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Genotoxic Effect of Phenol on the Cells of Onion (*Allium cepa*) Roots

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Abstract: The roots of onion (*Allium cepa*) stand out for having cells with large size and small number of chromosomes. These characteristics make them useful in bioassays for the measurement of a variety of cytogenetic and morphological parameters, in which they can be used as toxicity indicators of the induction and formation of micronuclei and chromosomal aberrations. Based on this background, the potential genotoxic effect of phenol concentration on cells of *A. cepa* roots was investigated either in terms of induced aberrations or micronuclei formation. The results demonstrated that the higher the concentration of phenol, the higher the incidence of abnormalities, thus confirming the genotoxicity of this pollutant.

Keywords: phenol; *Allium cepa*; toxicity; genotoxicity; roots

Introduction

Environmental pollution is a serious and global problem that affects the industrialized societies as well as the natural ecosystems mainly as results of human production activities and resource exploitation[1-2].

A lot of environmental pollutants are known to be carcinogenic, mutagenic, or toxic[3], and among the negative effects caused by chemical agents to exposed organisms, the genotoxicities have been proven to be especially hazardous. Genetic damage can be induced, leading to various health problems and even affecting future generations[4]. Furthermore, many enzymatic reactions as well as exogenous sources such as tobacco, smoke, radiations, auto exhaust emissions and pesticides are responsible for the formation of reactive oxygen species (ROS) as products or by-products. ROS can in turn oxidize nucleic acids, proteins, lipids and carbohydrates[5-7] and accelerate the development of many aging-related pathologies such as cancer and cardiovascular, inflammatory and neurodegenerative diseases[8-10], in addition to exerting a genotoxic effect[11-12].

Toxicity tests require the identification of the compounds that react with DNA[13] as well as the test-species useful to assess the effects of pollutants on both living beings and ecosystems.

According to Grant[14], the higher plants are considered to be excellent genetic models to detect the damage caused by environmental pollutants and

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are often used in monitoring studies to evaluate the mechanisms of genetic damage, ranging from mutations to chromosomal aberrations, in cells of different organs and tissues such as leafs, roots and pollen. Plant bioassays are known to be sensitive to directly active mutagens (such as herbicides, pesticides, heavy metals and radionuclides) and to have a broad detection spectrum[15]. In order to assess the environmental contamination, the most used plant species are *Allium cepa*, *Vicia faba*, *Zea mays*, *Tradescantia* spp., *Nicotiana tabacum*, *Crepis capillaris* and *Hordeum vulgare*[14].

According to Leme and Marin-Morales[16], the *A. cepa* bioassay is one of the most efficient, routinely-used approaches to assess the toxic effects of chemicals on the environment. Compared to other assays, it has some additional advantages including the possibility of measuring macroscopic and microscopic parameters and disturbances in the mitotic cycle, because of the presence of a reduced number ($2n = 16$) of large chromosomes in *A. cepa*[17]. Moreover, this assay allows to assess the action mechanisms of the tested agents on the DNA of the exposed organisms, and the obtained results can serve as a warning to other test systems[16]. Finally, it was successfully used to evaluate the genotoxicity of industrial wastewaters[18] and to assess the biodegradation of oil and other pollutants in contaminated soils and sludges[19-20].

Phenols and their derivatives are compounds commonly found in the environment as products of natural processes, constituents of chemical formulations such as dyes, polymers and drugs, or pollutants either occurring in industrial and municipal sewages or deriving from pesticide degradation. Phenols are ubiquitous environmental pollutants, so they are widely used as reference toxicants in bioassays[21]. Besides their ecotoxicity, peroxidative capacity and ability to generate organic radicals and ROS, phenols are harmful to living organisms. In particular, as far as humans are concerned, phenols were shown to be hematotoxic, hepatotoxic, mutagenic, carcinogenic[22] and even fatal when used in human therapy[23].

Based on this background, the objective of this study is to evaluate the genotoxic effects caused by different concentrations of phenol in phosphate buffer in cells of *A. cepa* roots, mainly in terms of incidence of micronuclei and aberrant cells in both anaphase and telophase.

1 Materials and methods

Sixty-six healthy *Allium cepa* bulbs of uniform size (3.0 to 3.5 cm in diameter, and mass = 15 g ± 400 mg) were obtained from a commercial source. After removal of skin, they were cut and put into glass beakers with their lower ends immersed in mineral water. They were then kept in a BOD incubator (model NT 708, Nova T cnica, Piracicaba-SP, Brazil) at controlled temperature (22 ± 2)℃ and 6/18 hours dark/light photoperiod. After 48 hours, grown roots with 2.0 cm average length were harvested for tests. Phenol solutions, prepared by adding phenol to 0.05 mol L^{-1} phosphate buffer (K_2HPO_4/KH_2PO_4 in distilled water, pH 6.6) up to the desired concentrations (0.01, 0.5, 1.0 mg·L^{-1}), were put into contact with 18 bulbs each. Six of the remaining bulbs were used to prepare the negative control in mineral water, and the other six ones the positive control in a 10 mg·L^{-1} methyl methanesulfonate (MMS) solution prepared using Milli-Q water. Incubations lasted for 48 hours in all tests except for the positive control (6 hours). This procedure was set up based on the international protocol for the use of *A. cepa* as test-species to monitor environmental mutagens and for statistical and comparative purposes, according to which the negative control has to be prepared in mineral water[16, 24]. On the other hand, exposure of roots to phosphate buffer was regarded, according to Ota et al.[25], as an experimental group with no cytotoxic action.

All the roots underwent a recovery period of 48 hours in the solution of Hoagland and Amon[26], which had the following composition in Milli-Q water: 60 mg·L^{-1} $CaSO_4$, 60 mg·L^{-1} $MgSO_4$, 96 mg·L^{-1} $NaHCO_3$ and 4 mg·L^{-1} KCl, and was renewed every 24 hours. After this period, 0.5 cm sized pieces of the apex of 6 roots were separated from each bulb and im-

mediately fixed in 1:3 (v/v) acetic acid-ethanol solution for 24 hours. Roots were washed in distilled water to remove the excess of this solution, hydrolyzed with 1N HCl at 60 C for 8 minutes and stained with aceto-carmine solution (2 g of carmine added to 100 mL of 45% (v/v) acetic acid) for the identification of nuclei, micronuclei and aberrations. The roots were then placed on a slide with the first millimeter removed from their apex, so that the meristematic region corresponding to 2 mm and F1 cells was isolated for analysis by optical microscope (model KF-2, Zeiss, Freiburg, Germany). One thousand cells were counted per slide at 400 (magnification, with 5 slides per treatment and control[4]. The mitotic division stages, aberrant cells in anaphase and telophase, and micronuclei frequency were quantified[4]. The mitotic index (MI) was calculated as the percentage of the total number of dividing cells (1 000 per slide). The analyses of chromosomal aberrations (aberrant cells in anaphase and telophase) and micronuclei frequency were performed according to the method previously described by Grant[27] and the adjustments made by Yildiz et al.[28].

2 Results

Table 1 shows the results of phenol genotoxicity tests on cells of *Allium cepa* roots at different phenol concentrations, namely 0.01, 0.5 and 1.0 mg·L^{-1}. It can be seen that exposed meristematic cells suffered a progressive increase in the frequency of aberrant cells with increasing phenol concentration, in both the anaphase and telophase (Table 1). Such a concentration-dependent action provides a first indication of the genotoxicity of phenol to the root cells of this test-species. The mitotic index (MI), which is well known to express the total number of dividing cells in the cell cycle, is a parameter routinely used to evaluate the cytotoxicity of various agents, and for this reason it was employed for phenol in this study. MI of the negative control (NC) was higher than those of both the positive control (PC) and the treament groups, probably due to phenol interference with the mitotic cell division. Moreover, since treated cells belonged to the apex of the primary root where the meristematic region is located, they exhibited high mitotic activity as a result of hormonal stimulation of cell division just in that region. Either a decrease or an increase in MI is regarded as an important indicator in environmental pollution control especially for the assessment of toxic contaminants that have potential toxicity and cytotoxicity[29]. According to Smaka-Kincl et al.[30], the reduction of MI of *A. cepa* meristematic cells can be considered as a sensitive method to detect cytotoxic agents in the environment, being very reliable for the estimation of the pollution levels.

The *A. cepa* test also allows evaluating the frequency of micronucleated cells, resulting from damages occurring in the DNA molecules. Accordingly, the exposure to phenol was responsible for the additional formation of micronuclei in the experimental groups compared with NC. In particular, since the higher the concentration of phenol, the higher the occurrence of micronuclei in cells of *A. cepa* roots (Table 2), a concentration-dependent trend was observed for this parameter as well.

Table 1　Genotoxic effect of phenol on cells of *Allium cepa* roots at three different concentrations

Phenol Concentration /(mg·L^{-1})	Mitotic Index (Mean±SD)/%	Number of Aberrations		Total Number of Aberrant Cells	Average Percentage of Aberrant Cells (Mean±SD)/%
		Anaphase	Telophase		
NC	13.92±1.90	19	19	38	0.76±0.19
0.01	2.10±0.79	22	3	25	0.54±2.40
0.5	1.57±1.07	37	4	41	1.06±5.60
1.0	1.64±0.62	46	16	62	1.24±4.24
PC	6.14±0.48	62	43	105	2.10±0.12

Note: NC, negative control in mineral water; PC, positive control in 10 mg·L^{-1} methyl methanesulfonate; SD, standard deviation.

Table 2 Number of micronuclei in cells of *Allium cepa* roots treated with different phenol concentrations

Phenol Concentration/(mg·L^{-1})	Number of Micronuclei (Mean±SD)
NC	1.0±0.25
0.01	3.6±0.61
0.5	4.0±0.08
1.0	5.2±0.84
PC	16.4±2.30

Note: NC, negative control in mineral water; PC, positive control in 10 mg L^{-1} methyl methanesulfonate; SD, standard deviation.

3 Discussion

The results showed that a progressive rise in phenol concentration induced an increase in the numbers of cells of *A. cepa* roots that exhibited chromosome aberrations and micronuclei formation, thus indicating the genotoxicity of this pollutant. Nonetheless, both numbers of cells with chromosome aberrations and micronuclei formation were lower than those observed in the positive control (PC), as expected by the well-known carcinogenic activity of methyl methanesulfonate[31].

The negative control (NC) always showed lower occurrence of micronuclei formation and chromosomal aberrations in comparison with phenol-treated samples, except that the average percentage of aberrant cells induced by phenol at the lowest concentration exhibited a value close to that of the NC. This result suggests that a minimum threshold of phenol concentration is probably required to provoke aberrations.

The mutagenic activity of industrial effluents and surface water was demonstrated by a variety of bioassays[32-33], and the presence of potentially genotoxic and mutagenic compounds can be easily detected by the use of *A. cepa*[19]. This is made possible by the fact that the meristematic and F1 cells of this plant have some favorable characteristics to carry out cytogenetic studies. Therefore, they are recommended to evaluate chromosomal aberrations and micronuclei formation for the assessment of environmental pollutants[20]. It is reported that the evaluation and determination of the frequency of micronuclei in *A. cepa* cells is one of the most powerful tools in biomonitoring studies[31,34-36].

The *A. cepa* biossay is considered to be very important in the analysis of the toxic potential of unregulated substances and chemical mixtures that are mainly found in the aquatic environment. In this respect, this method was applied to detect the presence of mixtures of benzene, toluene, ethylbenzene and xylenes (BTEX), which were shown to exert genotoxic and mutagenic effects on meristematic cells, even at low concentrations[37]. *A. cepa* is also reported to be an efficient test-species to evaluate the toxic effects induced by petroleum hydrocarbons such as BTEX, and chromosome aberration and micronucleus tests are found to be reliable tools to detect DNA damage induced by different compounds.

Fatima and Ahmad[38] investigated the potential of 7-ethoxy resorufin O-deethylase (EROD) as a biomarker of pesticide pollution using the *A. cepa* system. The enzyme activity of EROD was analysed after exposure to six model pesticides, viz. 2, 4-dichlorophenoxy acetic acid, hexachlorobenzene, malathion, carbaryl, dichlorodiphenyl trichloroethane and endosulphan, as well as to wastewater samples collected from the industrial areas of Aligarh and Ghaz-iabad cities of Uttar Pradesh, India. *A. cepa* EROD showed high sensitivity between the basal and the induced level in a wide range. Therefore, it was selected as a good biomarker for the detection of certain pesticide residues in water samples. The EROD assay in *A. cepa* system might be of great concern because it would allow to detect the presence of these pesticides in water samples before using expensive analytical techniques such as high performance liquid chromatography or gas chromatography.

Phenols were repeatedly indicated as toxicants for organisms including humans, where the major toxic manifestations include chemical burns, lethargy, coma and cardiac dysrhythmias[39]. Their toxicity has been recently investigated in recombinant bacteria[40], macrophytes[21] and human colonic epithelial cells[41], leading to the hypothesis of the occurrence of a radical-mediated process in bio-

logical interactions involving them[42]. Cytotoxicity of phenols to cultured cells[43], their activity at protein and gene levels[44] and their action on frond multiplication and colony disintegration in aquatic macrophytes[21] were suggested to be concentration-dependent.

Using the *A. cepa* test, Herrero et al.[45] evaluated the toxicity of 5-chloro-2-(2,4-dichlorophenoxy) phenol (triclosan), an anti-bacterial ingredient of many healthcare products and cosmetics that is found in the aquatic environment[46-47]. Triclosan was shown to cause dose-dependent reduction of onion roots length and chromosome stickiness in both anaphase and telophase[44], as well as reduction of their growth[48-49]. In addition, according to Fiskesjo[24], the chromosome stickiness is the most frequent abnormality detected in cells of *A. cepa* roots after triclosan treatment, leading to cell death as well as folding of chromosome fibres into single chromatids[50]. Anaphase and telophase bridges were also observed as the likely result of sticky chromosomes and impaired chromosome segregation, which suggested a mitotic spindle disturbance.

The micronucleus test is a cytogenetic test that allows investigating cells previously exposed to chemical agents, in order to detect possible chromosomal aberrations[51-52]. It is known that micronuclei are formed by a new membrane developing around the chromatin matter that fails to move to either pole of the cell during the mitotic anaphase. Such chromatin matter arises either from anomalous disjunction of chromosomes due to breakage of chromosomes spindle or abnormalities resulting in the formation of chromatin bridges, dicentric chromosomes and acentric fragments. Therefore, the induction of micronuclei suggests that pollutants might act either as spindle inhibitors or clastogens[53].

The results of this work demostrated that the occurrence of micronuclei in cells treated with phenol, a compound occurring in pesticides, was higher at all tested concentrations than that in the NC and always lower than that in the PC. As far as chromosome aberrations are concerned, with the exception of the lowest concentration, the higher the concentration of phenol, the greater the number of aberrant cells of *A. cepa* roots, thus confirming the genotoxicity of this compound.

The lower sensitivity of the chromosomal aberration test compared with the micronucleus one can be explained by the fact that DNA damages, laggards and bridges are more associated with chromosomal aberrations. On the other hand, the induction of micronuclei in *A. cepa* root meristems and the consequent increase in the number of aberrant cells are the likely result of chromosome damage and disturbance of mitotic processes induced by phenol[54].

In general, the results of the present study also suggested that special attention should be paid to the action of emerging contaminants in higher plants, in particular making use of suitable test systems so as to counter any underestimation of their environmental risks.

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