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A Modifying Impact of Temperature on Bacteria Response to Soil Treatment with Fungicides

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Abstract

In soil-incubation experiments the action of the fungicides Impact Super and Mirage on soil microorganisms at temperatures of 10°C and 25°C has been studied. Irrespective of the applied fungicide and temperature, the reduction of fungi occurrence was accompanied by proliferation of copiotrophic bacteria, especially of fluorescent pseudomonads. Changes in the number of copiotrophic bacteria caused no increase in the soil bacterial community as a whole. Qualitative differences in the action of temperature on the response to a fungicide were found in the case of bacteria defined as chlorocycline-resistant, which proliferated only at 25°C, as well as in the case of soil respiration activity, which intensified or became weaker depending on the type of fungicide. Moreover, it has been shown that the application of fungicides also affected a percentage of forms adapted to growth at low temperatures in fungal and bacterial communities. The arrangement of the experiments also permitted comparison of the survival rate of the examined groups of microorganisms under the lack of fresh organic matter inflow.

Keywords: soil, bacteria, Impact Super fungicide, Mirage fungicide

Introduction

Fungicides are toxic not only to phytopathogenic, **but** also to saprophytic fungi. It has previously been shown [7, 8, 9,10] that their introduction into the soil not only reduces the growth of fungi, but may also generate a secondary effect expressed by significant changes in the soil bacteria community as a result of the development of some ecotrophic or taxonomic groups and reductions of others.

It should, however, be emphasized that the above observations were made at 27°C, an optimal or close to optimal temperature for the development of mesophilic bacteria, at which soil microflora is chiefly investigated. However, in view of physiological differentiations in microorganisms, one may have doubts whether the same secondary effect will occur at considerably lower temperatures, occurring in the temperate climatic zone at the time of sowing at the beginning of plant vegetation and, among others, during the application of seed dressing.

To elucidate the above doubts if partially, soil-incubation experiments were conducted to compare changes in the number of microorganisms occurring in the soil after the application of two different fungicides at 10°C and **25°C.**

Materials and Methods

The experiments were carried out with limed loose sand of pH_{H2O} 6.0 containing 8% silt and clay jointly and 0.6% organic C. The soil was taken from the field, where maize was grown in the year preceding the experiments. The soil was sampled in the spring from a 30-cm plough layer after thawing, when positive temperature became settled. The soil sample was sieved (2-mm mesh), moistured up to 50% of its total WHC and divided into two portions. One of them was stored in a laboratory at 10°C and another at 25°C.

From the soil portions stored in this way, samples were simultaneously made for incubation with and without fungicide (controls). The applied fungicides were the Impact Super seed dressing (47 g flutriophol + 300 g chlor-thalonil, produced by Imperial Chemical Industries PLC) and Mirage preparation (450 g E.C. prochloraze produced by Makhteshim, Israel). They were used at a dosage of 100 mg/ 1 kg of dry wt of soil.

The action of each fungicide was observed in a separate experimental set. In the first set involving Impact Super, the soil was examined 2 weeks after being taken from the field, while in the second set with Mirage application the soil was examined 4 months later. The soil samples were put into jars (1 kg of fresh mass per jar) in duplicate for each experimental combination, and then incubated at the same temperature at which the soil had been previously stored. After 4, 7, 10, 14, 21 and 28 days of incubation the numbers of microorganisms were determined by the plate or microscopic method. The plate method was applied to determine:

- the number of fungi using Martin's medium [13];
- the number of copiotrophic bacteria using two media: full strength nutrient broth, the so-called NB-medium [16], and King's B medium [11]. Bacteria determined in this way were called further on NB-medium copiotrophic bac teria and King's B medium copiotrophic bacteria, corres pondingly. Among the latter ones, fluorescent pseudomonads were distinguished. The identification of all the bacteria growing on King's B medium as copiotrophic was supported by their rapid growth reaction to the abun dance of nutritive substances in the environment. Besides, the number of chlorocycline-resistant bacteria, which de veloped among the colonies of fungi grown on Martin's medium, was also determined. As mentioned before [8], these bacteria showed the tendency of growth reaction to some fungitoxic compounds.

Bacteria from the above groups were differed on the basis of their temperature requirements. For that purpose, one part of agar plates inoculated with these bacteria were incubated at 27°C and another at 4°C. In the case of incubation at 27°C, the colonies of chlorocycline-resistant bacteria were enumerated after 5 days, the colonies of NB-copiotrophic bacteria after 7 days and the colonies of King's B medium copiotrophic bacteria (including fluorescent pseudomonads) after 2 days. The incubation at 4°C always lasted for 14 days, irrespective of microorganism group. The application of just that temperature to culture psychrophilic microorganisms was determined by the fact that growth at this temperature is an important diagnostic feature in taxonomy of some bacteria [15], for instance, from Pseudomonas genus. Microorganisms grown at that temperature are recognized as psychrophiles, while those grown at 25°C or 27°C as mesophiles, but one should be fully aware of the involved simplifications resulting from non-determination of cardinal temperature points in isolates.

The microscopic method was applied to determine the total number of soil bacteria. The fluorescence microscope and acridine orange as a fluorochrome was applied according to the modified [4] method of Strugger [15].

Besides determinations of the numbers of microorganisms, soil respiration activity was also determined using the sorption method [5]. Measurements were made in soil-

Table 1. Microorganism mumber determined in soil immediately after being taken from the field and sieved.

Microorganisms	Number		Psychrophiles to mesophiles ratio
	Mesophiles	Psychrophiles	
Fungi (10^3/g dry wt of soil)	79.0	60.3	0.76
Copiotrophic bacteria determined using NB-medium (10^6/g dry wt of soil)	5.2	2.1	0.40
Copiotrophic bacteria determined using King's B medium (10^6/g dry wt of soil)	1.1	1.5	1.36
Fluorescent pseudomonads (10^3/g dry wt of soil)	53.3	140.4	2.63
Chlorocycline-resistant bacteria (10^3/g dry wt of soil)	9.3	18.7	2.01

Table 2. Effect of Impact Super fungicide in soil samples incubated at 25°C or at 10°C on the numbers of mesophilic and psychrophilic fungi (10^3/g dry wt of soil) and copiotrophic bacteria (10^6/g dry wt of soil).

Microorganisms	Determined Value	25°C				10°C			
		Control		Impact Super		Control		Impact Super	
		Mesophiles	Psychrophiles	Mesophiles	Psychrophiles	Mesophiles	Psychrophiles	Mesophiles	Psychrophiles
Fungi	Number	70.0	24.0	35.1 [2.0]*)	9.2 [2.6]	80.3	52.8	15.6 [5.1]	25.9 [2.0]
	Psychrophiles to mesophiles ratio	0.34		0.26		0.65		1.66	
Copiotrophic bacteria determined using NB-medium	Number	3.0	0.9	13.8 [4.6]	7.4 [8.2]	3.8	1.3	19.6 [5.2]	13.2 [10.1]
	Psychrophiles to mesophiles ratio	0.34		0.54		0.34		0.67	
Copiotrophic bacteria determined using King's B medium	Number	3.3	0.6	39.2 [11.9]	8.3 [13.8]	1.0	1.0	25.8 [25.8]	17.6 [17.6]
	Psychrophiles to mesophiles ratio	0.18		0.21		1.00		0.68	

*)- Manifold decrease in number as compared with control, for fungi, and increase, in number for bacteria.
Statistical calculations of microbial number: Values for temperature and fungicide treatment differ significantly at $P < 0.01$, only the number of fungi in control at 25°C differ insignificantly at $P = 0.05$.

-containing jars separately prepared for this purpose after 3, 9, 15, 21 and 27 days of incubation.

Due to the large number of collected data it was impossible to present all obtained values. Therefore, mostly mean values were given in the tables and figures of this paper.

Results

Microorganisms in Soil Immediately after Its Sampling

Determinations of the numbers of microorganisms in the soil directly after its sampling and sieving (Table 1) indicated that both in the fungal and in the NB-medium copiotrophic bacteria communities the number of psychrophiles corresponded to several tens of mesophile percentages, while in King's B medium the copiotrophic bacteria community (as a whole as well as among fluorescent pseudomonads and chlorocycline-resistant bacteria) that number was even larger.

The total number of the soil bacterial community determined at that time amounted to 1.7 x 10^9/g dry wt of soil (not shown). The soil respiration activity at 25°C and 10°C was 3.4 µg C-CO_2 /g dry wt of soil/24 h and 1.6 µg C-CO_2 /g dry wt of soil/24 h, respectively.

Microorganisms in Soil Samples of the Experimental Set with Impact Super Fungicide

The data in Tables 2 and 3 compared with those in Table 1 indicates that during the incubation period the number of microorganisms determined by the cultural method in the control samples showed a tendency to decrease. That concerned first of all psychrophilic forms. The number of psychrophiles decreased more at a higher than at a lower temperature. On the other hand, the number of the soil bacterial community at 10°C was not subjected to a change, whereas at 25°C it even increased to 1.9 x 10^9/g dry wt of soil. That difference, though not large, was significant at $P < 0.05$. The respiration activity of the control soil decreased by, on average, 40% at 25°C and 20% at 10°C (Fig. 1).

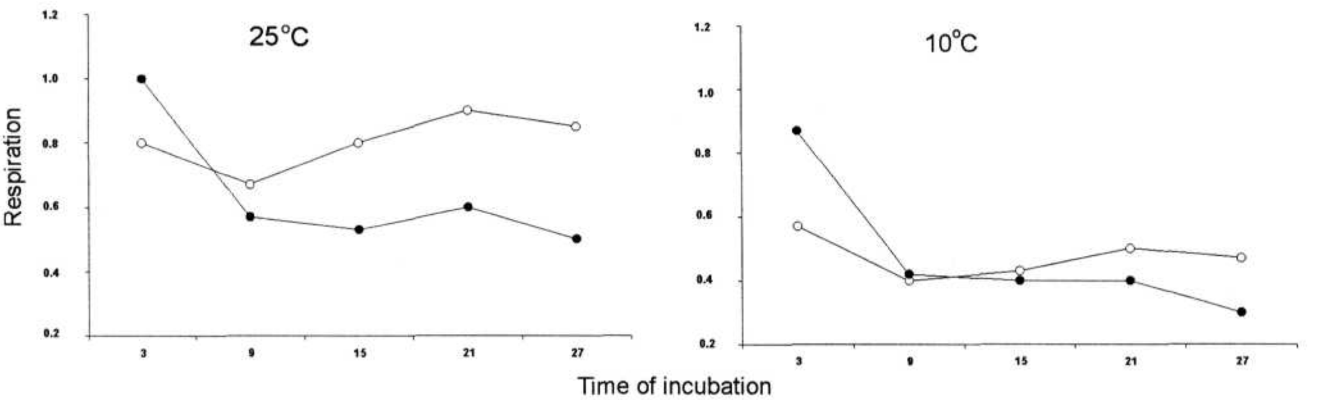

Fig. 1. Effect of "Impact Super" fungicide in soil samples incubated at 25°C or at 10°C on respiration activity µg C-CO_2 / g dry wt of soil / 24 hours), (O) - control, (●) - fungicide-treatment. Values for the treatments and for the time of analyze differ significantly at $P < 0.001$.

Table 3. Effect of Impact Super fungicide in soil samples incubated at 25°C or at 10°C on the numbers of mesophilic and psychrophilic fluorescent pseudomonads and on chlorocycline-resistant bacteria (10^3/g dry wt of soil).

Microorganisms	Determined value	25°C				10°C			
		Control		Impact Super		Control		Impact Super	
		Mesophiles	Psychrophiles	Mesophiles	Psychrophiles	Mesophiles	Psychrophiles	Mesophiles	Psychrophiles
Fluorescent pseudomonads	Number	27.0	42.8	1510 [55.9]*)	4480 [104.6]	52	179	2466 [47.4]	7846 [43.8]
	Psychrophiles to mesophiles ratio	1.6		3.0		3.4		3.2	
Chlorocycline – resistant bacteria	Number	6.4	10.6	32.3 [5.0]	84.4 [8.0]	7.7	15.6	8.7 [1.1]	16.6 [1.1]
	Psychrophiles to mesophiles ratio	1.7		2.6		2.0		1.9	

*)- Explanations as for Table 1, for bacteria.

Statistical calculations of microbial number: In the case of fluorescent pseudomonads all the values for temperature and treatment differ significatly at $P < 0.01$, but in the case of chlorocycline-resistant bacteria only the values for treatment at 25°C, because the others differ significantly at $P < 0.05$.

In comparison to the control samples, the fungal community in the soil samples treated with the Impact Super fungicide reduced by at least one half (Tables 1 and 2). At 10°C the number of its mesophilic forms was reduced even fivefold, as a result of which psychrophilic forms became quantitatively dominant.

Reduction of the fungal community after the Impact Super fungicide application was accompanied by an occurrence of bacteria from all groups (determined by cultural methods) more numerous than in the control samples (Tables 2, 3). That was already observed at the first date of analysis. The population of NB-medium copiotrophic bacteria increased 5-13-fold. Psychrophilic forms proliferated relatively more strongly, particularly at 10°C, but mesophiles continued to be dominant. The numbers of King's B medium copiotrophic bacteria increased still more significantly - 12-26 fold. At a lower temperature that effect was larger as in NB-medium copiotrophic bacteria, but mesophiles reacted more strongly than psychrophiles. Under the influence of Impact Super the number of fluorescent pseudomonads increased the most. Already 4 days after its application the number of these bacteria increased several tens of times. That multiplication was stronger at 25°C than at 10°C. Although mesophilic forms of pseudomonads proliferated more actively, the domination of psychrophilic forms continued to become evident.

Chlorocycline-resistant bacteria showed a positive growth reaction to the fungicide only at 25°C, and that reaction was relatively weak. Their number increased no more than twice.

Microscopic examination of soil showed no significant changes in the soil bacteria community after the fungicide application. Its mean number amounted to 1.8 x 10^9/g dry wt of soil at 25°C and at 1.6 x 10^9 /g dry wt of soil at 10°C. These numbers differed from those in the control samples by hardly several per cent.

Soil respiration activity (Fig. 1) after the fungicide application was initially more intensive, but later it declined and its mean value in the experimental period was lower than in the control samples.

Microorganisms in Soil Samples of the Experimental Set with Mirage Fungicide

As mentioned in "Materials and Methods," the soil samples in the experimental set with Mirage fungicide were prepared after a longer storage of the soil in the laboratory conditions than the experimental set with Impact Super.

When microbial count was made in the control samples of the experiment with Mirage (Tables 4 and 5), it was found that particular groups of microorganisms reacted differently to this prolonged lack of organic matter inflow as well as to the extension of the action of temperature higher than that under field conditions.

In the case of mesophiles their number in the fungal community did not change, whereas the number of copiotrophic bacteria estimated with the use of both applied media decreased. At 10°C and 25°C the discussed declines were more or less the same about several tens per cent. From among of mesophilic copiotrophs, fluorescent pseudomonads were eliminated particularly strongly. At 25°C their number in 1 g dry wt of soil was even below a thousand.

A declining tendency was shown also in mesophilic forms of chlorocycline-resistant bacteria, but they were continuously detectable in soil.

The occurrence of psychrophilic fungi in contrast to their mesophilic forms was still more reduced than in the first experimental set, and the reduction was of the same degree at both temperatures. The number of psychrophilic copiotrophic bacteria dropped also but more strongly at 25°C than at 10°C. Its declines repeated many times and, therefore, were more intense than those in the case of mesophilic forms of these bacteria. Psychrophilic, fluorescent pseudomonads at 25°C were reduced like mesophilic ones, whereas at 10°C they continued to be on the level of 10^4/g dry wt of soil.

The total number of soil bacteria community was on average 1.7 x 10°/g dry wt of soil at 10°C and 1.6 x 107g dry wt of soil at 25°C. These were only somewhat smaller

Table 4. Effect of Mirrage fungicide in soil samples incubated at 25°C or at 10°C on the numbers of mesophilic and psychrophilic fungi (107g dry wt of soil) and copiotrophic bacteria (10^6/g dry wt of soil).

Microorganisms	Determined Value	25°C				10°C			
		Control		Mirage		Control		Mirage	
		Mesophiles	Psychrophiles	Mesophiles	Psychrophiles	Mesophiles	Psychrophiles	Mesophiles	Psychrophiles
Fungi	Number	84.7	13.9	60.1 [1.4]*)	6.5 [2.1]	75.3	10.8	57.9 [1.3]	3.00 [3.6]
	Psychrophiles to mesophiles ratio	0.16		0.11		0.14		0.05	
Copiotrophic bacteria determined using NB-medium	Number	2.4	0.2	13.8 [5.7]	5.1 [25.5]	2.6	0.4	19.1 [7.3]	15.4 [51.3]
	Psychrophiles to mesophiles ratio	0.08		0.37		0.15		0.80	
Copiotrophic bacteria determined using King's B medium	Number	1.0	0.2	10.6 [10.6]	4.6 [23.0]	1.1	0.4	16.7 [15.2]	15.9 [79.5]
	Psychrophiles to mesophiles ratio	0.20		0.36		0.36		0.95	

*) - Explanations as for Table 1.

Statistical calculations for microbial number: Values for temperature and fungicide treatment differ significatly at P > 0.01, only the numbers of mesophilic fungi and mesophilic copiotrophic bacteria in control at 25°C differ insignificantly at P = 0.05.

values than those calculated for the first experimental set, but they were significant for the temperature of 25°C at $P < 0.05$. The decreases in soil respiration activity were more marked than in the experimental set with Impact Super application.

The applied dose of the Mirage fungicide was a dose reducing the development of fungi like that of the Impact Super fungicide. But Mirage unlike Impact Super reduced more strongly the development of mesophilic than psychrophilic forms of these organisms, particularly at 10°C (Table 4). Mirage as well as Impact Super also contributed to the propagation of copiotrophic bacteria, as was found irrespective of medium used for their enumeration. It was more intensive at 10°C than at 25°C. Psychrophiles propagated particularly strongly. Their community was at least only somewhat smaller than that characteristic of soil samples treated with Impact Super.

When making the above comparisons, it should, however, be taken into account that the Mirage and Impact Super fungicides were not applied at the same time and, therefore, bacterial communities responding to a definite fungicide were not identical.

Fluorescent pseudomonads after Mirage application also propagated considerably, more strongly than all the copiotrophic bacteria (Table 5). At a temperature of 25°C, at which initially they were not numerous in the soil, their numbers increased over 1000-fold, achieving 10^6/g dry wt of soil. Though at 10°C multiplication of their numbers was only over 100-fold, but since initially they were relatively more numerous, their absolute numbers continued to be larger than those at 25°C. At both applied temperatures, psychrophilic forms of fluorescent pseudomonads were more numerous than mesophilic forms so that nearly each colony of psychrophilic and each tenth colony of mesophilic King's B medium copiotrophic bacteria deriving from Mirage-treated soil fluorized in a way characteristic of pseudomonads.

Chlorocycline-resistant bacteria proliferated in the presence of Mirage as in the presence of Impact Super, i.e. exclusively at 25°C, and only mesophilic forms proliferated.

The application of Mirage had no significant influence on the total number of the soil bacterial community. At 25°C that number amounted to 1.6 x 10^9/g dry wt of soil, and 1.5 x 10^9/g dry wt of soil at 10°C, and these values insignificantly differed from those estimated in the control soil.

Soil respiration activity in the presence of Mirage was more intense (at least initially), beginning from the first analyses (Fig. 2). Mean amounts of CO_2 liberated during the whole experimental period were also increased.

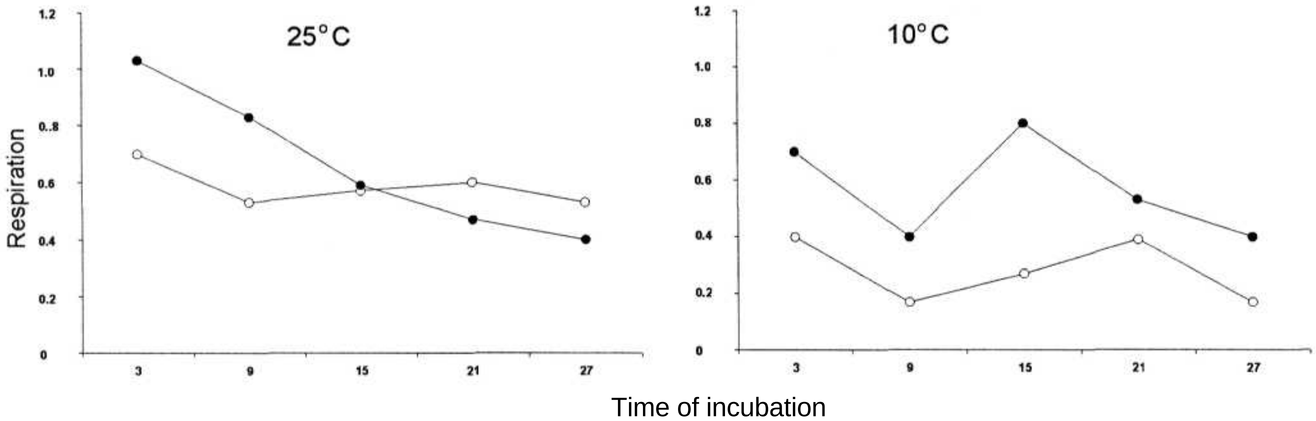

Fig. 2. Effect of "Mirage" fungicide in soil samples incubated at 25°C or at 10°C on respiration activity µg C-CO_2 / g dry wt of soil / 24 hours), (O) - control; (•) - fungicide-treatment. Values for the treatments and for the time of analyze differ significantly at $P < 0.001$.

Table 5. Effect of Mirage fungicide in soil samples incubated at 25°C or at 10°C on the number of mesophilic and psychrophilic fluorescent pseudomonads and chlorocycline-resistant bacteria (10^3/g dry wt of soil).

Microorganisms	Determined value	25°C				10°C			
		Control		Mirage		Control		Mirage	
		Mesophiles	Psychrophiles	Mesophiles	Psychrophiles	Mesophiles	Psychrophiles	Mesophiles	Psychrophiles
Fluorescent pseudomonads	Number	< 1	< 1	960 [> 960]*)	3180 [> 3180]	6	38	1450 [242]	13080 [344]
	Psychrophiles to mesophiles ratio	–		3.3		6.3		9.0	
Chlorocycline – resistant bacteria	Number	4.5	14.7	64.3 [14]	18.2 [1]	4.4	17.7	6.1 [1]	6.9 [< 1]
	Psychrophiles to mesophiles ratio	3.3		0.2		4.0		1.0	

*) - Explanations as for Table 1.

Statistical calculations of microbial number: In the case of fluorescent pseudomonads all the value for temperature and treatment differ significatly at $P < 0.01$, but in the case of chlorocycline-resistant only the values for treatment at 25°C, because the others differ insignificantly at $P < 0.05$.

Discussion

Though the subject of this paper was the reaction of soil microorganisms to fungicides, soil microflora analyses of the control samples not treated with these fungicides, permitted comparison to the response of their studied groups to temperature and starvation conditions.

The numbers of psychrophilic fungi and bacteria determined in the studied soil were rather significant. Results of earlier studies discussed by Stefaniak [18] suggest that psychrophiles constitute only several per cent of soil microorganisms. Nevertheless, it should be taken into account that in some of these studies microorganisms growing at 2°C or 0°, i.e. at the temperature below 4°C at which the isolated obtained in our studies were recognized as psychrophiles. The fact that in the soil taken from the field where the mean monthly temperature in Poland from November to April is below 9°C [14], and later stored in a laboratory at a temperature higher than that in the field, the percentage of psychrophiles gradually decreased attracts attention. This is not in agreement with some opinions [18] claiming that psychrophilic communities change a little in different seasons of the year.

As could be expected, microorganisms particularly well adapted to growth at a low temperature were bacteria from the Pseudomonas genus. Nevertheless, the fact that more pseudomonads were isolated at 4°C than at 25°C is not in line with the taxonomic characteristics of these bacteria [15], according to which the majority of them as a matter of fact grow at 4°C, but their optimal growth temperature is 28°C. It might also be suspected that a two-day incubation of plates at 28°C was too short to obtain the colonies of all fluorescent pseudomonads strains, but its extention for 4 days gave no effect.

Since there are available data [12] that a temperature of 28°C is not only reducing, but optimal for pyoverdin biosynthesis (a fluorescent pigment distinguishing colonies of fluorescent pseudomonads from the others), it could not be excluded that numerous strains among pseudomonads occurring in the studied soil had lower growth temperatures than more thoroughly known pathogenic strains found on food products, on the properties of which bacteria classification is chiefly based.

Though starvation conditions influenced psychrophilic microorganisms, the data permitting a show of significant differences in the response of particular microorganism groups to that agent were supplied first of all by changes in the number of mesophiles, particularly those occurring at 25°C, i.e. at optimal or close to optimal temperature for their metabolic activity. An extreme opposite of fungi, described already half a century ago as organisms capable of supporting growth only at trace amounts of food [17], as well as of the soil bacterial community as a whole with dominating oligotrophs in it [6] appeared to be fluorescent pseudomonads, which are distinguished among copiotrophs by a particularly bad tolerance to starvation. This not only supports the opinion [1] that the insufficient nutrient content in soil may be one of significant reasons of failures of the persistence of PGPR strains of pseudomonads introduced into the soil, but also indicates that in the soil the resource of nutritive substances should be larger for pseudomonads than for the other copiotrophs.

Observations concerning the ways of fungicide action on microorganisms in soil are in agreement with our earlier observations [7, 8, 9, 10]. Reduction of fungal communities was also accompanied by the propagation of copiotrophic bacteria, particularly fluorescent pseudomonads, which increased the soil bacterial community as a whole. It was shown that changes in soil respiration activity may have different directions under conditions of fungicide application, which may be explained by a different degree of copiotrophic bacteria activity compensating for reduction of fungal activity.

It seems that the above effects can be expected with a large approximation in the case of any fungicide application. Studies of 14 fungicides from different chemical groups in the present and previous papers [7, 8, 9, 10] showed that the total number of copiotrophic bacteria increased after application of 10 chemicals and that fluorescent pseudomonads propagated even in the case of 12 preparations.

Our observations on the influence of temperature upon the response of microorganisms to fungicides showed that copiotrophic bacteria propagate after their treatment not only at a temperature of 25°C favorable to mesophile development, but also at 10°C (i.e. a minimal temperature for these organisms, but suitable for the development of psychrophiles). An exception were chlorocycline-resistant bacteria propagating only at 25°C. These bacteria isolated from soil and tested in the additional experiment appeared to be fluorescent pseudomonads having physiological properties of M strain, isolated by us from the soil treated with Methiram fungicide [10], capable of growth at 41°C , but not at 4°C and, therefore, more adapted to a higher temperature than the majority of pseudomonads.

It is also worth mentioning that in other bacteria soil temperature was of no importance for their response to fungicides, but the noted differences had only a quantitative character and were differently directed. Thus, for example, the total numbers of copiotrophic bacteria increased after fungicide application more strongly at 10°C than at 25°C, whereas in the case of fluorescent pseudomonads the reverse was true. Temperature also significantly influenced the toxic fungicide effect to fungi.

Even at a 1000-fold increase of fluorescent pseudomonad numbers under the influence of fungicides, occurring at the same time, when these bacteria in the control soil disappeared as a result of starvation indicated that these preparations contributed to obtaining additional amounts of food by pseudomonads. That food was probably decayed fungal hyphae, but that could also be an active substance of the used fungicide or its carrier. Moreover, food competition of fungi was considerably reduced.

Fungicides not only changed the bacterial number but also their temperature requirements through increasing or decreasing the percentage of forms adapted to growth at 4°C, partially strictly psychrophilic and partially psychrotrophic. The direction of these changes depended on different factors, namely on microorganism group, applied fungicide and soil temperature.

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