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Cytotoxic evaluation of sodium hypochlorite, using *Pisum sativum* L as effective bioindicator

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ABSTRACT

The objective of this study was to evaluate the cytotoxic effect of different sodium hypochlorite concentrations, using apical root cells of *P. sativum* as a bioindicator. Initially, the seeds of *P. sativum* were exposed to different concentrations of sodium hypochlorite (0.1, 0.2, 0.4, 0.8, 1.6, 2 mg L^{-1}) and to a control solution based on distilled water. Next, root growth was measured during 24, 48 and 72 h. Subsequently, the mitotic index (MI) and cellular anomalies (5000 cells per treatment) were determined at 72 h. According to the results obtained, a decrease in root growth was observed at concentrations of 0.4, 1.6 and 2 mg L^{-1}. Likewise, it was evident that, among all the evaluated concentrations, an inhibition of mitosis higher than 50% was presented. Additionally, chromosomal anomalies were also generated, such as Nuclear notch, lagging chromosomes and Chromosomal break, which were present in all the concentrations evaluated. In addition, the presence of micronuclei at concentrations of 2.0 and 1.6 mg L^{-1} indicate that sodium hypochlorite is a highly cytotoxic substance. Therefore, *P. sativum* is a specie that offers a feasible experimental model to be implemented in the laboratory with the aim to evaluate the cytotoxic effect of any cytotoxic substance.

1. Introduction

Plants play an important role as bioindicators and are the ones that by modifying their structure or function, may present sensitivity to environmental changes and react to them as stimuli. The genetic structure of higher plants is extremely useful for monitoring and distinguishing toxic substances and contaminants in ecosystems (Ghosh et al., 2017; Pérez et al., 2017). Vascular plants are widely recognized as excellent genetic models to evaluate and detect compounds with morpho-toxic, cytotoxic and genotoxic potential (Fatma et al., 2018; Leme and Marin-Morales, 2009; Sommaggio et al., 2018), thus within this context, some terrestrial plants have been identified as bioindicators of toxins presence in both water and soil, among which are mainly, *Allium cepa, Vicia faba, Zea maiz* and *Lactuca sativa* (Dutta et al., 2018; Manrrique et al., 2011; Martins et al., 2016; Pérez et al., 2017).

Currently, the *A. cepa* test is the most widely used for the diagnosis of toxicity of various pharmaceutical, food, detergent and contaminant compounds (Garcia et al., 2017; Olusegun et al., 2010; Pedrazzani et al., 2012; Sommaggio et al., 2018), this is due to the fact that roots grow in direct contact with the substances of interest (Tedesco and Laughinghouse, 2012) and contain a high proportion of cells in mitosis (Prajitha and Thoppil, 2016). This allows the substance under study to be absorbed in a rapid and constant way during cell growth, making it easy to distinguish some chromosomal anomalies, product of the mutagenic agent (Ragunathan and Panneerselvam, 2007). On the other hand, worldwide sodium hypochlorite is used on a large scale in industries such as agriculture, chemical industries, food industries, pharmaceuticals among others, due to this compound presents disinfectant properties, having as main objective the destruction of harmful to health microorganisms (Manrrique et al., 2011). Likewise, in the asymbiotic germination of orchids, sodium hypochlorite is used for disinfection, scarification and to stimulate the germination of seeds in concentrations from 0.5% to 3% (Salazar and Cancino, 2012; Salazar, 2012; Salazar et al., 2013; Salazar and Vega, 2017). However, different authors have reported different genic effects that oxidants such as sodium hypochlorite can present in different tests carried out with plants and animals (Manrrique et al., 2011). This may be due to the fact that it is a highly toxic compound and may present tissue effects if it is not handled in a correct manner (Juárez and Lucas, 2001). In the plants, when the roots are exposed to toxic substances, it produces alterations

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in their morphology. The variation degree will depend on the type of substance and the exposure time (Khanna and Sharma, 2013). Consequently, the evaluation of the radicle and hypocotil development work as representative indicators to determine the plant´s capacity of establishment and development (Castillo, 2004).

A research carried out by Causil et al. (2017), evaluated the cytotoxic effect of sodium hypochlorite (NaClO) on apical cells of *A. cepa* roots. They found that, at concentrations of 5, 2, 1, mg L^{-1}, cellular abnormalities and inhibition of cell division occurred. Such anomalies can be present due to sodium hypochlorite has a cytotoxic and genotoxic potential in the plant cell cycle, since it blocks the G2 phase of the cell cycle (Haq et al., 2017) and inhibits the DNA synthesis (Berrocal et al., 2013), thus avoiding the normal development of the cell cycle (Ruíz et al., 2018).

Taking into account the above and because, in recent years, bioassays have gained interest in the field of research to evaluate the potential phytotoxic, cytotoxic and genotoxic effects of different compounds (Abdelsalam et al., 2018; Asare et al., 2012; Biruk et al., 2017; Bortolottoa et al., 2017; Kotelnikova et al., 2019; Liman et al., 2019). The present study aims to evaluate the cytotoxic activity of sodium hypochlorite in apical cells of peas roots (*P. sativum*) through a series of indicators such as: the number of cells in cell division, mitotic index and the frequency of cellular anomalies, taking into account the seeds of *P. sativum* as an effective alternative for cytotoxic and genotoxic studies.

2. Material and methods

2.1. Area of study

The present study was carried out in the Basic Sciences laboratory from the Francisco de Paula Santander University, which is located at 7°54′01.1″N and 72°29′15.6″W with a height of 297 masl, in Norte de Santander capital city, Cucuta. According to the Institute of Hydrology, Meteorology and Environmental Studies (IDEAM), the average temperature is 27.5 °C, with a relative humidity that ranges between 62% and 77%.

2.2. Test conditions

Six NaClO solutions diluted in distilled water were prepared at different concentrations (0.1, 0.2, 0.4, 0.8, 1.6, 2 mg L^{-1}) and one control solution based on distilled wáter. For the germination process, the seeds were arranged in a Petri dish with cotton together with their respective solution, Under controlled environmental conditions (15 ± 2 °C in darkness); once the growth of the radicle started, the growth of the radicle was registered at 24, 48 and 72 h. After 72 h, several tests were performed for mitosis in the root tips, the mitotic index was calculated, and finally the identification of cellular anomalies present. Ten pea seeds were needed per treatment.

2.3. Microscopic analysis

Mitosis tests were performed at 72 h, the root tips were cut 3–5 mm approximately, and immersed in hydrochloric acid for 15 min to break the cell walls (Causil et al., 2017). The staining was performed with Acetate-Orcein for 24 h. Once stained, the roots were arranged on slides and covered with a coverslip, where the squash technique was performed; with the yolk of the thumb a uniform pressure was exerted so that the cells dispersed and made visible each of the cellular phases (Valladolid et al., 2014). The prepared samples were analyzed by scanning electronic microscopes at 40× and 100×.

2.4. Mitotic index

For the evaluation of the mitotic index, 1000 cells per repetition and 5000 cells per treatment were analyzed. The evaluation was carried out using the following formulas: general mitotic index (MIg) = number of cells in division / number of total cells; mitotic index prophase (MIp) = number of cells in prophase / number of dividing cells; metaphase mitotic index (MIm) = number of cells in metaphase / number of dividing cells; anaphase mitotic index (MIa) = number of cells in anaphase / number of dividing cells; mitotic index telophase (MIt) = number of cells in telophase / number of dividing cells (Causil et al., 2017).

2.5. Cellular anomaly index calculation

The cellular anomalies are any abnormality or irregularity that occurs both in number and structure of the chromosomes. The appearance of these cellular anomalies is determined by the increase in concentrations of mutagenic agents such as NaClO (Dutta et al., 2018). For the calculation of anomalies present in this investigation the methodology proposed by (Akinboro et al., 2011) was used.

$$\text{Relative abnormality rate}(\%) = \frac{\text{Total number of abnormal cells}}{\text{Total number of cells observed}} \times 100$$

2.6. Experimental design and statistical analysis

The root growth analysis was carried by means of a factorial design (6 NaClO concentrations, one control and three exposure times: 24, 48 and 72 h) with 5 repetitions as well. As for the analysis of mitotic index and cellular anomalies, it was carried out by means of an experimental design of completely random blocks (6 NaClO concentrations, one control and a single exposure time of 72 h). The obtained data were evaluated using an analysis of variance (ANOVA) and a multiple range test through the of Tukey's HSD (Honestly Significant Difference) test, using the Statgraphic Centurion® version 17 software.

3. Results and discussion

3.1. Root growth

The exposure of roots to chemical substances produces alterations in their normal characteristics, such as shape, length and color. This degree of root alteration will depend basically on the nature and toxicity of the substances and the time they remain exposed. In this way, if there is a decrease in root growth above 45%, it indicates the presence of substances with toxic ffects on the plants, causing not only root growth delay, but also causing the cells to show genetic alterations (Khanna and Sharma, 2013).

Thus, during the exposure times 24, 48 and 72 h in which the pea seeds were maintained at different concentrations of sodium hypochlorite, it was observed that the highest root growth after 72 h was presented in the concentrations of 0.1 and 0.2 mg L^{-1} respectively. Likewise, it is possible to observe that for treatments with concentrations of 0.4 and 1.6 mg L^{-1} after the first 24 h had elapsed they had not presented any root growth (Table 1).

Through statistical analysis it was observed that there are statistically significant differences ($P \leq 0.05$) in treatments with concentrations of 0.4; 1.6 and 2 mg L^{-1}, which indicates that sodium hypochlorite inhibited root growth (Table 1).

These results differ from those reported by Causil et al. (2017), who in their test of sodium hypochlorite cytotoxic effect (NaClO), on apical cells of onion roots (*A. cepa*) they did not report significant differences in root growth for any of the evaluated concentrations. Likewise, these also contrast with the results obtained by Manrique et al. (2011) where only inhibition percentages of 45% were reported in higher concentrations of 5 mg L^{-1} of sodium hypochlorite in onion roots. On the other hand, these results do agree with the ones reported by Fusconi et al. (2006) in the cytotoxicity test with cadmium, they reported inhibition of roots of *P. sativum*, in which the degree of inhibition of root

Table 1
P. sativum root growth subjected to different concentrations of sodium hypochlorite.

Sodium Hypochlorite concentration: NaClO (mg L^{-1})	Root length (cm) 24 h	48 h	72 h
0	1.2 ± 0.29[a]	2.78 ± 0.22[a]	4.0 ± 0.30[a]
0.1	1.18 ± 0.34[a]	2.34 ± 0.46[a]	4.02 ± 0.69[a]
0.2	1.48 ± 0.36[a]	2.7 ± 0.50[a]	4.0 ± 0.38[a]
0.4	0[b]	1.0 ± 0.14[b]	2.84 ± 0.11[b,c]
0.8	1.22 ± 0.04[a]	2.9 ± 0.2[a]	3.8 ± 0.37[a]
1.6	0[b]	0.82 ± 0.16[b]	2.62 ± 0.27[b]
2	1.02 ± 0.49[a]	2.68 ± 0.51[a]	3.6 ± 0.29 [a,c]

The means ± SD values with different letter of each column indicate statistically significant differences, according to Tukey HSD test ($P \leq 0.05$). SD = Standard deviation.

growth was directly related to cadmium concentration, being the highest concentration of cadmium, the responsible of almost complete detection of root growth.

3.2. Mitotic Index

The mitodepressive action of sodium hypochlorite is evident. It was also found that at a higher concentration of chlorine the mitotic indexes were decreasing with statistically significant differences with respect to the control (Table 2). The control group was recorded with the highest general mitotic index value (19.2). In contrast, the lowest value corresponded to the concentration of 2 mg L^{-1} (6.0) with significant differences compared to the control group (Table 2).

The mitotic index (MI) has been widely used as a parameter to evaluate the cytotoxicity of several agents (Aybar and Zabala, 2017), this is because the levels of cytotoxicity of certain substances can be determined by the increase or decrease of MI (Fernandes et al., 2007). According to Hoshina (2002), MI significantly lower than those of the control treatment, can indicate alterations derived from the chemical action in the growth and development of the exposed organisms. On the other hand, MI higher than the control group results from an increase in cell division, affecting cells, since it leads to a disorganized cell proliferation and even to the formation of tumoral tissues.

Taking into account the results present in Table 2, it is possible to observe that the concentration that presented higher general mitotic index was the control treatment. The other concentrations presented a significant statistical difference for the general mitotic indexes, prophase and metaphase.

These results agree with the research carried out by Causil et al. (2017), where it is reported that in concentrations of 5, 2, 0.5 and 0.2 mg L^{-1} of sodium hypochlorite there is inhibition of the process of mitosis in *A. cepa*. The decrease in MI suggests a suppression of mitotic activity in pea, this is due to the fact that the components of sodium hypochlorite have a cytotoxic and genotoxic potential in the cell cycle of plants, blocking the G2 phase of the cell cycle (Haq et al., 2017) and inhibiting DNA synthesis (Berrocal et al., 2013), thus preventing the normal development of the cell cycle (Ruíz et al., 2018).

Table 3
Percentage of mitosis inhibition of *P. sativum* root cells, submitted to different concentrations of sodium hypochlorite.

Sodium hypochlorite concentration: NaClO (mg L^{-1})	Inhibition of mitosis
Contraste (Control)	–
0.1	55.2%
0.2	62.5%
0.4	57.2%
0.8	62.5%
1.6	61.45%
2	68.75%

Next, Table 3 evidence that the concentration where the highest percentage of cellular inhibition present was that of 0.2, 0.8 and 2 mg L^{-1} (Table 3). However, it is important to highlight that for all concentrations they were above 50% cell inhibition, which indicates that sodium hypochlorite is directly related to this inhibition.

3.3. Cellular anomalies

As comparison parameters between the normal phases of mitosis and cellular anomalies. Afterwards, in Fig. 1, its shows the interface, prophase, anaphase metaphase and telophase from *P. sativum* root cells found in the control.

In the present study, the presence of cellular anomalies was observed in all the evaluated concentrations except the control (Fig. 2; Table 4). However, the most frequent were those with nuclear cleft, lagged chromosomes and split chromosomes, which were present in all the evaluated concentrations (except the control). On the other hand, the treatment with the concentration of 2 mg L^{-1} presented the greatest number and variety of anomalies, being micronuclei and hyperchromasia the cellular anomalies of greater frequency with 15 and 9 respectively (Table 4).

Cellular anomalies are characterized by changes in structure or in the total number of chromosomes, which can occur spontaneously or as a result of physical exposure to a chemical agent (Hemachandra and Pathiratne, 2015; Restrepo et al., 2012; Russel, 2002). Structural chromosomal alterations can be induced by several factors, such as DNA breaks, inhibition of DNA synthesis and altered DNA replication (Morais-Leme and Marin-Morales, 2009). To evaluate the chromosomal anomalies, the different phases of the dividing cell (prophase, metaphase, anaphase and telophase) must be considered. However, this analysis is not simple to carry out, since it requires an accurate knowledge of the phases of cell division and their possible anomalies (Rank and Nielsen, 1993). Currently, the root tips of various plant

Table 2
Mitotic indexes of the cells from *P. savatum* tip of the roots, submitted to different concentrations of sodium hypochlorite. (5000 cells per treatment).

Sodium hypochlorite concentration: NaClO (mg L^{-1})	Mitotic index				
	GMI	PMI	MMI	AMI	TMI
0	19.2 ± 2.48[a]	11.6 ± 1.8[a]	4.4 ± 1.94[a]	1.8 ± 1.30[a]	1.4 ± 1.14[a]
0.1	8.6 ± 2.07[b]	5.2 ± 1.09[b]	0.6 ± 0.54[b]	1.4 ± 0.54[a]	1.6 ± 0.89[a]
0.2	7.2 ± 1.92[b]	3.2 ± 0.83[b,c]	1.8 ± 1.30[b]	1.4 ± 1.14[a]	0.8 ± 0.44[a]
0.4	8.2 ± 0.83[b]	2.4 ± 0.54[c]	2.0 ± 0.70[b]	2.2 ± 0.83[a]	1.6 ± 0.54[a]
0.8	7.2 ± 1.92[b]	3.4 ± 1.5[b,c]	1.0 ± 0.7[b]	1.4 ± 0.54[a]	1.4 ± 0.89[a]
1.6	7.4 ± 1.8[b]	3.0 ± 1.5[b]	1.6 ± 0.54[b]	1.8 ± 0.83[a]	1.0 ± 1.0[a]
2	6.0 ± 3.16[b]	3.4 ± 1.6[b,c]	0.6 ± 0.54[b]	1.0 ± 0.70[a]	1.0 ± 0.70[a]

The means ± SD values with different letter of each column indicate statistically significant differences, according to Tukey HSD test ($P \leq 0.05$). SD = Standard deviation. **GMI** = General mitotic index. **PMI** = Profhase mitotic index. **MMI** = Metaphase mitotic index. **AMI** = Anaphase mitotic index. **TMI** = Telephase mitotic index.

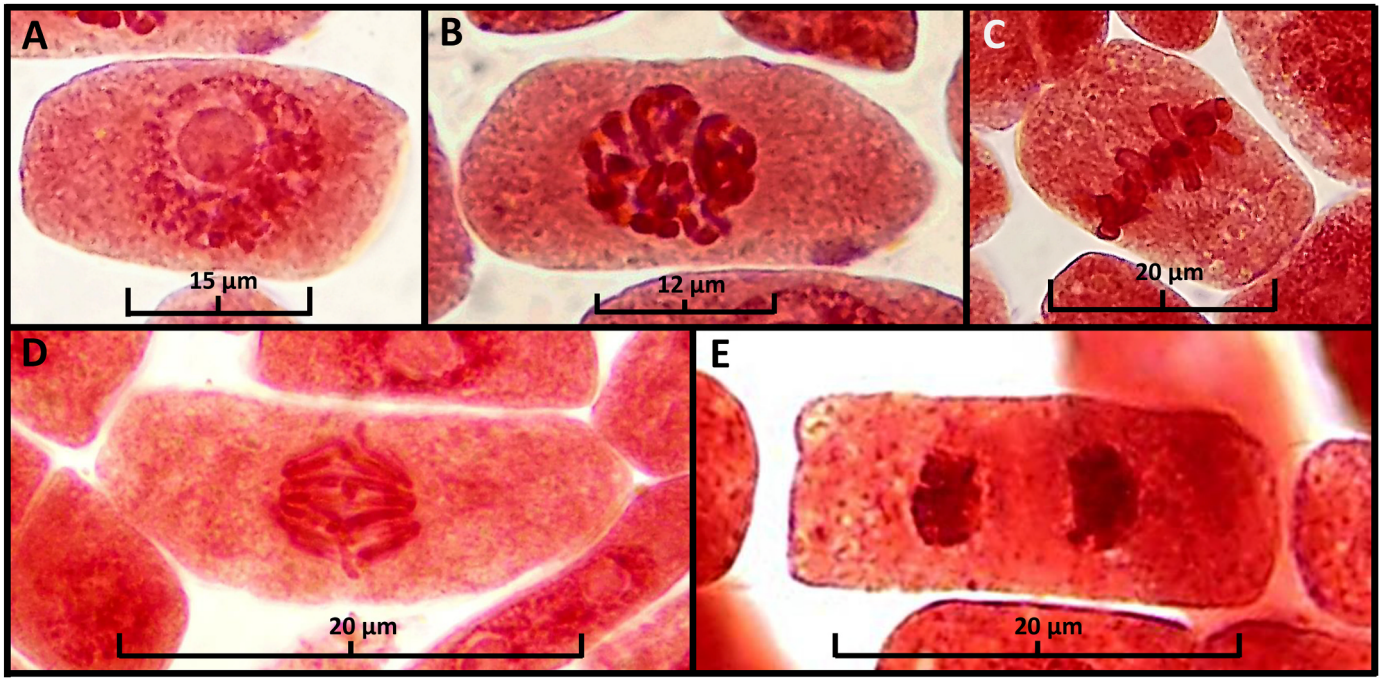

Fig. 1. Mitosis normal stages of *P. sativum* root cells. (A) Interphase. (B) Prophase. (C) Metaphase (D) Early anaphase (E) Telophase.

species are used to study chromosomal aberrations (CAs) and the presence of micronuclei (Mn); due to the fact that the root meristems contain a high proportion of cells in mitosis, which facilitates the incidence of toxic contaminants in plant cells (Prajitha and Thoppil, 2016). Pollutants are those that are capable of causing damage to the genetic material, because they have a direct action on the genetic material causing changes in the structure of the chromosome (Silva et al., 2018).

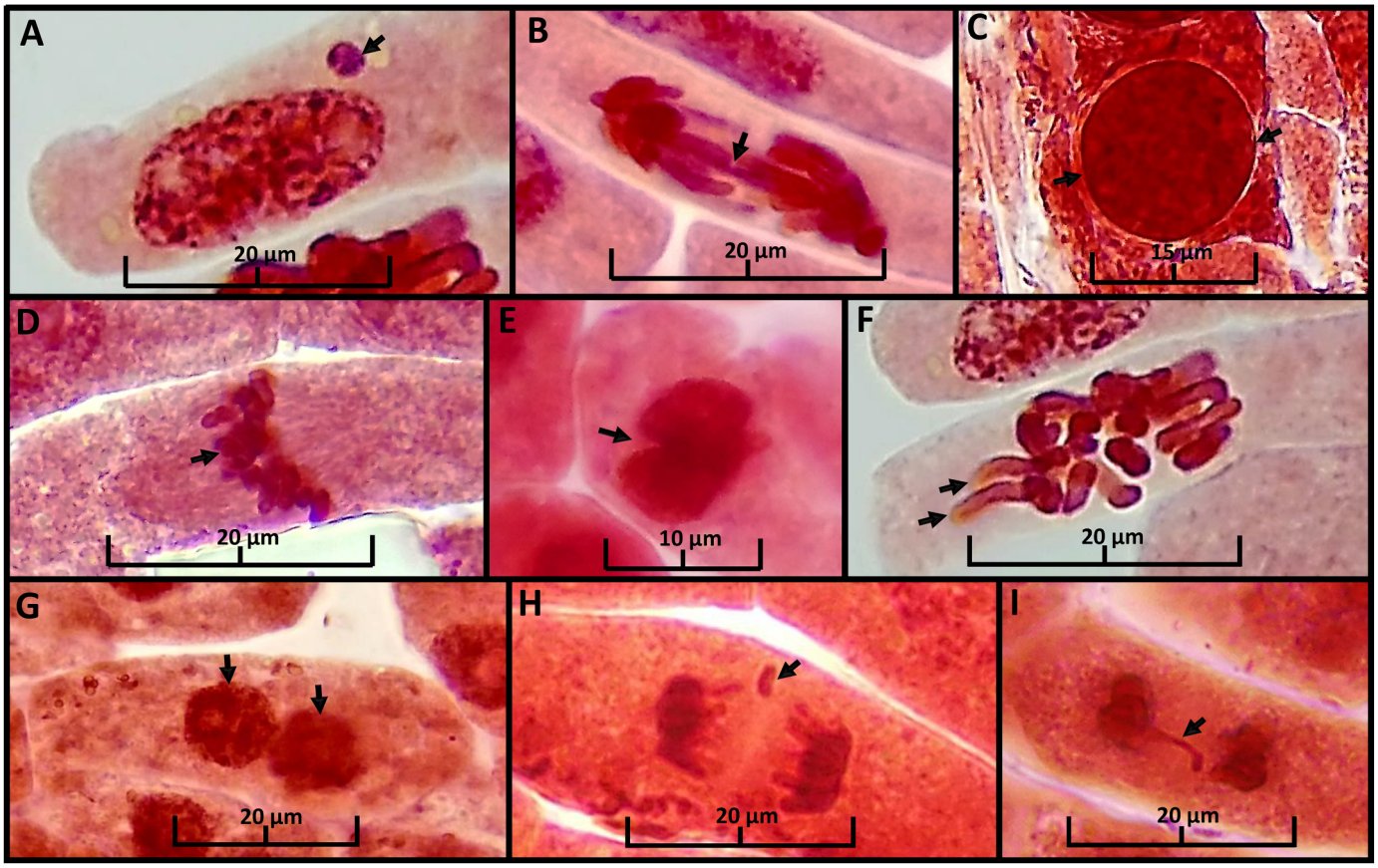

Fig. 2. Abnormalities in dividing cells of *P. sativum* root tip cells treated with different concentration of Sodium Hypochlorite. (A) Micronuclei. (B) Anaphase bridge. (C) Hyperchromasia. (D) Sticky chromosomes (E) Nuclear notch. (F) Lagged chromosomes (G) Binucleate cells (H) Split chromosomes. (I) Telophase bridge.

Table 4
Frequency of Chromosomal Anomalies at *P. sativum* tip of the root treated with different concentrations of sodium hypochlorite. The data are averages of 5 repetitions (5000 cells per treatment).

Concentración de Hipoclorito de sodio (mg L^{-1})	Frequency of Chromosomal Anomalies (Mean ± SD)								
	Mn	AP	HP	SC	Nn	LC	BC	SC	TB
0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0	0^{a}	0^{a}	0^{a}
0.1	0^{a}	0^{a}	0^{a}	$1.0 \pm 0.7^{a,b}$	0^{a}	2.0 ± 0.70^{a}	0^{a}	$1.0 \pm 0.7^{a,b}$	1.0 ± 1.2^{a}
0.2	0^{a}	4.0 ± 2.44^{c}	0^{a}	3.0 ± 0.7^{b}	0^{a}	3.0 ± 2.23^{a}	$1.2 \pm 0.8^{a,b}$	$3.0 \pm 1^{a,b,c}$	0^{a}
0.4	0^{a}	$1.0 \pm 0.7^{a,b}$	0^{a}	$1.2 \pm 1.3^{a,b}$	0^{a}	1.2 ± 0.44^{a}	0.4 ± 0.8^{a}	$3.8 \pm 1.9^{b,c}$	0.6 ± 0.54^{a}
0.8	0^{a}	$1.0 \pm 1.0^{a,b}$	0^{a}	3.0 ± 1.8^{b}	0^{a}	3.0 ± 1.87^{a}	$1.0 \pm 0.7^{a,b}$	$4.0 \pm 2.2^{b,c}$	0^{a}
1.6	1.0 ± 0.7^{a}	$0.8 \pm 0.83^{a,b}$	4.0 ± 2.64^{b}	$1.4 \pm 1.1^{a,b}$	1.2 ± 1.09^{b}	3.0 ± 2.82^{a}	$1.0 \pm 0.7^{a,b}$	4.2 ± 1.9^{c}	0^{a}
2	15 ± 3.5^{b}	3.0 ± 2.34^{c}	9.0 ± 2.54^{C}	3.0 ± 1.2^{b}	2.0 ± 0.70^{b}	3.0 ± 1.58^{a}	2.0 ± 1.0^{b}	$3.0 \pm 1^{a,b,c}$	1.0 ± 0.7^{a}

The means ± SD values with different letter of each column indicate statistically significant differences, according to Tukey HSD test ($P \leq 0.05$). SD = Standard deviation. **Mn** = Micronuclei. **AP** = anaphase bridge. **HP** = Hyperchromasia. **SC** = Sticky chromosomes. **Nn** = Nuclear notch. **LC** = Lagged chromosomes. **BC** = Binucleate cells. **SC** Split chromosomes. **TB** = Telophase bridge.

Table 5
Relative abnormality rate for each concentration of sodium hypochlorite. (mean ± standard deviation).

Sodium hypochlorite concentration (mg L^{-1})	Relative abnormality rate
Contraste (Control)	0.0
0.1	0.55 ± 0.86
0.2	1.57 ± 1.98
0.4	0.91 ± 1.39
0.8	1.2 ± 1.8
1.6	1.84 ± 2.0
2	4.55 ± 4.6

The anomalies here reported coincide with those found by several authors who used *P. sativum* as an agent for evaluating the cytotoxicity of different chemical substances with cytotoxic potential such as cadmium and chromium (Fusconi et al., 2006; Rai, Sangeeta Dayal, 2016). Likewise, the results obtained coincide with tests using *A. cepa* as toxicity bioindicator in sodium hypochlorite, in the herbicide pendimethalin and in Amaranthus spinosus Linn leaves aqueous extracts (Causil et al., 2017; Prajitha and Thoppil, 2016; Verma and Srivastava, 2018). However, there is an anomaly that is particularly striking, and it is the presence of micronuclei (Fig. 2A), since these are widely used as indicators of high degree of cytotoxicity (Bhatia and Kumar, 2013), due to the fact that they produce chromosomal damage and genome instability, since they show alterations in the chromatic spindle or in the structure of the chromosomes (Iarmarcovai et al., 2008). The micronuclei are formed during mitosis in the metaphase-anaphase transition and can be complete chromosomes lagged by damage to mitotic spindle (aneuploidgenic effect), or fragments of chromosomes without centromere (clastogenic damage); in any of the cases, these do not achieve to be incorporated into any of the nuclei of the daughter cells (Ruíz-Bernés et al., 2013).

3.4. Cellular Anomalies Index

Table 5 shows the rates of cellular anomalies present in each one of the evaluated concentrations. Sodium hypochlorite significantly increased the frequency of cellular abnormalities in all treatments compared to the control, where it is possible to observe that the concentration that presents a higher relative rate of abnormality is 2 mg L^{-1} with an average of 4.55. In a study conducted by Causil et al. (2017) on *A. cepa* apical root cells. They found that, in concentrations of 5, 2, 1, mg L-1 of sodium hypochlorite, a higher rate of cellular anomalies were found.

Based on the above, it is possible to highlight that these results corroborate those obtained by Verma and Srivastava (2018) in their research, where it is reported that an increase in the percentage of mitotic index inhibition is directly related to the increase in the abnormality rate, and where anomalous cells were also reported in all concentrations, even at the lowest concentrations.

4. Conclusions

It is possible to conclude that *P. sativum* is a specie that offers a feasible experimental model to be implemented in the laboratory with the aim to evaluate the cytotoxic effect of any cytotoxic substance. Sodium hypochlorite generated cellular (chromosomal) anomalies in all concentrations, being nuclear cleavage, lagged chromosomes and split chromosomes the ones with the greatest presence. Additionally, it showed that sodium hypochlorite caused inhibition in the cell division of all the evaluated concentrations. Finally, sodium hypochlorite could be determined as a highly cytotoxic substance, due to the fact that it generated cellular anomalies in concentrations lower than 2 mg L^{-1} in which there is even evidence of micronuclei.

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