Comet assay stained with Giemsa as a suitable method for evaluation of mutagenicity in maize

Fagiani, Marcela A.B.; Reis, Luís S.L.S.; Leite, Kerle A.S.;
Zandonato, Vivian V.; Fluminhan, Antonio
Universidade do Oeste Paulista, Presidente Prudente, SP, Brazil
Email: fluminhan@pq.cnpq.br

Abstract

Studies to evaluate the genotoxicity of agents with mutagenic potential have been performed widely, due to the recent concern about the impacts caused by human activities on the environment. Among the methods used for this evaluation, there are those that determine the genomic lesions, which can result in mutation in the different living beings, as is the case of the comet assay. In the present research, the methodology of the comet assay for the evaluation of possible genomic lesions caused by physical and chemical mutagenic agents on maize, which is considered an excellent biological model for this type of evaluation. Tissue samples from the leaves were collected and used to evaluate the two main protocols available for the comet assay test: the neutral version, which uses electrophoresis in buffer with neutral pH, detecting double breaks in the DNA molecule, and the alkaline version (pH> 13), which was developed from the original method and is performed using alkaline pH electrophoresis, detecting both single and double breaks in the DNA molecule. Numerous pilot tests were carried out and effectively allowed for the standardization of the methodology by using Giemsa dye and observation under light microscope. The implementation of this methodology opens broad perspectives for its use in the evaluation of the mutagenic potential of anthropic factors.

Introduction

The microgel electrophoresis assay (or simply: comet assay) is a test that detects primary damage in DNA, such as: single, direct or injury-induced breaks. This method is indicative of mutagenicity, even though such lesions may undergo repair by the intracellular repair system. The comet assay can be considered a simple, rapid, sensitive and low cost method that provides results at the individual cell level, with extremely small cell samples being required (Green et al. 1992, Betti et al. 1994). The advantage of the comet test is that it can be used to measure DNA breaks in any type of eukaryotic cells, not necessarily in proliferation, requiring only a good cell suspension (Ribas et al. 1995, Ross et al. 1995, Tice et al. 1996).

The test consists in passing an electric current through the lysed cells of the sample under alkaline solution condition and embedded in a low melting point agarose gel on slides for microscopy. The cells are gelled and spread over a slide and subjected to an electric current that promotes a migration of the free DNA segments resulting from breaks out of the nucleus. After electrophoresis, cells with round nuclei are identified as normal, with no detectable DNA damage. The damaged cells are visually identified by a comet-like tail-like species formed by DNA fragments that have been "dragged" by the electric current of the electrophoresis.

The analyzes can be performed on optical microscopes, when cells are usually stained with Silver nitrate or fluorochromes. The cells are classified according to the size of the "tail" with respect to "head" (nucleus), in four classes of damage: class 0: without tail, class 1: tail with smaller diameter than head, class 3: long tail, superior to twice the diameter of the head, class 4: long tail and mirrored, fan-shaped and absense of head. For some authors, the size of the tail is proportional to the size of the damage that was caused, but the simple visualization of the "comet" already means that varying degrees of damage are present in the DNA, and may involve: single-strand breaks, double strands, cross links, excision repair sites and / or labile alkali lesions (Tice et al. 2000).

The comet assay has been proposed for toxicogenic studies due to its peculiarities and advantages when compared to other tests for the detection of genotoxic substances (Koppen et al. 1999). It combines the simplicity of the biochemical technique of detecting breaks in DNA with the use of few cells, thus, it corresponds to a cytogenetic assay. Advantages of this technique include sensitivity in detecting DNA damage; data collection at the individual cell level; the use of a small number of cells for analysis and the possibility of application in any eukaryotic cell population (Singh et al. 1998); and especially the speed of results, that is important for clinical diagnoses (Burlinson et al. 2007). It is a promising technique, especially to evaluate effects caused by chemical agents (Ribas et al. 1995).

During the last two decades, the comet assay has been extensively used as a basic tool in many areas of research, reaching applications for environmental biomonitoring, biological radiation, and DNA repair and genetic ecotoxicology processes (Cotelle and Férard 1999). It has a potentially broad application in both:in vitro and in vivo genotoxicity tests in damage and repair studies in DNA and environmental biomonitoring (Tice et al. 2000).

The use of maize plants is fully justified because this species is sensitive to various types of mutagenic agents. Numerous studies indicate that this species is classified as ideal for on-site monitoring of environmental conditions, including water, soil and air pollution. In particular, the cells of almost all parts of the plant since from the root to the pollen mother cells provide excellent materials for cytogenetic studies.

Material and methods

The protocols were established from adaptations of methodologies originally employed for animal cells and with another plant model: *Tradescantia pallida* cv *purpurea* (Leite et al. 2013). Adult plants were exposed for 0, 10 and 30 minutes at 1m distance from a 30W ultraviolet germicidal irradiation (UVGI) lamp that was used as mutagenic treatment. Suspension cells removed from adult plant leaves were soaked in a low melting point (LMP) agarose solution and placed immediately on the already covered slide with agarose solution. After solidification, the slides were dipped in ice-cold lysis solution for at least one hour and then transferred to electrophoresis vessel with TBE buffer for 20 min.

The two main protocols available for the comet assay, which are established according to the pH: the neutral version (pH 7-8), where electrophoresis was used in buffer with neutral pH and appropriate for the detection of breaks in the DNA molecule. Then, we have tested in its alkaline version (pH> 13), that performs electrophoresis with alkaline pH, which allows the detection of bothtypes: single and double breaks in the DNA molecule (Tice et al. 1996).

According to the protocol used, the slides were immersed in the denaturation solution and incubated with slow agitation for 20 minutes at 4° C. As the agarose gel is very brittle, at the time the treatment is performed, the slides were immersed in the denaturation solution and held in the refrigerator for 15 minutes, followed by careful washing in distilled water.

The electrophoresis cell was filled with the TBE buffer solution until completely covering the slide containing the biological material under analysis. The time and running conditions used in the present study were, respectively, 20 minutes at 75V and 250mA. After the electrophoresis run the slides were treated with neutralizing solution. Finally, the slides were stained with Giemsa dye (1% v/v solution) for about 5 minutes, and washed with distilled water. After drying the slides in the oven at 37° C for 10 minutes, the slides were analyzed under the optical microscope to check for comets.

Results and Discussion

It was verified that the first method (neutral version) produced the best results. Cells removed from leaves of plants exposed to 10 and 30 minutes UV radiation emitted from the germicidal lamp allowed the visualization of extensive occurrence of damage to the genetic material (Figure 1 - 1b and 1c, respectively).

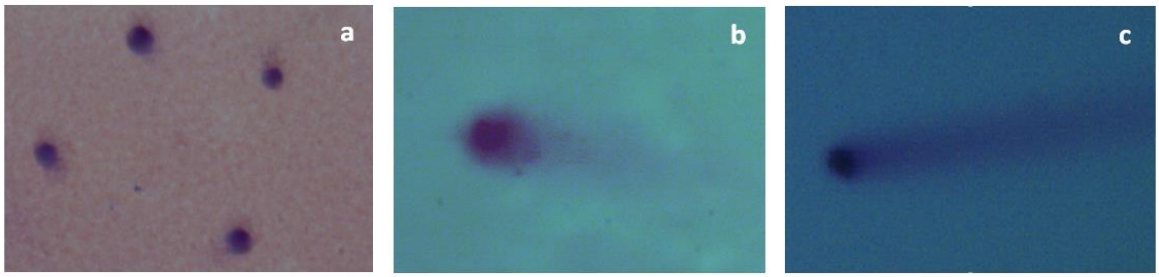

Figure 1: Cell nuclei removed from maize leaves and stained with 1% Giemsa solution, showing the chromosomes apparently devoid of nuclear envelope (a), forming the "tail" of the sharp comet under the optical microscope, after exposition for 10 (b) and 30 minutes (c) at 1m distance from a 30W ultraviolet germicidal irradiation (UVGI) lamp that was used as mutagenic treatment.

The Giemsa dye presents technical advantages over fluorochromes, since it uses the commom bright field optical microscope and over Silver nitrate, due to the ease of manipulation of samples and reagents. In this case, comet analysis can be done visually, but there is a disadvantage of the subjectivity of the results, which can be minimized by automated digital analysis.

It should be noted that the Comet assay is not used to detect mutations, but rather genomic lesions that, after being processed, may result in mutation. Unlike the mutations, the lesions detected by the Comet assay can be corrected. Thus, the Comet assay can also be used for DNA repair studies, providing important information about the kinetics and type of repaired lesion, although it is not possible to infer the reliability of the repair process (Albertini et al. 2000).

The implementation of this methodology opens up favorable perspectives for its use in the evaluation of the mutagenic potential of other anthropic factors. As an example of application of the methodology, it is intended to cultivate plants of this species in discrepant situations, as a result of the combinations of basic environmental factors, such

as: contaminated soils, water and polluted air. Also, we have considered to evaluate the seasonal effects due to variations of the weather conditions along the year.

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