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Changes in microbiological properties of soil during fungicide degradation

Abstract: Laboratory tests were performed on sandy loamy soil to establish the relations between bacterial diversity, soil enzyme activity and degradation of Amistar 250 SC, Falcon 460 EC and Gwarant 500 SC fungicides. Apart from carrying out microbiological and biochemical analyses, the residues of active substances from the tested fungicides were determined. Structural diversity of was determined based on the next-generation sequencing (NGS) method, and fungicide residues the liquid chromatography tandem-mass spectrometry (LC-MS/MS). It was found that changes in bacterial diversity occurred in the soil subject to fungicide treatment, particularly at the family and genus level. Proteobacteria, Firmicutes and Actinobacteria were prevailing in all soil samples. *Bacillus* occurred both in the control soil and in the soil treated with fungicides, while *Pseudonocardia* occurred only in the fungicide-treated soil. Of all the fungicides tested, the biggest changes in bacterial diversity were caused by Gwarant 500 SC. The preparations tested not only affected the composition of soil microbiota, but also contributed to changes in the biochemical properties of soil by inhibiting the activity of almost all tested enzymes, with the exception of alkaline phosphatase and β -glucosidase. Chlorothalnil was the fastest degraded in the soil and spiroxamine at the slowest.

Keywords: fungicides, pollution, soil microbiome, enzymes, degradation

INTRODUCTION

In order to meet the requirements related to farming production, chemization of agriculture has become increasingly more widespread (Winiarek and Kruk 2017). Pesticides are used to maintain plant health. Global pesticide production increases every year by about 11%, with herbicides, insecticides and fungicides dominating in the sales structure (Carvalho 2017). Long-term and intensive application of these agents can lead to extensive treated of soil ecosystems (Pose-Juan et al. 2017). As Ye et al. (2018) reports, even 80–90% of applied pesticides penetrate into the natural environment, and only a small percent (1–2%) operate against the target organisms, while the remaining share (10–20%) is situated on the plant surface. This can have a harmful effect on the quantity and quality of microorganisms inhabiting the soil, as well as on their physiological processes, which is related to unfavourable changes in soil productivity and fertility (Pose-Juan et al. 2017). It is very important to determine the response of microorganisms and soil enzymes to pesticides in soil in view of their participation in biogeochemical cycles and their role in maintaining proper soil functions and promoting

growth and development of plants (Grządziel and Gałązka 2018, Siwek-Ziomek and Koper 2018). All changes in soil microbial population can be of significant importance for the regeneration rate of soil subject to stress factors (Kosiorek and Wyszkowski 2017, Radziemska et al. 2018). Therefore, changes in the soil microbiome provide an appropriate warning and forecasting indicator, which can successfully be used for monitoring the condition of soil ecosystems (Ju et al. 2016). The aim of the present study is to establish of the effect of the tested fungicides on bacterial diversity and enzymes activity of the soil.

MATERIALS AND METHODS

The experiment was conducted on soil sample taken from an Eutric Cambisols soil of pH_{KCl} 7.0 at a depth of 0 to 20 cm, which texture was classified as sandy loam (fraction from 2000 to 50 μ m – 80.56%, fraction from 50 to 2 μ m – 17.98% and fraction < 2 μ m – 1.49%). The soil originated from Tomaszkowo, a locality in the Mazurian Lakeland, NE Poland, Central Europe (53.7161° N, 20.4167° E). Soil material used in the experiment was described in detail by Baćmaga et al. (2016). Three fungicides

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(Amistar 250 SC, Falcon 460 EC and Gwarant 500 SC) were tested, the characteristics of which are presented in Table. Those preparations were introduced per kilogram of soil in the following doses (as per active substances): Amistar 250 SC – 22.50 mg (azoxystrobin), Falcon 460 EC – 27.60 mg (spiroxamine – 15.00 mg, tebuconazole – 10.02 mg and triadimenol – 2.58 mg), Gwarant 500 SC – 49.80 mg (chlorothalonil).

A laboratory experiment was conducted in three replications. It was carried out in glass beakers (150 ml capacity), to which 100 g of dry soil matter was added. Fungicides in specific doses (Amistar 250 SC – 22.50 mg kg⁻¹ a.i, Falcon 460 EC – 27.60 mg kg⁻¹ a.i, Gwarant 500 SC – 49.80 mg kg⁻¹ a.i) were added to relevant objects in a single application, they were carefully mixed and the humidity of the entire sample was brought to 50% capillary water capacity. Under these humidity conditions, soil samples were incubated in a thermostat, at a constant temperature (25°C) for two months.

In the soil subject to fungicide treatment, bacterial diversity was determined using the next-generation sequencing method (NGS) by Genomed S.A. in Warsaw (Poland). The metagenomic analysis of 16S rRNA encoding gene was conducted based on the V3-V4 hypervariable region. Specific primer sequences 34F and 785R were applied for amplification of a selected region. The sequencing was conducted using the MiSeq sequencer (Illumina). Biochemical analyses included determination of dehydrogenase activity (Deh), catalase (Cat), urease (Ure), acid phosphatase (Pac), alkaline phosphatase (Pal), arylsulphatase (Aryl) and β -glucosidase (Glu). Determinations of the analysed soil enzymes were performed in three replications according to the procedure described in Borowik et al. (2017).

In the soil, residues of azoxystrobin, chlorothalonil, spiroxamine, tebuconazole and triadimenol were determined, using the Eksigent Ultra LC-100 liquid

chromatography system (Eksigent Technologies, Dublin, CA, USA) coupled with 6500 QTRAP mass spectrometer (AB Sciex Instruments, Foster City, CA), equipped with an electrospray ionization source (ESI) and atmospheric pressure chemical ionization (APCI). A detailed procedure applied to determine pesticide residues was described in Kaczyński et al. (2016) and Jankowska et al. (2016). Fungicide residues in soil samples were determined with limit of quantification of 0.005 mg kg⁻¹, and mean recoveries for the examined active substances ranged from 70.26% (spiroxamine) to 111.62% (chlorothalonil).

The results were statistically analysed using the ANOVA variance analysis at the level of $P \leq 0.01$, using the Statistica 13.1 software (StatSoft... 2018). Uniform groups were calculated using Tukey's test.

RESULTS AND DISCUSSION

The meta-analysis (Fig. 1) demonstrated the existence of differences in the diversity of prokaryotes in soil subject to fungicide application. In all soil samples, a prevalence of Protobacteria, Firmicutes and Actinobacteria was found. In the soil with Falcon 460 EC and Gwarant 500 SC, Bacteroidetes did not occur. As regards the family range, bacteria classified as *Bacillaceae* were the most numerous. Bacteria from the *Enterobacteriaceae* family, present in the control soil, disappeared in objects treated with fungicides. *Chromatiaceae* did not occur in soil treated with Amistar 250 SC, and *Alicyclobacillaceae* – in objects with Falcon 460 EC and Gwarant 500 SC. However, it was observed that in soil with Gwarant 500 SC preparation, bacteria belonging to *Intrasporangiaceae*, *Kouteothriaceae*, *Gemmationadaceae*, *Geodermatophilaceae*, *Bradyrhizobiaceae* and *Sinobacteraceae* occurred, which were not present in the control soil (soil without added fungicides). In samples of soil treated with fungicides, the identified bacteria genera also demonstrated changes in the microbiome composition.

TABLE. Characteristics of preparations used in the experiment

Trade name of the preparation	Name of active substance	Chemical group	Content of active substance in the preparation (g dm ⁻³)	Half-life (days)	Optimum dose (dm ³ ha ⁻¹)	Manufacturer
Amistar 250 SC	azoxystrobin	strobilurins	250	14–180	0.8–1.0	Syngenta
Falcon 460 EC	spiroxamine	ketoamines	250	37–44	0.6	Bayer CropScience AG
	tebuconazole	triazoles	167	49–610		
	triadimenol	triazoles	43	30–50		
Gwarant 500 SC	chlorothalonil	phthalates	500	18–100	1.0	Arysta LifeScience S.A.S

FIGURE 1. Composition of bacterial groups in soil (classification range $\geq 1\%$ sequence)

Bacillus dominated both in the control soil and in the soil treated with fungicides, while *Pseudonocardia* occurred in soils treated with fungicides. *Alicyclobacillus* and *Cohenella* bacteria present in the control soil, disappeared in objects with Falcon 460 EC and Gwarant 500 SC preparations. It was recorded that in soil samples subject to the effect of Gwarant 500 SC preparation, bacteria *Balneimonas*, *Kouleothrix*, *Gemmatimonas*, *Hyphomicrobium* and *Skermanella* appear, which were not present in the control soil (soil not treated with fungicides). Subrahmanyam et al. (2016) observed that Firmicutes, Acidobacteria and Actinobacteria were typical of chemically degraded soils, which can prove their high tolerance to the treated. As Paul et al. (2006) reports, in soils affected with parathion and methyl parathion, Acidobacteria, Proteobacteria, Planctomycetes, Bacteroidetes, Chloroflexi, Verrucomicrobia and Cyanobacteria are attributed the highest share. On the other hand, in soils subject to chlorantraniliprole, Proteobacteria, Crenarchaeota, Bacteroidetes, Acidobacteria and Actinobacteria are prevailing (Wu et al. 2018). In our study, in all soils samples dominated Protobacteria, Firmicutes and Actinobacteria. According to Jeffries et al. (2018) in soils originating from the areas treated with chlorpyrifos, *Koribacter*, *Hyphomicrobium*, *Burkholderia*, *Bradyrhizobium* and *Acidomicrobium* are the most numerous. In turn, Parte et al. (2017) provide that soil ecosystems treated with organochlorine pesticides are colonized by *Bacillus*, *Pseudomonas*, *Flavobacterium*, *Moraxalla*, *Acinetobacter*, *Arthrobacter*, *Paracoccus*, *Aerobacter*, *Alcaligenes*, *Burkholderia* and *Sphingomonas*. In the present study, it was observed that the soil samples treated with fungicide occurred bacteria of the genus *Bacillus* and *Pseudonocardia*.

A very important role in maintaining proper functions of soil ecosystems is played by biochemical activity, which is closely related to microorganisms. This is the reason why it is considered an appropriate indicator for estimating changes occurring under the effect of pesticides penetrating the soil (Wu et al. 2017). In our study, in all soils samples dominated Protobacteria, Firmicutes and Actinobacteria.

Our research demonstrated that fungicides contributed to changes in the biochemical properties of soils, leading to a reduction in the activity of the majority of the enzymes examined ($n=3$). The tested fungicides proved to be strong inhibitors of soil enzymes (Fig. 2). Amistar 250 SC and Falcon 460 EC inhibited to the highest extent the activity of catalase (by 61.06% and 69.26%, respectively), and Gwarant 500 SC – of dehydrogenases (by 77.88%). It was observed that fungicides did not contribute to significant changes in the activity of alkaline

phosphatase and β -glucosidase. A reduction of enzyme activity could be the effect of destroying microorganisms living in soil by an excessive amount of fungicides (Ye et al. 2018). Inhibition of soil enzyme activity was also proved by Onyszko et al. (2017). They reported that the application of excessive doses of napropamide demonstrated high toxicity towards dehydrogenases, acid phosphatase, alkaline phosphatase and urease. An unfavourable effect of tebuconazole on soil enzyme activity was proven by the research by Wang et al. (2016). Tebuconazole present in soils in treated doses (10 and 100 mg kg⁻¹) inhibited the activity of urease, alkaline phosphatase, dehydrogenases and invertase. A deactivating effect on amylase, cellulase and invertase was found for the mixture of buprofezin and acephate applied in doses of 7.5 and 10 μ g g⁻¹ (Raju and Venkateswarlu 2013).

Microbiological and biochemical activity of soil is of crucial importance for the process of pesticide degradation in soil environment. Microorganisms play a crucial role in pesticide transformation, leading to their neutralization (Alvarez-Martin et al. 2016). Our own research demonstrated that spiroxamine was the slowest to decompose in soil, since after two months it was degraded by 7.33% (Fig. 3). Chlorothalonil in the soil was degraded in 85.19%. Other active substances were degraded by 35.43% (triadimenol), 26.25% (tebuconazole) and 24.89% (azoxystrobin). The examined active substances were degraded to various extents, due to the fact that some pesticides can be used by microorganisms as carbon sources, which accelerates their decomposition (Chen et al. 2015). Others can be immobilized by microorganisms by being incorporated into cells, which can lead to a lower rate of degradation in case of these agents (Botero et al. 2017). In our research, the highest bacterial diversity was found in the soil contaminated with chlorothalonil and therefore it could have a significant effect on the rapid disappearance of this active substance in soil. Bacteria occurring in soil could demonstrate a high tolerance to chlorothalonil, which provided the source of energy for their growth and development. Diversified degradation of the examined active substances could also result from their chemical structure and persistence in the soil environment, which is proven by the research conducted by Muñoz-Leoz et al. (2013). They recorded that pesticides introduced at the dose of 5 mg kg⁻¹ degraded to a various extent (the amount of difenconazole decreased by 52.00%, deltamethrin – by 69.00% and ethofumesate – by 89.00%). On the other hand, Ju et al. (2016) reported that degradation of pesticides is determined both by their dose and the type of soil. That research concerned the dynamics

FIGURE 2. Effect of fungicides on soil enzyme activity: C – control, A – Amistar 250 SC, F – Falcon 460 EC, G – Gwarant 500 SC

FIGURE 3. Degradation percentage of active substances in soil

of myclobutanil degradation in alluvial soil and in so-called cinnamon soil. Within 90 days, myclobutanil degraded faster in alluvial soil (62.40%–95.80% degradation) than in so-called cinnamon soil (36.20%–47.50% degradation). According to Manoharan et al. (2017), an excessively high dose of pesticides can destroy indigenous microorganisms colonizing the soil ecosystem and therefore lead to their increased persistence in soil. In our research, changes in the community structure of bacterial groups and the enzyme activity may be evidence of the varied rate at which the tested fungicides disappeared from soil environment. Therefore, it can be concluded that bacterial diversity is closely related to the enzymatic activity of soil, as well as to the ability of microorganisms to degrade fungicides.

CONCLUSIONS

Contamination of soil with fungicides brings about a lot of unwanted effects, which manifest themselves in changes in the structure and activity of microorganisms. In order to prevent it, it is important to monitor the fate of these chemicals in the environment. Determination of the structure of bacterial assemblies by next-generation sequencing (NGS) allowed to obtain valuable information about the occurrence of changes in bacteria populations inhabiting the soil treated with fungicides. The sequence analysis has shown that changes of taxons in the soil samples under

study took place at the phylum level, with the greatest differences observed at the levels of families, genera and species. At the phylum level, *Proteobacteria*, *Firmicutes* and *Actinobacteria* dominated in all the samples. *Bacillus* occurred both in the control soil and in the soil treated with fungicides, while *Pseudonocardia* occurred only in the fungicide-treated soil. The tested fungicides proved to be strong inhibitors of soil enzymes, except for alkaline phosphatase and β -glucosidase. The bacterial diversity is closely related to the enzymatic activity of soil, as well as to the ability of microorganisms to degrade fungicides. Therefore, the active substances included in the tested preparations were decomposed in the soil at different degrees. Chlorothalonil degraded at the highest rate and spiroxamine at the lowest.

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Zmiany właściwości mikrobiologicznych gleby podczas degradacji fungicydów

Streszczenie: W celu ustalenia zależności pomiędzy różnorodnością bakterii, aktywnością enzymów glebowych i degradacją fungicydów Amistar 250 SC, Falcon 460 EC i Gwarant 500 SC wykonano badania laboratoryjne na próbce glebowej o uziarnieniu gliny piaszczystej. Oprócz analiz mikrobiologicznych i biochemicznych oznaczono pozostałości substancji aktywnych wchodzących w skład testowanych fungicydów. Oznaczenia różnorodności strukturalnej wykonano w oparciu o sekwencjonowanie następnej generacji (NGS), natomiast pozostałości fungicydów metodą chromatografii cieczowej sprzężonej ze spektrometrią mas (LC-MS/MS). Wykazano, że w glebie poddanej działaniu fungicydów pojawiły się zmiany w różnorodności bakterii, szczególnie na poziomie rodziny i rodzaju. We wszystkich próbkach gleby dominowały Proteobacteria, Firmicutes i Actinobacteria. *Bacillus* występował zarówno w glebie kontrolnej jak i zanieczyszczonej fungicydami, zaś *Pseudonocardia* tylko w obiektach zanieczyszczonych fungicydami. Spośród testowanych fungicydów do największych zmian przyczynił się Gwarant 500 SC. Testowane preparaty nie tylko wpływały na skład mikrobioty glebowej, ale również przyczyniły się do zmian właściwości biochemicznej gleby poprzez zahamowanie aktywności niemal wszystkich testowanych enzymów. Wyjątek stanowiła fosfataza alkaliczna i β -glukozydaza. Najszybciej degradowany był chlorotalonil, zaś najwolniej spiroksamina.

Słowa kluczowe: fungicydy, zanieczyszczenie, mikrobiom gleby, enzymy, degradacja