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Cytotoxic and genotoxic effects of silver nanoparticle/carboxymethyl cellulose on *Allium cepa*

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Abstract Several mutagenic agents may be present in substances released in the environment, which may cause serious environmental impacts. Among these substances, there is a special concern regarding the widespread use of silver nanoparticles (AgNP) in several products due to their widely known bactericidal properties, including in the medical field and the food industry (e.g., active packaging). The assessment of the effects of AgNP released in the environment, having different concentrations, sizes, and being associated or not to other types of materials, including polymers, is therefore essential. In this research, the objective was to evaluate the genotoxic and cytotoxic effects of AgNP (size range between 2 and 8 nm) on root meristematic cells of *Allium cepa* (*A. cepa*). Tests were carried out in the presence of colloidal solution of AgNP and AgNP mixed with carboxymethylcellulose (CMC), using distinct concentrations of AgNP. As a result, when compared to control samples, AgNP induced a mitotic index decrease and an increase of chromosomal aberration number for two studied concentrations. When AgNP was in the presence of CMC, no cytotoxic potential was verified, but only the genotoxic potential for AgNP dispersion having concentration of 12.4 ppm.

Keywords Mitotic test · Chromosomal aberration · Silver nanoparticles · Carboxymethylcellulose · *Allium cepa*

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Introduction

Packaging is indispensable for food preservation purposes by retarding food deterioration, increasing food shelf life, and maintaining food quality and safety. Thus, packaging provides protection against three main external classes of spoilage agents: chemical, biological, and physical (Becaro et al. 2015, Marsh and Bugussu 2007).

Coating fruits and vegetables with biodegradable films based on polysaccharides is of great interest to the packaging field due to increasing demands for enhanced food quality and shelf life. Moreover, coating can be an alternative to non-renewable materials conventionally used for the production of plastic films (Mayachiew and Devahastin

2010). Carboxymethyl cellulose (CMC) is a cellulose derivative (ether) usually presented in the form of sodium salt, being an anionic polymer that is soluble at low concentrations, biocompatible, biodegradable, and features great film-forming properties. Also, within the percentages in which CMC is typically used (1 to 5%), it does not cause interference in the sensory properties of the packaged product, being physiologically inert, nontoxic, and widely applied in food and pharmaceutical industries (Hebeish et al. 2013).

One of the promising systems intended to modify the characteristics of polymeric films for food preservation is the incorporation of nanostructures (Shin et al. 2014; Kanmani and Rhim 2014; Moura et al. 2011). Silver-based compounds are among the most promising inorganic antibacterial agents due to their remarkable biocidal effect against several bacteria and fungi. Therefore, these compounds arouse considerable interest in the food packaging field (Becaro et al. 2016; Siqueira et al. 2014; Derbalah et al. 2011).

However, the widespread use of AgNP and other nanostructures in several segments exposes humans and the environment to their effects through inhalation, ingestion, dermic contact, etc (Su et al. 2014; Gambardella et al. 2013; Becaro et al. 2015). Toxic compounds, including mutagenic agents, are present in pollutants that are inappropriately released into the environment, leading to severe impacts to the ecosystem. Thus, researches on their effects are highly demanded (Sobeh et al. 2016; Panda et al. 2011; Yu et al. 2013; Juchimiuk and Maluszynska 2005).

Cytotoxicity and genotoxicity tests are widely known and have been used since the 1920s to evaluate chromosomal aberration frequency of mutagenic potential and chromosomal breakages (Satapathy and Swamy 2013). International and national environmental entities, such as EPA (US Environmental Protection Agency), WHO (World Health Organization), and UNEP (United Nations Environment Programme), recommend the use of plants as test organisms for genetic tests (Ma et al. 1995).

The toxicity test using *Allium cepa* (*A. cepa*) was introduced by Levan in 1938 (Fiskesjö 1985) and has been largely used as a tool for environmental monitoring towards distinct micro- and nanomaterials (Leme and Marin-Morales 2009) due to the high sensitivity compared to other organisms tested. The use of *A. cepa* as test organism is favored by several characteristics such as rapid root growth, low cost, high tolerance to distinguished cultivation conditions, elevated number of cells in division, notable proliferation kinetics, reduced number of large chromosomes, chromosomes in good condition to study damage or disturbances in the division including the assessment of aneuploid risks, as well as easy availability and management (Belcavello et al. 2012; Turkoglu 2012). Therefore, *A. cepa* has shown outstanding position among the tests recommended by law to assess genotoxic agents (Mateuca et al. 2006). For instance, *A. cepa* has been used to evaluate the influence of contaminants in water resources (Christofoletti, Pedro-Escher, Fontanetti 2013; Mazzeo et al. 2015), textile effluent (Caritá and Marin-Morales 2008) sand, soil (White and Claxton 2004), fertilizer (Anacleto, Roberto, Marin-Morales 2017), dyes, and petroleum derivatives (Leme and Marin-Morales 2008), heavy metals (Qin et al. 2016; Deng, Wang, Xin 2016; Vargasa et al. 2017; Rajeshwari et al. 2016) and pesticides (Rodríguez et al. 2015).

Cytotoxicity and genotoxicity tests are based on atypical nuclear standard parameters, which consist of an elevated number of cells with heteromorphic pair of nucleoli. The presented results in these tests can be considered as an indicative that the sample represents also a biological threat for other organisms (Fiskesjö 1985). Its high sensibility allows that no contamination is undetected even in complex mixtures. In a study performed by Pakrashi et al. (2014), *A. cepa* cells were found to present several abnormalities (e.g., chromosomal breakage, alterations in anaphase, and micronuclei formation) upon exposure to 12.50 ppm of TiO_2 NPs. The effects of ZnO NPs on the mitotic index (MI) and chromosomal aberration index were determined through the hydroponic culturing of *A. cepa* by Kumari et al. (2011): *A. cepa* roots were treated with the dispersions of zinc oxide nanoparticles at four different concentrations (25, 50, 75, and 100 ppm). The authors observed that by increasing concentrations of ZnO NPs, MI decreased with the increase of pycnotic cells, while chromosomal aberration index increased. Therefore, it becomes of prime importance to evaluate the toxicity of nanostructured systems in plant models. For this purpose, this research aimed to evaluate the genotoxic (presence of chromosomal aberration) and cytotoxic (cell death and mitotic index) effects of AgNP (size range between 2 and 8 nm) in root meristematic cells of *A. cepa*. Tests were carried out in the presence of colloidal solution of AgNP and AgNP mixed with

carboxymethylcellulose (CMC), using distinct concentrations of AgNP.

Materials and methods

The methodology of the in vivo tests using *A. cepa* seeds to evaluate cytotoxicity and genotoxicity used in this study was based on the procedure proposed by Leme and Marin-Morales (2009). *A. cepa* L. seeds ($2n = 16$) were selected and germinated in ultrapure water at ambient temperature (25 °C). After achieving 1.50 to 2.00 cm in length, the roots were removed from the germination plate, and ten were elected and placed in contact with a colloidal solution of silver nanoparticles of 8–10 nm. The AgNP were synthetized according to Neto et al. (2008) and Mbhele et al. (2003) with concentrations of 1.50 ppm (A) (a less concentrated AgNP dispersion than employed by Pulate et al. (2011) and Kumari et al. (2009)) and 15 ppm (B) (concentration similar to the one employed by Patlolla et al. (2012)) for 24 h, and each vessel contained ten roots (test 1).

In test 2, seeds were deposited in contact with a CMC/(AgNP-PVA) film-forming solution with a silver concentration of 1.24 ppm (C1) and 12.40 ppm (C2) throughout 24 h. Silver nanoparticles were synthetized through a chemical reduction reaction of silver ions (using $AgNO_3$) by a reducing agent ($NaBH_4$) in the presence of a stabilizing agent (PVA). In order to obtain CMC/silver nanoparticle (CMC/AgNP-PVA) film-forming solution, 1% of CMC (*m*/*v*) was added to the silver nanoparticle solution. Ultrapure water was used as negative control, whereas the herbicide trifluralin (0.075 g/L) was used as positive control. After 24 h of contact with the solutions, roots were fixed with the Carnoy reagent (ethyl alcohol and acetic acid at a volume ratio of 3:1) for 6 h at room temperature. Once fixed, a new fixing solution was prepared and added and the rootlets were kept under refrigeration. Subsequently to the fixing period, the roots were removed from the reagent, washed with water, and subjected to acid hydrolysis using a 1 mol/L HCl solution at 60 °C for 9 min. Afterwards, the samples were washed in distilled water and placed in contact with the Schiff reagent for 2 h in an amber glass flask and in the absence of light. Excess dye was removed with distilled water. The root meristematic region was cut with a stainless steel blade, disposed on a glass slide, added by two drops of 2% acetic acid carmine, and covered with a cover slip. For the cell count, ten slides of each sample were selected and analyzed under a bright field light microscope with ×40 objective, in which cell divisions and abnormalities based on nucleus and chromosomal characteristics were observed. In these slides, at least 500 cells were analyzed, totaling approximately 5000 cells, which were used to calculate the mitotic index[1] (MI, Eq. 1) and the chromosomal aberration index (CAI, Eq. 3) according to methodology described by Lima et al. (Lima et al. 2012). These images were collected using an optical microscope (Olympus, model BX 50 DP2BSW). The relative mitotic index[2] (RMI, Eq. 2) and relative chromosomal aberration index (RCAI, Eq. 4) are obtained in relation to the negative control and generally used in graphs for better visualization.

$$\mathrm{MI} = \left(\frac{\textit{Total number of cells in division}}{\textit{Total observed cells}}\right) \times 100 \quad (1)$$

$$\mathrm{RMI} = \left(\frac{\mathrm{MI}}{\mathrm{MI}\ \textit{negative control}}\right) \quad (2)$$

$$\mathrm{CAI} = \left(\frac{\textit{Total number of altered cells}}{\textit{Total observed cells}}\right) \times 100 \quad (3)$$

$$\textit{RCAI} = \frac{\mathrm{CAI}}{\mathrm{CAI}\ \textit{negative control}} \quad (4)$$

Statistical analysis

Results of cytotoxicity and genotoxicity assays were analyzed using the BioEstat 5.8.3.0 software (AnalystSoft) through the non-parametric Mann-Whitney test, intended to compare two independent samples and one variable. The statistical significance analysis was defined as $p < 0.05$ (Christofoletti, Pedro-Escher, Fontanetti 2013).

[1] Mitotic index is the percentage of cells found in one of the four mitosis phases.

[2] Relative mitotic index indicates alterations observed in root growth induced by the tested substance (or material) compared to the negative control.

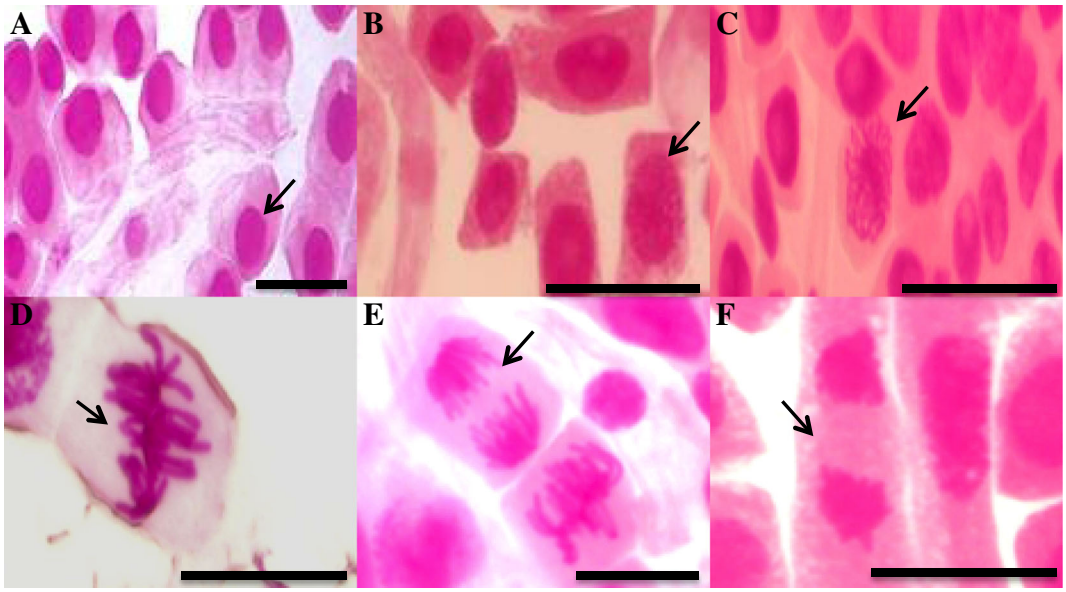

Fig. 1 Normal *A. cepa* meristematic cells. **a** Interphase (*arrow*). **b**, **c** Prophase (*arrow*). **d** Metaphase (*arrow*). **e** Anaphase (*arrow*). **f** Telophase (*arrow*). *Scale bar*—10 μm

Results and discussion

Test 1: effect of silver nanoparticles in the absence of CMC

After the treatments, the cells at the different phases (i.e., prophase, metaphase, anaphase, and telophase) of the cell division cycle (Fig. 1) as well as the number of cells carrying chromosomal aberrations (Fig. 4) were counted and used to calculate the MI (Eq. 1), RMI (Eq. 2) (Table 3), CAI (Eq. 3), and RCAI (Eq. 4) (Table 2).

Table 1 and Fig. 2 show the results obtained for the MI and RMI analyses in *A. cepa* cells after 24 h of exposure in AgNP-containing solutions. Table 1 shows that MI was inferior to concentration A, presenting a value of 5.01.

Regardless of the tested concentrations (1.50 and 15.00 ppm), a cytotoxic effect was induced upon the exposure to nanoparticles, as indicated by the significant reduction of MI of treated cells when compared to the negative control ($p = 0.01$ and $p = 0.04$, respectively) (Fig. 2). According to some authors, the cytotoxicity of AgNPs may occur due to the generation of ROS, fact observed in the study of Cvjetko et al. 2017.

Increase of nanoparticle concentration led to an independent enhance in MI, although Kumari et al. (2009), when studying different AgNP concentrations (25, 50, 75, and 100 ppm), reported that the cytotoxic effect is dependent on the increase in concentration. Some authors have demonstrated that alterations in the number of cell division occur due to the modifications caused by the mitotic cycle duration time with an

Table 1 Mitotic index (MI) and relative mitotic index (RMI) of *A. cepa* meristematic cells for two concentrations of the tested substance, negative control and positive control

Samples	Number of analyzed cells	Mean and SD of cells in division	MI (%)	RMI
Negative control	5235	194 ± 52	14.10	1.1
Positive control	5182	148 ± 20	13.33	1.0
A (1.50 ppm)	5058	43 ± 16	5.01*	0.4
B (15.00 ppm)	5572	59 ± 22	6.40*	0.5

*Statistically significant ($p < 0.05$) in relation to the negative control

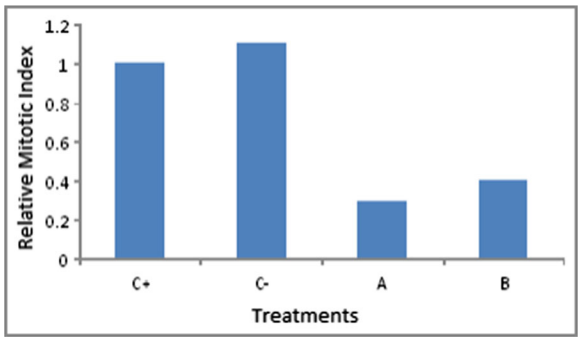

Fig. 2 Different treatments effect in relative mitotic index (RMI) in *A. cepa* cells, where C^+ is trifluralin 0.075 g/L, C^- is ultrapure water, *A* is 1.50 ppm silver nanoparticle, and *B* is 15.00 ppm silver nanoparticle

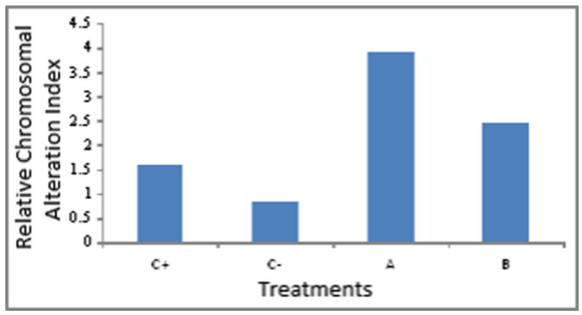

Fig. 3 Effects of different treatments on the relative chromosomal alteration index (RCAI) of *A. cepa* cells, where C^+ is trifluralin 0.075 g L, C^- is ultrapure water, *A* is 1.5 ppm of silver nanoparticles, and *B* is 15 ppm of silver nanoparticles

extension of the cell division S phase (Macleod 1969; Webster and Davidson 1969).

Like cytotoxic effects, significant DNA damages were also observed, as suggested by the comparison of CAI and RCAI values (Table 2 and Fig. 3) with those of the negative control, which characterizes genotoxic effect. However, this effect was only statistically significant for concentration A ($p = 0.006$) and was not observed for concentration B ($p = 0.06$). The CAI value of concentration B (2.0) was higher than that of the positive control (1.61), indicating a remarkable AC induction to concentration B. Nonetheless, the non-significant results may be associated with the standard deviation around the average value. The average number of CAs (chromosomal alterations) found for concentration A (26.80) was considerably higher than the value obtained for concentration B (17.20).

The CA types and amounts found after exposition are presented in Fig. 4. According to (Barbério 2013), contaminants can cause two alteration types: clastogenic, characterized by the breakdown of genetic material (bridges, for instance) and aneugenic, typified by compromising chromosome segregation throughout cell division (C-metaphase and adhesion, to mention a few). As shown in Fig. 4, CAs of both clastogenic (bridges) and aneugenic (chromosomal loss, C-metaphase, and chromosomal adhesion) types were observed.

The modification known as C-metaphase (Fig. 4e), according to (Fernandes et al. 2007), can result from the action of aneugenic agents that promote complete inactivation of mitotic cycle. This alteration, in turn, may generate alterations such as polypoid, multinucleate, and micronuclei cells (Kirsch-Volders et al. 2002). In accordance with Kuriyama and Sakai (1974), the mitotic cycle is impaired by the interaction between AgNPs and tubulin-SH groups.

Chromosomal bridges (Fig. 4b) can result from chromosomal adhesion (Fig. 4h), which in certain cases could be multiples and persist until telophase (Marcano et al. 2004). According to Kumari et al. (2009), chromosomal adhesion can be related to the effect caused by extrachromosomal intertwining of the chromatin fibers. The presence of this alteration is evidence of toxic effect and is considered irreversible.

Chromosomal fragments (Fig. 4b) could result from the interruption of chromosomal bridges (Fiskejo 1993), being chromosomal breakages (Fig. 4d, i) and micronuclei (MN) (Fig. 4c) excellent mutagenicity

Table 2 Chromosomal alteration index (CAI), and relative chromosomal alteration index (RCAI) of *A. cepa* meristematic cells for two concentrations of tested substance, negative and positive controls

Samples	Number of analyzed cells	Mean and SD of altered cells	CAI %	RCAI
Negative control	5235	8.4 ± 3.9	0.85	1.0
Positive control	5182	16.8 ± 4.1	1.61*	2.0*
A (1.50 ppm)	5058	26.8 ± 13.9	3.2*	3.9*
B (15.00 ppm)	5572	17.2 ± 11.7	2.0	2.4

*Statistically significant ($p < 0.05$) compared to the negative control

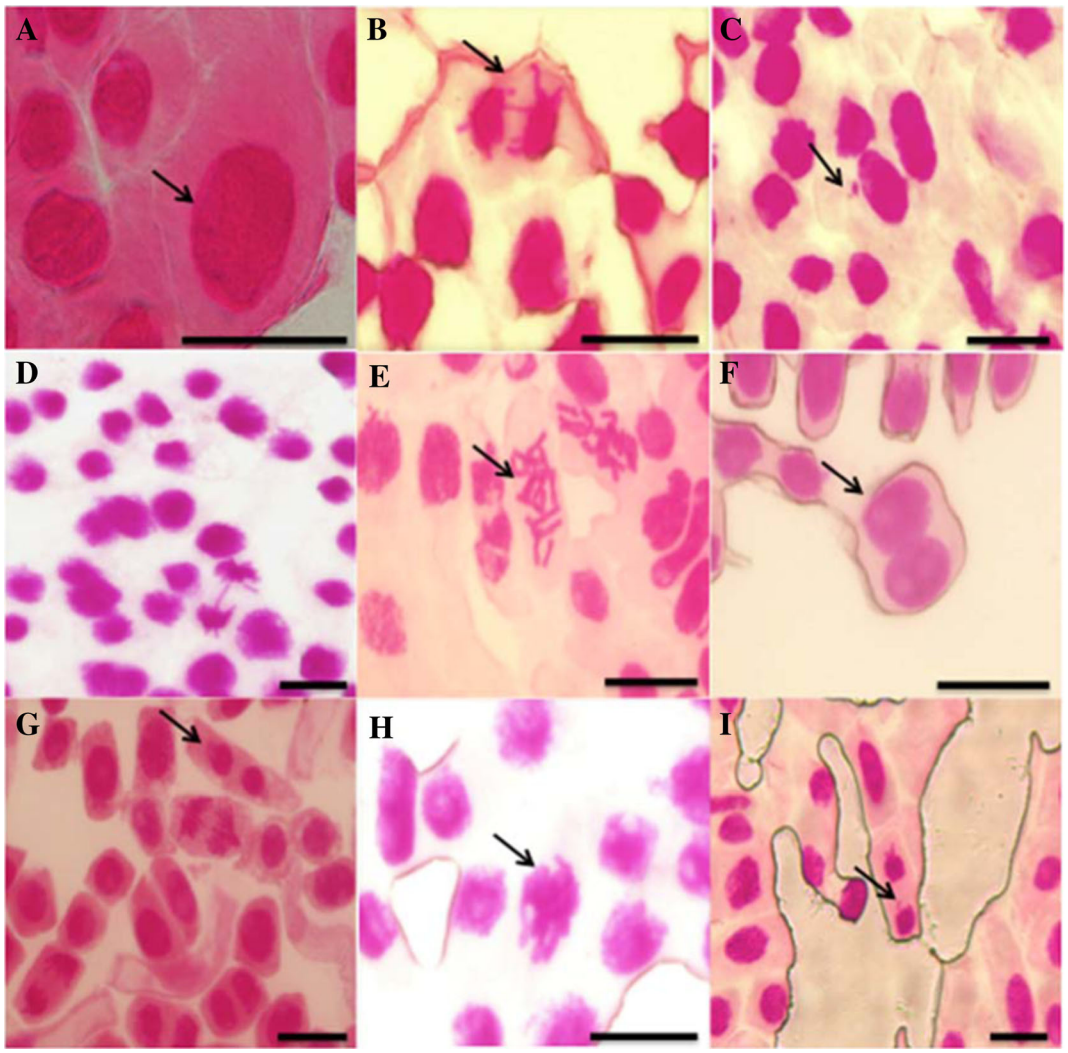

Fig. 4 Chromosomal aberrations found in *A. cepa* meristematic cells after exposure to AgNPs. **a** Polypoid cells (*arrow*). **b** Anaphase with bridge and chromosomal fragment (*arrow*). **c** Cell with micronuclei. **d** Belated anaphase and chromosomal fracture. **e** C-metaphase. **f** Binuclear cell. **g** Telophase with sprout (*arrow*). **h** Cell with chromosomal adhesion (*arrow*). **i** telophase with chromosomal fracture (*arrow*). *Scale bar*—10 μm

endpoints. Because such genetic material amendments cannot be repaired by cells, these may, therefore, be transmitted to new cell generations (De Campos Ventura et al. 2008). Consequently, MN are rarely reincorporated to chromosomal set after division by the mitotic cycle inactivation (aneugenic event—loss of

Table 3 Mitotic index (MI) and relative mitotic index (RMI) of *A. cepa* meristematic cells for both concentrations of the tested substance, negative control and positive control

Samples	Number of analyzed cells	Mean and SD of altered cells	MI (%)	RMI
Negative control	5235	132.1 ± 35.3	13.3	1.0
Positive control	5182	148.2 ± 28.8	14.2	1.1
C1 (1.24 ppm Ag)	5157	114.1 ± 35.1	11.1	0.8
C2 (12.40 ppm Ag)	5203	97.4 ± 20.3	9.28	0.7

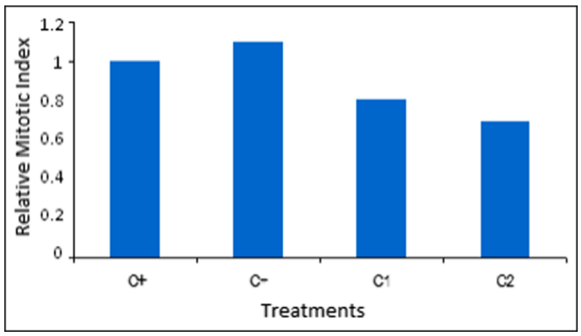

Fig. 5 Effect of different treatments on the relative mitotic index (RMI) of *A. cepa* cells, where C^+ is trifluralin 0.075 g/L, C^- is ultrapure water, C1 is CMC/AgNP-PVA 1.24 ppm Ag, and C2 is CMC/AgNP-PVA 12.40 ppm Ag

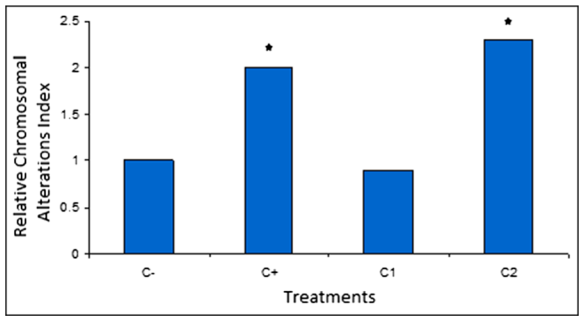

Fig. 6 Effect of different treatments in the relative chromosomal alteration index (RCAI) of *A. cepa* cells, where C^+ is triflurin 0.075 g/L, C^- is ultrapure water, C1 is CMC/AgNP-PVA 1.24 ppm Ag, and C2 is CMC/AgNP-PVA 12.40 ppmAg. * Statistically significant ($p < 0.05$) compared to the negative control

entire chromosomes) or chromosomal breakages (clastogenic events—loss of acentric fragments) (Fenech 2000). The induction of chromosomal breakages by AgNP indicates its clastogenic potential, which may engender the loss of genetic material (Cuicai et al. 1992).

Kumari et al. (2009) and Nair et al. (2010) reinforced the need for evaluating the potential citotoxic and genotoxic effects of AgNPs, taking into account nanoparticle properties (absorption, distribution, and translocation) in plant cell tissues. For instance, in certain plants, few negative effects are observed, including plant biomass and transpiration decreases. These outcomes were observed by Stampoulis et al. (2009) when studying the germination and growth of squash seeds in a AgNP-containing hydroponic solution. In contrast, effects related to necrosis, senescence, and cellular death are also noticed in plants as a result of the effect caused by the presence of Ag^+ (Navabpour et al. 2003).

In 2011, Park et al. (2011) verified that the citotoxic and genotoxic effects of AgNPs are correlated with their sizes, which results corroborate with the findings reported by Lima et al. (2013): AgNPs of 20 nm in diameter showed more toxic effects than their counterparts with diameters from 80 to 113 nm.

Test 2: analysis of silver nanoparticle effects in the presence of the polymer CMC

The cytotoxicity assessment tests did not indicate cell death induction. Taking the relative mitotic index calculations into account, no cytotoxic action of the AgNP-containing samples was observed. Therefore, the indices were not significantly decreased (Table 3 and Fig. 5).

Genotoxic effect analysis pointed out significant results after the exposure of *A. cepa* seeds to the herbicide trifluralin and to the highest tested AgNP-PVA concentration (Table 4 and Fig. 6).

Using *A. cepa* as a test organism for evaluating the cytotoxic and genotoxic effects of CMC/(AgNP-PVA) film-forming solutions allowed us to detect significant results for the highest AgNP-PVA concentration, which led to an enhanced chromosomal alteration index. Nonetheless, the lowest AgNP-PVA concentration caused no significant alterations when compared to the negative control, indicating that these materials were less toxic

Table 4 Chromosomal alteration index (CAI) and relative chromosomal alteration index (RCAI) of *A. cepa* meristematic cells for both tested substance concentrations, negative control and positive control

Samples	Number of analyzed cells	Mean and SD of altered cells	CAI %	RCAI
Negative control	5235	8.4 ± 3.9	0.82	1.0
Positive control	5182	16.8 ± 4.1	1.61*	2.0*
C1 (1.24 ppm Ag)	5157	7.5 ± 3.4	0.72	0.9
C2 (12.40 ppm Ag)	5203	19.6 ± 4.5	1.92*	2.3*

*Statistically significant ($p < 0.05$) compared to the negative control

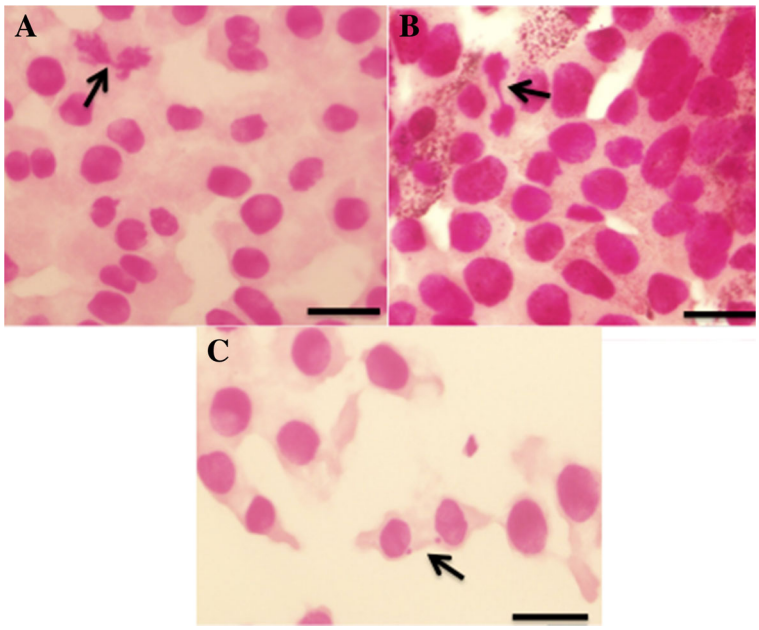

Fig. 7 Chromosomal aberrations in *A. cepa* meristematic cells upon exposure to film-forming solutions containing silver nanoparticles. **a** Anaphase with bridge. **b** Telophase with bridge. **c** Cell with micronuclei. *Scale bar*—10 μm

for *A. cepa* cells. The chromosomal alteration types encountered are presented in Fig. 7.

Among the registered aberrations, anaphase chromosomal bridges showed the highest occurrence in all treatments. The chromosomal bridges that appeared in the anaphase may have been originated from previous structural changes between either the chromatids of two different chromosomes or among the chromatids of the same chromosome. The chromosomal bridge induction mechanisms by agents that cause DNA damage in cells with normal telomeres are still not elucidated, but such mechanisms have been associated to defects in the repair system of DNA double strand (Zhu et al. 2002). Because the formation of anaphase chromosomal bridges was the main alteration observed experimentally, one may infer that AgNP-PVA at a concentration of 12.40 ppm features clastogenic properties.

Conclusion

AgNPs having size range between 2 and 8 nm for conditions in test 1 (AgNPs with no CMC) presented potential cytotoxic and genotoxic (clastogenic and aneugenic) effects at low silver nanoparticle concentrations (1.50 and 15.00 ppm). In test 2 (AgNPs associated with CMC), however, no cytotoxic effect was verified in any of the studied concentrations, although a clastogenic-type genotoxic potential was observed for 12.40 ppm of AgNp in the presence of CMC. The toxicity demonstrated here may interfere in intracellular components and lead to several problems during cell division. Because of the possibility of inducing harmful effects to the genetic material of the studied organism, the results indicate that, depending on the concentration and the combination with distinct materials, AgNP can be detrimental to the environment, which requires the control of the disposal of materials and packaging containing silver nanoparticles.

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