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Evaluation of the nuclear DNA Diffusion Assay to detect apoptosis and necrosis

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Abstract

We applied the nuclear DNA Diffusion Assay, described as an accurate tool to estimate apoptotic and necrotic cells [N.P. Singh, A simple method for accurate estimation of apoptotic cells, Exp. Cell Res. 256 (2000) 328–337] to tobacco root and leaf cells. In this assay, isolated nuclei are embedded in an agarose microgel on a microscope slide and low molecular-weight DNA fragments diffuse into the microgel. Exposure of the roots to hydrogen peroxide significantly increased the average nuclear area of isolated nuclei. After 4 and 24 h of recovery, all DNA damage was repaired. The data clearly demonstrate that the manifestation of diffused nuclei upon exposure to hydrogen peroxide is not the result of non-repairable apoptotic or necrotic DNA fragmentation, but represents repairable genotoxin-induced DNA damage. In contrast, treatment with the alkylating agent ethyl methanesulphonate (EMS) followed by 24 h of recovery produced a significant increase in the average nuclear area. The contribution of apoptosis to this increase cannot be excluded. Heat treatment of leaves at 50 °C for 1–15 min leading to necrosis, and treatment of isolated nuclei with DNase-I, which digests DNA to nucleosome-sized fragments as during apoptosis, also led to a dose-dependent increase in the nuclear area. The use of different fluorochromes (ethidium bromide, DAPI or YOYO-1) for DNA staining yielded similar results in the DNA Diffusion Assay. As all types and sizes of diffused nuclei were observed after EMS and hydrogen peroxide treatments, we were unable to differentiate, on the basis of the structure of the nuclei, between apoptotic or necrotic DNA fragmentation and other types of genotoxin-induced DNA damage in plants.
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1. Introduction

Apoptosis is a genetically regulated pathway of programmed cell death in animals and in plants. It plays an important role in tissue differentiation and aging and may also occur in response to cell injury, such as exposure to toxic agents. In contrast, necrosis represents unscheduled cell death [1,2]. Singh [3,4] described a 'DNA Diffusion Assay' that – according to the author – detects apoptotic cells and clearly distinguishes them from necrotic cells. The assay involves mixing cells with agarose and preparing a microgel on a microscope slide, lysing the embedded cells with salt and detergents, followed by an alkaline treatment. The processing of cells is similar to that of the single-cell gel electrophoresis or Comet assay [5,6], except that the nuclei are not subjected to electrophoresis. In the DNA Diffusion Assay, low molecular-weight DNA fragments are allowed to diffuse in the agarose in all directions, are then precipitated with a mixture of spermine and ethanol and stained with a DNA-binding fluorescent dye. It was proposed that diffused nuclei with apoptotic and necrotic DNA fragmentation could be distinguished according to their structure from diffused nuclei with genotoxin-induced DNA damage [3,4].

We applied the DNA Diffusion Assay to tobacco seedlings treated with (1) hydrogen peroxide inducing oxidative stress, (2) an alkylating agent, ethyl methanesulphonate, (3) hyperthermia inducing necrotic DNA fragmentation and (4) DNase-I, a nuclease involved in apoptosis [7], which digests DNA to nucleosome-sized fragments. The objective was to determine if the diffusion assay could distinguish apoptotic and necrotic nuclei according to their structure from nuclei exhibiting other types of DNA damage. The assay was performed either immediately after the treatment with the toxic stimuli or 4 or 24 h later, as apoptosis requires a certain expression period. In addition, a comparison was made of the size of the average nuclear DNA area after staining with the DNA-binding dyes ethidium bromide, DAPI and YOYO-1.

2. Materials and methods

2.1. Chemicals and media

4′,6-Diamidino-2-phenyindole dilactate (DAPI, CAS no. 28718-90-3), ethyl methanesulphonate (EMS,

CAS no. 62-50-0), hydrogen peroxide (H_2O_2, CAS no. 7722-84), ethidium bromide (CAS no. 1239-45-8), spermine (CAS no. 71-44-3), Phytagel, MS salts, normal melting-point (NMP) and low melting-point (LMP) agarose and general laboratory reagents were purchased from Sigma Chemical Co., St. Louis, MO. The YOYO-1 stain (CAS no.143413-85-8) quinolinium,1,1′ - [1,3-propanediylbis[(dimethyliminio)-3,1-propanediyl]]bis[4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]]-tetraiodide was purchased from Molecular Probes, Inc. Eugene, OR, and deoxyribonuclease-I (DNase-I) was from Worthington Biochemical Corporation, Freehold, NJ.

2.2. Tobacco growth

Leaves and roots of heterozygous *Nicotiana tabacum* (var. xanthi; $a_1{}^+/a_1$; $a_2{}^+/a_2$) plants [8] were used for the DNA Diffusion Assay and the Comet assay. Tobacco seeds were sterilized by immersion in 70% ethanol for 2 min followed by a 20-min treatment with a solution containing 4.5 ml of distilled water, 0.5 ml of 5.25% sodium hypochlorite and 5 μl of 10% Triton X-100. The seeds were then washed 5 × in sterile distilled water. Each seed was placed in a vented plastic container with 50 ml of sterile, solid growth medium and the plants were grown in a growth chamber at 26 °C with a 16-h photoperiod. A detailed description of plant growth conditions was previously published [9,10].

2.3. Treatment conditions

2.3.1. EMS and H_2O_2 treatment

At the 4–5 true-leaf stage, the seedlings were carefully removed from the containers, the roots rinsed in water and immersed in plastic vials containing 22 ml of a defined concentration of EMS or H_2O_2. For studies on root nuclei, the seedlings were treated for 30 min with H_2O_2 or 2 h with EMS, for analysis of leaf nuclei the seedlings were treated with EMS for 24 h in the dark at 26 °C. For recovery studies, the roots of seedlings were washed after treatment and the seedlings were kept in water for 4 or 24 h in the dark at 26 °C.

2.3.2. Heat treatment

Excised leaves from tobacco seedlings were immersed for 1–15 min in water at 50 °C Nuclei were isolated immediately or after 24 h, during which the

heat-treated leaves were kept in water in the dark at 26 °C.

2.3.3. DNase-I treatment

Agarose slides with nuclei from untreated tobacco leaf cells were prepared as outlined below. Each slide was exposed to 800 μl of DNase-I (0–0.5 U) dissolved in water for 1 min. After the treatment, the slides were dipped in water and subjected to the DNA Diffusion Assay procedure.

2.4. Diffusion assay

After treatment of the seedlings, the roots or leaves were processed immediately or after a recovery period of 4 or 24 h. The tissues were placed in a 60-mm petri dish containing 250 μl of cold 400 mM Tris buffer, pH 7.5 and kept on ice. Using a fresh razor blade, roots and leaves were gently sliced. The plate was kept tilted in the ice so that the isolated nuclei would collect in the buffer. Regular microscope slides were dipped into a solution of 1% NMP agarose in water at 50 °C, dried overnight at room temperature and kept dry in slide boxes until use. Nuclear suspensions (50 μl) and 1% LMP agarose (50 μl) in phosphate-buffered saline were gently mixed at 40 °C by repeated pipetting using a cut micropipette tip, placed onto each slide and covered with a coverslip to obtain a uniform layer. The gel was allowed to solidify by keeping the slide in a steel tray on ice for a minimum period of 3 min, the coverslip was removed and a final layer of 0.5% LMP agarose (100 μl) was placed on the slide.

A modified method of Singh [4] was applied. The slides with the isolated nuclei embedded in agarose were placed for 4 min in a Coplin jar containing a cold lysing and denaturing solution (2.5 M NaCl, 1% sodium sarcosinate, 100 mM Na_2EDTA, 10 mM Tris, pH 10 and 0.2% DMSO, 0.3 N NaOH freshly added) before rinsing in cold water. The slides were placed for 1 h in a solution of 50% ethanol and 50% Tris buffer (400 mM, pH 7.4), with a final concentration of 1 mg spermine/ml to remove the precipitated salts and detergents while retaining DNA in the agarose. The slides were air-dried at room temperature.

Air-dried slides were immersed for 10 min in cold water and then stained for 5 min with 80 μl of either ethidium bromide (20 μg/ml), DAPI (2 μg/ml) or YOYO-1 (2 μM). All the dyes were dissolved in water.

The slides were rinsed in cold water to remove the excess dye and covered with a coverslip. The nuclei were analyzed by fluorescence microscopy. For detection of ethidium bromide an excitation filter of BP 546/10 nm and a 590-nm emission filter were used, for DAPI and YOYO-1 the excitation filters were 330–385 nm and 470–490 nm, and the emission filters were 420 nm and 520 nm, respectively. A computerized image-analysis system (Komet version 3.1, Kinetic Imaging Ltd., Liverpool, UK) was employed.

The radius of each nucleus was determined from the center of the nucleus to its right hand edge using the tail-length parameter of the Komet version 3.1 software. The nuclear area of each nucleus, expressed in μm^2 was calculated. Fifty nuclei were scored per slide, two slides were evaluated per treatment and each treatment was repeated twice. From each replicate experiment, the percentage of diffused nuclei and the average nuclear area on each slide were used to express the nuclear diffusion.

2.5. Statistics

Data were analyzed using the statistical and graphical functions of Sigma plot 8.0 and SigmaStat 3.0 (SPSS, Inc., Chicago, IL). When a significant F-value of $P < 0.05$ was obtained in a one-way analysis of variance test, a Dunnett's multiple comparison test between the treated and control group was conducted. Differences between two groups were statistically evaluated by means of a t-test.

3. Results

3.1. DNA damage in root nuclei after H_2O_2 treatment

After treatment of tobacco seedlings for 30 min with 6 or 10 mM H_2O_2, nuclei from roots were isolated either immediately after the treatment, or after a recovery period of 4 or 24 h. Just after treatment, the DNA Diffusion Assay (Fig. 1) revealed an average nuclear area (±S.E.) of $9111 \pm 414 \, \mu m^2$ with 6 mM H_2O_2; with 10 mM H_2O_2 this increased to $11884 \pm 381 \, \mu m^2$. These values differed significantly ($P < 0.001$) from the negative control ($3614 \pm 152 \, \mu m^2$). The average nuclear area of roots treated with 10 mM H_2O_2 decreased to $3614 \pm 298 \, \mu m^2$ when evaluated 4 h after
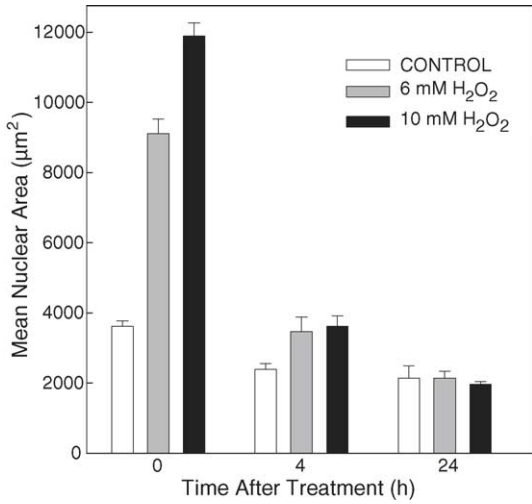
Fig. 1. The average nuclear area (μm^2) after hydrogen peroxide treatment (30 min) of nuclei from tobacco roots analyzed 0, 4 or 24 h after treatment. The nuclei were stained with ethidium bromide. The error bars represent the standard error of the mean.

treatment. In the above experiments, the period of exposure (30 min) and the period of recovery (4 h) may not have been sufficient for the expression of DNA fragmentation generated by apoptosis. Therefore, nuclei were also isolated 24 h after treatment. The average nuclear area of 10 mM H_2O_2-treated roots had further decreased to $1936 \pm 106 \, \mu m^2$ and there was no significant ($P = 0.89$) DNA damage compared with the concurrent negative control after 24 h. A similar trend was observed in the nuclei from roots treated with 6 mM H_2O_2, i.e. a substantial decrease after 4 h of recovery and a further decrease after 24 h of recovery.

The distribution of the level of DNA damage induced by 10 mM H_2O_2 in the DNA Diffusion Assay is presented in Fig. 2. The distribution of more severely damaged nuclei was detected when the nuclei were isolated from the roots immediately after the 30-min H_2O_2 treatment (Fig. 2B). When the H_2O_2-treated cells were recovered after 24 h, the frequency of highly diffused nuclei decreased and repair of genomic DNA was observed (Fig. 2E).
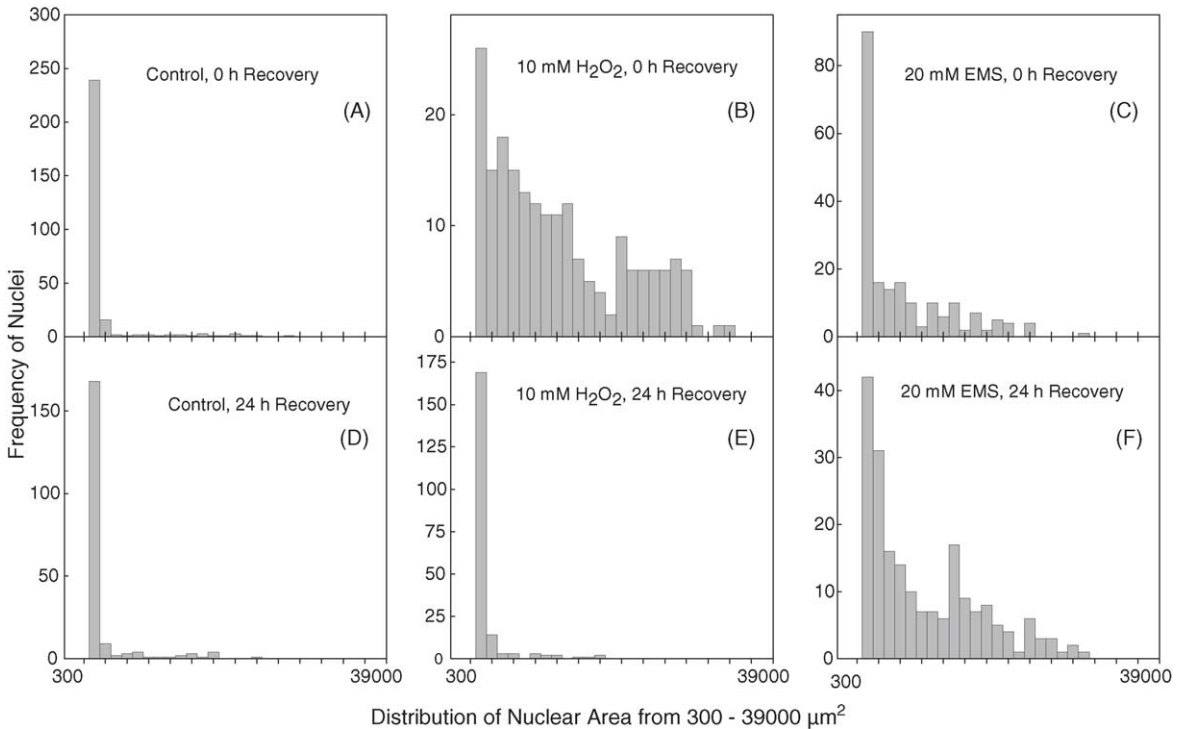

Fig. 2. Histograms illustrating the distribution of the nuclear area (μm^2) in tobacco root nuclei. Negative control with no (A) or 24 h (D) recovery; 10 mM hydrogen peroxide treatment with no (B) or 24 h (E) recovery; 20 mM EMS treatment with no (C) or 24 h (F) recovery.

The data demonstrate that the diffused nuclei induced by hydrogen peroxide are not of apoptotic origin as they can be completely repaired, whereas apoptotic DNA fragmentation cannot re-assemble under any condition [11,12].

3.2. *DNA damage in root nuclei after EMS treatment*

Fig. 3 illustrates the DNA-damaging effect on the nuclei of tobacco roots upon treatment with the direct-acting monofunctional alkylating agent EMS for 2 h. Nuclei were isolated from the EMS-treated roots at 0, 4 and 24 h after the termination of the treatment. Immediately after treatment, the average nuclear area increased significantly ($P < 0.001$) from $2007 \pm 63\ \mu m^2$ (negative control) to $4185 \pm 235\ \mu m^2$ with 10 mM EMS and $6023 \pm 428\ \mu m^2$ with 20 mM EMS. Nuclei isolated from 20 mM EMS-treated roots 4 h after treatment ($5887 \pm 333\ \mu m^2$) did not express a significant difference ($P = 0.8$) in the average nuclear area compared with nuclei isolated immediately after treatment, however, 24 h after the termination of treatment the average nuclear area had increased significantly ($P < 0.001$) to a value of $9073 \pm 344\ \mu m^2$. A similar trend was observed in the nuclei from roots treated with 10 mM EMS, i.e.

Fig. 4. The average nuclear area (μm^2) of leaf nuclei after 24 h ethylmethane sulphonate treatment of tobacco seedlings, analyzed 24 h after treatment. The nuclei were stained with ethidium bromide, DAPI or YOYO-1. The error bars represent the standard error of the mean.

no difference after 4 h of recovery and an increase after 24 h of recovery.

Fig. 2C and F illustrate the distribution of genomic DNA damage induced by 20 mM EMS in nuclei iso-

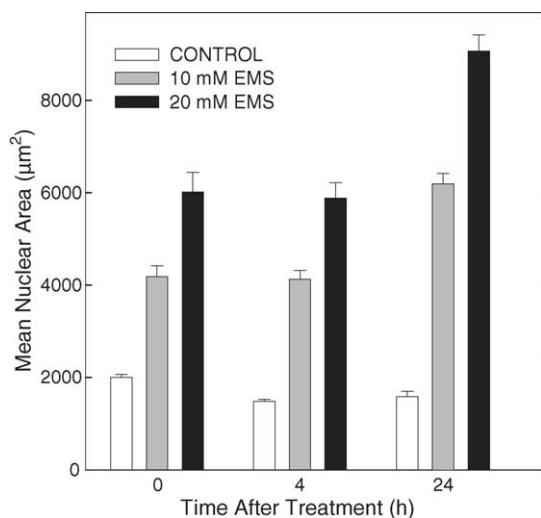Fig. 3. The average nuclear area (μm^2) after ethyl methane-sulphonate treatment (2 h) of nuclei from tobacco roots analyzed 0, 4 or 24 h after treatment. The nuclei were stained with ethidium bromide. The error bars represent the standard error of the mean.
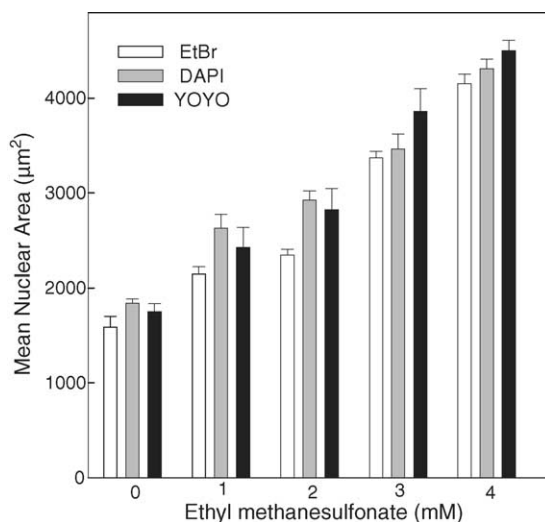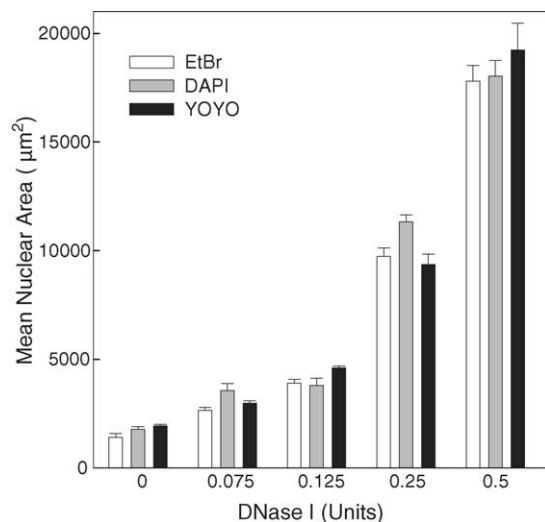

Fig. 5. The average nuclear area (μm^2) after treatment of isolated leaf nuclei embedded in agarose for 1 min with 0.075–0.5 U of DNase-I. The nuclei were stained with ethidium bromide, DAPI or YOYO-1. The error bars represent the standard error of the mean.

lated from roots. More highly diffused nuclei were observed after a 24-h recovery period (Fig. 2F) than immediately after treatment (Fig. 2C). In contrast to the results observed with H_2O_2, exposure to EMS caused a significant increase of DNA damage in root nuclei isolated 24 h after treatment. The contribution to this effect of apoptotic DNA fragmentation and/or the formation of DNA fragments due to the activity of repair enzymes [6,13] cannot be excluded.

3.3. DNA damage in leaf nuclei after EMS treatment

Leaf nuclei isolated from tobacco seedlings treated with EMS for 24 h were processed for the DNA Diffusion Assay and stained with one of three fluorescent dyes: ethidium bromide, DAPI and YOYO-1.

With increasing concentrations of EMS, the average area of nuclei stained with ethidium

bromide increased significantly ($P < 0.001$) from $1588 \pm 114 \, \mu m^2$ (negative control) to $4154 \pm 74 \, \mu m^2$ (4 mM) (Fig. 4). Staining with DAPI resulted in a significant ($P < 0.001$) increase in the average nuclear area from $1839 \pm 44 \, \mu m^2$ (negative control) to $4311 \pm 99 \, \mu m^2$ (4 mM). The area of nuclei stained with YOYO-1 increased significantly ($P < 0.001$) from $1752 \pm 82 \, \mu m^2$ (negative control) to $4503 \pm 107 \, \mu m^2$ (4 mM). There was no significant difference in the average nuclear areas between ethidium bromide and DAPI ($P = 0.130$) or ethidium bromide and YOYO-1 ($P = 0.122$) with 4 mM EMS.

3.4. DNA damage in leaf nuclei after DNase-I treatment

DNase-I generates an apoptosis-like morphology by digesting nuclear DNA to nucleosome-sized DNA
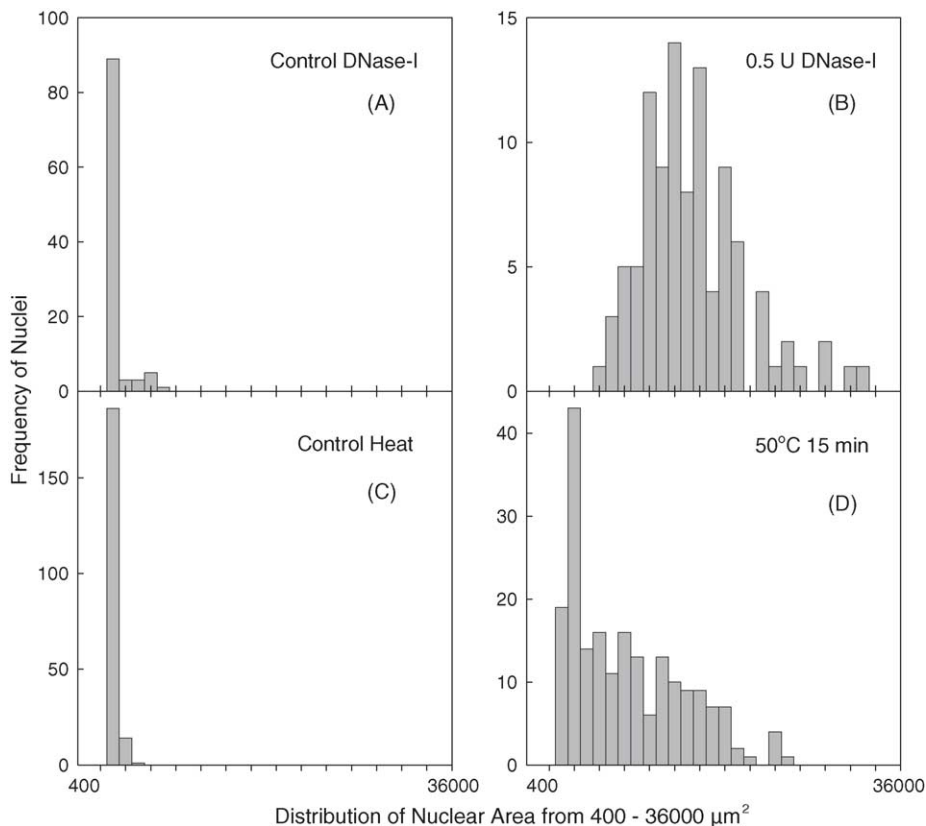

Fig. 6. Histograms illustrating the distribution of the nuclear area (μm^2) in tobacco cells. DNase-I negative control (A), 0.5 U of DNase-I treatment for 1 min (B). Heat-treated negative control (C) and heat treatment at 50 °C for 15 min (D).

fragments [3,4]. Diffused tobacco nuclei exposed to DNase-I for 1 min were stained with ethidium bromide, DAPI or YOYO-1 (Fig. 5). With increasing DNase-I activity (0.075–0.5 U), the average area of ethidium bromide-stained nuclei increased significantly ($P < 0.001$) from $1410 \pm 173 \, \mu m^2$ (negative control) to $17801 \pm 730 \, \mu m^2$ (0.5 U DNase-I). Staining with DAPI resulted in a significant ($P < 0.001$) increase of the average nuclear area from $1780 \pm 116 \, \mu m^2$ (negative control) to $18044 \pm 706 \, \mu m^2$ (0.5 U DNase-I). The area of DNase-I treated nuclei stained with YOYO-1 increased significantly ($P < 0.001$) from $1929 \pm 80 \, \mu m^2$ (negative control) to $19243 \pm 1230 \, \mu m^2$ (0.5 U DNase-I). The size of the average nuclear area after treatment with 0.5 U of DNase-I was not significantly different between ethidium bromide and YOYO-1 ($P = 0.267$), and between DAPI and YOYO-1 ($P = 0.471$).

The distribution histograms of the amount of DNA damage after treatment of isolated nuclei with DNase-I in the DNA Diffusion Assay are presented in Fig. 6B. The nuclease treatment of isolated nuclei resulted in extremely large diffused nuclei with areas of up to $20,000 \, \mu m^2$.

3.5. DNA damage in leaf nuclei after heat treatment

To induce necrotic DNA fragmentation tobacco leaves were incubated in water at $50 \,^{\circ}C$ for 1–15 min. Subsequently, the DNA Diffusion Assay was performed either immediately, or after a 24-h storage of the heat-treated leaves in water at $26 \,^{\circ}C$ in the dark.

In nuclei isolated immediately after treatment, the average nuclear area increased significantly ($P < 0.001$) from $1598 \pm 76 \, \mu m^2$ before treatment to $12648 \pm 452 \, \mu m^2$ after 15 min (Fig. 7). Evaluation of the nuclei 24 h after heat treatment showed that the average nuclear area had increased significantly ($P < 0.001$) from $1466 \pm 204 \, \mu m^2$ (negative control) to $13970 \pm 404 \, \mu m^2$ (7.5 min of heat treatment). After 10 and 15 min of heat treatment and 24 h of recovery, the nuclear fragmentation was so high that the nuclear area could not be evaluated. The distribution of the amount of DNA damage induced with heat treatment is presented in Fig. 6D.
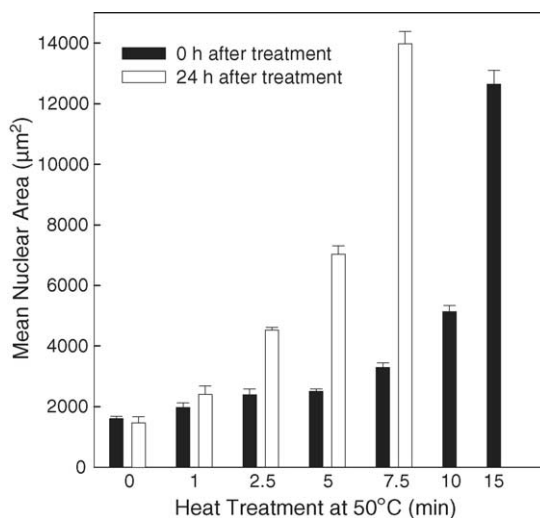

Fig. 7. The average nuclear area (μm^2) after heat treatment of leaves at $50 \,^{\circ}C$ for 1–15 min and analyzed at 0 or 24 h after treatment. The nuclei were stained with ethidium bromide. The error bars represent the standard error of the mean.

4. Discussion

Singh [3,4] proposed the DNA Diffusion Assay as a simple and accurate method to detect apoptosis, and as an alternative to other methods such as agarose gel electrophoresis to demonstrate the ladder pattern of DNA generated by the endonucleolytic cleavage of genomic DNA into nucleosomal fragments [14], morphological evaluation based on chromatin condensation, shrinkage of cytoplasm and blebbing of plasma membrane [15], or identification of externalized phosphatidylserine [16].

In this paper, we have tried to address the following issues:

The technical question of whether it is essential to use the fluorescent dye YOYO-1 for the DNA Diffusion Assay. YOYO-1 is recommended [3,4] for this assay, because of its high sensitivity in detecting small nucleosome-sized DNA fragments of 180 bp. The disadvantages of YOYO-1 are its high cost, low stability and the necessity to have a fluorescence microscope equipped with additional expensive filter sets. The data presented in this study and in a related study using mammalian cells demonstrate that the less expensive and commonly used fluorescent dye ethidium bromide
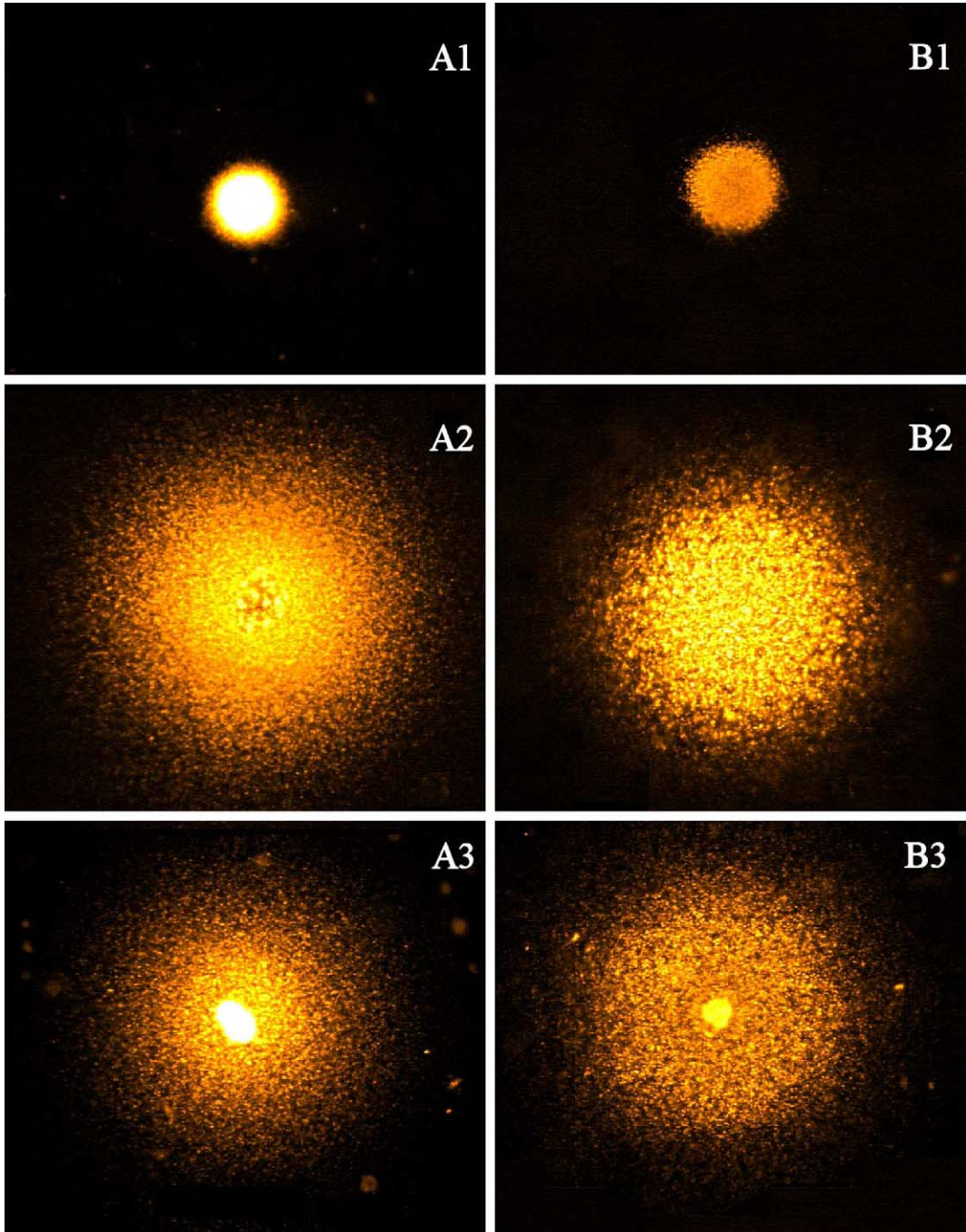
Fig. 8. Images illustrating normal (1), diffused nuclei after DNase-I treatment (2) and necrotic heat treatment (3). The nuclei were stained with ethidium bromide (A) or YOYO-1 (B).

can be used for the DNA Diffusion Assay with the same efficiency [Plewa et al. (2005) personal communication].

More importantly, the question was addressed whether the DNA Diffusion Assay is suitable for detecting apoptosis. According to Singh [3,4], apoptotic cells, when tested in the diffusion assay, show a circular gradient of granular DNA with a dense central zone and a lighter, hazy outer zone, giving the overall appearance of a halo due to nucleosome-sized DNA. By contrast, in diffused nuclei due to necrosis the DNA shows a clearly defined outer boundary and a relatively clear appearance. This sharp outline in necrotic cells may be due to larger sized DNA fragments, which do not diffuse as much as the smaller fragments in apoptotic cells. Nuclei with genotoxin-damaged DNA (but not necrotic or apoptotic) are clearly defined, according to Singh [3,4], and the nuclei are larger with projections of DNA all around.

The data in the present study show that there were only slight differences in the structure of diffused tobacco cell nuclei after digestion with DNase-I (simulating apoptotic DNA fragmentation) and after heat treatment (representing necrotic DNA fragmentation) (Fig. 8). Heat treatment led primarily to diffused nuclei with a dense central zone (Fig. 8, images A3 and B3), but only after short heating. After longer heat treatments, the nuclei displayed the same structure as after DNase-I digestion (Fig. 8, images A2 and B2). However, with the genotoxins hydrogen peroxide and EMS, all types and sizes of diffused nuclei were observed.

In conclusion, the DNA Diffusion Assay was unable to differentiate between apoptotic and necrotic DNA fragmentation and other types of genotoxin-induced DNA damage in plant cells.

Acknowledgements

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