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Comet Assay: A Strong Tool for Evaluating DNA Damage and Comprehensive Guidelines for Plant Cells

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Abstract

Heavy metals affect plant system in various toxic ways including morpho-physiological alterations and genotoxic damages inside a plant cell. The extent of DNA damage under any genotoxic agents can be effectively measured in single cells applying comet assay approach. Comet assay primarily measures DNA strand breakage in single cells and its use has increased in different areas: clinical applications, human monitoring, radiation biology and genetic ecotoxicology. This paper is a review of the detailed protocol and precautions to be taken while performing comet assay and may have been slightly modified from other original protocols according to the plant, organ, cell type, etc. In conclusion, the study reviewed in this paper demonstrate that the comet assay application in plants provides a reliable, sensitive and rapid system for the study of environmental genotoxicity caused by heavy metals.

1. Introduction

The single cell gel electrophoresis (SCGE) assay also known as Comet assay has become a reliable and sensitive method to appraise the extent of DNA damage in eukaryotic systems at a single-cell level. The principle of the comet assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleus whereas undamaged DNA migrates slower and remains within the confines of the nucleus under the influence of an electric field. Evaluation of the "comet tail" shape and migration pattern allows for the assessment of DNA damage. The neutral comet assay is typically used to detect double-stranded breaks, whereas the alkaline comet assay is more sensitive, and is used to detect smaller amounts of damage including single and double-stranded breaks, alkali labile sites, incomplete excision repair sites, and DNA cross links. The practice of this technique was initially limited to animal/ mammalian systems and was not feasible in plant cells. But, over a period of time, the continuous advancement in this technique, made it possible to perform the comet assay successfully in plant cells too. Higher plants have a long history of use in mutational research and the use of comet assay in plant tissues would significantly extend the utility of plants in basic and applied research in the field of environmental mutagenesis and bio-monitoring.

Plants being sessile organisms continuously exposed to various biotic and abiotic stresses in their lifetime, particularly toxic chemicals and/ or heavy metals due to anthropogenic activities (Hu *et al*., 2016). Therefore, plants are being used widely for the evaluation of toxicity and for the bio-monitoring of toxicants both in aquatic and terrestrial ecosystems. While rapid progress have been made in the past few decades to assess the impact of these environmental hazards in the induction of genetic damage in humans/ animals, little progress has been done to determine the impact of such factors on plants. Koppen and Verschaeve (1996) first reported comet assay analysis in plant (*Vicia faba*) treated with mutagens with isolated nuclei rather than whole cells. From this experiment, it became evident that comet assay is well suited to be applied in plants too. Nowadays, plant comet assay has been used in different adverse conditions and some recent work in this field (Gichner *et al*., 2006; Ventura *et al*., 2013) revised most recent advances in plant comet assay. Plant genetic assay systems are excellent *in situ* environmental monitors and SCGE analysis in plants could increase their utility as environmental monitors.

During the last decade, SCGE or comet assay has became one of the most promising technique used in the field of genetic ecotoxicology because of its simplicity, sensitivity, speed and the less number of cells required to obtain robust results (Dhawan and Anderson, 2016).

Over the years, its use was limited to a few model plant species, e.g., *Allium cepa* (Navarrete *et al*., 1997; Seth *et al*., 2008), *Vicia faba* (Koppen and Verschaeve, 1996), *Nicotiana tabacum* (Gichner and Plewa, 1998; Gichner *et al*., 2006) but this list got increased gradually with addition of *Arabidopsis thaliana* (Menke *et al*., 2001), *Brassica juncea* (Jana *et al*., 2017) etc.

In normal healthy cells, DNA is confined to the nucleus (comprised of high molecular weight DNA): undamaged DNA is supercoiled and thus, does not migrate very far out of the nucleus under the influence of an electric current (Fig. 1c). On the other hand, cells that have accrued DNA damage, migrating fragments (comet tail) comes out from the nucleus (comet head) under the influence of an electric field and thus are observed (Fig. 1d). The negatively charged DNA migrates toward the anode and the extrusion length indicates increasing relaxation of super coiling, which reflects DNA damage (Azqueta and Collins, 2013). The aim of this article is: (i) to define the optimal conditions for isolation of nuclei from leaf and root tissues and (ii) to calibrate the comet assay procedure for producing best, robust and authentic results in terms of tail length and tail moment values. The authors have successfully performed comet assay technique to evaluate the extent of DNA damage in leaf and root cells of Indian mustard (*Brassica juncea* L. Czern. cv. Varuna) caused by lead (Pb) and the ameliorating potential of salicylic acid (SA), a plant hormone. Therefore, the authors intend to publish detailed guidelines of this technique that may be useful to the new workers in this field across India and world. The following sections will cover in detail the procedure, precautions to be taken, importance of work

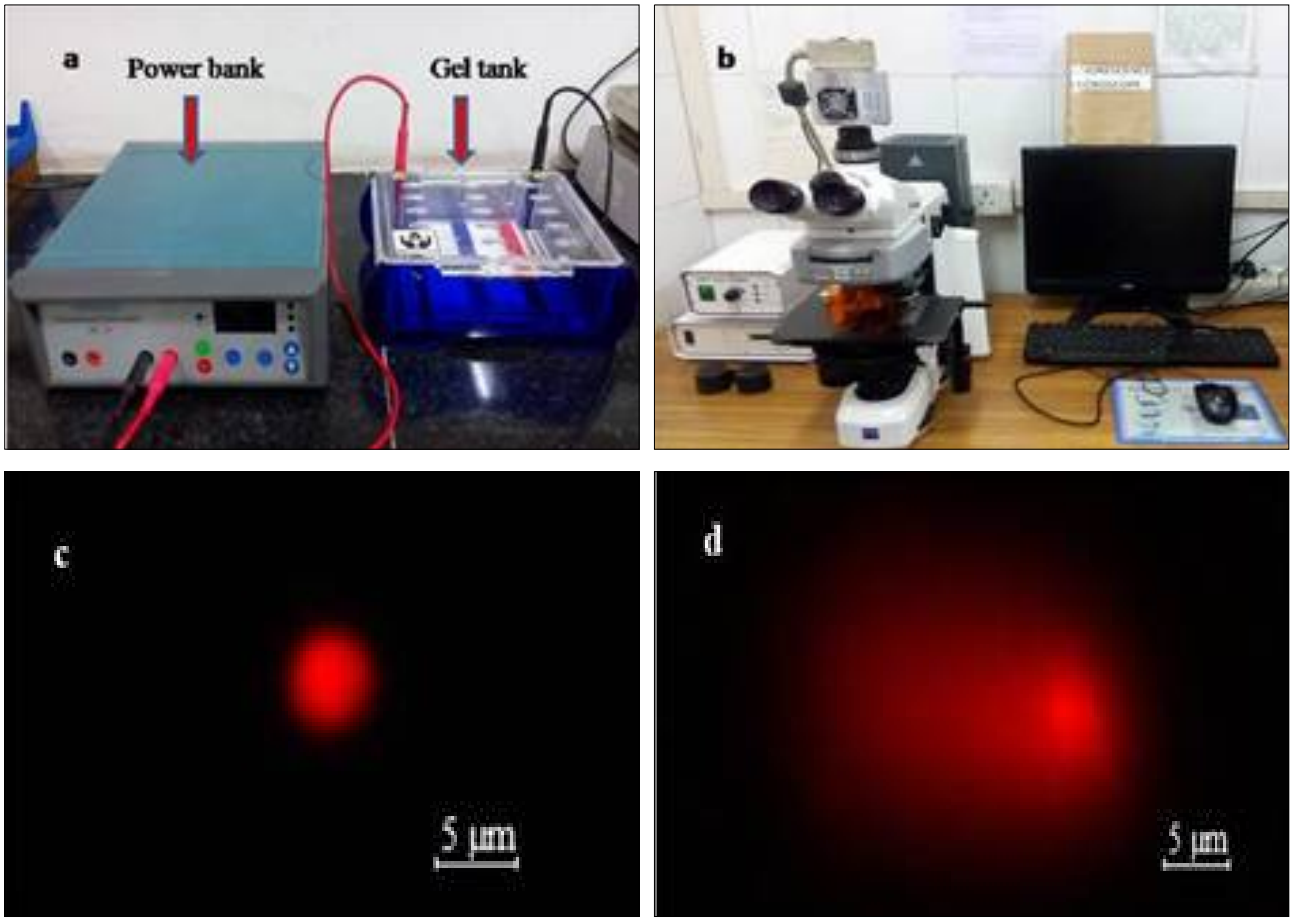

Fig. 1: Image showing **(a)** Horizontal gel electrophoresis tank (length 18 cm × width 15 cm × height 5 cm) with its power source, **(b)** Carl Zeiss Axio (Scope. A1, Germany) fluorescence microscope with all its components, **(c)** fluorescent microscopic image of nucleus of root cell of 45 days-old *Brassica juncea* (L.) cv. Varuna showing normal, clear, round with intense fluorescence, undamaged nucleus in control and **(d)** prominent comet tail (tail length = 17.98 µm and tail moment = 11.34 µm) indicating heavy damage in the nucleus of root cell in 2000mg Pb treatment (Images are taken at 40 x magnification)

and future perspectives of comet assay in plant cells.

2. Step by Step Complete Procedure

2.1. Plant sample collection

Fresh, healthy plants with intact leaves and roots should be uprooted carefully, washed with distilled water avoiding any mechanical stress and be immediately stored in liquid-N_2. All these steps are best when performed on a bright sunny day (9:00-11:00 am) and after freezing in liquid-N_2, stored in deep freezer (-80°C) for performing comet assay in future. Fresh plant material can also be used directly after proper harvesting and washing. It is suggested that always place fresh plant sample on ice prior to isolation of nuclei from plant cells.

2.2. Preparation of single cell gel electrophoresis (SCGE) slides

Regular, clean, non-frosted, glass microscopic slides should be first scratched with a diamond pen making a mesh-like structure so as to facilitate proper adherence of agarose base layer on it. Agarose coated "Comet Slides" can also be used provided by various companies in the market. On one edge side of the slide, small place should be left blank for marking/ numbering. Then, these slides should be dipped (3-5 seconds) into a solution of 1% normal melting agarose (NMA) prepared with distilled water @ 50°C in a beaker. The bottom of the slides should be wiped down to remove the agarose, placed horizontally on a level surface and dried overnight at room temperature. Slides should be kept dry in slide box until further use and face side of slide (top side) having layer of agarose should not be touched/ disturbed.

2.3. Nuclei isolation from plant cells

Traditionally, comet assay uses cell suspension embedded in agarose on a microscopic slide and exposed to lysis which removes membranes leaving behind supercoiled DNA. When subjected to electrophoresis, DNA fragment migrates towards anode forming a typical "Comet tail" which is proportional to the amount of DNA damage/ breaks. However, in plants, there are technical problems due to the presence of a rigid cell wall which acts as a resistant barrier (Pourrut *et al*., 2015). So, a simple and efficient mechanical extraction method to isolate the cell nuclei was developed and reported by some of the researchers worldwide.

Utmost care must be exercised while isolating the nuclei from plant tissues. All operations should be performed in a dark room under dim or yellow light. Individual leaf and root were harvested from the plant and immediately placed in a 60 mm petriplate kept on ice. Onto each plant tissue in a petriplate, 400 µl cold modified Sorensen buffer (50 mM sodium phosphate, pH 6.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% dimethyl sulfoxide (DMSO) or 400 mM Tris-HCL buffer, pH 7.5) were to be spread. Using a fresh sharp razor/ scalpel blade, each leaf should be chopped/ sliced such that a fringe produces across most of the leaf area. The fringe has to be spread on the bottom of the plate and the Sorensen buffer be rinsed over the chopped/ sliced leaf area several times using a cut plastic pipette tip. The plate should be kept tilted over the ice so that the isolated nuclei could be collected in the buffer on one side and approximately 20 µl buffer containing nuclei should be collected and stored at 4°C in microfuge tube for further analysis of DNA damage (Gichner *et al*., 2004).

Note:

(i) Nuclei isolation is the main bottleneck limiting step which determines the success of comet assay to a large extent. The slicing method used very often by most workers is efficient to obtain intact and non-damaged nuclei but exhibits very low extraction yield. On the other hand, chopping (30 seconds) is clearly a more efficient technique which increases yield by 5 to 10 times but could damage nuclei if done for longer time duration. Hence, it is important to determine the optimal chopping time leading to high nucleus extraction yield without causing DNA damage.

(ii) Several researchers have used a filtration step to clean up cellular debris and remove intact cells but this step reduces the yield of obtained nuclei and we also observed the same. Hence, this filtration step can be skipped as short, careful chopping for 15 to 30 seconds does not significantly produces more cellular debris than slicing method and it could be used as a whole without any need of filtration (Pourrut *et al*., 2015).

(iii) To prepare 100 ml of 50 mM Phosphate buffer (pH 6.8), weigh 0.489 g of NaH_2PO_4 (acts as acid) and 0.205 g of Na_2HPO_4 (acts as base), keep them in a beaker and then add about 50 ml of distilled water and dissolve the components. After dissolution, add more distilled water to the solution to make final volume of 100 ml and adjust pH to 6.8.

(iv) To check the effective isolation of nuclei from the plant tissue, we can stain them with DAPI and can see their size, shape and density using fluorescence microscope.

2.4. Electrophoresis of single cell gel electrophoresis (SCGE) slides

Onto each initially coated and dried slide prepared with 1% normal-melting agarose (NMA), the nuclear suspension (40 µl) and 60 µl of 1% low-melting agarose (LMA) prepared in PBS (phosphate buffer saline) should be added one after each onto the slide at 37°C. The nuclei and the LMA should be gently mixed by repeated pipetting using a cut micropipet tip over the coated slide and a cover slip placed on it keeping the mixture on ice surface for 5 minutes. Now, remove cover slip and again, a next and topmost layer of 90 µl of 1% LMA be placed on previously prepared slide and a cover slip be placed upon the slide keeping it on ice surface for 5 minutes. Finally, cover slip should be removed carefully by sliding without disturbing any of the layers and slides should be immersed in cold lysing solution prepared with 2.5 M NaCl, 1% sodium sarcosinate,100 mM Na_2EDTA, 10 mM Tris (pH 10) with 1% Triton X-100 and 10% DMSO (dimethyl sulphoxide). After a minimum of 1 h in lysing solution or overnight, the slides should be placed in a horizontal gel electrophoresis tank (Fig. 1a) containing freshly prepared cold electrophoresis buffer made up of 1 mM Na_2 EDTA and 300 mM NaOH, pH >13 (total volume 500 ml). The nuclei must be incubated for 20-30 minutes in the gel tank to allow the DNA to unwind followed by electrophoresis usually at 0.74 V cm^{-1} (25 V, 300 mA) for 20-30 min at 4°C. Following electrophoresis, slides should be rinsed 3 times with 400 mM Tris-HCL, pH 7.5 and should be stained with 60 µl ethidium bromide (EtBr, 20 µg ml^{-1}), a DNA staining dye, for 5 min. After staining the electrophored slides be dipped in ice cold water for 2 to 5 minutes to remove any excess EtBr and covered with a cover slip. For each slide, a minimum of 20 randomly chosen nuclei should be analyzed under a fluorescence microscope enabled with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. A computerized image analysis system Komet version 3.1, Kinetic Imaging, Liverpool, UK can be employed to measure various comet parameters.

In an alternative way, slides can be observed under fluorescence microscope (Fig. 1b) (Carl Ziess, Axioscope, Germany) in Tred/ Rhodamine filter (510-595 nm) and comet images can be visualized in the computer attached to the microscope. The tail length and tail moment of each comet images can be measured using Carl Zeiss Axiovision software using the stage micrometer as a reference for scaling at a particular magnification.

3. Camera Settings for Viewing Comet Slides under Fluorescence Microscope

Detailed steps are given here under for viewing and taking images of comet slides:

- Carl Zeiss Axio Vision fluorescence microscope with an excitation filter of BP 546/10 nm and barrier filter of 590 nm.
- ON fluorescence battery box source and OFF normal halogen light battery source.
- Set the filter of the microscope at Rhodamine for EtBr.
- Pull the camera knob for viewing live comet slides on the monitor using the camera software.
- Switch on the camera button as well.
- Always use a cover slip.
- Protect comet slides from direct light (should be covered inside some box).
- Avoid slides from drying (should be kept covered in moist blotting sheets).
- As an alternative for researchers who do not have access to a fluorescence microscope, silver staining allows standard light microscopy for comet tail analysis.

4. Precautions to be taken while Performing Comet Assay

- Individual leaf or root should be carefully and properly taken out from the stored samples and washed carefully. Freshly prepared modified cold Sörensen buffer or 400mM Tris-HCL buffer should be used keeping leaves/ root sample on ice.
- Utmost care must be exercised while isolating the leaf/ root nuclei. All operations should be conducted under dim or yellow incandescent light at a room temperature below 25°C to prevent DNA damage from ultraviolet light.
- Fresh, sharp razor/ scalpel blade should be used and leaves have to be chopped/ sliced in such a way that a fringe produces across most of the leaf area. Never crush the leaves/ roots sample, otherwise it will cause more damage to DNA and does not divulge actual result.
- The petriplate should be kept in tilted manner so that buffer collects on one side and could be taken easily via pipette. Use of razor blade is more recommended than scalpel as we obtained more nuclei using razor blade.
- Regular, clean microscope slides should be used free of any dirt/ markings over it and have to be

very well scratched with a diamond pen in both directions i.e. horizontally and vertically. Slides should be labeled accordingly on one side.

- The bottom of the slides should be wiped to remove the agarose, placed horizontally on a level surface so that agarose can spread evenly and uniformly and not on one side.
- After the slides were prepared, they have to be kept dry safely in slide boxes until their use without touching/ damaging them as touching them could damage the agarose layer.
- The nuclei and the LMA have to be gently mixed by repeated pipetting using a disposable, cut micropipette tip and a cover slip be placed on the mixture. Putting and removal of cover slip should be very gentle.
- Despite the fact that nuclei isolation and slide preparation have to be performed on ice, a strong increase in DNA damage was observed when temperature rises above 25°C due to heat shock. This could be explained by release of nucleases in the buffer solution during the nuclei isolation step. Hence, all these operations must be performed below 25°C to refrain endogenous damage during sample preparation and to inhibit repair in cells.
- Lysis should be at 4°C and can be for 1 h, overnight, or longer (but we have to be aware of prolonged lysis as it could detach agarose coating layers from the glass slides).
- Gently drain excess electrophoresis solution and gently immerse in ice cold water. Do not pour liquid directly over the slides.
- After electrophoresis, staining and washing, allow the slides to slightly dry at room temperature. When the slide becomes dry, it brings all the cells in a single plane to facilitate observation, and focusing on comets will become easier. But total dryness of slides should also be avoided and all the slides should be kept covered protecting them from direct light.
- Avoid scoring comets near the edge of the slide or near bubbles in the gel, as they tend to be anomalous.
- As the nuclei isolation step is clearly operator-dependent and comet assay parameters could be plant/ organ-dependent, we suggest that researchers should carry out their own tests to obtain optimal conditions in laboratory. Moreover, researchers should always specify their comet assay parameters, calibration procedures, plant stage (if relevant) and external factors (room temperature and light intensity) as part of the experimental conditions for getting successful results of comet assay.

5. Importance of Comet Assay

In plants, an increasing interest for the comet assay was shown in the last decade. This versatile technique appears to be promising, reliable and rapid approach to detect the genotoxic effect of hazardous pollutants and therefore to monitor the existing environmental/ soil conditions for human health and plant productivity perspectives. Comet assay is now successfully applied for basic and applied research from lower (algae, moss) to higher eukaryotic crop plants (rice, wheat, mustard, maize etc.).

In contrast with the comet assay in animal cells for which technical progress has been made to improve its reliability and reproducibility, only limited advancement has been made and there is a lack of understanding of the critical steps of this technique in plants system. The aim of publishing this article is to identify key factors affecting comet assay performance to improve its reliability and reproducibility in plant systems for monitoring of environmental hazards and their serious genotoxic consequences on humans, animals and plants.

6. Future Perspectives of Comet Assay

Single cell gel electrophoresis is currently used to investigate the cell response to genotoxic agents as well as to several biotic and abiotic stresses that lead to oxidative DNA damage. Different versions of single cell gel electrophoresis have been developed in order to expand the range of DNA lesions that can be detected and guidelines for their use in genetic toxicology have been provided. This technique is now emerging as a useful tool in assessing the potential of higher plants as stable sensors in ecosystems and source of information on the genotoxic impact of dangerous pollutants. Another interesting application of single cell gel electrophoresis deals with ''Mutation Breeding'' or the combined use of irradiation and *in vitro* culture technique to enhance genetic variability in elite plant genotypes.

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References

Azqueta, A. and Collins, A.R. 2013. The essential Comet assay: A comprehensive guide to measuring DNA damage and repair. *Archives of Toxicology* **87**:949-968.

Dhawan, A. and Anderson, D. (Eds.) 2016. The Comet Assay in Toxicology. *Royal Society of Chemistry*, Vol. 30.

Gichner, T. and Plewa, M.J. 1998. Induction of somatic DNA damage as measured by single cell gel electrophoresis and point mutation in leaves of tobacco plants. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **401**:143-152.

Gichner, T., Patková, Z., Száková, J. and Demnerová, K. 2004. Cadmium induces DNA damage in tobacco roots, but no DNA damage, somatic mutations or homologous recombination in tobacco leaves. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **559**:49-57.

Gichner, T., Mukherjee, A. and Velemínský, J. 2006a. DNA staining with the fluorochromes EtBr, DAPI and YOYO-1 in the comet assay with tobacco plants after treatment with ethyl methanesulphonate, hyperthermia and DNase-I. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **605**:17-21.

Gichner, T., Patková, Z., Száková, J. and Demnerová, K. 2006b. Toxicity and DNA damage in tobacco and potato plants growing on soil polluted with heavy metals. *Ecotoxicology and Environmental Safety* **65**:420-426.

Hu, Z., Cools, T. and DeVeylder, L. 2016. Mechanisms used by plants to cope with DNA damage. *Annual Review of Plant Biology* **67**:439-462.

Jana, A., Ghosh, M., Sinha, S., Jothiramajayam, M. and Mukherjee, A. 2017. Comprehensive analysis of fly ash induced changes in physiological/growth parameters, DNA damage and oxidative stress over the life cycle of *Brassica juncea* and *Brassica alba*. *Chemosphere* **186**:616-624.

Koppen, G. and Verschaeve, L. 1996. The alkaline comet test on plant cells: a new genotoxicity test for DNA strand breaks in *Vicia faba* root cells. *Mutation Research/Environmental Mutagenesis and Related Subjects* **360**:193-200.

Menke, M., Chen, I.P., Angelis, K.J. and Schubert, I. 2001. DNA damage and repair in *Arabidopsis thaliana* as measured by the comet assay after treatment with different classes of genotoxins. *Mutation Research/ Genetic Toxicology and Environmental Mutagenesis* **493**:87-93.

Navarrete, M.H., Carrera, P., Miguel, M. and de la Torre, C. 1997. A fast comet assay variant for solid tissue cells. The assessment of DNA damage in higher plants. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **389**:271-277.

Pourrut, B., Pinelli, E., Mendiola, V.C., Silvestre, J. and Douay, F. 2015. Recommendations for increasing alkaline Comet assay reliability in plants. *Mutagenesis* **30**:37-43.

Seth, C.S., Misra, V., Chauhan, L.K.S. and Singh, R.R. 2008. Genotoxicity of cadmium on root meristem cells of *Allium cepa*: Cytogenetic and comet assay approach. *Ecotoxicology and Environmental Safety* **71**:711-716.

Ventura, L., Giovannini, A., Savio, M., Donà, M., Macovei, A., Buttafava, A. and Balestrazzi, A. 2013. Single cell gel electrophoresis (Comet) assay with plants: research on DNA repair and ecogenotoxicity testing. *Chemosphere* **92**:1-9.