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The effects of the „Stomp“ herbicide application on the microbial prevalence in the soil

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Abstract

Agricultural production has benefited a lot from herbicides; however, the use of herbicides caused many environmental problems. Herbicide application can affect the biodiversity of an ecosystem by killing non-target organisms. Microorganisms in the soil are important factors for plant growth; they represent the biological factor of soil fertility. Herbicides can have a beneficial effect on the development of some microorganisms and a negative on others, leading to depletion of microbial diversity in soil. The objective of this work is to determine microbial activity in the soil and to isolate herbicide-resistant bacteria after the use of the “Stomp” herbicide. Agar plate method was used for the determination of microbial prevalence in the soil. The results showed an increase in the total number of bacteria, ammonifiers, fungi, and actinomycetes. Nine isolates, mostly Gram-positive spore-forming rods, showed an ability to grow in the mineral salt medium with different concentrations of “Stomp” herbicide. Isolates G1/1 and G1/2, showed high level of tolerance at the initial pendimethalin concentration of 25 mg/l. Those isolates have the potential to be used to decontaminate herbicide affected ecosystems.

Key words: “Stomp” herbicide, microbial prevalence, pendimethalin, soil

Introduction

Herbicides are a broad group of agrochemicals that, regardless of benefits they have on plants, may cause environmental problems (Kanissery and Sims, 2011). During the last several decades, herbicides are intensively rinsed through agricultural soils which caused contamination of the surface and subsurface water (Graymore et al., 2001).

Herbicide that widely used in plant production is Pendimethalin [N-(1-ethylpropyl)-3,4-dimethyl-2, 6-dinitrobenzenamine] is in the focus of presented research. Its brand name is “Stomp 330 E”.

Stomp belongs to the dinitroaniline group (Ni et al., 2016). This herbicide controls various weeds during the production of field crops, fruits and vegetables (Kočarek et al., 2016). Regardless of its low solubility in water, pendimethalin can enter the water ecosystems and exceed the concentration limits in groundwaters proposed by EU legislative (Kjær et al., 2011), furthermore, half-life of

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pendimethalin depends on the various abiotic and biotic factors; Rose et al. (2016), and Walker and Bond (1977) reported the half-life of 90, and 563 days, respectively. Several studies also showed that cancer incidence was associated with pendimethalin exposure (Hou et al., 2006; Andreotti et al., 2009). Meister (1992) and Strandberg and Scott-Fordsmand (2004) found the toxicity of pendimethalin for aquatic living organisms.

Although microorganisms play a key role in nutrient cycling and decay of organic residues (Kumar et al., 2016), they are also involved in biodegradation of xenobiotics, i.e. environmental pollutants such as nitroaromatic pesticides (Kulkarni and Chaudhari, 2007). Microbes have ability to grow on organic pollutants and degrade them to less toxic or non-toxic products (Diez, 2010). Several pendimethalin-degrading microorganisms have been isolated from various environments and described, including *Bacillus* (Megadi et al., 2010; Ni et al., 2016), *Pseudomonas* (Elsayed and El-Nady, 2013), *Clavispora* (Han et al., 2019) etc. Therefore, selection of indigenous microbial strains capable of rapid growth on pendimethalin as a unique carbon and energy source may have a practical application in contaminated environments.

The objective of this paper is to determine the microbial prevalence in soil after application of “Stomp” herbicide, containing pendimethalin as an active constituent, and to select autochthonous microbial strains capable of growth on pendimethalin as the sole carbon and energy source.

Material and Methods

The experiment was performed at Kakanj municipality Central Bosnian Canton, Bosnia and Herzegovina during spring 2019. “Stomp” herbicide (BASF, Germany) in the amount of 2 and 0.5 l/ha was applied by spraying the soil cultivated with onion (*Allium cepa* L.). Two samples of soil were taken, control sample (before herbicide treatment), and one composite sample (zero to 20 cm) after ten days from herbicide application.

Microbial presence in soil was determined using standard methodology, i.e. agar plate method. Tryptic soy agar (Torlak, Serbia) was used for determination of total bacterial number, Rose Bengal streptomycin agar (Peper et al., 1995) for fungal count, Nutrient agar (Torlak, Serbia) for estimation of ammonifiers, and starch-ammonia agar for actinomycetes prevalence. The experiment was performed in triplicate. Microbial count was expressed as colony forming units (CFU) per gram of dry soil. The obtained results were statistically processed using the software package SPSS 20. To determine the statistical significant differences of the obtained results was used the Independent Sample t-test ($p = 0.05$), as well as ANOVA post hoc Tuckey test ($p = 0.05$) were performed.

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Herbicide-tolerant bacteria were isolated using the modified Talaie et al. (2010) method, “Stomp” herbicide was added to Nutrient agar reaching the final concentrations of 0.5; 1.0 and 2.0 % (v/v). After isolation, bacteria were purified and stored at 4°C.

In order to describe bacterial growth, Nutrient agar supplemented with „Stomp“ at final concentrations of 1.3; 2.2; 3.5; 5.4 and 10 g/l (v/v) was used. Growth rate was estimated (0 without growth; + slow growth; ++ moderate growth; +++ intensive growth) after an incubation at 30°C for five days in incubator (Binder, Germany). The isolates with most pronounced growth were chosen for the testing in the presence of „Stomp“ as a unique carbon and energy source. The mineral salt medium (Talaie et al., 2010) supplemented with pendimethalin solution (up to final concentrations 25; 125; 250; and 500 mg/l) was inoculated by bacterial isolates suspended in saline solution containing 10^8 CFU/ml. Bacterial growth was measured using the spectrophotometer (T70 Ltd. Instruments, UK). Optical density (OD_{600}) was estimated at the start of the experiment, and after 24; 48; 72; 96; 120; and 144 h of incubation in orbital shaker (GFL-3005, Germany) at 30ºC and 150 rpm.

Results and Discussion

The presented results showed that the prevalence of major microbial groups depends on the herbicide application (table 1).

Our results show the increase of microbial abundance after ten days of “Stomp” application in all samples. In the control sample, total average number of bacteria was 150.0 x 10^5 CFU/g, while in soil treated with herbicide 340.0 x 10^5 CFU/g. A high increase of ammonifiers population after herbicide treatment was recorded: from 120.0 x 10^5 CFU/g in control to 270.0 x 10^5 CFU/g after the „Stomp“ treatment. A high increase of fungal prevalence after the herbicide application was observed (from 0.4 x 10^5 CFU/g in control to 3.0 x 10^5 CFU/g after the herbicide treatment). In contrast with other groups of microorganisms, statistically significant differences regarding average number of actinomycetes between control sample and the „Stomp“ treatment were not observed.

Table 1. Prevalence of the major microbial groups in soil sample

Microbial group		Control	Stomp-treated soil
	n	x 10^5 CFU/g dw	
		$\bar{x}$ ± SD	$\bar{x}$ ± SD
Total number of bacteria	3	150.0 ± 12.59^{aA}	340.0 ± 26.84^{aB}
Ammonifiers	3	120.0 ± 10.41^{bA}	270.0 ± 27.44^{bB}
Fungi	3	0.4 ± 0.06^{cA}	3.0 ± 0.36^{cB}
Actinomycetes	3	1.6 ± 0.26^{dA}	1.9 ± 0.29^{dA}

$^{a, b, c, d}$ - values of the same sample of different microbial groups marked with different letters, have a statistically significant difference (p<0.05), ANOVA, post hoc Tuckey test.

$^{A, B}$ - values of different samples of the same microbial group, marked with different letters, have a statistically significant difference (p<0.05), T-test.

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Table 2. Morphological characteristics of bacterial isolates

Isolates	Herbicide concentration (%)	Colony shape	Colony color	Colony diameter (mm)	Cell shape	Sporulation	Gram staining
G1/1	2.0	cocci	white	1.3	rod	+	+
E1/1	0.5	cocci	yellow	2.8	rod	+	+
G*	2.0	cocci	white	0.9	rod	+	+
G1/2	2.0	cocci	yellow	1.2	cocci	-	+
E1/2	0.5	cocci	yellow	2.6	rod	-	-
F2	1.0	cocci	yellow	1.7	rod	+	+
G1/3	2.0	cocci	yellow	1.0	rod	+	+
G2	2.0	cocci	yellow	0.9	rod	+	+
F1	1.0	cocci	yellow	1.1	cocci	-	+

Nine bacterial isolates with different morphologies are selected from the soil treated with „Stomp“. Macromorphological and micromorphological characteristics of isolates are presented in table 2. Most of them were isolated in the presence of the highest initial herbicide concentration (2.0 %), having small yellow colonies and Gram positive spore-forming rod-shaped cells.

Different growth rate of bacteria cultivated on Nutrient agar supplemented with herbicide was obtained. The results of this research are presented in the table 3.

Table 3. Bacterial growth rate on nutrient agar supplemented with herbicide

Isolate	Herbicide concentration (g/l)	Growth rate	Isolate	Herbicide concentration (g/l)	Growth rate	Isolate	Herbicide concentration (g/l)	Growth rate
G1/1	1.3	+++	G1/2	1.3	+++	G1/3	1.3	++
	2.2	+++		2.2	+++		2.2	++
	3.5	++		3.5	+++		3.5	+
	5.4	++		5.4	++		5.4	+
	10.0	++		10.0	++		10.0	0
E1/1	1.3	++	E1/2	1.3	+++	G2	1.3	++
	2.2	+		2.2	++		2.2	++
	3.5	+		3.5	++		3.5	++
	5.4	0		5.4	+		5.4	+
	10.0	0		10.0	+		10.0	0
G*	1.3	++	F2	1.3	+	F1	1.3	++
	2.2	++		2.2	+		2.2	++
	3.5	++		3.5	+		3.5	+
	5.4	+		5.4	0		5.4	+
	10.0	+		10.0	0		10.0	+

At the lowest initial concentration of the herbicide (1.3 g/l), pronounced bacterial growth was present in three bacterial isolates. Further increase of the „Stomp“ concentration in agar was followed by a decrease of bacterial growth rate in most isolates. Only the isolates G1/1 and G1/2 showed no differences in growth rate at the „Stomp“ concentrations of 1.3 and 2.2 g/l. These isolates showed moderate growth in the highest herbicide concentration (10 g/l) and are selected for further research.

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Bacterial growth (OD_{600}) in the presence of the pendimethalin is presented in tables 4 and 5.

At the lowest initial concentration of pendimethalin (25 mg/l), a decrease of OD_{600} of isolate G1/1 up to 24 h of incubation was observed (table 4). Further incubation resulted in an increase of OD_{600}, with maximal value after 96 h of incubation (0,199). The end of incubation (120 and 144 h) was characterized by a decrease of OD_{600}. Similar growth curve characteristics were noticed at the concentration of 125 mg/l. Inhibitory effects of the highest pendimethalin concentrations (250 and 500 mg/l) on bacterial growth were noted (table 4).

Table 4. Growth of isolate G1/1 in the presence of pendimethalin as unique carbon and energy sources (OD_{600}).

Herbicide concentration (mg/l)	Time of sampling (h)							
	0	8	24	48	72	96	120	144
25	0.122	0.101	0.084	0.142	0.178	0.199	0.187	0.152
125	0.164	0.155	0.134	0.142	0.139	0.155	0.147	0.122
250	0.174	0.166	0.162	0.142	0.133	0.130	0.141	0.131
500	0.184	0.175	0.161	0.149	0.127	0.102	0.088	0.082

Compared to G1/1, G1/2 isolate showed lower growth rate. At the lowest concentration of pendimethalin (25 mg/l), a decrease of OD_{600} up to 24 h of incubation was observed (table 5). Further incubation resulted in an increase of OD_{600}, with the highest value (0,139) after 120 h of incubation. The end of the incubation (144 h) was characterized by a decrease of OD_{600}. In other concentrations of pendimethalin, weak bacterial growth was noted. After initial adaptation, the highest value of optical density (OD_{600}) at concentration of 250 mg/l was obtained (table 5).

Table 5. Growth of isolate G1/2 in the presence of pendimethalin as unique carbon and energy sources (OD_{600}).

Herbicide concentration (mg/l)	Time of sampling (h)							
	0	8	24	48	72	96	120	144
25	0.055	0.048	0.033	0.061	0.079	0.101	0.139	0.101
125	0.075	0.055	0.036	0.041	0.045	0.051	0.047	0.033
250	0.082	0.074	0.070	0.072	0.065	0.062	0.056	0.059
500	0.088	0.081	0.074	0.064	0.052	0.048	0.045	0.041

Our results indicate the stimulatory effect of herbicide „Stomp“ on microbial prevalence in soil. Shetty and Magu (1998) confirm that soil microbial population is influenced by pendimethalin application. A significant increase in bacterial and fungal abundance after “Stomp” application was noticed. On the other hand, Singh and Singh (2020) found that pendimethalin did not influence the actinomycetes number in soil, which is confirmed in this research. As shown previously (Belal et al., 2008), microbes can utilize xenobiotics as nutrient and energy sources; these microbes can be used to

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alleviate environmental pollution caused by herbicide application, which leads to an increase in microbial diversity in soil (Yu et al., 2015).

Two isolates that were obtained using the enrichment method, have shown a low growth rate at all herbicide concentrations except at concentration of 25 mg/l. From this result, it was evident that both isolates were able to use low concentrations of herbicide as a unique carbon and energy source. Das et al. (2012) claimed that pendimethalin application led to the increase of aerobic bacterial populations in soil, which is in accordance with our results. In contrast, Nayak et al. (1994) noted a decrease in microbial abundance in soil after pendimethalin treatment; however, 15 days after the treatment, increase of the microbial activity was registered. Chikoye et al. (2014) also found the decrease of microbial activity after pendimethalin application. This finding is similar to our results, indicating that after the initial adaptation to the stress condition caused by the application of the herbicide, microbial populations in soil were capable of growth in the presence of pendimethalin.

Conclusion

The results of the study confirm that the application of „Stomp“ led to the increase of microbial abundance in soil, particularly the rapid increase in the prevalence of bacteria and fungi was observed. Nine different colonies and cell morphologies were observed, from which two isolates (G1/1 and G1/2) were capable of moderate grow on Nutrient agar with the addition of 10 g/l of „Stomp“. Our results showed an increase in bacterial growth during incubation on mineral salt medium supplemented with 25 mg/l of pendimethalin; this indicates that those two isolates have the potential for application on the soils contaminated with pendimethalin.

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Efekat primene herbicida „Stomp“ na zastupljenost mikroorganizama u zemljištu

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Izvod

U poljoprivrednoj proizvodnji, primena herbicida ima višestruke koristi. Međutim, njihova upotreba izaziva i probleme u životnoj sredini. Primena herbicida utiče na biodiverzitet ekosistema, uništavajući neciljane organizme. Mikroorganizmi u zemljištu su važan faktor za rast biljaka; oni predstavljaju biološki faktor plodnosti zemljišta. Herbicidi imaju korisni efekat za neke mikroorganizme, dok su za druge negativni, što dovodi do smanjenja mikrobnog diverziteta u zemljištu. Cilj ovog rada je determinacija mikrobne aktivnosti zemljišta i izolacija bakterija rezistentnih na herbicid nakon primene “Stompa”. Metod agarnih ploča je korišćen za determinaciju prisustva mikroorganizama u zemljištu. Rezultati pokazuju povećanje ukupnog broja bakterija, amonifikatora, gljiva i aktinomiceta. Devet izolata, uglavnom Gram pozitivnih sporogenih štapića, pokazalo je sposobnost rasta na mineralnoj podlozi obogaćenoj različitim koncentracijama pendimetalina. Izolati G1/1 i G1/2 pokazali su visoku toleranciju prema koncentraciji pendimetalina od 25 mg/l. Ovi izolati imaju potencijal za primenu u dekontaminaciji ekosistema kontaminiranih herbicidom.

Ključne reči: herbicid “Stomp”, zastupljenost mikroorganizama, pendimetalin, zemljište

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