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Toxic Effects of the Insecticide "Actara WG" on the Allium cepa Root Meristem

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Abstract. The effect of the insecticide "Actara WG" on the cell division rate and on the chromosomal apparatus of *Allium cepa* root meristem cells was studied. By applying anaphase analysis and micronucleus mutagenicity test, a control sample (tap water) and experimental samples of insecticide solutions with different concentrations - 100%, 50% and 25% ("Actara WG" SS; "Actara WG" 50; "Actara WG" 25) of the recommended by the producer were compared. Approximately 2,000 cells per individual and five individuals per sample were analyzed. Comparative analysis of mitotic indices showed a negative effect of the tested pesticide in solution with the recommended concentration and in 50% solution of it on the rate of the cell division during the root germination for 48 hours. The genotoxic effect of the studied insecticide was analyzed. The chromosomal structural changes observed during the investigation are classified into 7 categories. Chromosomal abnormalities such as pulverized chromosomes, diagonal anaphases, chromosome fragments, anaphase and telophase bridges - alone and in combination with fragments, wandering and lagging chromosomes and micronuclei were detected in meristem cells after treatment with all the tested solutions of "Actara WG", but the highest percentage of aberrations was found after treatment with "Actara WG" 50%. Some chromosome aberrations were found in the control sample, but in a significantly lower percentage. It was concluded that the insecticide "Actara WG" negatively affects the cell division rate and has a genotoxic effect on the *Allium cepa* root meristem cells.

Key words: Actara, insecticide, neonicotinoids, *Allium cepa*, chromosomal aberration.

Introduction

Neonicotinoids are neurotoxic insecticides widely used in modern agriculture to control pests such as ants, aphids, whiteflies, beetles, and some Lepidoptera species (Goulson, 2013). Their negative impact on various natural components and nature in general has attracted the attention of researchers from around the world in recent decades. "Actara WG" is one of the most widely used agrochemicals with the active substance thiamethoxam. Thiamethoxam is a second generation (class III - moderately toxic) neonicotinoid insecticide (Maienfisch et al., 2001; Nauen et al., 2003). Compared to another neonicotinoid insecticide, imidacloprid, thiamethoxam has a lower affinity for nicotinic receptors. While imidacloprid is effective at nanomolar concentrations, thiamethoxam acts in millimolar concentrations (Wiesner & Kayser, 2000). Its binding to biological receptors *in vivo* can cause a series of biochemical reactions (Copeland, 2000). According to Motohiro & John (2005), neonicotinoids are easily absorbed by plants and kill insect pests at very low doses, but at the same time have low toxicity to vertebrates. In contrast, Su et al. (2021) in their study on the binding mechanism of thiamethoxam with three protein models report that it is an environmental pollutant and due to its accumulation in the ecosystem, poses potential risks to the health of mammals and even humans. This view is also supported by studies by other researchers who provided data on the presence of thiamethoxam and its metabolites in the human body (Wang et al., 2019; Zhu et al., 2019).

Cytogenetic markers such as chromosomal aberrations and micronuclei have been used in screening studies for neonicotinoid genotoxicity (Yadav & Kaushik, 2002; Zeljezic & Garaj-Vrhovac, 2004). De Morais et al. (2019) investigated and compared the genotoxic capacity of different concentrations of thiamethoxam, acetamiprid, imidacloprid and fipronil by a micronucleus

test using the *Tradescantia pallida* test object. The authors reported genotoxic activity in the study subject at the highest concentrations of the studied pesticides. The genetic test object *Allium cepa* is widely used in biomonitoring studies on the genotoxicity of various factors due to its proven high efficiency (Saxena, 2010). Using this test object, Verma & Srivastava (2018) investigated the morphotoxic and cytogenotoxic effects of the pesticide pendimethalin, developing a system of morphological and genotoxic biomarkers. Datta et al. (2018) also used *Allium cepa* to compare the genotoxicity of soils treated with pesticide and vermicompost and reported significant differences in the established mitotic indices and the frequency of the established chromosomal aberrations with respect to the studied samples.

The present study aims to monitor and characterize the effect of the pesticide "Actara WG" on the rate of cell division and to analyze its genotoxic potential using the *Allium cepa* test system.

Material and Methods

Three concentrations of the broad-spectrum insecticide "Actara WG" (active substance thiamethoxam, 250 g.kg-1) were tested in the present study. Stock solution ("Actara WG" SS 250 g.kg-1) was prepared according to the manufacturer's recommendations. Working solutions with a lower concentration were prepared from it - 50% ("Actara WG" 50 125 g.kg-1) and 25% ("Actara WG" 25 62.5 g.kg-1) of the basic solution. *Allium cepa* bulbs of the variety "Asenovgradska kaba 5" were used as a test object. The sprouted roots were fixed at the 48th hour of the treatment. Bulbs grown in tap water were used for control. Five bulbs from every concentration (and control) and five roots per a bulb were used in the analysis. A temporary microscopic slide was prepared from each root after washing with distilled water, treatment with 3N hydrochloric acid, 45% acetic acid and staining with acetocarmine. About 500 cells from a microscope slide were analyzed - a total of about 2000 cells per a sample.

The effect of the pesticide in solutions with the indicated concentrations on the rate of cell division was studied by calculating the mitotic index and phase indices. The mitotic index (IM) was determined as a percentage of the number of dividing cells and the total number of cells analyzed. Phase indices are calculated as percentages of the number of cells in a particular mitotic phase and the total number of dividing cells. To evaluate the cytogenetic effect of the experimental concentrations, the IM found in the analysis of the test samples was compared with the IM calculated for the control sample.

The genotoxic potential of the tested concentrations was investigated by anaphase analysis and micronucleus mutagenicity test. The observed chromosomal aberrations are summarized in seven categories as follows: 1) pulverized chromosomes in metaphase and anaphase; 2) wandering and lagging chromosomes; 3) fragments in metaphase, anaphase and telophase; 4) anaphase and telophase bridges with additional presence of fragments; 5) diagonal anaphases; 6) K mitosis and 7) micronuclei. For categories 1-6, the encounter frequencies were calculated as a percentage of the total number of cells and the number of dividing cells. For the seventh category, the frequency is calculated as the ratio between the number of cells with micronuclei and the total number of cells. The data obtained in the experimental and control samples were statistically compared. Student's t-test (Stangroom, 2018) was used to assess statistically significant differences between each test group and the control. Established differences at $P < 0.05$ were considered statistically significant.

Results

The results on the mitotic index and phase indices reported in the control and experimental samples are presented in Table 1.

Table 1. Mitotic index (IM) and phase indices in (%) in Allium cepa treated for 48 hours with different concentrations of "Actara WG". *Legend:* $p < 0.05$*; $p < 0.01$**; $p < 0.001$***

Samples	Mitotic index IM	Prophase index IPph	Metaphase index IMph	Anaphase index IAph	Telophase index ITph
Control	53.09 ± 3.88	87.92 ± 1.46	6.09 ± 1.00	2.94 ± 0.71	3.05 ± 0.64
"Actara WG" SS 250 g.kg-1 thiamethoxam	33.36 ± 7.24***	82.84 ± 1.56***	6.28 ± 1.61	6.36 ± 1.89**	4.53 ± 1.01*
"Actara WG" 50 125 g.kg-1 thiamethoxam	40.10 ± 6.68**	82.85 ± 2.43**	7.12 ± 1.78	5.94 ± 0.47***	4.09 ± 0.96*
"Actara WG" 25 62.5 g.kg-1 thiamethoxam	47.98 ± 8.31	85.87 ± 2.06	5.45 ± 0.90	4.61 ± 0.82**	4.07 ± 1.11

The mitotic index is an indicator of the rate of cell division. In the present study, the highest intensity of cell division was recorded with respect to the control sample (IM - 53.09%). Statistically significant differences compared to the control were found after the treatment with the recommended concentration of the pesticide - "Actara WG" SS (IM - 33.36%) and after the treatment with the sample "Actara WG" 50 (IM - 40.10%). Although not statistically significant, the data for IM showed a decrease even after treatment with "Actara WG" 25. When analyzing the phase indices, it was found that the cells in prophase have the highest frequency when treated with the control sample – 87.92%. This frequency was statistically significantly higher in comparison with those found for the tested samples of "Actara WG" SS and "Actara WG" 50 (82.84% and 82.85%, respectively). Statistically significant differences compared to the control were found with respect to the anaphase index in the three experimental samples with solutions of "Actara WG" SS, as well as with respect to the telophase index in the two samples with higher concentration (stock solution and 50% dilution) of the insecticide studied (Table 1).

Data on the established frequency of chromosomal aberrations in the root meristem of *Allium cepa* (control and experimental concentrations) are presented in Table 2.

During the study, chromosomal aberrations from the seven mentioned categories were found in the analyzed cells from the experimental samples (Figure 1). Some chromosomal aberrations were also detected in the control sample (0.53% in total), but in a statistically significantly lower percentage than all tested concentrations of the insecticide Aktara. The statistically significant differences found when comparing the results obtained in the control and experimental samples are shown in Table 2.

The highest percentage of structural chromosomal changes in dividing cells when testing the analyzed experimental samples was found after treatment with "Actara WG" 50 (5.57%) where the aberrations detected were as follows: wandering and lagging chromosomes (1.44%); single chromosome fragments (0.77%); anaphase and telophase bridges with fragments (0.63%); pulverized chromosomes and K mitosis (0.31%) and diagonal anaphases (0.26%). The frequencies of the detected chromosomal aberrations among the dividing cells in the other two tested solutions of the "Actara WG" (4.05% and 2.83%) were statistically significantly higher in comparison with the control (1.0%). The highest percentage of micronuclei in the total number of cells was found when treated with the solution "Actara WG" 50 - 0.67%. The data from the present study showed the highest pronounced genotoxic effect on meristem cells of *Allium cepa* with respect to the tested "Actara WG" 50 solution.

Table 2. Frequency of occurrence of different types of chromosomal aberrations analyzed by the Allium test: *Legend:* $p < 0.05$*; $p < 0.01$**; $p < 0.001$*** For each sample, the data in the first row are calculated as % 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	Pulverized chromosomes in metaphase and anaphase	Wandering and lagging chromosomes	Fragments in metaphase, anaphase and telophase	Anaphase and telophase bridges + fragments	Diagonal anaphases	K mitosis	Micronuclei	Total
Control	-	0.33±0.16 0.60±0.25	-	0.04±0.04 0.07±0.07	-	0.08±0.07 0.14±0.14	0.09±0.08	0.53±0.11 1.00±0.17
"Actara WG" SS 250 g.kg-1 thiamethoxam	0.06±0.14 0.08±0.19	0.54±0.27 1.57±0.61**	0.05±0.06* 0.14±0.17	0.15±0.11* 0.43±0.38*	0.24±0.12** 0.78±0.56**	0.17±0.11 0.55±0.41*	0.14±0.21	1.34±0.46** 4.05±1.18***
"Actara WG" 50 125 g.kg-1 thiamethoxam	0.12±0.04*** 0.31±0.22**	0.71±0.03*** 1.44±0.33***	0.07±0.13 0.77±0.75*	0.12±0.05** 0.63±0.21***	0.13±0.01*** 0.26±0.09***	0.10±0.08 0.31±0.14*	0.67±0.55*	2.14±0.53*** 5.57±2.18***
"Actara WG" 25 62.5 g.kg-1 thiamethoxam	0.08±0.03*** 0.17±0.07***	0.46±0.09 0.97±0.18*	0.08±0.10 0.16±0.21	0.27±0.13** 0.57±0.27**	0.13±0.06*** 0.27±0.09***	0.24±0.15* 0.49±0.25*	0.11±0.23	1.38±0.43** 2.83±0.54***

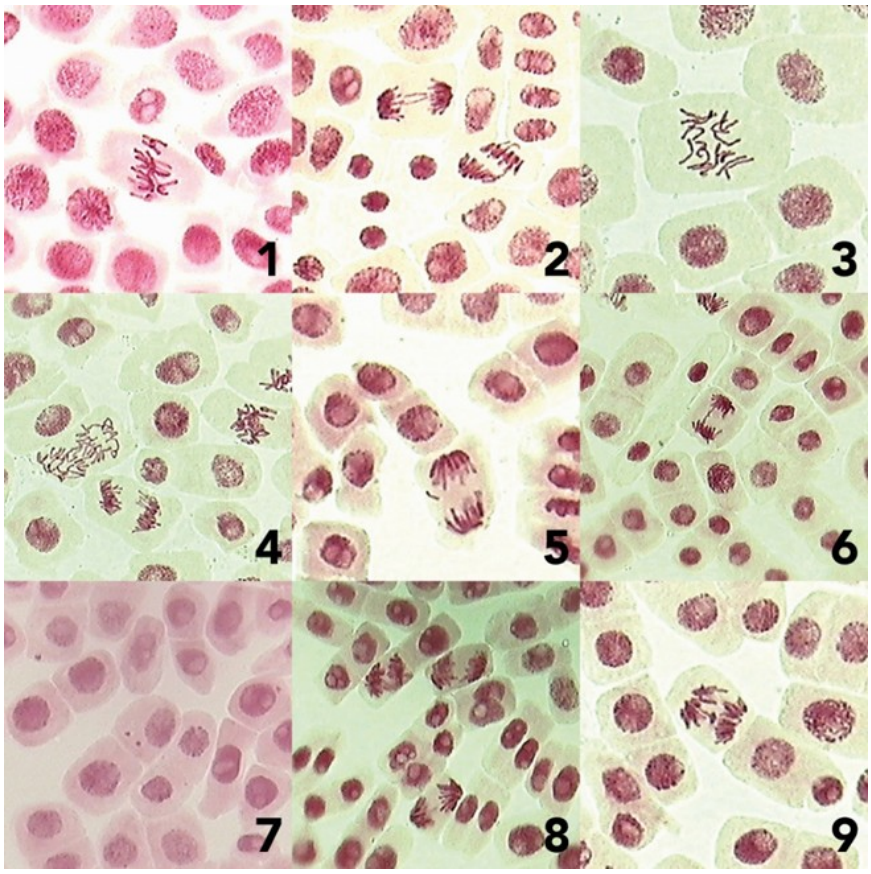

Fig. 1. Mutagenic effect of the pesticide "Actara WG" on the root meristem of *Allium cepa*. *Legend*: 1 - Wandering chromosomes; 2 - Anaphase and telophase bridges; 3 - K mitosis; 4 - Pulverized anaphase; 5 - Fragment in anaphase; 6 - Telophase bridge; 7 – Micronuclei; 8 - Diagonal anaphases; 9 - Fragment in anaphase.

Discussion

The negative impact of pesticides on the rate of cell division has been studied by many researchers (Singh, 2007; Liman et al., 2010; Asita & Mokhobo, 2013; Huan et al., 2016). There is evidence that treatment with certain agrochemicals adversely affects the course of the mitotic cycle, disrupting the regulation of its basic processes (Zabka et al., 2012). In accordance with these studies, the data from the present experiment showed reduced values of IM in meristem cells after treatment with the studied solutions in all of the three concentrations (Table 1). These results are an indication for the mitosis-depressive effect of the insecticide "Actara WG". Studies on the problem show that cell division can be negatively affected by various reasons – blocking the mitotic cycle in the interphase, inhibiting the synthesis of nuclear proteins and DNA, changes in the duration of individual mitotic phases and others (Mohanty et al., 2004; Chauhan & Gupta, 2005; Şekeroğlu et al., 2013; Önen et al., 2018).

The data from the present study show that the solutions of "Actara WG" in their different concentrations affect not only the rate of mitotic division, but also the distribution of dividing cells in different phases. Statistically significant differences compared to controls were available for both prophase and telophase (for "Actara WG" SS and "Actara WG" 50) and for anaphase (for all the three concentrations tested). It is noteworthy that in the experimental samples the prophase index was lower and the anaphase and telophase indices were higher (Table 1), which demonstrates a delay in the rate of mitosis during prophase and its intensification in anaphase and telophase. Changes in the duration of mitotic phases under the influence of various pesticides have been previously reported by Prasad & Das (1977) and Hanif & Davies (1998). According to Liman et al. (2011), the accumulation of pesticides in the cell could be highly toxic and this could affect the duration of the individual phases of mitosis. The decreased rate of cell division in *Allium cep*a meristem cells reported in the present study after treatment with "Actara WG" probably provokes mitotic stress and in compensation for this - a subsequent accelerated course of certain phases (anaphase and telophase).

The genotoxic potential of various pesticides, including neonicotinoids, has been studied by a number of authors (Karabay & Oguz, 2005; Jemec et al., 2007; Kreutzweiser et al., 2007; Rodríguez et al., 2015, etc.). The data from the present study show the highest levels of genotoxicity with respect to "Actara WG" 50. The observed overall incidence of structural chromosomal changes in the root meristem of *Allium cepa* was statistically significantly higher at all experimental concentrations of the tested pesticide compared to the control (Table 2). The great variety of reported chromosomal aberrations (Figure 1) is due to various damages - fragmentations, adhesion of chromosomes without telomeres, loss or damage in the centromere regions, damage to the dividing spindle and others. In the current study, the presence of micronuclei was found for all tested samples, but the highest frequency of cells with micronuclei was found after treatment with "Actara WG" 50 (Table 2). Their presence (Figure 1) is further evidence of the genotoxic activity of the studied insecticide. Micronuclei are the result of dropped chromosome fragments or the loss of whole chromosomes with damaged centromeres (Fenech, 2000). The results of the present study are consistent with those reported by Karabay & Oguz (2005) and Rodríguez et al. (2015), who report increased genotoxic potential of other neonicotinoid insecticides tested by *Allium cepa*.

Conclusions

The neonicotinoid insecticide "Actara WG" has a negative effect on the rate of cell division in the root meristem of *Allium cepa*. The wide range of chromosomal structural changes found with significantly higher frequencies in the experimental samples of the neonicotinoid insecticide "Actara WG" compared to the control are evidence for its clear genotoxic effect.

References

Asita, A. O. & Mokhobo, M. M. (2013). Clastogenic and cytotoxic effects of four pesticides used to control insect pests of stored products on root meristem of *Allium cepa. J. Environ. Sci. Nat. Resour. 3*: 133-145.

Chauhan, L. K. S. & Gupta S. K. (2005). Combined cytogenetic and ultrastructural effects of substituted urea herbicide and the synthetic pyrethroid insecticide on the root meristem cells of *Allium cepa*. *Pestic. Biochem. Physiol. 82*: 27-35.

Copeland, R. A., (2000). *Enzymes: a practical introduction to structure, mechanism, and data analysis*. Second Edition. Wiley-VCH, Inc. Retrieved from https://enzimo.files.wordpress.com/2012/09/enzymes-a-practical-guide2.pdf.

Fenech, M. (2000). The *in vitro* micronucleus technique. *Mutat. Res. 455*: 81-95.

Goulson, D. (2013). An overview of the environmental risks posed by neonicotinoid insecticides. *J. Appl. Ecol. 50*, 977e987. Doi: 10.1111/1365- 2664.12111.

Hanif, M. & Davies, M. S. (1998). Mitotic Activity in the Root Apical Meristems of *Secale cereale* (cv. K2) and *Triticum aestivum* (cvs. Chinese Spring, Lyallpur 73, Pak 81, Lu-26-S and Sandal) Under Elevated NaCI Treatment. *Pakistan Journal of Biological Sciences, 1(3)*: 163-166.

Huan, Z., Luo, J., Xu, Z. & Xie, D. (2016). Acute toxicity and genotoxicity of carbendazim, main impurities and metabolite to earthworm (*Eisenia foetida*). *Bull. Environ. Contam. Toxicol. 96*: 62-69.

Jemec, A., Tisler, T., Drobne, D., Sepcic, K., Fournier, D. & Trebse P. (2007). Comparative toxicity of imidacloprid, of its commercial liquid formulation and of diazinon to a non-target arthropod, the microcrustacean *Daphnia magna*. *Chemosphere, 68*: 1408-1418.

Karabay, N. U. & Oguz M. G. (2005). Cytogenetic and genotoxic effects of the insecticides, imidacloprid and methamidophos. *Genet. Mol. Res. 4(4)*: 653-662.

Kreutzweiser, D., Good, K., Chartrand, D., Scarr, T. & Thompson, D. (2007). Non-target effects on aquatic decomposer organisms of imidacloprid as a systemic insecticide to control emerald ash borer in riparian trees. *Ecotoxicol. Environ. Saf., 68*: 315-325.

Liman, R., Akyıl, D., Eren, Y. & Konuk, M. (2010). Testing of the mutagenicity and genotoxicity of metolcarb by using both Ames/Salmonella and Allium test. *Chemosphere, 80*: 1056-1061.

Liman, R., Cigerci, I. H., Akyıl, D., Eren, Y. & Konuk, M. (2011). Determination of genotoxicity of fenaminosulf by Allium and comet tests. *Pesticide Biochem Physiol., 99*: 61-64.

Maienfisch, P., Angst, A., Brandl, F., Fischer, W., Hofer, D., Kayser, H., Kobel, W., Rindlisbacher, A., Senn, R., Steinemann, A. & Widmer, H. (2001). Chemistry and biology of thiamethoxam: a second generation neonicotinoid. *Pest. Manag. Sci. 57*, 906e913. doi: 10.1002/ps.365.

Mohanty, S., Das, A. B., Das, P. & Mohanty, P. (2004). Effect of a low dose of aluminum on mitotic and meiotic activity, 4C DNA content, and pollen sterility in rice, *Oryza sativa* L. *Ecotoxicol. Environ. Saf. 59*: 70-75.

Morais, C. R. de, Pereiraa, B. B., Sousa, P. C. A., Santos, V. S. V., Campos, C. F., Carvalho, S. M., Spano, M. A., de Rezende, A. A. A. & Bonetti, A. M. (2019). Evaluation of the genotoxicity of neurotoxic insecticides using themicronucleus test in *Tradescantia pallida*. *Chemosphere, 227*/371-380.

Motohiro, T. & John, E. C. (2005). Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu. Rev. Pharmacol. Toxicol., 45*, 247–268.

Nauen, R., Ebbinghaus-Kintscher, U., Salgado, V. L. & Kaussmann, M. (2003). Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. *Pestic. Biochem. Phys. 76*, 55e69. doi: 10.1016/S0048-3575(03)00065-8.

Önen, Ö., Kiliçle, P. A., Adali, Y. & Beşeren H. (2018). The histopathological and genotoxic effects of neonicotinoid pesticides. *Bozok Med J. 8(1),* 139-147.

Saxena, P. N., Gupta, S. K. & Murthy, R. C. (2010). Carbofuran induced cytogenetic effects in root meristem cells of *Allium cepa* and *Allium sativum*: a spectroscopic approach for chromosome damage. *Pestic. Biochem. Physiol., 96*, 93-100.

Prasad, G. & K. Das. (1977). Effects of some growth substances on mitosis. *Cytologia, 42*: 323-329.

Rodríguez, Y. A., Christofoletti, C. A., Pedro, J., Bueno, O. C., Malaspina, O., Ferreira, R. A. & Fontanetti, C. S. (2015). *Allium cepa* and *Tradescantia pallida* bioassays to evaluate effects of the insecticide. *Chemosphere,120*: 438-442.

Şekeroğlu, V., Şekeroğlu, Z. A. & Kefelioğlu, H. (2013). Cytogenetic effects of commercial formulations of deltamethrin and/or thiacloprid on Wistar rat bone marrow cells. *Environ Toxicol., 28(9)*: 524-531.

Datta, S., Singh, J. & Singh S. (2018). Assessment of genotoxic effects of pesticide and vermicompost treated soil with Allium cepa test. *Sustainable Environment Research, 28*(4), 171-178.

Singh, P. (2007). Comparative sensitivity of barely (*Hordeum vulgare* L.) to insecticide and fungicide on different stages of cell cycle. *Pestic. Biochem. Physiol., 89*: 216-219.

Verma, S. & Srivastava, A. (2018). Morphotoxicity and cytogenotoxicity of pendimethalin in the test plant *Allium cepa* L. - A biomarker based study. *Chemosphere, 206*, 248-254.

Stangroom, J. (2018). T-Test Calculator for 2 Independent Means. Retrieved from https://www.socscistatistics.com/tests/studentttest/default.aspx?fbclid=IwAR2ID_DdZY0GUswWhUVICJmfvT8vsGVQgcUVP1d2jcBS-BENz6CSLtJOQTM.

Wang, A., Mahai, G., Wan, Y., Jiang, Y., Meng, Q., Xia, W., He, Z. & Xu, S. (2019). Neonicotinoids and carbendazim in indoor dust from three cities in China: spatial and temporal variations. *Sci. Total Environ., 695*, 13379.

Wiesner, P. & Kayser, H. (2000). Characterization of nicotininic acetylcholine receptors from the insects *Aphis craccivora, Myzus persicae* and *Locusta migratoria* by radioligand binding assays: relation to thiamethoxam action. J. *Biochem. Mol. Toxicol., 14*, 221e230. doi: 10.1002/(SICI)1099-0461(2000)14.

Su, X., Wang, L., Xu, Y., Dong, L. & Lu, H. (2021). Study on the binding mechanism of thiamethoxam with three model proteins:spectroscopic studies and theoretical simulations. *Ecotoxicology and Environmental Safety, 207*(2021), 111280.

Yadav, J. S. & Kaushik, V. K. (2002). Studies on the genotoxicity of an organophosphorous pesticide Baytex-1000. *Int. J. Hum. Genet., 2*: 19-25.

Zabka, A., Polit, J. T. & Maszewski, J. (2012). DNA replication stress induces deregulation of the cell cycle events in root meristems of *Allium cepa*. *Ann. Bot., 110*: 1581-1591.

Zeljezic, D. & Garaj-Vrhovac, V. (2004). Chromosomal aberrations, micronuclei and nuclear buds induced in human lymphocytes by 2,4-dichlorophenoxyacetic acid pesticide formulation. *Toxicology, 200*: 39-47.

Zhu, L., Wei, L., Zha, J., Na, L. & Wang, Z. (2019). Chronic thiamethoxam exposure impairs the HPG and HPT axes in adult Chinese rare minnow (*Gobiocypris rarus*): docking study, hormone levels, histology, and transcriptional responses. *Ecotoxicol. Environ. Saf., 185*, 109683.