

Review

A molecular-based fast method to determine the extent of DNA damages in higher plants and fungi

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Comet assay also called 'single cell gel electrophoresis is a technique for the detection of DNA damage and repair at the level of single cells, which is one of the most advanced techniques introduced to the agricultural sciences in recent years. The assay is one of the most popular tests of DNA damage detection (e.g. single and double-strand breaks, oxidative-induced base damage and DNA-DNA/DNA-protein cross linking) by electrophoresis. The assay is very sensitive, rapid, easy to handle, non-invasive, visual and inexpensive compared to most conventional techniques to detect DNA damage, there is also little amount of cell samples required and it is applicable for most eukaryotic cells, thus, it has rapidly gained importance in the fields of genetic toxicology, medicine, environmental studies and agriculture. Isolated DNA from cells are embedded in a thin agarose gel on a microscope slide and unwound in a suitable buffer and exposed to a weak electric field to attract broken, negatively-charged DNA towards the anode. After electrophoresis, migrated DNA fragments stained with a fluorescent dye would resemble a shape of a comet observed by a fluorescence microscopy. The extent of comet-like shapes would indicate the level of DNA damage in cells. The intensity of comet tail relative to the head would also reflect the extent of DNA damage in numerical.

Key words: The alkaline cell gel electrophoresis, comet assay, single cell gel electrophoresis, SCGE, DNA damage, DNA repair.

INTRODUCTION

As a result of rapid urbanization, air pollution and environmental quality deterioration have been affecting our daily lives as well as the nature. Under these circumstances, organisms might suffer from the damages and

show various defense responses. However, for the determination of stress levels in living organisms various molecular and biochemical tests are required. Some of them are quite expensive and need various meticulous and tedious works. The case, however, is not different in agricultural sciences. In fact, laboratory facilities and financial supports are poorer than the other areas of science in many places. For example, crop plants, as well as other living organisms are exposed to various types of abiotic and biotic stress factors such as disease, drought and salinity etc., either deliberately as in the case of wrong agricultural practices or accidentally as compounds present in polluted air, soil or water (Gichner and Mühfeldova, 2002). Assays to quick determination and measurement of the genotoxicity for these stress factors

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Abbreviations: TM, Tail Moment; NMP, Normal Melting Point; LMP, Low Melting Point; PBS, Phosphate Buffered Saline; tBr, Ethidium Bromide; MH, Maleic Hydrazide; EMS, Ethyl ethanesulfonate, SPSS, Statistical Package for the Social Sciences.

are not available at present for many plants. In fact, only specific tester lines could be used and these tester lines are not available for many of the plant species either (Gichner and Mühlfeldova, 2002). For example, only a few mutagenicity assays have been performed for a couple of plants such as *Arabidopsis thaliana* and *Nicotiana tabacum* (Gichner et al., 1994; Gichner and Plewa, 1998). This limitation, of course, hinders or in most cases, prevents the detection of levels of DNA damage and stress level in many crop plants and microorganisms living in soil.

In many biochemical and molecular tests, metabolites synthesized in living organisms such as hormones, enzymes, carbohydrates and stress proteins etc. have given the indication of severity of stress and the extent of organism resistance against such stress agents. However, the actual site, DNA, where these metabolites are encoded and triggered to be synthesized have long been ignored due to lack of technical facilities and skills. But, introduction of a new technique enabling a quick determination of the severity of stress, especially in DNA level, would open a new research area in many crop plants as well as microorganisms that have not been previously studied.

So far, a number of techniques for the detection of DNA damage have been used to identify substances with genotoxic activity. Of these, the most frequently used methods involved either the detection of DNA repair synthesis in individual cells, or the detection of DNA single strand breaks or alkali-labile sites in pooled cell populations using the alkaline elution assay (Tice et al., 2000). The first method provided information at the level of individual cells, however, the method is technically difficult to perform and requires the use of radioactivity and is not very sensitive. On the other hand, the second assay ignored the critical importance of intercellular differences in DNA damage and required relatively large number of cells (Tice et al., 2000). In recent years, a new molecular-based assay, the Comet or single cell gel electrophoresis (SCGE) has been introduced to plant and mycological sciences for detecting the induced DNA damage (Collins and Harrington, 2002; Gichner et al., 2003; Lin et al., 2007) to overcome this limitation. Although this technique has been primarily applied to human and animal cells (Sing et al., 1988; Mitchelmore and Chipman, 1998) such as sperm and blood cells, the incorporation of this technique with plant tissues has enabled us to fast determination of level of DNA damages in plants. Use of this technique also extends the utility of plants in basic and applied studies in environmental mutagenesis. In theory, comet assay can be applied to every type of eukaryotic plant cell. The basic principle of this assay is to determine the DNA breaks by measuring the DNA damage which is quantified by the proportion of DNA, which migrates out of the nuclei towards the anode when individual cells or isolated nuclei

embedded in a thin agarose layer (Menke et al., 2001). The formation of comet or "comet-like" shape (with a head, the nuclear region and a tail which contains DNA fragments) of nuclei followed by electrophoresis enables quantification of DNA in comet tails after staining with an appropriate fluorochrome such as propidium iodide or ethidium bromide (Bhanoori and Venkateswerlu, 1998; Olive and Banath, 2006). Diameter of nuclei of the studied species and the degree of DNA denaturation under assay conditions would indicate the condition of DNA, which is responsible for many metabolic activities.

Comet assay was first described by Swedish researchers Östling and Johansson (1984), then it was modified by Singh et al. (1988) as 'alkaline comet assay' and after that numerous modifications have been made to date (Fairbairn et al., 1995; Lin et al., 2007; Gichner et al., 2008). Comet assay has 2 commonly used versions; neutral (neutral unwinding/neutral electrophoresis, N/N) and alkaline (alkaline unwinding/alkaline electrophoresis, A/A). In recent studies, alkaline-neutral (alkaline unwinding/neutral electrophoresis, A/N) assay was also employed (Lin et al., 2007). The N/N assay (pH of lysing and electrophoretic solutions are approximately 9) is useful to assess DNA double strand breaks (Östling and Johanson, 1984). This method was then developed by Olive et al. (1990) to detect single strand breaks. Alkaline version of the comet assay, A/A, (pHs of lysing and electrophoretic solutions are 10 and 13, respectively) can quantitatively measure DNA damage, including single strand breaks, double strand breaks, alkali labile sites (primarily aprunic and apyrimidinic sites) incomplete excision repair sites and DNA cross links (Singh et al., 1988; Gichner and Plewa, 1998; Lin et al., 2007). The Singh and Olive methods are identical in principle and similar in practice, but Singh method appears to be one or two orders of magnitude more sensitive. The A/N method (pHs of unwinding and electrophoresis solutions are 10 and 8.5, respectively) also useful to measure both double- and single strand breakages of DNA (Lin et al., 2007).

In many works, various combinations of neutral and alkali pH solutions prior to and during electrophoresis or addition of antioxidant to the lysing/electrophoretic buffer, and precipitation of DNA with ethanol and the use of sensitive dyes (e.g. YOYO-1, DAPI) have enhanced the sensitivity of assay techniques to screen for low level DNA damages in variety of cells (Singh, 1996; Angelis et al., 1999).

Since its first application to *Vicia faba* (Koppen and Verschaeve, 1996), the comet assay has also been applied to *A. thaliana* (Menke et al., 2001), to onion (Navarrete et al., 1997), tobacco (Gichner and Plewa, 1998; Gichner et al., 2008), carrot (Jiang et al., 1998), barley (Jovtchev et al., 2001), agronomic plants (Gichner et al., 2003), weeds (Gichner and Mühlfeldova, 2002) and potato (Gichner et al., 2006) etc.

THE AREA OF USE OF COMET ASSAY

In environmental monitoring

Air pollutants generated from traffic and industrial plants are believed to be one of the major causes of DNA damage in living species. Effects of the combination of these pollutants on living organisms have not been clarified in detail. In many cases, some indicator plants have been chosen and their responses to stress agents have been evaluated. Particularly, their biomass values followed by the harvest were considered at first place. For example, bioindicators exposed to air pollutants and natural environmental stresses such as water stress, nutrient deficiency and temperature stress may undergo some biochemical and physical alterations. However, some of which are able to accommodate large amount of pollutants without undergoing damage. In this case, only a chemical analysis may allow the determination of toxic elements. However, in the growth season no valid tests, especially non-destructive tests, have been carried out *in situ* conditions. In some cases, biological stress agents such as fungi or bacteria might also suffer from the environmental stresses while they are infecting the host (Dikilitas, 2003). Under these circumstances, comet assay would be helpful to elucidate the each stress agents in detail on organisms while aiming to solve the negative sides of other methods. Therefore, the results obtained from the molecular and biochemical analysis would be more meaningful. For example, in a study carried out by Sriussadaporn et al. (2003), comet assay was performed on mature plants located in the road-side and non-roadside environments in which the road-side samples showed significantly higher degrees of DNA damage than non-roadside samples. The comet assay is also able to determine the low level of DNA damage, which cannot be determined by other assays.

In soil monitoring

Pesticides are by definition toxicants intended to control pest populations and although the benefits associated with their use in agriculture are unquestionable, many of their active substances have potentially adverse effects on living organisms including human and non-target organisms. However, their toxicity, at both the genetic and metabolic level, has not been adequately described. Preliminary results on a broad series of compounds belonging to different biological classes (herbicides, fungicides, insecticides) seem to indicate that pesticides are toxic as shown by negative or weakly positive results on different organisms (Garaj-Vrhovac and Zeljezic, 2000). In recent years, "environmentally-friendly" called substances have been on the market. The impression given from the suppliers is that these chemicals in right

concentrations and right amounts are not harmful to the human beings or crop plants. However, tests with comet assay showed that the DNA damages, even the lowest degree, were able to be detected in living organisms facing the low level concentrations of such chemicals (Piperakis et al., 2000, 2003).

In fungi biomonitoring

Single cell gel electrophoresis assay is also becoming more significant in this area because of its simplicity, sensitivity, speed and reliability.

Fungi are ubiquitous in natural environment and can be regarded as valuable biomonitors of genotoxic effects caused by environmental pollutants like toxic metals, metalloids, organometalloid compounds and salts (Bhanoori and Venkateswerlu, 1998; Dikilitas and Smith, 2004). Therefore, physiological and biochemical metabolisms such as growth and cell differentiations of fungi could be affected depending on the species of minerals and organisms (Gadd, 1993; Trevors et al., 1986). For example, the mechanisms by which cadmium induced toxicity are multiple and lead to increased lipid peroxidation level. Cadmium can also cause genotoxicity by directly binding to DNA bases or by enhancing reactive oxygen species production (Waalkes and Poirier, 1984). Interactions between toxic chemicals, pathogens and plants are quite complex. Each organism could be affected differentially by toxic elements while the pathogen could continue its infection. However, the effect of toxicants on the virulence of pathogens or on the resistance of plants would vary according to their concentrations and species. Under these circumstances, it would be more appropriate and beneficial to evaluate the biochemical mechanisms by studying the DNA damage in each organism. For example, studies carried out by Dikilitas and Smith (1997) and Dikilitas (2003) showed that the combined effect of NaCl and the wilt fungus *Verticillium albo-atrum* was more detrimental on tomato and lucerne plants than those of each individual stress agents. Devastating effects of the combined stress on those plants could be well studied by looking at the DNA damages both on the fungus and the plants under normal or saline conditions. Thus, biochemical pathways for the fungus and NaCl would be clarified both under normal and stressed conditions. It is also important to determine the level of damage occurred in DNA structures since the repairing could only be determined by knowing the sites of damaged areas. The efficacy of any ameliorative chemicals, on the other hand, no DNA would then also be evaluated via comet assay. In many studies, the application of comet assay to fungal protoplasts were used to estimate the bioavailability and genotoxic damage of pollutants to fungal and other microorganisms (Gadd, 1993; Bhanoori and Venkateswerlu, 1998).

Other areas of the use of assay can be arranged in order as apoptosis, genetic toxicology, DNA repair, nutritional toxicology, cell cycle analysis and clinical applications etc.

METHODOLOGY OF THE ASSAY

In general, comet involves the following steps (Gichner et al., 2006; Mancini et al., 2006; Lin et al., 2007; Baysal et al., 2009). As a general rule, all buffers and reagents used in the assay should be fresh if possible and should not exceed more than 2 weeks. All operations should be conducted under dim or yellow light to avoid DNA damage induced by light.

Isolation of nuclei from leaves or other plant parts

It is quite important to isolate the nuclei from plant parts mechanically since the plant cell walls cannot be removed as the animal cell membrane by lysis in high concentrations of detergents and salts. After experimental treatments, a small part of leaf or root is placed in a tilted petri dish kept on iced surface and spread with 200 - 300 μ l of cold 0.4 M tris buffer, pH 7.5, using a fresh razor blade. In some studies, 200 - 300 μ l of cold PBS buffer (130 mmol L⁻¹ NaCl, 7 mmol L⁻¹ Na₂HPO₄, 3 mmol L⁻¹ NaH₂PO₄, 50 mmol L⁻¹ Na₂EDTA, pH 7.5) was also used for the same purpose (Lin et al., 2007). The plant parts are then gently sliced into fringes so the nuclei should be collected in the buffer solution (Gichner et al., 2004). Here, slicing the leaves with a new razor edge rather than chopping is of utmost importance in obtaining low control values such as low comet tail moment (TM).

Isolation of nuclei from plant callus or cell cultures

After the treatment period, the cell suspensions are poured onto a pad of multiple layers of cheese-cloth which is placed onto absorbent paper. The cells are then rinsed with a suitable buffer and scraped from the cheese-cloth and placed into a micro centrifuge tube using a spatula. The cells are gently agitated with small amounts of sand and buffer. After the settlement, the mixture is filtered through the mesh nylon filter to obtain the nuclei which are then collected in a tube on ice for the comet assay (Stavreva and Gichner, 2002).

Isolation of nuclei from fungal cell cultures

Primarily, fungal protoplasts should be isolated from fungal cells to measure the extent of DNA damages. In this step, lysis buffer and cell wall degrading enzymes

(e.g. Novozyme 234) could be used to release protoplasts. Protoplasts viability should be tested by using trypan blue technique. A detailed protocol for fungal protoplast isolation was reported in detail in the works of Unkles et al. (1989) and Bhanoori and Venkateswerlu (1998). Enzymatic release of protoplasts from fungal or plants could be sometimes tedious and require an ample amount of time. DNA damage in nuclei obtained after the isolation of protoplasts could be too high to achieve good and reliable results. However, in the study of Hahn and Hock (1999) this step was omitted and the extent of DNA damages in fungal cells exposed to toxic chemicals or irradiations were directly measured.

Preparation of slides for plants

The ultimate goal of slide preparation is to obtain uniform and sufficiently stable gels which survive throughout the experimental procedure and to ensure easily visualized comets with minimal background noise. The slides should also be preserved for data collection. A number of different techniques have been used to prepare comet slides. Initially, fully frosted slides were used commonly due to their increased gel bonding and stability. However, either conventional microscope slides (Klaude et al., 1996; Hahn and Hock, 1999) or slides specifically modified to increase gel stability (frosted end slides) have also been used (Tatli et al., 2008). There are also commercially available comet assay slides, which shortens assay time and allowing rapid and reliable analysis for large number of samples (Trevigen Inc., 2000). There is couple of procedures for preparing the microscope slides.

In the single-layer procedure, cells are suspended in low melting point (LMP) agarose (generally at 37 - 40°C) and placed directly on a slide (Tice et al., 2000).

In the 3-layer procedure; microscope slides with frosted ends are dipped into a solution of 1% normal melting point (NMP) agarose prepared with water at 50°C (Gichner et al., 2006). The bottom of the slides are wiped to remove the agarose and placed horizontally on a level surface then and covered with a coverslip and finally, it is dried for 5 min at 4°C (Kocyigit et al., 2005). At this point, the slides could be stored in slide boxes at 4°C until use for unwinding and electrophoresis. After this, the coverslip is gently removed from the slide, subsequently, 50 μ l of the nuclear suspension and 50 μ l of 1% molten low melting point (LMP) agarose prepared in PBS (2 mmol L⁻¹ KCl, 1 mmol L⁻¹ KH₂PO₄, 136 mmol L⁻¹ NaCl, 8 mmol L⁻¹ Na₂HPO₄.12H₂O, pH 7.4) is added to the slide at 40°C. The nuclei and LMP agarose are gently mixed by repeated pipetting using a cut micropipette tip and immediately covered with a coverslip to flatten out each molten agarose layer. The slide is then placed horizontally on an iced surface for a minimum of 5 min to enhance gelling of the agarose after which the coverslip

is removed (Gichner, 2003; Gichner et al., 2004). Finally, a third layer of 100 μl molten 0.5% LMP agarose is placed on the second layer to fill any residual holes and to increase distance between cells and the gel surface and should be kept on for further 5 min. However, in a recent study of Kocyigit et al. (2005) and Gichner et al. (2008), the top layer of agarose in the 3 layer method was found unnecessary, so this step is omitted.

Preparation of slides for fungi

Although there are limited publications on DNA damages of fungi with comet assay (Bhanoori and Venkateswerlu, 1998; Hahn and Hock, 1999; Miloshew et al., 2002), here a detailed protocol was summarized.

It could be difficult to work with cell wall-degrading enzymes especially with such sensitive methods as comet assay. Therefore, microgels, a compact test system for growing, treating and analyzing fungi for DNA damage (Hahn and Hock, 1999) was introduced here. First, microgels are prepared by dipping microscope slides in 1% NMP agarose and following dryness of the slides 150 μl of 0.8% LMP agarose in a defined growth medium should be applied and covered with a coverslip and incubated for a period of 15 min at 4°C to allow gelling to occur. The coverslips should then be removed and the gels should be inoculated by transferring a small piece from a well-grown colony onto the gel film. The slides, if needed, are placed in petri dishes and incubated for a required period of time at optimum temperature for fungal growth. After this stage, chemicals or other test agents are applied directly onto the slides (90 μl) and covered with a coverslip and incubated depending on the treatment period, then washed off with distilled sterile H₂O. Slides are then submerged in lysis buffer (0.3 mol L⁻¹ NaOH, 30 mmol L⁻¹ Na₂EDTA, 0.1% SDS) for 30 min at room temperature, all subsequent steps until microscopic analysis should be performed at 4°C (Hahn and Hock, 1999).

If treatments are made on fungi in liquid cultures with various concentrations of toxic chemicals, the viability test should be performed on fungal cells. After the preparation of slides as described above, fungal cells must be collected by centrifugation in an Eppendorf microcentrifuge tubes and washed with distilled sterile water and resuspended in growth medium or sorbitol buffer (1 mol L⁻¹ Sorbitol, 25 mM KH₂PO₄ pH 6.5) to avoid from osmotic shock. Aliquots of cells should be mixed with LMP agarose containing cell wall-degrading enzymes and spread over the slides, covered with coverslips and incubated as a period of time (e.g. 20 min) at room temperature to disintegrate the cell wall and obtain protoplasts.

The concentrations of cells in agarose, as well as the concentration of agarose itself, are important issues for

ensuring a successful analysis. Higher cell densities can result in a significant proportion of overlapping comets, especially at high levels of DNA migration. Higher agarose concentrations can also affect the extent of DNA migration (Hartmann et al., 2003). On the other hand, DNA migration also depends on the pH, temperature and duration of unwinding and electrophoresis, as well as voltage and amperage.

Unwinding, electrophoresis and neutralization of DNA

In this stage, the slides are dipped into the related buffer and the nuclei are incubated for a required period of time to allow the DNA to unwind. The length of time for unwinding here mainly depends on the plant or fungi species used. After unwinding, the slides without coverslips must be placed on a platform in the electrophoresis tank and covered by electrophoresis buffer followed by the electrophoresis at a required voltage and time. In general, electrophoresis solution should be cooled if it is not specified (Gichner et al., 2003). Due to large variability in the size of commercially available electrophoresis units, the electric field strength should be expressed as V cm⁻¹ with accompanying amperage. Once the electrophoretic conditions have been established, the same electrophoresis unit and power supply should be used throughout the study. Slide to slide variation should be kept at minimum by maintaining a constant temperature during electrophoresis. The optimal electrophoresis duration depends on the extent of DNA migration desired in control cells.

Although there are several comet assay protocols for macro- and microorganisms as well as plants and fungi, here, 3 different comet assay protocols for plants and fungi were summarized.

Electrophoresis conditions for plant cells; in A/A comet assay protocol, the slides are put in freshly prepared cold alkaline buffer (300 mmol L⁻¹ NaOH, 1 mmol⁻¹ Na₂EDTA, pH > 13) at 4°C for 10 - 15 min to allow DNA to denature (Navaratte et al., 1997; Lin et al., 2007). Electrophoresis is generally conducted at 4°C in the alkaline buffer for 15 min at 300 mA. Following electrophoresis, if the conditions are alkaline during unwinding stage, the gels must be neutralized by rinsing the slides at least three times with a suitable buffer such as Tris (pH 7.5) for 5 min each. An increased rinsing may be useful in situations where a high background is seen during scoring (Rojas et al., 1999; Kocyigit et al., 2005; Gichner et al., 2008).

After electrophoresis, the slides must be neutralized with a neutralization buffer (0.4 mol L⁻¹ Tris-HCl, pH 7.5) at room temperature for 15 min.

In A/N Comet assay protocol, the slides are subjected to cold alkaline buffer (contents given above) at 4°C for 10 min to unwind DNA, then neutralized for 5 min in 0.4

mol Tris-HCl (pH 7.5) followed by equilibration in TBE buffer (90 mmol L⁻¹ Tris-borate, 2 mmol L⁻¹ Na₂EDTA, pH 8.4) at least three times for 5 min each. Electrophoresis is generally conducted in TBE buffer at room temperature for 4 min at 13 mA (Angelis et al., 2000; Lin et al., 2007).

In N/N protocol, the slides are initially subjected to lysis in high salt solution (2.5 mol L⁻¹ NaCl, 10 mmol L⁻¹ Tris-HCl, pH 7.5, 100 mmol L⁻¹ Na₂EDTA) for 20 min at room temperature. Equilibration is made in TBE buffer as above and the electrophoresis is conducted at room temperature in the same buffer for 6 min at 15 - 17 mA (Koppen et al., 1999; Lin et al., 2007).

Electrophoresis conditions for fungal cells; after the preparation of slides, a 10 min preincubation step is required in half strength of TBE electrophoresis buffer (50 mmol L⁻¹ Tris-borate, 10 mmol L⁻¹ Na₂EDTA, pH 8), then the slides were transferred to the electrophoresis tank and subjected to electrophoresis with a current ranging from 10 - 20 mA. Neutralization and washing steps in the half strength of TBE for 5 min is generally enough. The slides should be dried as described below (Hahn and Hock, 1999; Miloshew et al., 2002).

After neutralization, the slides should be stained and the comets should be scored within 6 h or so. If time is not convenient for immediate scoring, the slides should be incubated for 10 - 15 min in cold distilled water followed by dehydration in 70 and 100% ethanol before staining and left overnight to dry (Klaude et al., 1996; Gichner et al., 2008). The slides could then be stored up to several months in slide boxes in dry and dust-free conditions. The comets by this way do not suffer from high background noise (personal communication with Prof. Abdurrahim Kocyigit, 2009).

Staining and scoring

The most frequently used dyes are fluorescent dyes including ethidium bromide (Singh et al., 1988; Lin et al., 2007), propidium iodide (Olive et al., 1990), 4,6-diamidino-2-phenylindole (DAPI) and YOYO-1 (benzoxazolium-4-quinolinium oxazole yellow homodimer) (Singh et al., 1994; Gichner et al., 2006). Non-fluorescent techniques for visualizing comets based on staining with silver nitrate are also in use. The most common magnifications used have been between 200X and 400X. Slides with stained nuclei (e.g. 80 - 100 µl ethidium bromide, 20 µg ml⁻¹) should be washed at least 3 times with ice cold water to remove excess dye then scoring should be made on slides followed by covering with a coverslip within 6 h.

For scoring, the slide preparations should contain sufficient amount of cells. Generally, 50 randomly chosen cells per slide at least with 2 replicates should be analyzed with a fluorescence microscope with an excitation filter of BP 546/10 mm and a barrier filter of 590 nm (in case of staining DNA with ethidium bromide). Slides

should be coded before analysis unless fully automated analysis is used. However, in fully automated image analysis, which is commonly used, some parameters such as the percentage of DNA in the head (H-DNA, %), the percentage of DNA in the tail (% of migrated DNA), tail length (TL, µm) and tail moment (TM expressed in µm, which is the fraction of migrated DNA multiplied by the tail length divided by 100) are easily measured. Of these, tail moment and/or tail length measurements are the most commonly reported, but there is much to recommend the use of percent DNA in tail, as this gives a clear indication of the appearance of the comets and in addition, is linearly related to the DNA break frequency over a wide range of levels of damage (Gichner et al., 2008; Collins and Harrington, 2002). Several companies supply software which, linked to a closed circuit digital camera mounted on the microscope, automatically analyses individual comet images. The programs are designed to differentiate comet head from tail and to measure a variety of parameters including cell area, comet area, % H-DNA, % tail DNA, olive TM, TL, TM etc.

It is also possible to analyze comets quantitatively without image analysis software. The human eye can discriminate comets representing different levels of damage, therefore, visual scoring is performed due to its speed and simplicity. Comets must be selected without bias and must represent the whole gel, so it is important to scan the whole gel either in computer-based analysis or visual scoring. The migration of DNA could be categorized according to its head and tail shape by visually. For this, a generally accepted DNA damage-index has been used in many cited articles (Kobayashi et al., 1995; Gichner et al., 2003; Kocyigit et al., 2005). According to this; different levels of DNA damage is classified from 0 (no tail) to 4 (almost all DNA in tail). The scale used is as follows: 0 = no cometting; 1 = comet < 0.5 times the width of nucleus; 3 = Comet greater than width of nucleus; 4 = Comet > twice the width of the nucleus. Scoring cells in this manner has been shown to be as accurate and precise as using computer image analysis.

If 100 comets are scored and each comet assigned a value of 0 - 4 according to its class (Figure 1), the total score for the sample will be between 0 - 400 'arbitrary units' (Collins et al., 1997).

It is also possible to measure TL by using an eyepiece micrometer to come to a conclusion about the condition of DNA. The extent of DNA damage can also be evaluated quantitatively by determining the TM values for each treatment group. For example, in a study carried out by Gichner et al. (2006 and 2008), the DNA damage on tobacco plants was expressed in numerical by calculating TM values. They were able to determine the individual effect of genotoxic stress agents such as MH (maleic hydrazide), EMS (ethyl methanesulfonate) or lead nitrate (Figure 2).

The DNA-specific dye and the magnification used for comet visualization depend largely on investigator's needs

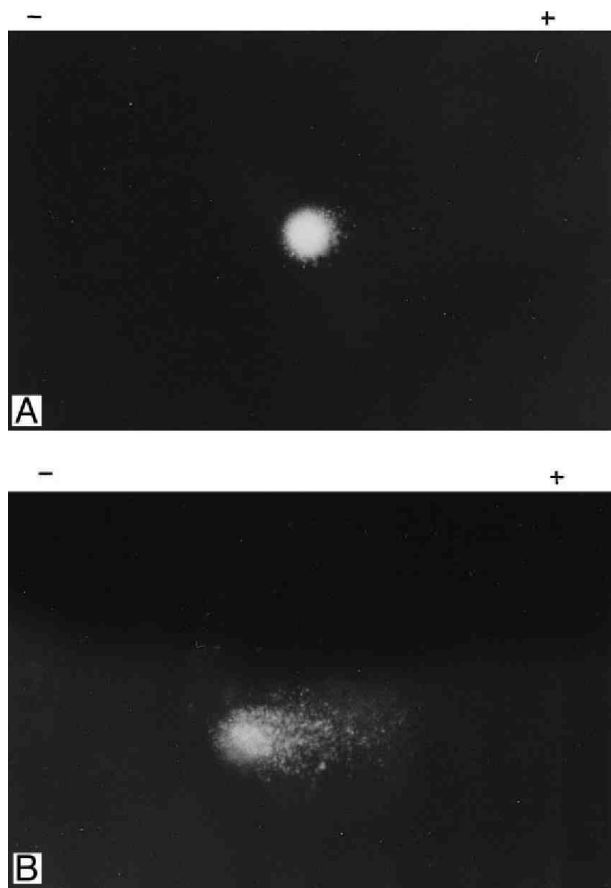


Figure 1. Photomicrographs of EtBr-stained DNA from protoplasts of *Neurospora crassa* processed for alkaline comet assay. (A) Untreated showing no DNA damage (length:width ratio 1:1) and (B) CdSO₄ (100 µM) treated showing DNA migration towards anode (length:width ratio 3:2). [Adopted from the work of Bhanoori and Venkateswerlu (1998) with permission of Elsevier].

and presumably have little effect on assay sensitivity or reliability. For some fluorescent dyes, anti-fade could be used to greatly reduce the rate of signal quenching (Tebbs et al., 1999), allowing the same slide to be scored multiple times.

Statistical analysis of comet assays

Generally data are analyzed using the basic versions of any statistical and graphical package programs such as sigmaplot or SPSS. TM or % of tail DNA values are analyzed with a 1-way analysis of variance test. If a significant F-value of $P < 0.05$ is obtained, then multiple comparison test between the treated and control group is conducted. Differences between 2 groups are statistically evaluated by using the paired *t*-test (Gichner et al., 2006; Mancini et al., 2006).

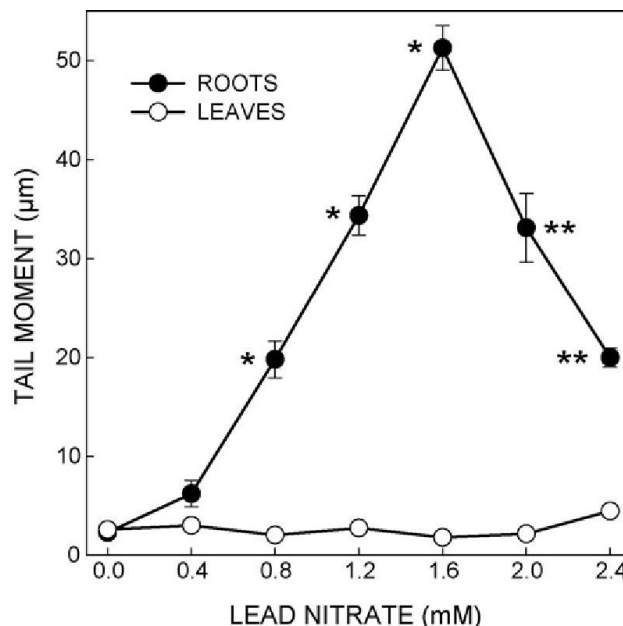


Figure 2. The average median tail moments (TMs) in root and leaf nuclei after a 24 h treatment of tobacco (*Nicotiana tabacum* var. *xanthi*) seedlings with lead nitrate. The error bars represent the standard error of the mean. *Significantly ($P < 0.05$) different from the control. **Significantly ($P < 0.05$) lower than the TM after treatment with 1.6 mmol L⁻¹ lead nitrate. [Adopted from the work of Gichner et al. (2008) with permission of Elsevier].

Preparation and storage conditions of the reagents used in the assay

- i) Tris buffer: 0.4 M, pH 7.5-store at 4°C.
- ii) NMP agarose (ROTH Germany or Sigma): dissolve NMP in dH₂O at 50°C store at 4°C.
- iii) LMP agarose (ROTH Germany or Sigma): dissolve LMP in PBS at boiling temperature- store at 4°C.
- iv) PBS: Contents were given in the text. Store at 4°C.
- v) Electrophoresis buffer: Store at 4°C.
- vi) Fluorescent DNA stains e.g. EtBr (80-100 µl, 20 µg l⁻¹). Store at 4°C.

Equipments

- i) Large-bed gel electrophoresis tank and a power supply unit.
- ii) Fluorescence microscope equipped with an excitation filter of 515 - 560 nm and a barrier filter of 590 nm.
- iii) Computerized image analysis system linked to a charge-coupled device (CCD) camera.
- iv) And general laboratory equipments such as water bath for melting agarose, haemocytometer for counting cells, frosted-end slides and coverslips for nuclei. Fully frosted-end slides could also be used but drying and storing after staining could damage to the cells.

Timing

- i) Preparation of reagents, 30 min.
- ii) Slide preparation, cell-sample preparation and agarose embedding: 1 h.
- iii) Unwinding and electrophoresis, neutralization: 2 h.
- iv) Staining: 30 min.
- v) Scoring and analysis: 2 h.
- vi) The above steps for the comet assay protocol are also illustrated in Figure 3.

LIMITATIONS OF THE METHOD

The main limitation to the application of this technique to all eukaryotic cells is the fact that solid tissues need a previous treatment to free the individual cells (Singh et al., 1988; Tice, 1995; Demirbag et al., 2005). Preparation of the samples for the assay is one of the most important steps. If this step is performed rapidly and accurately, then any DNA damage caused by the procedure could be minimized significantly. There are, however, practical limitations to the number of cells and samples that can be analyzed. If the automated system to be used maximally 500 - 600 comets per hour can be scored and analyzed (Olive and Banath, 2006). Manually, 50 slides per day could be evaluated. If there is heterogeneity in the sample, more slides should be counted. Viability of cell suspension could be checked if necessary. On the other hand, necrotic and apoptic cells could be mixed in a population, this could prevent the workers from having accurate information on detection of single and double strand breaks as well as base damage. If one is suspicious about this case, these cell types could be distinguished by using DNA diffusion assay method (Singh, 2000).

It is also difficult to come to a conclusion about the degree of severity of the stress in any organism by just looking at the extent of DNA damage under the microscope. Comparing comet assay results with other molecular or biochemical methods could be very useful to have an idea about the resistance of target organism and the proposed damaging agents. It is important to note that DNA damage is not only caused by a direct effect of genotoxicity (Olive and Banath, 2006) but membrane damage, oxidative stress or unsuitable isolation methods for nuclei could also cause extensive DNA damages.

Conclusion

The main issue in comet assay is to measure increased DNA migration values in treated cells without affecting the migration in controls and make dose-response comparisons between the groups and conclude the extent of damage in cells. Compared with other genotoxicity assays, the

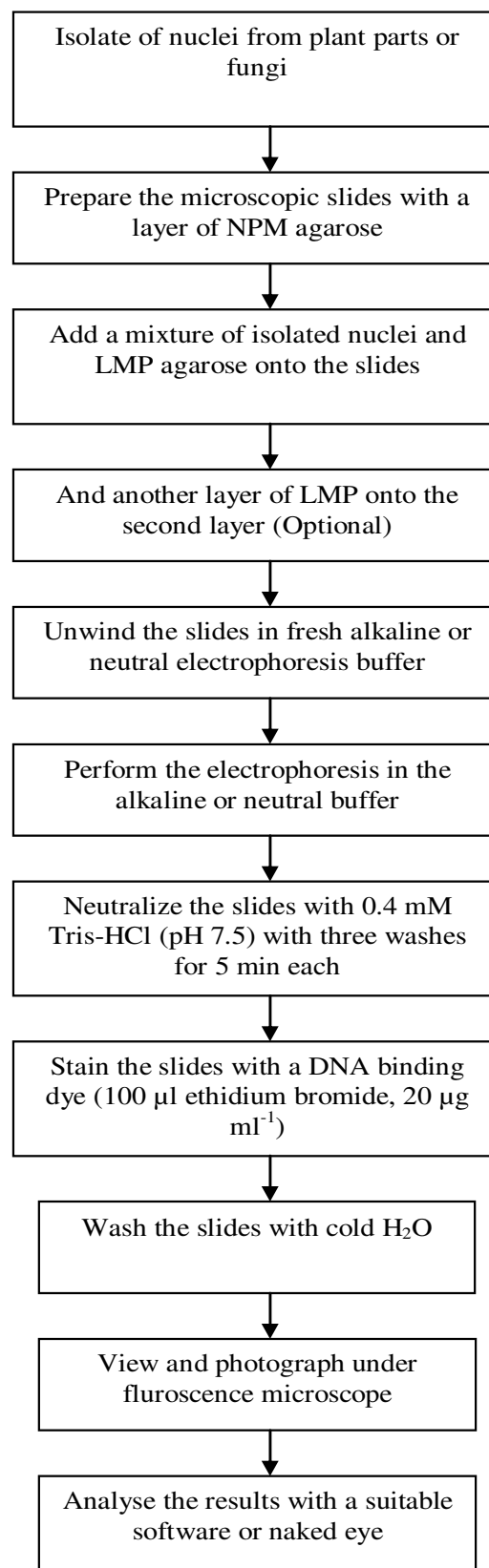


Figure 3. General illustration of the comet assay protocol.

advantages of the technique include: its demonstrated sensitivity for detecting low levels of DNA damage, the requirement for small numbers of cells per sample, flexibility, low costs, ease of application, the ability to conduct studies using relatively small amounts of a test substance and the relatively short time period (half a day) compared to most conventional cytogenetics which take a few days needed to complete an experiment (Lin et al., 2007; Gichner et al., 2008). For each species, e.g. plant, fungus, etc., the comet assay protocol has to be modified, especially during unwinding stage. Voltage and amperage of electrophoresis should be rearranged if needed. Not only seedlings, but also isolated leaves or roots of various mature plants could also be used in the assay. In many molecular assays, calli or cell cultures are preferred due to their sensitivity and required amount of work, which is less time consuming than working on whole-plant organisms. However, establishment of a laboratory with good facilities such as illuminated incubators and thermo-stable orbital shakers for studying cell and callus cultures is not always easy and quick, especially in the early stages of laboratory establishment. With this assay, research programs and projects would be more valuable and reliable. On the other hand, it is not always possible to work on callus or cell cultures for every plant species. In most cases, *in vivo* conditions have been preferred. Therefore, this assay would provide a new opportunity for scientists working on biochemical stress mechanisms and pathways.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

COMPETING INTEREST STATEMENT

The authors declare that no endorsement of named products is intended nor any criticism implied of similar products which are not mentioned.

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