique makes for effective and precise discrimination of the number of nuclei that have previously been isolated and marked with a fluorochrome.

Estimating the nuclear DNA content of a sample requires the comparison of the fluorescence of isolated nuclei from a reference standard with a known genome size. Therefore, it is important to include control samples (of the species and of known ploidy) each time an evaluation is made. As the labeled samples flow, they are entrained by a flow carrying the ploidy analyzer (Partec PA-II Ploidy Analyzer) against a detection system (mercury lamp emitting ultraviolet light of 488 nm wavelength). The stream of suspended nuclei passes through a quartz chamber (10 μm conduit that does not allow the simultaneous passage of two units), while it is illuminated by ultraviolet light. Fluorochrome DAPI (4,6-diamidino-2-phenylindole) fixed to DNA emits a fluorescence proportional to the amount of DNA in the nucleus, and this emission is recognized and captured by a photoreceptor. The system must be previously calibrated, placing the peak corresponding to a DNA content equal to 2C (diploid) over the value 100 on the abscissa scale (Gutierrez and Moreno, 2005). The pattern is then determined according to the relative area (in percentage) of the peaks corresponding to the different cell populations (2C, 4C, 8C, etc.).

Taking the sample

1. Collect approximately 40 to 50 mg of leaflets from the apical part of the plant and place them in Petri dishes (Fig. 10A).

Procedure

Preparation

2. Place the sample in a Petri dish and add 0.5 mL of the extraction buffer (CyStain UV Ploidy), which contains fluorochrome DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride) that stains the DNA.

3. Cut the leaflets with a blade, sectioning into small squares until the tissue disaggregates (use a new blade for each sample) (Fig. 10B).

4. Add 1.5 mL more of the extraction buffer and incubate for 5 minutes at room temperature.

   Once the mixture is resuspended, run it through a 30 µm nylon filter (Partec 50 µm, Cell TricsTM) allowing separation of nuclei, which pass into a receptor tube already placed in the analyzer (Fig. 10C and 10D).

Observation

The flow cytometer IT system converts each fluorescent signal into a result, presenting a histogram with logarithmic scale. The resulting graph sorts the data according to the DNA's nuclear content in the x-axis, and tallies the number of nuclei of each type on the y-axis (Fig. 11).

All cells belonging to a single peak have the same amount of DNA measured and this peak represents a level of ploidy. The noise consists of a small undesired signal and appears as a result of the cells' resulting fragments at the time of their preparation.

DNA quantification also allows for the discovery of existing aneuploidies, if it is observed that the histograms' curves are slightly displaced toward one side or the other of the expected ploidy.
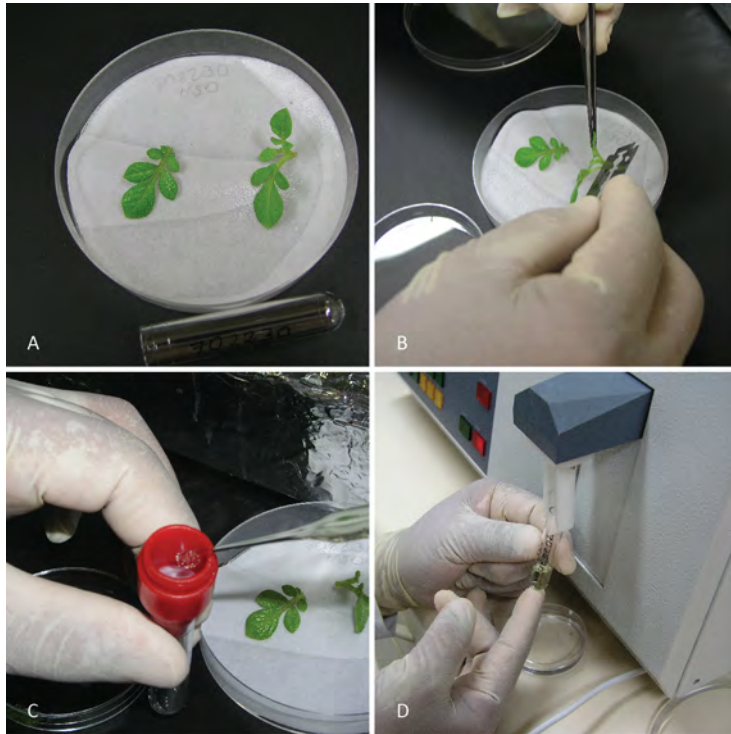
Fig. 10.
A) Samples for evaluation
B) Section the sample with a razor blade
C) Filter the sample for separation of the nuclei
D) Sample analysis in the flow cytometer

Fig. 11.
Histograms with results for ploidy levels HA) *Solanum goniocalyx* (2n=2x=24) and HB) *Solanum tuberosum spp andigena* (2n=4x=48)
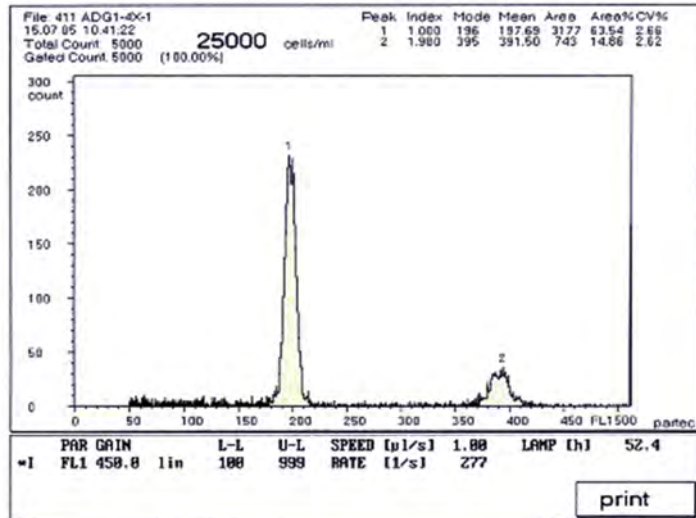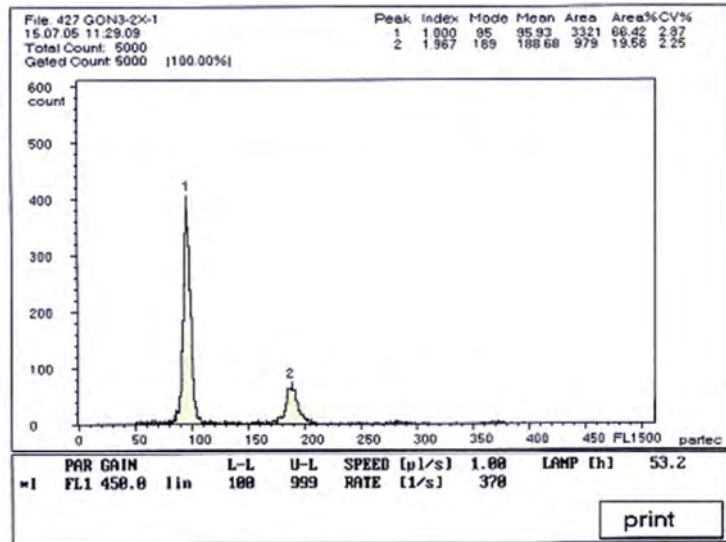
2. Chromosomal behavior in sexual cells

Microsporogenesis is studied in immature anthers, because the meiotic process quickly terminates in cell differentiation.

The method of staining pollen stem cells obtained from flower buds is used, as this is a tissue-specific process.

Obtaining appropriate flower bud samples depends on several factors, such as the environment, time of collection, and genetic factors. It should be recalled that the process of microsporogenesis usually lasts 7 to 14 days before flowering.

Meiosis can be observed only in the early primordia, in which differentiation has not advanced.

Taking the sample

Collect flower buds at different stages of development.

Fixation

1. Place the flower buds in small glass recipients containing a freshly prepared modified Carnoy solution (Annex 1f).

2. Make a cut with a scalpel blade on the flower buds in order for the fixative to penetrate completely into the tissues.

3. Leave the samples in this solution for 24 to 48 hours at room temperature.

Preparation

4. Select the buds previously set and place them in a watch glass containing 70% ethanol (Fig. 12A).

5. On a slide, dissect the buds by cutting the ends and pressing them with a dissecting needle until very small fragments of tissue are obtained.

6. Place one drop of propionic carmine on the sample and cover it with a coverslip (Fig. 12B).

Observation

7. Heat the sample by passing the slide over the flame of a Bunsen burner.

8. Remove excess dye by placing the slide between two filter papers, avoiding any movement of the slide.

9. Tap the coverslip gently with a pencil eraser to spread out the chromosomes.

10. Examine the preparation under an optical microscope at a magnification of 400 or 1000X, and identify the different stages (Fig. 13).
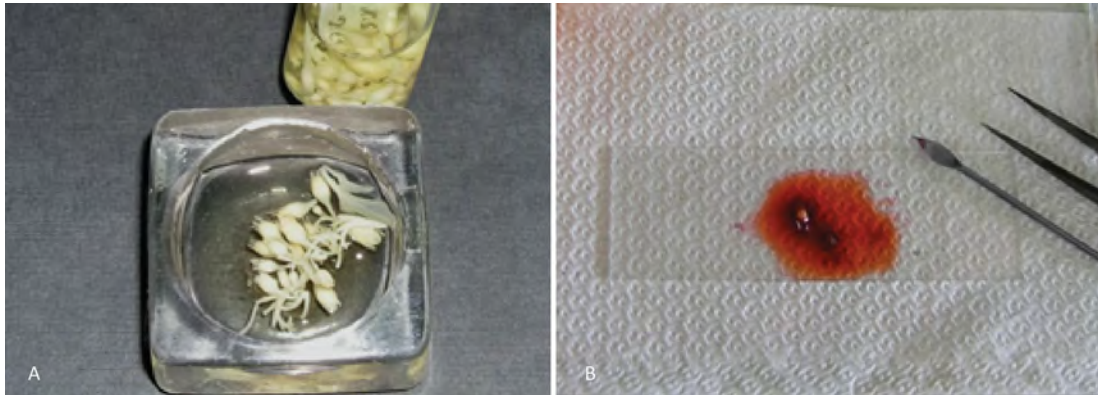
Fig. 12. A) Flower buds in fixative. B) Anthers stained with propionic carmine dye.
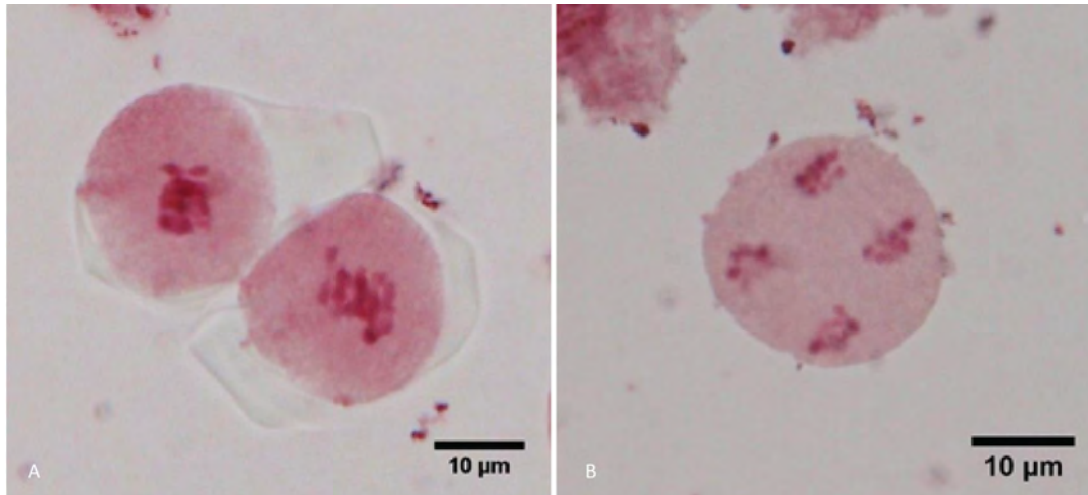
Fig.13. A) Anaphase I exhibiting delayed chromosomes, B) Anaphase II exhibiting parallel spindles. Magnification 600X. 10 µm Scale bar

3. Determination of Pollen Viability and Fertility

In breeding programs, determination of pollen viability is an essential factor in initiating the cross-breeding plan, to achieve successful targeted hybridizations, and thereby obtain hybrid seed. Evaluating pollen viability makes it possible to identify fertile male progenitors to be used as pollinators. The viability of the pollen can be estimated by calculating the percentage of viable pollen using a staining test or the percentage of pollen germinated with the *in vitro* germination test

Different methods can be applied to study the viability of pollen, including dye staining tests or *in vitro* germination. In addition, a scale based on pollen viability ranges taken from promising genotypes is proposed, to determine whether they can be used as male parent in breeding programs (Table 2 and Fig. 16).

Table 2. Scale allocation according to the percentage of viable pollen.

Scale	Ranges (%)	Viability Description
1	0	Sterile
3	0 < % ≤ 50	Low
5	50 < % < 80	Moderate
7	80 ≤ % ≤ 100	High

Fig. 14.
A) Extraction of pollen into a gelatin capsule
B) Place the dye on the slide
C) Spread the pollen in the dye.
D) Store samples at 4°C

3.1. Dye test (with acetocarmine glycerol jelly or with X-Gal)

The purpose of staining techniques is to determine the membrane's enzymatic activity in the pollen grain, as well as the integrity and color of the nucleus.

Pollen viability can be estimated by calculating the percentage of viable pollen, using the staining test with acetocarmine glycerol jelly or with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside).

Taking the sample

1. Collect flowers that are completely open or bloomed, with anthers near dehiscence or completely dehiscent.

2. Extract pollen from one or more anthers using a vibrator (Fig. 14A) or tap the anthers with a dissecting needle to drop the pollen directly onto a slide (if the pollen has been previously collected in gelatin capsules, a sufficient amount is placed in the same way with the tip of a needle or a wooden toothpick).

3. In the center of a previously identified slide, place 1 or 2 drops of 2% acetocarmine glycerol jelly (Fig. 14B) (Annex 1h). If X-Gal dye is used, incubate the pollen in the X-Gal solution (Annex 1i) for 30 min. in the dark at 37 °C.

4. Spread the pollen in the dye with light circular movements using a toothpick (Fig. 14C).

5. Let the sample stand for one minute and cover with a coverslip.

6. Keep the mounted slides horizontal for one or two days.

7. If the samples need to be stored, place them in boxes designed for this purpose, under refrigeration at 4 °C (Fig. 14D).

Observation

8. Observation of a uniformly colored cytoplasm is indicative of a viable or fertile pollen, whereas a non-colored, granular and/or retracted cytoplasm indicates a non-viable or sterile pollen.

9. In the case of staining with acetocarmine glycerol jelly, a pollen grain will be viable when it presents limpid bright red cytoplasm, and non-viable when the grains have a pink cytoplasm and/or are deformed (Fig. 15 B and D).

10. In the case of X-Gal staining, the pollen grain is considered viable if it shows a cytoplasm uniformly blue in color, and non-viable when the grains present a pale blue and/or constricted cytoplasm (Fig. 15A and C).

Fig. 15.
A and B) Viable pollen,
C and D) Non-viable pollen.
Staining with X-Gal and acetocarmine glycerol jelly, respectively.
Magnification 400X. Scale bar 10μm.
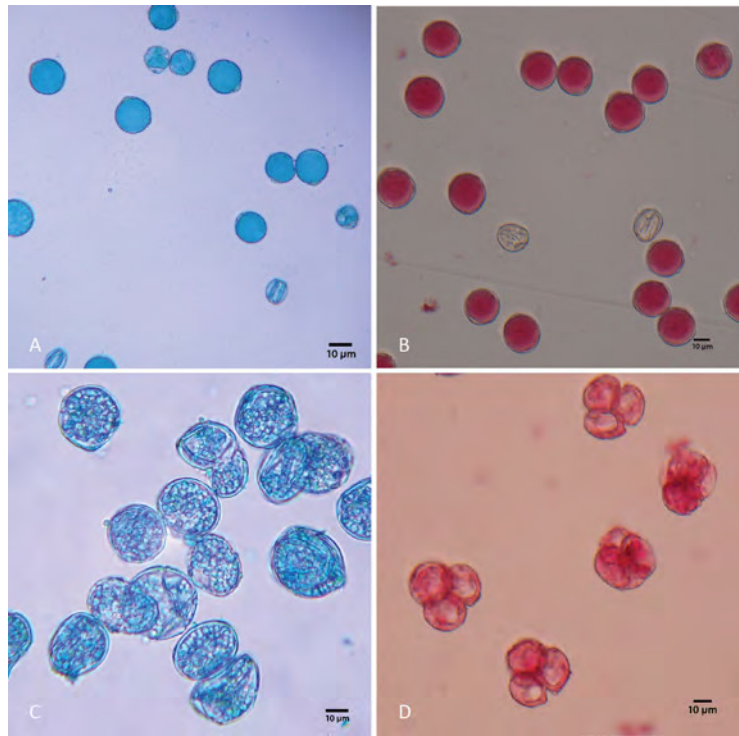
Using acetocarmine glycerol jelly staining, a sample showing moderate to high pollen viability is considered fertile (Fig. 16 C and D). Observation of abnormalities such as tetrads or pollen grains with four nuclei indicates that the pollen sample is infertile (Fig. 16A).

In pollen samples of diploid potatoes, grains of pollen with a non-reduced genetic load (2n) may also be observed, which appear 1.2 times larger than a normal grain (n) (Fig. 17).

Fig. 16. Different degrees of pollen viability, stained with Acetocarmine Glycerol.
A) Sterile tetrads (HER-3.1)
B) Low viability (HER-50.84)
C) Moderate viability (HER-56.13)
D) High viability (HER-44.8).
Magnification 200X. Scale bar 10 µm.
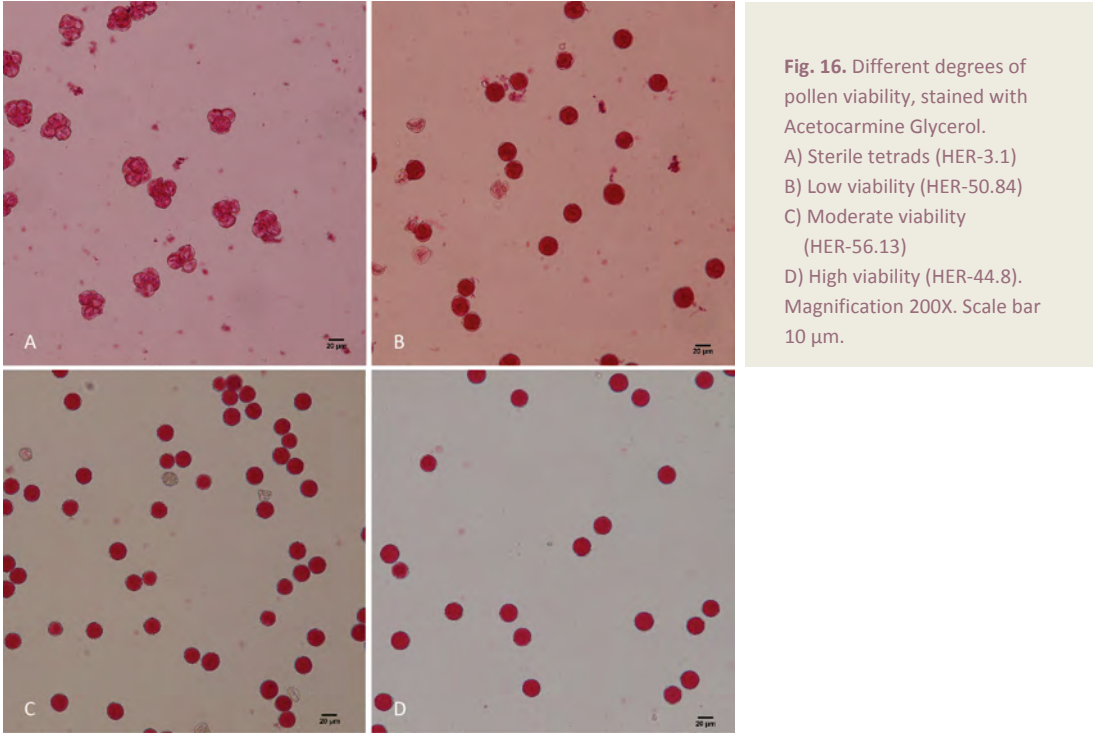
Table 3. Range of percentage of 2n pollen production.

Range	Description
0	Absent
$0 < \% \leq 1$	Low
$1 < \% \leq 5$	Medium
$\% > 5$	High
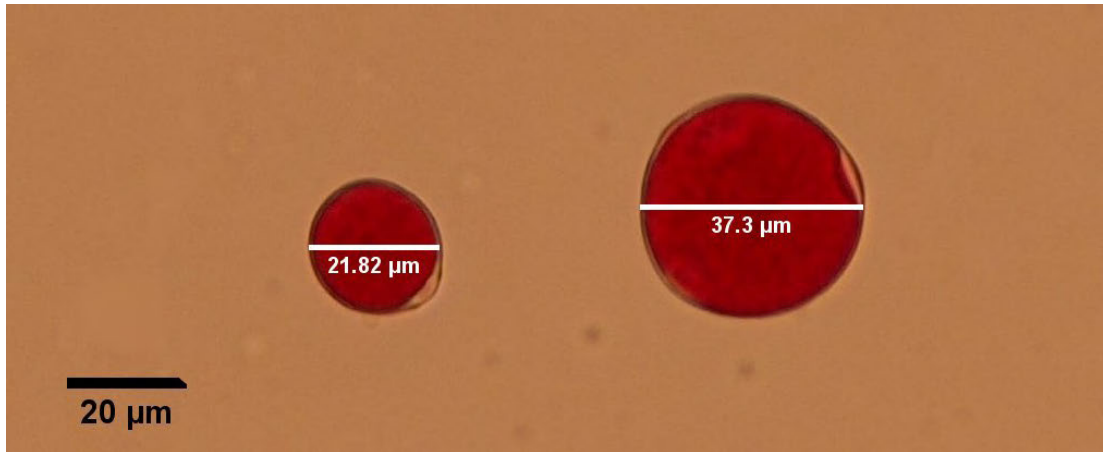
Fig. 17. Pollen sample of diploid genotype (BSLI-7.95), pollen n and 2n are observed. (21.82 µm and 37.3 µm, respectively). 200X Magnification, 20µm scale bar.

3.2. *In vitro* pollen germination test

The determination of *in vitro* pollen germination enables reliable estimates of fertility to be made, defined by the growth of the pollen tube in a suitable medium (Van Marrewijk, 1993). This is a reliable method; staining methods tend to overestimate viability. Thus, the *in vitro* pollen germination or culture simulates the development of the pollen tube in the style tissues, since the culture medium used resembles the stigma mucilage in its composition.

The culture medium used for the germination and growth of the pollen tube contains sucrose, boric acid (H_3BO_4) and polysorbate 20 (Tween 20) (Annex 1j).

Other authors recommend modifications in the culture medium. Thus, Mortenson *et al.* (1964) propose a solution of 20% sucrose and 50 ppm boric acid as the most suitable medium for *in vitro* pollen germination in *Solanum*. Bamberg & Hanneman (1991) consider that a medium of 20% lactose and 50 ppm of boric acid is superior to the medium containing sucrose. Trognitz (1991) finds a highly variable response for this same medium.

On the other hand, González *et al.* (2002) use a medium composed of 12% sucrose, 300 ppm calcium nitrate ($CaNO_3$), 200 ppm magnesium sulfate ($MgSO_4$), 100 ppm potassium nitrate (KNO_3) and 100 ppm boric acid in solution with a pH of 6.

Procedure

Pollen germination

1. Prepare a wet chamber by placing a filter paper moistened with distilled water in the bottom of the Petri dish 5.5 cm in diameter.

2. Place 4 drops of the culture medium on the inside of the lid of the Petri dish, distributing the drops at the corners of an imaginary square (Fig. 18A).

3. With the tip of a wooden toothpick, add small amounts of pollen from the same sample to each drop, spreading it with light circular movements (Fig. 18B).

4. Cover the Petri dish with its lid and leave at a temperature of 20 to 24 °C until the next day.

Observation

5. Put one drop of iodine-potassium iodide solution (I-KI) (Annex 1e) onto the pollen sample.

6. Cover with a coverslip of 22 × 40 mm, and observe it under an optical microscope at magnification 60X or 100X.

7. A pollen grain is considered to be successfully germinated when the pollen tube development reaches a size equal to or greater than the diameter of the pollen grain (Fig. 19).

   The counting of germinated and non-germinated pollen grains must be performed in a minimum of 10 fields, expressing the results as the percentage of the total number of pollen grains per field. An average percentage of 80 percent will indicate fertile pollen.
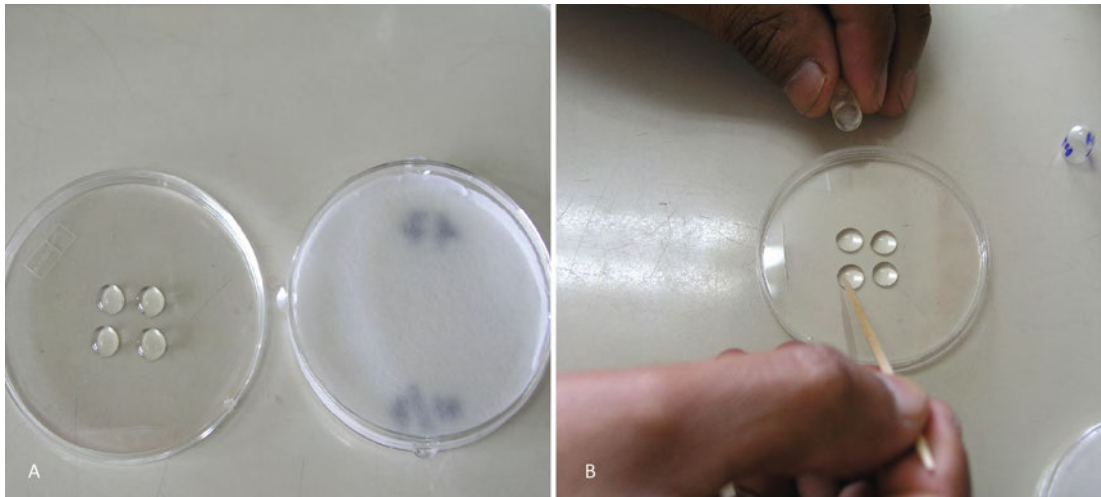
Fig. 18. A) Place culture medium in Petri dish. B) Add pollen to the culture medium using a toothpick.
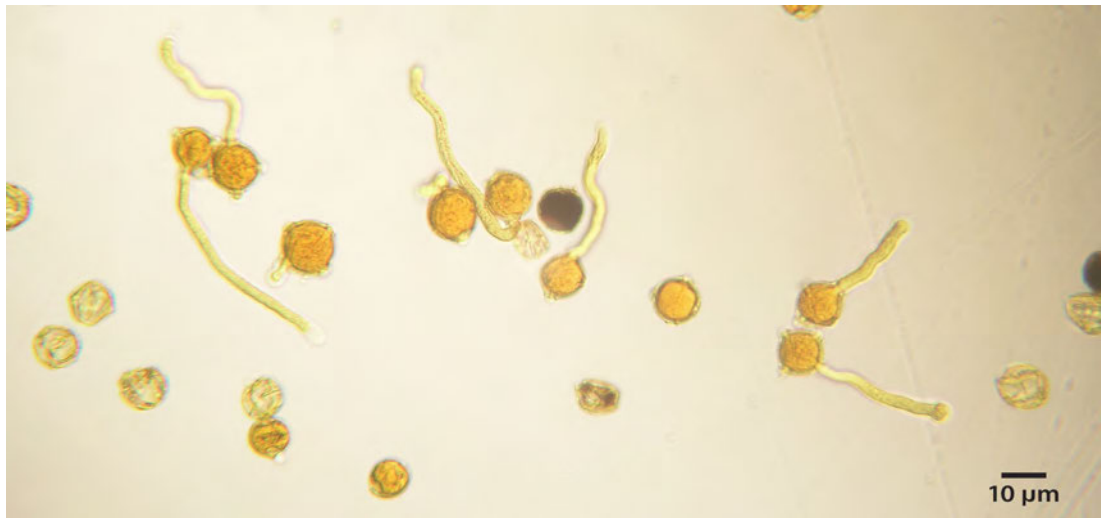
Fig. 19. Germination of pollen grains in culture medium. Magnification 400X. 10 µm Scale bar.

3.3 *In vivo* pollen tube growth test

The period of time between pollination and fertilization involves an intense process of interaction between the pollen tubes and the pistil. It is important to remember that successful sexual reproduction depends on the specific and successful interaction between pollen and pistil.

The analysis of pollen tube growth shows the existing differences in compatible, partial or completely incompatible crosses.

The determination of self-incompatibility and the characterization of the interspecific incompatibility is accomplished by observing the growth of the pollen tube in the pistil (Fig. 20).

The growth of the pollen tubes can be affected by environmental conditions, which can mask incompatibility results.

Pollen tubes, once originated from the pollen grain, grow along the pistil and toward the base of the style, leaving callose deposits to isolate the end of the growing tube from the older part. Those pollen tubes that stop their growth can leave a callose deposit on the end of the tube.

Another demonstration of incompatibility, in addition to those already known, is the production of morphological abnormalities, such as wrinkling of pollen tubes or an explosion releasing the contents of these. Abnormalities include callose plaques, spiral forms, etc.

The fluorescence staining method is based on selective absorption of aniline blue (a component of the Schreiter solution) by the callose deposited along the wall of the pollen tube, and they are identified by the presence of callose at random points of the tube (Aronne *et al.* Al., 2001).
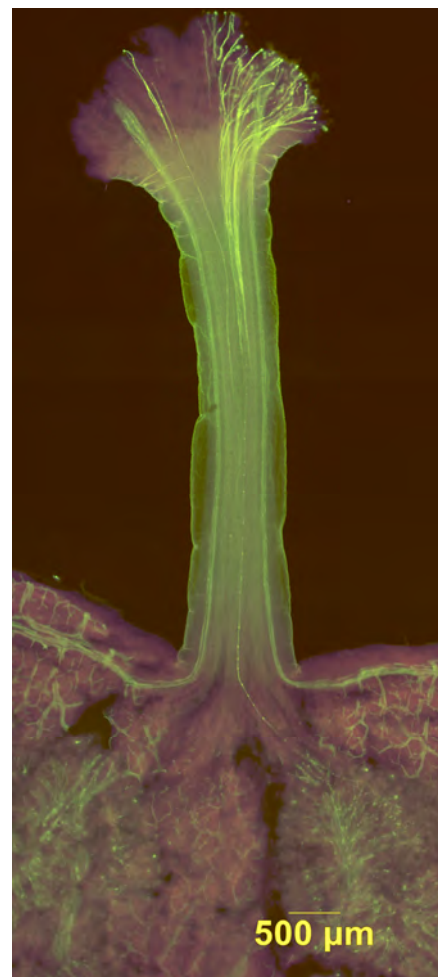
Fig. 20. Pollen tube development at the interspecific crossing of *S. stenotomum × S. chiquidenum*. 48 HDP. 500 Scale bar um.

Taking the sample

Collect 3 to 5 pistils per type of crossing, 48 hours after pollination (H.A.P). They should be carefully cut from the base with a scalpel blade.

Procedure

Fixation

1. Immediately immerse the collected pistils in an Eppendorf tube containing Schreiter solution (Annex 1k y1l).

2. Store samples at 4°C until ready to prepare.

   Samples may remain stored in that condition for up to 9 months.

Preparation

3. Place the Eppendorf tubes containing the pistils in a bain-marie water bath at 55 °C until the tissue is sufficiently clear and soft (Fig. 21 A). On average, leave them 30 min. However time will depend on the consistency of the tissue, which varies from one genotype to another.

   The pistils tend to float, so it is recommended to gently shake each vial for 3 to 5 seconds, so that they remain submerged for the longest time possible.

Observation

4. On a slide, add a drop of aqueous glycerol solution and distilled water in a 1 to 1 ratio. Extend the pistils into the drop of solution, cover with a coverslip and squash gently (Fig. 21 B).

5. Evaluate the pollen tube's growth under a fluorescence microscope with an HBO 200 UV lamp as a light source, an excitation filter (BG 12), a barrier filter (UG 1), and a protection filter (Y-455), at a magnification of 60X.

Pollen tube growth is evaluated using a combined scale or matrix of 18 qualitative levels (level to which the pollen tubes arrive) and quantitative levels (number of pollen tubes) (Trognitz, 1991) (Fig. 22). Many researchers evaluate the length of the pollen tube using free-access programs that allow measurements, such as the Image J program (Fig. 23).
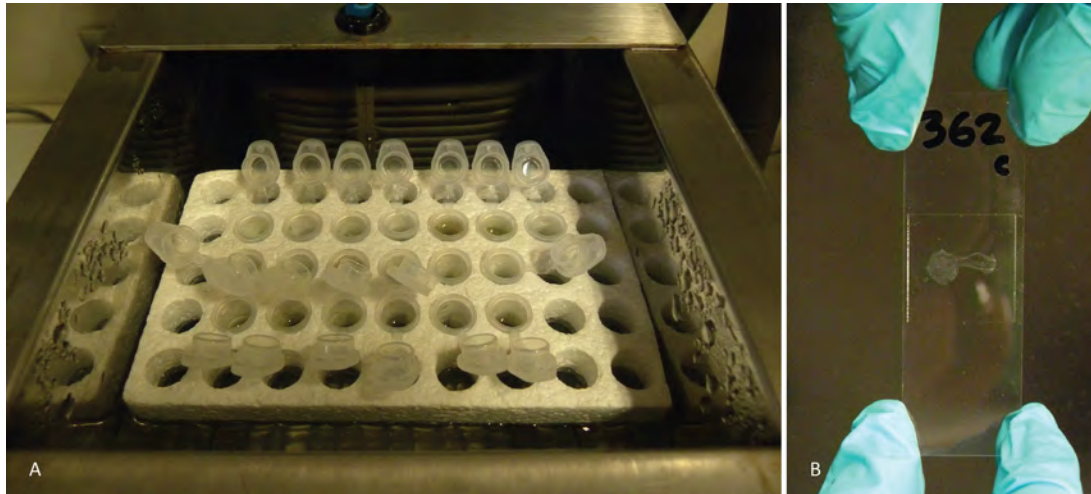
Fig. 21. A) Boil the pistils in a water bath for 30 min. B) Slide ready for observation.

Levels*	Quantity (Total number of polinic tubes in the style-ovary)		
	>30	10-30	<10
	Combined value of level and pollen quantity		
Not germinated 6	16	17	18
Stigma 5	13	14	15
Style-stigma 4	10	11	12
Style 3	7	8	9
Style (end) 2	4	5	6
Placenta 1	1	2	3

Fig. 22. Value matrix for qualitative and quantitative evaluation. Levels of pollen tube length:
6_pollen does not germinate;
5-stigma; 4_style-stigma;
3_style; 2_style end;
1_placenta.
SOURCE: Trognitz, 1991.

Fig. 23. Pollen tube length levels 48 HAP: A) 6_pollen does not germinate, B) 5_stygma; C) 4_stylo-stigma; D) 3_style; E) 2_style end; F) 1_placenta. Increase 200X. 500μm bar.

Another Procedure based on Covey *et al.*, 2010

Fixation

1. Place the pistils in Eppendorf tubes containing the Farmer solution (Annex 1c).

2. Store samples at 4°C overnight.

Preparation

3. Discard Farmer's solution using a pipette, rinse twice with distilled water to remove the fixative.

4. Immerse the pistils in 0.5 mL of 8N NaOH solution for 24 hours.

Staining

5. Remove the NaOH solution using a pipette (carefully, so as to not damage the pistils) and wash delicately 3 to 5 times with distilled water.

6. After removing the distilled water from the last wash, it should be replaced with the stain solution (Annex 1q).

7. Once the stain solution is added to the tubes, they are placed in the dark for at least 24 hours.

Mounting

8. After incubation in the stain solution, the pistils are removed. Place a drop of aqueous glycerol solution and distilled water at a ratio of 1 to 1 onto a slide. Extend the pistils into this solution, cover it with a coverslip and gently squash.

9. The staining solution contains a fluorescent substance that permits visualization of callose deposits present in the pollen tubes. The aniline blue contained in the solution stains the deposits so they can be easily detected with ultraviolet light (Dionne and Spicer, 1958).

3.3.1 Measurement of growth of the pollen tube using the Image J program

1) Open the file with the image using the Image J program.

2) First, you must established the scale. Do this as follows:

a. In the program toolbar, right-click the button that shows a diagonal line ( ).

b. Draw the **Bar scale**.

c. Click on **"Analyze"** and then click on **"Set scale"**.

Write down a known distance (for example, if the scale bar to be used represents 100 μm, set the known distance in micrometers for the length unit).

Click on **"Global"**. This will maintain the scale bar for as long as the Image J program is open.

3) Then, return to the program toolbar and right click on the button that shows a straight diagonal line

( ).Now you have to save the setting with the **"Freehand"** button ( ).

4) Trace the pollen tube from the edge of the pollen grain to the end of the pollen tube.
"Command + M" measures the length of the pollen tube.

5) There are two ways to measure the length of the pollen tube: through the keyboard button combination **"Ctrl + M"** or by going to the program toolbar and selecting **"Analyze"**.

6) The measurements will be displayed in a popup window called **"Results"**.

7) After measuring 10 pollen tubes for each of the 3 repetitions, perform the respective analysis.

3.3.2 Assembly of images of pistils using PanaVue Image Assembler 3 program.

PanaVue Image Assembler is a program for assembling microscopy images. It can be downloaded from the following link http://www.panavue.com/en/downloads/index.htm

Procedure

1. Open the program and create a new project with the option "Mosaic Stitching"

2. Select "**Add image**" and click on "**Add**"
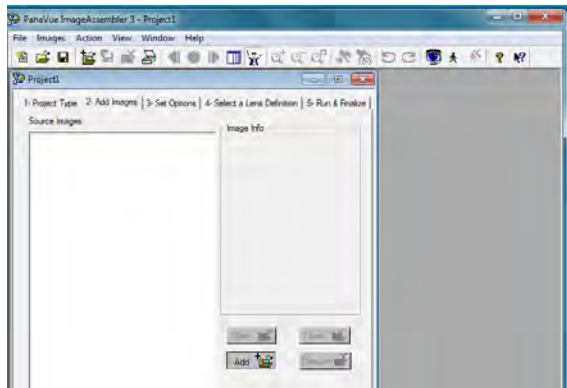
3. Add the images you want to assemble in order, from top to bottom, or from left to right. In the case of pistil photos, from the stigma to the base of the pistil. The first image added will appear at the top left, and the last one at the bottom right. Write the number of columns to be assembled.
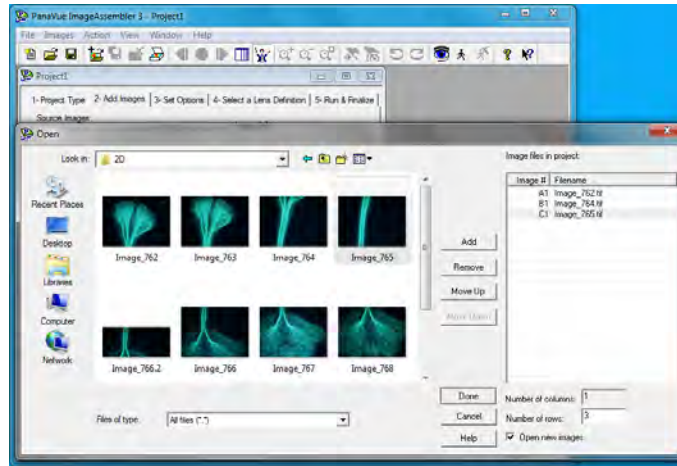
4. In "Set Options", place the following characteristics.
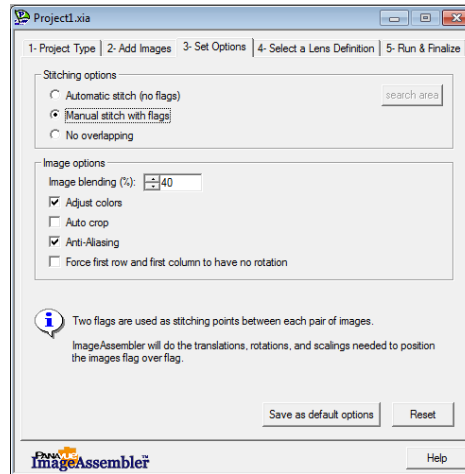
5. Click and drag the arrows with the cursor. Place them in common areas for all images and leave overlapping parts in the consecutive images, so that the assembly maintains that order.
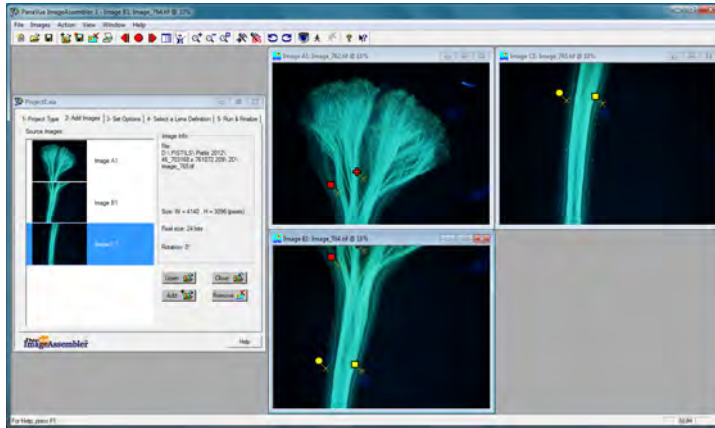
6. Click the "Full Run" button to start the assembly. It may take a few seconds for the final image to appear.
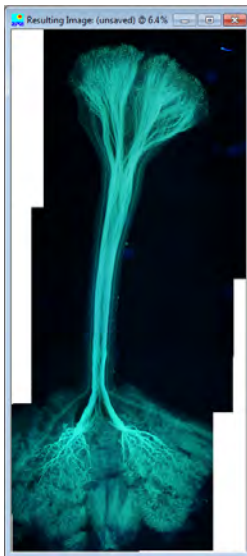
4. *In vitro* culture of immature embryos

The *in vitro* embryo culture technique has been very useful in obtaining interspecific and intergeneric hybrids. The technique allows embryos that fail to develop as normal, or that degenerate due to their immaturity, to progress until they form a seedling if they are placed in a suitable medium, successfully overcoming a lack of viability of seeds from difficult crosses (Brown & Thorpe, 1995). The success of an *in vitro* culture of immature embryos is influenced by the chemical composition of the growth medium, which must be able to induce the successful development of hybrid embryos, establishing itself according to the main nutritional requirements of the species (Pellegrineschi *et al.*, 1997).

Fig 24. Embryo rescue procedure. (Ordonez B., 2008)

Procedure

Depending on the type of crossing, berries can be collected between 19 and 27 days after pollination (D.A.P).

Preparation of berries

1. Place the collected berries, for approximately 10 min., in a solution containing the acaricides Azociclotin (Peropal 50 SC) and Imidacloprid (Confidor 35 SC) at 1 ‰, adding 6 drops of Tween 20.

2. Disinfect with 70% ethanol for 30 sec.

3. Wash twice with distilled water.

4. Sterilize the berries' surface with 2.5% calcium hypochlorite solution ($Ca(ClO)_2$) for 10 min.

5. Rinse the berries with sterile distilled water, inside a laminar air-flow (LAF) bench.

Rescue and culture of embryos

6. Open the berries along the longitudinal axis using a scalpel and a No11 scalpel blade (the procedure is performed inside a laminar air-flow bench with the aid of a stereoscope under conditions of asepsis).

7. Carefully separate the immature embryos from the seed coat and place them directly into 9 × 50 mm Petri dishes containing the culture medium (Annex 1m). (Fig. 24).

8. Place the Petri dishes with the immature embryos in a growth chamber (temperature: 18-22 °C, photoperiod: 16 hours, light intensity: 3000 lux) until germination (Ordoñez, 2008).

9. Once the seedling has developed, transplant into tubes containing the propagation medium (Annex 1n).

Annex 1. Preparation of reagents and solutions

a. **8-hydroxyquinoline solution.**

Weigh 0.29 g of 8-hydroxyquinoline and dissolve it in 25 mL of any solvent (usually ethanol). Add distilled water by sliding it very slowly through a glass rod, avoiding precipitation of the solution, to 1 L. Store this solution, 0.002M 8-hydroxyquinoline, in a dark or amber bottle at room temperature.

b. **Colchicine solution 0.05-0.5% w/v.**

Dissolve 0.05 to 0.5 g (according to the required concentration) of colchicine in 100 mL distilled water.

c. **Farmer's solution.**

Mix three parts of 96% ethanol and one part glacial acetic acid (CH_3COOH).

Important Note: The solution must be prepared and used, at the most, within half an hour after its preparation, because over time the acid will reduce and its fixative potential will diminish.

d. **Lacto-propionic orcein solution.**

To prepare 100 mL of a lacto-propionic orcein solution, dissolve 1 g of orcein in a mixture of 23 mL of lactic acid ($C_3H_6O_3$) and 23 mL of propionic acid ($C_3H_6O_2$) at room temperature. Add distilled water to arrive at a total of 100 mL. Shake well and filter.

e. **Iodine-potassium iodide solution.**

Dissolve 1 g of iodine (I_2) and 1 g of potassium iodide (KI) in 100 mL of 70% ethanol. Store in a dark colored container or jar at room temperature.

f. **Carnoy modified solution.**

Mix absolute ethanol and the saturated ferric acetate solution in propionic acid, at a ratio of three to one.

Saturated ferric acetate solution: Add 10 g of ferric acetate to 100 mL of pure propionic acid. Mix the solution well and decant carefully, preventing the precipitate from mixing into the final solution.

g. **Propionic carmine solution**.

Heat up a 45% propionic acid ($C_3H_6O_2$) solution to boiling point, add 1 g of carmine, let it boil, filter, and add five drops of saturated ferric acetate solution.

h. **2% glycerol acetocarmine jelly solution.**

Inside an extractor hood and under constant stirring, heat up a 45% acetic acid solution to boil. Add 2g carmine, wait until it is completely dissolved and there is approximately a 60 mL solution left. Leave to cool, filter and add an equal volume of glycerine to the solution (Marks, 1954). Store at 4 ºC.

i. **X-Gal solution.**

Dissolve 1 mg of x-Gal in 50 µl of N, N'-dimethylformamide and 1 mL of acetate buffer (50 mmol, pH 4.8).

j. **Culture medium used for pollen tube germination and growth.**

In a beaker, add 5 mL of a stock solution of 200 ppm boric acid, 20 g sucrose and 0.2 mL polysorbate 20, commercially known as Tween 20. Dissolve and homogenize in 100 mL of distilled water. Adjust the culture medium's pH to 5.5.

k. **Aniline blue aqueous solution (2%).**

Dissolve 2 g of aniline blue in 100 mL of distilled water, boil the solution and filter.

l. Schreiter fixing agent solution.

Set aside 100 mL of aniline blue solution. Add 700 mL of 0.2N aqueous solution of tribasic potassium phosphate (K3 PO4), 200 mL of 1N sodium hydroxide (NaOH) and 200 mL of Tween 20. Store the Schreiter solution in a dark or amber jar, under refrigeration at 4 °C (Schreiter & Tiemann, 1977). The solution can be reused for approximately ten cycles. If it becomes cloudy, it must be filtered again.

m. Modified pro-embryo culture medium Singsit and Hanneman (1991).

Set aside 4.6 g/L of Murashige and Skoog basal medium (Sigma). Add 4% sucrose, 100 mg/L myo-inositol, 0.001 mg/L adenine, 2 mg/L glycine, 1 g/L hydrolyzed casein, 0.1 mg / L thiamin HCl, 1 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 100 mg/L malic acid, 0.7% agar and 1 g/L activated charcoal. The medium's pH is adjusted to pH 5.6, then place in the autoclave at 120 °C for 20 min.

Remove from the autoclave. Add 0.1 mg L 3-indole-acetic acid and 0.001 mg/L kinetine through filtration. Homogenize and dispense into Petri dishes.

n. Propagation medium for embryos.

Set aside 4.6 g/L of Murashige and Skoog basal medium (Sigma). Add 25 g/L sucrose, 2.9 g/L phytagel (agar substitute) and 5 ml vitamin stock (0.025 g gibberellic acid, 0.5 g glycine and 0.125 g of nicotinic acid). Desired pH is 5.6.

o. 1N HCl solution.

Dilute 82.8 mL of concentrated hydrochloric acid (HCl) in 1 L of distilled water.

p. 1N NaOH solution.

Dissolve 4 g of the sodium hydroxide pellets (NaOH) in 100 mL of deionized water in a beaker.

q. Aniline blue dye solution.

Dissolve 1.42 g of 0.1N tribasic potassium phosphate aqueous solution (K3PO4) in 200 mL of distilled water. Add 0.2 g aniline blue and mix. Store in a dark or amber jar at 4 ºC (Covey *et al.*, 2010).

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Glossary

Achromatic spindle fibers: A group of microtubules extending from the centromeres of the chromosomes to the poles of the spindle, or from pole to pole, in a dividing cell.

Achromatic spindle: Structure of protein filaments, responsible for moving and holding chromosomes during mitosis.

Alternation of generations: Life cycle in which a diploid stage is followed by a haploid one and so on. The gametophyte (n) produces gametes (n) by mitosis, and the fusion of male and female gametes produces zygotes (2n). Each zygote gives rise to a sporophyte (2n) which through meiosis gives rise to haploid spores (n). Each haploid spore forms a gametophyte, thus closing the cycle.

Androecium: Male reproductive organ of a flower.

Autotetraploid: A tetraploid formed by the doubling of a single genome. In an autotetraploid all the chromosomes come from the same species.

Basic or monoploid number (x): Number of different chromosomes that make up a complete chromosomal set. It represents the minimum number of chromosomes of a polyploid series.

Callose: Polymer of glucose (bond 1-3, B-D glucopyranose) that is deposited as a thin film along the wall of the pollen tube.

Carpel: Female structures of the flower, comprising the ovary, the style and the stigma. Collectively the carpels are called gynoecium.

Cell plate: Plate that separates the plant cells dividing the cytoplasm in the telophase.

Centromere: Region in the chromosome where the fibers of the achromatic spindle are united during cellular division. In this region the two chromatids of the chromosome are joined.

Chromatid: One of the longitudinal units of a duplicate chromosome.

Chromatin: A substance that forms a chromosome, consisting of the combination of DNA and proteins.

Chromosome: Physical structure, made up of DNA and proteins; the chromosomes are visible only in cell division.

Cross-over: The process by which homologous chromosomes exchange genetic segments. It results in a new combination of genetic material in the gamete.

Cytokinesis: Division of cytoplasm.

D.A.P: Days after pollination (English acronym).

Diploid: Species or individual that possesses two sets of chromosomes in its somatic cells.

DNA (deoxyribonucleic acid): A molecule carrying genetic information, which enables its transmission from one generation to the next.

Double fertilization: A process whereby the egg cell and a spermatic nucleus form the zygote (2n), and the two polar nuclei (in the center of the embryo sac), and a spermatic nucleus form the endosperm (3n).

Endosperm: A reserve tissue (in angiosperms) that supplies nutrients to the developing embryo.

Gametes: Sexual cells resulting from meiosis.

Gametic or haploid number (n): Number of chromosomes that the gametes carry; it may or may not coincide with the basic number, depending on the level of ploidy of the species in question.

Gametogenesis: Meiotic process whereby the sexual gametes or cells are formed.

Gametophyte: In plants that present alternation of generations, the haploid stage (n) that produces female or male gametes (n) by mitosis, which after fertilization will give rise to the sporophyte.

Gene: Basic unit of inheritance. It contains genetic information that determines one or more characteristics of an organism. Sequence of DNA bases that usually encode for a polypeptide sequence of amino acids.

Genetic Variability: Variation in genetic material in a population or species, product of mutations, recombination, gene flow, migration, natural selection, or genetic drift.

Genome: The complete set of genetic information that a particular organism possesses.

Genotype: Genetic constitution of a cell or individual.

Germ cells: Cells of the reproductive organs of multicellular organisms, which are divided by meiosis to produce gametes.

Gynoecium: collective term applied to all carpels (or pistils) of a plant.

H.A.P: Hours after pollination (English acronym).

Haploid: A cell that contains half the genetic material. It has only one set of chromosomes.

Homologous chromosomes: Pair of chromosomes of similar characteristics and functions, one contributed by the mother and the other by the father; they mate during meiosis.

Interspecific hybridization: Crossing between two different species within the same genus. Example: *Solanum phureja* × *Solanum chomatofilum*.

Intraspecific hybridization: Crossing between different sub-species within the same species. Example: *L. culinaris ssp. Culinaris* × *L. culinaris ssp. Orientalis*.

Megaspores: The four haploid cells produced by meiosis in the flower's ovule. Usually three degenerate and the remaining cell becomes a gamete (n): ovocell.

Microsporangia: Structure of the sporophyte that produces, by meiosis, the microspores. In plants with flowers, known as pollen sacs of the anther.

Microspore stem cell: A cell that, through meiosis, produces microspores or pollen grains.

Microspores: The four haploid cells produced by meiotic division in the anther sacs of the flowers; they are divided by mitosis and surrounded by a thick wall to form the pollen grains.

Mitotic index: Relationship between the number of cells in mitosis and the total number of cells observed. It is a relative measure and varies according to each time zone, which makes it possible to determine the most appropriate time for the collection of roots with a greater number of metaphase cells.

Ovary: Lower part of the gynoecium that contains the ovules.

Petals: Non-reproductive structures that form the corolla of the flower.

Phenotype: Expression of the genotype in a given environment.

Ploidy: Number of groups or sets of chromosomes that an individual possesses.

Pollen tube: Structure originated in the vegetative cell of the pollen grain through which the spermatic nucleus travels until it reaches the ovule.

Pollen: In plants with seed, the male gametophyte surrounded by a protective cover.

Polyploid: Species or individual presenting more than two sets of chromosomes.

Polyploidy: Changes in the number of chromosomes, caused by accidents in the meiosis.

Receptacle: Part of the flower where the whorls are inserted.

RNA (ribonucleic acid): A molecule that directs the intermediate stages of protein synthesis.

S locus: Locus of self-incompatibility. It consists of a single polymorphic locus that controls self-incompatibility in many species.

Sepals: Modified leaves that protect the petals of the flowers and its reproductive structures.

Somatic or diploid number (2n): Total number of chromosomes in a somatic cell.

Sporophyte: Generation (2n) that produces spores by means of meiosis.

Stamen: Male structure of the flower that produces pollen, consisting of a filament that supports the pollen-producing anther.

Stigma: In the flowers, it is the region of the carpel that receives the grains of pollen that germinate on it. It secretes a moist, sticky substance to fix the pollen grains**.**

Style: Part of the flower carpel formed from the ovary wall. The tip of the style leads to the stigma. Part of the pistil that separates the stigma from the ovary**.**

Synergids: Cells in the embryo sac of angiosperms flanking the ovocell. The pollen tube grows through one of them (usually the smallest).

Turgidity: A phenomenon in which cells swell when they absorb water, exerting pressure against cell membranes, which become tense.

Value C: Amount of DNA per haploid genome (single chromosomal set).

Whorl: Set of parts (leaves, sepals, tepals, etc.) arising from the same node.

Zygote: Structure resulting from fertilization, fertilized ovum. Diploid cell (2n) resulting from the fusion of a male gamete with a female one**.**