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Qualitative Changes in the Community of Copiotrophic Bacteria Accompanying Reduction of the Fungal Community in Soil

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Abstract

Reduction of the fungal community in soil caused by the addition of the fungicide methiram and the antibiotic actidione was accompanied by an intensive development of the community of copiotrophic bacteria. Irrespective of the fungal toxic substances used, there was a particularly conspicuous increase in the numbers of bacteria belonging to fluorescent *Pseudomonas*, among which three morphologically and physiologically different groups were distinguished. All the isolated strains of *Pseudomonas* were mycolytic and antibiotic for typical bacteria and actinomycetes; most of them decomposed methiram, but none decomposed actidione.

Moreover, propagating rapidly in the presence of methiram were also bacteria from the species *Vibrio proteolyticus (Aeromonas proteolyticus)*, and in the presence of actidione, bacteria from the species *Sporocytophaga myxococcoides*. The *Vibrio* strains, but not those of *Sporocytophaga,* were also mycolytic, like fluorescent *Pseudomonas*. Contrary to *Pseudomonas*, no strain of *Vibrio* nor of *Sporocytophaga* was antibiotic for typical bacteria and actinomycetes or able to decompose methiram or actidione.

Keywords: soil, fungitoxic substances, bacteria.

Introduction

The decay of numerous cells of fungi as a result of the introduction of the fungicides captan and methiram, as well as the antibiotic actidione, into the soil was found to cause an increase in the number of copiotrophic bacteria [1, 2, 3]. However, taxonomical changes which accompanied this propagation remain unrecognized. It is only known that the discussed increase is of crucial importance for bacteria from other ecotrophic groups, because it involved a decline in the numbers of actinomycetes and the soil bacterial community as a whole.

In the present work, investigations were made of copiotrophic bacteria that developed in the soil especially intensely as a result of a reduction in the number of fungi. Attention focused on the response of fluorescent *Pseudomonas* because of their significance for biodegradation processes and for the health of plants. Studies of bacteria resistant to chlorocycline and capable of growth on Martin's fungus-selective medium were also conducted. Such bacteria are usually scarce in the soil, but, as our preliminary (unpublished) research has shown, sometimes they can become abundant in conditions especially favorable to bacteria in general.

Material and Methods

Experiments were carried out with soil incubated in laboratory conditions. The soil was limed loose sand of pH_{H_2O} 7.0, containing 6% silt and clay jointly and 0.8% organic C. It was taken from the field pluogh layer in autumn, after rye harvest. The soil was sieved (2 mm mesh) and moistured up to 50% of its total WHC. Thus prepared, a part of the soil was treated with methiram (preparation Polyram-Combi 80 WP, produced by BASF, Germany), another part with actidione (a product of Upjohn Company Kalamazoo, Michigan, USA), and the rest was left without any addition and considered control. The dose of methiram amounted to 100 $\mu g \cdot g^{-1}$ dry wt, and of actidione, 1000 $\mu g \cdot g^{-1}$ dry wt. With these doses the number of fungi in the soil samples dropped, respectively, to 10-20% and to over 60%, as in our previous work [2]. The soil, treated in such a way, was put into jars, 1 kg of fresh mass per jar, in two replicates for each experimental combination, and then incubated at 28°C at constant moisture of 50%. After various incubation times the soil samples were tested using the plate method and applying the following media: full-strength nutrient broth (the NB medium) for the total number of copiotrophic bacteria [7], King's medium B for

Table 1. Impact of methiram (100 μg · g^{-1} dry wt of soil) and actidione (1000 μg · g^{-1} dry wt of soil) on the number of copiotrophic bacteria (x10^6 · g^{-1} dry wt of soil) in incubated soil samples.

Experimental combination	Number					
	Day of incubation					
	0	2	4	7	10	20
Control – without no addition	36	37	32	27	33	24
+ methiram	36 [100]	36 [97]	67 [209]	67 [248]	43 [130]	30 [125]
+ actidione	36 [100]	43 [116]	86 [269]	97 [359]	114 [343]	61 [254]

Explanation: in brackets – percentage in relation to the control.

the number of fluorescent *Pseudomonas* [4], and Martin's [5] medium for number of bacteria resistant to chlorocycline. When the bacteria under study reached their maximum numbers in the soil, from colonies which grew up on the agar plates, the bacteria growing in pure culture were derived and 10 cultures for each of bacterial groups investigated. Next, following Bergey's manual [6], attempts were made to determine the taxonomic status of the isolated bacteria. Studies were also carried out on their mycolytic properties using the multi-layer plate method [8], their antibiotic activity towards the typical copiotrophic bacteria and actinomycetes isolated from the control soil, using the cross-streak test, and their ability to utilize methiram and actidione, and sometimes other fungicides, as their sole organic C source, using a modified Wallace-Lockhead medium [9] in which 1 g glucose per 1 dm^3 medium was replaced by 500 mg of fungicide. Only those bacteria were recognized as being capable of utilizing the preparation in question that still grew on the medium after the fifth cultivation.

Results and Discussion

The results presented in Table 1 are in agreement with the previous ones [1, 2, 3]. They prove that the treatment of the soil with antifungal substances helps copiotrophic bacteria propagate. They also indicate the application of methiram to have a weaker effect than that of actidione, which is a substance completely harmless towards bacteria. Thus, they confirm the option about the toxicity of methiram for some copiotrophs [3].

The morphology of colonies of bacteria isolated from the soil suggested that the application of both methiram and actidione caused the selection of some genera of species from the copiotrophic community. In bacteria isolated from the methiram-treated soil only two types of colonies were distinguished. The colonies of those isolated from the soil treated with actidione were more different but one type was still dominant, which was not observe in bacteria isolated from the control soil. This type had the morphology of one of the dominant types in bacteria isolated from the methiram-treated soil. The colonies of each dominant type contained gram-negative with polar flagellae that are characteristic for bacteria of the genus *Pseudomonas*, among others.

Table 2. Impact of methiram (100 μg · g^{-1} dry wt of soil) and actidione (1000 μg · g^{-1} dry wt of soil) on the number of fluorescent *Pseudomonas* (x10^3 · g^{-1} dry wt of soil) in incubated soil samples.

Experimental combination	Number					
	Day of incubation					
	0	2	4	7	10	20
Control – without no addition	90	40	20	20	20	20
+ methiram	90 [100]	1780 [4450]	1460 [7300]	540 [2700]	1390 [6950]	690 [3450]
+ actidione	90 [100]	160 [400]	360 [1800]	200 [1000]	110 [550]	180 [900]

Explanation: in brackets – percentage in relation to the control.

There is direct evidence of the propagation of *Pseudomonas* in the conditions of a limited number of fungi, viz. the results of quantitative determinations of fluorescent forms of the discussed bacteria performed using King's medium B (Table 2). The number of fluorescent *Pseudomonas* in the control soil without the addition of antifungal preparation maintained at the level of 10^4 · g^{-1} dry wt of soil. After application of this preparation the number amounted to 10^5 – 10^6 · g^{-1} dry wt of soil, depending on the kind of preparation and the time of analysis. In the methiram-treated soil, despite a comparatively weaker increase in the total number of the copiotrophic bacteria, the number of the fluorescent *Pseudomonas*, which belongs to this group, was multiplied much more intensely, up to 73 times, than in the actidione-treated soil, where it increased 18 times at most.

The determined chlorocycline-resistant bacteria also belonged to those copiotrophs that propagated particularly fast after the application of antifungal substances. They responded to them even more strongly than fluorescent *Pseudomonas* isolated on King's medium B (Table 3). Still, as in those *Pseudomonas*, that effect on methiram was greater than that of actidione. In 1 g control soil the number of chlorocycline-resistant bacteria was below 20 thousand, while in the soil treated with actidione it sometimes amounted to almost 100,000, and in that with methiram it ranged from 1.5 to over 3 milion.

Table 3. Impact of methiram (100 μg · g^{-1} dry wt of soil) and actidione (1000 μg · g^{-1} dry wt of soil) on the number of chlorocycline resistant bacteria (x10^3 · g^{-1} dry wt of soil) in incubated soil samples.

Experimental combination	Number					
	Day of incubation					
	0	2	4	7	10	20
Control – without no addition	3	19	12	1	10	2
+ methiram	3 [100]	2078 [10937]	1588 [13233]	3129 [312900]	3238 [32380]	1561 [78050]
+ actidione	3 [100]	58 [305]	94 [783]	91 [9100]	29 [290]	2 [100]

Explanation: in brackets – percentage in relation to the control.

Among fluorescent *Pseudomonas* isolated on King's medium B from the methiram-treated soil, two morphologically different groups could be distinguished. Out of 10 strains, 7 were assigned to a group we named K1, and 3 to one named K2. Colonies of the 10 strains isolated from the actidione-treated soil were identical, the same as K1 strains.

When carrying out taxonomic identification we only succeeded in establishing the species membership of chlorocycline-resistant bacteria. This varied and depended upon their origin. All isolates coming from the methiram-treated soil belonged to the species *Vibrio proteolyticus (Aeromonas proteolyticus)*, and those from the actidione-treated soil to the species *Sporocytophaga myxococcoides)*. In an additional experiment carried out solely with methiram, it was found that not only the sort of the applied fungitoxic preparation determined the species membership of the isolates in question. In this experiment, in which we used the same soil as in the basis experiments (not collected after rye harvest, but in its tilling phase during cultivation in the next vegetation season), a fast propagation of chlorocycline-resistant bacteria was observed. Their number increased after 2, 4, 7, 10 and 20 days of incubation 7.5, 61, 2823, 93 and 103 times, respectively, in comparison with the control soil. These bacteria, however, did not represent the species *Vibrio proteolyticus*, but they were the fluorescent strains of *Pseudomonas*, and we marked them with the symbol M.

We were unable to overcome the difficulties in the full identification of membership of the *Pseudomonas* isolates because their physiological properties, which we determined and listed in Table 4, showed that both K1 and K2 strains as well as M strains belonged, according to Bergey's Manual of Determinative Bacteriology, to the Vth group of *Pseudomonas*, including species that have not yet been described sufficiently well from newly isolated bacteria to be identified with them.

When considering the determined properties of the *Pseudomonas* isolates in terms of their importance for biochemical processes occurring in the soil, it is worth noting that all of them decomposed fats and lecithin. Some (K1 and K2 strains) were proteolytic, and other (K2 and M strains) reduced nitrates. K2 strains also decomposed chitin, and M strains decomposed starch or cellulose.

What was particularly significant in the research described was the fact that all the *Pseudomonas* isolates had mycolytic properties with respect to at least one of the two test fungi (Table 5). Also, strains with a negative reaction to chitinase activity dispolayed such properties. Another very significant feature was the ability of strains K1 and M to use methiram, but not actidione, as a sole organic C source.

The above data indicate that the propagation of fluorescent strains of *Pseudomonas* after treating the soil with fungicides was caused by supplying these microorganisms with additional nutrients contained in the fungal hyphae and the given preparation itself. In the soil with methiram the majority of strains, consisting of K1 and M strains, could use both these sources of food, and the minority (K2 strains) – only dead hyphae.

It is also worth noting that the fluorescent strains of *Pseudomonas* propagating in the soil treated with a specific antifungal substance may be able to decompose not only this preparation, but others acting in the same way. Thus, K1 strains propagated in the soil with actidione could decompose methiram. M stains isolated from the methiram-treated soil also made use of other fungitoxic preparations with active substances from a variety of chemical groups. These were the preparations: Ridomil (8% methylalaxyl + 64% mancozeb), Bayleton (5% triadimephon), Topsin M (70% methyl thiophanate), Cynkotox (65% zineb), Dithane M45 (80% mancozeb), suspension Captan (50% captan), Euparen (50% dilorfluamide), and

Table 4. Characteristics of fluorescent *Pseudomonas* strains multiplied in soil samples as a result of their methiram treatment.

Characteristics	Strains		
	K1[a]	K2[a]	M[b]
Cell diameter, μm	0.6	0.6	0.7
Cell length, μm	1.6	1.2	2.8
Number of flagella	1–2	1–2	1
Pyocyanine production	–	–	–
Pyoverdine production	+	+	+
Phenazine monocarboxylate pigments	–[c]	+[d]	+[e]
Others not carotenoids pigments[f]	–	–	–
Requirement of growth promoting substances	–	–	–
Cellular pigments	–	–	–
Oxidase reaction	+	+	+
Catalase reaction	+	+	+
Acid products from glucose	–	–	–
Levan formation from sucrose	–	–	–
Polihydroxybutyrate accumulation	–	–	–
$NO_3^- \rightarrow NO_2^-$	–	+	–
Denitrification	–	–	+
Arginine dihydrolase production	–	–	+
Arginine utilization as a single carbon source	+	+	+
Production of:			
Lipase[g]	+	+	+
Lecithinase[h]	+	+	+
Urease	–	–	+
Gelatinase	+	+	–
Amylase	–	–	–
Cellulase	–	–	–
Chitinase	–	+	–
H_2S	–	–	–
Indol	–	–	–
Pungent smell of toilet soap	–	–	+
Growth at 4°C	+	+	–
Growth at 41°C	–[i]	+[j]	+[k]
Optimal growth at 28°C	+	+	+

Explanations:
- [a, b] – isolation from soil on B King's and Martin's medium respectively,
- [c, d, e] – pigment absent in 6 week's cultures, present in 4 week's cultures, present in 3 week's cultures grown at 20°C on Burkholder's and Martin's medium, correspondingly,
- [f] – together with chloraphine and indigoidine (test on Gray's indol medium),
- [g] – Tween 80 hydrolase,
- [h] – positive reaction in egg-yolk test,
- [i] – no growth after 6 days,
- [j] – poor growth after 6 days,
- [k] – luxurient growth after 2 days.

Table 5. Ability to mycolysis, antibiotic activity towards copiotrophic typical bacteria, and towards actinomycetes and ability to utilization of methiram and actidione as a sole organic C source in bacteria which become multiplied in soil samples after their treatment with methiram and actidione.

Soil treatment	Medium for isolation	Predominant isolates	Lysis of		Antibiotic activity	Utilization of	
			Fusarium sambuccinum	*Penicillium* sp.		methiram	actidione
Methiram	King's Medium B	*Pseudomonas* sp. K1	+	+	+	+	–
		Pseudomonas sp. K2	+	+	+	–	–
	Martin's medium	*Vibrio proteolyticus**	+	+	–	–	–
		Pseudomonas sp. M	+	–	+	+	–
Actidione	King's medium B	*Pseudomonas* sp. K2	+	+	+	+	–
	Martin's medium	*Sporocytophaga*	–	–	–	–	–

* – Strain isolated from soil samples derived from under rye culture. The remainders strains were derived from soil samples taken from the field after harvest of rye.

Rubigan (12% phenarymol). They did not grow, however, if the only source of organic C was the seed dressing suspension T (75% thiram) or the preparations Bravo 500 SC (500 g chlorothalonil/1 l liquid) and Sillit (65% dodin). It should be emhasized, however, that it was not established whether the utilization by *Pseudomonas* of various fungicide preparation consisted in their metabolizing the active substance, or only its carrier.

In contrast to the fluorescent *Pseudomonas*, bacteria belonging to the species *Vibrio proteolyticus (Aeromonas proteolyticus)* found in great numbers in the methiram-treated soil, could propagate in it solely due to their mycolytic properties, because they were unable to utilize this fungicide as the sole C source. They were not able to utilize actidione as well. Bacteria from the actidione-treated soil belonging to *Sporocytophaga myxococcoides* were neither mycolytic nor capable of assimilating this antibiotic as a food. Thus, we failed to examine why these bacteria propagated in the soil under experimental conditions. What could additionally facilitate the selection of the fluorescent strains of *Pseudomonas* in methiram- or actidione-treated soil were the antagonistic properties of these organisms towards other typical copiotrophic bacteria and actinomycetes. This was established by examining three strains of *Pseudomonas* chosen at random from among the isolates obtained in the present study, one representative of each group, because it turned out that most of the tested typical copiotrophic bacteria and actinomycetes were sensitive to those strains (Table 6). Actinomycetes were sensitive to an even greater degree. This sensitivity may have been one of the causes of the already known [3] negative reaction of those microorganisms to fungitoxic substances stimulating the growth of the fluorescent *Pseudomonas*.

In contrast to fluorescent *Pseudomonas*, no antibiotic properties were found in isolates belonging to the species *Vibrio proteolyticus* and *Sporocytophaga myxococoides*.

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Table 6. Sesitivity of 20 strains of copiotrophic typical bacteria and of 20 strains of actinomycetes on various flourescent *Pseudomonas* and of *Vibrio proteolyticus* (*Aeromonas proteolyticus*) and *Sporocytophaga myxococcoides*, which multiply in soil samples after treatment with methiram and actidione.

Microbial group	Zone growth inhibition (mm)	Number of strains sensitive to				
		Pseudomonas species			*Vibro proteolyticus*	*Sporocytophaga myxococcoides*
		K1	K2	M		
Copiotrophic typical bacteria	2 – 10	12	13	8	0	0
	11 – 15	3	3	7	0	0
	> 15	5	2	2	0	0
	Total:	20	18	17	0	0
Actinomycetes	2 – 10	1	2	1	0	0
	11 – 15	12	7	8	0	0
	> 15	7	10	9	0	0
	Total:	20	19	18	0	0

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