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Full Length Research Paper

Effect of Carbamazepine on the *In Vitro* Propagation of *Zantedeschia aethiopica*

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***Zantedeschia aethiopica* is a vascular plant recently introduced in bioremediation treatments, which seeks to remove effluent or water bodies contaminants such as heavy metals and emerging contaminants, thus effect of carbamazepine on the in vitro propagation of *Zantedeschia aethiopica* was evaluated. Among these, the presence of the drug carbamazepine has acquired great importance, because there are no adequate methods to eliminate them. This allows them to move and increase their presence in surface or underground water bodies. *Z. aethiopica* presents an alternative for the treatment of these waters; however, there is little information about its response to the exposure to these drugs, carbamazepine, an antiepileptic commonly used. The effect of carbamazepine on Z. aethiopica was studied by quantifying three dependent variables: growth, weight and number of buds at three concentrations of carbamazepine (0, 25 and 50 µg/L), while the control group had a concentration of 0 µg/L of carbamazepine. The ANOVA statistical analysis was applied to compare the three groups and later the Tukey test to evaluate paired variation. It was found that the presence of carbamazepine in the test concentrations affects the development of *Z. aethiopica* in all three variables and in high concentrations it leads to senescence and cell death.**

Keywords: Emerging contaminants, *Zantedeschia aethiopica*, Carbamazepine, *In vitro* propagation, Bioremediation.

INTRODUCTION

Recently, *Z. aethiopica* has been used in studies of bioremediation processes of contaminated water for the uptake of various pollutants, including heavy metals (Suárez Escobar and Agudelo Valencia, 2014; Marín-Muníz, 2017). The use of Z. aethiopica in the treatment of wastewater has also been studied, from pig production systems (Figueroa Gallegos, 1999) to domestic effluents with a double purpose, such as the treatment of domestic wastewater and the production of cut flower (De Anda Sánchez et al., 2016); a market that is develops in European countries such as England, Germany, France and Italy among others. While in Asia, part of the market is developed in Japan and also found important in the US market. Moreover, in countries with developing economies such as Mexico, Argentina, Chile, Taiwan, Korea and others are also interested in cut flower consumption, which is expected to increase (Soto de Paz, 2014).

Ecotoxic effects of Chromium VI, a heavy metal present in sewage effluents, have been studied; its presence caused inhibition of *Z. aethiopica* germination from 100 ppm and an LD50 at 132 ppm, as well as a hormesis effect, with a greater development at low

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doses and phytotoxic effects at higher concentrations (Gualli Aragadvay and Mena Maldonado, 2017).

Within the Emerging contaminants (EC), wastewater contains drugs of these the most commonly used are analgesics, antihypertensive and antimicrobial (Barceló and López de Alda, 2008; Gil et al., 2012). The presence of carbamazepine (CBZ), a commonly used antiepileptic, has been detected in drinking water, with low rates of strippage in Sewage Treatment Plants (STPs) close to 7%, according to Ferrari et al. 2003. In studies of hospital wastewater conducted in Mexico, concentrations of CBZ were obtained in STP influences between <1 to 55.8 μg/L and effluents of <1-5.9 μg/L. While the data reported in other countries have been between <0.04 to 6.3 μg/L (Martínez Garay, 2013). ECs are substances that can be retained and accumulate in soils, or move to mantles or water sources, representing a risk to ecosystems and human health. There are water treatment systems that are not suitable for the elimination of these pollutants, so their removal is null or deficient, keeping them in the effluents even after the remediation treatments and in this way enter the water bodies (Delgado, 2011).

In the incorporation of the plant species use for the treatment of contaminated places or media, the plants are considered bio-indicators or bio-remediators, by providing monitoring information, or for the extraction of substances. However, these species, in turn, are susceptible to suffer the effect of the pollutants to which they are exposed which cause them biochemical, cellular, physiological variations and/or wide changes throughout the organism (Pernía et al., 2008).

Phytotoxic evaluations of drugs in macrophyte plants used in water treatment are scarce, so it is necessary to study the effect of emerging contaminants on the development of *Z. aethiopica* and other plant species. *In vitro* culture allows the propagation of explants in aseptic conditions, pathogens free and in predetermined chemical composition culture media, providing organic nutrients and phytohormonal balance, as well as controlled environmental conditions providing valuable information on the effect of pollutants in an isolated and controlled way (Jarquín Cordero and Flores Mora, 2002). This process can be developed through organogenesis or embryogenesis, the former is widely used in commercial reproduction, the latter is more oriented towards regeneration of plants of forest species (Martínez Ruiz et al., 2010).

MATERIALS AND METHODS

Collection and cleaning

Samples of Z. aethiopica were collected from a commercial nursery garden in the city of Ocotlán, Jalisco, Mexico. They were extracted from the pot and and cleaned mechanically, removing the aerial parts until the apex/bulb was obtaining, only healthy young bulbs were selected. Once the bulbs were cleaned, they were disinfected by submerging them in a 1.5% w/w chlorine solution for 25 minutes. They were then extracted and placed under orbital shaker in a new solution composed of 500 mL of sterile bidistilled water, 250 mg of streptomycin, 250 mg of oxytetracycline and 250 mg of captan fungicide for 60 minutes. Then bulb was bulbous at a concentration of ethyl alcohol at 70% for 30 seconds, ending with a triple wash in sterile bidistilled water. Finally, each bulb was placed in a 70% ethyl alcohol concentration for 30 seconds, ending with a triple wash in sterile bidistilled water.

In vitro establishment

The establishment was made in a culture medium Murashige and Skoog 1962, enriched with thiamine, myo-inositol 6-benzylaminopurine and sucrose according to (Sánchez et al., 2010), adjusting to a pH at 5.7 ± 0.01, finally 3 g/L of phytagel were added. 25 mL of the medium was placed in 250 mL glass bottles and sterilized in an autoclave for 20 minutes at 1.2 Kg/cm^2 and 121 ºC.

In a first step, previously sterilized explants of Z. aethiopica were subjected to expression and thus, plants were generated *in vitro*. For this process, a bulb per bottle was introduced and they were taken to a growth chamber at 28 ± 2.0 °C, and kept in darkness for 8 days. After this period, at the same temperature, they were exposed with a flow of photosynthetic photons of 38-47.5 μmol/m^2/s with exposure to light for 18 h and 6 h in darkness for 30 days. The obtained were extracted and placed in a flasks prepared with 25 ml of MS medium added with 25 or 50 μg/L of CBZ. A completely randomized block design was carried out with three treatments at: 0, 25 and 50 μg/L of CBZ; with 5 bottles each and three repetitions, while the control (treatment with 0 μg/L of CBZ) was carried out with 5 bottles and 2 repetitions.

The size (cm) and weight (g) of each bulb and number of buds were measured.

Optical Microscopy

A tissue sample of *Z. aethiopica* was taken at random from bottles subjected to the CBZ treatments. A cross-section was made and the tissue was exposed to a few drops of 70% methylene blue for 3 minutes; then, the excess of dye was removed with running water and the vegetable segment was placed under the microscope in order to observe the cellular structure developed in the new tissues of plants exposed to CBZ and contrast with those of the control.

Statistical Analysis

Data were analyzed using SPSS version 19.0 software, and descriptive statistical such as the means ± SD were obtained in order to compare whether there was significance effect on the different treatments, ANOVA test was applied, to check the theoretical assumptions of this test: equivalence, independence, normality and equality of variances; finding that for the three treatments there was correspondence in size (cm) and weight (g), not so for the number of buds or multiplication coefficient (MC). Due to this, the first two variables were evaluated by one-way ANOVA and the last one by nonparametric ANOVA (Kruskal-Wallis). Finally, a Tukey test was applied to find out if there was a significant difference by pairs of treatments. The significance levels for each test were $p < 0.05$.

RESULTS AND DISCUSSION

Development of *in vitro* bulbs

Table 1 shows the average values obtained, as well as the standard deviation (SD) of the samples of *Z. aethiopica* subjected to the three treatments of 0, 25 and 50 µg/L of CBZ. The measurements were made after thirty days of remaining in the growth chamber in aseptic and controlled conditions. Seedlings were extracted, washed by withdrawing the surplus growth medium and were subsequently measured, weighed and counted the number of sprouts (multiplication coefficient, MC).

The values in growth (cm) and MC coincide with data previously reported for *in vitro* reproduction of *Z. aethiopica* Treasure variety at 45 days culture, with a multiplication coefficient of 3.63, and with a height of 2.53 cm in semi-solid medium (Sánchez et al., 2010).

On the other hand, the size of the bulbs showed an increase in the group with 50 µg/L of CBZ (4.7 ± 0.8 cm) with respect to the control group (2.7 ± 0.3), while the weight showed a decrease ranging from 379.76 ± 14.4 to 95.7 ± 47.9 in the control group vs. the group with 50 µg/L of CBZ respectively. Similarly, the MC decreases from 4 ± 0.7 to 0.4 ± 0.5 as the concentration of CBZ increases. These same results can be seen in Figure 1, where the control group (Figure 1a), had a smaller size and/or growth with respect to the groups with 25, 50 and more µg/L of CBZ (Figures 1b, 1c and 1d respectively). It should be noted that exposure to CBZ, visibly affects the formation of new tissue and the multiplication coefficient. Although a greater longitudinal development of the explants is shown in the concentrations 25 and 50 µg/L CBZ, this is accompanied by a lower weight of the seedling, suggesting a possible cellular alteration; which is evidenced in Figure 1d where tissue necrosis is shown at concentrations of 50 µg/L CBZ.

This result contrasts with the behavior of the control sample where there is a smaller elongation and a higher weight. To check if the effect of the three treatments on the size, weight and MC of the seedlings is significant, one-way ANOVA test was applied, so it was necessary to check the theoretical assumptions of the test: 1) Equivalence: it is assumed that the three treatments were equivalent since the study consisted of n = 15 samples for each treatment (0, 25 and 50 µg/L of CBZ), distributed in 5 for each variable (Size, weight and MC). 2) Independence: it is assumed that the treatments were independent since each one had different samples with different concentrations of the exposure factor. 3) Normality: for this the Shapiro-Wilk test was applied, which showed that all the variables presented normality of the data ($p > 0.05$) except the MC treated with 50 µg/L of CBZ ($p = 0.006$). These results are congruent and expected, since the only treatment that presents a significant p value is due to the fact that, in this treatment, most of the samples did not develop buds (data not shown), possibly because they were at a concentration close to premature necrosis. Finally and the last assumption 4) Homogeneity of variances: from the Levene test it was observed that both the size and the weight of the buds fulfilled this assumption ($p > 0.05$) except the MC, which is consistent with the results of the normality test, and therefore this variable was analyzed with a nonparametric ANOVA (Kruskal-Wallis).

Once the theoretical assumptions of the ANOVA were verified, we obtained that the three treatments have a significant effect on the size ($F = 13.559$, $p = 0.001$), weight ($F = 145.501$, $p = 0.000$) and MC ($p = 0.003$) of the buds. However, we look for the paired significance, so we apply the Tukey test. For the size it is observed that the control group is statistically significant with the 25 µg/L and the 50 µg/L CBZ treatments ($p = 0.02$ in both cases). But there was no significance between the 50 µg/L and 25 µg/L of CBZ treatments ($p = 0.971$). Similarly, for the weight, the control group is statistically significant with both treatments 25 µg/L and 50 µg/L CBZ ($p = 0.00$). While there was no significance between the 50 µg/L and 25 µg/L CBZ treatments ($p = 0.678$). Finally, for the number of buds, it was observed that the control group is not statistically significant with 25 µg/L CBZ treatment ($p = 0.079$), but it is with 50 µg/L CBZ treatment ($p = 0.00$). There was also statistical significance between the 50 µg/L and the 25 µg/L CBZ treatment ($p = 0.00$). Due to these results we suggest that, as the concentration of CBZ increases in the treatments, the size (increases) and the weight (decreases) of the seedlings are affected. Similarly, we suggest that the increase in this substance affects the number of buds or MC to the extent to reach an inhibition threshold, so we advocate that emerging contaminants such as drugs, pesticides and other substances that pollute rivers and lakes should be regulated.

Table 1. Results obtained for CBZ exposure treatments, after a period of 30 days under controlled conditions in the growth chamber

Treatment (μg/L CBZ)	**Size (cm)** **Average ± SD**	**Weight (mg)** **Average ± SD**	**MC ± SD**
0	2.7 ± 0.3	379.76 ± 14.4	4 ± 0.7
25	4.8 ± 0.8	111.76 ± 8.7	3 ± 0.7
50	4.7 ± 0.8	95.7 ± 47.9	0.4 ± 0.5

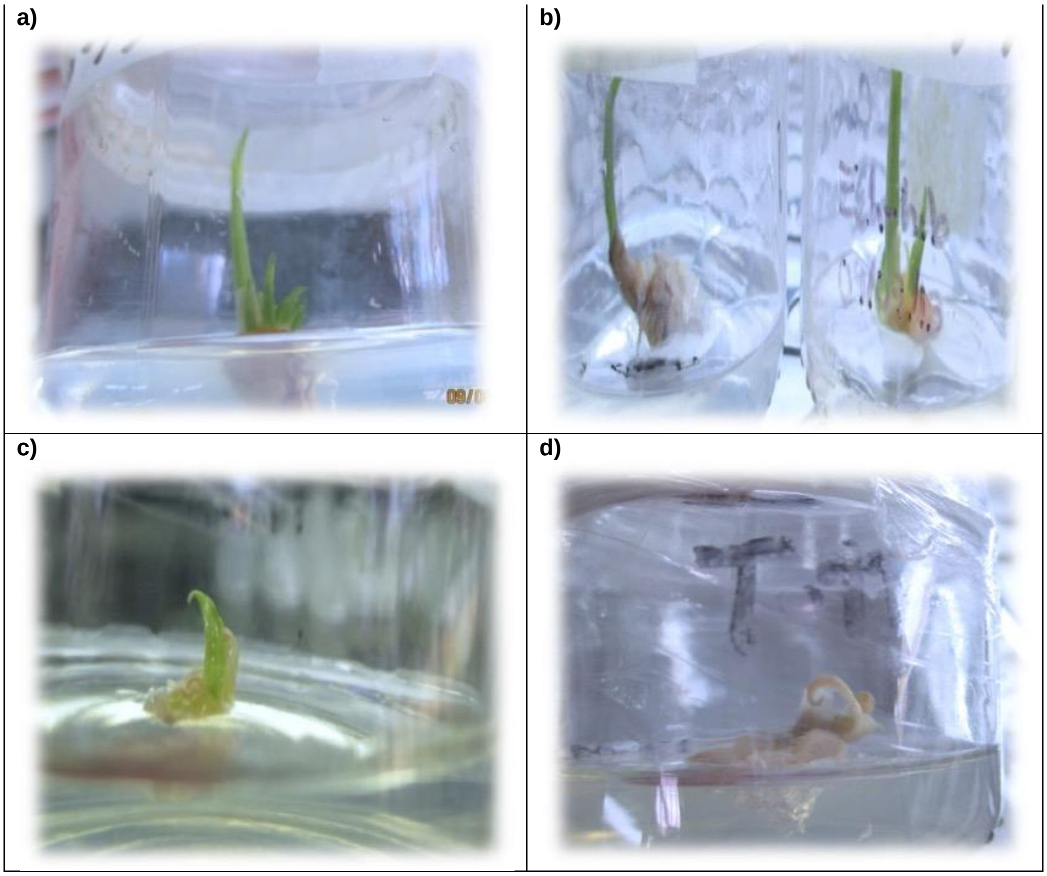

Figure 1. Photographs of Z. aethiopica after 30 days in growth chamber. a) growth of buds with 0 μg/L CBZ (control), b) growth of buds with 25 μg/L CBZ, c) growth of buds with 50 μg/L CBZ, d) Premature necrosis of *Z. aethiopica* with 50 μg/L CBZ.

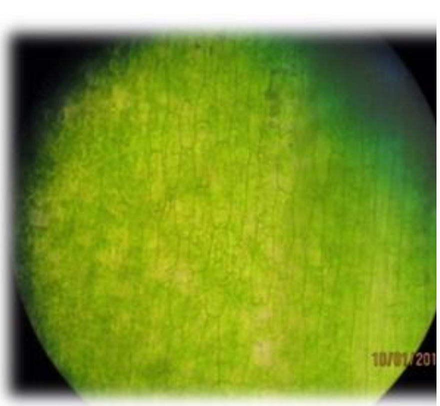

Figure 2. Control *Z. aethiopica* at 60x.

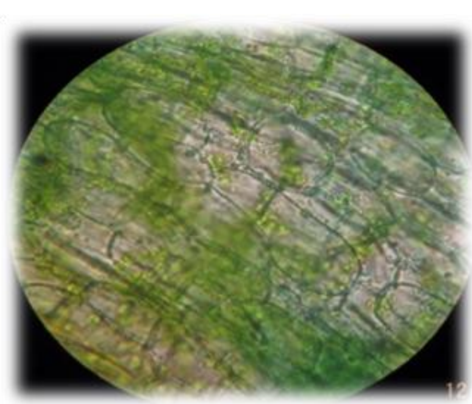

Figure 3. *Z. aethiopica* control at 100x.

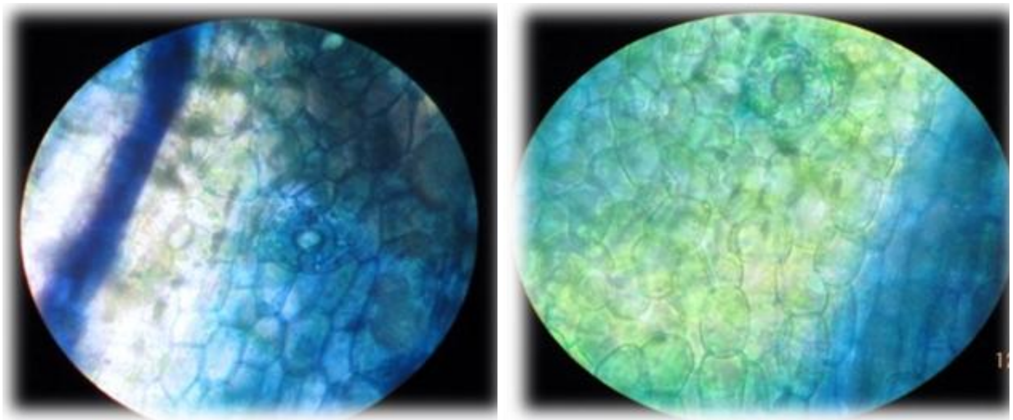

Figure 4. *Z. aethiopica* 25 µg/L CBZ at 100x.

Physiological observation under a microscope

In order to confirm the previous results, we made microscopic observations of tissues from the treatments at 0 and 25 µg/L CBZ, after 30 days of growth in the chamber under controlled conditions (Figure 2-4). They were not performed for treatment at 50 µg/L CBZ, because in that concentration there was cell damage and premature necrosis.

In these observations there is a variation in the size of the cells and organelles, which leads to conditions that prevent the cells from generating normal buds or tissues, the presence of CBZ in the concentration of 50 µg/L prevents the formation of new cells or cell de-differentiation, leading to premature tissue death.

Prieto-Mendez and colleagues point out some phytotoxicity effects of species subjected to heavy metals in the manifestation of chlorosis and necrosis, although accompanied by a decrease in root size with subsequent senescensia and death (Prieto Méndez et al., 2009).

Studies of elongation of adventitious buds under to Ca^{2+} and gibberellic acid, show that, the elongation has a cost on the number of sprouts or (MC); generating, even, considerable leaf deformations, along with a decrease in leaf area. Gibberellic acid promotes and increases the extensibility of the cell wall (Calderón-Baltierra, 1994) and threatens the integrity of the plant if the level of elongation is exceeded.

Z. aethiopica exposed to CBZ shows stimulation in its elongation, obtaining a larger size, however the number of its buds and therefore of MC is decreased; analogous behavior to the effect shown by vegetal species subjected to other growth regulators. (Robres-Torres et al, 2015; Miguel Luna et al., 2014).

CONCLUSION

Significant differences were found ($p<0.05$) in the three treatments 0, 25 and 50 µg/L of CBZ and for the three variables: size, weight and number of buds. This allows us to conclude that the presence of CBZ affects the size, weight and number of buds (MC) of *Z. aethiopica*.

In the studies under the microscope, variation in size and morphology was observed with respect to the treatment at 0 µg/L of CBZ; which suggests modification in the reproductive performance, even to present cellular damage. At higher concentrations of CBZ (50 µg/L) the plant shows senescence and death effects, showing chlorosis and necrosis, during the 30-day trial period.

It is possible to infer, that the repeated exposure of plant species to this type of EC can modify its characteristics and possibly affect the number of individuals present in an ecosystem.

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