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Cytogenetic Effects of the Neonicotinoid Insecticides Nuprid 200 SL and Calypso 480 SC on Plant Model System

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Abstract

Data from the present study indicate that the neonicotinoid insecticides Nuprid 200 SL (with the active substance imidacloprid) and Calypso 480 SC (with the active substance thiacloprid) reduce the mitotic activity by inhibiting cell division and increasing chromosomal aberrations in the cells of the root apical meristem of Allium cepa L. The tested solutions of pesticides cause a wide range of anomalies associated with disorders in forming the cell's division apparatus and the integrity of chromosomes. The comparative analysis of their mutagenic action shows a higher genotoxic potential of Calypso 480 SC. The established indices of chromosomal abnormalities for Calypso 480 SC and Nuprid 200 SL are 1.07 ± 0.38% and 0.70 ± 0.41% respectively. The mitosodepressant effect and a large number of chromosomal aberrations and mitotic abnormalities in Allium cepa cells when treated with Nuprid 200 SL and Calypso 480 SC are evidence of their high toxic potential and the significant risk of environmental pollution by their use in agriculture. Parts of this work was published in abstract form and presented as an oral presentation at the IV International Agricultural, Biological & Life Science conference, Edirne, Turkey, 2022 August 29-31, [1] and as a poster

presentation at the 5th Balkan Scientific Conference on Biology, Plovdiv, Bulgaria, 2021 April 15-16 [2].

Keywords
Allium test; chromosomal aberration; mitotic index; phase indices; cytotoxicity; genotoxicity

1. Introduction

The chemical properties and concentration of pesticides released in nature are essential in determining the degree of pollution in the environment. Pesticides are used to control pests in agriculture, but they can also adversely affect other organisms. Many studies have shown that chronic exposure to low levels of pesticides can cause mutations and carcinogenicity in experimental plant and animal organisms [3-11]. In this sense, studies on the toxic potential of these substances are critical. One of the possibilities for studying the genotoxic effect of pesticides is using plants as test objects. Plants are recognized as excellent genetic models in detecting mutagenic environmental agents and are often used for biomonitoring studies [12-16].

Neonicotinoids are a significant class of insecticides developed over the last 30 years to increase crop yields. They are neuroactive substances chemically similar to nicotine. Their use is associated with several adverse environmental effects, including increased mortality of honey bees in areas with arable crops. Despite the complex nature of the problem, it is primarily related to pesticide exposure. Pesticide residues are present in water, air, and food. In addition to the desired effects, such as protecting crops from diseases and pests, pesticides harm non-target organisms, including humans. Their incorrect use hurts the health of bees. In our research, the presence of neonicotinoids was found in samples of bees and bee stocks (honey, wax, and pollen) in regions in Bulgaria, with reported high losses of bee colonies, which is the reason for the present study.

The potential genetic risk of neonicotinoids requires extensive study of their genotoxicity, using a variety of approaches and test systems [17, 18]. In this study, we evaluated the cytostatic and mutagenic effects of the neonicotinoid insecticides Nuprid 200 SL (with the active substance imidacloprid) and Calypso 480 SC (with the active substance thiacloprid) on the cells of the root meristem of *Allium cepa* L.

2. Materials and Methods

2.1 Materials

Our study used bulbs from Allium cepa, provided by the Maritsa Institute of Vegetable Crops - Plovdiv. Bulbs with a diameter of 25-30 mm, which were not subjected to preliminary treatment, were selected. At the beginning of the experiment, the outer scales and old roots of the bulbs were carefully removed. The bulbs are protected from direct sunlight to minimize fluctuations in the rate of cell division. The experiment was performed in a laboratory environment at a constant temperature of 22 ± 0.5°C.

Nuprid 200 SL is a systemic insecticide with solid contact and gastric activity against pests. It has a quick initial effect and a prolonged after-effect. It is effective against many problems that have

acquired resistance to other groups of insecticides. Nuprid 200 SL is toxic to bees, fish, birds, and beneficial insects. The active substance of the insecticide is imidacloprid ($C_9H_{10}ClN_5O_2$), with a recommended dose of 200 grams per liter.

Calypso 480 SC is a systemic insecticide with a broad spectrum of action. It is effective in contact and ingestion by insects. The active substance of the insecticide is thiacloprid ($C_{10}H_9ClN_4S$), with a recommended dose of 480 grams per liter. Its mechanism of action is similar to other neonicotinoids. It involves selective binding to the insect's nicotinic acetylcholine receptor, initially stimulating postsynaptic receptors, then paralyzing nerve conduction, leading to rapid death.

2.2 Methods

2.2.1 Experimental Solutions

From the tested pesticides, stock working solutions (BS) were prepared according to the producers' instructions in a concentration recommended for use in agricultural practice: 0.05% for the insecticide Nuprid 200 SL and 0.02% for the insecticide Calypso 480 SC. Working solutions with lower concentrations - 75%, 50%, and 25% of the experimental solutions for each pesticide were prepared from the stock solutions. In the control variant, the roots were grown in distilled water.

2.2.2 Reading of Microscopic Parameters

The *Allium* test system [19] allows for a combined approach in cytotoxicity and mutagenicity studies by applying genetic methods. From each experimental variant, five microscopic preparations were prepared from the roots of different individuals to assess mitotic indices and chromosomal aberrations. Acetocarmine staining was applied. A minimum of 2000 cells from each microscope slide, or a total of at least 10000 cells for each test pesticide concentration tested, was analyzed. Calculating the total mitotic and phase indices determined the cytostatic action of pesticide solutions in different concentrations. They are calculated according to the following formulas:

$$\boldsymbol{IM}\ (\%) = \frac{\boldsymbol{N'}}{\boldsymbol{N}} \times \mathbf{100}$$

$$\boldsymbol{I\ Pph}\ (\%) = \frac{\boldsymbol{N\ Ppf}}{\boldsymbol{N'}} \times \mathbf{100}$$

$$\boldsymbol{I\ Mph}\ (\%) = \frac{\boldsymbol{N\ Mph}}{\boldsymbol{N'}} \times \mathbf{100}$$

$$\boldsymbol{I\ Aph}\ (\%) = \frac{\boldsymbol{N\ Aph}}{\boldsymbol{N'}} \times \mathbf{100}$$

$$\boldsymbol{I\ Tph}\ (\%) = \frac{\boldsymbol{N\ Tph}}{\boldsymbol{N'}} \times \mathbf{100}$$

Where: IM is the mitotic index, calculated in %,
N' is the number of dividing cells,
N is the total number of analyzed cells,

IPph is the prophase index, calculated in %,

NPph is the number of cells in prophase,

IMph is the metaphase index, calculated in в %,

NMph is the number of cells in metaphase,

IAph is the anaphase index, calculated in %,

NAph is the number of cells in anaphase,

ITph is telophase index, calculated in %,

NTph is the number of cells in telophase.

Genotoxicity was assessed by determining the frequency of chromosomal aberrations in *Allium cepa* meristem cells using an anaphase method (Fiskesjo, 1985) and a micronucleus test (Fenech, 2000). The spectrum of chromosomal abnormalities and the frequency of different disorders were analyzed, reflecting the specifics of the genotoxic action of the studied insecticides.

2.2.3 Statistical Analysis

Data on mitotic index and aberration frequency were analyzed statistically using Student's t-test to assess significant differences between each test group and the control.

3. Results

3.1 Cytostaticity of the Insecticides Nuprid 200 SL and Calypso 480 SC

By examining the mitotic index, cytotoxic contaminants in the environment can be reliably identified. This parameter allows for estimating the frequency of cell division [9, 11, 20, 21].

Table 1 gives data on the mitotic and phase indexes in the growing root tip of *Allium cepa* in the control and the experimental variants treated with Nuprid 200 SL and Calypso 480 SC.

Table 1 Mitotic index (IM) and phase indexes in (%) of *Allium cepa* treated for 72 hours with different concentrations of Nuprid 200 SL and Calypso 480 SC.

Samples	Mitotic index IM	Prophase index IPph	Metaphase index IMph	Anaphase index IAph	Telophase index ITph
Control	51.58 ±1.50	93.42 ±2.91	2.51 ±1.16	1.90 ±0.96	2.17 ±0.92
Nuprid 25 25 mg·l^{-1} imidacloprid	47.32 ±7.82	92.26 ±3.07	2.96 ±0.75	1.75 ±1.08	3.03 ±1.61
Nuprid 50 50 mg·l^{-1} imidacloprid	45.71 ±4.05***	93.61 ±3.76	1.86 ±1.11	1.84 ±1.35	2.70 ±1.69
Nuprid 75 75 mg·l^{-1} imidacloprid	46.54 ±1.83***	94.15 ±2.05	1.73 ±0.91	1.99 ±0.96	2.12 ±0.66
Nuprid BS 100 mg·l^{-1} imidacloprid	46.55 ±2.46***	90.90 ±1.70	3.23 ±1.08	2.63 ±0.72	2.63 ±0.72
Calypso 25 24 mg·l^{-1} thiacloprid	49.85 ±6.56	93.98 ±4.22	1.61 ±1.32	2.17 ±1.79	2.24 ±1.42
Calypso 50 48 mg·l^{-1} thiacloprid	49.04 ±10.79	93.87 ±2.73	1.74 ±1.03	1.81 ±1.10	2.58 ±1.26

Calypso 75 72 mg·l^{-1} thiacloprid	48.16 ±4.39	96.70 ±2.92	1.07 ±1.13	0.87 ±0.67	1.36 ±1.26
Calypso BS 96 mg·l^{-1} thiacloprid	46.60 ±4.35*	90.42 ±1.37	3.02 ±0.42	3.72 ±1.46	2.84 ±0.67

$p < 0.05$*; $p < 0.01$**; $p < 0.001$***

3.2 Genotoxicity of the Insecticides Nuprid 200 SL and Calypso 480 SC

Analysis of chromosomal and mitotic abnormalities is one of the most sensitive and effective methods for testing the genotoxic potential of various agents. The present study ascertained that different concentrations of Nuprid 200 SL and Calypso 480 SC caused various abnormalities, ranging from chromosomal fragmentation to division spindle disorganization.

Table 2 presents the frequency of observed abnormalities in the experimental and control samples treated for 72 hours with different concentrations of Nuprid 200 SL and Calypso 480 SC.

Table 2 Frequency of incidence of different types of chromosomal aberrations analyzed by the *Allium* test. For each sample, the data in the first row are calculated relative to the total number of cells (N), and the data in the second row - as% relative to the number of dividing cells (N').

Samples	C-mitosis	"Vagrant" chromosomes	Chromosomal bridges	Chromosomal fragments	Micronuclei	Asynchronous mitosis	Disturbed prophase	Multipolar mitosis	Total
Control	0.12 ±0.08	0.12 ±0.11	-	0.01 ±0.02	0.01 ±0.02	0.01 ±0.02	0.01 ±0.02	-	0.27 ±0.15
	0.23 ±0.16	0.23 ±0.23		0.02 ±0.04	0.02 ±0.04	0.02 ±0.04	0.02 ±0.04		0.53 ±0.29
Nuprid 25 25 mg·l^{-1} imidacloprid	0.12 ±0.04	0.15 ±0.04	0.06 ±0.06	0.04 ±0.04	0.04 ±0.09	0.01 ±0.02	-	-	0.45 ±0.15
	0.26 ±0.09	0.33 ±0.10	0.13 ±0.15	0.08 ±0.08	0.07 ±0.16	0.02 ±0.05			0.97 ±0.27*
Nuprid 50 50 mg·l^{-1} imidacloprid	0.19 ±0.07	0.18 ±0.10	0.06 ±0.07	0.02 ±0.03	0.02 ±0.03	0.01 ±0.02	-	0.01 ±0.02	0.48 ±0.20
	0.41 ±0.15	0.39 ±0.20	0.12 ±0.16	0.04 ±0.06	0.05 ±0.06	0.02 ±0.05		0.02 ±0.04	1.05 ±0.41*
Nuprid 75 75 mg·l^{-1} imidacloprid	0.26 ±0.10	0.20 ±0.12	0.07 ±0.10	0.02 ±0.03	0.05 ±0.09	-	0.03 ±0.04	0.02 ±0.03	0.65 ±0.27*
	0.56 ±0.20	0.44 ±0.28	0.15 ±0.20	0.04 ±0.06	0.11 ±0.19		0.07 ±0.10	0.04 ±0.06	1.41 ±0.60**
Nuprid BS 100 mg·l^{-1} imidacloprid	0.28 ±0.16	0.25 ±0.20	0.05 ±0.07	0.03 ±0.06	-	-	0.02 ±0.04	0.08 ±0.15	0.70 ±0.41***
	0.59 ±0.35	0.54 ±0.41	0.10 ±0.15	0.06 ±0.13			0.04 ±0.09	0.16 ±0.30	1.49 ±0.84***
Calypso 25 24 mg·l^{-1} thiacloprid	0.09 ±0.07	0.21 ±0.19	0.04 ±0.04	0.06 ±0.07	0.06 ±0.11	-	-	0.01 ±0.02	0.48 ±0.29

	0.18 ±0.14	0.40 ±0.34	0.09 ±0.08	0.12 ±0.13	0.11 ±0.19			0.02 ±0.05	0.92 ±0.49
Calypso 50 48 mg·l^{-1} thiacloprid	0.14 ±0.07 0.31 ±0.21	0.21 ±0.17 0.46 ±0.35	0.08 ±0.03 0.17 ±0.09	0.07 ±0.10 0.14 ±0.20	0.01 ±0.02 0.02 ±0.04	0.01 ±0.02 0.02 ±0.04	0.01 ±0.02 0.02 ±0.04	0.02 ±0.04 0.04 ±0.08	0.54 ±0.19* 1.17 ±0.47*
Calypso 75 72 mg·l^{-1} thiacloprid	0.29 ±0.09 0.59 ±0.18	0.23 ±0.07 0.47 ±0.12	0.01 ±0.02 0.02 ±0.04	-	0.01 ±0.02 0.02 ±0.04	-	-	0.01 ±0.02 0.02 ±0.04	0.55 ±0.21* 1.12 ±0.41*
Calypso BS 96 mg·l^{-1} thiacloprid	0.22 ±0.14 0.47 ±0.29	0.52 ±0.19 1.13 ±0.46	0.08 ±0.07 0.17 ±0.16	0.07 ±0.09 0.14 ±0.19	0.12 ±0.09 0.24 ±0.18	-	-	0.05 ±0.06 0.10 ±0.13	1.07 ±0.38*** 2.29 ±0.77***

$p < 0.05$*; $p < 0.01$**; $p < 0.001$***

The tested concentrations of Nuprid 200 SL and Calypso 480 SC cause a wide range of chromosomal aberrations and mitotic disorders. Deviations of the type: chromosome fragments and bridges, asynchronous and multipolar mitoses, 'vagrant' and lagging chromosomes, micronuclei, disturbed prophases, and K-mitoses were reported (Figure 1 A-H).

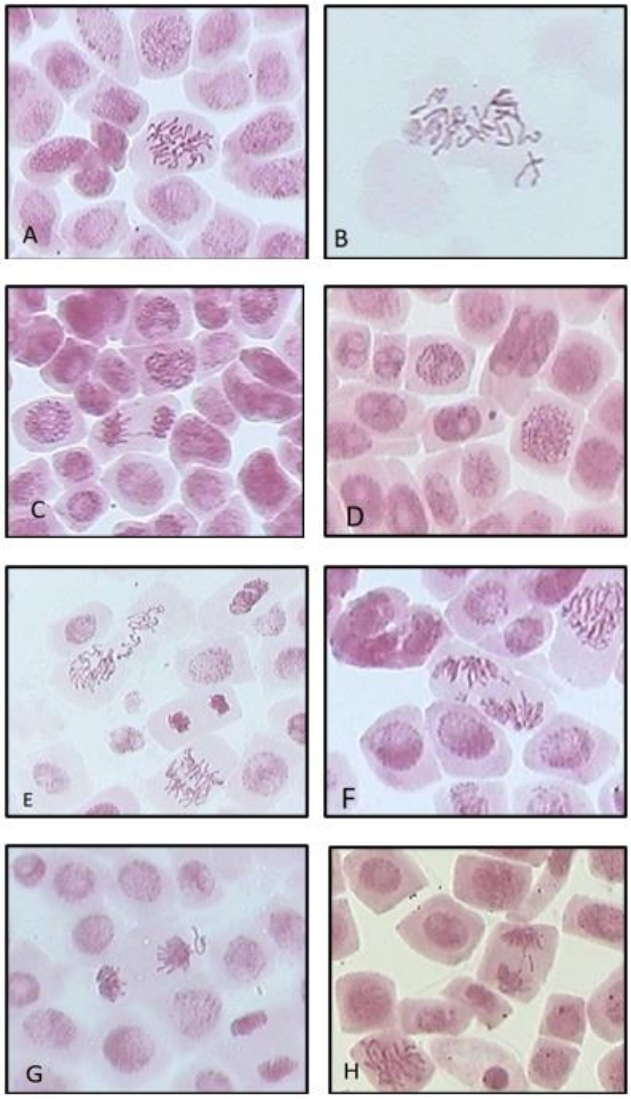

Figure 1 Types of chromosomal aberrations in cells of *Allium cepa,* treated for 72 hours with different concentrations of Nuprid 200 SL and Calypso 480 SC: C-mitosis (A, B), "vagrant" chromosome (B, G), chromosomal bridge (C), micronucleus (D), disturbed prophase (E), chromosomal fragments (F), lagging chromosome (H).

4. Discussion

4.1 Cytostatic Effect of Nuprid 200 SL

Treatment with certain chemicals disrupts the regulation of cell cycle processes and causes activation of mechanisms to control and inhibit cell division [18, 22]. In our study, a mitosodepressant effect of Nuprid 200 SL was established. The value of the mitotic index is significantly lower than the control variant at the three highest-tested pesticide concentrations

(Table 1). No dose-dependent inhibition of cell proliferation was observed. The base solution of Nuprid 200 SL and its 75% concentration showed a similar decrease in mitotic activity, while the maximum cytostatic effect was observed with the 50% pesticide solution. Suppression of mitotic activity has been reported as a combined effect of many pesticides [17, 23-26]. Decreased cell proliferation may be due to blockage of the mitotic cycle during interphase [17], inhibition of nuclear protein synthesis essential for the ordinary course of mitosis [27], inhibition of the synthesis of DNA [28], or a change in the duration of the mitosis stages [29]. Based on the analyzed phase indices, the influence of the tested concentrations of Nuprid 200 SL on the distribution of dividing cells in the individual phases of mitosis can be determined. The data from the present study show that the insecticide Nuprid 200 SL does not significantly affect the frequency of cells in the individual mitotic phases. In the stock solution of the pesticide, we reported the lowest percentage of cells in prophase compared to the control and other experimental variants (Table 1). In this solution, we also found the highest indices of metaphase and anaphase. The data obtained show that the maximum concentration of Nuprid 200 SL provokes some acceleration of the transition of dividing cells from prophase to the following stages of mitosis.

4.2 Cytostatic Effect of Calypso 480 SC

The value of the mitotic index is maximum in the control variant (51.58 ± 1.50) and significantly lower in the stock working solution of the pesticide (46.60 ± 4.35), demonstrating its cytotoxic potential. A weaker depressant effect on cell proliferation was reported for the experimental variants with a lower dose of the active substance thiacloprid (Calypso 25 and Calypso 50). Other authors have also written that the mitotic index decreased when exposed to the neonicotinoid thiacloprid [30, 31]. The present study found changes in cell frequencies in the various mitotic phases due to exposure to Calypso 480 SC. When pesticides penetrate and accumulate in the cell, they can be highly toxic and affect the phases of the mitotic division [32]. Treatment with Calypso 480 SC significantly reduced the percentage of cells in metaphase at all tested concentrations compared to the control, except for the stock solution. The lower metaphase index may be related to the accelerated course of metaphase in response to mitotic stress. At the same time, the solution with the pesticide concentration recommended in agricultural practice shows a different effect on the cell frequencies for the different phases of mitosis. The stock solution provoked a decrease in the relative duration of prophase compared to the other tested concentrations and controls, as demonstrated by the lowest prophase index reported (90.42 ± 1.37). This experimental variant found the highest frequency of dividing cells in metaphase, anaphase, and telophase (Table 1).

Analysis of the results of the mitotic index and phase indices showed an inhibitory effect of the tested insecticides Nuprid 200 SL and Calypso 480 SC on cell division in *Allium cepa*.

4.3 Genotoxic Effect of Nuprid 200 SL

The analysis of the type and frequency of chromosomal abnormalities allows interpretation of the mutagenic effects of the chemical compounds. The genotoxic effects of imidacloprid on various test organisms have been studied by several authors [17, 18, 33-38]. In the present study, the genotoxic effect of the insecticide Nuprid 200 SL was established, with the active substance imidacloprid manifested in the meristem cells of *Allium cepa*. The results show a significant dose-dependent increase in the frequency of chromosomal aberrations in onion cells (Table 2). The

reported values of chromosomal changes range from 0.27 ± 0.24% in the control variant to 0.70 ± 0.41% in the pesticide stock solution after 72 hours of exposure—all tested concentrations of Nuprid 200 SL cause a higher frequency of aberrations compared to the control. Disorders related to the formation and function of the division spindle are the most common type. A high percentage of cells with C-mitosis and 'vagrant' chromosomes was reported at all tested concentrations of Nuprid 200 SL. C-mitosis can occur due to disrupted microtubules [39] or due to problems with the formation of the filaments of the division spindle [40]. The stock solution of the pesticide provokes deviations with a frequency exceeding the control of the other experimental variants. In this variant, a high percentage of cells with C-mitosis (Figure 1A), 'vagrant' (Figure 1B), and lagging chromosomes, multipolar anaphases, and fragmented metaphases were found, which is an indicator of the aneugenic potential of the pesticide. Experimental variant Nuprid 75 provoked a high percentage of aberrations, with a maximum number of cells with chromosomal bridges (Figure 1C), micronuclei (Figure 1D), and disturbed prophases (Figure 1E) reported. The experimental variants Nuprid 50 and Nuprid 25 also induce a wide range of aberrations but with a lower frequency than the pesticide stock solution. In these test groups, chromosome fragments (Figure 1F), diagonal anaphases, and cells with varying degrees of compactification of genetic material through telophase were observed. Aberrations were established in the control cells, which resulted from an antimutagenic effect and had a lower frequency.

The results of the present study show that the insecticide Nuprid 200 SL has a mutagenic effect on Allium cepa cells, causing chromosomal aberrations and disorders during mitosis. These results are consistent with data from studies by other authors [17, 18, 34, 38], who reported significant levels of chromosomal aberrations in *Allium cepa* and *Pisum sativum* L. cells and the erythrocytes of imidacloprid-treated mice.

4.4 Genotoxic Effect of Calypso 480 SC

A proportional relationship was found between the concentration of Calypso 480 SC solutions and the frequency of aberrations and deviations during mitosis. As can be seen from the data presented in Table 2, Calypso 480 SC stock solution has a genotoxic effect by inducing disorders with a much higher frequency than the control. In the other experimental variants, the frequency of structural changes of the chromosomes also exceeds the the frequency in the control sample. Based on the identified disorders provoked by the action of the stock solution of Calypso 480 SC, 'vagrant' and lagging chromosomes predominate (Figures 1G and 1H), which are the result of an unrealized connection between the kinetochore of the chromosome and the filament of the division spindle. We also found many cells with C-mitosis (Figure 1B) and micronuclei in this variant. The presence of micronuclei demonstrates clastogenic and aneugenic pesticide activity. These structures can be formed from acentric fragments due to clastogenic actions or loss of an entire chromosome due to aneugenic activities [41]. In this study, micronuclei in the cells were observed in all tested samples. Still, at the maximum concentration of thiacloprid studied, their frequency was very high and demonstrated the mutagenic potential of this neonicotinoid (Table 2). For all Calypso 480 SC solutions, the presence of chromosomal bridges, which are missing in the control variant, was reported. The tested Calypso 50 variant shows a significant index of aberrations such as chromosome fragments, 'vagrant' chromosomes, and multipolar anaphases.

The results show that Calypso 480 SC has a pronounced genotoxic effect on the root meristem of *Allium cepa*, as evidenced by the higher number of chromosomal and mitotic disorders registered in the experimental variants compared to the control.

5. Conclusions

Data from the present study indicate that the insecticides Nuprid 200 SL and Calypso 480 SC have a cytostatic effect by inhibiting cell division from the root apical meristem of *Allium cepa*. The comparative analysis of the mutagenic action of the studied pesticides shows a higher genotoxic potential of Calypso 480 SC. Stock solutions of both pesticides induced chromosomal abnormalities in *Allium cepa* cells with a much higher frequency than the control, but the chromosomal aberration index for Calypso 480 SC (1.07 ± 0.38%) exceeded by value the index for Nuprid 200 SL (0.70 ± 0.41%).

The direct toxicity of neonicotinoids to environmental organisms requires an assessment of their long-term effects on ecosystems. The mitosodepressant effect we have reported, the appearance of a large number of chromosomal aberrations and mitotic abnormalities in *Allium cepa* cells when treated with the neonicotinoid insecticides Nuprid 200 SL and Calypso 480 SC is evidence of their high toxic potential and the significant risk posed by their use in agriculture from environmental pollution.

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Author Contributions

The authors contributed equally to this work.

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Competing Interests

The authors have declared that no competing interests exist.

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