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ISOLATION AND CHARACTERIZATION OF SOIL MICROORGANISMS DEGRADING THE HERBICIDE ISOXAFLUTOL

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Abstract

Chemical weed control is one of the most distributed weed management methods around the globe. As herbicide-resistant weeds often develop, dosage increases are required, leading to environmental pollution. An effective environmental strategy for the degradation of herbicides is to detect and apply microorganisms capable of breaking down and transforming the herbicides. The study aims to determine the degradation of the herbicide Merlin Flexx SC 480 (240 g/l isoxaflutole + 240 g/l cyprosulfamide - antidote) by the soil microorganisms. The current research was conducted with microbial communities from the maize rhizosphere that are resistant to isoxazoles. The soil was treated with two rates of isoxaflutole - 420 and 840 ml ha^{-1}. The biodegradation of isoxaflutole to diketonitrile and benzoic acid derivative was measured by HPLC. The main representatives of the microflora were bacteria, mold fungi, and nitrogen-fixing bacteria to a greater extent. A decrease in the number of bacteria and an increase in the number of mold fungi in the treated soils was found. The number of nitrogen-fixing bacteria increased by increasing the amount of Merlin Flexx in the soil.

Key words*: herbicide, herbicide degradation, soil microflora.*

INTRODUCTION

The most limiting factor in agricultural production is the weeds. They can decrease the maize yield, for instance, from 24% to 96.7% (Mukherjee and Debnath, 2013; Zhalnov and Raikov, 1996).In agriculture, the weed control is based mainly on chemical control (Tityanov, 2016; Tonev et al., 2007). The active substance izoxaflutol is izoxazole herbicide, which is applied for control of grass and broadleaf weeds in maize (Taylor-Lovell et al., 2002; Tonev et al., 2002).

The application of the herbicide Merlin Duo (37.5 g/l isoxaflutole + 375 g/l terbuthylazine) showed different efficacy against the weeds in comparison to the application rate. The most difficult-to-control annual broadleaf weed in the experiment was *Xanthium strumarium* L. (Mitkov et al., 2018). Very good efficacy against the weeds was found (94.6%) after the application of Merlin Flexx (240 g/l izoxaflutol + 240 g/l cyprosulfamide) in a rate of 42 ml/da (Dimitrova et al., 2013).

The herbicide izoxaflutol inhibits hydroxy-phenylpyruvate dioxygenase, an enzyme that is involved in the carotenoids biosynthesis, as by this way supress the weeds growth and development.

In soil, the izoxaflutol is hydrolysed diketonitrile derivative, which is the active form of the herbicide and benzoic acid analogue (Rice et al., 2004; Spundjueva et al. 2001).

There is a strong relationship between the contamination of pesticides and their residual detection and accept they have toxic for humans, there is a high risk of contamination in ecosystems (Veiga et al., 2006; Calderbank, 1989). Biodegradation is widely used for the treatment of xenobiotics in soil. It is employed in many countries because it is being ecofriendly (Enrica, 1994; Ritmann et al., 1988).

Although a number of techniques are available for biodegradation: (i) Bacterial degradation; (ii) Fungal degradation; (iii) Enzymatic degradation (Javaid et al., 2016).

Therefore, it is necessary to evaluate the effect of persistent soil herbicides and their degradation products by rhizospheric and soil microorganisms, to study the biochemical pathways for their degradation. It is also necessary resistant microorganisms to be isolated and a study of their biochemical pathways for their transformation to be conducted.

The aim of this study is to investigate the degradation of the herbicide Merlin Flexx SC containing the active substance isoxaflutol by soil microorganisms.

MATERIALS AND METHODS

Herbicide application

The experiment was conducted in two consecutive years (2016 and 2017) on the experimental field of the base for training and implementation of the Agricultural University of Plovdiv, Bulgaria. On the area where the trial was performed, maize was grown in the two years. The herbicide product Merlin Flexx SC 480 (240 g/l isoxaflutole + 240 g/l cyprosulfamide - antidote) was applied with a sprayer for plot experiments with a spraying solution volume 300 l/ha. Treatments of the experiment were: 1) Untreated control; 2) Merlin Flexx SC 480-420 ml/ha; 3) Merlin Flexx SC 480-840 ml/ha. The size of each experimental plot was 30 m^2.

Soil samples

The soil of the experimental field is with the following agrochemical properties in the layer of 0-30 cm depth: pH (H_2O) - 8.10; NH_4 N - 19.30 mg/1000 g; NO_3 N - 16.60 mg/1000 g; P_2O_5 - 32.75 mg/100 g; K_2O - 31.80 mg/100 g.
An average soil sample from the upper soil layer in depth of 5-15 cm was taken from all treatments. For greater accuracy of the experiment the average sample is obtained by taking soil by the diagonal of each plot.

Toxicity test

In 2016, in the scientific laboratory of the department of "Microbiology and ecological biotechnologies" at the Agricultural University of Plovdiv a pot trial with test crop lettuce (*Lactica sativa* L.) was conducted. This plant is very sensitive to the active substance izoxaflutol. The used pots were with 8 cm of diameter and were filled with soil taken from the experimental area. Treatment 1 was with soil taken from the untreated plots. Treatments 2 and 3 were with soil taken from the treated plots - Merlin Flexx SC 480-420 and 840 ml/ha respectively.
The pot trial was conducted with two lettuce varieties: "Cherna giumiurdjinska" (*L. sativa*) and "Lollo Rosso" (*L. sativa* var. *roso*). In ech pot 25 lettuce seeds were seeded. Each treatment was replicated three times.

Soil respiration

Fifty grams of air dry soil was weighted; it was previously sifted through a 2 mm sieve. The soil sample was placed in glass jar with a lid. At the bottom of the glassjar a Beakerglass with 20 ml 0.05 M KOH was placed. The soil was incubated for 6 hours at 25-27ºC temperature. After the six hours, the soil was taken out and was immediately treated with 5 ml 0.05 M $BaCl_2$ for the sedimentation of carbamates. Glucose for inducing is added. A titration with 0.1% HCl with the phenolphthalein indicator till obtaining white coloration was performed. As a control a sample with KOH only was used.

Soil pH and EC (electroconductivity)

Five gram soil sample is placed in 25 ml of distilled water (dilution 1:5) ad was well shaken for 10 minutes. It was followed by 120 minutes of rest for sedimentation of the soil particles. The sample was decanted and pH with pH meter was measured. The extract was also used to measure the soil EC.

Determination of β-glucosidase activity

One g soil sample was placed in 50 ml flask, 0.25 ml toluene, 4 ml buffer (21.1 g Tris, 11.6 g maleic acid, 14 g citric acid, 6.3 g boric acid, 500 ml 1 M NaOH and distilled water to 1 liter, as well as 1 ml p-nitrophenol-β-D-glucoside were added. It was intensively shaken for 1 hour at 37ºC on a water bath. After that $CaCl_2$, tris buffer with pH 12 was added and the solution was filtered. The color reaction was analyzed spectrophotometrically (400 nm).

Isolation of resistant microorganisms

The influence of Merlin Flexx SC 480 on different groups of microorganisms was evaluated. Important groups of microorganisms resistant to izoxaflutol were isolated.
For this purpose, 1 g soil sample is placed in 99 ml of sterile water (dilution 1:100) in flask. The solution is shaken for 10 minutes and is placed at resting for sedimentation of the soil particles. From the solution the other 3 dilutions by adding 1 ml of soil suspension to 9 ml sterile water (dilutions 10^3, 10^4, and 10^5). Petri dishes with different nutrient media were prepared beforehand. The nutrient medias are on Table 1.

Table 1. Chemical composition of the media used

Nutrient media	Content
Mineral nutrient media	K_2HPO_4 - 0.8 g/l, KH_2PO_4 - 0.2 g/l, $MgSO_4.7H_2O$ - 0.2 g/l, $CaSO_4$ - 0.1 g/l, $(NH_4)_6MoO_{24}.4H_2O$ - 0.001 g/l, $(NH)_4SO_4$ - 5 g, agar - 20 g/l
LB (Laura-Bertani)	Triptone - 10 g/l, yeast extract - 5 g/l, NaCl - 5 g/l, agar - 20 g/l, pH 7.0
Chapek	$NaNO_3$ - 3.0 g/l, KH_2PO_4-1.0 g/l, $MgSO_4$ - 0.5 g/l, KCl - 0.5 g/l, $FeSO_4$ - 0.01 g/l, sucrose 30 g/l, agar 20.0 g/l, pH 4.5-5
TSA	Caseinpeptone - 17.0 g/l, soypeptone - 3.0 g/l, NaCl - 5.0 g/l, K_2HPO_4 - 2.5 g/l, dextrose - 2.5 g/l, agar - 20 g/l
Nutrient media for molds with rosebengal	TSB - 6.8 g/l, yeast extract - 0.8 g/l, glucose - 9.4 g/l, $MgSO_4$ - 0.5 g/l, rose Bengal - 0.05 g/l, agar - 24 g/l
Yeast agar	Yeast extract - 3.0 g/l, malt extract - 3.0 g/l, peptone - 5.0 g/l, glucose - 10.0 g/l, agar - 20.0 g/l
Nutrientmediaf ornitrogen-fixingbacteria	Glucose - 5.0 g/l, mannitol - 5.0 g/l, $CaCl_2{\cdot}2H_2O$ - 0.1 g/l, $MgSO_4{\cdot}7H_2O$ - 0.1 g/l, Na_2MoO_4 - 0 5 mg/l, KH_2PO_4 -0.1 g/l, $FeSO_4{\cdot}7H_2O$ - 0.01 g/l, $CaCO_3$ - 5.0 g/l, agar - 20 g/l
Water agar	Agar 20 g was added per liter of distilled water
Nutri Agar	Meat extract - 1.0 g/l, yeast extract - 2.0 g/l, peptone - 5.0 g/l, agar - 15.0 g/l, pH - 7.4-7.5

Universal and selective nutrient media were used in order to isolate microorganisms from different groups that are resistant to izoxaflutol. For resistant microorganisms isolation, izoxaflutol (Merlin Flexx SC 480) was added to the nutrient media (mineral nutrient media, water agar and rose Bengal media) in concentrations of 0, 15, 75, 150 µg/L.

RESULTS AND DISCUSSIONS

Toxicity test

There are several methods for testing the toxicity of herbicides to test crops. Seed germination has been reported with acute herbicide toxicity. The reaction of the test plants to the two evaluated izoxaflutol rates was evaluated. The results are presented on Figure 1. The results of the pot trial showed that the rate of 420 ml/ha of izoxaflutol suppressed the growth and development of the test crop. The doubled rate of 840 ml/ha stopped the growth of the lettuce completely. The toxic effect was more severe for the plants of the "Lollo Rosso" lettuce variety than "Cherna giumiurdjinska" variety (Pictures 1 and 2).

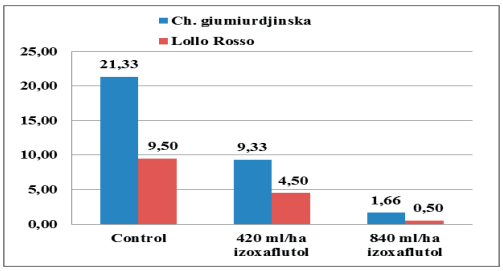
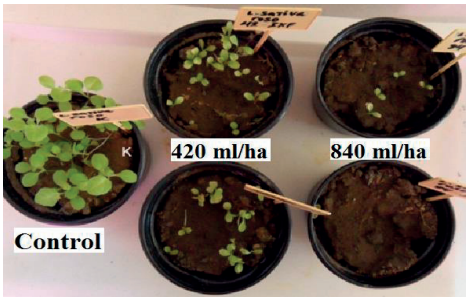
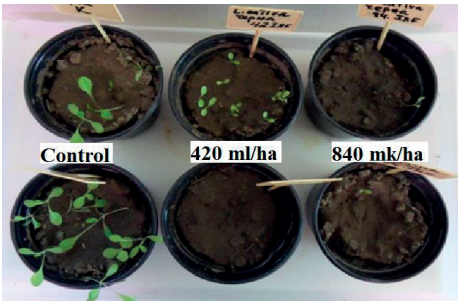


Figure 1. Test for toxicity - number of germinated lettuce plants of the two studied varieties



Picture 1. Test plants of "Lollo Rosso" lettuce variety



Picture 2. Test plants of "Cherna giumiurdjinska" lettuce variety

Soil respiration

To determine the influence of the herbicide rate on soil microorganisms, quantitative and functional characteristics by usage of soil respiration method were performed. Soil respiration indicates the current state of the soil by determining the amount of CO_2 released and indirectly serves to determine microbial biomass. The microorganisms present may not be active at this time, so the induction of microbial activity by glucose supplementation is used. The experiment was conducted in two stages - initial (immediately after field sampling) and a second period (after 6 months of soil storage). The obtained results indicated

that in the initial stage without induction, the total biological activity of the treated soils is higher than that of the control (Figure 2A). Glucose induction decreased the amount of CO_2 released from the treated soils (Figure 2 B). Differences in CO_2 emissions before and after induction are significant in untreated soil (Figure 2 C). In the second stage, the analyses performed with and without induction showed that the soils treated with Merlin Flexx SC 480 in both examined rates increased the biomass values twice as compared to the control (Figure 2 B). On Figure 2 D the difference in reported total biological activity reported at 180 days from sampling is presented. In the absence of glucose, there is a decrease and, after induction, an increase in the amount of microbial biomass is observed. On Figure 3 the differences in the biomass changes reported in the periods are presented. The amount of individual CO_2 is higher at the higher dose of the herbicide (840 ml/ha) compared to twice lower rate (420 ml/ha). This result proves that the microorganisms are resistant to izoxaflutol.

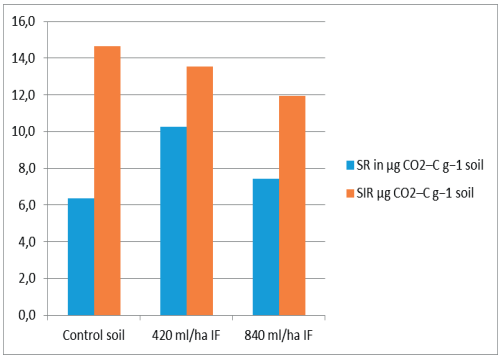


Figure 2A. CO_2 released from the treated soils without induction

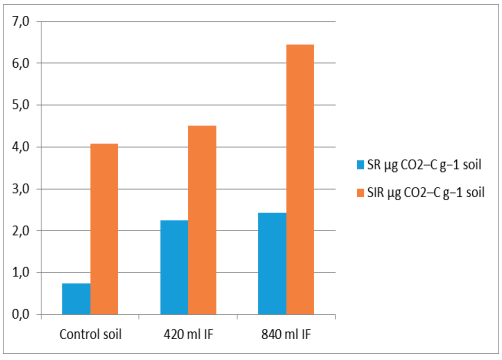


Figure 2B. Biomass increase after induction

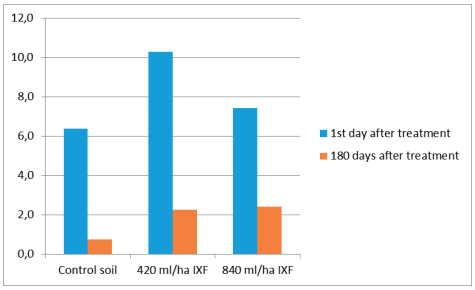


Figure 2C. Differences in CO_2 emissions before and after induction

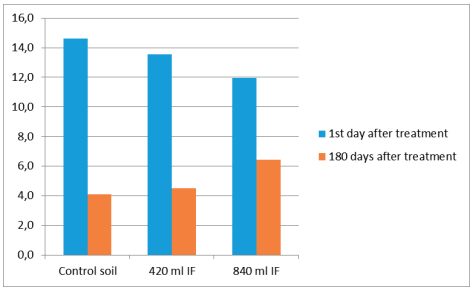


Figure 2D. Total biological activity

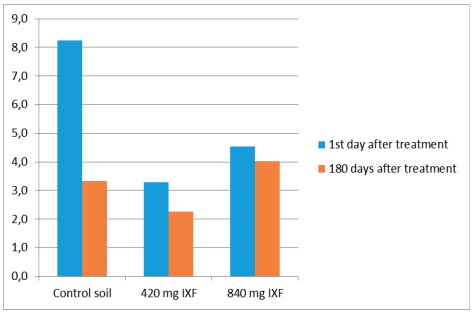


Figure 3. Differences in the biomass changes

Determination of β-glucosidase activity

The obtained data showed that the higher herbicide rate had simulative effect on the development and activity of the microorganisms (Figure 4).

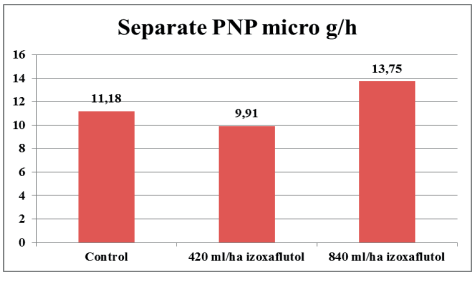


Figure 4. β-glucosidase activity

Soil pH and EC

The soil EC of treatment 2 (420 ml/ha izoxaflutol) was 103 mS/cm. For the doubled rate (840 ml/ha izoxaflutol) the EC values were 97 mS/cm. The data concerning the soil pH showed no considerable differences and varied from 8.23 to 8.32 (Table 2).

Table 2. Soil EC and pH

Soil samples	EC mS/cm	pH (H_2O)
Control	78	8.30
420 ml/ha izoxaflutol	103	8.32
840 ml/ha izoxaflutol	97	8.23

The obtained data regarding the biogenicity of the three evaluated soil samples showed that the maincomponents of the microfloraare, to a lesserextent, bacteria, molds, and to a much greater extent nitrogen-fixing bacteria. On Figure 5 it is shown that there is decrease of the quantity of bacteria and increased quantity of molds in the treated soils.

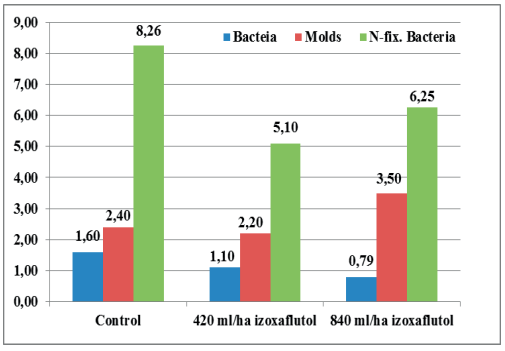


Figure 5. Number of colonies of bacteria, molds and nitrogen-fixing bacteria after treatments with izoxaflutol

The dynamics of the nitrogen-fixing bacteria are consistent with the results obtained from the total biological activity and β-glucosidase activity. It is worth to notice that the quantity of the nitrogen-fixing bacteria was increased with the increase of the izoxaflutol rate.

Isolation of resistant microorganisms

Theinfluence of the herbicide on the growth and development of different groups of microorganisms by cultivation on three different nutrient media was studied (Table 1) in the presence of increasing concentration of isoxiflutolfrom 0, 15, 75 to 150 µg/l.

The data obtained that is shown on Figure 6 were processed by three replicates and showed that as the concentration of isoxyflutol in the nutrient media increased, the number of soil microorganisms was decreased. The nutrient media with rose Bengal is used to cultivate molds. In the control soil, the quantity of the molds varies between 18.5 x 10^5.

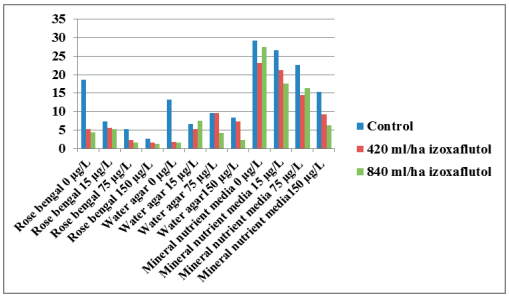


Figure 6. Total number of colonies forming unitsof microorganisms, isolated from the evaluated soils I three different medias

In the soil samples after treatment with 2 izoxaflutol rates (420 and 840 ml/ha) the number of microorganisms decreases with increasing dose of the herbicide added to the nutrient media.

Mold fungi developed on water agar in the presence of isoxyflutol, and bacterial number decreased. In the water agar the herbicide was used by the microorganisms as a carbon source. It was found that with the increase of the izoxaflutol rate in the nutrient media the total number of microorganisms was decreased.

CONCLUSIONS

The rate of 420 ml/ha suppressed the development of the test crop, and the doubled herbicide rate of 840 ml/da stopped the growth and development of the test culture.

The reported quantity of released CO_2 was higher at the higher izoxaflutol rate (840 ml/ha) in comparison to the lower rate (420 ml/ha). This result shows that the microorganisms are resistant to the studied herbicide.

In treated with izoxaflutol soils the quantity of the bacteria was decreased, and the quantity of the molds was increased.

The quantity of nitrogen-fixing bacteria was increased with the increasing the izoxaflutol rate.

With the increase of izoxaflutol rate in the nutrient media the total number of microorganisms decreased.

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