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Original Research

Biomarkers of Leguminous Plant Viability in Response to Soil Contamination with Diclofenac

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Abstract

Pharmaceuticals have become an emerging environmental protection problem due to their presence in water and soil ecosystems. Reliable cell viability biomarkers (mitochondrial and cytosol distribution of cytochrome c oxidase activity), germination, and growth of seedlings were assessed to be sensitive endpoints of diclofenac toxicity. Moreover, the content of soluble carbohydrates in seedlings of three leguminous plants was an additional indicator of germination. The tested diclofenac concentrations (from 0 to 12 mM) in the three plant species (lupin, pea, and lentil) resulted in increased activity of the enzyme in cytosol, and a decreased activity in mitochondrions. The increase of the cytochrome c oxydase activity in cytosol was most rapid in pea and slowest in lupin. The decrease in mitochondrions was gradual, yet in roots growing in the soil contaminated with 12 mM of diclofenac, from 35 to 68% of total enzyme activity leaked from the mitochondrion to the cytoplasm. The dynamics of seedling growth was a better parameter of soil contamination with diclofenac than germination. On the basis of the described morphological and biochemical features, it was found that diclofenac is decidedly less phytotoxic toward leguminous plants (lupin, pea, lentil) than e.g. sulfamethazine. The research has shown that carbohydrate metabolism is a good parameter of seedling growth, but it is not an indicator of contamination and thus cannot be applied to assess soil ecosystem contamination with medicines.

Keywords: seeds and seedlings of leguminous plants, soil, diclofenac, cytochrome c oxydase activity, soluble carbohydrates

Introduction

In recent years pharmaceuticals have been seen as an emerging environmental protection problem due to their presence in water and soil ecosystems [1]. Diclofenac (DCF, (2-[(2,6-dichlorophenyl)amino]benzeneacetic acid) is a synthetic non-steroidal anti-inflammatory drug (NSAD), mostly used as its sodium salt in medical care as an analgesic, antiarthritic, and antirheumatic. The medicine has been applied in human medicine for over 30 years [2]. Diclofenac sodium 3% gel (Solaraze®) gained US approval for the treatment of actinic keratosis (AK) more than 10 years ago. It has been proven that, applied in this form for over 10 years, the medicine is effective and safe.

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Its mechanism in AK involves cyclooxygenase-2 (COX-2) inhibition, inhibition of angiogenesis, and induction of apoptosis [3]. Diclofenac acts via inhibition of prostaglandin synthesis by inhibiting cyclooxygenase-1 (COX-1) and COX-2 with relative equipotency. Moreover, diclofenac may inhibit thromboxane-prostanoid receptor affecting arachidonic acid release and uptake, as well as inhibiting lipoxygenase enzymes, and activate the nitric oxide-cGMP antinociceptive [4]. It also has been proven that it shows bacteriostatic activity inhibiting DNA synthesis of bacteria [5].

The medicine is applied on skin as an ointment, gel, sticking plasters or sprays, as well as being administered as tablets, suppositories, and injections [3, 6-8]. It is available without prescriptions but also prescribed, used by patients of all ages all over the world [9, 10]. As an active ingredient (in the form of sodium or potassium salts) it is sold under many brand names. For instance, 85.8 tons of diclofenac were sold in Germany in 2001. Yet it is not much in comparison to such painkillers as acetylsalicylic acid (836.26 t), paracetamol (621.65 t), and ibuprofen (344.89 t), which together amount to 1,802.8 t taken by German patients in only one year [10].

Such a considerable consumption of medicines results in low levels of pharmaceuticals discovered in the natural environment [11]. Bioaccumulation of diclofenac in the food chain has contributed to a decrease in the population of vultures (Indian Vulture (*Gyps indicus*) and Slender-billed Vulture (*Gyps tenuirostris*)) in India by 95%. This decrease correlates with diclofenac's content in their kidneys, a content whose remains were found in all birds and ranged from 0.051 to 0.643 $\mu g \cdot g^{-1}$ [12]. A dose of 0.8 $mg \cdot kg^{-1}$ of diclofenac was highly toxic to a Eurasian (*Gyps fulvus*) and an African (*Gyps africanus*) species [13]. Found in fish livers, kidneys, and muscles, diclofenac is toxic also to fish [14]. It remains in biological fluids (joint fluids) for 11 hours [15]. Yet in the environment it undergoes photolysis under sunlight and its half-life is 39 minutes [16]. Photodegradation depends on light radiation, which is a function of the depth, season, geographic latitude, and weather.

So far diclofenac's influence on plants has not been assessed, although it enters soil with excrement and urine. Approximately 65% of the dosage of diclofenac is excreted through urine. However, the actual amount of metabolites in the faeces is still not clear [17]. It has been proven that medicines are taken up by plants [18, 19]. Cytochrome c oxydase (CcO) plays a key role in plants. Located in the inner membrane of mitochondria, it is the terminal enzyme of most respiratory chains [20]. It catalyzes the one electron oxidation of ferrocytochrome c and the four-electron/four-proton reduction of dioxygen to water. In people, deformations including genetic mutations of CcO, leading to serious metabolic disorders, and even death. CcO dysfunction is believed to be one of the most serious among many mitochondrial diseases [21]. Most CcO dysfunctions are related to nuclear-encoded protein mutations, including transcription and translation of mitochondrion-encoded subunits [22].

The aim of our study was to determine the effects of different concentrations of diclofenac on distribution of cytochrome c oxydase activity in-between mitochondria and cytosol of leguminous roots. Furthermore, the influence of DCF on germination and elongation of root and shoots of leguminous seedlings was examined. The content of soluble carbohydrates in seedlings of three leguminous plants was an additional indicator of germination.

Material and Methods

Seed Germination and Root and Shoot Growth Test

Seeds of yellow lupin (*Lupinus luteus*), pea (*Pisum sativum*), and lentil (*Lens esculenta*) were germinated for nine days in PHYTOTOXKIT™ plates (MicroBio Test, Inc., Belgium). Germination was carried out under controlled climatic conditions with temperature set at 25ºC and 90% relative humidity (RH) in darkness. Ninety ml of soil (sand, vermiculite, peat 1 :0.3:1, v/v/v) was placed in each plastic microbiotest plate. The soil was covered with Whatman No. 1 filter paper and watered with 27 ml distilled water supplemented with diclofenac (DCF) sodium salt (2-[(2,6-Dichlorophenyl)amino]benzeneacetic acid sodium salt; Sigma-Aldrich) at final concentrations of 0.06, 0.125, 0.25, 0.5, 1, 3, 6, 8, or 12 mM. The control plants were watered with pure distilled water. The root and shoot length was estimated using Image Tool for Windows. The experiment was carried out with four replicates.

Cytochrome c Oxidase Activity

Isolation of mitochondria was carried out on ice. Roots of DCF-treated or control (water) seedlings (9 days old) were ground in a mortar in homogenization buffer (0.4 M mannitol, 20 mM HEPES-KOH, pH 7.4, 1 mM EDTA, 0.2% BSA, 0.6% PVP, 8 mM cysteine). One ml of homogenization buffer was used per 200 mg root fresh weight. The homogenate was centrifuged for 10 min at 1000×g at 4ºC. The supernatant was removed and centrifuged at 15,000×g for 20 min to pellet mitochondria. The supernatant (cytosol) fraction was stored for further analysis. The crude mitochondrial pellet was washed three times and finally resuspended in washing medium (0.4 M mannitol, 20 mM HEPES-KOH, pH 7.4, 1 mM EDTA).

CcO was assayed with a cytochrome c oxidase assay kit (Cytocoxl, Sigma) in crude mitochondrial pellet and the supernatant (cytosol) fraction. The colorimetric assay in this kit is based on observation of the decrease in absorbance at 550 nm of ferrocytochrome c produced by its oxidation to ferricytochrome c by CcO. Activity of CcO was calculated in units per milliliter separately for mitochondrial and the supernatant (cytosol) fractions. One unit converts 1 μM reduced cytochrome c to oxidazed cytochrome c per minute at pH 7 and 25ºC. The activity in mitochondria and supernatant was combined and it was assumed that this comprised 100% of the activity for both

cell compartments. The activity determinations were conducted in three replicates.

Soluble Carbohydrate Content

Soluble carbohydrate contents in seedlings were analyzed by GC chromatography according to Piotrowicz-Cieślak [23]. Seeds (80-100 mg fresh mass) were homogenized in ethanol:water, 1:1 (v/v) containing 300 µg phenyl-α-D-glucose as internal standard. The homogenate and the wash were combined in a 1.5 ml microfuge tube, heated at 75ºC for 30 min and centrifuged at 15,000 g for 20 min. The supernatant was passed through a 10,000 MW cut-off filter (Lida, Kenosha, WI USA). Aliquots of 0.3 ml filtrate were transferred to silylation vials and evaporated to dryness. Dry residues were derived with 300 µl of silylation mixture (trimethylsilylimidazole:pyridine, 1:1, v/v) in silylation vials (Thermo Scientific) at 70ºC for 30 min, and then cooled at room temperature. One µl carbohydrate extract was injected into a split-mode injector of a Thermo Scientific gas chromatograph equipped with a flame ionization detector. Soluble carbohydrates were analyzed on a DB-1 capillary column (15 m length, 0.25 mm ID, 0.25 µm film thickness, J&W Scientific). Soluble carbohydrates were identified with internal standards as available, and concentrations were calculated from the ratios of peak area, for each analyzed carbohydrate, to the peak area of respective internal standard. Quantities of soluble carbohydrates were expressed as mean ±SD for 3-5 replications of each treatment.

Results

The effect of DCF concentration (0.06, 0.125, 0.25, 0.5, 1, 3, 6, 8, and 12 mM a conversion was added DCF in mg·kg^{-1}) on germination and roots and shoots elongation of seedlings of yellow lupin (*Lupinus luteus* L.), pea (*Pisum saivum* L.), and lentil (*Lens esculenta* L.) was analyzed. The seed germination, root and shoot elongation (Fig. 1A, B, C) measurements were taken nine days after DCF application. All seeds, irrespective of the concentration of diclofenac, germinated with frequency between 97 and 100% (Fig. 1A, B, C). The seeds were considered germinated when the radicle penetrated the seed coat.

With increasing diclofenac concentration there was an inhibition of root and shoot elongation in tested plants (Fig. 1A, B, C). After 9 d of growth in the soil without diclofenac (control soil), the shortest roots were observed in lentil (75 mm) and the longest in pea and lupin (120 mm). With increasing DCF concentration the root growth was inhibited more severely. After 9 d, with the highest diclofenac concentration, the rate of root growth retardation compared to roots of control seedlings amounted to 85, 83, and 80% for pea, lupin, and lentil, respectively.

Pea and lupin roots were more sensitive to DCF than lentil roots, since even the lowest of the tested concentrations of the medicine suppressed their elongation by as much as 25%. The same effect in lentil was caused by the third concentration (0.25 mM). The highest concentration reduced root elongation of the three species by 82.6% on average.

Similarly, the suppression of shoot elongation depended on the plant species and DCF concentration (Fig. 1A, B, C). The seedlings of lupin and pea had the longest shoots in the control soil (118 mm on average), while lentil had the shortest ones (60 mm). Lupin, pea, and lentil developed shoots shorter than their roots. Like in roots, also in shoots the percentage of elongation suppression was the highest in pea and lupin, while it was the lowest in lentil: 91%, 86%, and 77%, respectively.

Nine days after diclofenac application the soluble carbohydrates were analyzed in the seedlings. Both in the control soil and the contaminated soil, monosaccharides (fructose and glucose), disaccharides (sucrose and galactinol), cyclitols (myo-inositol, D-pinitol), and raffinose family oligosaccharides (raffinose and stachyose) were found in lupin, pea, and lentil seedlings (Fig. 3). The soluble carbohydrates indicated the rate of seed germination. The longest seedlings of the three analyzed plants included the highest

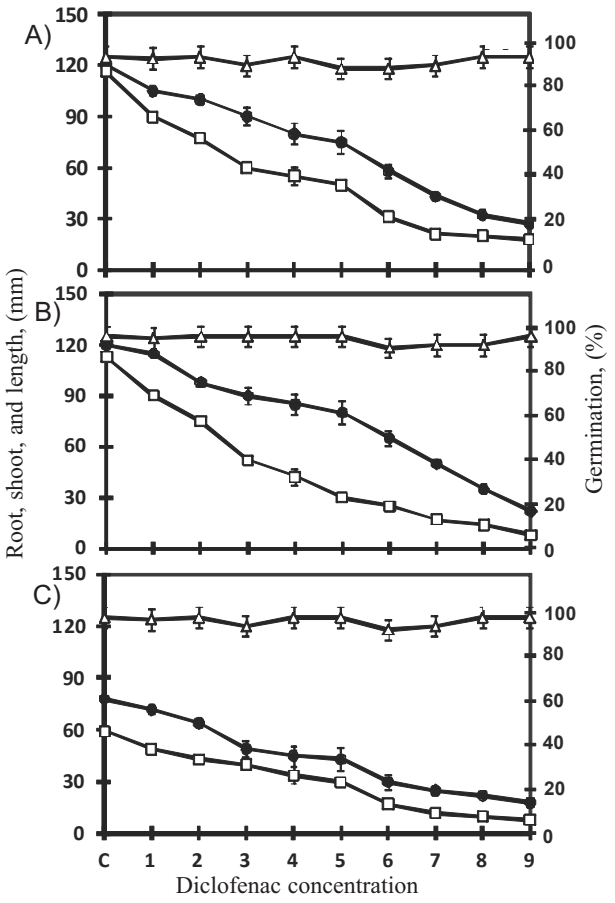

Fig. 1. Seed (Δ) germination (%) and root (●) and shoot (□) length (mm) of *Lupinus luteus* (panel A), *Pisum sativum* (panel B), and *Lens esculenta* (panel C) after nine days on soil supplemented with different diclofenac concentrations (c, control; 1 – 0.06 mM, 2 – 0.125 mM, 3 – 0.25 mM, 4 – 0.5 mM, 5 – 1 mM, 6 – 3 mM, 7 – 6 mM, 8 – 8 mM, and 9 – 12 mM i.e. 3, 6.25, 12.5, 25, 50, 150, 300, and 400 mg·kg^{-1} of soil dry). Data points represent the means ±SD for nine replicate samples.

content of monosaccharides and cyclitols, while the shortest seedlings had the highest content of oligosaccharides (raffinose and stachyose). The length of seedlings depended on diclofenac concentration in soil. In the tissues of the control plants of the three analyzed species the content of glucose was slightly higher than the content of fructose (25 mg/g dry weight, 14 mg/g dry weight, respectively). Lupin, pea, and lentil seedlings were growing in the soil contaminated DCF the content of monosaccharides decreased on average to 1.3 mg/g dry weight (fructose) and 9 mg/g dry weight (glucose). In all analyzed seedlings, there was the same tendency of slight lowering of the content of sucrose and myo-inositol. The analyzed content of raffinose and stachyose, as well as galactinol, increased in the plants in line with soil contamination. Yet, their content was 100 times lower than the content of monosaccharides and sucrose. It is worth emphasizing that in lupin seedlings raffinose and stachyose content was the highest (Fig. 2).

The analysis included distribution of cytochrome c oxidase activity between the mitochondrion and cytosol in lupin, pea, and lentil roots growing in soils supplemented with various diclofenac concentrations (Fig. 3). In line with the growing concentration of diclofenac in soil, the activity of the enzyme in the mitochondrion decreased, while it grew in the cytosol. Despite this, at the highest of the analyzed diclofenac concentrations there was still some CcO

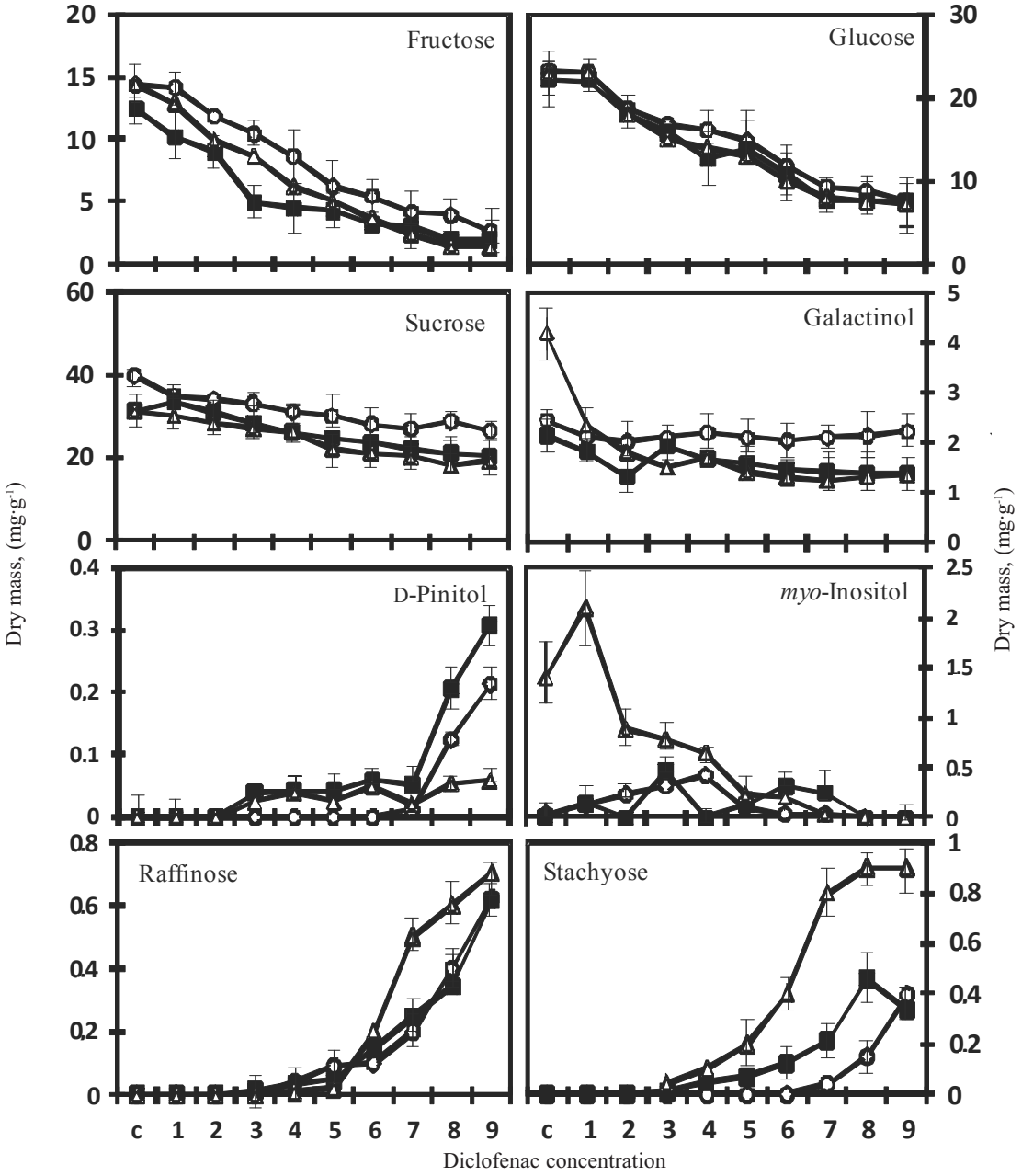

Fig. 2. Soluble carbohydrate sugars (fructose, glucose, sucrose, galactinol, *D*-pinitol, *myo*-inositol, raffinose, and stachyose) in seedlings of *Lupinus luteus* (Δ), *Pisum sativum* (■), and *Lens esculenta* (o) after nine days on soil supplemented with different diclofenac concentrations (c, control; 1 – 0.06 mM, 2 – 0.125 mM, 3 – 0.25 mM, 4 – 0.5 mM, 5 – 1 mM, 6 – 3 mM, 7 – 6 mM, 8 – 8 mM, and 9 – 12 mM i.e. 3, 6.25, 12.5, 25, 50, 150, 300, and 400 mg·kg^{-1} of soil dry). Data points represent the means ±SD for nine replicate samples.

activity in the mitochondrion. The highest activity of the enzyme at this DCF concentration was found in the lupin mitochondrion and the lowest in the pea mitochondrion. As much as 68% of the enzyme activity leaked from the pea mitochondrion to the cytosol, while for the lupin mitochondrion it was only 35%. The CcO distribution in 50% between the mitochondrion and cytosol was observed for 8 and 12 mM diclofenac concentration for pea and lentil, respectively (Fig. 3).

Discussion

In bioindication of the natural environment contaminated with chemical compounds vascular plant species definitely are used less often than animal organisms. The response of farm plants to the presence of medicines in the environment consists of disturbed biochemical and physiological reactions such as suppressed seed germination, hindered development of roots and shoots, and changes in cytochrome c oxidase activity in the mitochondrion and cytoplasm [19].

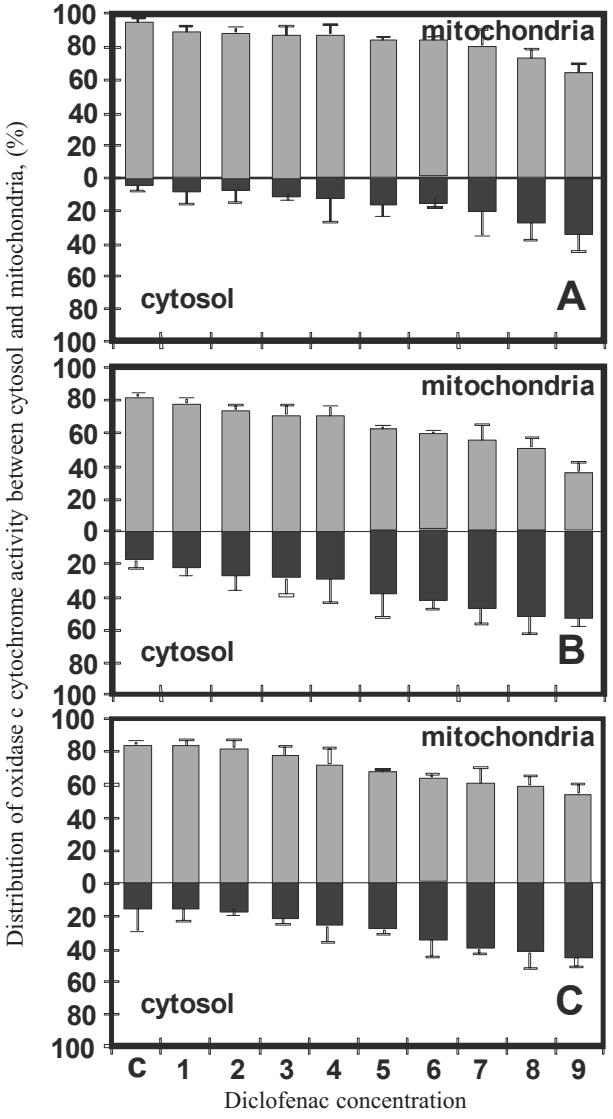

Fig. 3. The ratio (%) between cytochrome c oxidase activity in mitochondria and cytosol of root of 9-days-old leguminous plants: (A) *Lupinus luteus*, (B) *Pisum sativum*, (C) *Lens esculenta* on soil supplemented with different diclofenac concentrations (c, control; 1 – 0.06 mM, 2 – 0.125 mM, 3 – 0.25 mM, 4 – 0.5 mM, 5 – 1 mM, 6 – 3 mM, 7 – 6 mM, 8 – 8 mM and 9 – 12 mM i.e. 3, 6.25, 12.5, 25, 50, 150, 300, and 400 mg·kg^{-1} of soil dry). Data points represent the means ±SD for four replicate samples.

In ecotoxicology, there are two important morphological parameters of environmental stress: seed germination and root elongation. The latter have direct contact with contaminated soil, absorb water, and deliver it to the developing seedlings. The tested DCF concentrations from 0.06 to 12 mM did not suppress germination of lupin, pea, and lentil (Fig. 1). However, it was proven that seed germination is not a sensitive parameter for assessing xenobiotic presence in the environment [24, 25]. The length of lupin, pea, and lentil roots growing in soil contaminated with growing dicolfenac concentrations decreased. The highest concentration (12 mM) considerably limited the length of roots (by 85, 83, and 80% for pea, lupin, and lentil, respectively), as compared with plants in the control soil. The same tendency of limited root elongation was shown for enrofloxacin [18] and sulfamethazine [19]. Suppression of pea, lupin, and lentil root elongation caused by sulfamethazine was higher than the one observed for diclofenac. It is worth emphasizing that the same concentrations of sulfamethazine and diclofenac were analyzed for the same plants. The roots of pea, lupin and lentil which grew in the soil contaminated with diclofenac (12 mM), were longer than the roots growing in the soil contaminated with sulfamethazine [19]. The roots of lupin and pea did not grow in the soil contaminated with 1, 3, 6, 8, and 12 mM of sulfamethazine [19], while at the highest concentration of diclofenac (12 mM) roots of pea, lupin, and lentil were 30, 32, 28 mm long, respectively. The roots of such plants as: cucumber, lettuce, bean, and radish growing in the soil contaminated with enrofloxacin also were shortened [26]. Seedling reactions to soil contamination caused by diclofenac have not yet been thoroughly analyzed, although the medicine is found in the environment. In German rivers, the average content of diclofenac is 0.15 μg/L, yet contents even eight times higher have been reported [27]. Diclofenac also has been found in Swiss rivers and lakes, with highest contents amounting to 0.37 μg/L [28].

The length of seedlings is correlated with the content of monosaccharides and oligosaccharides. In seeds of leguminous plants, oligosaccharides (primarily RFO: raffinose, stachyose, and verbascose) are mobilized during germination and then an increase in monosaccharides (glucose and fructose) content, cyclitols, and sucrose contents are observed [29]. A limited growth of seedlings under increasing DCF concentrations resulted in a slower decomposition of raffinose family oligosaccharides. In 9-day seedlings in the control soil raffinose and stachyose were not found, and their highest content was observed at the highest DCF concentration (12 mM) in soil. The research showed that metabolism of sugars is a good parameter of seedlings' growth, yet it does not indicate contamination and cannot be applied to assess contamination of the soil environment with medicine.

Toxic effect of diclofenac on animals has been proven in numerous tests. In mice after oral application, it was shown to create conjugation with taurine and hydroxylation followed by conjugation to taurine, glucuronic acid, or glucose [30]. In plants, diclofenac undergoes quick metabolism, already 3 hours after exposure of plants to the medicine, its metabolites are found in their tissues. Similarly to the processes in mammalian cells, diclofenac in plants is activated by creating hydroxylated metabolite 4'OH-diclofenac. This metabolite then creates glucopyranoside conjugations in plants [31]. Cytochromes are engaged in detoxificating human tissues from medicines. Among cytochromes, the cytochrome P450 constitutes a big isozyme family responsible for biotransformation of medicines through oxidation [32, 33]. Microsomal and mitochondrial enzymes of the cytochrome P450 play a really important role in regulating the intensity and time of medicine activity [34]. Moreover, the hydroxylated derivatives of medicines especially strongly hinder cytochromes activity [35] when diclofenac enters plants as it is almost immediately hydroxylated [31]. In this research, one of the enzymes of cytochrome system in plants was analyzed. It was cytochrome c oxidase (the terminal oxidase of the respiratory chain), which can play a role in detoxificating medicines. CcO receives an electron from each of four cytochrome c molecules and transfers them to one oxygen molecule, converting molecular oxygen to two molecules of water. It is a basic chemical process in cell respiration [36]. In mitochondria of seedling roots of the three analyzed plants the activity of CcO was lowered. At the same time, there appeared an increase in the activity of CcO in the cytosol. The proportions of activity of CcO present in root cells of lupin, pea, and lentil seedlings growing in soil contaminated with sulfamethazine and diclofenac differed. When concentrations of sulfamethazine were growing, the activity of CcO was also growing very fast (up to 80%) in the cytosol of root cells of these plants. The increase was observed form 0.25 mM sulfamethazine, e.g. in lupin roots [19].

Conclusions

1. The different proportion of CcO activity in the mitochondrion and cytosol was observed in the analyzed plants when soil was contaminated with diclofenac.
2. The lowest concentrations of diclofenac (0.06 mM) caused an increase in activity of cytochrome c oxidase in the cytosol of the plants.
3. Diclofenac is definitely less phytotoxic towards leguminous plants (lupin, pea, and lentil) than sulfamethazine.

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