CYTOGENOTOXIC EFFECTS OF NITROGEN ON HYDROPONIC LETTUCE

EFEITOS CITOGENOTÓXICOS DO NITROGÊNIO EM ALFACE HIDROPÔNICA

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ABSTRACT: Nitrogen accumulation in hydroponically-grown lettuce may pose a health risk to consumers. Thus, the objective of this study was to analyze different concentrations of nitrogen applications in hydroponic lettuce cultivation and their effect on toxicity, cytotoxicity and genotoxicity. A nutrient film technique (NFT) hydroponic system was used to grow the lettuce variety "Vanda." The treatments consisted of different concentrations of nitrogen (in the form of calcium nitrate) in Furlani solution (75, 100, 125 and 150%), a negative and a positive control. The following commercial characteristics were measured: plant fresh weight (PFW), root fresh weight (RFW), shoot fresh weight (SFW), shoot diameter (SD), root dry weight (RDW), shoot dry weight (SDW) and leaf nitrogen (LN). Cytogenotoxicity was indicated by toxicity, cytotoxicity and genotoxicity, which were in turn determined by root length, the mitotic index, chromosomal aberrations and the presence of micronuclei. The nitrogen concentrations used in this experiment did not cause phenotypic toxicity or cytotoxicity in lettuce roots. The most severe genotoxicity was observed at the 125% nitrogen concentration, which nevertheless did not affect commercial characteristics. Although nitrogen fertilization provides great benefits to agriculture, such as greater yields, indiscriminate use should be avoided since concentrations above recommended rates may induce genotoxicity.

KEYWORDS: Cytotoxicity. Genotoxicity. *Lactuca sativa*. Nitrogen. Toxicity.

INTRODUCTION

Lettuce (*Lactuca sativa*) is inexpensive and produced year-round in Brazil. Consequently, it is one of the leading vegetables regarding production, sales and consumption. Like other crops, lettuce requires fertilization and, given that it is a leaf crop, responds especially well to nitrogen fertilization (RESENDE et al., 2010; PÔRTO et al., 2012; LOURENÇO et al., 2017).

Nitrogen (N) is a limiting nutrient for most crops while deficiency in lettuce crops is characterized by slow plant growth, misshapen heads and chlorosis and necrosis in older leaves (LI et al., 2016). To combat these symptoms and maximize yields, nitrogen is sometimes applied excessively, especially in developing countries (LI et al., 2016). Excessive N application is a source of great concern and may cause decreases in head lettuce yield, firmness and revenues during the last third of the crop cycle, (RESENDE et al., 2010; SANTOS et al., 2012). Another problem is rising nitrate (NO_3^-) levels in green leafy vegetables such as lettuce (LIU et al., 2014).

Ingesting more than 5.0 mg/kg/day of nitrates is a health hazard since nitrates can be reduced to nitrites during digestion and may also form nitrosamines in the blood, which are carcinogenic, mutagenic and teratogenic (TURAZI et al., 2006). Stivaktakis et al. (2010) evaluated the genotoxicity of the pesticide pimidacloprid in the absence or presence of potassium nitrate (KNO_3) and found that KNO_3 influenced the genotoxic behavior of pimidacloprid by increasing the permeability of the plasma membrane, which consequently increased the frequency of micronuclei induced by the pesticide. Thus, KNO_3 has been classified as a class III toxic agent by the Environmental Protection Agency (EPA).

It is essential to grow edible crops with low nitrate levels. The Joint Expert Committee on Food and Agriculture (JECFA) of the United Nations/World Health Organization and Europe's Scientific Committee on Food have established a daily nitrate limit to 0-3.7 $mg \cdot kg^{-1}$ of body weight, while the Environmental Protection Agency of the United States has determined a daily limit of 7.0 $mg \cdot kg^{-1}$ of body weight (LIU et al., 2014).

Despite the risks associated with excessive nitrogen fertilization, high application rates are still recommended or adopted by growers given the higher yields that can be achieve with relatively little investment (MASCARENHAS et al., 2008). Nitrate accumulation and its associated risks are

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even greater in hydroponically-grown lettuce than in conventionally-grown lettuce (LUCENA; SILVA, 2016).

Cytogenetic studies on plants can show chromosomal changes caused by mutagenic agents. These mutations may be caused by chemical, environmental and radioactive compounds and the intrinsic stability of nucleic acids. Evidence of mutagenic agents in cells may appear as cell cycle inhibition, metaphase interruption, numerical and structural chromosomal changes, and exchanges between sister chromatids (BAGATINI; SILVA; TEDESCO, 2007). Micronuclei provide evidence of mutagenesis (e.g. clastogenesis, aneugenesis and mitotic spindle damage) in eukaryotic organisms. Micronuclei can be found in any cell type, including plant cells (SILVA et al., 2011).

Ferreira et al. (2014) performed biotests with different nitrogen sources and application rates in *Allium cepa* and found that 300 Kg. ha^{-1} of a typical urea source and 150 kg ha^{-1} of coated urea produced toxicity. The same authors found that 150 and 240 kg ha^{-1} of N produced genotoxicity (micronuclei and chromosome anomalies) in *Allium cepa* roots.

The same authors also noted that while the *A. cepa* test showed how different nitrogen sources and application rates affect macroscopic parameters (root growth) and cytological parameters (presence of micronuclei and cellular aberrations that inhibit cell division) it did not show how the crop was affected. The objective of the present study was to evaluate the effect of different concentrations of nitrogen on hydroponic lettuce and determine if these application rates produced toxicity, cytotoxicity and/or genotoxicity.

MATERIALS AND METHODS

The experiment was conducted using an NFT hydroponic system (. It was set up with four benches (4.5 m long) and nine polypropylene profiles (100 mm), spaced 18 cm apart and 25 cm between holes. Each group of three tubes was fed by a 32-watt pump and a 100-liter plastic reservoir. Before transplanting, the roots of the leaf-lettuce seedlings (var. Vanda) were cleaned under running water and then placed in the hydroponic system, containing only city tap water, for 45 hours to remove any remaining substrate from the roots. The treatments were started after this cleaning process, at which point the mean shoot and root lengths were 9.3 and 6.3 cm respectively.

A completely randomized design (CRD) was used with six repetitions. The treatments consisted of various concentrations (75, 100, 125 and 150%) of the nitrogen (in the form of Calcium nitrate) in the hydroponic solution proposed by Furlani et al. (1999). Three controls were also used: a negative control (city tap water), a positive control (750 $mg.L^{-1}$ paracetamol solution) and a treatment control (100% nitrogen solution - FURLANI et al., 1999).

The nutrient solution was prepared using city tap water (MARTINEZ, 1997) that was left to stand for 24 hours to eliminate chlorine. The nutrient solution was prepared using a “basic” hydroponic kit from Gioplanta – Comércio e Representação Agrícola Ltda.

The nutrient solution was replenished and checked (electrical conductivity - EC and pH) every two days. EC was corrected whenever the value dropped 25% below the starting point (EC adjustment kit, Gioplanta – Comércio e Representação Agrícola Ltda), while the pH was maintained between 6.0 and 6.5 using either 1M Potassium Hydroxide (KOH) or 5M Hydrochloric Acid (HCl).

A timer was used to activate the pumps and circulate the nutrient solution at 15 minutes intervals (15 minutes on / 15 minutes off) from 6am to 6pm, and for 15 minutes on and 45 minutes off from 6pm to 6am.

Evaluation of commercial characteristics

Thirty-five days after transplant (when the plants were ready for harvest), the six center-most plants from each plot were harvested in the afternoon and evaluated for the following characteristics: plant fresh weight (PFW), shoot fresh weight (SFW) and shoot diameter (SD).

Afterwards, the samples were dried at 65°C to a constant weight, yellowed and dead leaves were discarded, and the following were evaluated: root dry weight (RDW), shoot dry weight (SDW) and leaf nitrogen (LN).

Toxicity analysis

After applying the treatments (72 hours), a sampling of six repetitions per treatment was taken to determine toxicity and root length. Root length of six individuals was compared to the negative and positive controls. Toxicity was indicated by significant differences between treatments.

Cytotoxic and Genotoxic Analysis

The various nitrogen treatments were continued until completing the hydroponic crop cycle (35 days). Afterwards, root samples were collected, bathed in Carnoy 3: 1 (ethyl alcohol /

glacial acetic acid) at room temperature for 24 hours, then transferred to 70% alcohol and kept in the freezer until slide preparation.

Before preparing the slides, the roots were removed from the fixative and bathed three times in distilled water (5 minutes each), hydrolyzed for 20 minutes in 5N HCl at room temperature and then washed twice in distilled water. Afterwards, the root tips were mounted on slides with a drop of 45% acetic acid. The root caps were removed with the aid of a stereomicroscope, leaving only the meristems.

The meristem was fragmented into small pieces with a needle and covered with a coverslip. A blunt-tipped needle was then used to separate the cells. This process was monitored using a common microscope with the condenser diaphragm partially closed or the microscope condenser lowered. After preparation, the slides were placed in liquid nitrogen for subsequent removal of the coverslip.

A fluorescent stain was used that consisted of Hoechst 33258 intercalating agent (a stain that interacts with DNA and becomes fluorescent when excited at 365 nm) in solution with PBS (phosphate buffered saline). The slides were immersed in this staining solution for 20 minutes, at 37° C, in the dark and then washed with distilled water and dried in an oven. Finally, coverslips were placed on the slides with 40 µl of mounting solution.

After staining, the scanning technique (1000 cells per treatment) and a fluorescence microscope (Olympus® Model DP70) with an ultraviolet filter (340 nm) were used to detect toxicity (evidenced by reduction in the mitotic index – MI) and genotoxicity (evidenced by the presence of chromosomal aberrations in mitotic cells - CA and micronuclei in interphasic cells– MN). The chromosomal aberrations analyzed were lagging chromosome, anaphase Bridge and disturbed metaphase. The mitotic index was determined by dividing the number of mitotic cells by the total number of cells observed and multiplying by one hundred to express the index as a percentage.

The commercial characteristics and toxicity data were submitted to analysis of variance and, in cases where the F test was significant, the means were compared by the Scott-Knott test ($p < 0.05$). SISVAR 5.3 (Ferreira, 2011) was used for statistical analysis while the cytotoxicity and genotoxicity data were evaluated by one-way analysis of variance (ANOVA) with Bonferroni's posttest using GraphPad Prism, v. 8.

RESULTS AND DISCUSSION

Analysis of commercial characteristics

Plant fresh weight (PFW), shoot fresh weight (SFW), root dry weight (RDW), shoot dry weight (SDW) and leaf nitrogen did not differ significantly among the various nitrogen concentrations (Table 1).

Shoot diameter did not differ significantly among the 100% (control), 125% and 150% concentrations, but did differ at the 75% concentration ($p<0.05$). This confirms the results of Schmidt et al., (2001), who demonstrated that Furlani's full-strength nutrient solution was the most efficient for lettuce production.

Table 1. Commercial characteristics of lettuce with different N concentrations.

N Concentration (%)	SD (cm)	PFW	RFW	SFW	RDW	SDW	LN (mg)
		(gr)					
75	33.9[b]	215.1[a]	21.3[b]	193.8[a]	4.0[a]	3.6[a]	41.2[a]
100	38.9[a]	252.7[a]	22.6[b]	230.1[a]	4.1[a]	3.6[a]	38.1[a]
125	38.3[a]	283.7[a]	27.4[a]	256.3[a]	4.3[a]	4.3[a]	39.0[a]
150	37.2[a]	279.3[a]	27.5[a]	251.8[a]	4.3[a]	4.1[a]	40.5[a]
C.V. (%)	7.61	24.25	14.41	25.59	17.39	22.63	7.83

Means followed by the same letter within a column do not differ by the Scott-Knott test (p = 5%). Plant fresh weight (PFW), root fresh weight (RFW), shoot fresh weight (SFW), shoot diameter (SD), root dry weight (RDW), shoot dry weight (SDW), leaf nitrogen (LN) and coefficient of variation (C.V.).

Root fresh weight (RFW) was significantly lower at the 75 and 100% concentrations than at the 125 and 150% concentrations ($p<0.05$). This result supports other findings that nitrogen is a determining factor in the length, surface area and dry weight of roots (BATISTA; MONTEIRO, 2006).

According to Lucena and Silva (2016), excessive nitrogen levels from mineral or organic fertilizers or nutrient solutions cause problems in plants related to nitrogen accumulation. However, nitrogen is essential for building the organic molecules used in photosynthesis (LOURENÇO et al., 2017).

An adequate nitrogen supply is associated with high photosynthetic activity and vigorous vegetative growth in hardwoods. In lettuce, high application rates of nitrogen yield greater mass and higher leaf accumulation of macronutrients and thus lower nitrate concentrations (DAPOIGNY et al., 2000; LUZ et al., 2008).

Nitrogen absorbed by the roots can be reduced, translocated to the shoot or stored in foliar vacuoles (SILVA et al., 2011). The nitrate accumulation capacity of a cell vacuole is influenced most significantly by genetics and ion availability but also by light intensity, molybdenum availability, temperature, relative humidity, cultivation method, growing season and harvest time (LUZ et al., 2008).

Toxicity Analysis

Statistical analyzes of Mitotic Index and Root Length did not show significant differences from nitrogen treatments to negative control. However, when analyzing cytogenetic aberrations, a statistically significant difference was observed between 125% nitrogen concentration and negative control (Table 2).

Table 2. Results of analysis of variance and comparison of means for the different concentrations of nitrogen on lettuce.

Source of variation	DF	Mean Squares		DF	Mean Square
		MI	CA		RL
Treatments	5	26.619*	0.00018*	5	4.307*
Residue	12	4.75	0.00001	30	1.029
C.V. (%)		36.47	27.41		13.14
Treatments		Mean of variables			
		MI	CA	RL	
Nitrogen 75%		7.2[a]	0.009[a]	7.3[b]	
Nitrogen 100%		6.3[ab]	0.013[a]	7.1[b]	
Nitrogen 125%		9.2[a]	0.028[b]	7.6[ab]	
Nitrogen 150%		5.3[ab]	0.012[a]	9.4[a]	
Negative control		7.4[a]	0.007[a]	7.8[ab]	
Positive control		0.5[b]	0.019[b]	7.1[b]	

*significant according to the F test. C.V.: coefficient of variation. Means followed by the same letter in the columns do not differ significantly according to the Bonferroni test at 0.05 probability. DF - Degrees of freedom; MI – mitotic index; CA – Cytogenetic Aberrations; RL – Root Length.

Macroscopic observations showed that the Paracetamol 750 mg.L^{-1} treatment (positive control) produced fewer roots and significant leaf necrosis (Figure 1A), suggesting that this application as a positive control could substitute other dangerous substances such as sodium azide (RIBEIRO et al., 2016) and trifluralin (ALMEIDA et al., 2016).

Figure 1B shows that root development was consistent regardless of nitrogen concentration, suggesting that these nitrogen concentrations did not cause toxicity in the hydroponically grown lettuce (Figure 1B).

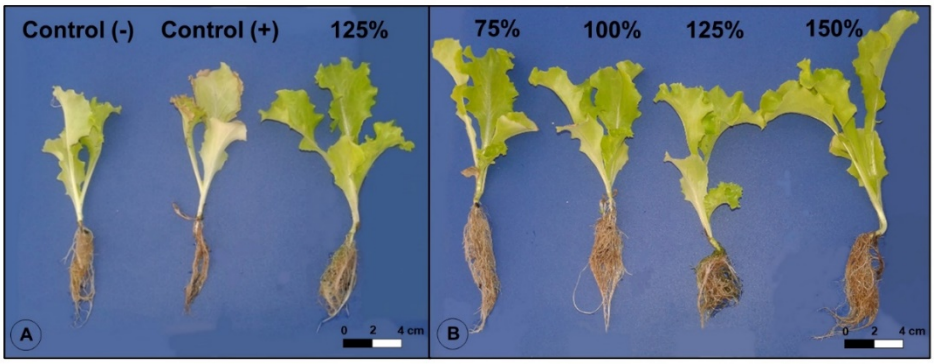

Figure 1. Toxicity from different concentrations of nitrogen in hydroponically grown lettuce. Uberlândia, MG, Brazil, 2016. (A) Comparison of 125% N concentration with a negative control (city tap water) and a positive control (paracetamol solution, 750 mg.L^{-1}). (B) Nitrogen concentrations.

Figure 2 shows the mean root lengths used to evaluate toxicity. Statistically significant differences ($p<0.05$) in root length were found between 75 and 150%, 100 and 150% and 150% and the positive control. However, no differences were found between the negative control and various nitrogen concentrations.

Higher nitrogen concentrations did not affect root growth and the analysis of root dry weight confirmed that higher nitrogen concentrations did not cause phenotypic toxicity in hydroponic lettuce cultivation.

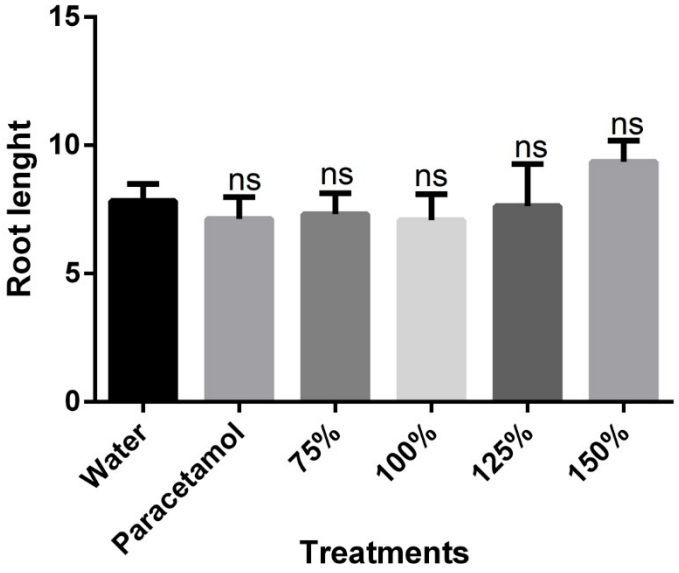

Figure 2. Toxicity in root of lettuce treated with different nitrogen concentrations in hydroponically grown. (ns) $p > 0.05$ (not significant), by one-way analysis of variance (ANOVA) with Bonferroni's posttest comparing with vehicle control (water).

The roots are the first organ to contact the nutrient solution and therefore the main system of nutrient entry and accumulation and the first organ to show sensitivity to these elements (SILVA et al., 2016). However, according to Shan et al. (2012) most incorporated nitrogen is allocated to the leaves, regardless of source, which should prevent the direct effects of this element in the roots, as seen in the present study.

Nitrogen is needed for amino acids, proteins, enzymes, coenzymes, vitamins and pigments (e.g. chlorophyll) and is therefore essential to plant structure and consequent plant development and yield. Nitrogen is linked to greater foliage size and as such is used to fertilize and improve yields of leafy crops such as lettuce. In hydroponic systems, as in the soil, nitrogen is mostly supplied as nitrate, which must be reduced to ammonium before forming organic compounds (LUZ et al., 2008). Most of the nitrogen absorbed by the roots can be reduced or stored in vacuoles or translocated to the shoot system where it is reduced or stored in foliar vacuoles (SILVA et al., 2011).

Cytotoxic and Genotoxic Analysis

Cytotoxicity was measured using the mitotic index (MI), which is related to the rate of root cell division. An MI significantly lower than that of the negative control indicates that nitrogen is affecting root growth and development while an MI greater than that of the negative control results from increases in cell division, which may harm cells and lead to disordered cell proliferation and eventually tumor development (MACEDO et al., 2014).

In the present study, MI did not differ significantly among the various treatments and the negative control, indicating that higher concentrations of nitrogen did not cause cytotoxicity in the hydroponically grown lettuce (Figure 3). These results agree with those found in the commercial and toxicity evaluations.

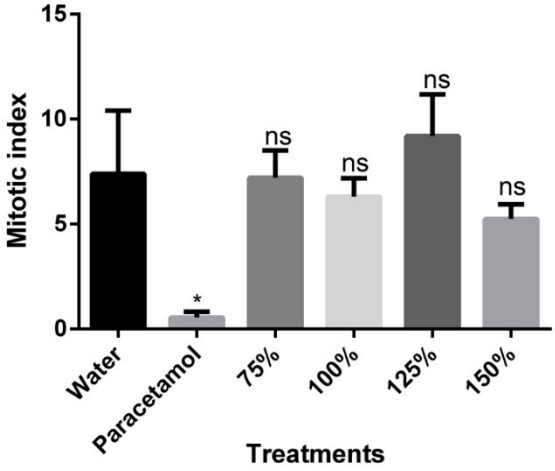

Figure 3. Cytotoxicity (indicated by reduction of mitotic index) in root of lettuce treated with different nitrogen concentrations in hydroponically grown. (ns) $p > 0.05$ (not significant) and *$p<0,05$ by one-way analysis of variance (ANOVA) with Bonferroni's posttest comparing with vehicle control (water).

Several studies have reported on cytotoxicity caused by substances such as agrochemicals and secondary metabolites in plants (BALIGA et al., 2011; MOREIRA et al., 2014; FERREIRA et al., 2016; SANTOS et al., 2016); however, little is known about the toxicity of the nutrient solutions used in hydroponic crops. This may be directly related to the fact that the morphology and composition of plant metabolites in artificial environments can be accurately adapted to the physical and chemical conditions of this environment (MIYAGI et al., 2017; SARDARE; ADMANE, 2013).

Macedo et al. (2014) suggest that mitotic indices, whether higher or lower than a negative control, indicate the changes in root growth and development caused by nitrogen, which may also be detrimental to cells and lead to disordered cell proliferation and eventual tumor formation. The number of chromosomal aberrations (CA) and presence of micronuclei (MN) were determined to evaluate the genotoxic effects of nitrogen in lettuce roots (Figure 4). These evaluations showed significant differences in genotoxicity between positive control (paracetamol) and 125% treatment to negative control ($p < 0,0004$). Cytogenetic aberrations were also seen in the control at low levels, and according to Verma, Arora, And Srivastava (2016) it may be due to errors occurring in mitosis under natural conditions.

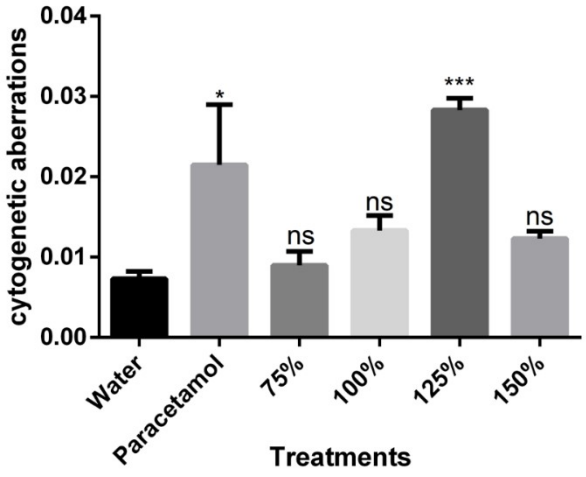

Figure 4. Genotoxicity (indicated by cytogenetic aberrations) in root of lettuce treated with different nitrogen concentrations in hydroponically grown. (ns) $p > 0.05$ (not significant), *$p<0,05$ and ***$p<0,0007$ by one-way analysis of variance (ANOVA) with Bonferroni's posttest comparing with vehicle control (water).

The highest level of genotoxicity in the roots of the hydroponically-grown lettuce was found at the 125% concentration; however, nuclear bud and micronuclei were also present at the 75, 125 and 150% treatments (Figure 5). In these concentrations there is a tendence in increasing anomalies frequency, but it was not statistically different from negative control ($p>0,05$).

Damage caused by toxic substances interferes with the genetic material of a cell, causing cytotoxicity and mutations (ANCIA; ROMÃO, 2016). In the present experiment, higher nitrogen concentration did not cause toxicity or cytotoxicity; however, chromosomal aberrations and micronuclei were observed, which may indicate possible genotoxicity in the roots of this hydroponically-grown lettuce.

According to Verma, Arora, And Srivastava (2016), ammonium nitrate (an inorganic fertilizer) contains nitrogen as both nitrate (NO_3^-) and ammonium (NH_4^+). The same experiment evaluated the effects of ammonium nitrate and urea (the two most commonly used nitrogen fertilizers) on *Allium cepa* L. and concluded that indiscriminate use of ammonium nitrate and urea can cause irreversible cytological damage in plants. The authors suggest that nitrogen fertilizers should be used judiciously given that they may induce chromosomal variations and genotoxic damage in plants.

Several authors have stated that excessive nitrogen fertilization can diminish soil fertility by reducing microbial enzymes and causing cytogenotoxicity (ARORA et al., 2014; VERMA; SRIVASTAVA, 2017). According to Verma and Srivastava (2017), nitrogen-based fertilizers can cause chromosomal damage, cytological changes and other abnormalities during *Allium cepa* cell division.

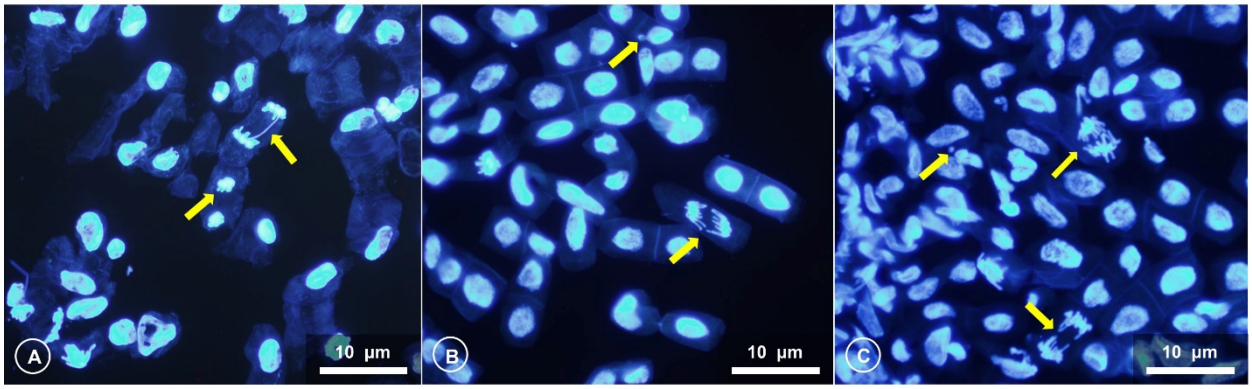

Figure 5. Genotoxicity at different nitrogen concentrations in hydroponically-grown lettuce. A) Anaphase Bridge and nuclear bud - 75%; B) Lagging chromosome and micronucleus - 125%; C) Lagging chromosome and micronucleus - 150%.

The most observed cytogenetic alteration was the micronucleus in interphase cells. Micronucleus is a small nucleus consisting of genetic material that has been lost by the main nucleus because of genetic damage caused by physical, chemical or biological agents that may interfere with the binding of chromosomes to spindle fibers, or that may induce losses of genetic material (RÉGIS; PESENTI, 2015).

Micronuclei are generated on the telophase and result from acentric chromosomal fragments that originate from isochromatids or chromatids breaks, or mitotic spindle dysfunctions, and may appear more than once in cells (ANCIA; ROMÃO, 2016). The presence of micronuclei in lettuce grown in Furlani (1999) solution with 125% of Nitrogen reflects the induction of structural or numerical chromosome aberrations during mitosis and therefore indicates chromosomal mutagenesis (clastogenesis), aneugenesis or mitotic spindle damage (RÉGIS; PESENTI, 2015). According to Verma, Arora, And Srivastava (2016), the indiscriminate use of Nitrogen fertilizers disturbs the balance between NH_4^+ and NO_3^- and this unbalance may impose genotoxic risks.

CONCLUSION

The nitrogen concentrations used in the current study did not cause toxicity or cytotoxicity in the roots of hydroponically-grown lettuce. The strongest genotoxicity was found at the 125% nitrogen concentration, which nevertheless did not affect the commercial characteristics of the crop. Although nitrogen fertilization provides important agricultural benefits, such as increased yield, indiscriminate use should be avoided since higher concentrations may induce genotoxicity. Greater

frequencies of chromosomal aberrations and micronuclei in fertilized plants, as seen in the present study, may be extrapolated to the final consumers of these plants.

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RESUMO: O acúmulo de nitrogênio em alface cultivada hidroponicamente pode representar um risco à saúde dos consumidores. Assim, o objetivo deste estudo foi analisar diferentes concentrações de aplicações de nitrogênio no cultivo de alface hidropônica e seus efeitos na toxicidade, citotoxicidade e genotoxicidade. Em sistema hidropônico do tipo filme de nutrientes (NFT) foi usado para cultivar a variedade de alface "Vanda". Os tratamentos consistiram em diferentes concentrações de nitrogênio (na forma de nitrato de cálcio) na solução Furlani (75, 100, 125 e 150%), um controle negativo e um positivo. Foram medidas as seguintes características comerciais: peso fresco da planta (PFW), peso fresco da raiz (RFW), peso fresco da parte aérea (SFW), diâmetro da parte aérea (SD), peso seco da raiz (RDW), peso seco da parte aérea (SDW) e nitrogênio foliar (LN). A citogenotoxicidade foi indicada por toxicidade, citotoxicidade e genotoxicidade, que por sua vez foram determinadas pelo comprimento da raiz, índice mitótico, aberrações cromossômicas e presença de micronúcleos. As concentrações de nitrogênio utilizadas neste experimento não causaram toxicidade fenotípica ou citotoxicidade em raízes de alface. A genotoxicidade mais severa foi observada na concentração de nitrogênio de 125%, que, no entanto, não afetou as características comerciais. Embora a fertilização nitrogenada traga grandes benefícios à agricultura, como maiores rendimentos, o uso indiscriminado deve ser evitado, pois concentrações acima das taxas recomendadas podem induzir genotoxicidade.

PALAVRAS-CHAVE: Citotoxicidade. Genotoxicidade. *Lactuca sativa*. Nitrogênio. Toxicidade.

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