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Assessment of genotoxicity, mutagenicity, and cytotoxicity of diclofenac and sulfamethoxazole at environmental concentrations on *Vicia faba*

J. Drzymała[1] · J. Kalka[2]

Abstract

The contamination of the environment with pharmaceuticals and their residues has become a global issue. The main objective of study was to assess the genotoxicity, mutagenicity, and cytotoxicity of two drugs, diclofenac, sulfamethoxazole, and their binary mixture. The research focused on conducting a micronucleus assay using *Vicia faba* in water and soil environments. In the experiment, several parameters were monitored: mitotic index, presence of micronuclei, and chromosomal aberrations. The antioxidant enzymes activity in the plants leaves was measured. The concentrations of the drugs used in the analysis were representative of those currently detectable in the environment. The results indicated that diclofenac and sulfamethoxazole caused a reduction in the mitotic index by 45% and 47% in hydroponic, and 46% and 22% in soil cultures, respectively. Micronuclei and chromosomal aberrations were observed at the tested environmental concentrations (0.008–0.5 mg L^{-1}). In the case of the drug mixture, the observed toxic effects in both cultivation were less significant than the predicted effects based on the Concentration Addition and Independent Action models. The tested compounds had an impact on the activity of enzymes. Even at environmental concentrations, the pharmaceuticals caused changes in catalase activity, with an average decrease of 39% in water and 10% in soil cultures, and in superoxide dismutase activity, showing an increase of 286% and 1835%, respectively. Overall, this study highlights the potential adverse effects of pharmaceutical contamination, even at low environmental concentrations. The findings underscore the importance of monitoring the presence of pharmaceutical residues to minimize their impact on ecosystems.

Keywords Cytotoxicity · Diclofenac · Genotoxicity · Mutagenicity · Sulfamethoxazole · *Vicia faba*

Introduction

For several decades, the presence of environmental pollutants has been a growing concern. Pharmaceuticals are a unique group of contaminants with therapeutic effects that can significantly impact the environment. The primary sources of pharmaceutical compounds are medical and veterinary products. These compounds can enter the environment through both point sources and diffuse sources. Point sources include locations such as sewage treatment plants, hospitals, and septic tanks, while diffuse sources refer to agricultural runoff from farmlands or effluents from wastewater treatment systems (Martínez Bueno et al. 2012; Li 2014). Diffuse sources are generally considered to have less environmental importance due to lower pollutant loads as well as the natural remediation processes occurring in the soil (Murray et al. 2010).

The main reason for the presence of pharmaceuticals in the environment is the increasing annual consumption of over-the-counter and prescription drugs. In the European Union, the average drug consumption per person per day is for example: 1.336 mg for erythromycin, 0.820 mg for sulfamethoxazole, and 0.418 mg for trimethoprim (Johnson et al. 2015). When analyzing antibiotic consumption data in the European Union between 2011 and 2020, it is evident that the intake of most drugs has decreased. This reduction is

✉ J. Drzymała
justyna.drzymala@polsl.pl

[1] The Biotechnology Centre, Silesian University of Technology, Krzywoustego 8, 44-100 Gliwice, Poland

[2] Environmental Biotechnology Department, Faculty of Energy and Environmental Engineering, Silesian University of Technology, Gliwice, Poland

influenced, among other factors, by legal regulations (European Centre for Disease Prevention and Control 2021). But still annual domestic consumption of diclofenac ranged from 2 to 60 tons between 2010 and 2013, while global consumption averaged 1443 ± 58 tons in 2012 (Acuña et al. 2015), and an estimated nearly 2200 tons per year in 2020 (Acuña et al. 2020). Such high pharmaceutical intake leads to the release of drug residues and metabolites into the environment. Once released, both parent compounds and metabolites may undergo structural changes due to biotic and abiotic processes, altering their physicochemical and biological properties. Organic pollutants can be transformed by living organisms, such as bacteria or fungi, and their decomposition can be facilitated by light, ozonation, and chlorination. However, structural changes can result in the formation of new substances with different and unpredictable properties (Kümmerer 2010).

Pharmaceuticals are commonly used in the treatment of animals, and their excrements, which contain parent compounds or their decomposition products, are often utilized as fertilizers in agricultural fields (Navrátilová et al. 2021). This uncontrolled introduction of drugs into the natural environment makes it challenging to predict the potential effects. In many cases, low concentrations of drugs may not result in visible changes in the natural environment. However, genetic-level alterations, which are crucial due to their potential multi-generational impact, can be observed (Henry et al. 2022).

One significant concern arising from the presence of drugs or residues of their decomposition in the natural environment is the potential enhancement or of their harmfulness through mixtures (Gworek et al. 2021; Nason et al. 2019). This poses a serious threat as the combined effects of different pharmaceuticals or their breakdown products can lead to unpredictable outcomes. The interaction between these compounds in the environment can create synergistic or antagonistic effects, amplifying or diminishing their individual toxicity levels.

Understanding the complex dynamics of pharmaceuticals in the environment is crucial for effective risk assessment and the development of appropriate mitigation strategies. As the usage and disposal of pharmaceuticals continue to increase, comprehensive studies are necessary to evaluate their long-term effects and devise sustainable approaches to minimize their environmental impact.

Another critical issue stemming from the significant presence of pharmaceuticals in the natural environment is the rise in antibiotic-resistant bacteria and antibiotic resistance genes (Felis et al. 2020; Osińska et al. 2020; Zieliński et al. 2021). Antibiotic resistance poses a global threat to both human and animal health. As a result, the treatment of an increasing number of infections becomes increasingly challenging, as the effectiveness of antibiotics diminishes. This not only escalates treatment costs but also contributes to higher rates of human mortality (Felis et al. 2020).

Diclofenac (DCF) and sulfamethoxazole (SMX) were chosen as representatives of two widely used pharmaceutical groups. DCF is a non-steroidal anti-inflammatory drug (NSAID) available over the counter, commonly prescribed for treating inflammation and pain (Alfaro and Davis 2022). SMX, on the other hand, is a sulfonamide antibiotic effective against aerobic Gram-positive and Gram-negative bacteria, protozoa, and certain fungal infections (Minato et al. 2018). Both of these pharmaceuticals have been detected in the natural environment.

The maximum concentrations of DCF found in surface water, groundwater, seawater, and soil are reported as 0.057 mg L^{-1} (Sathishkumar et al. 2020), 0.013 mg L^{-1} (Sathishkumar et al. 2020), 0.843 μg L^{-1} (Bonnefille et al. 2018), and 0.257 mg kg^{-1} (Ashfaq et al. 2019), respectively. As for SMX, the maximum concentrations detected are 0.022 mg L^{-1} (Straub 2016), 1.11 μg L^{-1} (Lapworth et al. 2012), and 0.048 μg L^{-1} (Baran et al. 2011) in surface water, groundwater, and seawater, respectively. In soil, SMX has been detected at a maximum concentration of 0.055 mg kg^{-1} (Cycoń et al. 2019).

There is a considerable body of literature addressing the acute toxic effects of the aforementioned drugs (Cleuvers 2004; Marciocha et al. 2009; Białk-Bielińska et al. 2017; Drzymała and Kalka 2020). However, it is also crucial to investigate the cellular-level effects of these substances in order to determine their genotoxicity, cytotoxicity, and mutagenicity. Studying the effects of DCF and SMX at the cellular level will provide valuable insights into the potential risks associated with their presence in the environment. By understanding their impact on cells, we can better evaluate the long-term consequences of exposure to these pharmaceuticals and make informed decisions regarding their management and mitigation.

Damage to genetic material can lead to various detrimental effects, including the development of cancerous changes and premature cell aging. Organisms originating from aquatic environments can experience growth inhibition, abnormal development, and reduced survival due to genetic damage. Importantly, alterations in genetic material can be transmitted to future generations, resulting in long-term harm and complex, unpredictable consequences (Barbosa et al. 2010). Microorganisms, plant cells, and animal cells are commonly employed in studying the genotoxic potential of substances (Iqbal 2016). Among these options, plants offer several advantages. Notably, they share morphological similarities with mammalian chromosomes and exhibit analogous responses to genotoxic factors. These characteristics make plants a preferred model organism for researchers (de Souza et al. 2016). Furthermore, plant-based tests present cost and

time efficiency compared to tests involving bacteria or animal cells, making them an attractive alternative. Additionally, plants can be used directly at contaminated sites, providing a practical advantage (Radić et al. 2010).

Genotoxicity tests aim to identify substances that have the potential to damage the genetic material of living organisms. Such damage can manifest as point mutations or breakage of DNA strands. In the case of organisms residing in aquatic or soil environments, exposure to genotoxic agents can result in growth inhibition, abnormal development, or decreased survival (Barbosa et al. 2010).

The micronucleus test is a comprehensive tool widely used to assess the cytotoxicity, genotoxicity, and mutagenicity of chemicals. By examining the presence of micronuclei, the test enables the evaluation of a substance's mutagenic potential. Additionally, genotoxicity can be assessed through the detection of chromosomal aberrations, while changes in the mitotic index indicate cytotoxic effects (Iqbal 2016). The mitotic index (MI) is a parameter that measures the number of cells undergoing division in the cell cycle and is commonly used to assess the cytotoxicity of chemicals. Micronuclei (MNs) are fragments of genetic material that are not incorporated into the main nucleus and can result from chromosome breakage or disruption of the mitotic apparatus. Chromosomal aberrations (CAs) can arise from various sources, such as DNA strand breaks, inhibition of DNA synthesis, errors in replication mechanisms, or abnormal chromosome segregation (de Souza et al. 2016). These processes can occur spontaneously or due to the action of xenobiotics (de Souza et al. 2016; Kwasniewska and Bara 2022).

The micronucleus test can be conducted using different plant species, including *Allium cepa, Vicia faba*, and *Tradescantia pallida*, among others (Gupta et al. 2018; Campos et al. 2019; de Morais et al. 2019; Iqbal et al. 2019; Alvarenga et al. 2020; Chen et al. 2020; Klein et al. 2021). In this study, *Vicia faba* beans were selected as the test organism due to their affordability, ease of cultivation, and their widespread use as a model organism in genotoxicity studies (Ma 1982). *Vicia faba* is also a popular leguminous crop consumed primarily in the Mediterranean region (De Cillis et al. 2019).

Through this study, we sought to gain insights into the potential effects of the selected pharmaceuticals on *Vicia faba*, providing valuable information regarding their genotoxic, mutagenic, and cytotoxic properties. Furthermore, the investigation explored the influence of these substances on antioxidant enzyme activity specifically catalase (CAT) and superoxide dismutase (SOD), shedding light on their potential impact on oxidative stress mechanisms in *Vicia faba*.

Materials and methods

Genotoxicity test for *Vicia faba*

The micronucleus test was conducted following ISO 29200 (2013) guidelines. The values of the mitotic index (MI), presence of micronuclei (MN), and presence of chromosomal aberrations (CA) were determined using the following equations:

$$MI = \frac{DC}{N} \cdot 100\% \tag{1}$$

$$MN = \frac{MC}{N} \cdot 100\% \tag{2}$$

$$CA = \frac{AC}{N} \cdot 100\% \tag{3}$$

where

DC – it is the total number of dividing cells;

MC – is the total number of cells with micronuclei;

AC – is the total number of cells with chromosomal aberration (for example: chromosome bridges, visible chromosome fragments, chromosome lagging, acentric fragment lagging or vagrant chromosome);

N – is the total number of counted cells;

Seed preparation

The seeds of the Windsor White variety, provided by Eden Company, were used. The seeds were soaked in distilled water for 24 h and then allowed to germinate on a moist layer of lignin. The germination process took place in the dark at a temperature of 20 ± 2 °C. After 3 days of germination, seeds with a primary root length ranging from 3 to 5 cm were selected. To stimulate the growth of secondary roots, approximately 0.5 cm of the primary root was trimmed. The seeds were then placed back onto a moist layer of lignin for an additional 3 days to allow for the growth of secondary roots.

Hydroponic culture

The toxicity of two pharmaceuticals, DCF and SMX, as well as their binary mixture (MIX), was assessed. To conduct the experiment, various concentrations were prepared: for DCF and SMX: 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, and 0.008 mg L^{-1}, and for MIX: 4, 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031, and 0.016 mg L^{-1}. Distilled water was used as the negative control (K-), while maleic hydrazide (CAS number: 123-33-1, purchased from Sigma-Aldrich) served as

the positive control (K+) at a concentration of 1.12 mg L^{-1} (following ISO 29200 (2013) guidelines). The test containers were filled with the prepared solutions, with a volume of 0.3 L in each container. In each container, six selected *V. faba* seeds with approximately 10 mm secondary roots were placed. The plants were then incubated under constant conditions of 20±2 °C temperature and a 16 h light/8 h dark cycle for a duration of 2 days.

Soil culture

To conduct the micronucleus test, the reference soil was prepared following the guidelines provided in OECD 222 (2016), including the determination of total water capacity. The detailed composition of the reference soil can be found in Table SI.1 of the Supplementary Information section. Test containers were filled with 250 g of soil, and the appropriate concentrations of the tested pharmaceuticals were added while maintaining the water holding capacity (WHC). The soil was contaminated with the following concentrations of DCF and SMX: 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, and 0.008 mg kg^{-1}, and for the MIX: 4, 2, 1, 0.5, 0.25, 0.125, 0.063, 0.031, and 0.016 mg kg^{-1}. Distilled water was used as the negative control (K−), and maleic hydrazide at a concentration of 1.12 mg kg^{-1} served as the positive control (K+). Six carefully selected plant samples were washed with distilled water and then gently planted in the contaminated soil, paying special attention to the delicate secondary roots of the *V. faba* seeds. The test containers were incubated under constant conditions of 20±2 °C temperature with a 16 h light/8 h dark cycle for a duration of 5 days. The test containers were periodically weighed, and distilled water was added twice a week to maintain a constant soil moisture level at 50% of the WHC.

Samples collection

For the water culture, the incubation period was 48 h (2 days), while for the soil culture, it was extended to 120 h (5 days). In the case of hydroponic culture, secondary roots were collected at three different time intervals: after 44, 46, and 48 h to capture the approximate duration of the cell cycle. However, in the soil cultivation, only secondary roots were collected after 120 h (5 days) of incubation. This approach was chosen to minimize the risk of damaging the secondary roots when removing the plants from the soil.

Regardless of the cultivation method, six secondary roots that showed no physical damage were collected for each concentration analyzed. The collected roots were carefully washed with distilled water and then placed in Carnoy's solution, which is a mixture of glacial acetic acid and 96% ethyl alcohol in a ratio of 1:3. The roots were left in Carnoy's solution for 24 h at a temperature of 4 °C. Afterward, the

roots were rinsed with distilled water and stored in 70% ethyl alcohol at a temperature of 4 °C.

Microscopic observations

For microscopic observation, the roots were carefully rinsed with distilled water to remove any traces of 70% ethyl alcohol. Subsequently, they were subjected to hydrolysis in 1 M HCl at a temperature of 60 °C for 6 min, followed by another round of washing with distilled water for 3 min. To facilitate staining, root fragments were treated with a 2% orcein solution in acetic acid for 1 min. A coverslip was then placed over the stained fragments, and they were examined under a microscope at a magnification of 1000×.

To ensure comprehensive analysis, six preparations were made for each drug concentration, corresponding to six individual test plants. In each preparation, a minimum of 1000 cells were observed and counted to determine the mitotic index (MI), which represents the number of cells undergoing division. Consequently, a total of 6000 cells were analyzed for each test concentration. Additionally, the number of micronuclei (MN) and chromosomal aberrations (CA) were recorded during the observation process. Following chromosomal aberrations were recorded: chromosome bridges, visible chromosome fragments, chromosome lagging, acentric fragment lagging or vagrant chromosome. Sample photographs of observed chromosomal aberrations have been presented in Fig. 1. Readers interested in exploring the types of aberrations that can be observed during studies using plant cells can find detailed information in the articles of Kumar and Nagpal (2015), Kaur et al. (2019) or Saxena (2022).

Enzymatic analysis

After harvesting the secondary roots, the leaves of the test plants were collected to assess the activity of antioxidant enzymes, namely catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1). The leaves chosen for enzymatic analysis, both in hydroponic and soil culture, were at the same developmental stage. However, the difference in time between sampling depending on the type of culture should be taken into account (3-days difference between soil and hydroponic culture). The top portion of the plant was removed, and the first fully developed leaf was selected for testing.

To extract the enzyme homogenates, a Pro200 homogenizer (Pro Scientific Inc., USA) was utilized along with appropriate buffers. A 0.06 M sodium phosphate buffer (pH 7.4) was used for CAT activity, while a 0.05 M carbonate buffer (pH 10.2) was employed for SOD activity. The prepared homogenates were subjected to centrifugation (20 min, 4000 rpm, 4 °C) and subsequently stored at −45 °C until further analysis.

Fig. 1 Examples of observed abnormalities in *Vicia faba* cells: **A** chromosomal aberration: chromosome bridge; **B** micronuclei
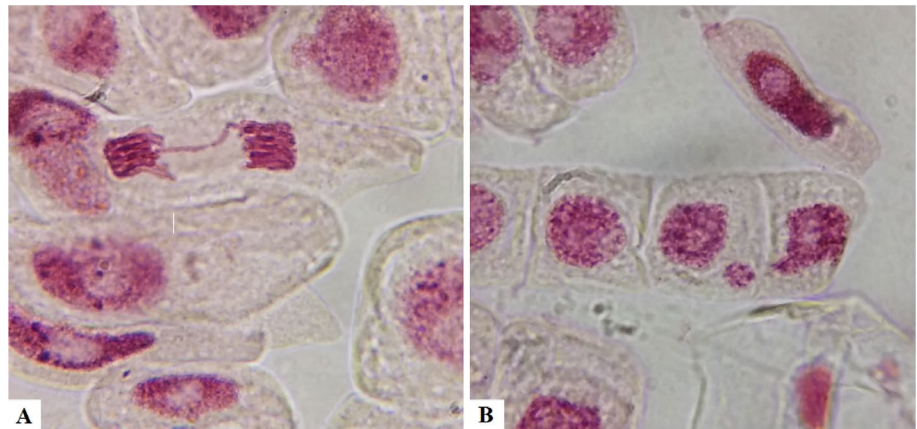
Enzyme activity was determined using the spectrophotometric method, with CAT activity measured at 405 nm and SOD activity at 480 nm. The enzymatic activity assays were conducted following the Góth method (Góth 1991) for CAT and the Misra and Fridovich protocol (Misra and Fridovich 1972) for SOD. The protein content of each sample was determined using the Bradford method (Bradford 1976).

Statistical analysis

Statistical analyses were conducted using STATISTICA 13 software (StatSoft Inc., 2016). The Shapiro–Wilk test was performed to assess the normal distribution of the examined variables. Subsequently, one of two tests was selected for further analysis: Student's t test ($\alpha = 0.05$) or Mann–Whitney U test ($p < 0.05$).

For groups with a normal distribution, Student's t test ($\alpha = 0.05$) was utilized. The data must meet certain criteria for the t-test to be applied, including values measured on a ratio or interval scale, homogeneity of variance, an adequate sample size, and a normal distribution of data. Conversely, if the data did not meet these criteria, the Mann–Whitney U test was employed.

Statistical significance was determined based on a *p*-value of less than 0.05. The Mann–Whitney U test is suitable when variables are measured on an ordinal or continuous scale, observations belong to independent categorical groups, and data do not follow a normal distribution.

Results and discussion

Results

Micronucleus test

Figure 2 displays the mitotic index (MI) of *V. faba* root cells exposed to DCF, SMX, and MIX in hydroponic and soil cultivation. The pharmaceuticals and their mixture had an impact on the number of cell divisions in *V. faba* root cells. Even at the lowest tested concentrations in water culture, all pharmaceuticals inhibited cell divisions, as confirmed by statistical analysis (Student's t test, $\alpha = 0.05$). Detailed statistical analyses can be found in the Supplementary Information, specifically in Tables SI.2–SI.4.

In the case of soil cultivation, no statistically significant differences were observed in relation to the negative control at the lowest concentrations of pharmaceuticals (DCF: 0.008 mg kg^{-1}, SMX: up to 0.016 mg kg^{-1}, MIX: up to 0.063 mg kg^{-1}) (Tables SI.2–SI.4). However, as the concentrations of the tested drugs increased, a decrease in cell division was observed.

In hydroponic culture, the highest MI values were observed for DCF at a concentration of 0.016 mg L^{-1} (MI = 8.75%), SMX at 0.008 mg L^{-1} (6.03%), and MIX at 0.016 mg L^{-1} (8.23%). The lowest MI values were observed for DCF and SMX at 2 mg L^{-1} (4.88% and 2.12%, respectively), and for MIX at 4 mg L^{-1} (6.18%). In soil culture, the corresponding highest MI values were observed for DCF and SMX at a dose of 0.008 mg kg^{-1} (9.42% and 9.55%, respectively), and for MIX at 0.016 mg kg^{-1} (9.27%). The lowest MI values were observed for DCF at 1 mg kg^{-1} (5.00%), SMX at 2 mg kg^{-1} (5.08%), and MIX at 4 mg kg^{-1} (6.52%).

Statistically significant differences were observed between the results obtained for water and soil cultivation in the case of SMX and MIX. The toxic effect (inhibition of cell division) was lower in the soil environment. However, for cells exposed to DCF, no statistically significant differences were found in the MI when comparing hydroponic and soil cultures (Table SI.2).

Figures 3 for MN and Fig. 4 for CA and Tables SI.5–SI.7 present the number of micronuclei (MN) and chromosomal aberrations (CA) in cells obtained from hydroponic and soil culture. Notably, no presence of MN was observed in any of the experiments involving the negative control. In hydroponic cultivation, the occurrence
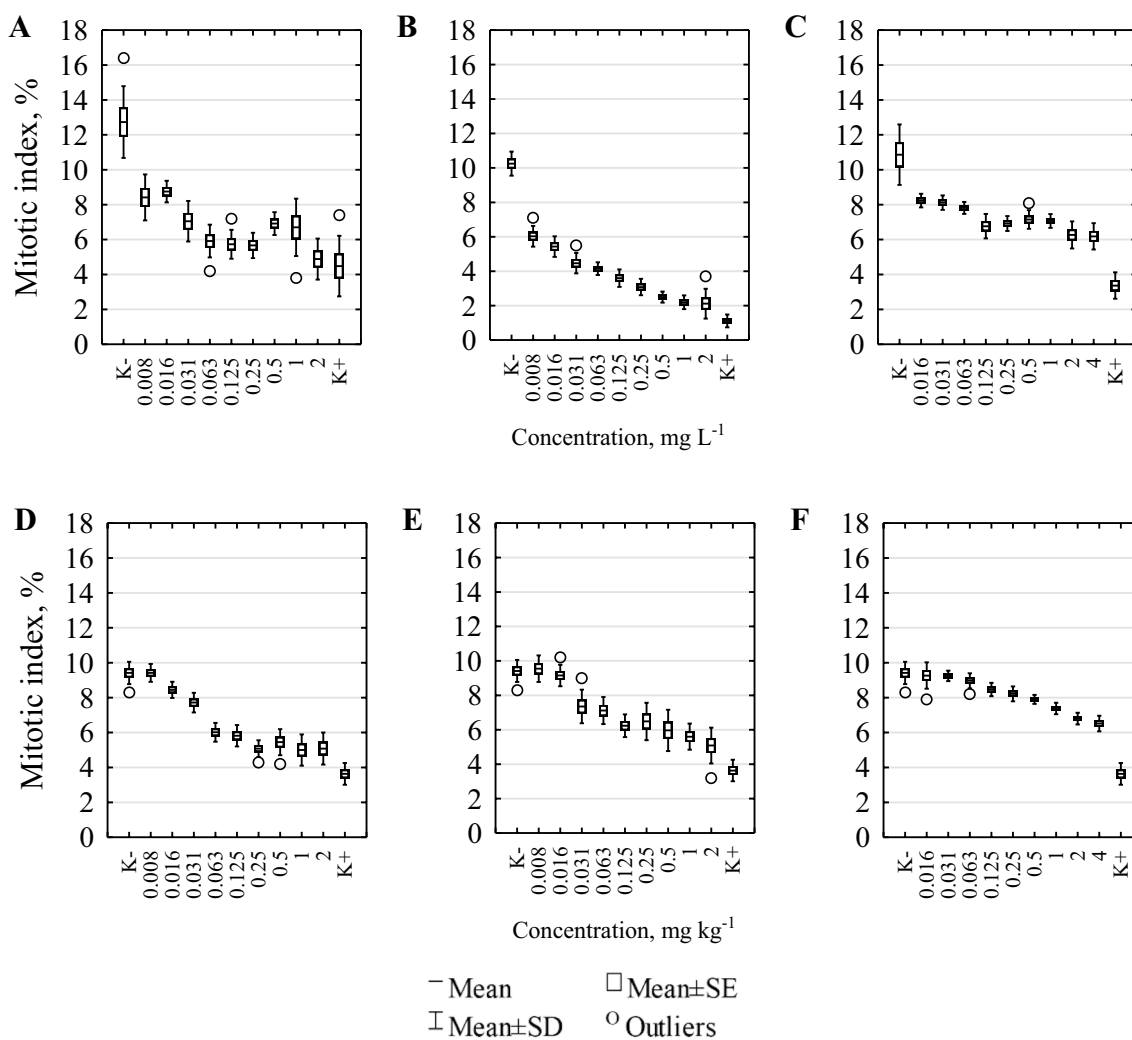
Fig. 2 Effect of the substances tested on MI in *V. faba* cells: **A** effect of DCF in water culture; **B** effect of SMX in water culture; **C** effect of MIX in water culture; **D** effect of DCF in soil culture; **E** effect of SMX in soil culture; **F** effect of MIX in soil culture

of MN was dependent on the mitotic index (MI). Lower drug concentrations resulted in increased genetic material damage, while the number of MNs decreased due to the inhibition of cell division. It is worth mentioning that the positive control (maleic hydrazide), which exhibits a strong genotoxic effect, still yielded significant amounts of MN despite low MI values. In the case of soil culture, genetic material damage was observed only at the highest tested concentration (DCF: 1 and 2 mg kg^{-1}; SMX: 2 mg kg^{-1}; MIX: 4 mg kg^{-1}). Detailed statistical analyses can be found in Tables SI.5–SI.7.

The highest MN values were observed for DCF at a concentration of 0.125 mg L^{-1} (0.15%) in water culture and at 2 mg kg^{-1} (0.02%) in soil culture. For SMX, corresponding values were observed at 0.063 and 0.125 mg L^{-1} (0.25%) in water culture and at 2 and 1 mg kg^{-1} (0.02%) in soil culture. In the case of MIX, the highest MN values were observed

at 0.25 mg L^{-1} (0.07%) in water culture and 0.125 mg kg^{-1} (0.30%) in soil culture.

Regarding chromosomal aberrations (CA), the highest values were observed for DCF at 0.063 mg L^{-1} (0.30%) and 2 mg kg^{-1} (0.08%), for SMX at 0.063 mg L^{-1} (0.18%) and 2 mg kg^{-1} (0.08%), and for MIX at 0.125 mg L^{-1} (0.30%) and 4 mg kg^{-1} (0.08%) in hydroponic and soil culture, respectively.

In terms of chromosomal aberrations (CA), damage was observed in all hydroponic samples and in some samples from soil cultures. As with the negative control, no presence of CA was observed in any of the experiments. In hydroponic cultivation, CA was observed at low concentrations: 0.008 mg L^{-1} for DCF and SMX, and 0.063 mg L^{-1} for MIX. However, statistical analyses revealed a statistically significant increase ($p < 0.05$) in CA compared to the negative control for DCF at 0.063 mg L^{-1} and higher
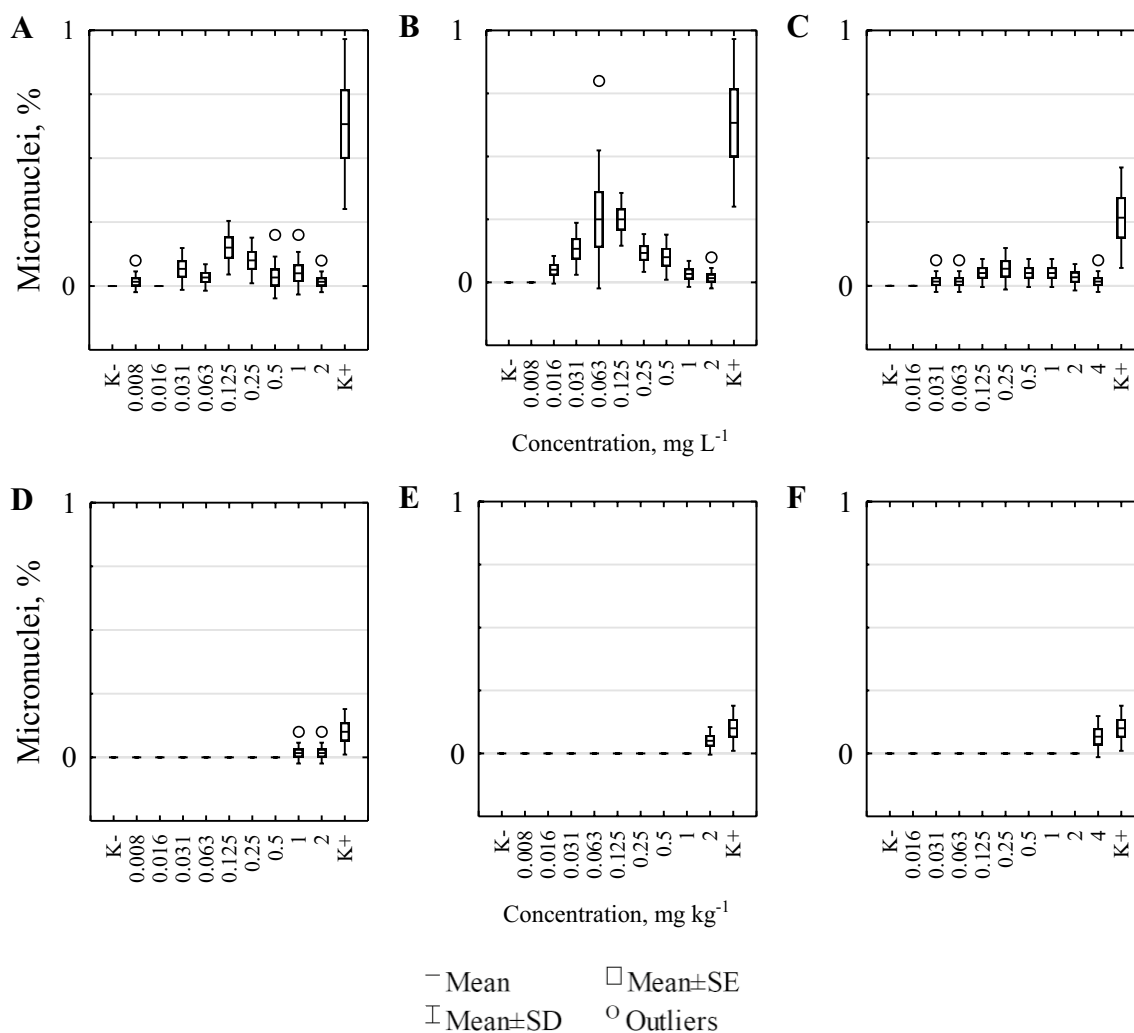
Fig. 3 Effect of the substances tested on MN in *V. faba* cells: **A** effect of DCF in water culture; **B** effect of SMX in water culture; **C** effect of MIX in water culture; **D** effect of DCF in soil culture; **E** effect of SMX in soil culture; **F** effect of MIX in soil culture

concentrations, and for SMX only at a concentration of 0.5 mg L^{-1} (Table SI.7). No statistically significant differences ($p < 0.05$) were observed for MIX, likely due to the very low frequency of genetic material damage and high standard deviations.

The occurrence of CA, similar to MN, was dependent on the MI. Statistically significant differences ($p < 0.05$) were observed in all experiments between the positive control (K +) and the negative control (K −). In the case of soil cultivation, CA was observed at higher concentrations: 0.5 mg kg^{-1} and above for DCF and MIX, and 0.25 mg kg^{-1} and above for SMX. Statistical analyses indicated a significant increase in CA compared to the negative control for DCF only at 2 mg kg^{-1}, for SMX at 1 and 2 mg kg^{-1}, and for MIX at 2 and 4 mg kg^{-1} ($p < 0.05$) (Tables SI.6–SI.7).

Fitting the functions to the results of the genotoxicity test

Based on the conducted analyses, it was observed that the MI decreased in accordance with a power function as the concentration of pharmaceuticals increased (Fig. 5, Table SI.8). This indicates a rapid inhibition of cell division due to the direct contact of xenobiotics with plants. As for the results obtained for MN and CA, the best fit was an initial linear increase in genetic material damage, followed by a decrease in the number of MN and CA according to a power function (Fig. 6).

Cytotoxicity of pharmaceuticals to *Vicia faba*

The CAT activity in *V. faba* leaves exposed to DCF, SMX, and MIX is presented in Tables SI.9–SI.10 and in Fig. 7.
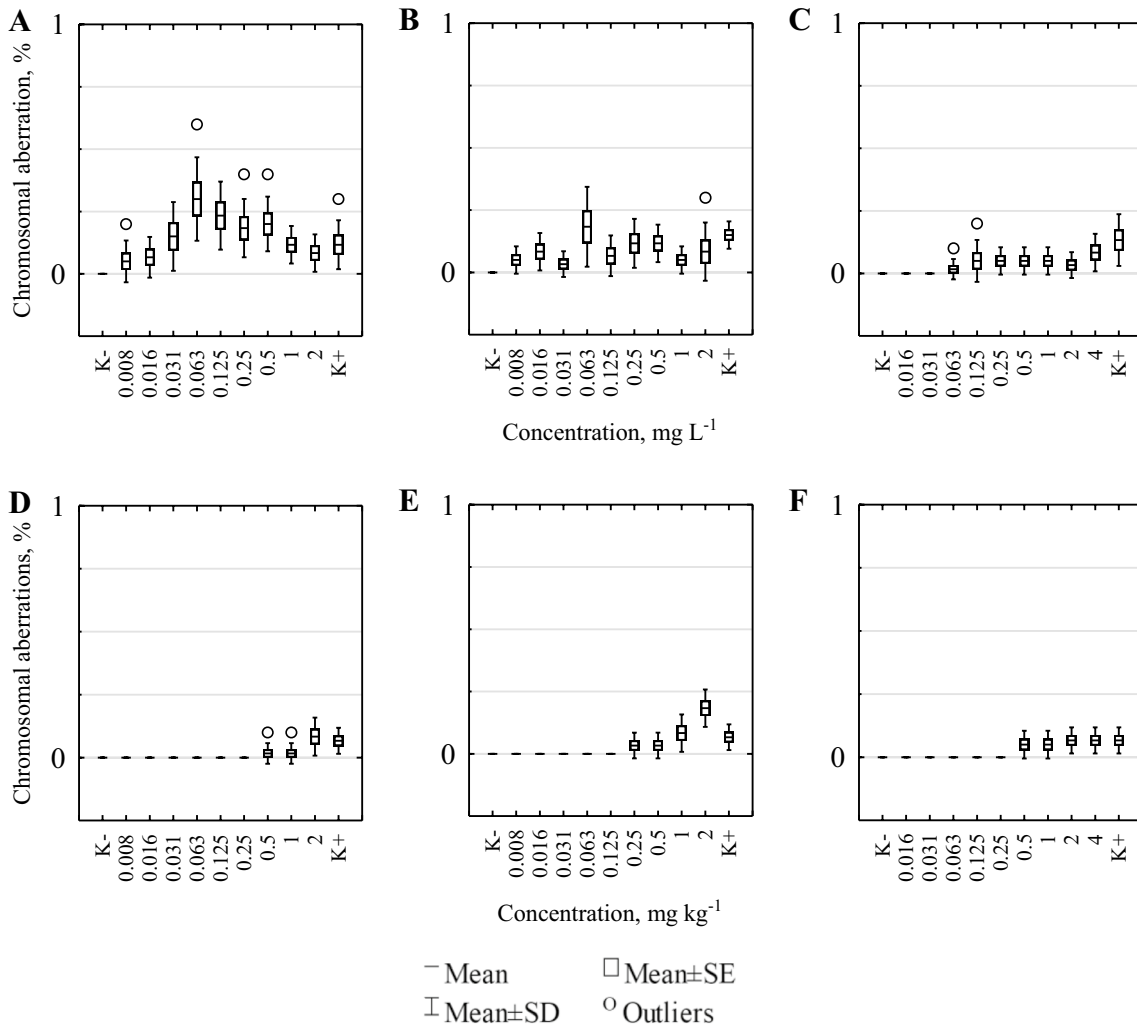
Fig. 4 Effect of the substances tested on CA in *V. faba* cells: **A** effect of DCF in water culture; **B** effect of SMX in water culture; **C** effect of MIX in water culture; **D** effect of DCF in soil culture; **E** effect of SMX in soil culture; **F** effect of MIX in soil culture
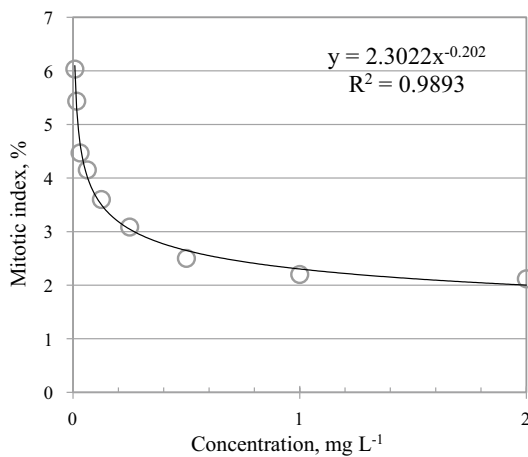

Fig. 5 An example of fitting the power function to the MI results for SMX in water culture

The results of the statistical analyses are shown in Tables SI.8–SI.9. The CAT activity tests revealed an increase in enzyme activity with increasing concentrations of pharmaceuticals. The highest increase in activity compared to the negative control (K-) was observed at the highest analyzed concentrations: for DCF (2 mg L^{-1} and 2 mg kg^{-1}), it was 141.74% for water culture and 42.84% for soil culture; for SMX (2 mg L^{-1} and 2 mg kg^{-1}), it was 245.65% for water culture and 18.45% for soil culture; and for MIX (4 mg L^{-1} and 4 mg kg^{-1}), it was 94.85% and 72.98% for water and soil culture, respectively. Statistically significant differences ($\alpha = 0.05$) were also observed in the enzymatic activity between plants derived from water and soil cultivation. In hydroponic culture, the pharmaceuticals present in the water induced a much higher effect (increase in enzyme activity) compared to the equivalent concentrations in soil cultivation. The average percentage difference in CAT activity between water and soil cultivation was as follows: for

Fig. 6 An example of fitting the linear and power functions to the results of **A** MN and **B** CA for MIX in water culture
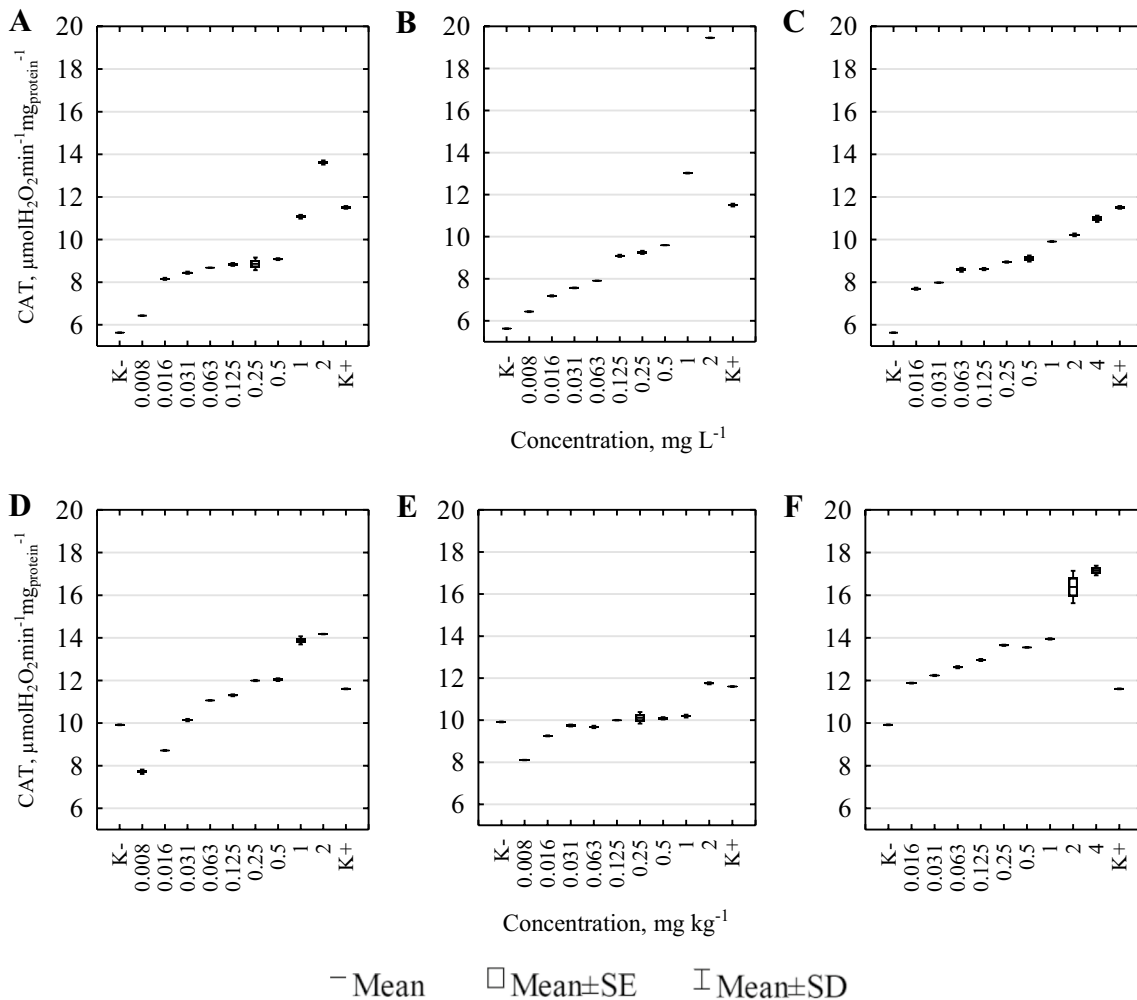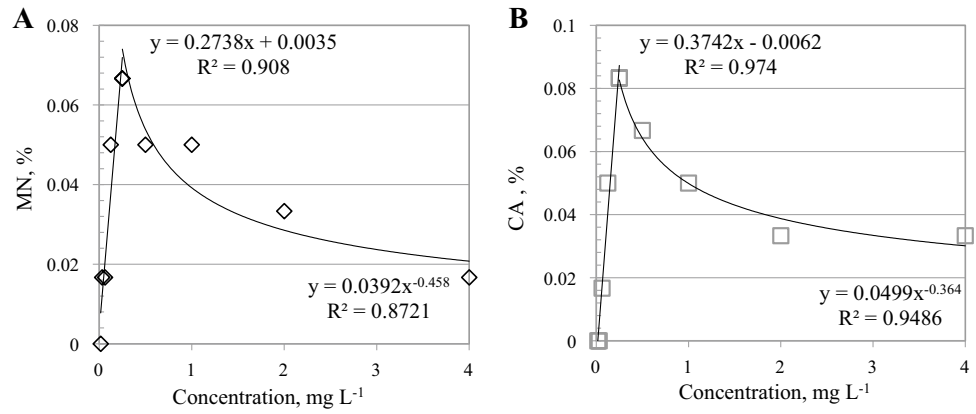

Fig. 7 Effect of the substances tested on the activity of CAT in *V. faba* leaves: **A** DCF effect of DCF in water culture; **B** effect of SMX in water culture; **C** effect of MIX in water culture; **D** effect of DCF in soil culture; **E** effect of SMX in soil culture; **F** effect of MIX in soil culture

DCF: 51%, for SMX: 77%, and for MIX: 23%. It should be noted that the CAT activity in *V. faba* leaves from soil cultivation contaminated with DCF and SMX at the lowest concentration was lower than under control conditions. This relationship was observed for the following concentrations: for DCF: 0.008 mg kg^{-1} and 0.016 mg kg^{-1}; and for SMX: from 0.008 to 0.063 mg kg^{-1}. However, such a relationship was not observed in the case of MIX. Even the lowest

concentrations analyzed caused an increase in CAT activity (for example, 0.016 mg kg^{-1} of MIX resulted in a 19.76% increase in CAT activity compared to control conditions).

The SOD activity in *V. faba* leaves exposed to DCF, SMX, and MIX is presented in Tables SI.10–SI.11 and Fig. 8. The results of the statistical analysis are shown in Tables SI.10–SI.11. In the analysis of SOD activity, an increase in enzyme activity was observed in all analyzed samples under the influence of the drugs. The highest increase in SOD activity compared to the negative control (K −) was observed for DCF (1 mg L^{-1} and 2 mg kg^{-1}), with values of 614.79% for water culture and 3330.50% for soil culture. For SMX (1 mg L^{-1} and 2 mg kg^{-1}), the increase was 572.97% (water culture) and 2644.82% (soil culture). For MIX (4 mg L^{-1} and 4 mg kg^{-1}), the increase was 401.43% and 3102.78% for water and soil culture, respectively. Moreover, the highest concentrations of the drugs analyzed caused higher SOD activity

than the positive control (K +). SOD activity was higher than K + at concentrations such as 0.25, 0.5, 1, and 2 mg L^{-1} for DCF in water culture, and 0.5, 1, and 2 mg kg^{-1} for DCF in soil culture. For SMX, the higher activity compared to K + was observed from 0.125 to 2 mg L^{-1} in water culture. For MIX, it was observed in 2 and 4 mg L^{-1} in water culture and 2 and 4 mg kg^{-1} in soil culture. In the case of soil cultivation in the SMX experiment, the SOD activity in all tested samples was lower than in the positive control (K +). Statistically significant differences ($\alpha = 0.05$) were also observed in the enzymatic activity between plants derived from water and soil cultivation. For samples exposed to DCF and MIX, the SOD activity was higher in soil-grown plants compared to hydroponic culture. For samples exposed to SMX, the SOD activity was statistically significantly lower in soil-grown plants compared to water culture ($\alpha = 0.05$).
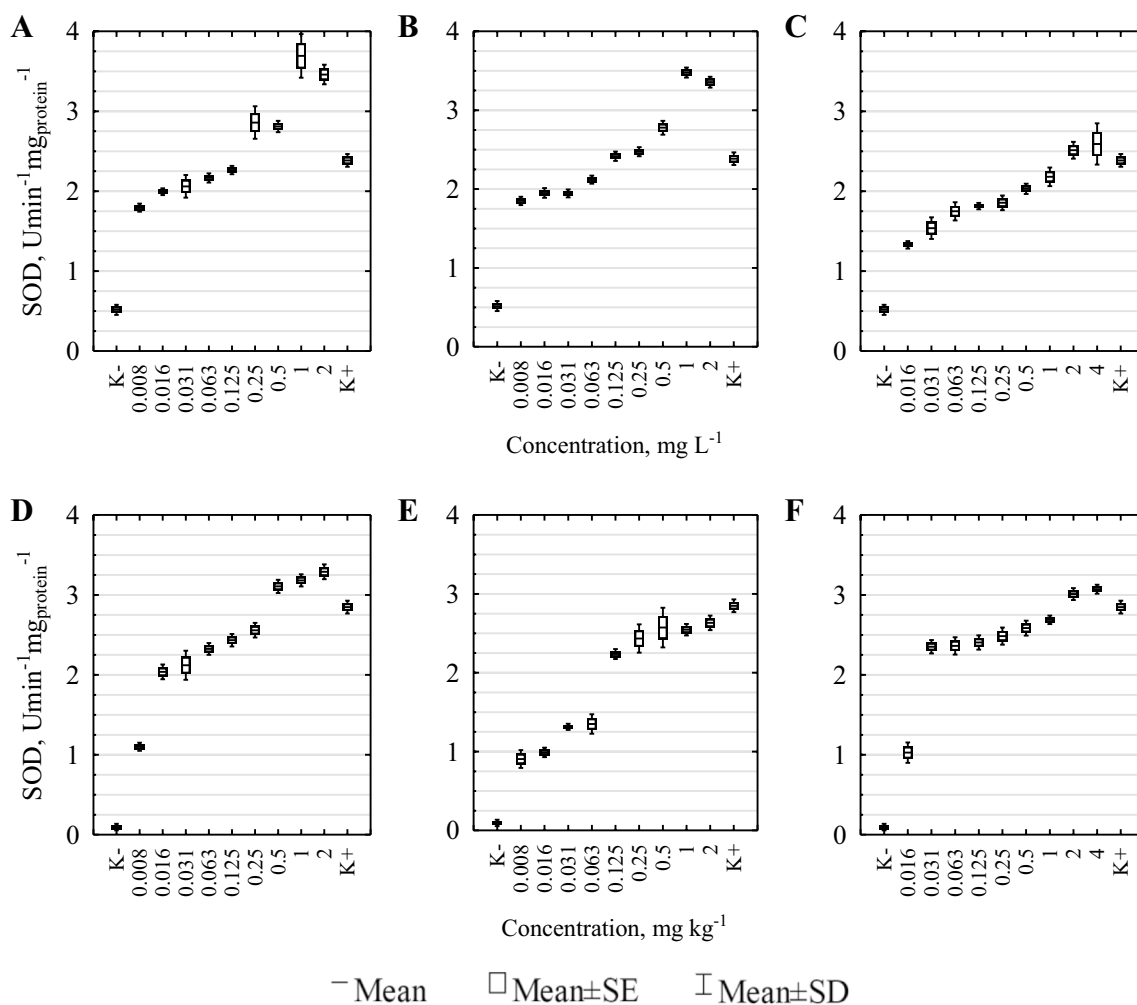

Fig. 8 Effect of the substances tested on the SOD activity in *V. faba* leaves: **A** effect of DCF in water culture; **B** effect of SMX in water culture; **C** effect of MIX in water culture; **D** effect of DCF in soil culture; **E** effect of SMX in soil culture; **F** effect of MIX in soil culture

Discussion

The presence of pharmaceuticals in the natural environment poses a threat to animals, plants, and humans. To assess their toxicity, basic tests are conducted to determine acute and chronic effects on organisms across different trophic levels (Ortiz de García et al. 2014). However, it is important to note that concentrations found in the natural environment often do not cause immediate effects on bioindicators. Therefore, it becomes necessary to examine the impact of xenobiotics on genetic material, cellular structures (Youssef and Elamawi 2020), and the reproductive capabilities of model organisms (Drzymała and Kalka 2022) in order to comprehensively assess their effects.

DCF, SMX, and their equilibrium mixture exhibited cytotoxic, genotoxic, and mutagenic effects on *V. faba* broad bean root cells. All tested drugs demonstrated a decrease in the mitotic index (MI) and an increase in the number of micronuclei (MN) and chromosomal aberrations (CA). To accurately assess the toxicity, the most suitable mathematical function was chosen to analyze the data. The MI was found to decrease following a power function. In the case of MN and CA, the correlations were more complex. These parameters were influenced not only by the concentrations of the drugs but also by the number of cell divisions (MI). When the MI decreased, indicating impaired cell division, the damage to genetic material during division also decreased. The best-fit model showed an initial linear increase in genetic damage, followed by a decrease in the number of MN and CA according to a power function. Similar results were observed in studies on arsenic conducted by Wu et al. (2010). The reduction in MN and CA could be attributed to the high physiological toxicity of the analyzed compounds. Gupta et al. (2018) also observed a decrease in MI and an increase in CA in root cells of *Allium cepa* exposed to chromium and arsenic. However, their study did not show the specific relationships illustrated in our research, with an initial increase in genetic damage followed by a decrease due to a decline in MI. Possible reasons for this disparity include the use of too low metal concentrations in their experiment, different plant species (*A. cepa*) with varying reactions to harmful substances, or distinct mechanisms of action. Nevertheless, regardless of the observed function relating to changes in MI or MN and CA, the reduction in the mitotic index and the increased occurrence of genetic damage clearly indicate the harmful nature of the analyzed substances.

Similar results were obtained when testing the genotoxicity of commonly used cytostatic drugs (5-fluorouracil, etoposide, cisplatin, carboplatin, vincristine sulfate, and cyclophosphamide monohydrate) against *A. cepa* root cells (Mišík et al. 2014). The authors observed the effects of the tested drugs on the mitotic index of cells, the number of chromosomal aberrations, and a negative impact of the drugs on the length of *A. cepa* roots (Mišík et al. 2014).

Genotoxicity analyses are frequently employed to assess the potential harm of intricate mixtures, such as wastewater. In their study, Kumari and Tripathi (2019) utilized genotoxicity assays with *A. cepa* root tip cells to analyze effluents from the pharmaceutical industry. The analyzed wastewater led to a decrease in the mitotic index and an increase in observed chromosomal aberrations. The authors concluded that the substances present in the wastewater interfere with the normal mitotic cell cycle by blocking mitosis during interphase, thereby preventing cells from entering prophase (Kumari and Tripathi 2019).

Bakare et al. (2009) also assessed the genotoxicity of wastewater containing pharmaceuticals. In their research, the authors employed various methods, including a micronucleus test using *A. cepa* onion. The obtained results indicated the genotoxic potential of the analyzed wastewater, as evidenced by a decreased mitotic index and an increased occurrence of chromosomal aberrations. These findings were further corroborated by other genotoxicity tests performed on mouse sperm and bone marrow cells (Bakare et al. 2009).

Confirmation of the genotoxicity of pharmaceuticals or pharmaceutical wastewater can also be obtained through other commonly used genotoxicity tests, such as the GreenScreen Assay utilizing the yeast *Saccharomyces cerevisiae* (Zounková et al. 2007), the *Escherichia coli* SOS-chromotest (Isidori et al. 2005), the Ames test (Sharif et al. 2016), or the SOS/umu tests based on the *Salmonella typhimurium* strain (Xie et al. 2017). However, analyzing drug mixtures presents a challenge due to the numerous factors influencing the observed toxic effects, including the composition of the mixture, which often fluctuates in the natural environment.

DCF and SMX are widely detected pharmaceuticals in the natural environment. Maximum concentrations found in surface water are 0.057 mg L^{-1} for DCF (Sathishkumar et al. 2020) and 0.022 mg L^{-1} for SMX (Straub 2016). In soil environments, the maximum concentrations detected are 0.257 mg kg^{-1} for DCF (Ashfaq et al. 2019) and 0.055 mg kg^{-1} for SMX (Cycoń et al. 2019). The concentrations detected in the environment fall within the range tested in this study. Therefore, it can be inferred that the concentrations observed in the environment can lead to cytotoxic effects, as well as genotoxic and mutagenic effects.

When comparing the results obtained from water and soil cultivation, it is important to note that the toxic effect was generally lower in soil cultivation. This difference is particularly noticeable in the case of SMX and the mixture of drugs (MIX) (Tables SI.3 and SI.4). This finding highlights the protective function of soil. Microcontaminants tend to adhere to soil particles, making them less accessible to plants. Soil acts as a complex matrix and serves as a reservoir for contaminants due to its sorption properties. Harmful

substances can be retained in the soil, preventing their leaching into surface water or groundwater (Rabot et al. 2018; Rinot et al. 2019). The ability to accumulate xenobiotics in soil is highly dependent on their physicochemical properties. Two crucial parameters are the octanol–water partition coefficient ($logK_{OW}$) and the acid dissociation constant (pKa) of the compound. DCF has a $logK_{OW}$ of 4.51 and a pKa of 4.15, while SMX has a lower $logK_{OW}$ of 0.89 and a pKa in the range of 5.6–5.7 (Pal et al. 2010). According to the Technical Guidance Document on Risk Assessment (2003), hydrophobic compounds with $logK_{OW} > 3$ tend to accumulate easily in the natural environment through sorption into soil organic matter. As for pKa, the accumulation and dissociation of a compound will depend on the pH of the soil. When the soil pH is lower than the pKa of the substance, the compound tends to accumulate, whereas when the soil pH is higher than the pKa, the compound dissociates and accumulates to a lesser extent (Revitt et al. 2015).

Based on the available data, it can be concluded that DCF readily binds to soil particles, whereas SMX does not accumulate in the soil. In our study, we observed that DCF exhibited lower toxicity to *V. faba* compared to SMX, particularly in terms of cytotoxicity and genotoxicity. However, the number of observed MNs in the mutagenicity analysis was too low to draw a meaningful comparison. These findings align with our previous research (Drzymała and Kalka 2022), where we investigated the toxicity of the same substances in soil on *Eisenia fetida* earthworms and found that DCF exhibited lower toxicity compared to SMX. This suggests that DCF was less accessible to soil organisms than SMX (Drzymała and Kalka 2022).

One aspect commonly explored in mixture studies is the analysis of interactions between the components. In our analysis of MI, the effect of pharmaceuticals was quantified as the percentage decrease in the number of dividing cells relative to the control conditions (K−). The effect of the drug mixture was also assessed using the Concentration Addition (CA) and Independent Action (IA) models. However, neither of these models adequately fit the observed data (Fig. 9). The observed effect, both in water and soil cultivation, was lower than the predicted effect based on the results obtained for the individual pharmaceuticals tested.

The selected drugs for this research exhibit different modes of action. DCF is a non-steroidal drug with analgesic and anti-inflammatory properties, while SMX is a bacteriostatic antibiotic. In our previous studies (Drzymała and Kalka 2020), conducted on these substances but using different bioindicators, we found that the mixture showed higher toxicity compared to its individual components. Tests conducted on *Aliivibrio fischeri* (bacteria), *Daphnia magna* (crustaceans), and *Lemna minor* (plants) demonstrated partially additive or synergistic effects of DCF and SMX when combined. However, as demonstrated in this study, the results from acute tests cannot be directly extrapolated to predict the interaction of mixtures in more specific genotoxicity tests. Surprisingly, the observed toxicity of the mixture was lower than what was predicted by the Concentration Addition (CA) and Independent Action (IA) models. These findings are in line with those of other researchers who have also investigated DNA damage and observed similar unexpected dependencies. López González et al. (2021) conducted a study on a ternary pesticide mixture comprising chlorpyrifos, cypermethrin, endosulfan, and glyphosate. They observed an interesting antagonistic effect, where the genotoxic effects observed for each individual compound were no longer visible in the mixture. However,
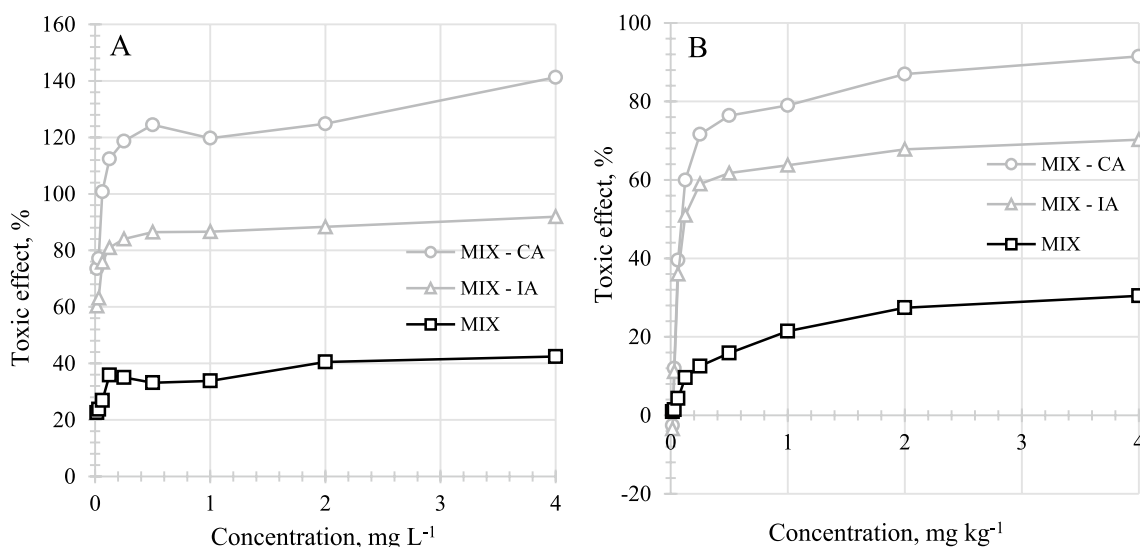

Fig. 9 Predicted effect of MIX on MI in **A** water and **B** soil culture

it is important to note that the study focused on higher organisms such as *Caiman latirostris*. Blood samples were collected from animals treated with a mixture of pesticides and individual compounds for a period of 20 days, and genotoxicity tests were performed. The authors suggested that the antagonistic effect observed in the mixture may be attributed to the interference between its components, leading to a reduction in the overall toxic effect (López González et al. 2021). Odetti et al. (2020) similarly reported antagonistic effects of toxins in mixtures. The researchers observed a reduction in the overall genotoxicity of binary and ternary pesticide mixtures, specifically combinations of cypermethrin and chlorpyrifos, as well as cypermethrin, chlorpyrifos, and glyphosate. According to the authors, it was hypothesized that the tested pesticides interfere with each other, leading to a diminished effect in the observed toxicity of the mixture. The biomarkers used in tests with a mixture of various substances play a crucial role in determining the interactions that occur. Depending on the concentrations employed, these biomarkers may exhibit varying degrees of sensitivity to xenobiotics. Therefore, it is imperative to adopt an integrated approach in biomonitoring during scientific research. On one hand, utilizing multiple parameters and diverse endpoints enhances the reliability and facilitates better interpretation of the results. On the other hand, it is challenging to anticipate all the factors that can influence such interactions in environmental conditions, including the presence of other stressors.

The studies conducted as part of this research have revealed that the interaction among mixture components is influenced not only by the individual mechanisms of action of each component but also by their environmental occurrence, bioavailability, and the specific test organisms selected. Interestingly, the harmfulness of the analyzed mixture to *V. faba* plants was found to be lower than predicted, both by commonly used models for predicting mixture activity and our previous results obtained with other bioindicators. These aspects warrant further investigation to gain a more precise understanding of the mechanisms of action for these and other mixtures. Subsequent findings will undoubtedly contribute to the development of more accurate prediction models for mixture toxicity, which will better reflect the actual behavior of mixtures under different conditions and in relation to various bioindicators.

Oxidative stress occurs in cells when there is an imbalance between the production of reactive oxygen species (ROS) and their removal by antioxidant systems. Organisms have developed mechanisms to eliminate excess ROS from cells. The primary antioxidant enzymes include catalase (CAT) and superoxide dismutase (SOD), along with other enzymes such as glutathione peroxidase (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR). Additionally, cells possess non-enzymatic antioxidants like ascorbic acid, glutathione, phenols, tocopherols, and carotenoids, which also play a role in regulating ROS levels (Sharma and Gupta 2020).

In our research, we observed a significant increase in the activity of CAT and SOD in the leaves of *V. faba* exposed to the lowest tested concentrations ($\alpha = 0.05$). These findings support the conclusions drawn from the micronucleus test and MI analysis. The elevated CAT activity indicates an accumulation of ROS in the cells, which can cause damage to genetic material. Similar results have been reported by Ma et al. (2014) and Shahid et al. (2014). Another potential mechanism of genotoxicity is the formation of DNA adducts. Complex structures may form as a result of endogenous chemicals or ionizing radiation, leading to genetic material damage. Chemical compounds containing free methyl or ethyl groups can readily bind to nitrogenous bases in DNA, resulting in genetic material damage (Theodorakis 2008). Regarding SOD activity, Jiang et al. (2019) have also observed similar results. They reported a statistically significant increase in SOD activity in response to polystyrene microplastics at concentrations of 10, 50, and 100 mg L^{-1}. Furthermore, the researchers suggested that high concentrations of toxic substances could inhibit the oxidative system, leading to a reduction in enzyme activity (Jiang et al. 2019).

A notable finding emerged from the analysis of CAT activity in the leaves of soil-cultivated plants exposed to DCF and SMX. Initially, the lowest concentrations of the tested drugs caused a decrease in CAT activity compared to the control conditions (K−). This may be attributed to a delayed activation of cell protective systems or plant acclimatization to adverse conditions (Sofo et al. 2015). However, similar dependencies were not observed for SOD activity. Determining the activity of oxidative enzymes is a very sensitive tool for defining the harmfulness of pollutants in water and soil. Changes in enzyme activity occur much earlier than other visible markers of toxicity, such as stunted growth or yield reduction. Long-term exposure of plants to contaminants can cause extremely important cytotoxic effects, especially in plants used in the food industry. Scientific research carried out at low environmental concentrations provides much more information than classical acute tests. They indicate that even very low concentrations can induce cytotoxicity, mutagenicity, and genotoxicity in test plants.

Conclusion

The micronucleus test employed in these studies serves as a versatile tool, allowing for the assessment of genotoxicity, cytotoxicity, and mutagenicity of xenobiotics. The inclusion of oxidative enzyme activity analyses further supported the conclusions drawn from the micronucleus test.

The tested pharmaceuticals, DCF and SMX, as well as their binary mixture, exhibited mutagenic, cytotoxic, and genotoxic effects on *V. faba* broad bean root cells. Furthermore, these substances led to an increase in the activity of two key enzymes, CAT and SOD, in *V. faba* leaves. CAT and SOD are essential components of the cell's protective mechanism against oxidative stress, thus confirming the cytotoxic effects of the analyzed drugs and their mixture.

The conducted analyses encompassed concentrations of pharmaceuticals that are currently detectable in the natural environment, making the obtained results highly significant. It was revealed that the environmental concentrations detected can reduce MI in *V. faba* cells, and in the case of genetic damage such as MNs and CAs, these abnormalities can occur at environmental concentrations. Consequently, the concentrations presently observed in the environment have the potential to exhibit mutagenic, cytotoxic, and genotoxic effects on the tested organisms.

Surprisingly, the conducted studies demonstrated a low level of toxicity in *V. faba* broad beans exposed to the pharmaceutical mixture. These findings suggest the existence of additional factors beyond those considered that may influence the interaction between the mixture's components. This aspect is of significant importance and calls for further extensive research.

Genotoxicity, cytotoxicity, and mutagenicity studies hold great importance as such changes remain imperceptible externally, while high concentrations of pharmaceuticals often result in visible alterations in bioindicators such as growth inhibition, reduced yield, leaf discoloration, and evident damage, micronucleus and enzyme.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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