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RESEARCH ARTICLE

Genotoxicity evaluation of environmental pollutants using analysis of nucleolar alterations

Dânia Elisa Christofoletti Mazzeo · Maria Aparecida Marin-Morales

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Abstract Nucleolar alterations resulting from the action of either chemical or physical agents can serve as important genotoxicity biomarkers. In this study, the efficiency of AgNOR banding technique to identify the presence of nucleoli in micronucleus and assess nucleolar alterations in aberrant cells of *Allium cepa* was evaluated. Seeds of this plant were exposed to both water samples from a river that receives untreated urban effluent and to the trifluralin herbicide (0.84 mg/L concentration), both analyzed in two different seasons (summer and winter seasons). Samples induced significant frequencies of chromosomal and nuclear aberrations and micronuclei, as observed in cells submitted to conventional chromosomal staining. The herbicide caused a significant increase in the number of nucleoli and micronuclei, interpreted as due to the elimination of excessive nucleolar material resulting from polyploidization. The use of the AgNOR technique enabled the identification of both the presence of the nucleolus in some micronuclei and the nucleolar organizer region (NOR) behavior of aberrant cells. The NOR-banding technique showed to be an efficient tool for studying the genotoxic effects caused by a xenobiotics and a complex environmental sample.

Keywords Nucleolus · *Allium cepa* · Micronucleus · Urban effluent · Trifluralin herbicide · AgNOR staining

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D. E. C. Mazzeo · M. A. Marin-Morales (✉)
Department of Biology, Institute of Biosciences, UNESP — Univ Estadual Paulista, Av. 24-A, 1515, 13506-900 Rio Claro, SP, Brazil
e-mail: mamm@rc.unesp.br

D. E. C. Mazzeo
e-mail: decm@rc.unesp.br

Introduction

Human activities have been causing great impacts on the environment. Pesticides and untreated effluents are among the main sources of environmental pollution that can lead to the deterioration of water resources (Murray et al. 2010; Lokhande et al. 2011; von der Ohe et al. 2011; Guillén et al. 2012; Martínez Bueno et al. 2012). Thus, nowadays, there is increasing need to investigate these contaminants in order to understand how they affect wildlife and can impact the environment. Several techniques are used to evaluate effects of environmental pollution on organisms, and among them cytogenetic assays are considered useful tools to evaluate the genotoxicity of these pollutants (Thiriot-Quiévreux 2002; Caritá and Marin-Morales 2008). In order to provide reliable and accurate information about the composition and arrangement of DNA along the chromosomes, chromosomal staining techniques have been applied (Kim et al. 2002; Leme and Marin-Morales 2009). One technique that may provide interesting contribution to the genotoxicity study of chemical agents as it enables identification of chromosomal composition is the silver-staining technique (AgNOR), which detects the presence of the nucleolar organizer region (NOR) (Teerarak et al. 2009). The AgNOR banding has been widely used in cytological studies in order to evaluate both the nucleolar cycle and organization in several organisms (Liu and Jiang 1991), including *A. cepa* (Liu and Jiang 1991; Arkhipchuk et al. 2000; 2004; Arkhipchuk and Garanko 2002; Qin et al. 2010).

According to Boulon et al. (2010), nucleoli are apparently the main structures involved in the activation of cellular stress responses. As a consequence, both structure and function of the nucleolus are directly affected by cellular stress. Such alterations may be involved in the coordination of procedures for the association and modification of RNA and proteins in proliferating cells. Therefore, the nucleoli deserve special

attention because alterations in these structures can serve as a powerful cytological marker, which can be considered an important parameter to use in environmental monitoring studies (Liu et al. 1994).

Nucleolar parameter alterations, such as the number of nucleoli per nucleus, nucleolar volume, and the number of cells with heteromorphic-paired nucleoli (PNhet) have been extensively used in genotoxicity assays both in animals (Arkhipchuk and Palamarchuk 1996; Çavas and Ergene-Gözükara 2002; 2003a; 2003b; Arkhipchuk and Garanko 2005) and plants (Arkhipchuk 1995; Arkhipchuk et al. 2000; Arkhipchuk and Garanko 2002). The choice of using these parameters is related to the amount and quality of information that can be collected, the speed at which results are obtained, the simplicity, high reproducibility, and low cost of the technique (Arkhipchuk and Garanko 2002; Arkhipchuk et al. 2004).

Among higher plants, *Allium cepa* has been considered an efficient test organism to monitor the genotoxicity of environmental pollutants (Matsumoto et al. 2006; Fernandes et al. 2007; Caritá and Marin-Morales 2008; Leme and Marin-Morales 2009; Mazzeo et al. 2011). *A. cepa* has satellites in the chromosome 6 pair, which bears the NOR (Kim et al. 2002; Leme and Marin-Morales 2009). In this species, according to Panzera et al. (1996), in addition to the presence of NOR in the chromosomes with satellite, there is another NOR located on the long arm of chromosome 8, which shows no secondary constriction. According to Arkhipchuk (1995), cells presenting a small number of NORs with fixed nucleolar characteristics are more indicated for cytological evaluations of alterations in functional activities of the rRNA gene than species with cells bearing many nucleoli. Thus, as *A. cepa* presents a small number of NORs, it can be considered a good biological indicator for the study of alterations in nucleolar parameters induced by environmental pollutants.

In the current study, the AgNOR method was used to evaluate the nucleolar activity of aberrant cells as a marker for genotoxicity, and to test its efficiency in identifying the presence of nucleolus in micronucleus (MN). Additionally, the variation of the number of nucleoli in *A. cepa* meristematic cells exposed to water samples from a river where untreated urban effluent is discharged, as well as model chemical commonly used in genotoxicity studies, the herbicide trifluralin, were also evaluated.

Materials and methods

Characterization of the studied area and water sampling

The study was conducted with water samples collected from the Ribeirão Claro stream, which belongs to the Corumbataí river basin. It flows through the city of Rio Claro, São Paulo State, Brazil. In the year during which this study was performed (2006), the municipality of Rio Claro had a total population of 188,109 inhabitants, and only 30 % of the sewage was treated before it was released into the stream (CETESB 2007). Water from the Ribeirão Claro stream represents the source for the public water supply for 40.3 % of the population in the region. However, along its course the stream flows through rural areas with pastures and cultivation of sugarcane, and receives discharges of industrial effluents and untreated domestic sewage, which is considered its main source of pollution (Gertel et al. 2003; Fonseca 2008).

Water samples were collected during two different periods: January 2006—summer (a month characterized by high temperatures and frequent rainfall) and July 2006—winter (a month characterized by lower temperatures and low rainfall). Meteorological data of the studied area for the periods of sampling were provided by Centro de Análise e Planejamento Ambiental (CEAPLA), Instituto de Geociências e Ciências Exatas from UNESP, Campus Rio Claro (Table 1).

Samples were collected at two sites along the Ribeirão Claro stream as follows: site 1, Granja Ipê reservoir (latitude 22° 18′ 04.7″ S and longitude 47° 32′ 13.7″ W), which is surrounded by riparian forest, was used as the reference site; site 2, CECAP (state company public housing) urban emissary (latitude 22° 24′ 43.2″ S and longitude 47° 31′ 19″ W), where the river receives the discharge of the untreated urban effluent.

The water sampling procedure was performed according to the method of superficial water collections proposed by CETE SB (1987). The samples were protected from light, refrigerated at 4±2 °C for transportation to the laboratory and then immediately used in the experiments.

Herbicide

The herbicide trifluralin (CAS 1582-09-8) was used at a concentration of 0.84 mg/L, taking into consideration the amount of the active compound present in the commercial product. This herbicide is referred to as a putatively carcinogenic for humans (USEPA 1999), and according to Fernandes et al. (2009) this concentration can be considered as a positive control for genotoxicity assays with *A. cepa* meristematic cells,

Table 1 Meteorological data for the periods of collection of the water samples of Ribeirão Claro stream, Rio Claro, SP, Brazil

Sampling	Maximum temperature (°C)	Minimum temperature (°C)	Mean temperature (°C)	Rainfall (mm)	Relative humidity of the air (%)
January 2006	30.59	19.61	25.10	158.2	72.24
July 2006	27.05	10.13	18.59	22.0	66.71

The values were obtained by calculating the averages of the daily values

because it shows high effect on the induction of cell irregularities, such as chromosomal and nuclear aberrations.

Test organism

Seeds of *A. cepa* (2n=16 chromosomes) of the same stock (TopSeed—Agristar, Petrópolis, Rio de Janeiro, Brazil) and cultivar (Baia Periforme) were used as the test organism because they are genetically and physiologically homogeneous, besides being available throughout the year—features that ensure a reliable assay.

Test procedure

About 100 seeds of *A. cepa* were germinated at room temperature, in separate Petri dishes, with filter paper embedded in ultra-pure water. The treatments were performed following the protocol proposed by Matsumoto et al. (2006) with some modifications. When the roots reached about 1 cm in length, they were transferred to other dishes with filter paper soaked in 10 mL of the respective sample (water from the stream or the 0.84 mg/L herbicide concentration), and kept there for 20 h (20-h treatment). Petri dishes were covered with aluminum paper to avoid degradation of the herbicide (photosensitive). Afterwards, half of the roots were collected and fixed, while the remaining roots were transferred to new Petri dishes containing ultra-pure water for a 48-h recovery period before collection (recovery treatment) to evaluate the persistence of the possible effects induced by the tested samples. The negative control test was performed with seeds submitted to germination in ultra-pure water. After termination of each exposure experiment root tips collected were fixed with Carnoy's fixative 3:1 (ethanol: acetic acid, *v*/*v*) for 6–12 h at room temperature. Afterward, they were transferred to a freshly prepared Carnoy's fixative and stored at 4 °C until utilization.

Conventional chromosomal staining

Conventional chromosomal staining methodology was applied to investigate the genotoxic effect of the tested samples, serving as a reference for the different types of cellular alterations induced by the test samples that were determined by the AgNOR technique.

Roots were submitted to the Feulgen reaction (Mello and Vidal 1978) and the slides with the meristematic regions of *A. cepa* were prepared according to the protocol described by Matsumoto et al. (2006). In order to characterize and quantify cellular irregularities five slides per treatment were prepared. Approximately 1000 cells were analyzed per slide, totaling 5000 cells per test in each treatment.

The analyses were performed under a light microscope (Carl Zeiss Standard Binocular Microscope 95 3465) at 400 x magnification. Any chromosomal and nuclear aberrations (adherence; chromosomal bridges, laggard, breaks and losses; C-metaphase; minicells; nuclear buds; polyploidy, polynucleated and multipolar cells; irregular nuclei and micronuclei) present during interphase and in cells undergoing division were counted in order to assess the genotoxic effects induced by the tested samples.

The results were compared to the negative control applying the Mann–Whitney statistical test with a 5 % significant level, using the BioEstat 5.0 statistical program (Mamirauá Institute for Sustainable Development, Tefé, Amazonas, Brazil).

Silver-stained nucleoli and nucleolar organizer region

Fixed roots were exposed to acidic hydrolysis in HCl 1 N at 60 °C for 8 min and then washed in distilled water. Individual meristematic regions were cut on slides, covered with a drop of 45 % acetic acid solution and a coverslip and then carefully squashed. The coverslip was removed with liquid nitrogen and the slides were dried at room temperature. The slides were stained with 50 % $AgNO_3$ solution at 60 °C until they reached a brown color, in accordance with the technique of Hizume et al. (1980). The material was analyzed under a light microscope (Carl Zeiss Standard Binocular Microscope 95 3465) at 400 x magnification.

The variation of nucleoli number of each cell was obtained by counting approximately 5000 cells per treatment (1000 cells per slide), comprising a total of five slides.

In order to evaluate the induction of nucleoli proliferation per treatment, cells with different nucleoli numbers were categorized into different classes (from 1 to 14), according to the amount of nucleoli present in each nucleus, where class 1 represents cells with one nucleolus per nucleus; class 2 represents cells with two nucleoli per nucleus and so on up to class 14, which represented the greatest number of nucleoli observed per nucleus.

A count of the number of nucleoli present in 1000 cells / slide for five slides / treatment was performed. The score of each slide was obtained by multiplying the number of cells found in each class by the number of the corresponding class, as described above. The score of each treatment was obtained by the mean of the scores of each one of the five slides/ treatment.

For the comparison among the scores of the treatments and the negative control, the t Student statistic test was applied, with a 5 % significant level, using the BioEstat 5.0 statistical program.

Results and discussion

Among the effects observed by the conventional chromosomal staining, the presence of chromosomal and nuclear aberrations indicated highly significant genotoxic effects in

meristematic cells of *A. cepa* exposed to the trifluralin herbicide, both after the 20-h treatment and the 48-h recovery period, during summer and winter seasons (Table 2, Fig. 1). However, the presence of these alterations was much higher and highly significant after the recovery treatment, indicating the persistence of the effect of the herbicide. From these results, it can be inferred that the observed cellular aberrations may be related to the formation of micronuclei in subsequent divisions of the cells exposed to trifluralin herbicide. According to Fernandes et al. (2007, 2009), chromosomal aberrations derived from the aneugenic action of the herbicide, such as chromosomal losses, C-metaphases, and polyploid cells, also observed in the present study, can give rise to micronuclei.

For the tests performed with the water samples from site 2, highly significant increases in the frequency of cells with chromosomal aberrations were observed in the 20-h treatment group in both summer and winter samples and for the recovery treatment after exposure to summer samples when compared to the negative control (Table 2, Fig. 1). Significant increases in this parameter were also observed for the reference site in summer (water samples from site 1), although the values were much lower than those observed for site 2 and the herbicide.

Significant results on the increase of micronuclei were observed for assays carried out with the trifluralin herbicide for 20-h and after recovery during both seasons. This parameter was also significantly increased after exposure to water samples from site 2 for 20-h during summer.

Regarding the different results observed for the water samples collected from site 2 during summer and winter, they can be explained by the different characteristics related to seasonality. Meteorological data for the periods of sampling showed a great variation, mainly related to the monthly temperature mean and rainfall index (Table 1). According to Manzano et al. (2014), the frequent rainfall observed in summer in tropical regions can resuspend river sediments, thus increasing the availability of contaminants that were entrapped into the sediment. Moreover, rainwater can wash contaminants present in the surrounding soil into the river. Thus, these events could explain the higher values of cellular aberrations observed for samples collected during the summer period when rainfall was greater.

Also, an increase in temperature, as observed in summer, can elevate the dissolution of substances in water (Delpla et al. 2009), including some metals and herbicides (Cairns et al. 1975), and increase the cellular membrane permeability (Cravalho et al. 1992; Oldenhof et al. 2010), which facilitates the transport/diffusion of these contaminants into the cell. This fact must have occurred in the present study because the experiments were carried out at room temperature (mean temperature: summer 25.10 °C; winter 18.59 °C). The explanation about membrane permeability was also proposed by Bianchi et al. (2011) who reported elevated genotoxic effects during summer in water samples from rivers in tropical regions.

Previous studies performed with water samples from the same site of the stream where untreated urban effluent is discharged (Hoshina 2002) and the 0.84 mg/L concentration of the trifluralin herbicide (Fernandes et al. 2007; 2009) showed similar results, indicating that contaminants with genotoxic proprieties present in the water samples as well as the trifluralin herbicide induce cellular and chromosomal damages in meristematic cells of *A. cepa*. Thus, from the literature data and the results obtained with the conventional chromosome staining, it can be concluded that the water samples as well as the substance studied here are potentially genotoxic and mutagenic and are usable samples for assessment of nucleolar damages induced by xenobiotics.

The analysis of meristematic cells by the NOR-banding technique indicated, in all the assays carried out, a variation in the number of nucleoli in the *A. cepa* interphase cells (Fig. 2). For the tests performed with ultra-pure water (negative control) and for water samples from site 1 (environmental control), both in summer and winter, a variation of one to four nucleoli per nucleus was observed (Fig. 2). In the assays carried out with water samples from site 2 collected in summer, variations from 1 to 6 nucleoli were observed for the cells exposed to a 20-h treatment as well as 1 to 5 nucleoli for the cells submitted to a recovery treatment (Fig. 2). For the tests carried out with samples from the same site collected in winter, a maximum number of 5 and 7 nucleoli were observed for cells exposed to the 20-h and to the recovery treatment, respectively (Fig. 2).

Root tips from seeds germinated at room temperature in the trifluralin solution (summer) showed a variation of 1 to 12 nucleoli for the cells submitted to the 20-h treatment; and 1 to 14, for the recovery treatment. For the assays conducted in the winter, the maximum nucleolus number observed for cells submitted to the 20-h treatment was 8, whereas for the recovery treatment it was 13 (Fig. 2). According to Hawxby et al. (1972), absorption of trifluralin in plants can be directly influenced by variations in temperature. Thus, as the present experiment was not conducted under controlled temperature conditions, the difference in the results obtained during summer and winter seasons could be related to the differences in temperatures registered for these periods (mean temperature: summer 25.10 °C; winter 18.59 °C).

Table 2 shows the score of nucleolus induction in the assays performed in this study. The assays carried out with the environmental reference water samples (site 1) and the ones collected near the urban effluent (site 2) were not statistically different from the negative control, considering the total number of nucleoli. However, exposure to trifluralin induced a significant increase in nucleolus number for the tests carried out in both summer (both for 20-h treatment and for recovery treatment) and winter (recovery treatment).

Thus, although an increase in the number of nucleoli in *A. cepa* meristematic cells was observed for both tests

Table 2 Mean and standard deviation of micronuclei (MN), chromosomal and nuclear aberrations (AB), and nucleolar score (NU) evaluated in *A. cepa* meristematic cells, after exposure to water samples from a river contaminated with urban effluents and to the trifluralin herbicide analyzed in two different seasons

		Trifluralin herbicide		Site 2		Site 1		Negative control	
		20-h treatment	Recovery treatment	20-h treatment	Recovery treatment	20-h treatment	Recovery treatment	20-h treatment	Recovery treatment
Summer 2006	MN	6.60±6.58*	43.80±37.09**	4.20±2.77*	3.60±2.97	1.80±1.10	1.40±1.67	0.80±0.45	1.40±1.14
	AB	38.20±27.51**	355.40±315.02**	25.60±15.21**	18.00±6.67**	7.80±2.59*	3.60±3.13	2.00±2.12	2.80±1.64
	NU	2,431.38±428.66*	3,563.7±1,015.95*	1,862.90±238.16	1,952.52±310.56	1,819.04±367.68	2,041.54±383.54	1,802.24±181.93	1,879.22±170.00
Winter 2006	MN	12.40±11.24*	28.40±15.10**	2.00±1.41	1.00±0.71	1.80±1.30	0.80±1.79	1.00±1.00	1.20±1.30
	AB	29.40±21.48**	110.40±54.07**	11.40±3.05**	5.00±3.00	2.00±1.58	2.00±2.34	2.60±2.30	1.40±1.67
	NU	1,749.26±389.43	2,187.84±272.04*	1,617.46±194.15	2,096.81±500.34	1,672.52±88.49	1,829.14±164.05	1,592.44±99.38	1,744.67±153.00

*Statistically different from negative control ($p<0.05$); ** Statistically different from negative control ($p<0.01$)

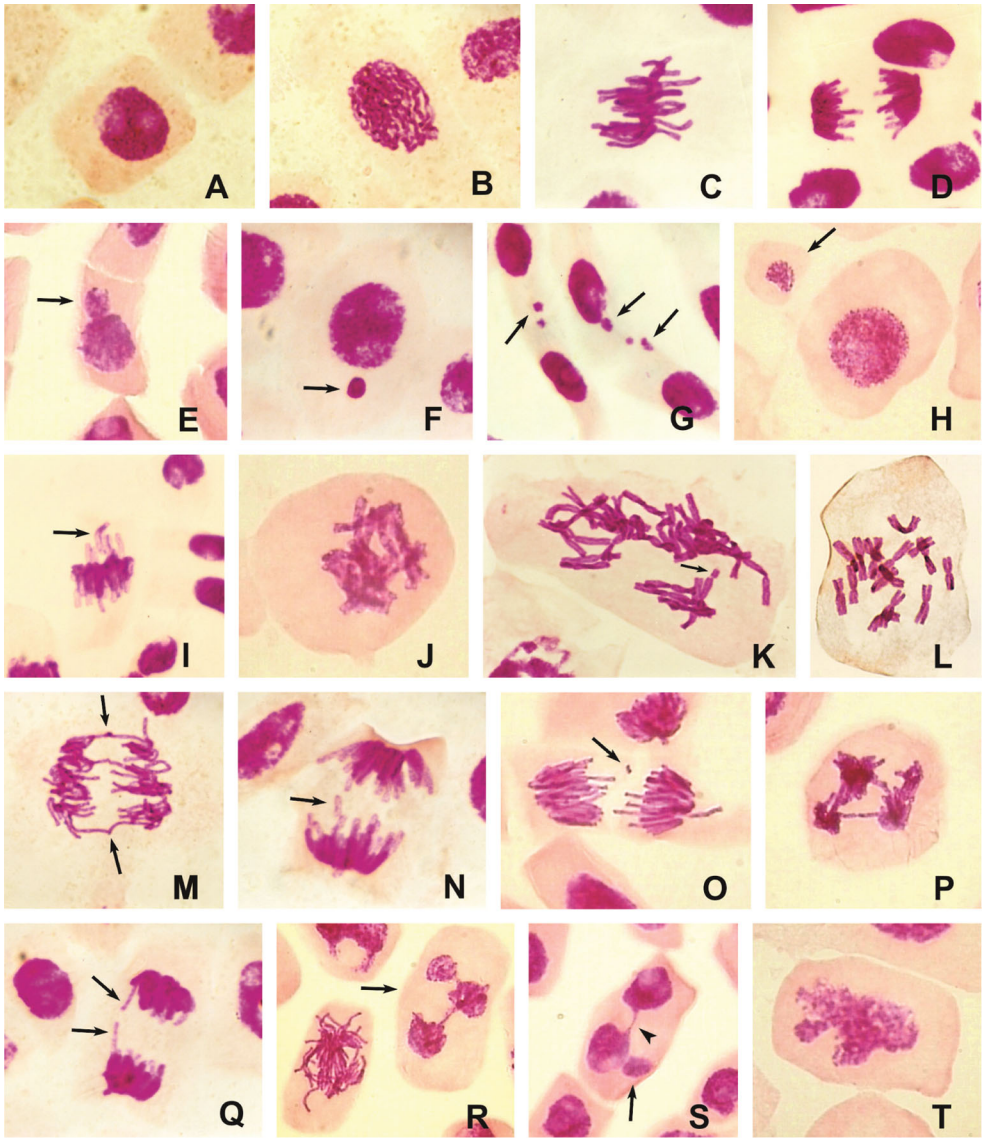

Fig. 1 Meristematic cells of *A. cepa* germinated in: ultra-pure water and water samples from site 1 (**a**–**d**); water samples from a stream contaminated with urban effluents (site 2) and trifluralin herbicide (**e**–**t**) submitted to the conventional chromosomal staining. **a** Normal interphase; **b** Normal prophase; **c** Normal metaphase; **d** Normal anaphase; **e** Cell bearing nuclear bud (*arrow*); **f** and **g** Cells with micronuclei (*arrows*); **h** Minicell (*arrow*); **i** Metaphase with chromosomal loss (*arrow*); **j** Metaphase with adherence; **k** Polyploid metaphase with chromosomal break (*arrow*); **l** C-metaphase; **m** Anaphase with chromosomal bridges (*arrows*); **n** Anaphase with chromosomal break (*arrow*); **o** Polyploid anaphase with chromosomal break of the telomeric region (*arrow*); **p** Multipolar anaphase with chromosomal bridges; **q** Telophase with chromosomal laggards (*arrows*); **r** Multipolar telophase with chromosomal bridges (*arrow*); **s** Telophase with chromosomal bridge (*arrowhead*) and micronucleus (*arrow*); **t** Cell with irregular nucleus. The alterations represented by figures K, P, R and S were observed only in assays performed with trifluralin herbicide (**a**–**d**, **f**, **h**, **j**–**q**, and **t**: original magnification 1,000×; **e**, **g**, **i**, **r**, and **s**: original magnification 400×)

performed (water receiving urban wastewater and trifluralin), the effect caused by the herbicide was more pronounced and significant due to a potentiating effect of this chemical, even when conditions returned to normal (after recovery period). These data corroborate the results reported by Fernandes et al. (2007; 2009), who observed that the effects of this substance were maintained even after 48 h of the exposure period. According to the authors, the observed effects are due to the persistence of this herbicide in the cell or to the injury caused to the cellular repair system. Mallory and Bayer (1972) also described a persistent effect of the trifluralin herbicide on the development of cotton roots after a treatment period of 96 h.

The significant increase in nucleolus quantity observed for the cells exposed to trifluralin may be related to the effect of the herbicide itself promoting the polyploidization of the genetic material due to its depolymerizing action on the microtubules (Anthony et al. 1998; Anthony and Hussey 1999; Fernandes et al. 2007; 2009).

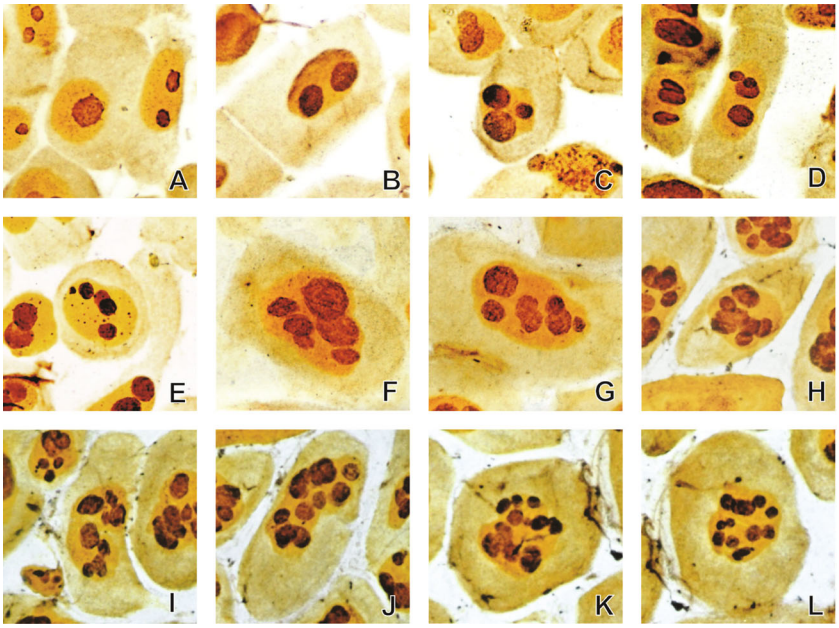

Fig. 2 Variation of the nucleoli number in meristematic cells of *A. cepa*. **a** Cell containing one nucleolus; **b** Cell containing two nucleoli; **c** Cell containing three nucleoli; **d** Cell containing four nucleoli; **e** Cell containing five nucleoli; **f** Cell containing six nucleoli; **g** Cell containing seven nucleoli; **h** Cell containing eight nucleoli; **i** Cell containing nine nucleoli; **j** Cell containing ten nucleoli; **k** Cell containing eleven nucleoli; **l** Cell containing fourteen nucleoli. **a**, **b**, **c**, and **d** represent cells found in all assays. **e**, **f**, and **g** represent cells found in assays performed with water samples from site 2 and trifluralin. **h**–**l** represent cells found in the assays performed with trifluralin only (Fig. **a**–**c**, **e**–**g**: original magnification 1,000×; **d**, **h**–**k**, and **l**: original magnification 400×)

Although the result found for trifluralin in the winter for the 20-h treatment assay was not significant in relation to the controls, it can be explained by the presence of a larger quantity of cells containing one nucleolus per nucleus, which presented an enlarged size (Fig. 3e). That would probably correspond to the sum of nucleoli present in the polynucleated cells caused by amphiplasty which, according to Cermeño et al. (1984), is a characteristic commonly observed in nucleoli. As shown in Fig. 3e, the large and single nucleoli have similar dimensions to those of the cell nucleus itself (an evidence of a polyploidization process), which are different from a nucleolus of normal size observed in the control cells (Fig. 3a).

In this study, the application of the NOR-banding technique in *A. cepa* meristematic cells exposed to a genotoxic herbicide and a complex environmental sample allowed identifying alterations in the number of nucleoli per nucleus,

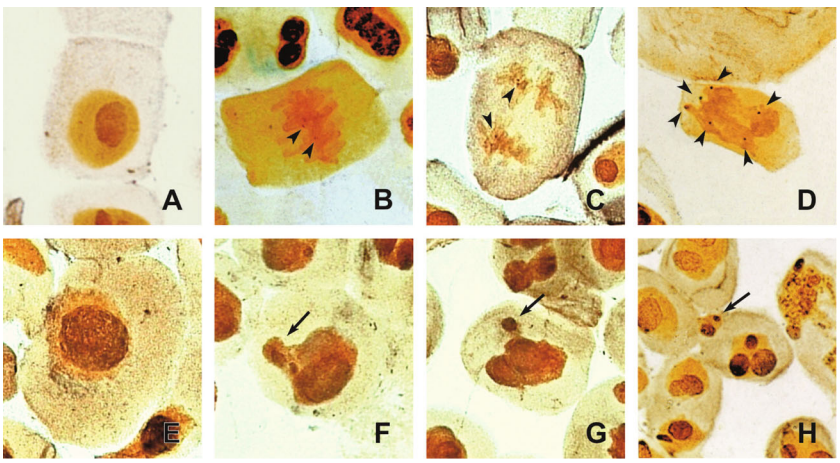

Fig. 3 Meristematic cells of *A. cepa* resulting from germination in negative and environmental controls (**a**–**c**) and in trifluralin (**d**–**h**) submitted to the AgNOR banding staining. **a**. Normal prophase with normal size nucleolus; **b** Normal metaphase with marked satellite regions; **c** Normal anaphase with marked satellite regions; **d** Polyploid anaphase (characterized by the presence of 6 AgNOR); **e** Polyploid interphase containing one nucleolus with enlarged size; **f** and **g** Elimination of polyploidized material containing nucleolus by the nucleus (*arrow*); **h** Minicell with nucleolus resulting from the expulsion of excessive genetic material (*arrow*). The *arrowheads* indicate the NORs (**a**–**c**, **e**–**g**: original magnification 1,000×; **d** and **h**: original magnification 400×)

as well as in their size. According to Leek et al. (1991) and Arkhipchuk and Palamarchuk (1996), the number of nucleoli present in each cell corresponds to the active centers of the rRNA synthesis in the interphase nucleus. Çavas and Ergene-Gözükara (2003b) reported that the number and size of nucleoli are directly related to cell proliferation and differentiation. Therefore, alterations in the nucleolar number and size would be reflecting damages in the activation/inactivation of ribosomal RNA synthesis. A significant increase in the number and size of nucleoli in *A. cepa* cells exposed to acetylsalicylic acid solutions and metamizole sodium for short periods (30 and 90 min) was reported by Arkhipchuk et al. (2004). According to these authors, alterations in the number and size of nucleoli affect the functional activities of the nucleolus, such as ribosomal gene transcription.

The increase in the nucleolus size and number observed in assays performed with trifluralin may be related to an increase in rRNA synthesis, which consequently led to an amplification of the ribosomal genes in these polyploid cells. Therefore, by the results presented here it can be inferred that the trifluralin herbicide, at the concentration tested, also affects functional activities of the nucleolus, even after the recovery treatment.

When conducting studies with erythrocytes of fish submitted to a pyrethroid pesticide, Çavas and Ergene-Gözükara (2003a) stated that the variation of the nucleoli number is a valuable parameter to be used in genotoxicity studies of chemical substances. These authors (Çavas and Ergene-Gözükara 2003b) reported that nucleolus number and size alterations in interphase cells are indicative parameters of functional genome changes, characterizing a sensitive genotoxicity test. According to Karpova et al. (2006), nucleolus size increase, resulting from exposure to chemical agents, is related to alterations in nucleolar metabolism. These alterations produce a ribosomal gene amplification and/or a high transcriptional activity, generating an accumulation of transcription products in the nucleolus.

In addition to the visibly enlarged size of the nucleolus and the increase in the number of nucleolus per cell, the use of the AgNOR technique with cells undergoing division also enables the identification of polyploidized cells because any alteration in the NOR quantity can indicate a possible polyploidization since *A. cepa* has a fixed number of chromosomes with NOR (Panzera et al. 1996; Kim et al. 2002; Leme and Marin-Morales 2009) (Fig. 3b and c). Thus, polyploid cells are easily identified by the increase in the amount of chromosomes containing NORs (Fig. 3d).

Cells that had their nucleolar material amplified due to the action of toxic agents (e.g., trifluralin) can present nuclear buds—structures that may be related to an expulsion response because of the excess of genetic material. According to Leach and Jackson-Cook (2004), studies carried out with human lymphocytes suggested that DNA excess in a cell might result in the occurrence of a nuclear bud, which may be expelled from the nucleus generating a micronucleus, which is finally expelled from the cell as minicells. In the present study it was observed that cells exposed to the trifluralin herbicide expelled the excess of genetic material as a minicell, which in most cases was composed of nucleolus (Fig. 3f–h).

The same event was also reported by Fernandes et al. (2007) for *A. cepa* meristematic cells submitted to different trifluralin concentrations. Therefore, it reinforces the indication of micronucleus induction by amplification of genetic material of the cell. According to these authors, the elimination of excessive material is related to maintenance of the cell's physiology since any elimination activity of a given cell structure suggests that it has shown a physiological response to the aggressive agent. Fiskejö (1983) observed the elimination of nucleolar material from the nucleus into the cytoplasm of *A. cepa* meristematic cells exposed to $AlCl_3$ aqueous solutions. The same phenomenon was also described by Liu et al. (1994), when *A. cepa* meristematic cells were exposed to nickel sulfate. However, in addition to the expulsion of the nucleolus from the nucleus, these authors noticed a disintegration of the nucleolar material in the cytoplasm, thus evidencing an even more serious effect of the chemical agent studied.

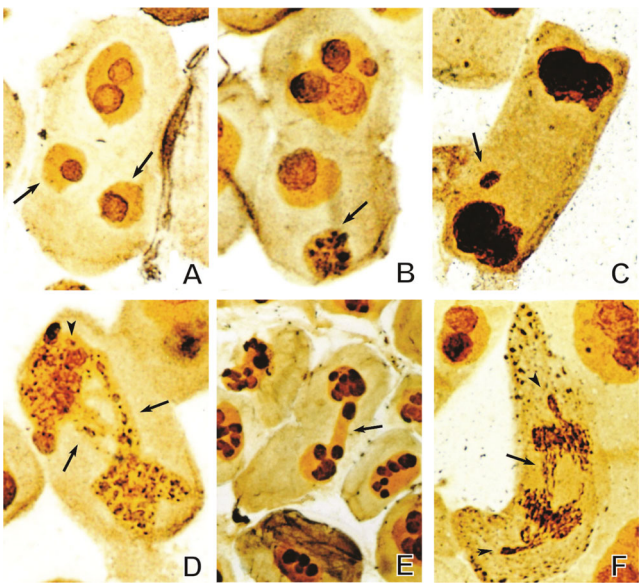

Fig. 4 AgNOR banding in meristematic cells of *A. cepa*, resulting from germination in water samples from a stream contaminated with urban effluents (site 2) and in the trifluralin herbicide. **a** Interphase bearing two micronuclei, both containing nucleoli (*arrows*); **b** Interphase containing micronuclei with acid proteins (*arrow*); **c** Final telophase showing loss of nuclear material with acid proteins (*arrow*); **d** Telophase with chromosomal bridges (*arrows*). Note in **d** the presence of nucleoli in only one of the cellular poles (*arrowhead*); **e** Binucleated cell with nuclear bridge (*arrow*). Note an irregular distribution of nucleoli in the nucleus; **f** Anaphase with chromosomal bridges (*arrow*) with irregular disposition of acid proteins between the poles. The *arrowheads* indicate the NORs (**a**–**d**, and **f**: original magnification 1,000×; **e**: original magnification 400×)

The elimination of the nucleolar material can also be due to the fact that the integrity of the nucleolus is related to the presence of calcium in the cell (Wang 1988). According to Hertel et al. (1981), Sree et al. (1988), Hansen et al. (1998), and Vidaković-Cifrek et al. (2002) the trifluralin herbicide interferes with and deregulates the intracellular concentration of calcium. These alterations in cell calcium level were hypothesized to contribute to the expulsion of the nucleolus from the nucleus as was also suggested by Liu and Jiang (1991) and Liu et al. (1994) that studied the effects of metals on the nucleoli of *A. cepa* root tip cells.

The necessity to know the composition of the MN in order to understand the mechanisms of their formation and the fate of these MN was highlighted by Stopper and Müller (1997) and Leach and Jackson-Cook (2004). Although MN can be formed by the elimination of amplified genetic material (Fernandes et al. 2007; Fenech et al. 2011), they can also result from chromosomal breaks (clastogenic action) or losses (aneugenic action) that were not incorporated to the main nucleus during the cell division cycle (Sudhakar et al. 2001). As observed with conventional chromosomal staining, both the water samples from site 2 and the trifluralin herbicide (0.84 mg/L concentration) were able to induce a significant increase in frequency of MN in *A. cepa* cells. Among the micronucleated cells observed in this study by applying the AgNOR technique, it was possible to identify the content of each micronucleus in order to characterize the presence of nucleolus. In this study, micronuclei with nucleolus (Fig. 4a), micronuclei without nucleoli and micronuclei containing a great amount of acid proteins (Fig. 4b, c) were observed. The variation noted in the content of MN indicated that the studied contaminants do not exert their effect in a specific region of the DNA. However, the presence of MN with nucleoli indicates that the affected chromosome was the one bearing rDNA, and effects on which can disrupt transcription and translation processes (Ventura-Camargo et al. 2011).

By using the AgNOR method it was also observed that the trifluralin herbicide concentration tested caused cellular alterations not observed in the negative control, such as chromosomal bridges in the anaphase and errors in the segregation of sister chromatids, which resulted in irregular division of nucleoli between daughter cells. Figure 4d shows a cell in telophase where only one of the nuclei contains nucleoli. Thus, the unviability of the cell, which was forming without any nucleolus, is evident. If this alteration is frequently observed in cells, it can lead to a serious impairment of an organism. Furthermore, cells containing aberrant nuclei such as bi- or multinucleated cells that had their nuclei connected by a nucleoplasmic bridge were observed after exposure to trifluralin (Fig. 4e). These alterations resulted from chromosomal bridges that were established in the first cell division phases, which were not terminated at the end of the cycle. Moreover, the daughter cells presented different numbers of nucleoli inside the nuclei, proving the impact of the trifluralin herbicide on the nucleolar domain of the cell.

Some proteins present in the nucleoli were described as important nucleolar components of the cells and are present in all the stages of the cellular cycle (Angelier et al. 1982). These proteins, more appropriately called acidic nucleolar proteins, are associated with ribosomal genes (Sirri et al. 1995). The migration of acidic proteins during the cellular cycle was easily observed using the AgNOR staining method. The acidic proteins were found both attached to chromosomes and free in the cytoplasm. The free proteins can migrate together with the chromosomal migration or not (previous, joint or later). In tests performed with the water samples from site 2 and the trifluralin herbicide, a differential quantitative migration of free acidic proteins was observed between the poles of cells in metaphase and anaphase (Fig. 4f), while in the controls these proteins revealed a parallel migration. From this unequal distribution it can be inferred that the substances present in urban sewage and the trifluralin herbicide interfered with the normal distribution of these proteins, characterizing them as agents with cytotoxic action on *A. cepa* cells. Ventura-Camargo et al. (2011) also observed a variable location of the acid nucleolar proteins in *A. cepa* cells exposed to different concentrations of azo dyes, indicating that some chemicals can interfere in the migration pattern of these proteins.

Conclusion

Based on the results presented here, it can be concluded that the chromosome banding method by AgNOR (NOR banding) represents a useful approach in support of genotoxicity studies by evaluating the nucleolar alterations induced by xenobiotics. This methodology proved to be a valuable tool to characterize the constitution of micronuclei including the presence or absence of nucleoli, as well as alterations in the distribution of nucleoli and acidic nucleolar proteins during cell division cycle. This technique was also able to detect increases in the quantity of chromosomes with positive AgNOR marking, providing an additional parameter in studies that assess alterations resulting from xenobiotic-induced polyploidization. Thus, it constitutes an efficient method for environmental genotoxicity assays, completing information obtained by other methods used for the same purpose. Additionally, trifluralin concentration of 0.84 mg/L seems to be appropriate to be used as a positive control in assays that evaluate the variation of the nucleoli number in *A. cepa* meristematic cells.

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