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Nonfluorescent Pseudomonads in Soils and Hortical Substrates, their Numbers and Properties

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Abstract

Nonfluorescent pseudomonads have been studied by estimating their numbers in different soils and hortical substrates as well as some physiological properties and antagonistic relationships between them and fluorescent pseudomonads and actinomycetes. Nonfluorescent pseudomonads, depending on the studied soil or substrate, constituted 7-10% of the total number of bacteria from genus *Pseudomonas,* and the dominant among them were pectinolytic psychrotrophs. Antagonism of nonfluorescent pseudomonads was less frequent and less intense than that of fluorescent pseudomonads as counter-partners. On the other hand, in the system of interrelationships with actinomycetes, the antagonism of nonfluorescent pseudomonads was relatively more frequent, though its intensity was lower.

Keywords! soil, nonfluorescent pseudomonads, fluorescent pseudomonads, actinomycetes, antagonism

Introduction

Fluorescent pseudomonads are bacteria known for their inhibitory activity. This results, among other things, from their ability to produce siderophores [8, 11, 13], antiobiotics [4, 5, 20, 21] and cyanides [1, 11, 16]. In studies of interrelationships between these bacteria and soil actinomycetes [6] it was found that the first of them on some media can have a stronger inhibitory effect than the others.

However, besides fluorescent forms intensively studied in soil nowadays, nonfluorescent forms are also referred to the genus *Pseudomonas.* In the VHI-th edition of Bergey's Manual of Determinative Microbiology [2], only 44 out of 215 bacterial species, whose properties coincided or seemed to coincide (on account of a not quite accurate examination) with properties characteristic of the genus *Pseuodomonas,* were found to have the properties of producing diffusible fluorescent pigment. In the next edition of the mentioned manual [15] - the number of such bacterial species constituted 19 out of 45 distinguished ones. The occurrence of nonfluorescent *Pseudomonas* in soil and physiological properties of these bacteria soil strains, in contrast to fluorescent pseudomonads, are, however, very little known.

The above situation within the genus *Pseudomonas* has encouraged us to undertake studies to compare the numbers of nonfluorescent and fluorescent forms of these bacteria in different soils and hortical substrates. Strains of nonfluorescent pseudomonads isolated from these environments were examined in a physiological aspect, taking into account first of all properties important for biochemical processes occurring in the soil, as well as their manner of action and response (inhibition and sensitivity) to fluorescent pseudomonads and soil actinomycetes.

Materials and Methods

Nonfluorescent pseudomonads of 8 soils and 3 hortical substrates were examined. Soil samples were taken

from the plough layer (0-20 cm). Hortical substrates were commercial articles. To obtain the isolates of pseudomonads serial dilutions of the tested material were made in 0.5% peptone [23] and a selective Grant-Holt's agar medium [3] was spread with 0.1 ml portion of each dilution and incubated at 30°C for 30 h. That medium was trypticase soil agar (TSA) with the addition of 9 mg of basic fuchsin, 100 mg cycloheximide, 140 mg TTC, 10 mg nitrofurantoin and 23 mg nalidixic acid and of final pH 7.2.

The number of bacterial colonies grown up on the plates with that selective medium was recognized as the total number of pseudomonads. To distinguish nonfluorescent from fluorescent forms, bacteria from 100 randomly selected colonies in each experimental combination were transferred onto King's B medium [8] and after 3 days of culture at 30°C examined for the lack of fluorescence under a source of ultraviolet light. The nonfluorescent bacteria were grown in pure cultures and subjected to further tests. Only those strains, which were motile, gram-negative slender rods, oxidase and catalase positive, forming no chains, flocks and pleomorphic forms, not growing at pH 4.5 and growing in the presence of 0.1% triphenyl tetrazolium chloride were recognized as nonfluorescent pseudomonads. Their portion in the whole community of pseudomonads was calculated by a comparison of their number with that of fluorescent strains.

Using the above technique, 68 nonfluorescent *Pseudomonas* strains were isolated in pure cultures. All these strains were examined for the ability to grow at temperature of 4°C after 7 days and at 42°C after 48 hours (King's B medium), the ability to decompose starch (on starch-broth medium), pectins [9], lipids [22] and proteins (on gelatine medium). They were also examined for heterotrophic nitrification [14], NO_3 reduction to NO_2 (Griess reagent) and gas reduction (bubbles production on nitrate medium, no reaction with Griess reagent after Zn reagent application) as well as for cyanide production on King's B medium enriched with glycine and $FeCl_3$ [16].

Studies on the phenomenon of antagonism and sensitivity were performed with 20 nonfluorescent strains randomly selected among all isolates, with 20 strains of fluorescent pseudomonads and with 19 strains of soil actinomycetes.

Some fluorescent pseudomonads and all actinomycetes were previously described by us [6]. The remaining fluorescent pseudomonads were freshly isolated from the soil treated with impact fungicide [7]. Identification with the use of the API 2ONE numerical test has classified them to the species *Pseudomonas fluorescens,* but they significantly differed from one another by physiological properties, which made some of them similar to representatives of the species *Pseudomonas aureofaciens.*-

All strains were maintained and proliferated to obtain an inoculum on Burkholder's medium containing g/dm^3 of extract from 300 g of peeled potatoes: $NaHPO_4$ x 12 H_3O, 2; sodium nitrate, 10; dl-asparganine, 1.0; bactopeptone, 5; dextrose, 6; agar, 15 g. The pH was adjusted to 7.0.

Inhibitory activity was determined by the conventional streak method. The media applied in this test had a composition, which on the basis of our previous investigations [6], could be recognized as optimal for manifestation of inhibitory properties of the studied microorganisms. Pridham-Gotllieb's medium [19] (PGM) and King's B medium [8] (KBM) were simultaneously used for both fluorescent and nonfluorescent pseudomonads, while potato-dextrose agar medium (PDA) was used for actinomycetes.

The inocula were prepared from a 24-h culture of pseudomonads and a 7-day culture of actinomycetes. Indicator microorganisms were perpendicularly inoculated to a 2- or 5-day old streak growth of the strain examined for inhibitory activity, depending on whether it was *Pseudomonas or Actinomycetes* strain. Growth-inhibition zones of these indicator strains were measured after 2 and 3 growth days, respectively. The lack of growth at a distance of no less than 3 mm was taken as a positive result.

All the bacteria were grown at 28°C.

Table 1. Occurence of nonfluorescent forms in pseudomonads communities of various soils and hortical substrates.

Soil or substrate	Total number of pseudomonads (10^3/g dry wt of soil)	% of nonfluorescent pseudomonads
Muck	40	7
Black earth	37	9
Loamy soil	28	8
Loose sand	13	10
Loose sand treated with "Impact" Fungicide*	58	9
Loose sand treated with "Zaprawa nasienna" seed dressing*	47	8
Loose sand treated with "Dithane" fungicide*	34	7
Garden soil manured with compost	138	10
Peaty – bark substrate	72	8
"Seedling – soil" substrate	47	8
"Palm soil" substrate	4	6

* Soil samples incubated for 14 days with 100 mg fungicide per kg dry wt.

Table 2. Some characteristics of 68 isolated strains of nonfluorescent pseudomonads.

Charactristics	Number of		
	typical positive reactions	weak reactions	typical negative reactions
Optimal growth at 28°C, 2 days	68	0	68
Growth at 4°C, 6 days	52	6	10
Groth at 41°C, 2 days	3	0	65
Acid produced from glucose	3	0	65
Starch hydrolysis	15	0	53
Pectin hydrolysis	49	11	8
Lipase (Tween 80 hydrolysis)	0	0	68
Gelatin hydrolysis	27	3	38
NO_3 reduction only to NO_2	26	3	39
Denitrification	20	2	46
Heterotrophic nitrification	0	0	68
Cyanide formation	9	12	47

Table 3. Antagonism and sensitivity of 20 nonfluorescent to 20 fluorescent pseudomonads growing on Pridham - Gottlieb's medium (PGM) and King's B medium (KBM).

Strains of nonfluorescent pseudomonads	Antagonism						Sensitivity								
	Number of inhibited fluorescent pseudomonads		Inhibition zone (in mm)				Number of inhibitory fluorescent pseudomonads					Inhibition zone (in mm)*			
			Range		Average							Range		Average	
	Total on PGM	Total on KBM	On PGM	On KBM	On PGM	On KBM	Total on PGM	Only on PGM	Total on KBM	Only on KBM	Both on PGM and KBM	For PGM	For KBM	For PGM	For KBM
1N	4	0	3-6	0	4	0	16	9	8	1	7	3-12	3-17	8	11
2N	16	0	4-12	0	8	0	18	11	8	1	7	6-14	3-18	10	11
3N	0	0	0	0	0	0	16	1	18	3	15	4-18	3-12	8	11
4N	6	0	4-11	0	6	0	17	13	5	1	4	5-15	4-17	9	13
5N	20	0	4-20	0	10	0	17	10	8	1	7	3-14	4-18	8	12
6N	18	0	3-20	0	9	0	15	11	4	0	4	4-16	8-21	10	14
7N	6	0	3-7	0	5	0	14	10	4	0	4	4-15	11-20	9	16
8N	13	0	5-12	0	9	0	17	12	5	0	5	4-14	3-20	8	12
9N	18	0	3-15	0	7	0	13	0	20	7	13	3-12	4-21	8	11
10N	4	0	3-6	0	4	0	3	0	17	14	3	4-12	3-18	8	10
11N	15	0	4-13	0	8	0	18	13	6	1	5	3-14	10-16	9	14
12N	0	0	0	0	0	0	11	11	0	0	0	3-11	0	5	0
13N	0	0	0	0	0	0	8	7	1	0	1	3-10	3-3	5	3
14N	1	0	3-4	0	4	0	5	1	14	10	4	3-11	3-14	6	9
15N	4	0	4-9	0	6	0	5	3	5	3	2	3-13	4-15	7	11
16N	20	0	5-18	0	11	0	17	8	10	1	9	3-14	4-16	10	10
17N	2	0	4-5	0	5	0	13	6	11	4	7	3-12	5-13	8	11
18N	20	0	5-18	0	9	0	10	9	2	1	1	3-12	6-10	8	9
19N	20	0	5-19	0	12	0	11	9	2	0	2	3-13	3-11	10	6
20N	14	0	3-12	0	6	0	15	4	13	2	11	5-15	7-19	10	13
Average	10	0	3-12	0	6	0	13	7	8	3	6	4-13	5-15	8	11

* Standard deviation ± 1 mm

Results and Discussion

In studied soils as well as in hortical substrates the total number of pseudomonads ranged within 10^3 - 10^5/g dry wt of examined material and was mostly of the order of 10^4/g dry wt of examined material (Table 1). These numbers were within the range determined by different authors in non-rhizosphere soils [7, 16, 17, 24].

In the discussed communities of pseudomonads, the fluorescent forms were always predominant in number, irrespective of the kind of soil or hortical substrate. The number of nonfluorescent pseudomonads, despite the abundance of species of these organisms occurring in nature, was hardly of the order of 10^3/g dry wt of examined material, except the garden soil manured with compost, where it was of the order of 10^4/g dry wt of examined

material. With such numbers, the nonfluorescent forms constituted only 6-10% of the community of pseudomonads, and their portion, therefore, was small, and more or less equal.

When determining physiological properties of nonfluorescent pseudomonads isolates, it was found that they were mainly psychrotrophs (Table 2). Though an optimal temperature for all isolates was 28°C, only few of them did not grow luxuriantly at 4°C after 6 days.

Despite the fact that all the isolates had oxidative metabiolism, 3 strains produced acid compounds from glucose. Starch decomposers were 22% of strains. Attention is drawn by the frequency of pectinolytic forms, which constituted over 82% of the isolates. No strain was found to have lipolitic properties, whereas half of them hydrolyzed proteins. Nitrates were reduced by $^{3/4}$ of strains, either only to NO^{-}_{2}, or with production of gas products. However, no ability of heterotrophic nutrification was found. Some strains were cyanogenic, but produced cyanides mostly in trace amounts.

As follows from Table 3, nonfluorescent pseudomonads under suitable conditions may show inhibitory properties towards fluorescent forms of these bacteria. The ability to inhibit their growth was found in cultures on PGM, but did not occur on KBM. On PGM, most nonfluorescent strains (17 out of 20) inhibited fluorescent strains, but the spectrum of that inhibition was differential. Some nonfluorescent strains inhibited only several fluorescent pseudomonads, others - over a dozen or so, or even all of them. The inhibition zones were from 3 to 20 mm wide, on average, of the order of 6 mm.

It might be suggested that the fact that inhibitory properties of nonflurescent pseudomonads were unrevealed in the case of their culture on KBM was caused by Fe^{+++} deficiency in that medium. Different microorganisms require iron for production of antibiotics [11]. Enrichment of KBM in Fe^{+++} by addition of 15 µg FeCyml, however, did not cause the inhibition of fluorescent strain growth by nonfluorescent strains of pseudomonads (not shown). An exception was two nonfluorescent pseudomonads. These were strains N13 and N14, which in 2 and 5 fluorescent strains, respectively, after 24 hours of a joint culture, caused the occurrence of 8 - 10-mm growth-inhibition zones, that being only a delay of growth, which occurred during the further, 2-day incubation.

Although nonfluorescent pseudomonads were inhibitory towards fluorescent strains of these bacteria, they also could be inhibited by them. That sensitivty, in contrast to antagonism, was manifested by all nonfluorescent pseudomonads on PGM. Moreover, the inhibition of their growth was relatively stronger. Particular nonfluorescent strains were inhibited, on average, by more fluorescent strains than those by their counter-partners (13 in comparison to 10). Besides, they were sensitive to all or almost all fluorescent strains, towards which they could be inhibitory (not shown).

Generally speaking, the above results permit the suggestion that in antagonistic interrelationships within pseudomonads, fluorescent forms dominate over nonfluorescent ones. This viewpoint is supported by the statement that fluorescent pseudomonads, unlike nonfluorescent ones, were inhibitory also on KBM, although in a narrower spectrum than on PGM. Individual nonfluorescent strains on KBM had only several, but not a dozen or so antagonists, each. In most cases these fluorescent pseudomonads, which inhibited a certain nonflorescent strain on KBM, inhibited it also on PGM. Nevertheless, some nonfluorescent strains were inhibited by fluorescent strains only on KBM. Noteworthy is the fact that their growth-inhibition zones were generally wider on KBM than on PGM. These differences could result from the production of a siderophore under these conditions in the form of a yellow-green fluorescent dye. Besides, nonfluorescent pseudomonads could be inhibited by strains from their own group (Table 4). Such inhibitory properties were manifested themselves in the bulk of tested nonfluorescent strains (13 out of 20).

Table 4. Antagonism between nonfluorescent pseudomonads.

Strain	Self – inhibition	Inhibition of other strains		
	Inhibition zone (in mm) if occurs*	Number of inhibited strains	Inhibition zone (in mm)*	
			Range	Average
1N	–	1	6-10	12
2N	16	14	5-18	10
3N	–	0	–	–
4N	–	9	3-12	6
5N	14	14	7-17	13
6N	11	14	4-12	9
7N	4	11	4-12	8
8N	11	10	5-14	8
9N	10	14	3-16	10
10N	–	0	–	–
11N	12	13	5-16	10
12N	–	0	–	–
13N	–	1	8	8
14N	–	0	–	–
15N	–	0	–	–
16N	13	15	8-25	13
17N	–	0	–	–
18N	8	16	5-18	13
19N	11	15	6-21	15
20N	–	4	7-13	10
Average	11	8	5-15	8

* Standard deviation ± 1 mm.

These were chiefly the same strains, which were strongly antagonistic to fluorescent pseudomonads. Each of them had a relatively wide antagonistic spectrum, it inhibited the development of most of counter-partners or even all of them. Moreover, the phenomenon of self-inhibition was encountered in 10 out of 13 such strains. Studying antibiotic activity of such a strain using the streak method, it was found that when the agar plates were at first inoculated with it as an inhibitory organism and then, after two days incubation as an indicator organism growth-inhibition zone was observed after further incubation of plates. This zone generally was not smaller than those found in other indicator organisms, different from an inhibitory organism. This showed that inhibitory strains of nonfluorescent pseudomonads do not tolerate

Table 5. Antagonism and sensitivity of 20 nonfluorescent pseudomonads strains to 20 strains of actinomycetes.

Strains of nonfluorescent pseudomonads	Antagonism			Sensitivity		
	Number of actinomycetes strains inhibited on PGM**	Inhibition zone (in mm)*		Number of actinomycetes strains inhibitory on PDA***	Inhibition zone (in mm)*	
		Range	Average		Range	Average
1N	0	0	0	2	15-17	16
2N	10	3-14	9	5	10-21	15
3N	0	0	0	8	3-17	8
4N	2	5-7	6	2	15-24	20
5N	8	3-17	11	2	12-18	15
6N	10	3-13	8	3	7-12	10
7N	4	4-11	8	4	5-11	8
8N	13	5-15	10	3	9-21	15
9N	13	5-15	9	3	5-14	8
10N	3	4-11	9	1	13-14	14
11N	13	5-22	11	4	14-20	17
12N	3	5-7	6	1	12-15	14
13N	2	4-7	6	2	5-10	7
14N	3	5-13	10	3	6-24	14
15N	3	4-10	8	3	6-25	14
16N	16	6-21	13	6	3-25	13
17N	3	6-7	7	4	6-16	14
18N	17	5-22	14	3	3-10	8
19N	14	5-23	14	5	7-18	9
20N	5	5-11	9	4	6-19	11
Average	7	4-11	8	4	7-18	13

* Standard deviation ± 1 mm, ** Pridham – Gottieb's medium, *** Pepton – glucose medium.

better than their counter-partners its own toxic metabolites gradually accumulated in the medium.

The phenomenon of antagonism was also characteristic of interrelationships between nonfluorescent pseudomonads and soil actinomycetes (Table 5). Inhibitory towards actinomycetes were 18 out of 20 nonfluorescent pseudomonads. Sensitivity to these pseudomonads was displayed by 17 out of 19 actinomycetes. Like in the action on fluorescent pseudomonads, particular nonfluorescent strains had a different antagonistic spectrum towards actinomycetes. More or less half the strains inhibited the growth of 10 to a dozen or so actinomycetes, while another half inhibited the growth of only several strains. Growth-inhibition zones ranged from several, to dozen or so millimeters. Inhibitory effect in relation to actinomycetes was not so frequent as in relation to fluorescent pseudomonads. In our system of experiments (20 nonfluorescent strains, 20 fluorescent strains and 19 actinomycetes), the inhibitory effect towards fluorescent pseudomonads was found in 201 cases out of 400 theoretically possible records (i.e. in 50% of such cases), whereas that towards actinomycetes was found in 142 cases out of 380 possible (i.e in 37%).

It has also been found that actinomycetes, similar to fluorescent pseudomonads, could not only be sensitive towards nonfluorescent pseudomonads, but could also be antagonistic to them. However, that antagonism, in contrast to sensitivity, was displayed only by part of actinomycetes, i.e. by only 12 studied strains. Sensitivity towards actinomycetes was observed in all examined nonfluorescent pseudomonads, but from the other hand their particular strains responded only to no more than several (1-8) strains of actinomycetes.

Unlike interrelationships between nonfluorescent and fluorescent pseudomonads, the sensitivity of nonfluorescent forms to actinomycetes was noted more rarely than their inhibition, i.e. only in 18% of theoretically possible cases in comparison with 35%. However, when measuring the growth-inhibition zones produced by actinomycetes, attention was drawn by their relatively significant width, larger than those produced by nonfluorescent pseudomonads.

If, as mentioned above in the description of results, the occurrence of a strictly two-sided antagonism between nonfluorescent and fluorescent pseudomonads was quite frequent, such relationship between nonfluorescent pseudomonads and actinomycetes occurred only sometimes. There were only 6 actinomycetes, which were simultaneously sensitive and antagonistic to 4 nonfluorescent strains of pseudomonads.

We agree that evaluation of results obtained in studies with actinomycetes should be done cautiously in view of the fact that both antagonism and sensitivity of these organisms were investigated under different conditions than antagonism within pseudomonads. As reported in the description of the applied methods, they were grown on PDA, not on PGM or KBM which as found previously [6], were unsuitable for demonstration of inhibitory properties of our strains. For that reason there is, among others, no certainty whether such two-sided antagonism,

described above, would also occur, if antagonism of pseudomonads and that of actinomycetes were revealed under similar conditions of culture.

Summarizing, it seems that despite the above reservations, it will not be a mistake to state that relations between nonfluorescent and fluorescent pseudomonads and actinomycetes are highly complicated, even only on the account of mutual antagonism between them. In relations between representatives of the genus *Pseudomonas*, the inhibitory effect of fluorescent forms was more frequent and stronger than that of nonfluorescent forms, which in contrast to initial assumptions, was not only caused by the ability to produce siderophore. It may, therefore, be suggested, that such a system of relations between these nonfluorescent and fluorescent forms is one of the reasons of the minority of nonfluorescent forms in *Pseudomonas* communities of soil environment. The occurrence of self-inhibition phenomenon in nonfluorescent pseudomonads may be taken as another reason. Contrary to fluorescent pseudomonads, actinomycetes appeared to be more frequently, but generally not more sensitive than inhibitory organisms in interactions with nonfluorescent pseudomonads. As a matter of fact, the ability of actinomycetes to produce antibiotics is particularly widespread, but already in the previous paper [6] studying interrelationships between actinomycetes and fluorescent pseudomonads, it was found that antagonism in actinomycetes occurs not as frequently as in nonfluorescent pseudomonads.

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