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Action of Ponceau 4R (E-124) food dye on root meristematic cells of *Allium cepa* L.

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ABSTRACT. This study aimed to evaluate the toxicity of Ponceau 4R food dye on the cell cycle in root meristematic cells of *Allium cepa* L. at three concentrations: 0.25, 0.50 and 0.75 g L^{-1}, at exposure times of 24 and 48 hours. For each concentration, we used a set of five onion bulbs that were first rooted in distilled water and then transferred to their respective concentrations. Radicles were collected and fixed in acetic acid (3:1) for 24 hours. The slides were mounted with the crushing technique and stained with 2% acetic orcein. Cells were analyzed throughout the cell cycle, totaling 5,000 cells for each control and exposure time. The calculated mitotic indices were subjected to the Chi-square test ($p < 0.05$). From the results, we observed that the concentrations of 0.25 and 0.50 g L^{-1} at the 48-hour exposure, and the concentration of 0.75 g L^{-1}, the two exposure times time significantly reduced ($p < 0.05$) the cell division rate. Importantly, all the three concentrations at the two exposure times tested caused cellular aberrations in significant numbers in this testing system. Therefore, under the conditions studied, the Ponceau 4R was cytotoxic.

Keywords: azo dye, cell division, cellular aberrations, cytotoxicity.

Ação do corante alimentar Ponceau 4R (E-124) sobre as células meristemáticas de raízes de *Allium cepa* L.

RESUMO. Este trabalho teve por objetivo avaliar a toxicidade do corante alimentar Ponceau 4R sobre as células meristemáticas de raízes de *Allium cepa* L., em três concentrações: 0,25; 0,50 e 0,75 g L^{-1}, nos tempos de exposição de 24 e 48 horas. Para cada concentração utilizou-se um grupo de cinco bulbos de cebolas, que primeiramente foram enraizados em água destilada, e em seguida transferidos para as suas respectivas concentrações. As radículas foram coletadas e fixadas em ácido acético (3:1) por 24 horas. As lâminas foram preparadas pela técnica de esmagamento e coradas com orceína acética a 2%. Analisaram-se células em todo ciclo celular, totalizando 5.000 para cada controle e tempo de exposição. Os índices mitóticos calculados e as aberrações celulares observadas foram submetidos à análise estatística do Qui-quadrado ($p < 0,05$). A partir dos resultados observou-se que as concentrações 0,25 e 0,50 g L^{-1}, no tempo de exposição de 48h, e a concentração 0,75 g L^{-1}, nos dois tempos de exposição avaliados reduziram de forma significativa ($p < 0,05$) o índice de divisão celular do sistema teste em questão. Observou-se também que todas as três concentrações, nos dois tempos de exposição analisados, ocasionaram aberrações celulares em número significativo a este sistema teste. Portanto, nas condições analisadas, o Ponceau 4R foi citotóxico.

Palavras-chave: corante azo, divisão celular, aberrações celulares, citotoxicidade.

Introduction

Many additives are used in the food industry to enhance the sensory aspects and acceptance of food by the population. As an example, one can cite the synthetic food dyes, additives that do not add nutritional value, but directly influence consumer acceptance by restoring or intensifying the color of the food (SILVA; REED, 2011). However, these dyes are not recommended by experts in the health field as they contribute to the impoverishment of the diet and often cause adverse effects to the body (CHEESEMAN, 2012).

Azo dyes, a class of synthetic organic dyes that provide more vivid colors to foods, have in their composition a naphthalene ring bonded to a benzene ring by an azo bond (N = N), and the Ponceau 4R, also known in the food industry as New Coccine, Coccine Red and Food Red, is a representative of this class (PAN et al., 2011). This dye gives a red color, is marketed as a powder, has high stability in the presence of light, heat and acids in general, and has good water solubility (PARK et al., 2009). It is widely used in the coloring of candy, lozenges, sweets, gummy candies, gelatin candies, jellies, chewing gum, soy-based beverages,

carbonated soft drinks and in almost all diet products (FAVERO et al., 2011).

The control of the use of food dyes is based on the Acceptable Daily Intake (ADI), which, in turn, is based on the research findings and the recommendations of the Codex Committee on Food Additives and Contaminants (CCFAC) (GANESAN et al., 2011). In Brazil, permission to use and the establishment of maximum tolerable levels of food additives is responsibility of the National Agency for Sanitary Surveillance (ANVISA) in partnership with the Ministry of Health (MOH), which performs this activity through the Standing Committee on Food Additives (CPAA) (BRASIL, 2005; FENG et al., 2012). According to Freitas (2012), the ADI established for the Ponceau 4R dye is 0.10 mg kg^{-1}.

Despite the required limits, the use of synthetic dyes in foods still raises a number of questions regarding their toxicity (FENG et al., 2012; YADAV et al., 2013). In the case of Ponceau 4R, according to Hamerski et al. (2013), this additive can cause hypersensitivity in the skin, severe anemia and hyperactivity in children, and glomerulonephritis in children and adults. However, these same authors reported the urgent need for studies to properly evaluate the actual toxicity of this dye at the cellular level. According to Prado and Godoy (2007), these evaluations are extremely important in terms of establishing the potential of chemicals to cause cytotoxicity, genotoxicity and mutagenicity, conditions that can greatly contribute to the development of cancer.

According to Polônio and Peres (2009), toxicity evaluations, in addition to warning about the permitted tolerance limits of dyes, have banned the use of some synthetic dyes worldwide, such as solid yellow (formerly used in jellies); orange GGN (formerly used in ice cream), solid red (formerly used in fillings and cookie coatings), alizarin blue (formerly used in emulsified oils and gelatins) and scarlet GN (formerly used in confectionery fillings), because these additives revealed cytotoxicity, genotoxicity and mutagenicity in various test systems. These same authors also noted that it is of great importance that the Ponceau 4R, as well as other food dyes, is evaluated in cells of various system tests, for example, animal and plant cells and cell cultures.

Bioassays with plants have been considered highly sensitive, rapid and simple for the monitoring of toxic effects of chemicals at the cellular level (USEPA) (IGANCI et al., 2006) and root meristem cells of *Allium cepa* (onion) have been shown as an efficient plant test organism for this type of evaluation (CARITÁ; MARIN-MORALES, 2009) for their kinetic properties of proliferation, large chromosomes few in number (2n = 16), which facilitates their analysis (HERRERO et al., 2012), for enabling verification of alterations in the cell division rate (mitotic index) and cell aberrations (TABREZ et al., 2011), and for demonstrating satisfactory similarity to the results obtained with other bioassays, such as in animals and in cell cultures (GERA'KIN et al., 2011).

Peron et al. (2008), Fachinetto and Tedesco (2009) and Geras'kin et al. (2011) reported that even though the plant metabolism is different from that of animals, the results obtained by this system test are good toxicity analysis parameters at the cellular level, and have long been used to alert the population about the consumption of some foods and some synthetic and natural medicines.

Therefore, due to the wide use of Ponceau 4R dye in the food industry, the need for additional studies evaluating its toxic effects at the cellular level, and considering the *A. cepa* system as a suitable bioassay for cytotoxicity evaluation of chemicals, this study aimed to evaluate the toxicity of this food dye on the root meristem cells of *A. cepa*.

Material and methods

This work was developed at the Plant and Animal Cytogenetics Laboratory, Senator Helvidio Nunes de Barros Campus, Federal University of Piauí, Municipality of Picos, Piauí State, from May to September 2013.

Obtaining the Ponceau 4R dye and definition of concentrations

The pure Ponceau 4R dye (E-124) was purchased from a distributor specialized in national and international marketing of food synthetic additives located in Northeastern Brazil. Each vial contained 50 g of the dye product, and as described on the label, it was recommended to dilute 0.25 g of Ponceau in 1l of water. Comparing with other manufacturers, this dilution is that recommended by most of the Brazilian industries manufacturing this dye.

Thus, for this study, the first concentration analyzed was 0.25 g L^{-1}. The second and third concentrations, 0.50 and 0.75 g L^{-1}, were established doubling and tripling the amount of the first Ponceau 4R concentration.

Obtaining root meristematic cells of *A. cepa* for cytogenetic analysis

Onions were allowed to root in flasks containing distilled water at room temperature (± 25°C) and aerated until obtaining roots of about 2.5 cm in length. For analysis of each concentration, an experimental group with five onion bulbs was established.

Before placing the roots in contact with their respective solutions, some roots were collected and fixed to serve as a control (CO) of the bulb itself. The remaining roots were then placed in their respective concentrations for 24 hours, a procedure denominated 24 hour-exposure time (24h ET).

After, some roots were removed and fixed. This procedure being completed, the remaining roots from each bulb were returned to their respective solutions where they remained for 24h, which is denominated 48 hour-exposure time (48h ET). Next, roots were again collected and fixed. Exposure times of 24 and 48h were chosen in order to evaluate the effect of the three concentrations along more than one cell cycle.

In the flasks for each bulb analyzed, 30 mL of its concentration were added, taking care to make sure that all roots were in proper contact with the test solution. The fixation of the roots was in Carnoy 3:1 (ethanol: acetic acid) at room temperature for 24h. For each root collection, three roots per bulb on average were removed.

Preparation and reading of the slides, and data analysis

The slides, 3 per bulb on average, were prepared following the protocol proposed by Guerra and Souza (2002). Each slide was stained with two drops of 2% acetic orcein and examined under an optical microscope at 40X. For each bulb 1,000 cells were analyzed, totaling 5,000 cells for each control and exposure time.

Cells were observed in interphase, prophase, metaphase, anaphase and telophase. We counted the number of cells in interphase and under division for each control and exposure time and the Mitotic Index (MI) was calculated. We also evaluated the potential of cell concentrations to cause cellular aberrations, such as micronuclei, colchicine metaphases, telophase and anaphase bridges, gene amplifications, cell adhesions, nuclear buds and multipolar anaphases. The statistical analysis was performed by the Chi-square (χ^2) test, with a probability level < 0.05, by means of the BioEstat 3.0 statistical software (AYRES et al., 2007).

Results and discussion

Given the variety of staining dyes, the list of dyes permitted for foods in each country contrasts substantially. By current legislation, Resolution 388 from August 9, 1999, ANVISA, in Brazil only eleven synthetic dyes in food and beverages is permitted. They are: Erythrosin included in the class of xanthene dyes; Blue indigotine included in the indigotin class of dyes, Patent Blue V, Fast Green and Brilliant Blue in class of triphenylmethane dyes, Bordeaux Red, Ponceau 4R, Red 40, Azorubine, Tartrazine Yellow and Sunset Yellow, included in the class of azo dyes (POLÔNIO; PERES, 2009).

In agreement with Rutkunas et al. (2010), studies on the adverse health effects at the cellular level caused by synthetic dyes, especially the azo class, are quite insufficient and contradictory, and almost all these evaluations are on Tartrazine, Sunset Yellow and Red Bordeaux dyes, which warrant further studies on the effect of Ponceau 4R at the cellular level.

In this present work (Table 1), the concentrations of 0.25 and 0.50 g L^{-1} of Ponceau 4R at 48h ET, caused a statistically significant reduction in MI when compared with the MI obtained from their respective CO and 24h ET. However, the cell division rates obtained for the CO and 24h ET for the two concentrations did not differ significantly to each other ($p < 0.05$).

At a concentration of 0.75 g L^{-1} (Table 01), we observed that the MI obtained in the 24 and 48h ETs were statistically different compared with the MI of their respective controls, with a significant reduction in cell division in these two exposure times. The MI obtained for their ETs were not significant among themselves. From these results, it is worth noting that the inhibition of cell division had already occurred at the lowest concentration, 0.25 g L^{-1}, the concentration suggested by the manufacturer on the label of the dye used for this work. It is also important to emphasize that the cell division rate decreased dramatically with increasing ET, for the three concentrations investigated.

Regarding cellular aberrations (Table 2), all the three concentrations studied, at both ET evaluated, showed a statistically significant number of cellular aberrations compared with their respective controls. Within each concentration, the number of aberrations at each ET was not significant to each other. The aberrations observed were micronuclei, gene amplifications, anaphase bridges and telophase bridges.

Table 1. Number of cells in interphase and at different stages of cell division in the cell cycle of *A. cepa* root tips treated with water and Ponceau 4R dye at concentrations of 0.25, 0.50 and 0.75 g L^{-1}, at exposure times of 24 and 48h. 5,000 cells were analyzed for each CO and ET.

Ponceau 4R (g L^{-1})	ET	Cells in Interphase	P	M	A	T	Cells in division	MI (%)
	CO	3163	1284	178	174	201	1837	36.7[a]
0.25	24h	3388	1227	119	105	161	1612	32.2[a]
	48h	4421	465	19	16	79	579	11.6[b]
	CO	3841	834	151	70	104	1159	23.2[a]
0.50	24h	3826	969	123	64	08	1164	23.3[a]
	48h	4511	451	22	12	04	489	9.8[b]
	CO	3688	822	284	88	118	1312	26.2[a]
0.75	24h	4291	400	133	85	91	709	14.2[b]
	48h	4608	215	69	53	55	392	7.8[b]

CO – Control; ET – Exposure time; h – hour; P – prophase; M – metaphase; A – Anaphase; T – Telophase; MI – Mitotic Index. Means followed by the same letter, within the same concentration, are not significantly different at 5% by the χ^2 test.
Source: Pessoal.

Table 2. Total number of cells analyzed, micronucleated cells, gene amplifications and telophase and anaphase bridges, and total number of aberrant cells found in each control and at concentrations of 0.25, 0.50 and 0.75g L^{-1} of Ponceau 4R dye, at exposure times of 24 and 48h. 5,000 cells were analyzed for each CO and ET.

Concentration (g L^{-1})	ET	Micronucleated	Gene amplifications	Metaphase and telophase bridges	Total aberrant cells
	CO	00	00	00	00[a]
0.25	24h	19	31	23	73[b]
	48h	22	21	29	72[b]
	CO	00	00	00	00[a]
0.50	24h	34	22	29	85[b]
	48h	28	17	27	72[b]
	CO	00	00	00	00[a]
0.75	24h	20	20	45	85[b]
	48h	45	20	34	99[b]

CO – Control; ET – Exposure time. Means followed by the same letter are not significantly different at 5% by the χ^2 test.
Source: Pessoal.

Under the conditions studied, therefore, the Ponceau 4R was cytotoxic to root meristematic cells of *A. cepa*. As reported in the literature, Shimada et al. (2010) applied the Comet Assay to evaluate the effect of Ponceau 4R, at 1 and 10 mg kg^{-1} in chronic intraperitoneal treatment, on the bone marrow cells of mice and Wistar rats, and found that the higher concentration caused significant damage to DNA molecules of these organisms, evidencing its genotoxicity. Based on a few studies conducted in the 60s and 80s, Xie et al. (2012) reported that the Ponceau 4R dye, at high concentrations and long exposure, is carcinogenic, teratogenic and mutagenic. Nevertheless, despite the findings of these authors, these were the only reports found on the action of this dye at the cellular level.

Freitas (2012) observed that, in the 90s, the Ponceau 4R dye was a topic of discussion in the food industry worldwide, due to the lack of studies proving its safety to the human body. This controversy has led some states of Japan and the United States to restrict the use of this dye in foods. However, in Brazil, the use of this additive has never been restricted, even with the lack of studies on its toxicity. According to Xie et al. (2012), this situation is worrying, as this author states that almost all foods consumed by Brazilians currently consist of azo dyes, with emphasis on Tartrazine, Ponceau 4R and Red 40 dyes.

Corroborating Freitas (2012), Polônio and Peres (2009) pointed out that although the limits established by national regulatory bodies for the use of food dyes, they do not actually control how much of these additives the food industries add to food and how much the manufacturing industries of these dyes suggest to be added to food. This was verified by Prado and Godoy (2007), who assessed the levels of artificial dyes in food through liquid chromatography and found that almost all artificial food dyes used by the food industry in Brazil, including the Ponceau 4R dye, show levels above those allowed by Brazilian law. They also reported that most processed imported foods marketed in Brazil do not comply with Brazilian law as to the permitted food dye limits.

Thus, the Ponceau 4R dye, as well as all food coloring, must be kept under observation and be periodically reassessed on cells of various test organisms, such as mammals, plants and *in vitro* cell cultures at various concentrations and exposure times. It is expected that cytotoxicity results obtained with this dye in this study arouse the interest of other researchers to evaluate its toxicity. These studies are important because they can greatly assist the regulators and additive manufacturing companies in establishing optimal and safe concentrations for use by the population.

Conclusion

Considering the conditions of this study, the concentrations 0.25 and 0.50 g L^{-1} at 48h ET, and the concentration 0.75 g L^{-1} at 24 and 48h ET, reduced the cell division rate in a statistically significant number of root meristematic cells of *A. cepa*. All concentrations analyzed caused a significant number of cellular aberrations in the cells of this test system. Thereby, under the conditions studied, the tested concentrations of the Ponceau 4R dye were cytotoxic.

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