Chapter 13
Mitotic Index

Elisa Graña

1 Introduction

The cell division cycle is a highly controlled process, essential for plant growth, whose purpose is to generate two identical daughter cells. Vegetative cell division, or mitosis, encompasses four sequential steps: two gap (G) phases separate the DNA replication (S phase) and chromosome segregation (M or mitosis) (Fig. 13.1). The first gap (G1) is the first step of cell division. It is located between cell division and DNA synthesis, and at this stage, each chromosome appears as a single chromatid with a single DNA molecule. G1 ends when cell moves into S phase (or synthesis phase), which corresponds to DNA duplication, obtaining the double of genetic material ready to be distributed between the two new daughter cells. Once achieved, cell enters in G2, the second gap previous to mitosis that differs from G1 in showing the double of DNA (two identical chromatids). Finally, cell division occurs in M phase or mitosis, which usually ends in cytokinesis. Resulting cells can continue to divide, remaining at meristematic zones, or leaving the cell cycle to undergo differentiation. In plants, mitosis specifically occurs in meristems, localized in leaves, stems and roots (Sánchez-Moreiras et al. 2008; Dewitte and Murray 2003; de Souza Junior et al. 2016).

Successful progression of cell division requires of different checkpoints, especially at the G1/S and G2/M transitions, to ensure that the previous phase has been correctly completed (Van't Hof 1985). These controlling checkpoints, which regulate the order and timing of cell division, are crucial for maintaining genomic integrity and a balanced growth and division.

Determination of mitotic index (or cell division rate) in meristematic zones results very useful to know the health status and meristematic activity of the cells

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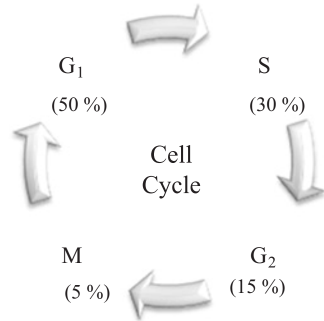

Fig. 13.1 Schematic representation of mitotic cell cycle. Data between brackets indicate the percentage of time spent by one cell in each phase. (Redrawn from Sánchez-Moreiras et al. 2001)

(Fiskesjö 1985). That is the main reason why this simple method has been widely used, especially when root growth inhibition is observed (Dayan et al. 2000), although as has been said, it can be also used to measure the mitotic activity of other organs.

Mitotic index is used to measure cytotoxicity in living organisms (Smaka-Kincl et al. 1996), based on the increase/decrease of the rate of cell division (Debnath et al. 2016; Jain et al. 2016). It can be simply calculated as follows and is given in percentage:

$$\mathrm{MI} = \frac{\text{Prophase} + \text{Metaphase} + \text{Anaphase} + \text{Telophase}}{\text{Total No. of cells}} \times 100$$

As a measure to trace cytotoxic substances, significant decrease in mitotic activity is an evidence of genotoxic potential. When mitotic index reaches values below **50%** of a negative control are considered sub-lethal effects (Sharma and Vig 2012; Jain et al. 2016), and this point is known as **cytotoxic limit value** (Panda and Sahu 1985; Sharma et al. 2012); while lethal effects are considered when inhibition decreases below **22%** (Jain et al. 2016; Sharma and Vig 2012). Reductions in mitotic index are usually attributed to an inhibition in DNA synthesis or a stop in the G2 phase (Sudhakar et al. 2001).

On the other hand, an increase in mitotic index can be the consequence of a reduction of the time necessary for DNA repair (Evseeva et al. 2003). It can reveal an uncontrolled cell proliferation being able to end in tumor formation (Hoshina 2002), or be indicative of shortening of the duration of the mitotic cycle (Al-Ahmadi 2013). Any of these options are characterized as being detrimental to cells.

In addition to Mitotic Index, it can be also calculated the Active Mitotic Index, which gives additional information about the percentage of actively dividing cells (cells at metaphase and anaphase) (Borah and Talukdar 2002; Madaan and Mudgal 2011):

$$\mathrm{AMI} = \frac{\text{Metaphase} + \text{Anaphase}}{\text{Total No. of cells observed}} \times 100$$

2 Mitotic Index and Its Applications

Mitotic index is widely used to evaluate the genotoxic potential of many substances in studies of environmental biomonitoring (Smaka-Kincl et al. 1996), manly using *Allium cepa* or *Lactuca sativa* as testing organisms due to the big size of their cells, which makes easier the visualization of different mitotic phases. Besides, higher plant bioassays are quick, cheap and easy to handle. This test is especially successful for screening, monitoring and detection of clastogenicity of environmental mutagens, including atmospheric, water and soil pollutants (Fiskesjö 1993; Ma et al. 1995).

Thus, MI has been used to test the toxic potential of many pollutants: overexposure to chromium (Rai and Dayal 2016), zinc oxide nanoparticles (Kumari et al. 2011), copper chloride (Can et al. 2016), aluminum (Salabert de Campos and Viccini 2003), insecticides (Panda and Sahu 1985), radioisotope-contaminated air in Chernobyl area (Cebulska-Wasilewska 1992; Ichikawa et al. 1996), chlorpyrifos, benzene, nitrogen oxide, nitric oxide, ozone or sulfur dioxide (Schairer et al. 1978).

Another area where the calculation of mitotic index is also very useful is allelopathy. Many of the allelopathic studies are focused on evaluating the ability of plant extracts or plant naturally-occurring isolated compounds to act as plant growth regulators. In this way, mitotic index is used to test the anti-proliferative profile of different plant extracts such as *Terminalia arjuna* and *Moringa oleifera* (Debnath et al. 2016); *Brassica juncea* (Sharma et al. 2012), *Zanthoxylum limonella* (Charoenying et al. 2010) or *Schinus* spp. (Pawlowski et al. 2012). Besides, mitotic index is also inhibited by plant isolated compounds like BOA (Sánchez-Moreiras et al. 2008), citral (Graña et al. 2013), 1,8-cineole (Romagni et al. 2000), or cinmethylin (El-Deek and Hess 1968). All these works are just an example, as there are many works of this type in the bibliography.

As well, this technique has been also used to test the suitability of cell culture media, and to be sure that it does not compromise cell viability (Maisch et al. 2016), or to verify that applied electric fields can enhance apical root regeneration (Kral et al. 2016).

3 Chromosomal Aberrations

At the same time that cells are visualized using a brightfield microscope, chromosomal aberrations can be easily observed, especially when *Allium* or *Lactuca* specimens are used. Alterations in mitotic index are usually accompanied by cytogenetic instabilities, and most of them are lethal and may cause genetic disorders (Debnath et al. 2016). Atypical number of chromosomes or structural abnormalities in chromosomes are very common. The most common chromosomal aberrations are summarized below and are also schematically represented in Fig. 13.2:

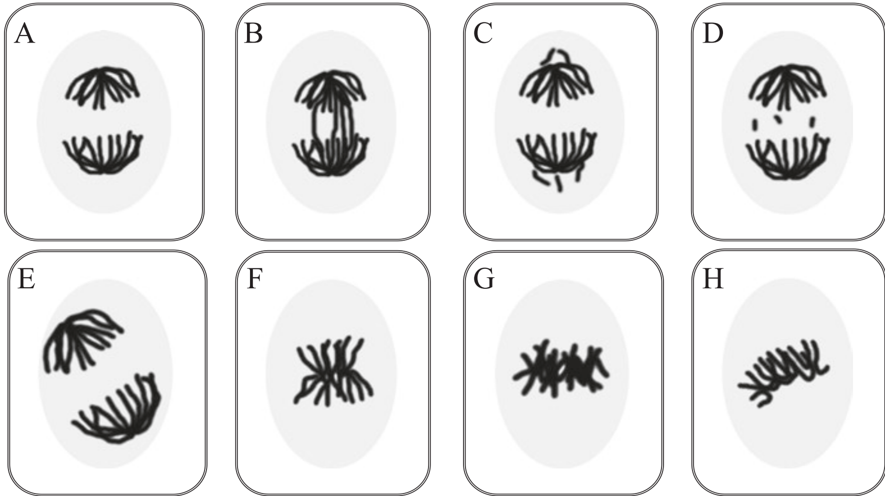

Fig. 13.2 Schematic representation of most common chromosomal aberrations: (**a**) Normal anaphase; (**b**) Chromosomal bridges; (**c**) Laggard chromosomes; (**d**) Fragmented chromosomes; (**e**) Diagonal anaphase; (**f**) Normal metaphase; (**g**) Sticky chromosomes at metaphase; (**h**) c-Mitosis

- **Chromosomal bridges**. The twin chromatids fail to separate (or there is a delay in separation); and as a result, chromosomes are subjected to an abnormal separation. Bridges are the result of stickiness of the chromosomes or due to the presence of dicentric chromosomes (Vorobjev et al. 1993; Debnath et al. 2016). Chromosomal bridges are usually observed in anaphase.
- **Laggard chromosomes** are the result of the inhibition of chromosome movement in anaphase, mainly due to a disturbance on the microtubule arrangement. Part of genetic material is 'left behind', causing an unequal distribution of chromosomes in the daughter cells. Besides, in some cases laggard chromosomes lead to micronuclei formation, since them reorganize later than other chromosomes (Kozgar 2014; de Storme and Mason 2014; Debnath et al. 2016). Micronuclei are the simplest indicator of DNA damage (Migid et al. 2007).
- **Fragmented chromosomes**, also known as banded chromosomes, are small chromosome pieces, mainly observed as fragments in anaphase (Fiskesjö 1988). Chromosome fragmentation causes unbalanced chromosome patterns and uneven distribution of chromatids (Nordenskiöld 1963). It is believed that fragments originate from fragmentation of terminal regions of regular chromosomes (Sheikh et al. 1995), or that are the result of the rupture of chromosome bridges (Liu and Makaroff 2006).

- **Diagonal anaphase** refers to the abnormal location of the spindle fibers, located in opposite corners instead of parallel to the cell division plate. It is also known as polar shifting (El-Bayoumi et al. 1979; Pandey and Sakya 2009).

- **Sticky chromosomes** are characterized by clustering at any phase of the cell cycle. Chromosomes lose their sharpness and tend to clump (Salabert de Campos and Viccini 2003; Rai and Dayal 2016). Apparently, they lose their individuality and can be observed as a mass made of chromatic substance (Kabarity and Malallah 1980).
- **c-Mitosis**. It happens when all chromosomes remain at cell division plate level, but no separation of chromatids occurs. It can be observed as collapsed chromosomes in the central part of the cell (Vorobjev et al. 1993). It is consequence of the inactivation of the spindle apparatus together with a delay in centromere division (Mann and Storey 1966; Jackson 1969; Shehab 1980).

4 The Method

The method here presented consists on an adaptation of the classical squash techniques from Armbruster and collaborators (1991), and can be used for plant seedlings, but also for cell suspension cultures. It is based on division synchronization and nuclear staining to study the effect of a given substance on the division behavior of actively dividing plant cells (Fig. 13.3).

Cycling cells are characterized by dividing asynchronously in meristems. To obtain a large number of cells in the same phase of the cell cycle, it is necessary to induce synchronization through the use of chemical agents. This kind of compounds generally act preventing the formation of the mitotic spindle or inhibiting the synthesis of DNA to block cell cycle progression (Sánchez-Moreiras et al. 2001). Since this method is widely used in genotoxicity studies or to assess the potential as cell cycle inhibitor of a given substance, the most common procedure is to compare the data from a control treatment with those obtained for the tested compound. Staining cells at different mitotic phases can be seen in Fig. 13.4.

The method for mitotic index calculation uses hydroxiurea (HU) as cell cycle arresting agent, it is focused on the analysis of seedlings root apexes and it consists in the following steps (Protocol is summarized in Fig. 13.3):

1. Treat plant seedlings with 2 mM HU during 14 hours to obtain cell cycle synchronization. HU is applied especially to radicles, ensuring that they are completely covered with treatment.
2. Remove HU through three consecutive washes with distilled water. Synchronization progressively decreases once HU treatment is released.
3. Immediately, apply the treatment of the studied compound. At this time, the control treatment (usually distilled water) is also treated. For a better asses of cell division under a potentially genotoxic substance it is advisable to do treatments at different times to observe progression. A powerful cell division blocker can act in minutes, but it is also interesting to know what happens after several hours.

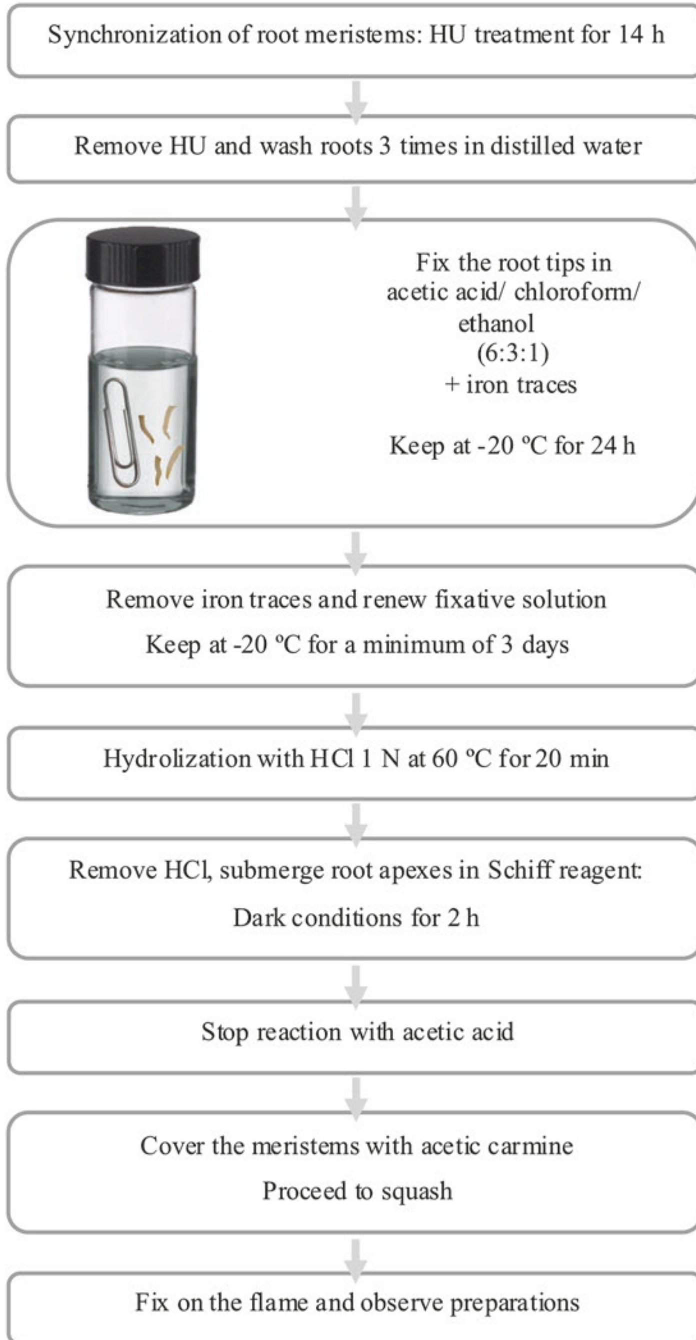

Fig. 13.3 Representative scheme of the mitotic index protocol

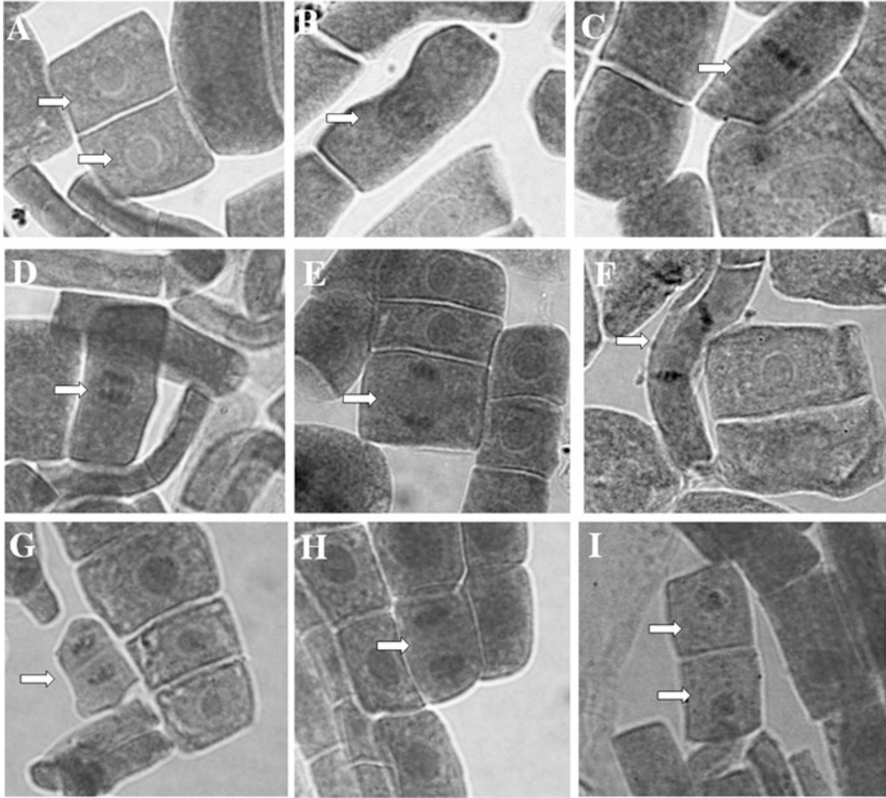

Fig. 13.4 Microphotographs of different phases of mitosis on *Arabidopsis* seedlings meristems. (**a**) Interphase cells; (**b**) Nucleus at incipient prophase; (**c**) metaphase; (**d**–**f**) different stages of anaphase; (**g** and **h**) telophases; (**i**) recently separated daughter cells. (Images belong to control-treated meristems. Experiment done by Graña et al. 2013)

4. Collect plant material. Root apexes are excised about 1 cm above the end of the roots. Then, they are submerged in a fixative medium consisting of acetic acid/chloroform/ethanol (6:3:1). This step is done in small crystal vials (5–10 mL) with hermetic lids to avoid evaporation, introducing some iron traces in them. This metal acts as a mordant and changes the isoelectric point and accelerates the nuclear staining (Sánchez-Moreiras et al. 2001).
5. Store vials at −20 °C for 24 h.
6. Remove iron traces, renew fixative solution and store again at −20 °C for a minimum of 3 days.
7. After that time, hydrolyze plant material with 1 N HCl at 60 °C for 20 min. Ensure that lids are well closed to avoid the evaporation of HCl.
8. Remove HCl and submerge root apexes in Schiff reagent in dark conditions for 2 h. Chromosomes will be stained with pink to violet color.

9. Stop reaction: put plant material on a slide and cover it with a drop of acetic acid. At this point, root apexes can be cut with a razor blade.
10. Put a drop of acetic carmine over the meristems and squash them. This step will separate cells and form a monolayer, essential to observe the nuclei of all the cells.
11. Set the preparation by passing the slide over a flame for 1–3 s. It has to be done very carefully avoiding t burn it.
12. Fix the slide with the cover using nail polish and observe preparations using bright-field microscope.
13. Score the number of mitotic cells and also the total number of cells.

5 Tricks and Recommendations

– Step 4: A very easy way to get iron traces is just using a clip, a staple or an iron pushpin. Besides, at this step, it is not necessary to prepare big amounts of fixative solution: just prepare the enough solution to cover all radicles.
– Step 10: A good idea to uniformly squash the meristems is pressing the cover with an eraser.
– Step 11: The time of exposure to flame is relative; it depends on the thickness of the tissue. It is very common to overexpose, burn the meristems and leave them useless. I recommend do it in small fractions of time and view the meristems to check fixation.
– Step 13: It is generally recommended to score about 1000 cells per replicate, which can represent 3–5 meristems depending on their size.

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