Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *79* (9) 1075–1084 (2014)
JSCS–4648
UDC 632.954+547.544+631.4:577.15–188
Original scientific paper

The effect of chlorsulfuron and MCPB-Na on the enzymatic activity of microorganisms

MARIOARA NICOLETA FILIMON[1,2], SORIN OCTAVIAN VOIA[3]*, ROXANA POPESCU[4], DESPINA-MARIA BORDEAN[5], DIANA LARISA VLADOIU[1,2], MIHAI MITULETU[1,2] and VASILE OSTAFE[1,2]

[1]West University of Timişoara, Faculty of Chemistry-Biology-Geography, Department of Biology-Chemistry, Pestalozzi, 16, Timisoara, 300115, Romania, [2]West University of Timisoara, Laboratory of Advanced Research in Environmental Protection, Oituz 4, Timisoara 300086, Romania, [3]Banat University of Agricultural Sciences and Veterinary Medicine, Faculty of Animal Science and Biotechnology, Calea Aradului, 119, Timisoara, 300645, Romania, [4]University of Medicine and Pharmacy "Victor Babes", Department of Cellular and Molecular Biology, E. Murgu, 2, Timisoara, 300041, Romania and [5]Banat University of Agricultural Sciences and Veterinary Medicine from Timisoara, Faculty of Food Processing and Technology, Calea Aradului, 119, Timisoara, 300645, Romania

(Received 15 January, revised 25 March, accepted 27 March 2014)

Abstract: Sulphonylureic herbicides have a broad spectrum effect on weeds in relatively low doses and with a much reduced toxicity to livestock. In this study, two herbicides: dacsulfuron with the active substance chlorsulfuron (0.005–0.035 μg g^{-1} soil) and butoxone with the active substance MCPB-Na (0.005–0.035 mg L^{-1} g^{-1} soil) were investigated. The samples were collected from a depth of 0–20 cm from chernozem soil. The effects of the herbicides were estimated by measuring the activities of catalase, actual and potential dehydrogenase, urease and cellulase. All samples were incubated for 10 days at 27 °C using Stapp medium for the isolation and study of cellulosolytic bacteria. The inhibitory effect of the tested herbicides was the most intense on the enzymatic activities of urease and dehydrogenase. The most resistant cellulosolytic bacteria to the effects of dacsulfuron were *Cellfalcicula fusca*, *C. viridis*, *Cellvibrio fulvus* and *Cellfalcicula* sp., and for butoxone *C. mucosa*, *C. viridis* and *C. fulvus*.

Keywords: herbicides; soil; cellulosolytic bacteria.

INTRODUCTION

Soil microorganisms play an important role on soil processes, influencing soil structure, plant cultivation, medium resources and soil quality.[1] Soil structure and stability are strictly related to the presence and activity of microorganisms.

* Corresponding author. E-mail: voia@animalsci-tm.ro
doi: 10.2298/JSC140115031F

Microbial activity in soil, being susceptible to xenobiotics, can be a useful tool to assess soil quality.[2] Xenobiotics can have both direct and indirect effects upon the enzymatic activities in soil. Enzymes such as hydrolases (invertase, protease, phosphatase and urease) and oxidases (dehydrogenase, catalase and peroxidase) can be used as sensitive bioindicators of soil pollution.[3,4] Enzymatic activities of soil microorganisms are of major interest in assessing the effects of herbicides on the quality of soil. Studies have shown that dehydrogenase activity may represent an important indicator of the secondary effects associated with the administration of sulphonylureic herbicides.[5–7] Depending on the employed type of herbicide, other enzymatic activities present in soils undergo quantitative and qualitative variations. Thus, urease, amylase and protease activities are inhibited by some sulphonylureic herbicides. Herbicides from the glyphosate group inhibit enzymatic activities in soil with increasing herbicide dose.[8,9]

Herbicide degradation in soil is due to intra- and extracellular enzymes produced by soil microorganisms, especially bacteria, but also fungi. Most studies on herbicide degradation were conducted on bacteria.[10,11] However, the role of fungi in herbicide degradation must also be considered.[12–14]

Herbicide impact on microorganism communities in soil, especially their metabolic activity, depends on various factors, such as: soil structure and texture,[15,16] physical and chemical factors (pH, temperature, humidity and organic matter content), intensity and activity spectrum of the herbicide and herbicide persistence in the soil.[16–18]

Chlorophenoxy derivatives, most commonly γ-phenoxybutyratic acids, are selective herbicides used against broadleaf weeds. The application of herbicides to destroy weeds in crops has advantageous effects on agricultural production but negatively influences soil microorganism growth,[19,20] population dynamics[19–21] and metabolic activity.[15,17,20]

The detection of bacterial ecophysiological groups is useful for identifying structural changes that occur in soil due to xenobiotic substances. Cellulosolytic bacteria are a group with a very important role due to the fact that cellulose is present in soil in large amounts, being the main component of plant organic matter. The isolation and identification of cellulosolytic bacteria and the effect of different xenobiotics on these bacterial species led to different studies.[6,22]

Sulphonylureic herbicides are a group of herbicides frequently used to destroy weeds in crops; their large scale administration is due to the need for relatively low doses, high efficiency and low toxicity for small mammals.[23]

The present study aimed at establishing the effects of the two herbicides on soil quality, based on the fact that soil enzymes could be considered as early indicators in soil quality change in the context of land management. The present study brings an important contribution in establishing the effects of anthropo-

genic influences (the use of herbicides) on soil quality, and their relation to enzymatic activity and their potentials.

EXPERIMENTAL

Materials

Chlorsufuron (CAS 79793-81-0-64902-72-3) also known as 1-(2-chlorophenylsulphonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea, may also be found in commercial herbicides under the trade name Dacsulfuron 750SP (Strand Group Holdings Ltd.). MCPB-Na also known as sodium 4-(4-chloro-2-methylphenoxy)butanoate (CAS 6062-26-6), may also be found in commercial herbicides under the trade name Butoxone M40 (Nufarm Ltd., UK). Microorganisms were isolated from chernozem soil samples collected from a depth of 0-20 cm, in the spring, before sowing and fertilization. The collected soil samples were treated with chlorsulfuron and MCPB-Na and analyzed in the Laboratory of Advanced Research in Environmental Protection.

Soil treatment with herbicides

The soil was sieved through a 2 mm sieve and placed in polyethylene bags in order to ensure soil moisture. The conversion rate: pesticides g^{-1} of soil applied in the field was calculated according to a uniform distribution of herbicides in the soil.[24]

An untreated sample was preserved as a control sample, while the experimental samples were treated with increasing doses of herbicide. The following experimental variants were obtained after applying the herbicides: normal doses (ND, 0.2 µg chlorsulfuron, 0.2 mg MCPB-Na), 2 times the normal doses (2×ND, 0.4 µg chlorsulfuron, 0.4 mg MCPB-Na), 5 times the normal doses (5×ND, 1 µg chlorsulfuron, 1.0 mg MCPB-Na) and 7 times the normal doses (7×ND, 1.4 µg chlorsulfuron, 1.4 mg MCPB-Na). The samples were incubated for 7 days at 24 °C.

The enzymatic activity

The experimental variants were considered for comparative studies of the activities of enzymes. The following enzymatic activities were chosen for assay: dehydrogenase (DA, EC 1.1.1.1), urease (EC 3.5.1.5), catalase (CA, EC 1.11.1.6) and cellulase (Cel A, EC 3.2.1.91). The enzymatic activity was determined using a T90 UV–Vis spectrophotometer (PG Instruments, UK).

The actual dehydrogenase activity (*ADA*) was measured using 2,3,5-triphenyltetrazoliumchloride (TTC), incubating soil samples (5 g) mixed with distilled water Tris-buffer (2 mL, 1 M, pH 7.6) at 37 °C for 48 h. To determine the potential dehydrogenase activity (*PDA*), glucose was added to the reaction mix. The formed triphenyl formazan was extracted with acetone and the absorbance of the supernatants measured at 485 nm. The activity of dehydrogenase was expressed as mg triphenyl formazan g^{-1} soil.[25]

The urease activity was determined in accordance with the method described by Alef and Nannpieri.[26] Reaction mixtures consisting of 3 g soil, 2 mL toluene, 5 mL phosphate buffer (0.6 M, pH 6.8) and 5 mL of a 3 % urea solution were incubated at 37 °C for 24 h. The absorbance was measured at 445 nm. The activity was expressed as mg NH_4 g^{-1} soil.

The catalase activity was determined using the permanganometric method described by Dragan-Bularda.[27] The reaction mixtures consisted of 3 g soil, 2 mL H_2O_2, 10 ml of a 3 %, phosphate buffer solution (0.4 M, pH 6.7). After 1 h incubation at 37 °C, the catalase activity was recorded as mg H_2O_2 decomposed by 1 g of soil in 1 h.

The cellulase activity (Cel A, EC 3.2.1.91) was determined by assessing the amount of cellulose consumed through decomposition.[28] On the basis of the difference between the initial and the final quantity of decomposed cellulose, specifically related to the amount of analyzed soil sample. All the assays of the enzymes activities were performed in triplicate, in a controlled laboratory environment, by the same researcher during the same day.

Isolation and identification of cellulosolytic microorganisms was made from 10^{-3} dilution soil experimental variants inoculated on a solid growth medium (Stapp medium). The inoculated Petri dishes were incubated for 10 days at 27 °C. After the incubation, the main cellulosolytic bacterial species were identified based on the specificity of the substrate degradation and the morphologic aspects of the colonies.

Statistical data interpretation

The data were analyzed using analysis of variance (ANOVA) of simple correlations as 2nd degree polynomial regression equations. The software MINITAB 14 was employed.[29] All data are presented as mean values with standard deviation ($X \pm SD$). Significant differences in variables were tested using the *F*-test at the 0.05 level of probability. Cluster analysis was performed using the Past Statistical Program, version 2.12, employing the Algorithm Single Linkage and Bray-Curtis Indices[30] were used as a similarity measure.

Cluster analysis divides the data into groups (clusters) that have an important similar effect. The clusters confine the meaningful groups and present the natural structure of the data as well as data summarization. "The greater the similarity within a group and the greater the difference between groups, the better or more distinct is the clustering".[31] The use of cluster analysis in microbiology and molecular microbial ecology was mentioned by de Bruijn in 2011[32] as a possible method to analyze microbiological data.

The distance between two clusters using the algorithm single linkage clustering, is defined as the distance between the closest members of the two groups.[33] Bray-Curtis is a popular similarity index for abundance data:

$$y_{jk} = 1 - \frac{\sum_i \left| x_{ji} - x_{ki} \right|}{\sum_i \left(x_{ji} + x_{ki} \right)}$$

Similarly, y_{ik} is the count for the i^{th} species in the k^{th} sample, representing the entry in the i^{th} row and j^{th} column of the data matrix, *i.e.*, the abundance for the i^{th} species in the j^{th} sample ($i = 1, 2, \ldots, p$; $j = 1, 2, \ldots, n$).[34]

RESULTS AND DISCUSSION

The statistical analyses of the values of the enzymatic activities from the soil samples treated with herbicides are presented in Table I.

The values of the catalase activity measured in the soil samples decreased with increasing herbicide dosage: 3.13 % decrease when dacsulfuron was applied and 9.24 % on application of butoxone. From a statistical point of view, the observed decrease in the experimental variants was significant, the *p* value being < 0.05. Thus, the catalase activity was more negatively influenced by butoxone than by dacsulfuron, probably due to the effect of other non-enzymatic catalysts on the catalase activity in soil.

The dehydrogenase activity reflects the respiratory processes in the soil, which are directly proportional to the number of microorganisms in the soil; the greater the number of microorganisms in the soil, the higher is the intensity of the dehydrogenase activity. Thus, analysing the effect of dacsulfuron in the experimental samples, a decrease of 39.82 % for the actual dehydrogenase activity and a 41.12 % decrease in the potential dehydrogenase activity were observed. The treatment with the herbicide butoxone induced a 51.37 % decrease in the actual dehydrogenase activity and a 25.44 % decrease in the potential dehydrogenase activity. Therefore, butoxone shown a stronger effect based on the fact that the potential dehydrogenase activity was significantly lower compared to the dehydrogenase activities of corresponding untreated (batch) and dacsulfuron-treated samples. Similar results, with decreased dehydrogenase activity of up to 25 or 50 % in soils treated with herbicides (metsulfuron-methyl and 2,4-D, glyphosate) were obtained in previous studies, such as those of Araújo *et al*. and Zabaloy *et al*.[5,7]

TABLE I. Enzymatic activity values in the analyzed soil samples; statistic analysis: *X*±*SD*; D – dacsulfuron; B – butoxone; ND – normal dose; 2×ND – 2 times normal dose; 5×ND – 5 times normal dose; 7×ND – 7 times normal dose; *X* – mean value; *SD* – standard deviation; *SEM* – standard error of mean; * – the mean difference is significant at the 0.05 level between the batch and the experimental variants

Exp. variants	CA[a]	ADA[b]	PDA[c]	UA[d]	Cel A[e]
Batch	3.485±0.085	1.421±0.019	0.800±0.013	2.660±0.010	0.091±0.001
D ND	3.126*±0.143	1.371±0.019	0.696*±0.019	1.132*±0.011	0.071*±0.001
D 2×ND	3.094*±0.093	1.238*±0.013	0.625*±0.013	0.945*±0.012	0.062*±0.003
D 5×ND	3.082*±0.128	1.054*±0.059	0.563*±0.013	0.720*±0.011	0.004*±0.001
D 7×ND	3.028*±0.128	0.825*±0.012	0.375*±0.013	0.218*±0.007	0.001*±0.000
SEM	0.0508	0.0588	0.0381	0.2196	0.0062
B ND	3.094*±0.131	0.763*±0.013	0.621*±0.019	1.143*±0.004	0.005*±0.000
B 2×ND	3.060*±0.085	0.643*±0.006	0.587*±0.013	0.987*±0.002	0.003*±0.000
B 5×ND	3.021*±0.045	0.446*±0.019	0.500*±0.025	0.966*±0.003	0.002*±0.000
B 7×ND	2.808*±0.085	0.371*±0.019	0.463*±0.013	0.760*±0.003	0.000*±0.000
SEM	0.0619	0.0890	0.0295	0.1842	0.0021

[a]Catalase activity (mg H_2O_2 not decomposed per g soil); [b]actual dehydrogenase activity (mg triphenyl formazan g^{-1} soil); [c]potential dehydrogenase activity (mg triphenyl formazan g^{-1} soil); [d]urease activity (mg NH_4 g^{-1} soil); [e]cellulase activity

Dehydrogenase activity was proved to be an important indicator of the secondary effects following the administration of the two herbicides. Similar studies on the influence of herbicides on dehydrogenase activity of soil microorganisms also revealed that herbicides cause a decrease in the activity of this enzyme.[5–7]

The urease activity registered significantly lower values after application of the herbicides compared to the untreated sample, showing that this enzymatic activity seems to be the most sensitive to the herbicides. Experimental values,

showed a 80.74 % decrease in the urease activity in soil samples treated with 7×normal dose (7×ND, 1.4 μg chlorsulfuron) of dacsulfuron, compared to the soil sample treated with the normal dose (ND, 0.2 μg chlorsulfuron) of dacsulfuron. The experimental variants treated with butoxone presented a of 33.50 % decrease in the urease activity compared to the control sample.

For both herbicides, a decrease in urease activity with increasing herbicide dose was observed, the effect of dacsulfuron being stronger than that of butoxone. Similar studies on the influence of herbicides on the enzymatic activities of soil microorganisms confirmed the effect of herbicides (glyphosate, gluphosinate and sulphonylureic) on urease activity.[8,9,35]

The cellulase activity decreased in the experimental samples with the increased dose of the applied herbicides. In the case of butoxone, for the (7×ND, 1.4 mg MCPB-Na) experimental sample, the cellulase activity could not be measured on the experimental level. Significantly reduced cellulase activities were also identified in other studies which analyzed the effects of herbicides on microorganism communities in soil.[6,22,36]

The sensitivity of different enzymatic activities to dacsulfuron and butoxone decreased in the following order: urease activity > potential dehydrogenase activity > actual dehydrogenase activity > catalase activity > cellulase activity.

The correlations between the cellulase activity and the number of cellulosolytic bacterial colony forming units showed a positive correlation (r = +0.809) for the herbicide dacsulfuron and a negative correlation (r = –0.838) for the herbicide butoxone. Therefore, it could be argued that the number of cellulosolytic bacteria is less important than the bacterial species present and their enzymatic capacity.

In the control samples, the following species of cellulolytic bacteria were identified: *Cellfalcicula fusca*, *C. mucosa*, *C. viridis*, *Cellvibrio fulvus*, *Cellvibrio ochreceus*, *Cytophaga aurantica* and *Sporocytophaga congregata*. In the soil experimental variants qualitative and quantitative variations of cellulolytic bacteria species were recorded. *Cellfalcicula fusca* was well represented in soils treated with dacsulfuron compared to those treated with butoxone, while *C. mucosa* showed the opposite distribution. *C. viridis* was found in soils treated with dacsulfuron and absent in those treated with butoxone. *Cellvibrio fulvus* showed large quantitative variations in the experimental variants compared to the control sample, which demonstrated its increased sensitivity to the action of herbicides. Toxicity of dacsulfuron was manifested on *Cytophaga* sp., as shown by its absence in the experimental variants treated with dacsulfuron (Fig. 1).

Cluster analysis showed, in the soil samples treated with increasing doses of chlorsulfuron, the species *Cellvibrio flavescens*, *Cellfalcicula fusca*, *C. mucosa*, *Cellulomonas pusilla*, *Cellfalcicula viridis* and *Cellvibrio fulvus* had a similar behaviour on exposure to dacsulfuron (0.329, Fig. 2). For the soil samples treated

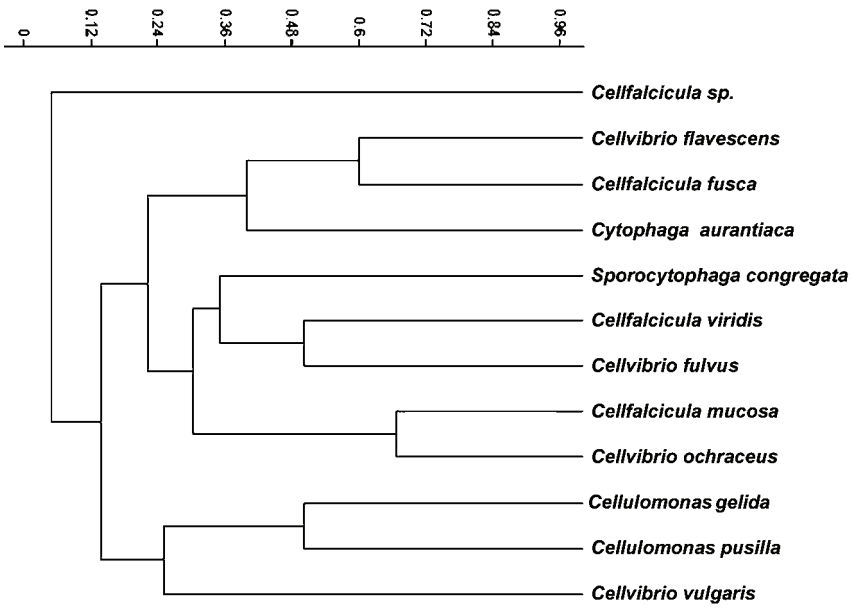

Fig. 1. Cluster analysis of cellulosolytic bacteria from soil samples treated with chlorsulfuron.

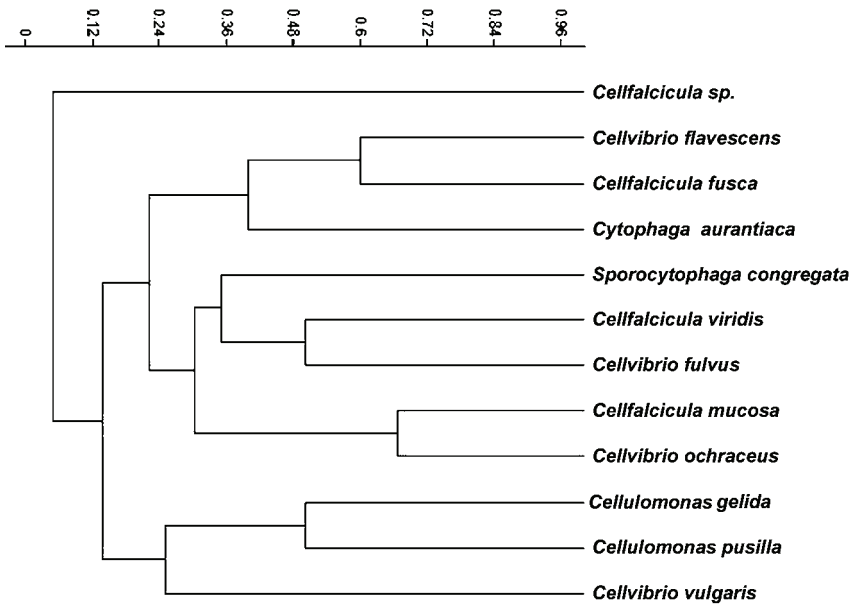

Fig. 2. Cluster analysis of cellulosolytic bacteria from soil samples treated with MCPB-Na.

with MCPB-Na, cluster analysis showed that the species *C. fulvus*, *Cytophaga aurantiaca*, *C. fusca* (0.365), *C. mucosa* and *Cellvibrio ochraceus* (0.259), as

well as *Cellfalcicula viridis* and *S. congregata* (0.517), also exhibited similar behaviour when exposed to the herbicide butoxone.

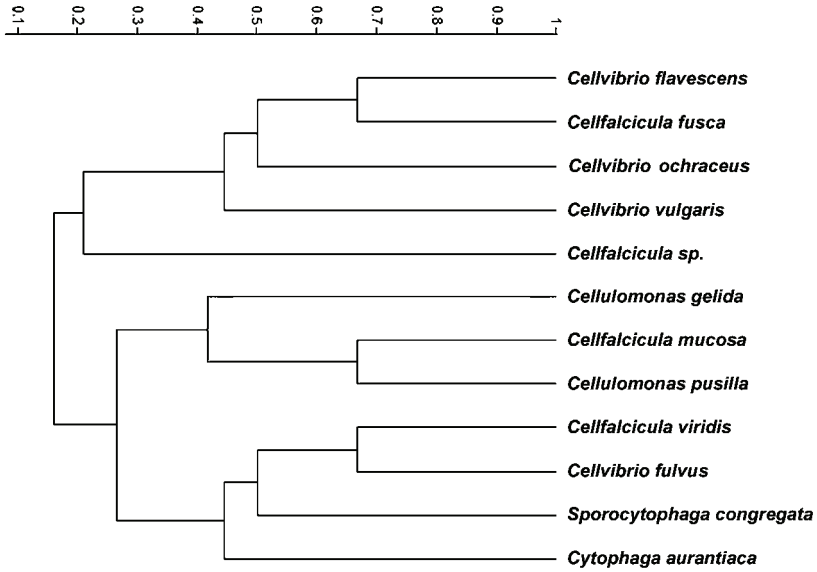

Fig. 3. Cluster analysis of cellulosolytic bacteria from soil samples treated with herbicides.

Based on cluster analysis, it was established that *C. flavescens*, *Cellfalcicula fusca* (0.400), *C. mucosa* and *Cellvibrio ochraceus* (0.345), *Cellfalcicula viridis* and *Cellvibrio fulvus* (0.513), *Cellulomonas pussila* and *Cellvibrio vulgaris* (0.533) presented a similar behaviour on exposure to dacsulfuron and butoxone (Fig. 3).

CONCLUSIONS

In conclusion, the use of microorganisms in monitoring programs is necessary because the changes in the structure of the microorganism communities may indicate changes in environment quality. In order to accurately identify the possible changes caused by the use of xenobiotics, many impact indicators must be considered – key microorganisms, quantitative and qualitative variations in microorganism groups and metabolic activities (enzymatic).

The urease and dehydrogenase enzymatic activities were the most sensitive to the action of chlorsulfuron and MCPB-Na. The inhibition of dehydrogenase activity indicates to the toxic effect of chlorsulfuron and MCPB-Na on the microorganism communities in soil.

The most resistant species to the effect of dacsulfuron were *Cellfalcicula fusca*, *C. viridis*, *Cellvibrio fulvus*, *Cellfalcicula* sp., *Cellfalcicula mucosa*, *C. viridis* and *Cellvibrio fulvus* to the action of butoxone.

ИЗВОД

ЕФЕКТИ ХЛОРСУЛФУРОНА И MCPB-Na НА ЕНЗИМСКУ АКТИВНОСТ МИКРООРГАНИЗАМА

MARIOARA NICOLETA FILIMON[1,2], SORIN OCTAVIAN VOIA[3], ROXANA POPESCU[4], DESPINA-MARIA BORDEAN[5], DIANA LARISA VLADOIU[1,2], MIHAI MITULETU[1,2] и VASILE OSTAFE[1,2]

[1]West University of Timişoara, Faculty of Chemistry-Biology-Geography, Department of Biology-Chemistry, Pestalozzi, 16, Timisoara, 300115, Romania, [2]West University of Timisoara, Laboratory of Advanced Research in Environmental Protection, Oituz 4, Timisoara 300086, Romania, [3]Banat University of Agricultural Sciences and Veterinary Medicine, Faculty of Animal Science and Biotechnology, Calea Aradului, 119, Timisoara, 300645, Romania, [4]University of Medicine and Pharmacy "Victor Babes", Departament of Cellular and Molecular Biology, E. Murgu, 2, Timisoara, 300041, Romania и [5]Banat University of Agricultural Sciences and Veterinary Medicine from Timisoara, Faculty of Food Processing & Technology Calea Aradului, 119, Timisoara, 300645, Romania

Хербициди имају широк спектар дејства у сузбијању корова у релативно ниским дозама, и показују смањену токсичност код животиња. У истраживању су примењени хербициди даксулфурон, са активном супстанцом хлорсулфурон (0,005–0,035 μg g^{-1} земљишта) и бутоксон, са активном супстанцом MCPB-Na (0,005–0,035 mg L^{-1} g^{-1} земљишта). Земљиште типа чернозем је узорковано до дубине од 20 cm. Ефекат хербицида је био оцењен преко анализе ензимске активности: каталитичке, тренутне и потенцијалне дехидрогеназне, уреазне и целулолитичне активности. За изоловање и развој бактерија целулолитика коришћена је подлога *Stapp*. Инкубација је трајала 10 дана на 27 °C. Инхибициони ефекат тестираних хербицида био је најинтензивнији у случају дехидрогеназне и уреазне ензимске активности. Најотпорније бактерије целулолитици на дејство даксулфурона биле се *Cellfalcicula fusca*, *C. viridis*, *Cellvibrio fulvus* и *Cellfalcicula* sp., а на деловање бутоксона *Cellfalcicula mucosa*, *C. viridis* и *Cellvibrio fulvus*.

(Примљено 15. јануара, ревидирано 25. марта, прихваћено 27. марта 2014)

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