



Concerning the Potential Toxic Effect of Coumaphos and Oxalic Acid Applied in Beekeeping

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Abstract: The potential mutagenic and cytotoxic effects of the beekeeping insecticide Coumaphos and oxalic acid in *Allium cepa* test object were investigated. The results showed that Coumaphos in the concentration recommended for agricultural practice has a cytotoxic effect and acts as a suppressor and blocker of seed germination. At the same time, the oxalic acid stock solution slows germination. The lack of cells in metaphase, anaphase, and telophase in treated material showed that the oxalic acid suppressed mitosis. Chromosomal aberrations (micronuclei, anaphase/telophase bridges, and acentric fragments/chromosomes) in the highest percentage were observed after Coumaphos treatment and indicate the obvious mutagenic effect of the acaricide even at the 20-fold dilution of the recommended concentration. This study reveals a potential toxic effect of the studied agrochemicals used in beekeeping against the Varroa mite. Significantly, Coumaphos in recommended and lower concentrations has a general toxic and genotoxic effect. Oxalic acid applied in organic beekeeping exhibits lower general toxicity and affects the cell division in the model object but has no distinct genotoxic effect. Given the fact that residues of Coumaphos have been found in samples of food stocks in some of the studied bee colonies, investigations of its toxicity should continue *in situ* as well.

Key words: Anti Varroa insecticide, mutagenic effect, cytotoxic effect, micronuclei, *Apis mellifera*

Introduction

In recent years, an alarming increase in mortality and a decrease in the number of honey bees (*Apis mellifera* L.) has been reported worldwide. Researchers are of the opinion that among the main reasons for losses of bee colonies in Europe are Varroa species (e.g. *Varroa destructor*), problems with the queen bee and drones, Nosema, starvation, theft between bee colonies, overwintering and losses during this period, other diseases, as well as non-iden-

tified causes (including pesticides and other chemical agents) (COSTA et al. 2012, MEIXNER et al. 2013, FRANCIS et al. 2014, MEIXNER et al. 2015).

Varroaosis is a bee disease caused by the *Varroa destructor* mite, which migrates from the Asian honey bee (*Apis cerana*) to the European honey bee (*Apis mellifera*) and is emerging as a major problem for beekeeping worldwide. Honey bees suffer from Varroa infestation at the individual, colony, and population levels (NOËL et al. 2020). Varroa is also a highly efficient vector of honey bee viruses and

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leads to changes in the distribution and virulence of these viruses. Some viruses, such as the Deformed wing virus (DWV) and Acute bee paralysis virus (ABPV), have a clear Varroa-vector relationship (DE MIRANDA et al. 2010, MARTIN & BRETTELL 2019, FLORES et al. 2021). For other viruses, the connection to Varroa is indirect, and the disease facilitates infection (TRAYNOR et al. 2020). Increasing resistance to acaricides has been observed in the Varroa population, and it has been suggested that the mites or their vector viruses are becoming more virulent. Strongly infected weak colonies make it easier for mites to spread and transmit disease to stronger, healthier colonies (TRAYNOR et al. 2020, KULHANEK et al. 2021).

Beekeepers use various methods to combat Varroa in honey bee colonies, such as monitoring varroa infestation, removing infested broods, treating with various acaricides (varroacides), applying oxalic acid and formic acid, etc. About 62% of bee colonies in Europe are reported to be treated with amitraz, followed by oxalic acid (BRODSCHNEIDER et al. 2023).

A widely used varroacide for bee colony treatment is Coumaphos – an organophosphorus pesticide that suppresses acetylcholinesterase and affects the functions of the nervous system. It is effective in controlling mites resistant to tau-fluvalinate and other pesticides, but coumaphos-resistant Varroa has been reported as well (SPREAFICO et al. 2001, PETTIS 2004, MAGGI et al. 2009, 2011, HIGES et al. 2020, HERNÁNDEZ-RODRÍGUEZ et al. 2021).

As an alternative effective method against varroa infestation in beekeeping, the natural acaricide oxalic acid is used, which reduces the sucking abilities of the mite and causes death from starvation (MUTINELLI et al. 1997, CHARRIÉRE & IMDORF 2002, NANETTI et al. 2003, RADEMACHER & HARZ 2006, GREGORC et al. 2017, KANELIS et al. 2003). Oxalic acid is used also against *Nosema ceranae* (NANETTI et al. 2015).

Frequent treatment of hives with Coumaphos or oxalic acid can negatively affect the bee colonies and cause increased mortality or smaller sizes of queen bees, lowering the amount of sealed brood, reducing the reproductive ability of drones, etc. (HIGES et al. 1999, HAARMANN et al. 2002, PETTIS et al. 2004, HATJINA & HARISTOS 2005, GREGORC 2012, CHAIMANEE et al. 2016, RADEMACHER et al. 2017, GREGORC et al. 2018, TERPIN et al. 2019, JACK et al. 2020).

It has been reported that after beehives were treated with Coumaphos, the pesticide was detected in bee brood, beeswax, and honey. Accumulation of

Coumaphos in beeswax was found not only in treated hives but also in nearby hives where it was not used. Coumaphos contamination of honeycombs is reported to persist even after commercial wax recycling (THRASYVOULOU & PAPPAS 1988, MARTEL et al. 2007, BALAYIANNIS & BALAYIANNIS 2008, PREMROV BAJUK et al. 2017, KAST et al. 2020, PETROV et al. 2022, LUNA et al. 2023).

The presence of residual amounts of oxalic acid was found after treatment of the hives in the hemolymph, rectum, Malpighian tubules, and digestive tract of the treated bees (NOZAL et al. 2003). The effect of oxalic acid on bees in treated hives is thought to depend on the way it is applied (PAPEŽÍKOVÁ et al. 2017).

The present study aimed to investigate the possible cytotoxic and genotoxic effects of Coumaphos and oxalic acid in laboratory conditions at concentrations applicable in beekeeping and below by usage of the classical genetic test object *A. cepa*.

Materials and Methods

Test object

The *Allium cepa* L. test system (FISKESJÖ 1985) was applied to screen for the mutagenic activity and cyto- and genotoxicity of the studied chemicals. For the purposes of the study, one-year-old seeds of *Allium cepa* (cultivar Ispanska Kaba 482) germinated in humid chamber conditions in distilled water (control) or in solutions of Coumaphos or oxalic acid.

Chemicals

In the course of the current study, the following concentrations were investigated: used in beekeeping practice 0,06% Coumaphos (Perizin 3,2%) and oxalic acid (30 g/l) (stock solutions, SS), and 10- and 20-times lower concentrations (10x and 20x respectively).

Coumaphos is an organothiophosphate insecticide with ectoparasiticide properties, widely used for both farm and domestic animals to control ticks, mites, flies, and fleas. It acts as an agrochemical, an acaricide, an antinematode drug, and an avicide. In beekeeping practice, Coumaphos is applied for treatment against Varroa (Perizin, CheckMite +). However, it is also an active substance in other chemicals such as Asuntol (against external parasites in dogs), Azunthol (insecticide against flies, ants, mosquitoes, cockroaches, ticks, and fleas), Muscatox (against flies), and others.

Oxalic acid, also called ethanedioic acid, is used in beekeeping as an alternative method against Varroa and *Nosema ceranae*.

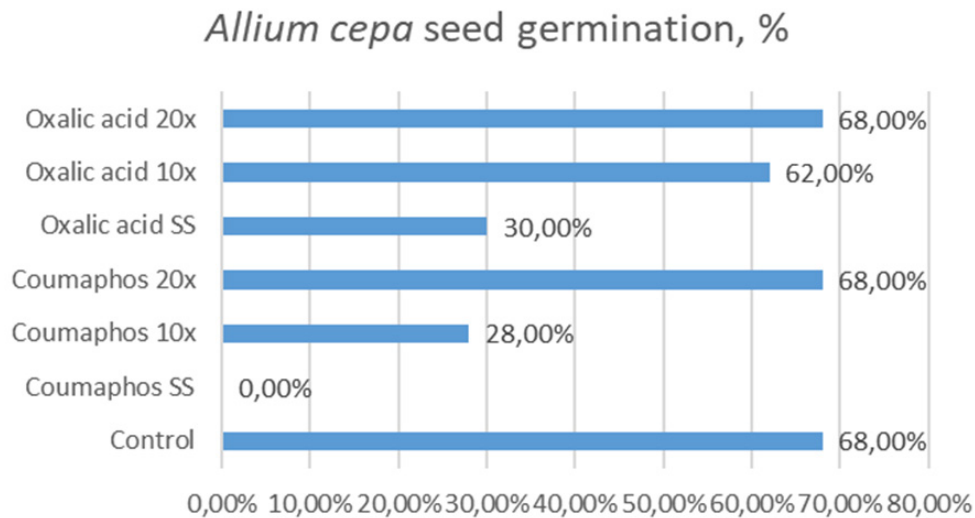


Fig. 1. Effects of studied concentrations of Coumaphos and oxalic acid on seed germination of *Allium cepa*.

Experimental Design

Fifty seeds of *Allium cepa* were cultivated in humid conditions in Petri dishes with a chemical solution or distilled water (in the control) till seed germination. The number of germinated seeds was registered during a 30-day period. Total toxicity was calculated as a percentage of seeds germinated from the total number of seeds in the control and the test samples.

From the fixed root apices, temporary squash preparations were prepared (POPOVA et al. 2021) to study the effects of the tested Coumaphos and Oxalic acid. At least 5000 cells of each variant (magnification 400x) were analyzed (5 microscope slides prepared from 5 different roots of each – control and treated variants).

The mitotic index (IM) and phase indices, which are indicators of cell proliferation, were calculated to evaluate the cytotoxic effects of Coumaphos and oxalic acid in the studied concentrations. The results were compared with the control (POPOVA et al. 2021).

The genotoxic effects (mutagenic action) of the tested chemical concentrations were analysed by anaphase analysis and the micronucleus test. For this purpose, the number of cells with chromosomal fragments and lagging chromosomes, anaphase- and telophase-bridges, and micronuclei in the treated cells and the controls were registered, and the frequencies in percentages were compared.

Statistical analysis

Comparisons between treated samples and the control sample were submitted to statistical analysis using Student's t-test to assess significant differences between them.

Results

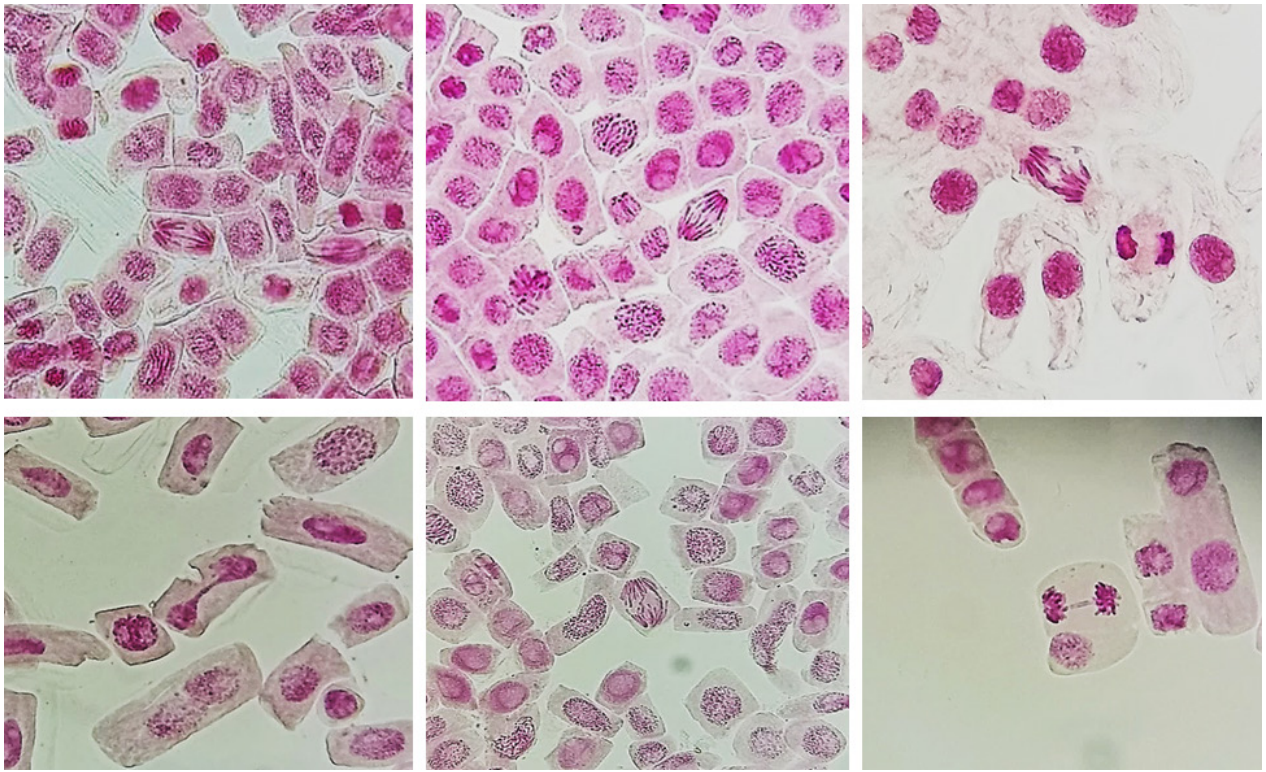
The data concerning the total toxicity found by the *Allium cepa* seed germination are presented in Figure 1.

The results show that onion seed germination varies from 68% (the control and treated with lowest concentrations seeds) to 0% (Coumaphos stock solution treated seeds). This indicates a total cytotoxic effect of 0,06% Coumaphos used in beekeeping practice. A strongly pronounced reduced seed germination was recorded after treatment with both oxalic acid stock solution and Coumaphos 10x (30% and 28% respectively).

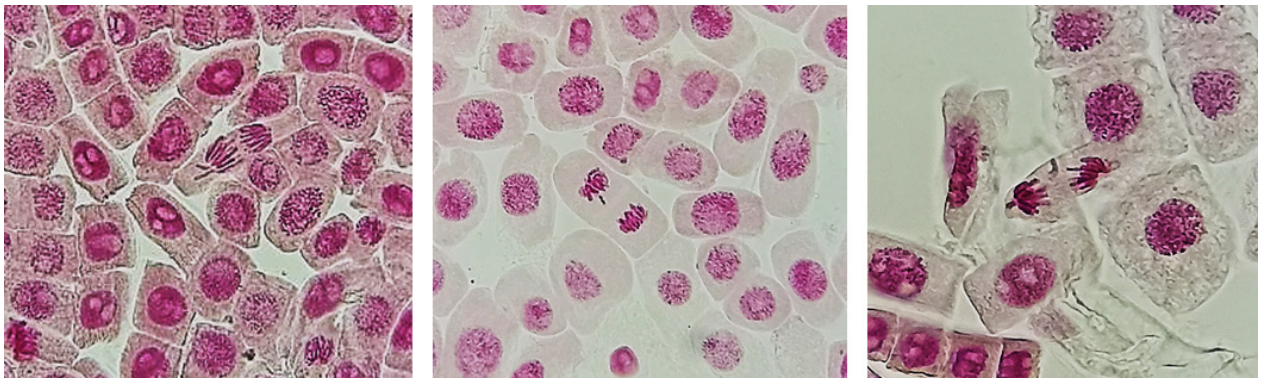
The mitotic index indicates the rate of cell division in the analysed tissue. Table 1 presents the results concerning the mitotic index and phase indices in the control and treated *Allium* root meristem cell samples.

The presented data show significantly lower mitotic indices in samples treated with oxalic acid 10x and 20x concentrations (38.96% and 32.14%) compared to the control (56.12%). The same concentrations of oxalic acid arrested mitotic division in prophase, and no metaphase, anaphase, or telophase cells were observed. The treatment in a stock solution of oxalic acid did not affect the mitotic index but resulted in a significantly lower presence of anaphase and telophase cells and a lack of metaphase cells.

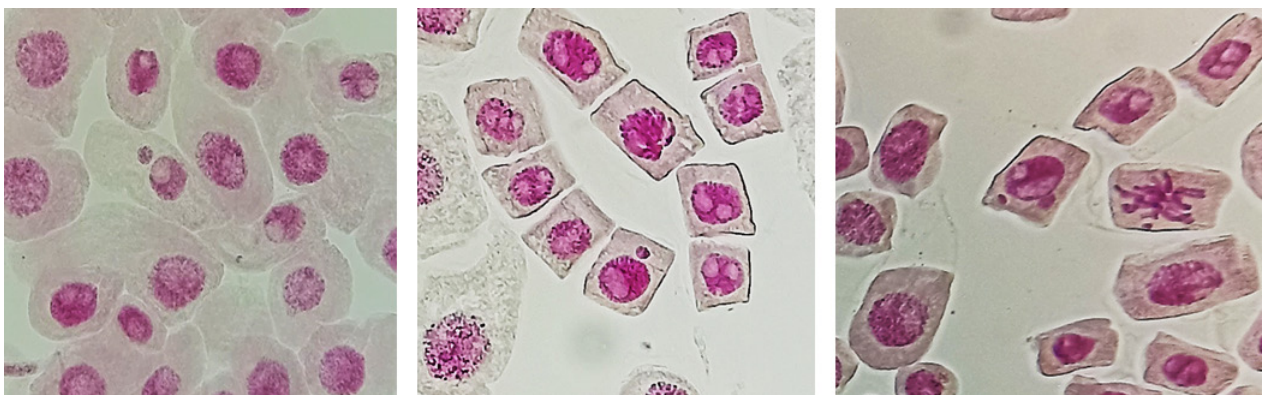
In contrast, treatment with Coumaphos 10x and 20x concentrations has no such drastic effect on the mitosis. However, significantly lower values of metaphase, anaphase, and telophase were observed as well as higher prophase values, compared to the control. This indicates a delay in cell division following Coumaphos treatment.



a



b



c

Fig. 2. Anaphase and telophase bridges (a), lagging acentric chromosomes and chromosomal fragments (b), and micronuclei (c) in the apical root meristems of *Allium cepa* treated with Coumaphos or oxalic acid (magnification x400).

Table 1. Mitotic index (IM) and phase indices (%) in *Allium cepa* treated with Coumaphos and oxalic acid.

Sample	Control	Coumaphos 10x	Coumaphos 20x	Oxalic acid SS	Oxalic acid 10x	Oxalic acid 20x
Mitotic index, IM	56.12±4.4	55.46±3.17	56.52±4.39	57.77± 9.77	38.96±12.92*	32.14±13.53**
Prophase index, Ipph	87.35±1.77	97.21±1.87***	94.35±1.56***	99.95±0.12***	100±0.00***	100±0.00***
Metaphase index, Imph	5.46±0.67	1.07±0.75***	2.66±0.62***	0***	0***	0***
Anaphase index, Iaph	4.18±0.49	0.88±0.53***	1.84±0.71***	0.03±0.06***	0***	0***
Telophase index, Itp	3.01±0.85	0.85**±0.61	1.15***±0.47	0.03***±0.06	0***	0***

p<0,05*, p<0,01**, p<0,001***

Table 2. Average number and frequency of chromosomal aberrations (%) (X±SD) in *Allium cepa* treated with Coumaphos and oxalic acid.

Sample	Control	Coumaphos 10x	Coumaphos 20x	Oxalic acid SS	Oxalic acid 10x	Oxalic acid 20x
Average number of chromosome aberrations	1±0.42	3.4±0.42**	15.6±4.54***	0.6±0.2	1.6±0.92	0
Frequency of chromosomal aberrations in % of N	0.1±0.07	0.27±0.19**	1.56±0.86**	0.05±0.07	0.12±0.17	0
Frequency of chromosomal aberrations in % of N'	0.17±0.12	0.47±0.33	2.80±1.67*	0.10±0.16	0.26±0.36	0

p<0.05*, p<0.01**, p<0.001***

Table 3. Incidence of different types of chromosomal aberrations as a result of Coumaphos and oxalic acid treatment (First row – % relative to the total number of cells N, second row – % relative to dividing cells N').

Sample	Control	Coumaphos 10x	Coumaphos 20x	Oxalic acid SS	Oxalic acid 10x	Oxalic acid 20x
Micronuclei	0.08	0.12	1.01	0.03	0.13	0
	0.14	0.22	1.79	0.06	0.33	0
Anaphase or telophase bridges	0.00	0.06	0.19	0	0	0
	0.00	0.11	0.34	0	0	0
Acentric fragments or lagging chromosomes	0.02	0.08	0.31	0.02	0	0
	0.03	0.14	0.55	0.03	0	0
Total	0.10	0.26	1.51	0.05	0.13	0
	0.17	0.47	2.69	0.09	0.33	0

The following types of chromosomal aberrations were registered in treated cells: anaphase (or telophase) bridges (intact or broken) (Figure 2a), lagging acentric chromosomes and/or chromosomal fragments (Fig. 2b), and micronuclei (Fig. 2c).

The results concerning the number and frequency of cells with aberrations are presented in Table 2.

The highest number of cells with chromosomal aberrations were observed in *Allium cepa* root meristems treated in Coumaphos 20x and 10x con-

centrations (15.6 and 3.4 respectively). The highest total frequency of registered aberrations in mitotic cells was 2.80% (after Coumaphos 20x treatment, Table 2). For the tested concentrations of oxalic acid, no statistically significant differences were found when compared to the control, although a slight increase in mutagenicity could be noted at the 10x concentration.

The results for frequency of various types of aberrations are exposed in Table 3.

The most frequently registered aberrations

were micronuclei in the highest percentages of cells treated with Coumaphos 20x (1.79% of mitotic cells and 1.01% of all analyzed cells, Table 3). The same Coumaphos concentration also provoked the highest frequencies of anaphase/telophase bridges (0.19% and 0.34%) and acentric fragments/lagging chromosomes (0.31% and 0.55%, Table 3). The Coumaphos 10x concentration also resulted in the same kinds of aberrations but in lower values. The tested concentrations of oxalic acid do not provoke anaphase or telophase bridges, and micronuclei were observed to have a higher value after treatment in 10x concentration (Table 3).

Discussion

According to ASITA & MATEBESI (2010), a pesticide concentration is considered to be cytotoxic when the mitotic index (IM) of treated cells is $\leq \frac{1}{2}$ of the control IM. The obtained results in this study indicated that lower concentrations of Coumaphos (10x and 20x dilution) are not cytotoxic on the *Allium cepa* cells, but beekeeping-relevant concentrations are cytotoxic. Concerning oxalic acid treatment, the provoked effect on cell division is near cytotoxicity, and the dividing spindle apparatus is probably destroyed or arrested.

Mutagenic damage to genomic DNA often leads to many diseases, such as cancer, neurobehavioral disorders, infertility, etc. Mutagenic effects might result from chronic exposure to pollutants. The results of this study show that Coumaphos possesses a clear mutagenic effect even in lower concentrations, which suggests that residual amounts in the honey and other bee products are not safe. Oxalic acid, used in organic beekeeping, also causes micronuclei in *Allium* cells that show a mutagenic effect.

Pesticide residues, including coumaphos, in honey and other bee products have been reported (MENKISSOGLU-SPIROUDI et al. 20001, AL-WAILI et al. 2012). According to NEKOEI et al. (2023), the main practice for now aimed to minimize the impact of Varroa mites and other pathogens on bee colonies is the application of an effective miticide (Coumaphos, fluvalinate-tau, amitraz, etc.), but this has a negative effect on bees. To maintain the health of beehives and improve the quality and honey productivity, it is very important to use new alternative biological control methods (NEKOEI et al., 2023). The application of management practices such as frequent mother changes as well as brood removal have a statistically significant effect on Varroa infestation rates. It is possible to maintain infestation

at acceptable low levels by applying Integrated Pest Management (IPM) programs and using Good Beekeeping Practices (GBP) (BAVA et al. 2023).

Conclusion

The insecticide Coumaphos, used in beekeeping against Varroa, in concentration 0.06%, stopped *Allium cepa* seed germination and a 10 times lower concentration of the chemical significantly reduced the germination process. Consequently, Coumaphos possess a clear cytotoxic effect. The lower concentrations (10- and 20 times lower) have mutagenic effects on *Allium cepa* apical root meristem and cause aberrations as micronuclei, anaphase/telophase bridges, and acentric fragments/chromosomes.

Oxalic acid, an alternative organic method against varroa, significantly reduced *Allium cepa* seed germination when applied in the concentration used in beekeeping. The lower tested concentrations reveal a mitostatic effect and a low mutagenic effect expressed as micronuclei formation.

Given that Coumaphos residues have been found in samples of food stocks in some of the studied bee colonies, investigations of its toxicity should continue *in situ* as well.

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