Chapter 7

Cyto(Geno)Toxic Endpoints Assessed via Cell Cycle Bioassays in Plant Models

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72997

Abstract

Environmental pollution is a matter of great concern. Therefore, researches that aim to access the risk of toxicity of these potential pollutants are welcome in the scientific community. The most common strategy to detect toxic agents is through chemical analysis. However, in the last years, the biological assays are often important for risk assessments. Among the bioassays using living organisms to detect toxicity of a compound, plant models have been highlighted as it is easy to be conducted, has low cost, high sensitivity and presents good correlation with other test systems, including mammals. Besides, it is in accordance with the Toxicology Guidelines for the twenty-first century, which claims for bioassays that could substitute the ones that use animals as models. At cellular level, the cytotoxicity, genotoxicity, and mutagenicity are the parameters determined by the endpoints as mitotic index, DNA fragmentation, induction of cell death, and malfunction of cellular structures leading to chromosome and cell cycle alterations. Each of these endpoints will be presented in details in this chapter.

Keywords: cell cycle analysis, chromosome alterations, DNA fragmentation, TUNEL assay, comet assay

1. Introduction

Concerns of the world society and authorities over the environment are imperative. Hereby the growing environmental pollution and how to slow down or mitigate it are key points of discussion. Various aspects need to be approached as regards understanding the whole process and dynamics of pollutants in the environment. Among the first actions required to

ensure the quality and health of the environment, in both the short and long terms, it is fundamental to obtain information about contaminating agents.

Overall, research in the environmental area is based on analyses and physicochemical characteristics of pollutants. However, it has been recognized that the effects of these compounds on living organisms, as well as their toxicity mechanisms, are excellent tools to complement the obtained physicochemical data [1], being important for decision-making and in the search for preventive, mitigating measures as well as alternatives to this scenario.

In this sense, the biological effects of pollutants can be assessed in vivo (in situ and ex situ) and in vitro via bioassays using test organisms, allowing to evaluate their toxic potential in a rapid and efficacious manner and at relatively low cost. Overall, the response in relation to toxicity can be given a different organization level, such as behavior, physiology, anatomy, cell, and DNA, among others, with each organism and test representing a certain endpoint. The integrity of the genetic material and its consequence for the proliferation and reproduction of model organisms are the most targeted outcomes and estimate the dimension of the risks of compounds to the environment and living beings in a real and functional manner [2–4].

Among the different bioassays performed in living organisms, those that use higher plants as models to evaluate the biological effects of environmental pollutants stand out. Besides being validated by the US Environmental Protection Agency (US EPA) as efficacious in the determination of toxicological risks in toxicity monitoring programs, they present important characteristics such as high sensitivity, fewer false-negative responses, low cost, not requiring approval from ethics commissions, and being as efficient as assays performed in animal models or even human cells [5–8]. In addition, they are in accordance with the Toxicology Guidelines for the twenty-first century, which calls for models that substitute animal ones to assess toxic risks [9].

Among the assays using higher plants highlighted by the Genetic Toxicology (Gene-Tox) program of the US EPA described by Ma [10], one of the most widespread is the *Allium* test. It was developed and described in 1938 by Levan [11] and consists in the evaluation of alterations in the mitotic phases of root meristem cells of *Allium cepa* [12]. In general terms, the test can be applied to any plant model that presents chromosomes of easy visualization under the microscope. It is employed to evaluate the cell cycle in meristematic root tip cells, observing disturbances in the frequency of cells in division as well as induction of alterations in the mitotic phases or in the interphase nucleus, arising from action of the tested pollutant.

In this chapter, the main characteristics of the assay based on evaluation of the cell cycle will be presented, as well as the endpoints that can be assessed and used for evaluation, determination of cyto(geno)toxicity, and understanding of the mechanisms of action of potential environmental pollutants.

2. Cytogenetic analyses applied to environmental toxicology

Plants constitute a system of great importance as bioindicators of pollution, having long been used for this end. International institutions such as the United Nations Environmental

Programme (UNEP), the World Health Organization (WHO), and the US EPA approve the use of bioassays with plants to investigate toxic effects of chemical agents released into the environment [13].

The use of plants as models to evaluate the toxicity and mutagenicity of substances or pollutants enables the analysis both in the natural environment (in situ) and in the laboratory (ex situ). They are excellent tools to complement the physicochemical analyses of investigated compounds, as they allow a practical confirmation of the theory developed in studies on the physicochemical properties of the potentially dangerous materials [1, 14, 15].

Tests ex situ commonly use meristematic root tip cells as biological material for analysis. In the natural environment, the root is the first part of the plant to be exposed to toxic agents dispersed in the soil and water. Therefore, the analysis of root cells represents a rapid method for the monitoring of toxicity. Moreover, the observed damage to the DNA and/or chromosomes of plant cells can be extrapolated to further organisms based on the universality of the DNA structure and genetic code [16]. This way, if a chemical substance causes damage to the DNA of one plant, it should also be considered potentially damaging to the DNA of other organisms [17].

The assay with meristematic root tip cells is based on cytogenetic evaluations involving the movement of chromosomes during the mitotic division, which allows deriving the mechanisms of action of the pollutant. The root of a propagule (bulb, seeds, cutting, etc.) is exposed to the agent that shall be tested. By the end of the exposure interval, the meristem is separated from the root and fixed; a slide is subsequently prepared, generally by squashing technique, and the meristematic cells are stained with acetic orcein and/or Schiff's reagent (for the detailed methodology, see [18]). The slide is observed under light microscope, and various parameters of the cell cycle are evaluated. The cell cycle stages, including interphase and mitotic division (prophase, metaphase, anaphase, and telophase), are observed, and the alterations detected in each phase are recorded. Based on the results, the assessed endpoints are (1) frequency of dividing cells or mitotic index (MI), given by the sum of cells in phase M (mitosis) divided by the total number of observed cells, being expressed in number of dividing cells out of every 100 observed cells; (2) total frequency of chromosome alterations (CA), given by the sum of all observed alterations, independently of type and division phase, divided by the total number of observed cells, expressed as number of altered cells out of 100 observed cells; or (3) nuclear alterations (NA), related to the presence of abnormal interphase nuclei, with unusual form or extremely condensed appearance, also given by the sum of total observed alterations by the total number of counted cells, and expressed as the number of alterations out of 100 cells (for calculations, see [19]).

In summary, the tested agent can be characterized as cytotoxic when it alters the normal MI (increase or reduction) of the used plant model, hence causing malfunctioning of cell structures and possibly leading to cell death, and/or genotoxic if the alterations observed throughout the cycle are related to DNA breakage, including the formation of micronuclei. These bodies are considered a mutagenicity parameter as they represent damage not corrected by the cell repair system and, thus, permanent and transmissible to the subsequent cell generations. An alteration can also be classified as aneugenic, when it is related to malformation or malfunctioning of the mitotic spindle or the attachment of the chromosomes on the spindle and leads to gain or loss of one or more chromosomes, or clastogenic, when associated to

breakages and rearrangements in the DNA or chromosomes [16, 20, 21]. Each of these endpoints and possible alterations that can be observed throughout the mitotic cell cycle, and their consequences, will be detailed next.

3. What can cell cycle analyses reveal?

Evaluation of the cell cycle, which comprises the interphase (G1, S, and G2) and the M phase (mitosis-prophase, metaphase, anaphase, and telophase), allows gaining knowledge about the organizational structure of the chromosomes and how they behave during the cell division. As mentioned previously in this chapter, such assessment can be employed to determine the toxicity of a chemical compound. Alterations in the mitotic index help determine the degree of cytotoxicity of an agent, whereas chromosome alterations observed in the cell cycle define the genotoxicity of the agents and their capacity of causing damage to the DNA, which may or may not be repaired by the cellular repair mechanisms. Together, the cell cycle alterations express the cyto(geno)toxicity of chemical compounds and environmental pollutants and are used to investigate their toxic potential.

Several endpoints can be monitored in the division of meristematic cells, such as the chromosomal and nuclear aberrations previously described, besides the formation of micronuclei.

To better understand the alterations observed in the cell cycle, it is necessary to remember that the movement of chromosomes for segregation of the DNA into the daughter cells relies on the mitotic spindle, formed by microtubules. The whole dynamics of the mitotic process thus depend on the binding of the microtubules to the chromosome centromeres, besides microtubule polymerization and depolymerization mechanisms. In this sense, alterations in these dynamics affect the segregation of chromosomes to the daughter cells and may be considered the first origin of alterations observed in the cell cycle. Hence, as consequences of alterations in the spindle and correct attachment of the chromosomes, we can cite the interruption of the cell cycle in metaphase, originating c-metaphases (**Figure 1A**) and formation of polyploid cells (**Figure 1B**), as consequence, and multipolar anaphase (**Figure 1C**), non-oriented chromosomes at the equatorial plan (**Figure 1D**) or delayed segregation of the chromosomes/chromatids in anaphase/telophase (**Figure 1E** and **F**) [21–24].

When interference in the polymerization and depolymerization of the microtubules occurs, the cell cycle may be paralyzed in metaphase, and the chromosomes are visualized as well condensed, with well-defined centromere and spread inside the cell [22]. In the laboratory, this situation is caused with substances' denominated blockers, such as colchicine, which gives this alteration its name: colchicine metaphase or c-metaphase (**Figure 1A**). These extremely condensed and separated chromosomes are used in karyotype studies of the species, as they allow observing the morphology of each chromosome individually.

Polyploidy emerges as a consequence of the prolonged effect of a substance or toxic compound in the cells. In the absence of the spindle, the cell with duplicated DNA, represented by the chromosome with two chromatids, returns to interphase, initiating a new cell cycle. In the G1

http://dx.doi.org/10.5772/intechopen.72997

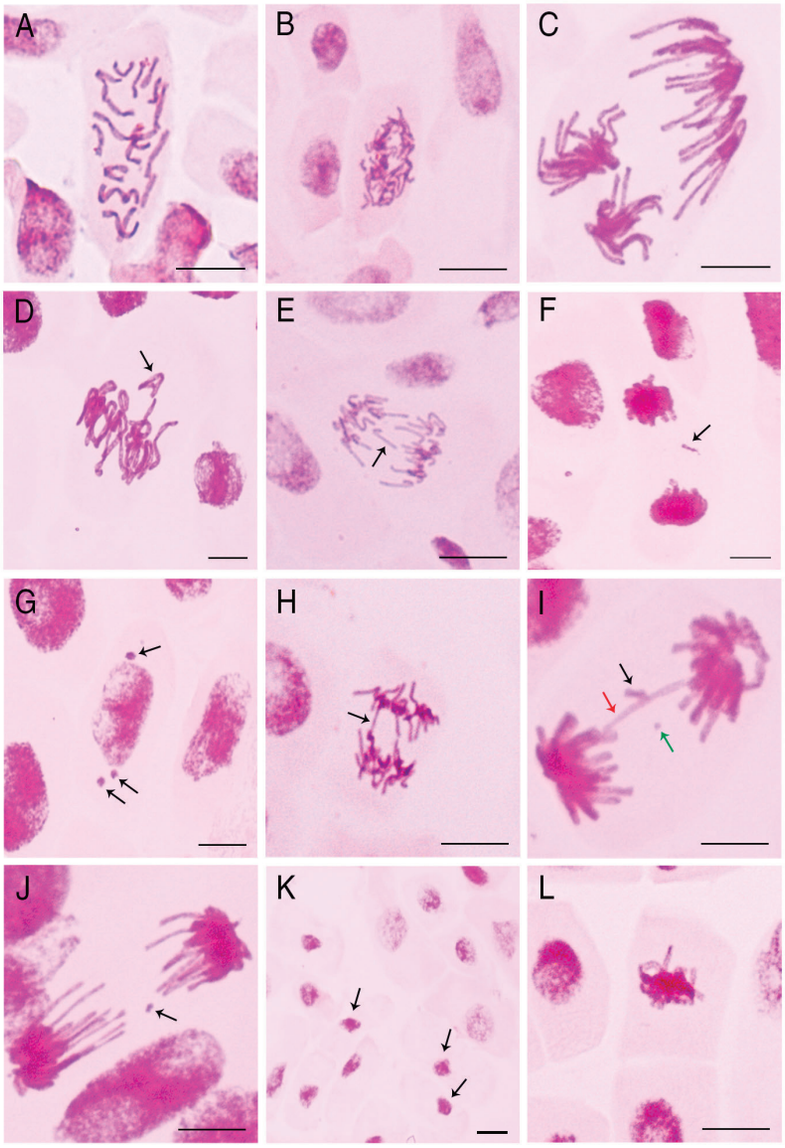

Figure 1. Example of cell cycle alterations observed in meristematic cells of *Allium cepa* (onion) and *Lactuca sativa* (lettuce) root tips. (A) C-metaphasis in lettuce exposure to methyl methanesulfonate (MMS); (B) polyploidy metaphasis in lettuce exposure to cadmium; (C) multipolar anaphases in onion exposure to atrazine herbicide; (D) non-oriented chromosome (black arrow) in onion metaphasis exposure to cadmium; (E) not normal/laggard segregation (black arrow) in lettuce anaphase exposure to cadmium; (F) not normal/laggard segregation (black arrow) in onion telophase exposure to MMS; (G) micronuclei in onion exposure to MMS; (H) anaphase bridge (black arrow) in lettuce exposure to spent Potliner (SPL); (I) anaphase bridge (red arrow) in onion exposure to MMS with a fragment (black arrow) and a micronucleus (green arrow); (J) chromosome fragments (black arrow) in onion exposure to MMS; (K) condensed nuclei (black arrow) in lettuce exposure to atrazine herbicide; and (L) stickiness chromosome in lettuce exposure to SPL. Images obtained in a light microscope at oil objective (100×). Bars 10 μm.

nucleus, each chromatid of the chromosome starts representing one DNA molecule of the cell that will be replicated in the S phase. Upon initiating a new mitotic cycle, after G2, the proteins of the chromosome's protein scaffold keep the sister chromatids united, and the cell starts mitosis with a duplicated number of chromosomes, characterizing polyploidy. Under light microscope, a cell is characterized as polyploid when an excess number of chromosomes and/or cell volume larger than usual can be observed at the end of prophase or beginning of metaphase (**Figure 1B**).

Multipolar anaphase and abnormal segregation of chromatids in anaphase/telophase also arise from the action of chemical substances on the organization of the microtubules. These alterations are observed as a consequence of incorrect binding of the mitotic spindle to the centromere of the chromosomes [24] or from the shortening and elongation of some microtubules of the mitotic spindle out of synchrony with the other microtubules. Unequal disjunction of the chromosomes may thus occur (non-oriented chromosomes, **Figure 1D**), giving rise to micronuclei (**Figure 1G**) when these chromosomes cannot be reincorporated into the main nucleus along with the other chromosomes [25].

So far, some alterations of the cell cycle have been demonstrated which arise from effect of the toxic agent on the malformation of a cellular structure. Together, these alterations characterize an aneugenic effect and mechanism of the toxic agent, as they can have as consequences the increase or decrease in the number of chromosomes of the species. All these aneugenic alterations represent the cytotoxicity of a given substance, as they relate to a cell structure.

The action of toxic substances can also occur directly on the DNA. In this case, they are observed in the cell cycle as alterations in chromosome structure. Since the effects occur on the genetic material in this case, the mechanism of action of the substance is clastogenic and represents its genotoxicity.

The most evident clastogenic effect when observing the cell cycle under the microscope is the presence of bridges (**Figure 1H** and **I**) and chromosome fragments (**Figure 1I**) arising from breakages in the DNA molecule. Overall, one of the consequences of the breakage is the loss of telomeres, a region in the terminal extremity of the chromosomes that has the function of ensuring the chromosome protection and stability. With the loss of the chromosome stability, fusion of the terminal portion of two chromosomes may occur. Upon division, chromosomes with two centromeres are observed as bridges in anaphase/telophase (**Figure 1H** and **I**), where each of the centromeres is linked to the spindle of one of the cell poles.

The chromosome region originated from the breakage that is devoid of centromere is denominated acentric fragment (**Figure 1J**). These chromosome fragments, due to containing parts of the genetic material, are recognized by the cell and involved in membrane during cell division, giving rise to micronuclei, which are easily observed in cells of the F1 generation [7].

Several authors highlight and affirm that micronuclei are the most effective and simple endpoint for the analysis of mutagenic effects caused by chemical compounds, owing to their arising from non- or incorrectly repaired damage in the parental cells. They are easily observed in daughter cells as a structure similar to the main nucleus but with smaller size (**Figure 1G**). Indeed, the micronucleus is easily recognized in the cell visualized under the microscope, particularly if the preparation was accomplished using a DNA-specific dye. In several cytological study models, including human blood cells in culture, the micronuclei assay is applied as a marker of mutagenicity. However, as explained here, it can originate from both acentric fragments and entire chromosomes that were not bound to the spindle. Since each of these causes of micronuclei formation originates from a distinct mechanism of action, assessment of the entire cell cycle, if possible, together with evaluation of micronuclei induction is seen as the cheapest strategy to determine the mechanism of action of the studied substance or compound.

All these reported alterations, if persistent and deleterious, activate the cell death mechanisms. Under light microscope, the evidence for occurrence of cell death is the observation of highly condensed interphase nuclei (**Figure 1K**), with very heterochromatic chromatin, appearing well rounded, darker, and smaller than the normal interphase nuclei [26, 27].

The cell death process due to abiotic stress is cytologically characterized by condensed nuclei and molecularly by DNA fragmentation [26]. This death mechanism is related to destruction and subsequent elimination of damaged cells [28].

Toxic substances can also trigger the formation of sticky chromosomes (**Figure 1L**). Overall, they are characterized by alterations in the physicochemical structure of the DNA, proteins, or both, formed from complexes with phosphate groups of the DNA, inter- and intrachromatid linkages, and DNA condensation [22, 29]. These factors promote loss of the normal characteristics of condensation, causing the formation of agglomerates [22, 30]. Chromosome stickiness is considered a highly toxic alteration [31] that hinders the segregation of the chromatids and the normal continuation of the cell division, which may trigger the cell death process, avoiding that the toxic effect be passed onto the following generation.

Of the observed alterations, chromosome stickiness is considered the most intriguing as regards the classification in aneugenic or clastogenic, in function of the mechanisms involved in their occurrence in the cell cycle. Here, it is considered a complex cyto(geno)toxic effect arising from previous events, for instance, polyploidy or excessive breakages and bridges in the DNA molecule, present at different levels. As regards the consequences of stickiness to the cell, some authors like Andrade et al. [31] cite that the high frequency of stickiness may activate the cell death mechanisms. Thus, the induction of severe stickiness cannot be repaired by the cells, having as consequence the heterochromatinization of the whole nucleus.

4. Investigation of cell death mechanisms and DNA damage applied to environmental toxicology

DNA fragmentation, previously reported as the clastogenic effect of a toxic agent in the cell, is one of the mechanisms related to the cell death process. It can be evaluated through application of techniques available as kits containing a marker for fragmentation.

The Terminal d-UTP Nick End Labeling (TUNEL) assay is one of the tests used for the analysis of DNA fragmentation and investigation of the cell death mechanisms. It is based on incorporation of nucleotides (d-UTP = 2′-deoxyuridine, 5′-triphosphate) marked with a fluorochrome (fluorescein isothiocyanate, FITC) in the free 3′OH region of the breakages in the DNA chain by the enzyme terminal deoxynucleotidyl transferase (TdT) [26, 32]. This reaction relies on the capacity of the enzyme TdT of coupling a deoxy-uracyl-fluorescein (d-UTP) conjugated to the 3′OH end of the broken DNA [32, 33]. The incorporation of fluorescein-12-d-UTP is then amplified by various enzymatic reactions [34]. These nucleotides can be marked with a fluorescent dye and detected by fluorescence microscopy or the laser of a cytometer [35].

Under fluorescence microscope, the cells can be visualized with different fluorescence intensities in function of the marker, fluorescein. They are then classified as (A) not marked (**Figure 2**), thus without fragmentation; (B) weakly marked (**Figure 2**), therefore presenting light damage that can still be recovered, since the cell death process involves several steps and only the final ones represent a "one-way road"; and (C) strongly marked (**Figure 2**), associated to cells with high frequency of fragmented DNA and in advanced stage of cell death [18].

The comet assay or single cell gel electrophoresis (SCGE) is another technique very useful to identify DNA damage. It allows the detection of damage to the genetic material caused by rupture of chains, alkali-labile sites (ALS), incomplete excision repair sites, and reticulations, induced by alkylating or intercalating agents and oxidative damage, even before the cell repair system acts. Further, it allows verifying the damage present after the cell repair process.

In plants, it is broadly used in ecotoxicological studies of environmental pollutants and is characterized by its high sensitivity and specificity, low cost, and rapidness in detecting the genotoxic effects, requiring small sample size, and allowing for simple analysis. The evaluation can be performed at individual cell level or applied to any cell population, without requirement of cell division. In summary, it can be executed in three versions and detect a broad spectrum of damage to the genetic material [36, 37].

The three possible versions of the comet assay differ with regard to the pH of the electrophoresis buffer, which can be neutral, slightly alkaline, and alkaline-alkaline. In the neutral method, ruptures of the DNA double strand are detected. In the moderately alkaline version, simple breakages in the DNA and the double helix are observed. In turn, in the alkaline-alkaline approach, used in the majority of the studies owing to its greater sensitivity, breakages of single and double strands as well as alkali-labile sites and crosslinks are quantified. The choice of the comet assay version depends on the type of damage that shall be observed [38].

The comet assay can be used to complement the cytogenetic data obtained from the cell cycle analyses, as it detects genomic lesions caused to the DNA arising from the action of mutagens. Unlike mutations, the lesions identified by the comet assay are prone to repair. The technique

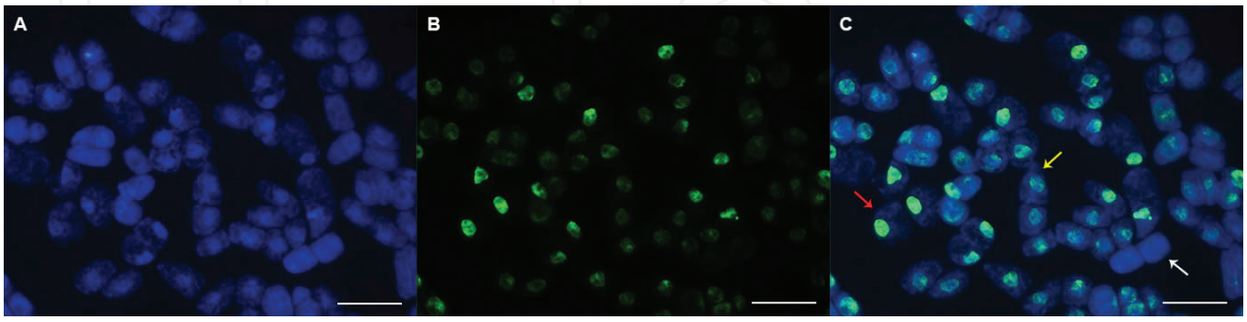

Figure 2. Meristematic cells of *Lactuca sativa* L. (lettuce) treated with MMS submitted to the TUNEL test. (A) Image captured with filter at wavelength of 345–358 nm (for DAPI). (B) Image captured with filter at wavelength of 488–495 nm. (C) Result of overlapping of images A and B made through the AxioVision program, where it is possible to observe unmarked nuclei, without damage (white arrow); weakly marked nuclei, with slight damage (yellow arrow); and strongly marked nuclei, with severe damage (red arrow) to DNA. Images obtained in a microscope of fluorescence (Olympus BX 60) on 40× objective. Bars 50 μm.

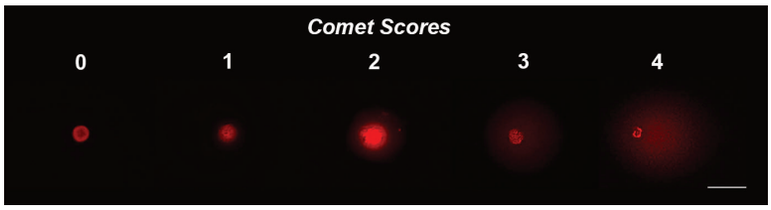

Figure 3. Meristematic cells of *Allium cepa* L. (onion) treated with water (*score* 0) and spent Potliner (SPL) (*scores* 1–4) submitted to the comet assay. The *scores* 0–4 are attributed according to visual analysis of nucleoids. Images obtained in a microscope of fluorescence (Olympux BX 60) on 40× objective. Bars 50 μm.

consists in the immersion of viable cells in agarose gel, lysis of the cell membrane by detergents and alkaline salts, and subsequent electrophoresis. Under alkaline electrophoresis conditions, cell DNAs that have suffered damage present higher rate of migration toward the anode, owing to breakages of simple or double strands and alkali-labile sites, simulating the appearance of a comet (head and tail) [39]. The level of damage is measured by observing the degree of fragmentation (score) of the genetic material in the electrophoresis, whereby the damaged DNA presents higher rate of migration toward the anode, and the least damaged shows greater migration rate. The four scores most commonly used in the visual identification under the microscope are presented in **Figure 3**.

5. Final considerations

Bioassays with plants usually comprise macroscopic tests, which involve evaluations of germination and initial plantlet development (growth of root and aerial part), as well as microscopic evaluations, including the observation of alterations during the cell cycle in meristematic cells exposed to the tested chemical agent. The results of these bioassays allow determining the phytotoxicity, cytotoxicity, genotoxicity, and mutagenicity of the pollutant or chemical compound in question.

Both in the macroscopic and microscopic evaluations, the root is the plant organ used in the tests. It is particularly useful in these eco(geno)toxicological tests, as it is the first part of the plant to be exposed to environmental pollutants. For macroscopic assessment, the observation of root growth is a rapid and sensitive method for environmental monitoring, but does not contribute to the understanding of toxicity mechanisms.

Root tips contain meristematic cells that present intensive cell division, allowing a rapid and adequate evaluation of the cell cycle, constituting the microscopic assessments whose measurable parameters have been described here. Besides providing important information to determine the mode of action of a given agent, the described microscopic evaluations are directly related to the growth parameter assessed in the macroscopic assay. In plants, as sessile organisms, the growth of an organ is closely related to the increase in the number of cells in the tissue composing it. This way, alterations in the endpoint mitotic index, assessed in the microscopic assay, explain what can be seen with the naked eye. In turn, the endpoint associated to the malfunctioning of cell structures like the mitotic spindle explains alterations

in the mitotic index as well as cell death, which is an endpoint characterized by increase in condensed nuclei and which contributes to the reduction of the mitotic index. Nevertheless, the endpoint associated to DNA fragmentation can be assessed not only by direct observation of the cell cycle but also through more specific techniques such as the TUNEL or comet assays. These methods ultimately explain and confirm the induction of cell death, which, as already reported, contributes for a mitodepressive and phytotoxic effect on root growth.

In conclusion, based on the endpoints that can be assessed through cell cycle analyses, cytotoxicity as well as genotoxicity and mutagenicity of environmental pollutants can be determined in a rapid and reliable manner. Such tests are highly useful to monitor and assess the risk of potentially toxic substances that are released into the environment.

Acknowledgements

We would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) for providing postgraduate scholarships to the authors of this work.

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