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Original scientific paper

Effects of herbicides on growth and number of actinomycetes in soil and *in vitro*

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SUMMARY

This study was conducted under laboratory conditions to investigate the effects of herbicides (nicosulfuron, metribuzin and glyphosate) on the number of actinomycetes in soil and growth of several isolates of actinomycetes *in vitro*. The lowest tested concentrations equalled the recommended rates (1X), while the other three were five-fold (5X), ten-fold (10X) and fifty-fold (50X). Samples were collected for analysis 3, 7, 14, 30 and 45 days after herbicide application. Treatment with the two highest concentrations of herbicides (10X and 50X) caused a significant inhibition of the number of actinomycetes in soil and growth of the isolates *in vitro*. The obtained data indicated that the effect depended on the type of herbicide, application rate, duration of activity and actinomycetes isolate. The study suggests that herbicide applications in soil caused transient effects on the growth and development on actinomycetes community in soil.

Keywords: Herbicides; Actinomycetes; Soil

INTRODUCTION

Herbicides are used in agriculture to destroy weeds and pests, and their use may result in irreversible soil pollution. There are a number of parameters that can indicate changes in fertility and health of treated soils. Microbiological parameters, compared to chemical and physical, are the most sensitive to changes in the environment. In particular, herbicides have a detrimental effect on soil microbial activity and change the abundance and community structure of soil microflora (Benslama & Boulahrouf 2013; Zhang et al., 2011). One of the new perspectives concerns the importance of soil microorganisms in modulating the interaction between weeds and crops (Massenssini et al.,2014).

Nicosulfuron, (2-[(4,6-dimethoxypyrimidin-2-yl)carbamoylsulfamoyl]-N,N-dimethylpyridine-3-carboxamide) is a herbicide of the sulfonylurea group and it has a good selectivity, characterized by broad-spectrum weed control for many cereal crops, sugar beet, and maize. This class of herbicides is characterized by high biochemical activity at low application rates (Radivojević et al., 2012). Metribuzin, (4-amino-6-tert-butyl-3-methylsulfanyl-1,2,4-triazin-5-one) belongs to the triazine class. Metribuzin is incorporated as a pre-emergent soil-applied herbicide in control methods for weeds in wheat, potato, tomato and others crops. Some microorganisms have the capacity to degrade metribuzin in soil very fast and to utilize it as a nutrient (Singh, 2014).

Glyphosate (2-(phosphonomethylamino)acetic acid) is a broad-spectrum, post-emergence and non-selective herbicide. It is widely used in soil cultivation, forestry and control of weeds (Bennicelli et al., 2009). The side effects of this herbicide on microorganisms are a cause for environmental concerns.

Actinomycetes are aerobic, gram-positive bacteria that constitute a major group of soil microbial population. Their number and dominance in soil are greatly influenced by soil temperature, pH, organic carbon content, aeration, moisture content and soil condition. Most reports suggest that the application of different herbicides decreases the abundance of this microbial population (Pampulha et al., 2007; Abbas et al., 2015). However, some authors consider actinomycetes as an important microbial group in soil which constitutes a complex biochemical system capable of producing enzymes. Also, they degrade a large number of toxic substances, including herbicides and use them as nutrients and source of energy (Milošević & Govedarica, 2002; Omar & Abdel – Sater, 2001).

Microbial degradation of herbicides applied to soil is important because it prevents the accumulation of those chemicals in the environment, and is therefore an important goal. Some researchers have pointed to biodegradation of herbicides and proposed ways to reduce the persistence of these chemicals in soil (Pal et al., 2006; El Hussein et al., 2012;Tamilselvan et al., 2014).

Determining the impact of herbicides on microbial growth and structure of their populations is of considerable interest. Assessment of the impact of herbicides on microbial communities, especially actinomycetes, is important for deepening the knowledge of herbicide risk management in soils. The present study was undertaken to assess the response of actinomycetes populations to different rates of herbicide applications *in vitro* and in the soil environment.

MATERIALS AND METHODS

Herbicide treatments

The herbicide nicosulfuron, tested in the experiment, was the product Motivell (BASF, Germany). The rates of application were 0.3, 1.5, 3.0 and 15.0 mg kg^{-1} soil. Another herbicide, metribuzin, was the product Sencor WG-70 (Bayer Crop Sciences, Germany), and its rates of application were 12.0, 60.0, 120.0 and 600 mg kg^{-1} soil. The herbicide glyphosate was the product Roundup (Monsanto, USA), and its rates of application were 32.6, 163.0, 326.0 and 1630.0 mg kg^{-1} soil. The lowest tested concentrations equalled the recommended rates (1X), while the other three were five-fold (5X), ten-fold (10X) and fifty-fold (50X).

Experiment in soil

The experiment was performed in loamy soil with sand 49.80%, silt 33.40, clay 16.80, total carbon 2.30%, total nitrogen 0.25%, organic matter 3.96% and pH 7.64. Soil samples were collected from the upper layer (0-10 cm), and were carefully dried, sieved to pass 5 mm mesh, and stored at 4°C. Before using them, the soils were air-dried at room temperature for 24 h. Each herbicide concentration was pipetted to the surface of 1 kg of soil before homogenization on a rotary stirrer for 30 minutes. After homogenization by mixing, the soil was portioned out in pots. Untreated soil served as a control. The experiments were conducted in four replications. The pots were kept in a controlled-environment chamber at 20±2°C, 50% air humidity and 12/12 h day/night photoperiod throughout the experiment. The samples were collected for analysis 3, 7, 14, 30 and 45 days after the application of herbicides. Populations of actynomicetes were enumerated using selective media and the standard spread plate dilution technique. Synthetic agar with sucrose was used for enumeration of actynomicetes populations in soil treated with different herbicides, as well as the control (Jarak & Đurić 2006).

In vitro experiment

Several isolates of actinomycetes (3/7, 2/7, 7/3 and 14/3) obtained from loamy soil were used in the *in vitro* experiment. These isolates were stored in an incubator at 4°C on potato dextrose agar (PDA). In examining their sensitivity to herbicides (nicosulfuron, metribuzin and glyphosate) top surfaces of Petri dishes were inoculated with 0.5 ml per inoculum isolate (10^{-2}) and kept in an incubator at 27°C. After five days, wells (Ø 10 mm) were made on the media with overgrown colonies. The wells were filled with 100 μl of different concentrations of the herbicides and the plates were returned to incubation. Sterile distilled water was applied to the wells in the control variant. After seven days, the zones of inhibition were measured.

Data were statistically processed in Statistica 8.0 STATSOFT software. A two-way ANOVA analysis of variance was used to compare means of the examined microbial parameters: the number of actinomycetes and growth of actinomycetes isolates. Tukey's test was used to compare treatments and assessments of each parameter when differences in F-values were statistically significant ($p<0.05$).

RESULTS

Herbicide treatments with nicosulfuron, metribuzin and glyphosate showed significant effects on actinomycetes growth and development *in vitro* and in soil. The effects of various concentrations of nicosulfuron on the number of actinomycetes are shown in Figure 1. For the two highest concentrations tested (10X and 50X the recommended field applicaton rates) a decrease in actinomycetes counts was observed after the application of nicosulfuron. The number of these microorganisms then decreased, and the reduction was significant (between 13 and 31% compared to the control) from the 3rd to 30th day after treatment. This reduction was greater for the higher herbicide concentrations. However, after 45 days these values again increased over time and returned to the control levels. The population of actinomyctes was significantly inhibited within 14 days after application of metribuzin (Figure 2),

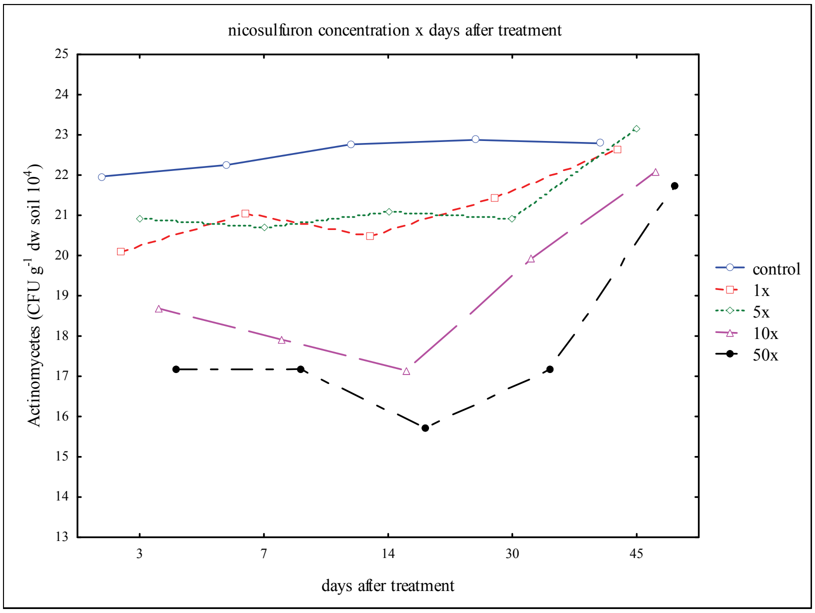

Figure 1. Effect of nicosulfuron on the abundance of actinomycetes (10^4g-^{1}dry soil)

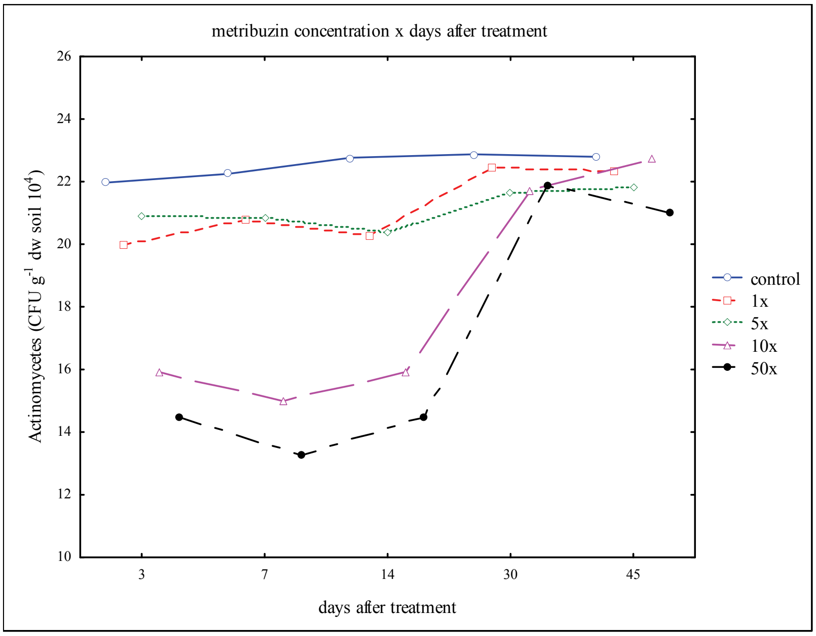

Figure 2. Effect of metribuzin on the abundance of actinomycetes (10^4g-^{1}dry soil)

compared to the control. Within this period, their number was reduced between 27 and 40% at the 10X and 50X recommended field application rates. A recovery of this population was verified from the 30th to 45th day. A similar trend in actinomycetes counts was observed in glyphosate treated soil (Figure 3). In all treatments with 1X and 5X recommended field application rates no significant difference was observed in the number of actinomycetes. The effect of glyphosate (10X and 50X recommended field application rates) showed a decreasing trend from the 3rd to 30th day after treatment and their number was reduced between 19 and 45%, compared to the control. After 45 days, the number of actinomycetes was not significantly different from the control treatments.

Herbicide treatments of the growth media *in vitro* significantly restricted growth of the actinomycetes isolates. Growth inhibition became more severe with increasing herbicide concentrations.The growth of isolate 3/7 significantly decreased in treatments with metribuzin and glyphosate (Figure 4). In contrast, nicosulfuron caused no inhibition at any concentration . Metribuzin was less toxic and inhibited growth by 18% with the recommended field application rate (1X), and by 70% with the highest concentration (50X). Glyphosate treatment at 1X, 5X,

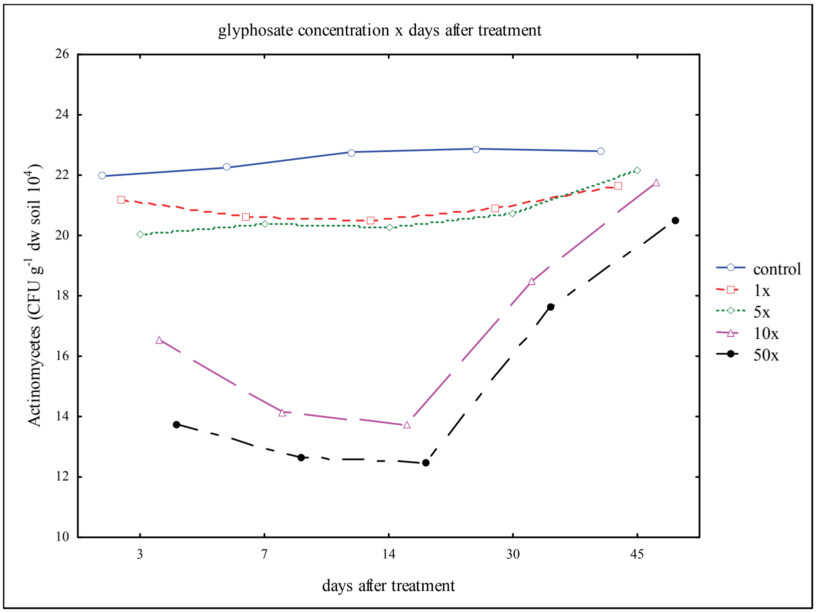

Figure 3. Effect of glyphosate on the abundance of actinomycetes ($10^4 g^{-1}$dry soil)

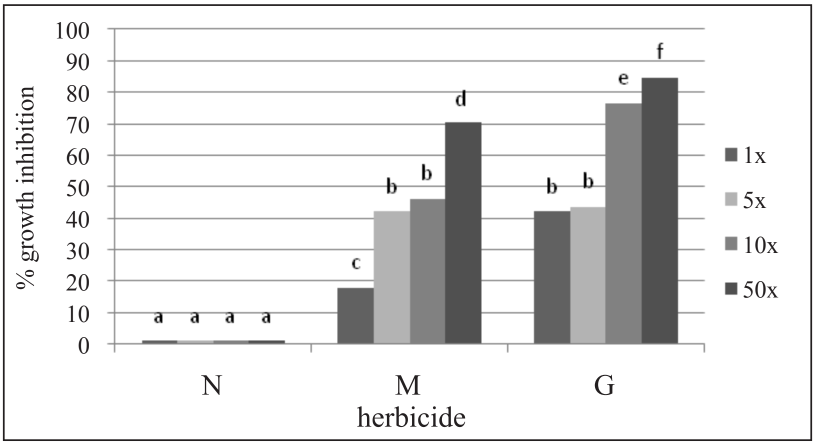

Figure 4. Effect of herbicide treatments *in vitro* on the growth of isolate 3/7

(N-nicosulfuron; M-metribuzin; G- glyphosate)
Bars marked with the same letter within each herbicide treatment (four concentrations) are not significantly different ($p<0.05$)

10X and 50X recommended field application rates caused 42-84% inhibition of the 3/7 isolate. A similar trend was observed for the growth of 14/3 isolate (Figure 5). None of the concentrations of nicosulfuron or the 1X recommended field application rates of metribuzin and glyphosate caused inhibition. The other three concentrations of metribuzin and glyphosate caused 20-45% and 46-77% growth inhibition of this isolate, respectively. The growth of isolate 2/7 was not reduced in any of nicosulfuron treatments (Figure 6). Metribuzin (1X) and glyphosate (1X and 5X recommended field application rates) also causeed no inhibition.The other concentrations of metribuzin and glyphosate significantly reduced growth by 23-45%, and 43-83%, respectively. The impact of the herbicides on the growth of isolate 7/3 is presented in Figure 7. Nicosulfuron inhibited its growth by 19% only with the highest concentration (50X). The other concentrations of nicosulfuron (1X, 5X and 10X) and the recommended field application rate (1X) of metribuzin and glyphosate caused no inhibition. Metribuzin treatment (5X, 10X and 50X recommended field application rates) caused 20%, 23% and 55% inhibition of the actinomycetes isolate 7/3. At the same rate of application, however, glyphosat caused 30%, 47% and 67% inhibition.

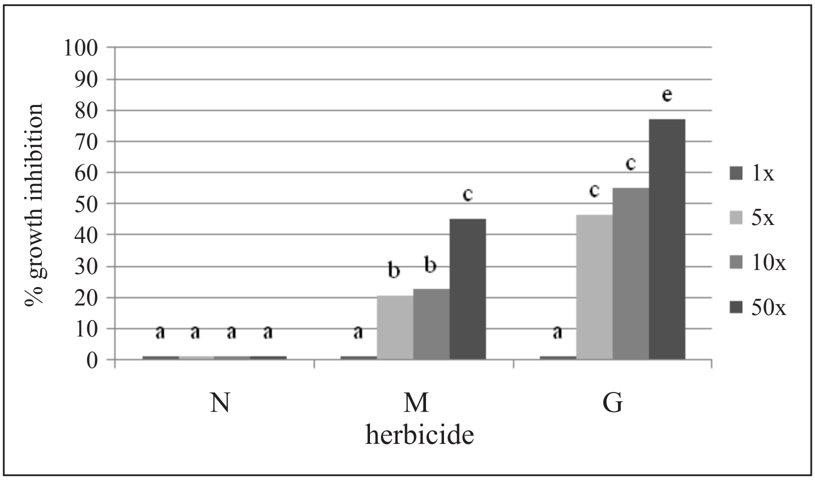

Figure 5. Effect of herbicide treatments *in vitro* on the growth of isolate 14/3
(N-nicosulfuron; M-metribuzin; G- glyphosate)
Bars marked with the same letter within each herbicide treatment (four concentrations) are not significantly different (p<0.05)

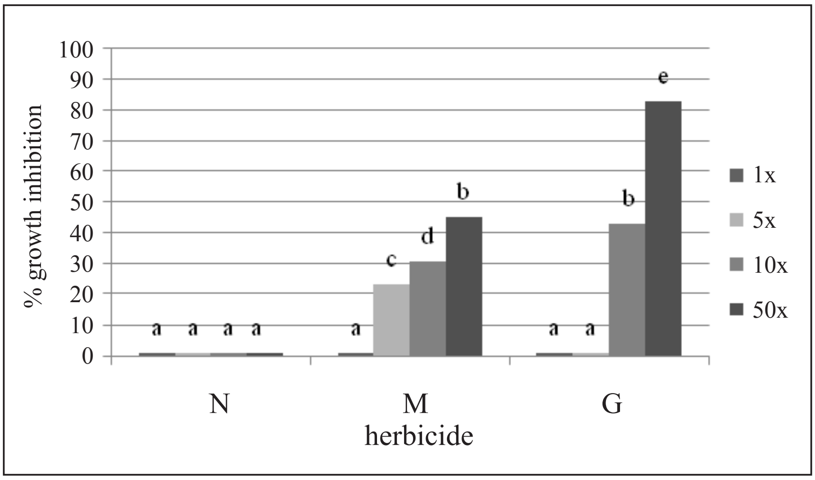

Figure 6. Effect of herbicide treatments *in vitro* on the growth of isolate 2/7
(N-nicosulfuron; M-metribuzin; G- glyphosate)
Bars marked with the same letter within each herbicide treatment (four concentrations) are not significantly different (p<0.05)

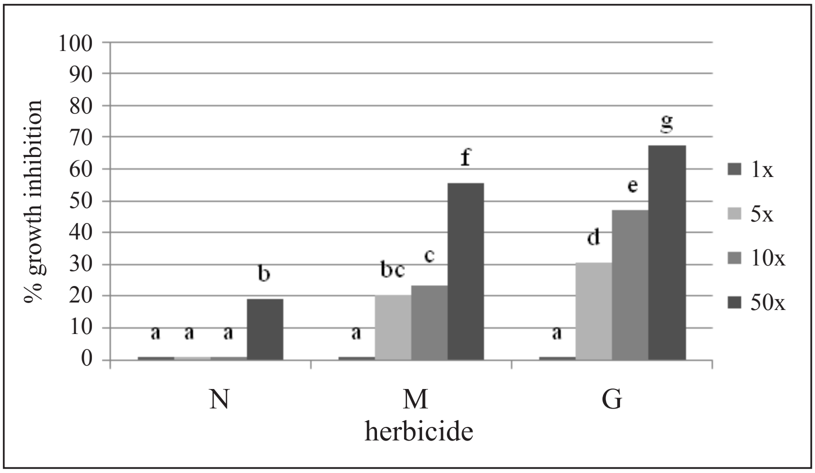

Figure 7. Effect of herbicide treatments *in vitro* on the growth of isolate 7/3
(N-nicosulfuron; M-metribuzin; G- glyphosate)
Bars marked with the same letter within each herbicide treatment (four concentrations) are not significantly different ($p<0.05$)

DISCUSSION

The choice of laboratory research enabled us to study the influence of herbicides on soil microorganisms without the variability of a field study, providing a better understending of the potential effects of these chemicals on soil microbes (Pampulha at al., 2007). In this study of possible effects of herbicides (nicosulfuron, metribuzin and glyphosate) on actinomycetes two important aspects were considred: their number and their growth *in vitro* on PDA medium.

In the experiment in soil environment, the exposure of actinomycetes to the herbicides caused short-term inhibitory effects on their population. In this study, the application of nicosulfuron at the highest concentrations (10X and 50X) decreased the number of actinomycetes from the 3rd to 30th day after treatment. On the last 45th day, those values increased and returned to control levels. In a study by Filimon et al. (2015), bacteria of *Clostridium* genus were the most sensitive bacteria to nicosulfuron impact, the strongest effect being reported when the exposure dose was seven-fold higher. Also, the application of metribuzin (10X and 50X) significantly inhibited growth of actinomycetes for 14 days. Similar results were reported by Mohiuddin & Mohammed (2013), indicating that the application of metribuzin drastically reduced the number of actinomycetes over the initial days. Also, Lone et al. (2014) reported that the field rate of metribuzin (up to 7th day) decreased actinomycetes population. In our study, glyphosate decreased the number of actinomycetes from the 3rd to 30th day with its 10X and 50X recommended field application rates. After 45 days, all values were at the control level. Our resultes are comparable to the results of Baboo et al. (2013) who found that the number of actinomycetes on different days after glyphosate application significantly differed, the lowest being on the 7th and highest on the 28th day after treatment. Zobiole et al. (2011) noted that glyphosate applied to GR soybean had a negative impact on complex interactions of microbial groups and biochemical activity. The recovery of microbial populations after initial inhibition were due to microbial adaptation to these herbicides or due to their degradation (Vandana et al., 2012).

In the *in vitro* experiment, actinomycetes growth inhibition increased significantly with growing herbicide concentrations. Actinomycetes growth inhibition due to the effects of herbicide treatments also varied among their isolates and the types of herbicide. Glyphosate was found to be more inhibitory then metribuzin and nicosulfuron, causing 67-84% growth inhibition to the isolates. Meriles et al. (2006) in an *in vitro* study conducted with glyphosate (100 ppm and 140 ppm) observed growing inhibitory effects on soil fungi. In our study, nicosulfuron caused the least inhibitory effects on actinomycetes isolate growth. Nicosulfuron-provoked inhibition was found in isolate 7/3 at the highest concentration (19%). The least inhibition of growth in response to metsulfuron-methyl was also observed for fungal species (Yu et al., 2005; He et al., 2006; Zain et al., 2013). Some investigators pointed to biodegradation of herbicides and proposed to reduce the persistence of these chemicals in soil. Zaki et al. (2014) were focused on the persistence of Sencor as a herbicide in soil inoculated with some streptomycetes. In their study the number of actinomycetes progressively increased to a peak on the 30th day, then declined thereafter. Also, similar was a study by Castillo et al. (2006), who reported that the herbicide diuron degraded the activity of 17 streptomyces strains obtained from agricultural and non-agricultural soils.

The inhibitory effect of herbicides on actinomycetes growth in soil treatment however, was lower than it was after direct exposure (*in vitro*). This indicates that herbicides in soil may undergo some different processes, which could reduce their toxicity to microorganisms (Zain et al., 2013).

In conclusion, the application of nicosulfuron, metribuzin and glyphosate (1X, 5X, 10X and 50X their recommended field application rates) variably inhibited the number of actinomycetes and growth of actynomicetes isolates**.** The extent of inhibition depended on the type of herbicide, their application rates, and period of exposure. The results of this experiment revealed that the application of herbicides reduced the population of actinomycetes. Glyphosate caused the highest population reduction and the effect was stronger as its concentrations increased. However, actinomycetes populations recovered from all herbicide treatments within 45 days and the number of actinomycetes was not significantly different from the control treatments. Actinomycetes growth inhibition due to herbicide treatments also varied among their isolates**,** application rates and types of herbicide. While the highest inhibitory effect was demonstrated by glyphosate at the 50X recommended field application rate, nicosulfuron showed the lowest effect in all treatments. Isolate 2/7 showed the greatest resistance to the herbicides. The study suggests that the application of herbicides to soil induced transient effects on the growth and development of actinomycetes community in soil.

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Delovanje herbicida na rast i broj aktinomiceta u zemljištu i *in vitro*

REZIME

Ogled je postavljen u laboratorijskim uslovima radi ispitivanja uticaja tri herbicida (nikosulfurona, metribuzina i glifosata) na broj aktinomiceta u uslovima zemljišta i porast izolata aktinomiceta u *in vitro* uslovima. Najniža ispitivana koncentracija odgovara preporučenoj količini primene (1X), dok su ostale bile pet puta (5X), deset puta (10X) i pedeset puta (50) veće. Uzorci za analizu uzimani su 3, 7, 14, 30 i 45 dana posle primene herbicida. Primena većih koncentracija herbicida (10X and 50X) izazvala je značajnu inhibiciju broja aktinomiceta u zemljištu i porasta izolata *in vitro*. Dobijeni rezultati su pokazali da je uticaj zavisio od vrste herbicida, primenjene količine, dužine delovanja i vrste izolata aktinomiceta. Istraživanje pokazuje da je primena herbicida izazvala prolazne efekte na rast i razvoj zajednice aktinomiceta u zemljištu.

Ključne reči: Herbicidi; Aktinomicete; Zemljište