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Original Article

Impact of Exposure Duration on DNA Damage Scores Through Comet Assay in *Allium cepa* L. and *Lactuca sativa* L.

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Abstract

The single-cell gel electrophoresis (SCGE), or comet assay, is a simple method that uses microgel and electrophoresis to evaluate damage in the DNA of the cells. The first protocol adapted for plants was described in the 1990s, however, a universal standardized procedure is not available to date. This study aimed to evaluate the influence of exposure time in the comet assay in plant models *Allium cepa* L. and *Lactuca sativa* L. Root tips of both plant models were exposed to methyl methanesulfonate (MMS), a known genotoxicant, for 2 h to 36 h. The alkaline version of the comet assay for plant samples was performed, and the DNA damage was assessed. Arbitrary units (AU) were calculated and compared to the negative control. MMS induced DNA damage ($P < 0.05$) after 2, 6, 10, 14, 28, 32 and 36 h of exposure in *A. cepa*, and at 2, 8, 10, 14 and 36 h for *L. sativa*. The highest AU values for *A. cepa* were observed after 4 h of exposure to MMS and for *L. sativa*, after 24 h of exposure. The optimal exposure times considering both genotoxic and mutagenic risk were indicated.

Keywords: Alkaline comet assay; Genotoxic; Methyl methanesulfonate; Mutagenic; Root tips; Single cell gel electrophoresis.

INTRODUCTION

The comet assay (single-cell gel electrophoresis) is a sensitive technique to detect DNA damage caused by exposure to genotoxic chemical and physical agents, and, is widely used in eco-geno-toxicology studies (Bolognesi *et al*., 2019). This promising technique is characterized as sensitive, versatile, rapid, economic, relatively simple and, considered the most popular method used in the last few years (Gutzkow *et al.*, 2013; Pourrut *et a*l., 2015; Dusinska *et al.*, 2017). According to a bibliometric study by Neri *et al.* (2015), Brazil is one of the countries where the comet assay has seen considerably increased application as reflected in the number of articles published by Brazilian workgroups using this approach. In a recent review, Brazil stood out as the fifth country most frequently engaged in research involving the comet assay with a plant model (Alias *et al.*, 2023). Moreover, it has been broadly applied to assess the toxic effects of environmental pollutants, for biomonitoring of both aquatic and terrestrial ecosystems, and even in basic research to understand the mechanisms of DNA damage and repair systems (Azqueta *et al*., 2011; Pourrut *et al*., 2015).

A brief review of the assay highlights its development, from studies in nuclear structure in the 1970s to the first application to quantify DNA damage in the 1980s. The first applied version of the comet assay was performed under neutral conditions (Dhawan *et al.*, 2009; Collins, 2015). Later, some adaptations were made to expand the range of DNA lesions that could be detected by the procedure (Ventura *et al*., 2013). Now, the comet assay applying alkaline conditions is the most used version of the test. It is performed under denaturing conditions and detects both

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single- and double-strand DNA breaks, induced by the tested chemical/substance/pollutant (Lanier *et al.*, 2015).

Mammalian cells were the first source materials on which the comet assay was applied. Nowadays, an increasing number of works have been focusing on the development of a high throughput version of the assay for human cells, as it is adopted in tests to screen novel drugs, cosmetics, potential carcinogenic substances, and disease inducers, as well as in occupational toxicology (Gutzkow *et al*., 2013; Neri *et al.*, 2015; Bolognesi *et al*., 2019). Despite this emphasis on application in human cells, the comet assay could be employed as a model in various cell types (from bacteria to human cells, including plants), considering that the sample is prepared from a single-cell suspension (Dhawan *et al*., 2009; Neri *et al.,* 2015).

It is well-documented and established that plants are excellent models for ecogenotoxic, cytogenotoxic, and mutagenic tests (Leme & Marin-Morales, 2009; Lanier *et al.,* 2015). Moreover, plant species are attractive models due to their low cost and ease of collection or cultivation. Besides, treatment effects in plant cells are strongly correlated with those in human cells; therefore, plant cells could be used alternatively to animals in screening projects tests (Ventura *et al.*, 2013; Andrade-Vieira *et al*., 2014; Reis *et al.*, 2017).

The comet assay in plants was developed in the early 1990s using a neutral version of this assay. The alkaline version was created in 1997, and the protocol was described by Koppen and Cerda using *Vicia faba* as a model (Lanier *et al.*, 2015; Pourrut *et al*., 2015). In their report on the comet assay in terrestrial plant models, Lanier *et al.* (2015) presented an extensive review that includes 45 plant species used in the assay. However, only three plant models could be highlighted as the most frequent: *V. faba*, *Allium cepa* and *Nicotiana tabacum*.

Higher plants offer significant value as toxicological models, allowing versatile experimentation (*in vitro, in vivo, in situ*) across diverse organs. They facilitate multi-endpoint analysis, cross-system correlations, and cost-effectiveness (Alias *et al*., 2023). Notably, the comet assay has gained considerable acceptance with plant models, utilizing different plant parts (leaves, shoots, roots) as cell sources. This approach has sparked dedicated efforts to refine the application of the comet assay in plant models. Among others, Pourrut *et al.* (2015) evaluated various factors related to the test, such as mechanical extraction, conditions such as temperature and time of exposure, lysis influence, and the concentration of the chemical tested associated with the time of exposure, on the frequency of damage assessed.

So far, a concise and reliable procedure for comet assay using plants as a model has not been defined. Thus, considering the efficiency of this method in detecting genotoxic compounds and its extended use for toxic risk assessment, in addition to the fact that plant models are reliable for this purpose, the present study aimed to contribute to an accurate comet assay protocol in higher plant models. For this, *A. cepa* L. and *Lactuca sativa* L. were selected as plant model systems and cell sources. The influence of exposure time was chosen as a variable to score damage, and genotoxicity or mutagenicity. Overall genotoxicity is considered when the damage occurred before a complete cell cycle as it could be repaired by cell repair system, and mutagenicity occurs when a cell cycle ends and the DNA damage persists to daughter cell lines (Collins & Horváthová, 2001; Aitken *et al.*, 2020).

A. cepa is the high plant model most widely used by laboratories investigating the cytotoxicity and genotoxicity of chemical compounds and environmental pollutants (Leme & Marin-Morales, 2009), including those that perform the comet assay (Lanier *et al.,* 2015). However, *L. sativa* was recently shown to be as efficient and sensitive as *A. cepa* for this same purpose, representing a simple eudicot to be applied as a model (Silveira *et al.*, 2017).

MATERIAL AND METHODS

Chemical compounds

Methyl methanesulfonate (MMS) is an alkylating agent that acts on DNA by preferentially methylating guanine and adenine bases. It is a frequently used agent in the field of genome integrity and a model agent to characterize and understand how alkylating agents work, including those with comet assay as an approach to study DNA damage (Bankoglu *et al*., 2021; Ovejero *et al.*, 2021). The MMS solution applied to the assay was prepared from the pure MMS compound Sigma® at the concentration of 4×10^{-4} mol L^{-1} (Caritá & Marin-Morales, 2008). Ultrapure water obtained in MilliQ equipment was used as the negative control.

Plant model material

Seeds of *A. cepa* L. var. *baia periforme* (onion) were acquired at agricultural supply stores, whereas the seeds of *L. sativa* L. var. *Verônica* (lettuce) was obtained from the Germplasm Bank of the Department of Agriculture at the Federal University of Lavras (DAG/UFLA), State of Minas Gerais, Brazil.

Exposure conditions

The experiment was conducted in the Laboratory of Cytogenetics of Biology Department of the Federal University of Lavras (DBI/UFLA), State of Minas Gerais, Brazil.

Exposure of the seeds to the treatments followed a completely randomized experimental design, with three repetitions (Petri dishes) of each treatment for each model (onion and lettuce). To obtain treated roots, 30 seeds of each plant model were spread in each Petri dish (9 cm in diameter) containing filter paper moistened with 3 mL of ultrapure water. Upon reaching lengths of 1 to 2 mm (48 h for onion and 16 h for lettuce), the seedlings were transferred to new Petri

dishes containing filter paper moistened with 3 mL of the test solutions (MMS or MilliQ). After 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, 28, 32, and 36 h of exposure, the roots were collected for performance of the assay.

The dishes were maintained under a controlled temperature of 22 ± 2°C, without photoperiod, in a biochemical oxygen demand (BOD) chamber throughout the exposure procedure.

Alkaline comet assay

After each exposure time, the roots were collected and the comet assay was performed according to the procedures and conditions described by Silveira *et al.* (2017). Briefly, the treated root tips (5 to 10 mm in length) were chopped in 300 µL of 1X PBS buffer at 4°C with the help of a new razor blade. The suspension was filtered in a CellTrics strainer (Partec®) of 50-µm diameter to yield the nuclei suspension.

Three slides, previously coated with a thin layer of 1% normal melting point agarose, were prepared for each treatment. To each slide were added 30 µL of a mixture (1:1) of the obtained nuclei suspension and 1% low melting point agarose (60°C), spread with a coverslip. The slides were kept under refrigeration (10°C) for 5 min for agarose hardening and then immersed in lysis solution for 10 min in a refrigerated room (20 ± 4°C). Subsequently, the slides were washed in cold 1X TBE buffer.

The electrophoresis was carried out in horizontal chambers containing cold NaOH–EDTA buffer. The gel was run under a current of 25V at 300 mA for 15 min in a refrigerated room (20 ± 4°C). Subsequently, the slides were fixed in absolute ethanol (Merck®) for 5 min and dried for 1 h at 10°C.

For microscopic evaluation, the slides were stained with 15 µL of propidium iodide solution (2 µg/mL), covered with a glass coverslip, and analyzed under an epifluorescence microscope (Olympus BX 60) at a wavelength of 530–550 nm and magnification of 400×. Three slides were prepared for each treatment and 100 nucleoids were evaluated per slide, totaling 300 nucleoids observed per exposure time and treatment (MMS and MilliQ).

The nucleoids were evaluated and classified into 0 to 4 by visual score, according to DNA damage level, following the patterns proposed by (Reis *et al.*, 2017). The percentage of damage and arbitrary unit (AU) values were calculated as described by (Collins, 2004). The arbitrary units were calculated according to the formula in Eq. 1:

$$AU = (Nx0)+(Nx1)+(Nx2)+(Nx3)+(Nx4) \quad \textbf{Eq. 1}$$

N: number of cells counted on the microscopic slide in each class of DNA damage

Statistical analyses

The obtained data were subjected to analysis of variance ($\alpha = 0.05$) and the means were compared by Tukey test at a 5% significance level. The statistical analyses were performed in the program R (R Development Core Team, 2014).

RESULTS

The percentage of DNA damage for both species in MilliQ water, considered as negative control, ranged from 0 to 27.67% for both species at all exposure times (Tables 1 and 2), depicting the basal damage that exists in cells. For this treatment, the nucleoids were classified as 0 and 1, characterized by low levels of damage, i.e. of less than 20% (Figure 1, Tables 1 and 2).

MMS exerted basal DNA damage similar to that observed for the negative control ($P < 0.05$) after 2, 6, 10, 14, 28, 32, and 36 h of exposure in *A. cepa* (Table 1), and at 2, 8, 10, 14 and 36 h for *L. sativa* (Table 2). The further tested MMS exposure times significantly increased DNA damage in relation to the control (Tables 1 and 2). These treatments presented a percentage of DNA damage above 30% (Tables 1 and 2) and nucleoids scoring from 0 to 4, comprising all damage classes (Figure 1, Tables 1 and 2).

Table 1. Damage observed in nucleoids of *Allium cepa* (onion) exposed to methyl methanesulfonate (MMS).

Treatment	Exposure time (h)	Scores					Percentage of damage (%)		
		0	1	2	3	4	Means	SD*	
Negative control – MilliQ water	2	96	4	0	0	0	4.00 a	1.00	Genotoxic
	4	86	11	0	0	0	13.67 b	4.16	
	6	100	0	0	0	0	0.00 a	0.00	
	8	77	22	1	0	0	22.67 c	1.52	
	10	99	1	0	0	0	0.67 a	1.15	
	12	100	0	0	0	0	0.00 a	0.00	
	14	99	1	0	0	0	1.00 a	1.73	
	16	85	15	0	0	0	14.67 b	0.57	
	20	89	10	1	0	0	11.00 ab	7.54	Mutagenic
	24	73	25	1	0	0	26.67 bc	4.93	
	28	100	0	0	0	0	0.33 a	0.57	
	32	74	21	6	0	0	26.33 bc	6.65	
	36	89	10	1	0	0	10.67 a	9.23	
Methyl methanesulfonate	2	89	11	0	0	0	11.00 ab	4.58	Genotoxic
	4	4	5	11	20	60	96.00 e	1.73	
	6	81	4	9	3	3	19.00 b	4.00	
	8	35	30	20	12	3	65.33 d	8.54	
	10	94	0	3	2	0	5.67 a	3.05	
	12	35	23	13	11	17	65.00 d	9.64	
	14	100	0	0	0	0	0.00 a	0.00	
	16	51	22	24	3	1	49.33 c	5.59	
	20	38	12	12	23	16	62.00 d	9.68	Mutagenic
	24	21	23	12	18	25	79.00 d	7.93	
	28	86	0	2	3	9	14.00 b	1.57	
	32	75	3	2	11	9	24.67 bc	2.08	
	36	76	2	7	7	9	24.00 bc	2.64	

* SD - Standard deviation.

Means followed by the same letter in the column do not differ by Tukey test ($P < 0.05$).

Table 2. Damage observed in nucleoids of *Lactuca sativa* (lettuce) exposed to methyl methanesulfonate (MMS).

Treatment	Exposure time (h)	Scores					Percentage of damage (%)		
		0	1	2	3	4	Means	SD*	
Negative control – MilliQ water	2	100	1	0	0	0	0.33a	0.57	Genotoxic
	4	88	12	0	0	0	12.33a	1.52	
	6	96	4	0	0	0	4.00a	1.00	
	8	89	12	0	0	0	12.00a	3.60	
	10	100	0	0	0	0	0.00a	0.00	
	12	83	17	0	0	0	16.67a	4.93	
	14	97	3	0	0	0	3.00a	2.64	
	16	91	9	0	0	0	9.33a	5.03	
	20	86	12	0	0	0	12.33a	6.59	Mutagenic
	24	72	24	4	0	0	27.67b	5.50	
	28	95	4	1	0	0	4.67a	4.16	
	32	88	12	0	0	0	12.00a	5.56	
	36	73	27	0	0	0	26.67b	8.73	
Methyl methanesulfonate	2	91	9	0	0	0	9.33a	5.50	Genotoxic
	4	49	47	4	0	0	50.67c	1.52	
	6	59	38	3	0	0	40.67bc	2.08	
	8	69	30	0	0	0	30.33b	3.51	
	10	98	2	0	0	0	2.33a	2.51	
	12	30	37	24	8	2	70.33d	8.38	
	14	93	7	0	0	0	7.33a	5.13	
	16	63	37	0	0	0	37.00bc	6.08	
	20	38	61	1	0	0	61.67c	7.57	Mutagenic
	24	18	10	16	25	32	83.33d	8.08	
	28	55	30	14	0	0	44.67bc	8.15	
	32	51	31	15	2	1	49.00c	6.65	
	36	38	30	23	7	3	62.33c	2.08	

* SD - Standard deviation.

Means followed by the same letter in the column do not differ by Tukey test ($P < 0.05$).

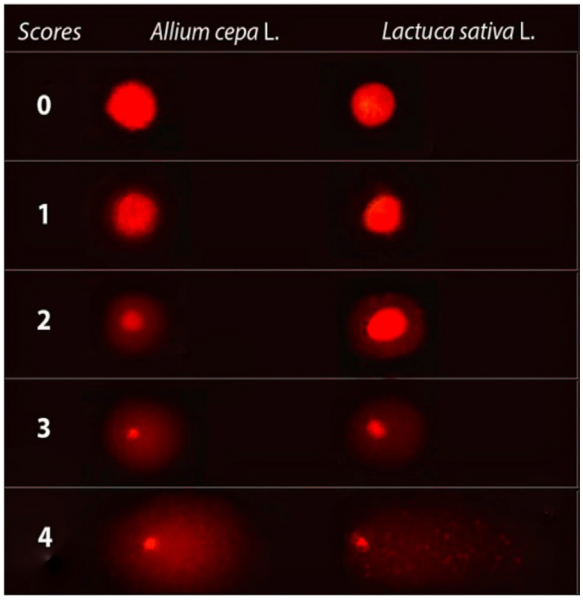

Figure 1. Standard visual scores used to classify the nucleoids of *Allium cepa* (onion) and *Lactuca sativa* (lettuce) exposed to MMS (methyl methanesulfonate). Score 0 – no observed damage (damage ≤ 5%); Score 1 – low level of damage (5–20%); Score 2 – medium level of damage (21–40%); Score 3 – high level of damage (41–85%); Score 4 –totally damaged DNA (> 86%).

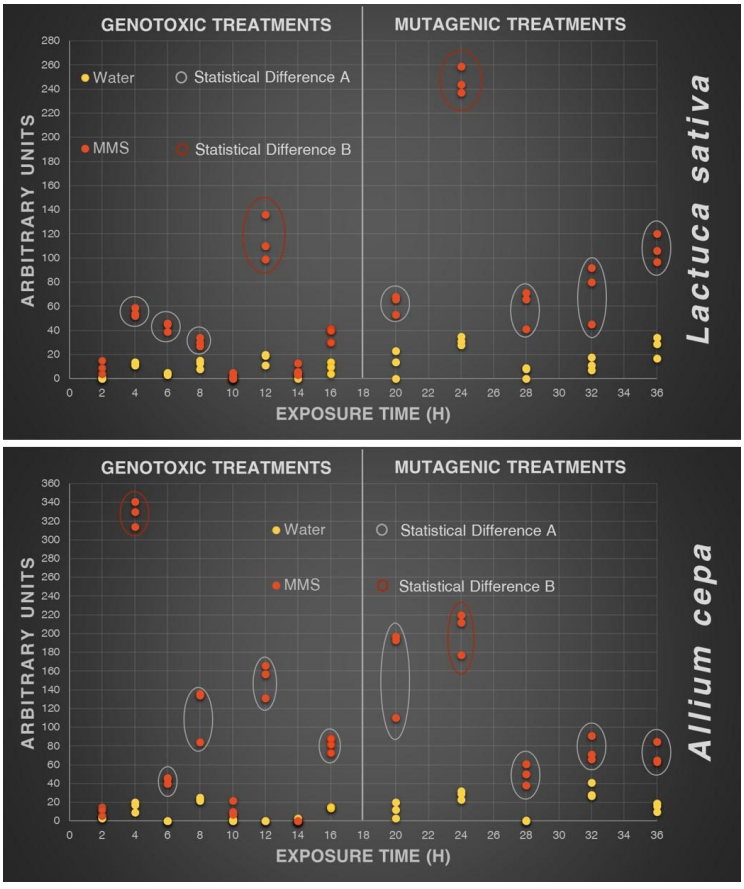

Figure 2. Arbitrary unit (AU) values obtained in nucleoids of *Allium cepa* (onion) and *Lactuca sativa* (lettuce) exposed to MMS (methyl methanesulfonate) with genotoxic (2 h to 16 h of exposure) and mutagenic treatments (20 h to 36 h of exposure). Each plotted point is the AU value for one repetition of 100 nucleoids scored in one slide. Therefore 3 points per time point. Statistical difference referred to as A indicates treatments with lower DNA damage. Statistical difference referred to as B distinguishes treatments with higher DNA damage levels.

DISCUSSION

The conditions adopted in the preparation of the samples followed the recommendations of Pourrut *et al*. (2015). Accordingly, to obtain the nuclei suspension a razor blade was used to chop the roots into small slices for at least one minute. This chopping time was addressed by those authors as important to guarantee an adequate amount of nuclei in the final slides.

In this work, nuclei isolation was accomplished in the presence of a lysis buffer, as it facilitates the isolation of the nuclei when cell walls are present. Pourrut *et al*. (2015) report that, despite some influence of lysis on the percentage of damage, as observed in *Lolium perenne* leaves exposed to ethyl methanesulfonate (EMS), this increase in damage was not significant.

Another recommendation by Pourrut *et al.* (2015) followed here concerns the temperature and luminosity during the processing of the samples. The temperature was controlled by an air conditioner set to 22°C, and only natural light was used, with no lamps. In addition, the concentration of MMS was chosen based on the report of Silveira *et al.* (2017) regarding its damage potential as measured by comet assay in *A. cepa* and *L. sativa*.

To evaluate DNA damage, we used the visual scoring method based on the classification of damage following the patterns presented in Figure 1. The efficiency of the scoring method was tested previously by Azqueta *et al.* (2011). These authors compared the visual scoring used in the present work, based on the classification of Collins *et al.* (1997) and containing five classes (0 to 4), with the so-called tail moment, a semi-automated and automated method based on image analysis that uses a computer program to determine (among other parameters) the percentage of DNA in the tail. They concluded that both scoring methods provided acceptable variation limits.

The exposure times applied in this work allowed evaluation of both genotoxic and mutagenic effects. In a review by Lanier *et al*. (2015), the exposure period in studies applying comet assay to higher plants varied from 2 h to more than 24 h. In doing so, all of these studies were regarded as genotoxic assessments of a chemical or an environmental agente. However, if one considers the action of the DNA repair system and the duration of the cell cycle, the exposure time in discussion here has a direct influence on the endpoint assessmed. In this sense, we assume that an exposure time that falls within the period of one cell cycle demonstrates DNA damage endpoints that correspond to genotoxic effects, as they could be repaired during the cell cycle (Collins & Horváthová, 2001). Nevertheless, if the adopted exposure time is longer than the duration of the cell cycle, we suggest arguing that the DNA damage observed in the comet assay will correspond to an endpoint for mutagenic effects, as it represents not primary damage, but non-repaired inherited damage.

An influence of exposure time on the scored damage using the comet assay in plant samples was also observed by Pourrut *et al.* (2015). The shortest exposure time to EMS they tested was 12 h, which was sufficient to increase the DNA damage in *Miscanthus* leaves. At 24 h, a decrease in DNA damage was observed, with a subsequent increase after 48, 72, and 96 h of exposure. The duration of a common plant cell cycle does not exceed 20 h; therefore, the damage at 12 h of exposure observed by these authors reflects the genotoxicity of EMS. Accordingly, the decrease in damage after 24 h reflects the action of the DNA repair system, with the observed damage corresponding to that which could not be repaired, indicating the mutagenic action of EMS.

In our study, the applied exposure periods allowed the evaluation of both genotoxic and mutagenic effects on DNA exposed to MMS. Moreover, an influence of exposure time on root tip cells treated with MMS was also observed for both plant species. Exposure times between 2 h and 16 h were considered as genotoxic in both cases, as the cell cycle duration in *A. cepa* and *L. sativa* is shorter than 16 h. The evaluations took place at 2-h intervals, and exposure-time dependent an independent time exposure variation in DNA damage was observed, considering the AU values with regard to the percentage of damage. Nevertheless, a great peak of damage was observed in *A. cepa* after 4 h of exposure, where more than 90% of the cells were impaired. For *L. sativa*, the highest AU values were obtained after 12 h of exposure to MMS, totaling 70% of damaged nucleoids. Further, the exposure times from 20 h to 36 h, evaluated every 4 h, were considered as mutagenic treatments. Similarly, the effects of MMS vary, and an accumulation of DNA damage was noticed in both species after 24 h of exposure.

Therefore, considering that AU values range from 100 to 160 after 12 h of exposure to genotoxic treatments, we recommended this exposure time for works that aim to assess the genotoxicity of a tested compound. For mutagenic effects, the recommendation is 24 h of exposure when the AU values range from 180 to 260 in these species. The AU values found here demonstrate that DNA damage in the recommended peaks accumulates, being 1.5 higher for mutagenic exposure compared to genotoxic exposure. In the work of Silveira *et al.* (2017), the exposure time of 48 h was applied and the AU values observed after MMS exposure were 300 for *A. cepa* and 130 for *L. sativa*. At any rate, the AU values observed after 12 h and 24 h of exposure are considered quite high (Jia *et al*., 2013) to determine genotoxic and mutagenic effects. Hence, MMS proves once again to be a reliable chemical to be applied as a positive control Tan *et al*. (2014), in risk assessment experiments using the comet assay technique.

CONCLUSION

Exposure time is a parameter that requires attention in the design of comet assay experiments, as it may influence the conclusions regarding the potential damage of the tested chemical compound. The DNA damage induced by MMS in onions and lettuce was different considering the exposure time. This difference between the species is related to the cell cycle of each. In addition, the difference in damage within the same species is due to the time of exposure to the genotoxic agent. For lettuce, the maximum damage was seen after 12 and 24 h of exposure, while for onions it was seen after 4 and 24 h of exposure. To assess the genotoxic effects of a given compound with the comet assay, an exposure time of up to 12 h hours should be considered, while to access mutagenic damage, more than 20 h of exposure is required. The chosen exposure time should be 12 h for genotoxic effects, whereas for mutagenic effects 24 h of exposure is recommended. At last, MMS can be considered an efficient chemical to demonstrate both genotoxic and mutagenic effects and could be applied as positive control in comet assays of plant cells.

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AUTHOR CONTRIBUTIONS

LFAV, SM: concepted and design the work; **FAC:** organized the experiments, collected the data and drafting the article; **FAC, GLS:** analyze and interpreted the data collected; **LFAV:** edited and revised the writing; **LFAV:** critical review the article and approved the final version to be published.

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