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Ivelina Nikolova [1]
Mariyana Georgieva [1]
Lubomir Stoilov [1]
Zornica Katerova [2]
Desisslava Todorova [2]

Authors' addresses:
[1] Department of Molecular Genetics, Institute of Plant Physiology & Genetics**,** Bulgarian Academy of Sciences, Sofia, Bulgaria.
[2] Department of Regulation of Plant Growth and Development, Institute of Plant Physiology & Genetics**,** Bulgarian Academy of Sciences, Sofia, Bulgaria.

Correspondence:
Mariyana Georgieva
Department of Molecular Genetics, Institute of Plant Physiology & Genetics, Bulgarian Academy of Sciences
Acad. G. Bonchev Str., Bldg. 21
1113 Sofia, Bulgaria,
Tel.: +359 2 974 62 28
e-mail: mimy80@abv.bg

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Optimization of Neutral Comet Assay for studying DNA double-strand breaks in pea and wheat

ABSTRACT

This study describes an adaptation of the Comet assay under neutral conditions for mono- and dicotyledonous plants pea (*Pisum sativum* L.) and wheat (*Triticum aestivum* L.). Modifications concern lysis and electrophoresis steps, respectively. Electrophoresis was carried out varying the intensity of the electric field. A linear relationship between the percentages of DNA in the tail from control background with alteration of intensity was found. Trypan blue dye exclusion test was used in order to determine the intactness of nuclear membrane of the isolated nuclei from both plant model systems. Assessment was conducted on non-irradiated and irradiated nuclei on a monolayer with three doses of UVC. It was found that the share of intact nuclei (trypan blue negative ones) is about 95% in controls. Gradual dose-related increase of damaged nuclei was observed in both species, reaching statistical significance only at the higher dose applied.

Key words: neutral Comet assay, trypan blue dye exclusion test, UVC.

Introduction

A wide range of methods are presently used for detection of early biological effects of DNA-damaging agents in the environment, irrespective of whether they are toxic, mutagenic, teratogenic and carcinogenic. Plant mutagenicity bioassays have taken their place as screening and biomonitoring tools for detection of DNA damage induction. Most higher-plant tests are based on detection of point mutations, chromosomal rearrangements, DNA fragmentation (Maluszynska & Juchimiuk, 2005; Gady et al., 2009; Uhl et al., 2003). Quantification methods of DNA strand breaks induction, such as Comet assay, have been developed and became increasingly popular during the last 10 years along with other cytogenetic assays in plant biomonitoring. The Comet assay was introduced by Östling & Johanson (1984) under neutral lysis and electrophoresis (pH 9.5) conditions. This technique has been developed in an empirical way, with two basically different alkaline protocols described in the literature by Singh et al. (1988) and Olive (1989), concerning animal cells. The respective protocol was developed to measure low levels of strand breaks with high sensitivity. The subsequent protocols were optimized to detect a subpopulation of cells with variable sensitivity to drugs or radiation (Singh et al., 1988).

In plants, this technique has been modified and extensively validated over the years. Primary applications of plant Comet assay were used to determinate the effect of ionizing radiation on seed disinfection and preservation of diseases and pathogens (Cerda et al., 1993, 1997; Koppen & Cerda, 1997). Since the radiation doses used in food irradiation extensively damage DNA, a neutral pH was chosen, combined with a low voltage and short electrophoresis time (Cerda et al., 1993). Neutral pH limits the amplitude of DNA unwinding as reported by Singh (1988). Comet assay under these conditions detects mainly

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double strand breaks (DSB). Alkaline conditions (pH 10 or above) were required for unwinding and detection of single strand breaks (SSB). However, as shown in *Nicotiana tabacum* and *Vicia faba*, low sensitivity of the neutral version creates problems in detecting of induced DSB, even after adaptation of the basic assay (Koppen & Angelis, 1998; Koppen et al., 1999). Additionally, neutral comet assay proved to be adequate for estimation of DNaseI-induced DSB in the presence of $MnCl_2$ (Menke et al., 2000a), although being less sensitive than alkaline lyses/neutral gel electrophoresis (A/N), used to follow repair of damage induced by N-methyl-N-nitrosourea (MNU) and UVC in barley genome (Jovtchev et al., 2001; Armalytė & Žukas, 2003).

Another limitation of the assay in plants was the relatively high value of comet parameter "% DNA in tail" in the controls, that could mask the effect of the treatment (Koppen & Angelis, 1998; Angelis et al., 1999; Menke et al. 2000a, 2000b). Moreover, a statistical requirement of this method is a low background level of breaks in untreated cells (i.e. between 10 and 20% DNA in tail) (Collins, 2004; Lovell & Omori, 2008).

The objectives of this study were: (1) to determine and compare the nuclear membrane intactness on non-irradiated and UVC irradiated pea and wheat nuclei by trypan blue exclusion test; (2) to define the parameters for neutral Comet assay by alteration of lysis duration and electrophoresis conditions using nuclei isolated from pea and wheat leaves.

Materials and Methods

Plant growth conditions and isolation of nuclei

Seeds of *Pisum sativim* and *Triticum aestivum* were surface-sterilized by immersion in 0.1% $KMnO_4$ for 15 min followed by 30 min in distilled water. Germination was performed on wet filter paper and seedlings were subsequently transferred to a container with water and kept in a plant growth chamber at 26/22ºC day/night temperature, 16/8 h (day/night) photoperiod, 150 µmol m^{-2} s^{-1} photon flux density and 60% relative air humidity.

Individual leaf samples were removed from the plant and placed in a petri dish with Sörensen buffer (50 mM sodium phosphate, pH 6.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% dimethyl sulfoxide (DMSO) kept on ice. The leaf tissue was gently sliced by a razor blade and the resulting material was repeatedly dipped in the cold Sörensen buffer. The suspension with released nuclei was filtered through a 30 µm disposable filter (Partec, Műnster, Germany) to remove most of the debris and centrifuged at 550 g for 5 min at 4ºC.

UVC irradiation

UVC irradiation at 254 nm was delivered by BLX 254 UV crosslinker (Life Technologies TM, GIBCO BRL UV Crosslinker). In a precooled, plastic petri dish nuclear suspension was irradiated with doses of 5, 7 and 9 kJ/m^2 for pea and 14, 16 or 18 kJ/m^2 for wheat, respectively. After treatment, nuclei were kept on ice and shielded from the ambient light until subsequent use for the viability test.

Measurement of nuclear membrane intactness by Trypan blue dye exclusion test

The nuclear membrane integrity was determined by trypan blue exclusion test. Briefly, nuclei were incubated in 0.4% trypan solution (1:1 vol/vol) for 5 min and scored as membrane damaged if the nuclei were stained blue. As a negative control for the trypan blue exclusion test were used non irradiated nuclei incubated in Sörensen buffer. All samples were examined and counted in duplicates under light microscope at 25x (Zeiss Jenamed-2). The nuclear membrane intactness was expressed as percentage of the number of nuclei excluding trypan blue from the total number of nuclei for each plant (pea and wheat). Statistical analysis was performed by the Kruskal-Wallis test. Subsequently the Duncan test was applied for calculations concerning pair-wise comparisons. The level of statistical significance was set at $p<0.05$. The data obtained are presented as mean with standard error (±SEM).

Neutral Comet assay

For the neutral Comet assay, the protocol of Georgieva & Stoilov (2008) with modifications was followed. Microscope slides were coated with 0.5% normal melting agarose and dried at room temperature. 40 µl of the nuclei suspension was mixed with 40 µl of 0.1% low melting agarose, spread on the slide surface and subjected to gel formation for at least 10–15 min on a cooling plate at 4°C. Lysis was carried out in 2.5 M NaCl, 10 mM Na_2EDTA (pH8), 10 mM Tris-HCl (pH8), 1% N-lauroylsarcosine sodium salt, 1% TritonX-100, 10% DMSO for 5 min for pea and 15 min for wheat at 4°C in the dark. Electrophoresis was performed in *ex tempore* prepared TAE buffer (pH8) at 0.5, 1, 2 and 5 V/cm for 10 min for pea nuclei. Lysis of embedded wheat nuclei was performed in the same lysis buffer for 15 min before electrophoresis at 1 V/cm at 4°C for 15 min. The slides were dehydrated in 70% and 96% ethanol for 5 min and dried at room temperature.

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Staining of the comets

The slides were covered with solution of the fluorescent dye acridine orange (10 μg/ml). Visualization of the stained comets was carried out using a fluorescence microscope (Zeiss Jenamed-2) coupled with a digital camera (Samsung Digimax V50). The percentage of DNA in tail was calculated as a measure of DNA damage. Three independent experiments were performed and 50 comets were analyzed per point in each experiment.

Results

Trypan blue dye exclusion test for nuclear membrane intactness after exposure to UVC

To determine the dose effect of UVC exposure on pea and wheat nuclei, a series of dose-response experiments were performed. Figures 1 and 2 demonstrate the response of the plant nuclei to the various doses.

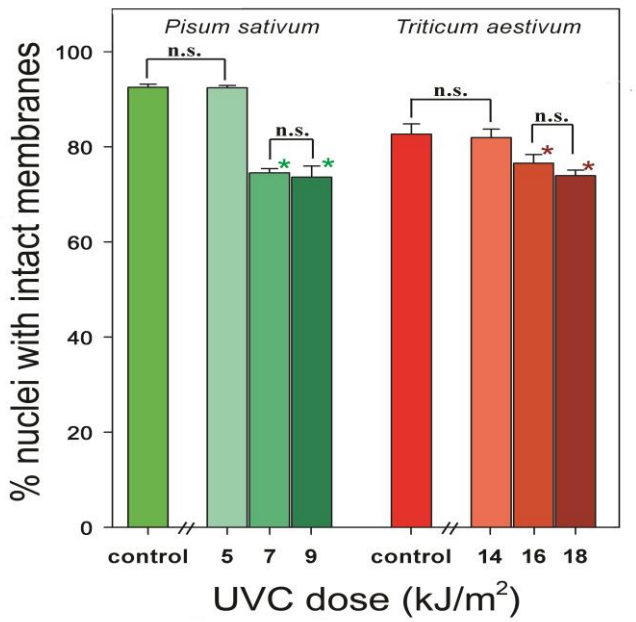

Figure 1. *Nuclei membrane integrity of pea and wheat cells exposed to UVC (Pisum sativum – 5, 7 and 9 kJ/m^2; Triticum aestivum – 14, 16 and 18 kJ/m^2) as indicated by staining with Trypan blue. Each result indicates the mean±SE from at least 3 independent experiments.*

** significant difference using the Kruskal-Wallis test compared to the control ($p<0.05$); n.s. no significant differences.*

The nuclear membrane intactness determined by Trypan blue dye exclusion test decreased significantly (approximately 20%) in pea nuclei irradiated with 7 kJ/m^2 UVC radiation as compared to untreated control. The intactness of wheat nuclei after 16 kJ/m^2 UVC treatment also decreased by approximately 8% compared to control (Figure 1). Significant differences were not observed between intact nuclei in control and nuclei irradiated with the lowest UVC dose. Untreated nuclei displayed a bright-blue color indicating an intact membrane. Dark-blue colored nuclei with membrane corruption and blebbing were also present (Figure 2).

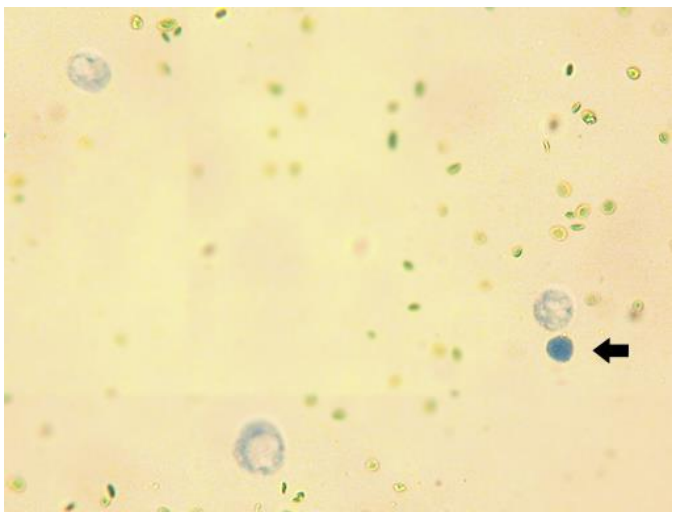

Figure 2. *Representative images of Trypan blue stained nuclei isolated from pea leaves. The intact nuclei which provide a negative test are bright and colored in pale blue. Trypan blue-positive nuclei are dark blue as indicated by arrow.*

Detection of background level of DNA damage in control samples by neutral Comet assay

For successful accomplishment of the neutral Comet assay a sufficient quantity of intact nuclei is needed. Besides, a neutral Comet assay under different conditions was performed. The methodological changes concerned mainly the lysis duration (data not shown) and electrophoresis conditions – duration and intensity of the electric field. Percentage of DNA in tail as a parameter was most accurate and quantative indicator of DNA damage since it reflects the total intensity of the tail and the total intensity of the comet being not dependable on the tail length.

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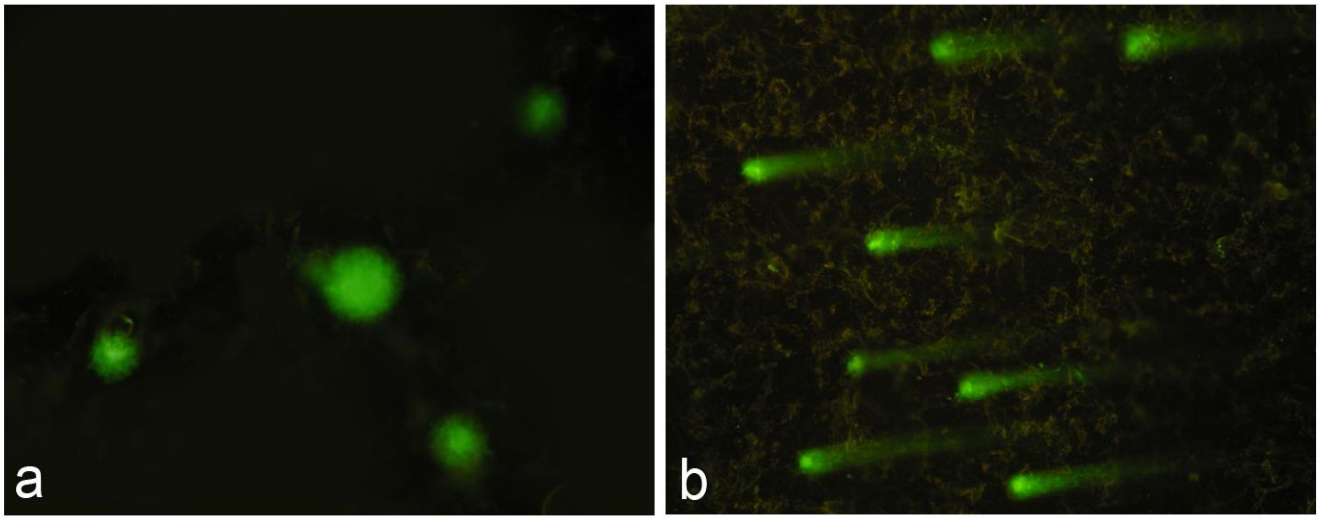

Figure 3. *Representative images of control pea nuclei stained with acridine orange isolated after electrophoresis at: a) 0.5 V/cm; b) 5 V/cm.*

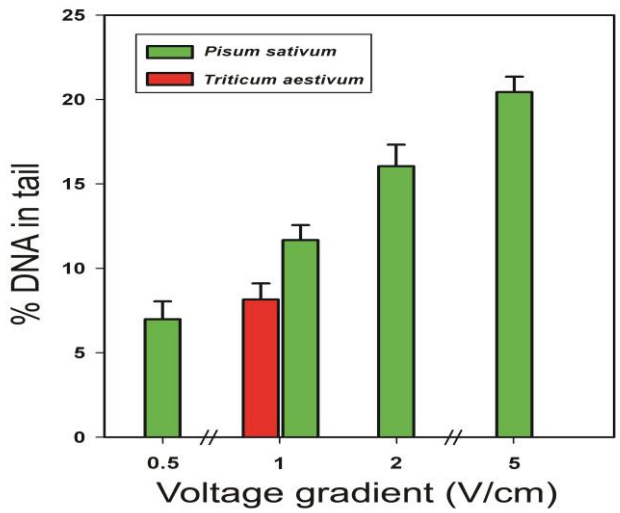

Figure 4. *Values of the parameter % DNA in comet tails under different intensities of the electric field.*

Nevertheless, pea nuclei showed high level of DNA migration in control samples at 5 V/cm and 10 min lysis (Figure 3b). Under these experimental conditions DNA fragments migrated out of the head to form a comet tail thus increasing the percentage of DNA in the tail. It is obvious that these conditions were not appropriate to quantify the amount of DNA damage in pea accurately. As shown on the Figure 4, at 5 V/cm electrophoresis conditions the DNA damage expressed by % DNA in tail was comparatively high and reached 20.44%. At 1 V/cm it was decreased to 11.67%. At 0.5 V/cm and 5 min lysis the amount of damages was reduced to 6.98% for pea (Figure 4). The releasing fragments that diffuse away from the center of the nucleoid create a "halo" with a small compact origin (Figure 3a). The most optimal conditions found to be most suitable for wheat were 15 min lysis and 15 min (1 V/cm) electrophoresis time (Figure 4).

Discussion

DSB are considered to be the most biologically important lesions, producing critical DNA damage, mainly due to the close relationship with chromosomal damage, genome instability, apoptosis etc. (McMillan et al., 2001). Several different experimental protocols have been established for the detection of DSB: ultracentrifugation in neutral sucrose gradients (Iliakis et al., 1991), neutral filter elution (Goutham et al., 2011), pulse-field gel electrophoresis – PFGE (Hong et al., 2010), clamped homogeneous electrical field electrophoresis – CHEF (Blöcher et al., 1989; Blöcher & Kunhi, 1990), static field gel electrophoresis – SFGE (Taucher-Scholz et al., 1995). One of the most sensitive methods is considered to be the single cell gel electrophoresis or the comet assay (Menke et al., 2000a; Collins, 2004).

Both versions of the comet assay protocol under neutral and alkaline conditions can measure a broad range of damage

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as SSB, DSB, alkali labile sites, oxidative DNA base damage, DNA-DNA/DNA-protein/DNA-drug crosslinking (Collins et al., 2008). In all model systems, alkaline version of this analysis is considered to be more sensitive, but SSB are quickly repaired, therefore they are not of significant importance. It is known that DSB are more essential than SSB (Collins et al., 1997) and they can be detected after application of the neutral version.

A number of Comet assay protocols have been developed for different type of studies in plants. Guidelines, recommendations and modifications of the both versions have been already published (Wojewódzka et al., 2002; Christofoletti et al. 2009; Lovell & Omori, 2008). Up to now, 3 protocols of the Comet assay are available in plants: A/A (alkaline lysis and electrophoresis), N/N (neutral lysis and electrophoresis) and A/N (alkaline lysis and neutral electrophoresis) protocol. The alkaline variant (A/A) (Klaude et al., 1996; Angelis et al., 1999) has a wider range of SSB and DSB detection. The minimal technical requirements to conduct the alkaline comet assay in pea and wheat have been established (Gichner et al., 2003; Peycheva et al., 2011).

Even though Comet assay in plants is already routinely applied, only few studies have examined DSBs under neutral conditions. Restrictive conditions of the neutral Comet assay in plants can be surmounted by performing the analysis in alkaline lysis and neutral or mildly alkaline electrophoresis (A/N) conditions. In A/N protocol the comet formation is supported by alkaline unwinding of DNA before electrophoresis that allows the expression of free single-stranded DNA fragments in the tail (Menke et al., 2000a). Relevant *in vivo* studies on DNA damage were reported for plants, such as *Allium cepa* L., *Arabidopsis thaliana* L., *Hordeum vulgare* L., *Nicotiana tabacum* L., *Vicia faba* L. However, classical neutral variant (N/N) without the unwinding DNA step was applied only for barley, broad bean and *Arabidopsis* (Koppen & Angelis, 1998; Menke et al., 2000b; Georgieva & Stoilov, 2008; Kozak et al., 2009).

As mentioned above, N/N protocol of the plant comet assay possesses minimal sensitivity (Menke et al., 2000a). For that reason the neutral version of the method needs adaptation for the individual cell types (Wojewydzka et al. 2002). Adaptation concerns the main steps of the analysis, including the nuclei isolation as different cytoplasmic components (nucleases and hydrolytic proteins) which influence the background level of induced DSB (Armalytė & Žukas, 2002). The nucleus as an experimental model is more sensitive than the intact cell (Armalytė & Žukas, 2002). One of the most significant requirements of the analysis is the low level of initial DSB background that could facilitate statistical approaches (Lovell & Omori, 2008). The amount of primary induced DSB is in close relationship with the induction of programmed cell death (Huang et al., 2005; Kuthanova et al., 2008). Studies have shown that plant response after UV irradiation affects nucleic acids, photosynthetic apparatus and membrane lipids (Yao et al., 2012; Kalbina & Strid, 2006; Rao et al., 1996; Hidema et al., 2000; Strid et al., 1990; Chow et al., 1992). Besides typical damages resulting from UV irradiation, direct induction of direct DSB is often discussed. For that reason the quality of the isolated pea and wheat nuclei and the membrane intactness was verified immediately after isolation and after irradiation by applying the Trypan blue dye exclusion test. This blue acid dye contains two azo chromophores group, but it is not capable to enter into the cell wall of plant cells grown in culture (Sini et al., 2012). Previous studies have shown the cell death as result of "classical" ozone symptoms in tobacco cv Bel W3 plants by Trypan blue test (Pasqualini et al., 2003).

Other analytical limitations of the neutral comet assay in plants are connected with migration of DNA under neutral conditions. Often the differences depend on the size of plant genomes and protein content (Armalytė & Žukas, 2002), which impose variation in methodology concerning lysis time, temperature during lysis, and electrophoresis. As shown in previous studies (Singh et al., 1999) we also detected dependence on the length of DNA migration and intensity on the electric field. In addition, these conditions reflect on quantity of DNA damage represented as mean of % DNA in tail of the comets, observed in control samples of pea and wheat nuclei. Similarly to the alkaline comet assay protocol elaborated on *Oreochromis niloticus* (Christofoletti et al., 2009), our results also revealed gradual increase in the intensity of DNA migration due to the effects of electrophoretic conditions on the morphology of comets in control samples. We consider this fact directly related to DNA organization within higher order chromatin structure in the form of 'matrix attachment sites' and 'loops' (Ventura et al., 2013). The kinetics of the comet tail formation during the alkaline A/A protocol is related to ssDNA fragments, the ends of which are pulled out from the comet head by electric force. In contrast, in N/N protocol the comet tail is formed by extended DNA loops. It is clearly shown that the rate of DNA release is dependent on the topological state of DNA (Afanasieva et al., 2010).

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Conclusion

In this study we found appropriate experimental conditions for neutral Comet assay, specific for pea and wheat by variation in the main steps of the protocol – lysis and electrophoresis. The optimal conditions were specified as: 5 min lysis and electrophoresis at 0.5 V/cm for pea and 15 min lysis and electrophoresis at 1 V/cm for wheat. The amount of nuclei with an intact membrane was decreased after application of high UVC doses in both model plants.

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