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The use of the Cytogenetic to Identify Mechanisms of Action of an Azo Dye in Allium Cepa Meristematic Cells

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Abstract

In the present study, cytotoxic, genotoxic and mutagenic actions of different concentrations (1, 10, 100 and 1000 µg/L) of an azo dye (BDCP – Black Dye Commercial Product) were evaluated using different cytogenetic techniques [(conventional dye, C banding, NOR banding, base-specific fluorochrome banding and fluorescent *in situ* hybridization (FISH)] applied to the *Allium cepa* test-organism. The use of conventional cytogenetic staining allowed us to determine that the azo dye induced cell death, chromosomal aberrations, nuclear alteration and micronuclei. By means of chromosome bandings and the FISH technique, it was possible to notice both cell and nucleolar alterations induced by BDCP, whose effects, in a general way, were not specific for any chromosome sites. The abnormalities observed made it possible to infer both aneugenic and clastogenic actions caused by the dye analyzed. The cytotoxic, genotoxic and mutagenic effects of BDCP were not completely eliminated, even after the recovery treatment of the *A. cepa* roots in water, showing a cumulative potential effect of the referred dye. Due to these results, we could infer that the BDCP may be a dangerous contaminant to the environment and, consequently, to the lives of the organisms exposed to it.

Keywords: BDCP (Black Dye Commercial Product); chromosome aberrations; chromosome bandings; FISH (Fluorescent *in situ* hybridization); genotoxicity

Introduction

Azo dyes – compounds characterized by the presence of one or more azo groups (-N=N-) – constitute the most important class of dyes in the textile industry [1, 2], because they are one of the most easily synthesized dyes, showing both excellent fixation properties and permanence in fibers. Moreover, they enable a wide variety of colors to be obtained in contrast to natural dyes [3]. However, during the textile process, staining inefficiency results in a large quantity of residues, all of which are directly discharged into bodies of water, consequently contaminating the environment [4-5], creating serious environmental impacts [6].

According to several authors, tests with microorganisms and mammal cells indicate that azo dyes are compounds of toxic [7-10], genotoxic and mutagenic activities [11-18].

Studies using the *Salmonella* test [15, 19] indicated the presence of textile azo dye components (BDCP – Black Dye Commercial Product) in both raw and treated industrial effluents, showing a mutagenic activity for that compound. By means of crypt foci, some researchers [20] even showed that there was an increase in pre-neoplasic lesions in the colon of rats exposed to different concentrations of those effluents containing BDCP. Studies of chromosome aberrations using the *A. cepa* test-organism showed mutagenicity for several tested concentrations of an industrial effluent contaminated with the BDCP azo dye [16]. Studies demonstrated that azo dyes are cytotoxic to hamster cells, because they induced the formation of micronucleated cells, and multilobulated and extremely condensed nuclei, besides inducing endoreplication and binucleated cells [21]. According to some scientists, *p*-dimethylaminobenzene azo dye (*p*-DAB) induced cytotoxic and genotoxic effects on bone marrow cells and rat sperms [22].

All the cytotoxic effects observed for azo dyes might be due to the direct action of dyes on the cells or, especially, to the formation of metabolites resulting from the azo bond reduction [1, 23]. Metabolites can react with the DNA molecule, damaging both its structure and function [15, 18, 24]. Because of the significant increase in chemical compounds being discharged into the environment, bioassays have been carried out using different organisms in order to identify and evaluate the harmful effects of various agents at their different concentrations and exposure periods [25].

Higher plants constitute an important material for testing genetic alterations brought about by environmental pollutants. They are also currently recognized as excellent bioindicators of cytotoxic, genotoxic and mutagenic effects of environments contaminated by toxic substances [26, 27]. The use of plants as test-organisms has been recommended by several environmental agencies such as the UNEP (United Nations Environmental Program), the WHO (World Health Organization), and the US EPA (US Environmental Protection Agency) [28]. The *Allium cepa* species has been used as an efficient standard organism to run genetic tests for cytoxicity, especially cytogenetic and chromosome aberration tests [16, 26, 29-35].

The emergence of new techniques for chromosome staining has lead to an increase in information about the DNA composition and disposition of different constitutions along the chromosomes, allowing for an in-depth analysis of the chromosome structural organization in contrast to the conventional staining techniques [36-38]. C banding, AgNOR banding and base-specific fluorochrome banding are techniques that stand out from the existing cytogenetic ones. All of these techniques may be used as efficient and auxiliary tools to detect the mechanisms of action of chemicals in different organisms.

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As described above, azo dyes and their metabolites are known for potentially causing serious DNA damage. There is a great need, however, to better evaluate the modes of action of these dyes on biological materials. The BDCP, widely used in textile processing industries, was tested in this study using chromosome aberration and micronuclei tests in *A. cepa*. In the study, different cytogenetic techniques were used [(conventional staining: chromosome and nuclear aberrations test; chromosome bandings: C banding, NOR banding, base-specific fluorochrome banding CMA_3/DAPI; fluorescent *in situ* hybridization (FISH)] in the *A. cepa* meristematic cells. The main purpose of this study was to analyze the cytotoxic, genotoxic and mutagenic potentials of BDCP, as well as the possible associations of this dye with the genetic material, and hence prove its action on the cells of exposed organisms.

Material and Methods

Chemical tested

The commercial azo dye evaluated was the BDCP, a product which is composed of three dyes belonging to the nitroaminoazobenzene group: C.I. Disperse Blue 373 ($C_{21}H_{21}BrN_6O_6$; CAS nº 51868-46-3); C.I. Disperse Violet 93 ($C_{18}H_{19}BrN_6O_5$; CAS nº 268221-71-2); and C.I. Disperse Orange 37 ($C_{17}H_{15}Cl_2N_5O_2$; CAS nº 13301-61-6) [15].

Treatment solutions

The BDCP concentrations used in the assays were: 1000 µg/L, 100 µg/L, 10 µg/L and 1 µg/L, and the highest concentration – determined with the help of pilot-tests – consisted of indicated solution for the viable application and development of cytogenetic techniques used. The remaining concentrations were obtained from successive dilutions of the highest dye concentration in ultrapure water. Summarizing the pilot-tests, 100, 10 and 1 mg/L concentrations were tested, and the *A. cepa* seeds germination rates and the roots development were analyzed in order to obtain a concentration at which the germination rate was above 60% and the roots were not so fragile for handling and for subsequent cytogenetical application. All concentrations used in the assays are close to those observed in environmental samples (river impacted by BDCP, raw and treated effluent samples) obtained from the aquatic environment after dying process, studied by some researchers [39].

Test organism

The *Allium cepa* species was used to evaluate the mechanisms of action of the chemical tested. *A. cepa* seeds (2n=16) of the Baia Periforme variety were used herein as a test-organism, since they are both genetic and physiologically homogeneous, besides being available throughout the year [16].

Assays

The *A. cepa* seeds (one hundred per Petri dish) were previously germinated in ultrapure water at room temperature. When the seeds reached about 1.5 cm in length, they were transferred to Petri dishes containing the different concentrations of the commercial dye (1000 µg/L, 100 µg/L, 10 µg/L and 1 µg/L), using one dish per concentration. The seeds were held in those dishes for a 20-h period. The negative control (NC) was prepared by exposing the seeds to ultrapure water only, whereas the positive control (PC) was exposed to the 9×10^{-5} M concentration of methylmetasulphonate (MMS, Sigma-Aldrich, CAS 66-27-3). As time went by, 1/3 roots were collected from each dish and the remaining roots were transferred to dishes containing water only, for the 48 and 72-h periods (recovery treatments). After those periods, the remaining roots were collected.

The roots collected were fixed in Carnoy's solution (Ethanol-Acetic Acid 3:1 - v/v) for 18 hours at room temperature [34, 40]. Preparation of slides was done using the conventional staining technique (chromosome and nuclear aberrations assay), chromosome bandings [(C banding, NOR banding, base-specific fluorochrome banding CMA_3/DAPI) and Fluorescent *in situ* hybridization (FISH)].

Conventional staining assay: For the conventional staining assay, the fixed roots were stained by means of the Feulgen reaction, as described by Feulgen and Rossenbeck in one study [41]. However, some changes were made. After staining with the Schiff reagent, root meristems were covered with a coverslip and slightly smashed in one drop of 2% acetic carmine. Coverslips were carefully removed in liquid nitrogen, and the slides were mounted in synthetic resin (Mounting Media, Permount®, Fisher Scientific). The slides analyses were carried out using light microscopy.

In the analyses, the following chromosome and nuclear aberrations (CNA) were considered: genetic material loss, chromosome adherence, C-metaphase, chromosome bridge, multipolarity, and polyploidy. To better interpret the results, CNA were classified into a single category, regarded as genotoxicity endpoint [16, 33, 35]. The presence of micronuclei and chromosome breaks (MN/B) observed in the *A. cepa* meristematic cells were regarded as mutagenicity endpoint [34]. Cells under death process, both apoptosis and necrosis (AP/NE), were analyzed separately and considered as cytotoxicity endpoint [34]. Mitotic Index (MI) – the ratio of number of cell division over total cells analyzed – was another category analyzed. MI was also an indicative of cytotoxicity [16, 34, 35].

All the experiments were conducted in duplicate. About 10,000 cells were counted per tested concentration (5,000 for each assay) and in the three treatments (20 hours, and recovery periods of 48 and 72 hours). 500 cells per slide were counted, comprising a total of 20 slides. The same number of cells was analyzed in the negative and positive control tests. After obtaining the present results, a statistical analysis using the Kruskal-Wallis test was carried out, accepting the 0.05 probability in order to indicate a significant effect.

Chromosome bandings and FISH (Fluorescent in situ hybridization)

In order to prepare the C, NOR, CMA_3/DAPI bandings and FISH slides, root meristems were previously treated with a cellulase/pectinase enzyme solution (2:20 – v/v) for 60 minutes, followed by a two-minute wash in distilled water. The meristems were slightly smashed in a drop of an acetic acid solution at 45%. The slides were kept aside to age for 7, 12 and 28 days, in the case of the C and CMA_3/DAPI bandings, and the FISH technique, respectively. The NOR banding experiments were carried out without previous aging of the slides.

a) **C Banding:** C banding was developed following the instructions on the technique protocol [42]. Slide staining was done with the Giemsa solution at 4% for 20 minutes. In order to be analyzed by light microscopy, the slides were mounted in synthetic resin (Mounting Media, Permount®, Fisher Scientific). The same types of cellular alterations considered in the conventional staining cytogenetic assays were qualitatively analyzed on the C banding slides. All the experiments were carried out in duplicate, and a total of ten slides both per concentration tested and per treatment were prepared (20 hours, and 48 and 72-hours recovery periods).

b) **NOR Banding:**The AgNOR banding experiments were conducted according to the protocol [43]. The slides were mounted in synthetic

resin (Mounting Media, Permount®, Fisher Scientific), so that they could be analyzed by light microscopy. They were also evaluated as for the presence of possible alterations in the cells and interphase nuclei, as well as the quantification of the nucleolar number variation in the cells, comparing the different concentrations and treatments. All the experiments were carried out in duplicate and a total of 5,000 cells were counted per concentration tested and per treatment (20 hours, and 48 and 72-hours recovery periods), being 500 cells per slide, comprising a total of ten slides. Statistical analysis was performed using the Kruskal-Wallis test, with a 0.05 significance level.

c) **CMA_3/DAPI Banding:** The band experiments conducted by means of base-specific fluorochromes (CMA_3/DAPI) followed the method proposed in 1982 [44]. The slides were observed and analyzed by fluorescence photomicroscopy (450-490 nm filters for CMA_3, and 320-380 nm for DAPI), at least 15 days following their preparation. All the assays performed with fluorescent staining were developed in a dark room. The slides resulting from CMA_3/DAPI banding were qualitatively analyzed, considering possible cell alterations such as those evidenced on the occasion that the conventional staining cytogenetic technique was used. All the experiments were carried out in duplicate, and a total of ten slides both per concentration tested and per treatment were prepared (20 hours, and a 48 and 72-hours recovery treatment).

d) **FISH (Fluorescent in situ hybridization):** The FISH technique were performed by means of the protocol described in 1986 [45] with modifications [46]. A 45S rDNA probe prepared from the *Passiflora* genome was used. The probe detection was carried out with a solution of 0.07% avidin-FITC (in buffer solution C) for one hour in a stove at 37°C. Preparations were mounted in 20 µL of antifading (Vectashield antifade vector), and chromosome counterstaining was performed with 50 g/mL propidium iodide. The slides were observed and quantitatively analyzed by fluorescence photomicroscopy (filter 450-490 nm), so that the best images with cell alterations were photodocumented.

Results and Discussion

Conventional staining assay

The cytotoxicity rate of a chemical compound can be determined by either the increase or the decrease in its MI, being the case that these values may be used in environmental toxicology studies [33, 47]. The MI results of the *A. cepa* meristematic cells exposed to four BDCP concentrations, to ultrapure water (NC) and to MMS (PC) did not show any significant differences among one another (Table 1). Nonsignificant results of MI alterations were also recorded [16] when the authors studied dilutions of water from a river that received wastewater contaminated with the same BDCP. Therefore, considering the nonsignificant results for this parameter, recorded both in this study and another [16], we believe that the MI endpoint may not be a good cytotoxicity indicator for this kind of chemical compound.

The sum total of alterations (CNA, MN/B and AP/NE) observed for the *A. cepa* meristematic cells exposed to the BDCP azo dye, for both the 20-hour treatments and the 48 and 72-hours recovery periods, indicated that only three major concentrations of the dye in the 20-hour treatment exhibited significant values in relation to the NC test results (Table 2).

In the current study, the CNA were considered as genotoxicity endpoints, since they reflect in damage to the genetic material of the cells that were not necessarily fixed in the organism, because they are liable for repair or they can lead to complete cell death. This way, such alterations do not make the effect heritable to descendant cells; therefore, not indicating a mutation. CNA were observed at all stages of the cell cycle: interphase (polyploid cells), prophase (genetic material loss) and anaphase (chromosome adherences, chromosome losses, C-metaphases and polyploidies), and anaphase and telophase (multipolarities, bridges and chromosome losses) (Figure1). CNA frequencies were always higher than the ones found in the NC tests for all the treatments performed with the dye, being most of them statistically significant, except for the 100 µg/L concentration (48-hour recovery treatment) and the 1 µg/L concentration (72-hour recovery treatment). The decrease in CNA frequencies for the 1 µg/L concentration was significant only after the 72-hour recovery treatment. It was also observed that only the 1,000 µg/L concentration showed a significant decrease in CNA frequencies after both the 48 and 72-hours recovery treatments (Table 2).

From the high and significant CNA frequencies recorded for the *A. cepa* meristematic root tips exposed to the different azo dye concentrations (Table 2), we may infer that this dye presents a genotoxic action. This corroborates the data presented by some authors [16], describing the presence of chromosome aberrations in the *A. cepa* meristematic cells exposed to water samples from rivers that received the azo dye (BDCP). It is essential to consider that the significant decrease in CNA frequencies for the higher azo dye concentration tested (1,000 µg/L) – after the 48 and 72-hour recovery treatment periods – may have resulted from a possible recovery of the *A. cepa* cells, following normalization of environmental conditions, indicating a non-cumulative genotoxic effect of the referred dye upon the *A. cepa* cells.

Statistical analysis of the CNA kinds were also performed separately, once each kind of aberration might have been induced by specific mechanisms, making it possible to better evaluate the modes of action of the BDCP components on the cells.

Chromosome losses that result from failure of the mitotic spindle formation potentially produce aneuploid cells. In the present study, the significant frequency of genetic material losses, recorded at the

Treatments	NC	PC	Black Dye Commercial Product Concentrations (µg/L)			
			1	10	100	1000
20 h	24.450 (0.009)	20.130 (0.009)	19.670 (0.010)	20.550 (0.009)	18.400 (0.011)	19.760 (0.010)
Recovery-48h	25.850 (0.014)	19.650 (0.009)	20.170 (0.012)	21.750 (0.015)	21.290 (0.011)	20.950 (0.009)
Recovery-72h	17.210 (0.013)	13.570 (0.013)	15.690 (0.015)	16.470 (0.015)	17.950 (0.010)	15.310 (0.015)

Note. NC. Negative Control; **PC.** Positive Control.
10,000 cells analyzed by concentration and treatment. Average (Standard Deviation)
The results didn't present significant differences ($p < 0,05$), according to the Kruskal-Wallis test.

Table 1: Mitotic Indexes (MI) observed in *A. cepa* meristematic cells exposed to the different concentrations of the Black Dye Commercial Product, Negative Control Test (ultrapure water) and Positive Control Test (MMS), before and after recovery treatments.

Treatment time		NC	PC	Black Dye Commercial Product Concentrations (µg/L)			
				1	10	100	1000
20 h treatment							
CNA	GML	0.040 (0.011)	1.090 (0.013)[a]	0.880 (0.023)	0.860 (0.021)	0.980 (0.019)	1.430 (0.021)[a]
	CL	0.040 (0.011)	0.500 (0.021)	0.480 (0.017)	0.630 (0.018)[a]	0.910 (0.026)[a]	0.940 (0.013)[a]
	CA	0.050 (0.012)	0.660 (0.024)[a]	0.640 (0.019)[a]	0.760 (0.010)[a]	0.580 (0.014)[a]	0.550 (0.014)[a]
	CM	0.100 (0.016)	0.090 (0.016)	0.130 (0.015)	0.180 (0.016)	0.290 (0.018)	0.350 (0.016)
	PM	0	0.010 (0.000)	0.030 (0.001)	0.050 (0.001)	0.040 (0.001)	0.120 (0.002)
	PI	0	0.040 (0.001)	0.030 (0.001)	0.060 (0.002)	0.060 (0.002)	0.130 (0.003)
	CB	0.020 (0.008)	0.170 (0.019)	0.240 (0.014)	0.380 (0.016)[a]	0.310 (0.021)	0.160 (0.012)
	MU	0	0.070 (0.002)	0.050 (0.001)	0.230 (0.010)	0.240 (0.010)	0.250 (0.011)
	CNA	*0.250 (0.017)*	*2.630 (0.012)[a]*	*2.480 (0.015)[a]*	*3.150 (0.026)[a]*	*3.410 (0.020)[a]*	*3.930 (0.021)[a]*
MNB	MN	0.290 (0.013)	2.010 (0.014)[a]	1.570 (0.017)[a]	1.390 (0.013)[a]	1.230 (0.017)	1.610 (0.014)[a]
	CBr	0.050 (0.014)	0.690 (0.025)[a]	0.310 (0.016)	0.190 (0.014)	0.560 (0.020)[a]	0.390 (0.019)[a]
	MNB	*0.340 (0.013)*	*2.700 (0.014)[a]*	*1.880 (0.017)[a]*	*1.580 (0.013)[a]*	*1.790 (0.017)[a]*	*2.000 (0.014)[a]*
AP NE	AP	0	0	0.010 (0.004)	0	0	0.190 (0.012)[a]
	NE	0	1.370 (0.023)[a]	1.030 (0.020)[a]	1.150 (0.021)[a]	2.730 (0.035)[a]	1.850 (0.024)[a]
	APNE	*0*	*1.370 (0.023)[a]*	*1.040 (0.022)[a]*	*1.150 (0.021)[a]*	*2.730 (0.035)[a]*	*2.040 (0.033)[a]*
TOTAL		**0.590 (0.011)**	**6.700 (0.011)[a]**	**5.400 (0.018)**	**5.880 (0.012)[a]**	**7.930 (0.014)[a]**	**7.970 (0.021)[a]**
48 h treatment							
CNA	GML	0.190 (0.021)	1.200 (0.011)	0.390 (0.014)	0.380 (0.017)	0.320 (0.016)	0.320 (0.012)
	CL	0.090 (0.017)	1.030 (0.013)[a]	1.000 (0.016)[a]	0.600 (0.026)	0.430 (0.020)	0.880 (0.020)[a]
	CA	0.170 (0.014)	0.540 (0.022)	0.660 (0.018)	0.780 (0.028)[a]	0.730 (0.017)[a]	0.650 (0.013)
	NB	0	0	0	0.110 (0.000)	0	0
	CM	0.120 (0.016)	0.380 (0.012)[a]	0.390 (0.021)[a]	0.260 (0.022)	0.220 (0.021)	0.330 (0.018)
	PM	0	0.080 (0.012)	0.130 (0.014)	0.080 (0.013)	0.100 (0.017)	0.090 (0.019)
	PI	0	0.100 (0.023)	0.160 (0.017)	0.140 (0.022)	0.150 (0.021)	0.140 (0.017)
	CB	0.030 (0.010)	0.270 (0.011)	0.260 (0.021)	0.550 (0.029)[a]	0.410 (0.015)[a]	0.320 (0.016)[a]
	MU	0.050 (0.013)	0.060 (0.019)	0.120 (0.022)	0.180 (0.033)	0.210 (0.031)	0.140 (0.025)
	CNA	*0.650 (0.019)*	*3.660 (0.012)[a]*	*3.110 (0.012)[a]*	*2.970 (0.019)[a]*	*2.570 (0.013)*	*2.870 (0.011)[ab]*
MNB	MN	0.440 (0.023)	1.730 (0.011)[a]	1.260 (0.019)	0.760 (0.023)	1.660 (0.030)[a]	1.470 (0.017)[a]
	CBr	0.010 (0.008)	0.330 (0.009)[a]	0.270 (0.023)[a]	0.200 (0.026)	0.160 (0.031)	0.170 (0.022)
	MNB	*0.450 (0.025)*	*2.060 (0.018)[a]*	*1.530 (0.028)*	*0.960 (0.027)*	*1.820 (0.037)[a]*	*1.640 (0.029)[a]*
AP NE	AP	0	0	0	0	0	0[b]
	NE	0	0.040 (0.023)[b]	0.020 (0.011)[b]	0.010 (0.015)[b]	0.030 (0.026)[b]	0.800 (0.029)[ab]
	APNE	*0*	*0.040 (0.013)[b]*	*0.020 (0.005)[b]*	*0.010 (0.005)[b]*	*0.030 (0.009)[b]*	*0.800 (0.024)[ab]*
TOTAL		**1.100 (0.025)**	**5.760 (0.017)[a]**	**4.660 (0.014)**	**3.940 (0.011)**	**4.420 (0.013)**	**5.310 (0.017)**
72 h treatment							
CNA	GML	0.040 (0.015)	0.740 (0.016)	0.220 (0.020)[b]	0.310 (0.023)	0.330 (0.018)	0.390 (0.026)
	CL	0.110 (0.016)	0.420 (0.033)	0.400 (0.019)	0.570 (0.027)	0.400 (0.023)	0.520 (0.036)[b]
	CA	0.100 (0.014)	0.150 (0.023)[b]	0.380 (0.025)	1.030 (0.029)[a]	0.360 (0.016)	0.400 (0.030)
	CM	0.050 (0.027)	0.130 (0.019)	0.160 (0.022)	0.190 (0.024)	0.090 (0.015)	0.280 (0.025)
	PM	0	0.220 (0.034)	0.010 (0.029)	0.020 (0.012)	0.090 (0.019)	0.050 (0.021)
	PI	0.010 (0.012)	0.240 (0.025)	0.040 (0.022)	0.080 (0.028)	0.110 (0.034)	0.120 (0.022)
	CB	0.010 (0.024)	0.190 (0.025)	0.110 (0.036)	0.180 (0.023)	0.200 (0.055)	0.080 (0.013)
	MU	0.010 (0.027)	0.050 (0.051)	0.090 (0.042)	0.040 (0.016)	0.090 (0.035)	0.070 (0.032)
	CNA	*0.330 (0.020)*	*2.140 (0.021)[a]*	*1.410 (0.021)[b]*	*2.420 (0.020)[a]*	*1.670 (0.017)[a]*	*1.910 (0.019)[ab]*
MNB	MN	0.440 (0.027)	1.350 (0.036)[b]	0.730 (0.027)[b]	0.520 (0.031)[b]	0.860 (0.023)[b]	0.550 (0.029)[b]
	CBr	0.010 (0.003)	0.160 (0.021)[b]	0.080 (0.013)[b]	0.060 (0.014)[b]	0.120 (0.004)[b]	0.040 (0.007)[b]
	MNB	*0.450 (0.027)*	*1.510 (0.036)[b]*	*0.810 (0.027)[b]*	*0.580 (0.031)[b]*	*0.980 (0.024)[b]*	*0.590 (0.030)[b]*
AP NE	AP	0	0	0.110 (0.014)[b]	0.040 (0.026)	0	0.010 (0.019)[b]
	NE	0.070 (0.023)	0.120 (0.021)[b]	0.100 (0.023)	0.200 (0.018)[b]	0.610 (0.022)[ab]	0.640 (0.026)[ab]
	APNE	*0.070 (0.023)*	*0.120 (0.021)[b]*	*0.210 (0.013)[b]*	*0.240 (0.019)[b]*	*0.610 (0.022)[ab]*	*0.650 (0.029)[ab]*
TOTAL		**0.850 (0.021)**	**3.770 (0.037)[ab]**	**2.430 (0.018)[b]**	**3.240 (0.023)[b]**	**3.260 (0.029)[b]**	**3.150 (0.040)[b]**

Note. NC. Negative Control; **PC.** Positive Control; **CNA.** Chromosome and nuclear aberrations**; GML.** Genetic material losses; **CL.** Chromosome losses; **CA.** Chromosome adherences; **CM.** C-metaphases; **PM.** Polyploidized metaphases; **PI.** Polyploidized interphases; **CB.** Chromosome bridges; **MU.** Multipolarities; **MNB.** Micronuclei and chromosome breaks; **MN.** Micronuclei; **CBr.** Chromosome breaks; **APNE.** Apoptotic and necrotic cells; **AP.** Apoptotic cells; **NE.** Necrotic cells; **Total.** Total of CNA, MNB and APNE. / 10,000 cells analyzed by concentration and by control. Average (Standard Deviation) / [a]Significantly different from the NC ($p < 0,05$) and [b] Significantly different from the 20h treatment ($p < 0,05$), according to the Kruskal-Wallis test.

Table 2. Frequency (%) of alterations (CNA, MN/B e AP/NE) observed in *A. cepa* meristematic cells exposed to the different concentrations of the Black Dye Commercial Product, and to the Negative and Positive Control Tests, for the 20, 48 and 72h treatments.

prophases, metaphases, anaphases and telophases of the meristematic cells exposed to the highest BDCP concentrations (20-h treatment) (Table 2), provided evidence for a mechanism of aneugenic action of the azo dye.

Although metaphases with chromosome adherences have been found in all the treatments carried out with the azo dye, significant NC-related results were only recorded for the four concentrations tested in the 20-hour treatment, for two 48-hour treatment concentrations (10 and 100 µg/L) and for the 10 µg/L concentration of the 72-hour treatment (Table 2). These data showed that the BDCP produced chromosome adherences in the *A. cepa* cells. This effect, however, did not persist after the 72-hour recovery treatment (simulating normalization of environmental conditions), indicating that the dye did not have a cumulative effect on the cells. Except for the 10 µg/L concentration, once it induced an increase in the number of cells with chromosome adherences.

The presence of chromosome adherences may be a sign of genotoxic effect of the damage inducer, whose consequence of the action might lead to irreversible cell damage – including its death [34, 48, 49] – an effect also observed herein. The presence of chromosome adherences reinforces the evidences of the aneugenic action of BDCP, previously described for the chromosome losses. Inactivation of the mitotic spindle (resulting from the BDCP aneugenic effect) prevents the chromosomes from migrating towards the cell poles, consequently blocking

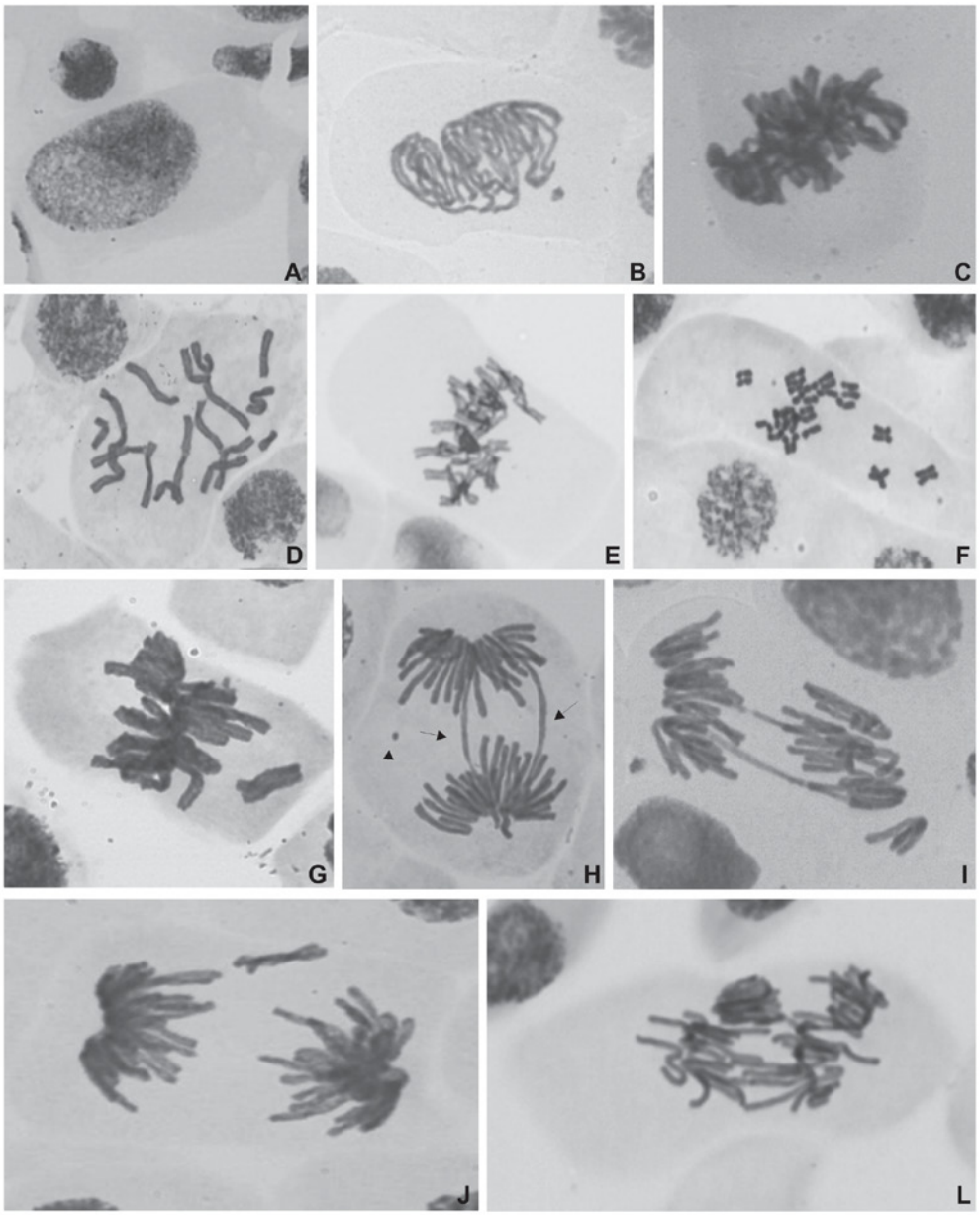

Figure 1: Genotoxic damages observe in meristematic cells of the *Allium cepa* roots treated with the Black Dye Commercial Product. A. Polyploidized intherphasic nucleus; **B.** Prophase with genetic material loss; **C.** Chromosome adherence; **D-F.** C-metaphases; **G, I, J.** Chromosome losses; **H.** Chromosome bridges (arrow) and chromosome fragment (arrow head); **L.** Multipolar anaphase.

Treatment	Nucleolus per cell	NC	PC	Black Dye Commercial Product Concentrations (µg/L)			
				1	10	100	1000
20 h	1	8.810 (0.042)	10.070 (0.042)	11.960 (0.035)	7.190 (0.024)	18.430 (0.061)	10.240 (0.040)
	2	47.560 (0.038)	47.030 (0.034)	56.300 (0.031)	32.710 (0.022)	39.390 (0.049)	43.680 (0.032)
	3	40.470 (0.056)	34.480 (0.030)	27.860 (0.045)	43.550 (0.027)	34.300 (0.044)	36.480 (0.038)
	4	2.450 (0.061)	7.250 (0.090)	3.480 (0.098)	14.180 (0.127)	6.570 (0.058)	7.380 (0.066)
	5	0.720 (0.027)	0.940 (0.046)	0.350 (0.029)	2.060 (0.037)	1.190 (0.031)	1.570 (0.051)
	6	0	0.160 (0.021)	0.060 (0.019)	0.160 (0.029)	0.110 (0.034)	0.530 (0.036)[a]
	7	0	0.070 (0.001)	0	0.110 (0.000)	0	0.120 (0.003)
	8	0	0	0	0.040 (0.001)	0	0
	9	0	0	0	0	0	0
	10	0	0	0	0	0	0
48 h	1	8.360 (0.045)	11.430 (0.032)	8.050 (0.030)	9.790 (0.028)	6.580 (0.036)	10.290 (0.043)
	2	38.410 (0.029)	44.920 (0.038)	37.130 (0.026)	37.580 (0.031)	24.980 (0.026)	38.130 (0.039)
	3	37.770 (0.040)	26.020 (0.051)	43.790 (0.021)	35.430 (0.036)	43.060 (0.022)	39.140 (0.046)
	4	14.780 (0.099)[b]	11.890 (0.202)	9.530 (0.060)	10.440 (0.090)	22.150 (0.051)[b]	11.320 (0.082)
	5	0.680 (0.198)	4.960 (0.040)	0.980 (0.038)	4.940 (0.036)[ab]	2.990 (0.024)[ab]	0.690 (0.027)
	6	0	0.780 (0.032)[a]	0.340 (0.011)[ab]	1.780 (0.022)[a]	0.230 (0.005)	0.300 (0.033)
	7	0	0	0.170 (0.011)	0	0	0.130 (0.024)
	8	0	0	0	0	0	0
	9	0	0	0	0	0	0
	10	0	0	0	0.040 (0)	0	0
72h	1	13.390 (0.028)	16.150 (0.030)	12.670 (0.029)	9.830 (0.025)	15.200 (0.026)	13.620 (0.024)
	2	41.630 (0.023)	37.610 (0.024)	42.820 (0.028)	26.260 (0.028)	29.350 (0.021)	30.270 (0.023)
	3	37.640 (0.029)	38.030 (0.025)	36.650 (0.026)	41.190 (0.020)	36.360 (0.024)	33.710 (0.020)
	4	6.120 (0.075)	7.040 (0.045)	6.170 (0.042)	20.170 (0.027)[a]	17.140 (0.032)[ab]	19.760 (0.022)[ab]
	5	1.170 (0.031)	0.850 (0.044)	1.500 (0.032)[b]	2.260 (0.061)	1.730 (0.045)	2.090 (0.024)
	6	0.050 (0.019)	0.320 (0.021)	0.110 (0.019)	0.190 (0.029)	0.170 (0.034)	0.430 (0.035)
	7	0	0	0.080 (0.002)	0.040 (0.016)	0.050 (0.013)	0.120 (0.001)
	8	0	0	0	0	0	0
	9	0	0	0	0	0	0
	10	0	0	0	0.060 (0)	0	0

Note. NC. Negative Control; **PC.** Positive Control / 5,000 cells analyzed by concentration and by control. Average (Standard Deviation).
[a]Significantly different from the NC ($p < 0,05$) and [b] Significantly different from the 20h treatment ($p < 0,05$), according to the Kruskal-Wallis test.

Table 3. Frequency (%) of nucleolus quantity observed in *A. cepa* meristematic cells exposed to the different concentrations of the Black Dye Commercial Product, and to the Negative and Positive Control Tests, for the 20, 48 and 72h treatments.

metaphase. Non-migrating chromosomes remain in a condensation process. They get closer and closer to one another, characterizing the so-called chromosome adherences [40], a cytogenetic phenomenon widely described in plants.

C-metaphases may result from the action of aneugenic agents on the cells whose compounds promote a complete inactivation of the mitotic spindle [29, 33, 50]. Such alterations may generate other types of cell abnormalities such as polyploid cells [33, 51], multinucleated and micronuclei-bearing cells [33, 52]. One study [16] showed the presence of both C-metaphase and micronuclei-bearing cells, after *A. cepa* seeds were exposed to water samples that received effluents contaminated with the BDCP. Our analyses indicated the presence of low frequencies of C-metaphase in the *A. cepa* meristematic cells exposed to tests using the BDCP azo dye; except for the statistically significant values shown by meristems submitted to the 1 µg/L concentration after a 48-h recovery treatment (Table 2), a fact which already suggests a possible aneugenic action of this dye on the *A. cepa* cells. However, the incongruous data observed between the results presented herein and our previous results [16] may be related to the possible synergy of the dye with other components of the water from the river investigated by those authors.

Chromosome bridges might result from cohesive chromosome terminations or structural rearrangements [50], or even from chromosome adherences [53, 54] which, in the last case, may be multiple and persist to telophase according to the authors mentioned. Frequencies of chromosome bridges, always higher than the ones found in the NC tests, were observed. However, significant frequencies of this alteration were only found at the anaphase and telophase of the cells exposed to the 20 (10 µg/L concentration) and 48-hour treatments (10 µg/L, 100 µg/L and 1000 µg/L concentrations) (Table 2), reinforcing the aneugenic action of this chemical, as previously suggested for chromosome adherence and losses.

In all the experiments, non-significant values of multipolar cells during anaphase and telophase, as well as of lobulated nuclei and polyploid cells, during interphase and metaphase, were observed for the *A. cepa* cells exposed to the different azo dye concentrations and treatments (Table 2).

With the help of the joint analysis of the CNA, considered herein as genotoxicity endpoints (losses, adherences, C-metaphases, polyploidies, bridges and multipolar cells), it was possible to detect that there is a statistically significant difference between the *A. cepa* cells exposed to the dye and the ones exposed to the NC (Table 2). Coincidentally, all the alterations considered result from the mechanisms of aneugenicity, which, once again, confirms the aneugenic action of BDCP.

Micronuclei and chromosome breaks are excellent mutagenicity-related endpoints, because they are genetic material alterations

(whether in the chromosomes or in the DNA) that can no longer be repaired by the cells. However, these can again be passed on to the new cell generations, and are therefore easily fixed in the organisms [55]. The MN/B frequencies registered in this study for all the treatments carried out with the dye were always higher and statistically more significant compared to the ones found in the NC test; except for the two smaller concentrations tested in the 48-h treatment (1 and 10 µg/L) and for the concentrations tested in the 72-h recovery treatment (Table 2, Figure 2A). These results showed that the BDCP is mutagenic in the concentrations mentioned. The significant decrease in the micronucleus frequencies and chromosome breaks – observed after the 72-h recovery treatment in ultrapure water (Table 2) – indicates that the azo dye promotes mutagenic effects on *A. cepa* while there is exposure to the dye; although it does not seem to act cumulatively in the cells.

From the significant micronucleus frequencies observed for three of the dye concentrations tested (20-h treatment), and for the two highest concentrations (48-h treatment) (Table 2), it is possible to assert that the BDCP presents a mutagenic action. These data confirm the mutagenic activity described by researchers in the test with *Salmonella* [15] and in tests with *A. cepa* [16], after evaluating the effects of water samples that received effluents from a textile company contaminated with such chemical.

Chromosome fragments may result from breaks in the chromosome bridges, the ones which may originate from either translocations or cohesive chromosome terminations [50]. Significant frequencies of chromosome breaks were observed at the metaphase, anaphase and telophase (Figure 2B-C) of the two highest BDCP concentrations tested (20-h treatment) and at the smallest 48-h treatment concentration, which confirmed a direct action of the azo dye on the DNA molecule of the *A. cepa* cells, reinforcing both the dye mutagenic potential and clastogenic action.

The action of chemical agents on the cells might lead to a complex sequence of events, which may result in cell death [56]. High frequencies of cell death might therefore be considered endpoints related to cytoxicity, once they are alterations directly interfering in cell viability, consequently damaging different physiological processes of the organisms. The current study showed significant frequencies of cells under death process (apoptosis and necrosis) in the *A. cepa* meristems exposed to four azo dye concentrations tested in the 20-h treatment, to the highest concentration in the 48-h treatment and to the two highest concentrations in the 72-h treatment (Table 2, Figure 3). These results show that the BDCP was cytotoxic to the concentrations mentioned. The significant decrease in the cell death frequencies (apoptosis or necrosis), observed after the recovery treatments in ultrapure water shows that, in relation to the cytotoxic damage, the effect of the dye may be minimized once exposure conditions are back to normal. In addition, the azo dye does not present cumulative effects on the meristematic cells of the *A. cepa* test-system.

Statistically significant differences were detected when only frequencies of necrosis were considered. It is a phenomenon that simultaneously involves many cells and is a result of cell injuries – a process which leads to metabolic damage and, finally, to cell death. In plants, it may be of several kinds: spontaneous; triggered by oxidative or stress mechanisms; induced by infectious agents or by toxic chemical components, and by hypersensitivity [57]. During necrosis, the cells first swell. Next, the plasma membrane bursts, consequently leading to fast cell lysis [58].

Unlikely necrosis, the term apoptosis may be attributed to the cell elimination process without an apparent burst of the plasma membrane [59]. Morphologically speaking, the cells are first reduced and their nuclei condensate. They then self-disintegrate, consequently forming structures named apoptotic bodies [58]. Concerning the apoptotic cell frequencies, significant differences in relation to the NC test were only observed for the highest concentration (1000 µg/L) in the 20-h treatment with the azo dye. After performing the recovery treatments for the 1000 µg/L concentration, a clear decrease in the frequencies of the apoptotic cells was observed. The decrease showed statistically significant differences in contrast to the 20-h treatment (Table 2), demonstrating that the meristems are able to recover from the azo dye cytotoxic action after exposure conditions are back to normal.

Data related to all cell abnormalities (CNA, MN/B and AP/NE) – observed for the 20-h treatment (Table 2) – show that the higher the azo dye concentration tested, the higher the frequency of damaged cells, which are characterized by a positive dose-response ratio of the *A. cepa* test-organism. These data confirm a cytotoxic, genotoxic and mutagenic action of the BDCP azo dye studied herein. Since the highest concentration induced significant frequencies of cell abnormalities (CNA+MN/B+AP/NE), it was regarded as the highest toxic potential of the test-organism. Furthermore, the gradual decrease in the total frequency of the altered cells – after the 48 and 72-hour recovery treatments (Table 2) – indicates that for *A. cepa*, the BDCP azo dye does not show any cumulative effects (a fact proven by the 72-hour recovery treatment), since they presented frequencies of CNA+MN/B+AP/NE significantly lower than the ones observed for the 20-h treatment.

In view of what was exposed, it can still be inferred that the main mechanism of action of the BDCP is the one of aneugenic nature. Some scientists [33, 40, 60] showed that the trifluralin herbicide has an attributed aneugenic action, especially due to the presence of an NO_2 group, which connects to tubulin molecules, avoiding its polymerization and, consequently, microtubule formation. According to one research

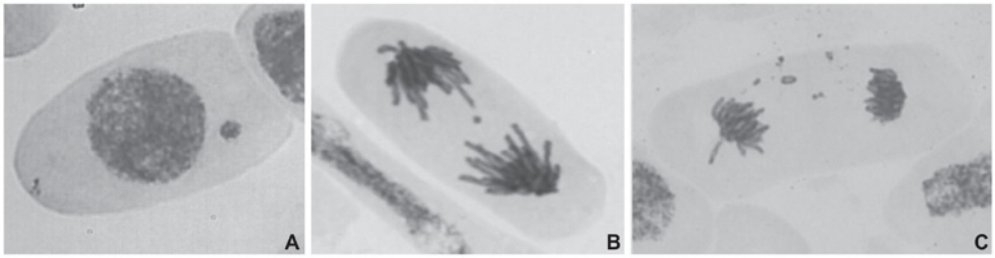

Figure 2: Mutagenic damages observed in meristematic cells of the *Allium cepa* roots treated with the Black Dye Commercial Product. A. Cell with a micronuclei; **B.** Cell with one chromosome fragment; **C.** Cell with chromosome fragments in varied number and size.

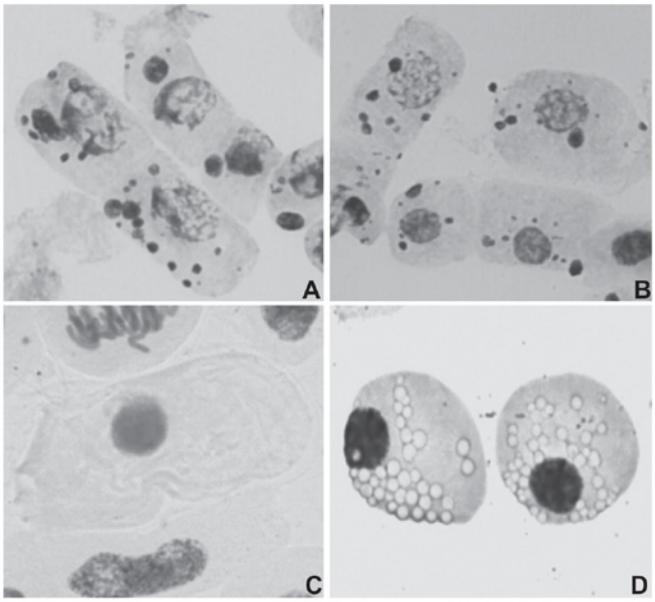

Figure 3: Cytotoxic damages observed in meristematic cells of the *Allium cepa* roots treated with the Black Dye Commercial Product. A-B. Apoptotic cells; **C-D.** Necrotic cells.

[61], the mutagenic potential of azo dyes is directly related to the kind and position of substituents such as the aromatic ring and the nitrogen atom in the amino portion. Knowing that the BDCP has three different components – the blue one (C.I. Disperse Blue 373), the violet one (C.I. Disperse Violet 93) and the orange one (C.I. Disperse Orange 37) – presenting the NO_2 groups [15], the components supposedly act in a similar way on the herbicide mentioned. Therefore, those groups must bind to the tubulin molecules, causing disturbances in the mitotic spindle formation during the cell cycle. This contributes to CNA formation, such as chromosome losses, chromosome adherences, C-metaphases, chromosome bridges, polyploidies and multipolarities, besides micronuclei formation.

C Banding

The NC tests developed in this study showed blocks of heterochromatin spread throughout the nucleus of interphase cells and C-positive labeling in the telomere regions of *A. cepa* mitotic cells, confirming the some studies, who observed such C-positive location in the telomere region of chromosomes of this species [37]. Amongst the cell alterations analyzed (the ones resulting from the action of the different BDCP concentrations and treatments) using the conventional staining method, some of them showed greater details after the application of the C banding method, such as chromosome bridges (Figure 4A-B), micronuclei (Figure 4C), chromosome breaks (Figure 4D), and chromosome loss (Figure 4E).

Chromosome breaks may be related to a higher affinity between some DNA regions and mutagenic agents, whether by the presence of higher fragility sites or by the composition of specific DNA sequences, possibly connected to the nuclear matrix [62]. Studies conducted by many scientists [63-68] showed a preferential location of chromosome breaks induced by alquilant agents, frequently associated with heterochromatin and the defined chromosome bands. In the present study, small chromosome fragments with C-positive labeling (Figure 4D) were observed in the *A. cepa* meristematic cells exposed to the two highest azo dye concentrations in the 20-h treatment. The fragments seem to have come from breaks in terminal regions of chromosomes, corroborating other studies [69], who assert that most of the *A. cepa* chromosome breaks take place in the telomere regions composed of heterochromatin. Larger chromosome fragments were also detected in the cells exposed to the highest azo dye concentration, which showed to be composed by typical euchromatin, indicating that the break might have taken place in a region more interspersedly disposed in the chromosome. According to some scientists [70], any loss of the chromosome portion – even though it is composed of material without direct gene expression – might lead to cell inviability.

The absence of C-positive labeling in the median region of most chromosome bridges (Figure 4A) indicates that these alterations may result from breaks occurring in the telomere regions of the chromosomes. The breaks lead to cohesive terminations which joined and, consequently, connected chromatids to one another. This result might confirm assertions by one author [50], in which he says that chromosome bridges may originate from translocations, or simply from cohesive terminations.

Among the micronuclei-bearing cells, it was possible to notice that some of them did not present micronuclei with C-positive labeling, whereas others presented. The presence of MN without C-positive labeling (Figure 4C) may still demonstrate a more serious effect of the dye on the exposed cells. The fact is that, if the MN do not have heterochromatin in their composition, it is because they do not have the telomere portion of the chromosomes, meaning they resulted from double chromosome breaks. Additionally, it was possible to detect nuclei with entirely condensed chromatin in the meristematic cells exposed to the highest azo dye concentration tested, in all the treatments. This might be considered as a strong sign of cell death induction, confirming the cytotoxic action of the dye studied herein.

NOR Banding

The results obtained from the AgNOR staining showed that a variable location of the acidic nucleolar proteins was found during cell division. The proteins were detected both near the chromosome peripheries and distributed in the cytoplasm during prophase and telophase, corroborating other studies using the *A. cepa* root cells [71].

After exposing the meristematic cells to all the BDCP concentrations and treatments, the presence of MN with and without a nucleolus organizer region (NOR) was confirmed (Figure 4F-G). The presence of NORs in MN shows that the azo dye may act directly on the regions related to rDNA sites, which are indispensable in processes of transcription and protein translation. Studies showed that either NOR loss or inactivation might be related to facts such as deletion or translocation [72]. On the other hand, the presence of MN-bearing cells without NORs shows herein that the azo dye does not influence the regions associated with the rDNA sites only, indicating that there are non-specific regions of the action of this chemical compound.

Additionally, a great variation of the nucleolus number in the *A. cepa* meristematic cells was observed. A size variation of these nucleoli both inside one single cell and among the cells was also observed (Table 3, Figure 4 H-J). According to some scientists, the variation in the number of nucleoli in plants results from the action of genotoxic agents [73]. This study showed a substantial variation within the quantity of nucleoli in the *A. cepa* meristematic cells after exposure to the azo dye concentrations. It also showed that only the PC tests, as well as the ones with the azo dye, were capable of inducing cell formation containing

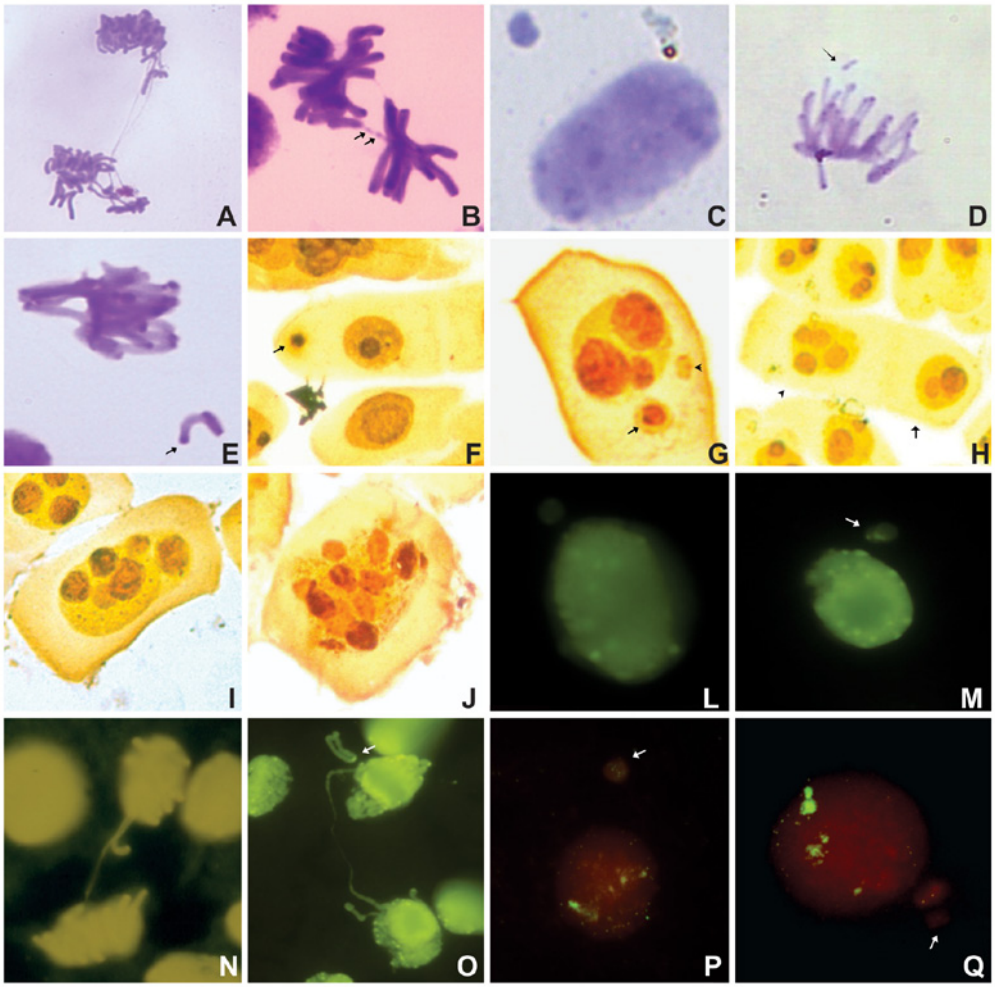

Figure 4: ***Allium cepa*** **meristematic cells treated with the Black Dye Commercial Product and submitted to the Chromosome Bandings and Fluorescent** ***in situ*** **Hybridization (FISH). A-E. C-Banding. A-B.** Anaphases with chromosome bridges: **A.** without C-banded region and **B.** with C-banded regions (arrows); **C.** Cell with micronuclei without C-banded region; **D.** Chromosome fragment with one C-banded region (arrow); **E.** Metaphase with chromosome loss, presenting C-banding in telomeric regions; **F-J. NOR-Banding. F-G.** Micronuclei: **F.** with nucleolus organizer region (arrow); **G.** with nucleolus organizer region (arrow) and without nucleolus organizer region (arrow head); **H.** Cells with two (arrow) and four nucleoli (arrow head); **I.** Cell with six nucleoli; **J.** Cell with ten nucleoli; **L-O. CMA_3 Banding. L-M.** Micronuclei: **L.** without CMA_3-banded region and **M.** with CMA_3-banded regions (arrow); **N.** Anaphase with chromosome bridge without CMA_3-banded region; **O.** Telophase with chromosome bridge without CMA_3-banded region and with chromosome loss showing CMA_3-banded region (arrow); **P-Q. FISH. P.** Micronuclei with various small hybridization signals (arrow); **Q.** Micronuclei without hybridization signal (arrow).

over six nucleoli, although there was not a proportional ratio for the dose-response of the dye. This way, it was possible to consider that the nucleolus number and size variation (Table 3, Figure 4 H-J) might be associated with the genotoxic action of the BDCP azo dye. The recovery treatments do not seem to have been efficient at minimizing the damage the dye caused to the nucleolar domains. This inefficiency lies in the fact that after the 48 and 72-hour periods, frequencies of cells containing many nucleoli were still detected, probably resulting from cell polyploidization processes.

CMA3/DAPI Banding

Analyses of the slides resulting from the CMA_3/DAPI chromosome banding demonstrated that the terminal regions of the *A. cepa* chromosomes presented positive CMA_3 labeling, i.e., rich in C-G, corroborating the results of other cytogenetic studies already conducted for this species [37].

Detection of MN-bearing cells was made possible both with and without positive CMA_3 labeling (Figure 4 L-M). The presence of positive CMA_3 labelings in the MN of the *A. cepa* meristematic cells indicates that these MN result from the genetic material containing terminal portions of the chromosomes – regions that are rich in satellite DNA composed of C-G repetitions. The MN without CMA_3 labelings, on the other hand, might result from breaks taking place in two chromosome regions, because they bear more decondensed regions of the chromosomes.

Most of the chromosome bridges did not present fluorochrome CMA_3 labeling (Figure 4 N-O). This result demonstrates that the chromosome bridges might result from either cohesive chromosome terminations or structural rearrangements, concurring with what was mentioned by other study [50]. They might also result from chromosome adherences [53] at previous phases, corroborating our C banding results.

FISH (Fluorescent in situ hybridization)

Fluorescent *in situ* hybridization (FISH) is a technique that enables locating DNA specific sequences both on metaphase chromosomes and in the interphase nucleus [74]. Among the different repetitive DNA sequences used in the *in situ* hybridization technique, the 45S rDNA sequence is one of the most employed probes, because it is highly preserved among the organisms.

Analyses of the slides submitted to the FISH technique (for all the tests and treatments performed) helped detect the presence of several labelings smeared throughout the nuclei (Figure 4 P-Q). Cell alterations were observed for both the conventional staining method and the chromosome banding resulting from the different BDCP concentrations and treatments. Some of those alterations could be better evaluated through the FISH technique with the 45S rDNA probe, such as the MN.

Among the MN, it was possible to notice a variation in their constitution – some of them presented several small signals (Figure 4P), whereas others showed no signals at all (Figure 4Q). The signals detected in the micronuclei are associated with the ends of chromosomes, since some studies conducted using the FISH technique showed that the rDNA sequences in *A. cepa* are located right in these chromosome regions [75, 76]. Additionally, some studies that applied FISH in *A. cepa* showed that rDNA loci are present on the satellite chromosome 6 and the smallest chromosome 8 of this species [77]. The presence of hybridization signals in the MN of the *A. cepa* meristematic cells indicates that these MN bear 45S rDNA sites, presenting regions from chromosome 6 and/or 8. However, this chemical compound also induced the formation of micronuclei without hybridization signals, indicating that the target sites of the azo dye action might vary and be non-specific.

Finally, knowing that the BDCP induces MN with hybridization signals, we concluded that FISH using 45S rDNA probe provided us a useful chromosome marker. This marker helped identify specific chromosomes involved in aneugenic and clastogenic effect of such chemical, since it is known that chromosomes 6 and 8 in *A. cepa* karyotype bear 45S rDNA loci.

Conclusions

Considering all the types of cell alterations discussed herein, we can conclude that all the BDCP dye concentrations tested were cytotoxic, genotoxic and mutagenic to the *A. cepa* test-organism. The alterations observed indicate the sort of mechanism of action of the azo dye (aneugenesis or clastogenesis).

The different cytogenetic techniques were useful and efficient in determination of the different mechanisms of action of the BDCP. By aid of chromosome bandings (C, NOR and CMA_3/DAPI) and FISH, it was possible to infer a relation between events like chromosome breaks and losses and varied chromosome sites. This leads us to suggest that these techniques must be associated with the one of conventional cytogenetic analysis (chromosome and nuclear aberrations assay) in order to evaluate genetic damage to organisms exposed to environmental contaminants.

Although the recovery treatments reduced the frequencies of cytotoxic, genotoxic and mutagenic damages in nearly all the assays performed, the effects caused by the dye were not entirely eliminated after the exposure conditions of the test-organism were back to normal.

From the results obtained, we may conclude that the associations of different cytogenetic methods may fully clarify the modes of action of environmental pollutants. Furthermore, they might be useful for eventual studies carried out with other textile dyes, or even with other chemicals potentially harmful to the environment.

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References

1. Chung KT, Stevens JR (1993) Degradation of azo dyes by environmental microorganisms and helminths. Environ Toxicol Chem 12: 2121-2132.
2. Kunz A, Peralta-Zamora P, Moraes SG, Durán N (2002) Degradation of reagent dyes for the system iron metal/hydrogen peroxide. New Chem 25: 78 (in Portuguese).
3. Griffiths J (1984) Developments in the Chemistry and Technology of Organic Dyes, first ed. Oxford; 2-30.
4. O'neill C, Hawkes FR, Hawkes DL, Lourenco ND, Pinheiro HM, et al. (1999) Colour in textile effluents – sources, measurement, discharge consents and simulation: a review. J Chem Technol Biotechnol 74: 1009-1118.
5. Pearce CI, Lloyd JR, Guthrie JT (2003) The removal of color from textile wastewater using whole bacterial cells: a review. Dyes and Pigments 58: 179-196.
6. Hao OJ, Kim H, Chiang P-C (2000) Decolorization of wastewater. Crit Rev Environ Sci Technol 30: 449-505.
7. Clarke EA, Anliker R (1980) Organic dyes and pigments, in: Hutzinger, O. (Ed.), The handbook of environmental chemistry, v. 3, part. A. Anthropogenic Compounds. United States of America: Springer-Verlag. 215 p.
8. Holme I (1984) Ecological aspects of color chemistry. Developments in the Chemistry and Technology of Organic Dyes, first ed. Society of Chemistry Industry, Oxford; 128.
9. Michaels GB, Lewis DL (1985) Sorption and toxicity of azo and triphenyl methane dyes to aquatic microbial populations. Environ Contam Toxicol 34: 323-330.
10. Amin KA, Abdel Hameid II H, Abd Elsttar AH (2010) Effect of food azo dyes tartrazine and carmoisine on biochemical parameters related to renal, hepatic function and oxidative stress biomarkers in young male rats. Food Chem Toxicol 48: 2994-2999.
11. Ames BN, Mccann J, Yamasaki E (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res 31: 347-364.
12. Chung KT, Fulk GE, Andrews AW (1981) Mutagenicity testing of some commonly used dyes. Appl Environ Microbiol 42: 641-48.
13. Clonfero E, Vernier P, Granell N, Levis AG (1990) Leather azo dyes: mutagenic and carcinogenic risks. Med Lavoro 81: 222-229.
14. Garg A, Bhat KL, Bock CW (2002) Mutagenicity of aminoazobenzene dyes and related structures, a QSAR/QPAR investigation. Dyes and Pigments 55: 35-52.
15. Umbuzeiro GA, Freeman H, Warren SH, Kummrow F, Claxton LD (2005) Mutagenicity evaluation of the commercial product C.I. Disperse Blue 291 using different protocols of the Salmonella assay. Food Chem Toxicol 43: 49-56.
16. Caritá R, Marin-Morales MA (2008) Induction of chromosome aberrations in the Allium cepa test system caused by the exposure of seeds to industrial effluents contaminated with azo dyes. Chemosphere 72: 722-725.
17. Chequer FMD, Angeli JPF, Ferraz ERA, Tsuboy MS, Marcarini JC, et al. (2009) The azo dyes Disperse Red 1 and Disperse Orange 1 increase the micronuclei frequencies in human lymphocytes and HepG2 cells. Mutat Res 676: 83-86.
18. Oliveira GAR, Ferraz ERA, Chequer FMD, Grando MD, Angeli JPF, et al. (2010) Chlorination treatment of aqueous samples reduces, but does not eliminate, the mutagenic effect of the azo dyes Disperse Red 1, Disperse Red 13 and Disperse Orange 1. Mutat Res 703: 200-208.
19. Oliveira DP (2005). Dyes as an important class of environmental contaminants – a case study. PhD. Thesis (Toxicology and Toxicologic Analyses) – São Paulo University, SP. 121 p. (in Portuguese).

20. Alves de Lima RO, Bazo AP, Salvadori DMF, Rech CM, Oliveira DP, et al. (2007) Mutagenic and carcinogenic potential of a textile azo dye processing plant effluent that impacts a drinking water source. Mutat Res 626: 53-60.

21. Matsuoka A, Tada A, Terao Y, Nukaya H, Önfelt A et al. (2001) Chromosomal effects of newly identified water pollutants PBTA-1 and PBTA-2 and their possible mother compounds (azo dyes) and intermediates (non-CIPBTAs) in two Chinese hamster cell lines. Mutat Res 493: 75-85.

22. Biswas SJ, Khuda-Bukhsh AR (2005) Cytotoxic and genotoxic effects of the azo-dye p-dimethylaminoazobenzene in mice: a time-course study. Mutat Res 587: 1-8.

23. Lin YH, Leu JY (2008) Kinetics of reactive azo dye decoloration by Pseudomonas luteola in a biological activated carbon process. Biochem Eng J 39: 457-467.

24. Arlt VM, Glatt H, Muckel E, Papel U, Sorg BL et al. (2002) Metabolic activation of environmental contaminant 3-nitrobenzanthrone by human acetyltransferases and sulfotransferases. Carcinogenesis 23: 1937-1945.

25. De-Serres FJ (1992). Preface: Higher plants as effective monitors of environmental mutagens. Mutat Res 270: 1-3.

26. Grant WF (1994) The present status of higher plants bioassays for the detection of environmental mutagens. Mutat Res 310: 175-185.

27. Yi H, Meng Z (2003) Genotoxicity of hydrated sulfur dioxide on root tips of Allium sativum and Vicia faba. Mutat Res 537: 109-114.

28. Ma TH, Xu C, Mcconnell H, Rabago EV, Arreola GA et al. (1995) The improved Allium/Vicia root tip micronucleous assay for clastogenicity of environmental pollutants. Mutat Res 334: 185-195.

29. Fiskesjö G (1985) The Allium test as a standard in environmental monitoring. Hereditas 102: 99-112.

30. Rank J, Nielsen MH (1994) Evaluation of the Allium anaphase-telophase test in relation to genotoxicity screening of industrial wastewater. Mutat Res 312: 17-24.

31. Matsumoto ST, Marin-Morales MA (2004) Mutagenic potential of the water river that receives tannery effluent using the Allium cepa system. Cytologia 69: 399-408.

32. Matsumoto ST, Mantovani MS, Malagutti MIA, Dias AL, Fonseca IC et al. (2006) Genotoxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using Oreochromis niloticus and chromosome aberrations in onion root-tips. Genet Mol Biol 29: 148-158.

33. Fernandes TCC, Mazzeo DEC, Marin-Morales MA (2007) Mechanism of micronuclei formation in polyploidizated cells of Allium cepa exposed to trifluralin herbicide. Pestic Biochem Phys 88: 252-259.

34. Leme DM, Angelis DF, Marin-Morales MA (2008) Action mechanisms of petroleum hydrocarbons present in waters impacted by an oil spill on the genetic material of Allium cepa root cells. Aquatic Toxicol 88: 214-219.

35. Leme DM, Marin-Morales MA (2009) Allium cepa test in environmental monitoring: a review on its application. Mutat Res 82: 71-81.

36. Rowland RE (1981) Chromosome banding and heterochromatin in Vicia faba. Theoretical and Applied Genetics: International J Plant Breed Res 60: 275-280.

37. Kim ES, Punina EO, Rodionov AV (2002) Chromosome CPD (PI/DAPI) – and CMA/DAPI – Banding Patterns in Allium cepa L. Russ J Genet 38: 392-398.

38. Çavas T, Ergene-Gözükara S (2003) Micronuclei, nuclear lesions and interphase silver-stained nucleolar organizer regions (AgNORs) as cyto-genotoxicity indicators in Oreochromis niloticus exposed to textile mill effluent. Mutat Res 538: 81-91.

39. Oliveira DP, Carneiro PA, Sakagami MK, Zanoni MVB, Umbuzeiro GA (2007) Chemical characterization of a dye processing plant effluent – Identification of the mutagenic components. Mutat Res 626: 135-142.

40. Fernandes TCC, Mazzeo DEC, Marin-Morales MA (2009) Origin of nuclear and chromosomal alterations derived from the action of an aneugenic agent – Trifluralin herbicide. Ecotoxicol Environ Safety 72: 1680-1686.

41. Mello MLS, Vidal BC (1978) The Feulgen Reaction. Sci Cult 30: 665-676 (in Portuguese).

42. Schwarzacher T, Ambros P, Schweizer D (1980) Application of Giemsa banding to orchid karyotype analysis. Plant Systematic Evol. 134: 293-297.

43. Hizume M, Sato S, Tanaka A (1980) A highly reproducible method of nucleolus organizing regions staining in plants. Stain Technol 55: 87-90.

44. Deumling B, Greilhuber J (1982). Characterization of heterochromatin in different species of the Scilla siberica group (Liliaceae) by in situ hybridization of satellite DNAs and fluorochrome banding. Chromosoma 84: 535-555.

45. Pinkel D, Straume T, Gray T (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Academy Sci 83: 2934-2938.

46. Martins C, Galetti PM (1999) Chromosomal localization of 5S rDNA genes in Leporinus fish (Anostomidae, Characiformes). Chromosome Res 7: 363-367.

47. Smaka-Kincl V, Stegnar P, Tornam MJ (1997) The evaluation of waste, surface and ground water quality using the Allium cepa test procedure. Mutat Res 368: 171-179.

48. Marcano L, Del Campo A (1995) Ultraestructural study of the nucleolus in Allium cepa L. onion meristematic populations treated com metabolic inhibitors. Sciences 3: 73-82 (in Spanish).

49. Türkoglu S (2007) Genotoxicity of five food preservatives tested on root tips of Allium cepa L. Mutat Res 626: 4-14.

50. Fiskesjö G (1993) Technical Methods Section. Allium test I: A 2-3 Day plant test for toxicity assessment by measuring the mean root growth of onions (Allium cepa L.). Environ Toxicol Water Quality: An International J 8: 461-470.

51. Odeigah PGC, Nurudeen O, Amund OO (1997) Genotoxicity of oil field wastewater in Nigeria. Hereditas 126: 161-167.

52. Krisch-Volders M, Vandhauwaert A, De Boeck M, Decordier I (2002) Importance of detecting numerical versus structural chromosome aberrations. Mutat Res 504: 137-148.

53. Giacomelli FRB (1999). Evaluation of the meiotic behavior in varieties of oat (Avena sativa) recommended for the south area. Dissertation (Master's degree in Genetics) - State University of Maringá, PR; p. 131 (in Portuguese).

54. Marcano L, Carruyo I, Del Campo A, Montiel X (2004) Cytotoxicity and mode of action of maleic hydrazide in root tips of Allium cepa L. Environ Res 94: 221-226.

55. Ventura BC, Angelis DF, Marin-Morales MA (2008) Mutagenic and genotoxic effects of the Atrazine herbicide in Oreochromis niloticus (Perciformes, Cichlidae) detected by the micronuclei test and the comet assay. Pestic Biochem Phys 90: 42-51.

56. Kaioumova D, Süsal C, Opelz G (2001) Induction of apoptosis in human lymphocytes by the herbicide 2,4-dichlorophenoxyacetic acid. Hum Immunology 62: 64-74.

57. Zakeri Z, Lockshin RA (2002) Cell death during development. J Immunological Methods 265: 3-20.

58. Majno G, Joris I (1995) Apoptosis, oncosis, and necrosis: an overview of cell death. Am J Pathol 146: 3-15.

59. Proskuryakov SY, Konoplyannikov AG, Gabai VL (2003) Necrosis: a specific form of programmed cell death? Experimental Cell Res 283: 1-16.

60. Anthony RG, Hussey PJ (1999) Dinitroaniline herbicide resistance and the microtubule cytoskeleton. Trends Plant Sci 4: 112-116.

61. Tsuboy MS, Angeli JPF, Mantovani MS, Umbuzeiro GA, Ribeiro LR (2007) Genotoxic, mutagenic and cytotoxic effects of the commercial dye CI Disperse Blue 291 in the human hepatic cell line HepG2. Toxicol in vitro 21: 1650-1655.

62. Natarajan AT, Raposa T (1975) Heterochromatin and chromosome aberrations. A comparative study of three mouse cell lines with different karyotype and heterochromatin distributions. Hereditas 80: 83-90.

63. Kihlman BA (1966) Actions of chemical on dividing cells. First ed. New Jersey: Prentice-Hall, Englewood Cliffs; p. 88.

64. Bourgeois CA (1974) Distribution of mitomycin induced damage in human chromosomes with special reference to regions of repetitive DNA. Chromosoma Biol Nucl 48: 203-211.

65. Pathak S, Mcgill M, Hsu TC (1975) Actinomycin D effects on mitosis and chromosomes: sticky chromatids and localized lesions. Chromosoma 50: 79-88.

66. Reeves BR, Margoles C (1974) Preferential location of chlorambucil-induced breakage in the chromosomes of normal human lymphocytes. Mutat Res 26: 205-208.

67. Kaina B (1977) The action of N-methyl-N-nitrosourea on non-established human cell lines in vitro. II. Non-random distribution of chromatid aberrations in diploid and Down's cell. Mutat Res 43: 401-413.

68. Gupta P, Sharma T (1981) Non-random distribution of aberrations and identification with C and G-banding of the position of breakage points on Muntjac chromosomes induced by Mitomycin C, Bromodeoxyuridine and Hydroxylamine. Mutat Res 81: 63-74.

69. Nuti Ronchi V, Bonatti S, Turchi G (1986) Preferential localization of chemically induced breaks in heterochromatic regions of Vicia faba and Allium cepa chromosomes. I. Exogenous thymidine enhances the cytologic effects of 4-epoxyethyl-1,2-epoxy-cyclohexane. Environ Exp Bot 26: 115-126.

70. Hatch FT, Bodner AJ, Mazrinas JA, Moore DH (1976) Satellite DNA and cytogenetic evolution. DNA quality, satellite DNA and karyotic variations in kangaroo rats. Chromosome 58: 155-168.

71. Medina FJ, Cerdido A, Fernández-Gomes ME (1995) Components of the nucleolar processing complex (pre-rRNA, fibrillarin, and nucleolin) colocalize during mitosis and are incorporated to daughter cell nucleoli. Exp Cell Res 221: 111-125.

72. Cermeño MC; Orellana J, Santos JL, Lacadena JR (1984) Nucleolar activity and competition (amphiplasty) in the genus Aegilops. Heredity 53: 603-611.

73. Arkhipchuk VV, Malinovskaya TT, Garanko NN (2000) Cytogenetic study of organic and inorganic toxic substances on Allium cepa, Lactuca sativa, and Hydra attenuate cells. Environ Toxicol 15: 338-344.

74. Maluszynka J, Heslop-Harrison JS (1993) Molecular cytogenetics of the genus Arabidopsis: in situ localization of rDNA sites, chromosome numbers and diversity in centromeric heterochromatin. Ann Botany 71: 479-484.

75. Schubert I, Wobus U (1985) In situ hybridization confirms jumping nucleolus organizing regions in Allium. Chromosoma 92: 143-148.

76. Pich U, Fuchs J, Schubert I (1996) How do Alliaceae stabilize their chromosome ends in the absence of TTTAGGG sequences? Chromosome Res 4: 207-213.

77. Ricroch A, Pefley EB, Baker RJ (1992) Chromosomal location of rDNA in Allium: in situ hybridization using biotin- and fluorescein-labelled probe. Theor Appl Genet 83: 413-418.