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EXPERIMENTAL ARTICLES

Dynamics of Abundance of Antifungal Strains of *Pseudomonas* in the Rhizosphere of Hydroponic Cucumbers Grown on Greenhouse Mineral Substrate

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Abstract—Data were obtained on the dynamics of the abundance of the biocontrol strains of *Pseudomonas chlororaphis* SPB1217 and *Pseudomonas fluorescens* SPB2137 with antifungal activity. These strains are able to develop in the rhizosphere of cucumbers grown on mineral substrate under hydroponic conditions in industrial greenhouses. After four weeks of vegetation of plants, the abundance of the inoculated strains was 19–28% of the total bacterial numbers determined by inoculation onto solid medium. The investigated strains spread together with the young, actively growing and exudating roots; they reached a stable level of abundance in deep layers of the greenhouse substrate. A significant difference in the abundance of fungi in the tested variants was observed after 20 days of vegetation: the abundance of fungi in the control was two times higher than in the variant inoculated with strain SPB2137.

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The application of rhizobacteria able to actively colonize plant rhizosphere and rhizoplane using the nutrients supplied by plants as exometabolites of roots is presently an efficient method of control of phytopathogenic microorganisms [1]. Such antifungal bacteria are able to control the development of phytopathogens in the plant rhizosphere both by competing for the ecological niche (carbon and energy sources) [2] and by producing various antifungal metabolites or hydrolytic enzymes that decompose fungal cell walls [3].

Biological control of phytopathogens is an actively developing field of biotechnology. During the recent decade, positive results have been obtained on biological control of phytopathogens in industrial greenhouses; new biocontrol agents are explored for the creation of efficient biopreparations based on rhizobacteria (bacteria associated with plant roots) [4]. A necessary stage in the screening of rhizobacteria is the estimation of their capacity for colonization of the root system and for competing successfully with other representatives of the complex of rhizosphere microorganisms. In all cases, the survival and stability of the introduced rhizobacteria in the root zone plays a significant role in the efficiency of their action and often is a limiting factor in biocontrol [5].

The goal of the present study is to investigate the dynamics of colonization with antifungal strains of the rhizosphere of cucumbers grown on a mineral substrate under conditions of hydroponics in industrial greenhouses.

MATERIALS AND METHODS

Antifungal strains from the ARRIAM collection, *Pseudomonas chlororaphis* SPB1217 and *Pseudomonas fluorescens* SPB2137 were used in the present study; they possess a high level of activity against various phytopathogenic fungi and intensively colonize the root system of plants [6]. Strains *Escherichia coli* S17-1 with plasmid λ pm Tn5 SS gus A40 (Str^{100}) and *P. fluorescens* PCL1500, a Tn5-*lacZ* marked derivative of the strain *P. fluorescens* WCS 365 [7] from the collection of the Institute of Biology (Leiden University, The Netherlands) were also used. The colonizing activity of the *Pseudomonas* strains was investigated on cucumber plants (*Cucumis sativus* L.) of the Ventura variety.

Colonization of the root surface by the investigated bacteria was studied in the gnotobiotic system under competition with the standard strain *P. fluorescens* PCL1500 [7]. The system contained sterile quartz sand with the addition of 10% (v/w) of nutrient solution (PNS) of the following composition: 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, and trace elements. The cell suspension for inoculation of seeds was prepared from 12 h bacterial culture grown on an LC medium by washing off the cells with PNS solution. The seeds were inoculated by soaking of seedlings in the cell suspension (10^8 cells/ml) for 15 min-

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Table 1. Colonization of the roots of cucumber of Ventura variety by rhizobacteria in competition with *P. fluorescens* PCL1500

Strain	Number of bacteria,* CFU × 10^4/cm of root		Ratio of abundance**
	Tested strain	PCL1500	
SPB1217	206.0 ± 23.0	39.0 ± 6.0	5.3
SPB2137	14.5 ± 5.3	4.0 ± 2.1	3.6

Note: Numbers in the table are the means of three replications. The value of measurement errors is shown as standard deviation.
* Number of bacteria per 1 cm long root tip after seven days of vegetation.
** Ratio of the number of cells of the tested strain to the number of cells of the standard strain PCL1500 after seven days of vegetation. The initial ratio of strains was 1 : 1.

utes. After inoculation, the seeds were aseptically placed to the depth of 5 mm into quartz sand. The plants were grown in the phytotron at 21°C and 16 h illumination (10000 lx) for 7 days. For determination of bacterial numbers, the tips of roots (1–2 cm long) were separated and intensively homogenized with quartz sand in 10 ml of water. Serial dilutions of the obtained suspension were prepared and the numbers of bacteria were determined by plating on LC medium. For calculation of the numbers of bacteria in the mixture of the tested strain of rhizobacteria with the standard Tn5-*lacZ* marked strain *P. fluorescens* PCL1500, X-Gal (40.0 μg/ml) was added to the agarized medium. The colonies of bacteria were counted after 1–2 day incubation at 28°C.

To obtain *gus* marked strains, the method of Tn5 mutagenesis was used. Strain *E. coli* S17-1 was used as a donor. The recipient strains (preliminarily tested for their inability to grow in the presence of 50–100 μg/ml streptomycin) were grown on the agarized LC medium [8] at 28°C for 24 h. The donor strain was grown under similar conditions but with the addition of 100 μg/ml streptomycin to the medium. The grown cultures were suspended in sterile water and the suspensions of the donor and recipient were mixed at the ratio 1 : 1 on a membrane nitrocellulose filter. The filter was placed on a dish with LC agar and incubated for 36 h at 28°C to perform conjugation. After conjugation, the mixture of bacteria was washed from the filter with 5 ml of sterile water and spread on the surface of the plates with LC medium containing 200 μg/ml of streptomycin and 20 μg/ml of rifampicin (blocking the growth of *E. coli* S17-1 and not affecting the recipient strains). The grown colonies were subcultured on dishes with the YMB medium (0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l NaCl, 2 g/l mannitol, 0.4 g/l yeast extract, and 2.5 g/l sodium succinate, pH 7.0) and 50 μg/ml X-gluc (Sigma) causing blue staining of the colonies of the *gus* marked strains. Further selection of *gus* marked strains was performed by comparison of motility of the marked and initial strains; the derivatives with motility indices closest to the initial ones were selected. Bacterial motility was estimated from the diameter of the migration zone after 24 h incubation on 20-fold diluted semisolid (0.3% agar) LC medium. The colonizing activity of the derivatives selected in such a way was determined in gnotobiotic systems by the method of Simons et al. [7] in competition with the parental strain. By the results of gnotobiotic experiments, the *gus* marked derivatives were selected with the indices of colonizing activity closest to those of the initial strains.

Dynamics of the abundance of strains SPB1217 and SPB2137 in the rhizosphere was investigated in industrial greenhouses of the Vyborzhets agricultural company (St. Petersburg). The *gus* marked derivatives of the corresponding strains were used as inoculants. The bacteria were grown for 24 h in liquid LC medium on a shaker (110 rpm) at 28°C. The grown cultures were centrifuged and the pellet was resuspended in physiological saline (0.85% NaCl). The optical density of the suspensions was adjusted to OD_{620} = 0.2 which corresponded to 10^8 CFU/ml. Immediately prior to seeding, the seeds were soaked for 5 minutes in a mixture of rhizobacteria (10^8 CFU/ml) with 1% methyl cellulose solution and then dried. The treated seeds were planted in the greenhouse in the mineral substrate (Rockwool mineral wool, Grodan Co., The Netherlands) and covered with a layer of vermiculite. For microbiological analyses, samples of the substratum with plant roots were taken in weeks 1, 2, and 4 of the growing of the plants. The total numbers of bacteria in the substrate were estimated on the LC medium, and the numbers of the introduced *gus* marked strains, on the YMB medium with the addition of X-gluc (50 μg/ml).

The number of fungi in the greenhouse substrate was estimated on Czapek medium (g/l): sucrose, 20; $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.1; pH 6.0).

RESULTS AND DISCUSSION

The requirement of a high colonizing activity in antifungal strains makes necessary its investigation in all rhizobacteria considered as prospective biocontrol agents. In the present study the colonizing potential of rhizobacteria in the cucumber rhizosphere was initially studied in gnotobiotic conditions under competition with a Tn5-*lacZ* marked strain of *P. fluorescens* PCL1500; the latter strain is highly competitive in the plant rhizosphere [7]. The data presented in Table 1 show that strains of *P. chlororaphis* SPB1217 and *P. fluorescens* SPB2137 demonstrated a high ability for competition on cucumber roots, with numbers 3.6–5 times higher than PCL1500.

Analysis of the competitive ability of rhizobacteria in gnotobiotic experiments is an initial stage in the investigation of their colonizing properties. Antifungal strains should possess the ability to survive in the plant rhizosphere under field or greenhouse conditions as

Table 2. Number of microorganisms in the greenhouse substrate after inoculation of cucumber plants of the Ventura variety with rhizobacteria

Strain	Vegetation time, weeks	Bacteria, CFU × 10^7/plant	*gus* marked strain	
			CFU × 10^7/plant	% of total number of bacteria
SPB1217	1	180.0 ± 19.4	60.0 ± 9.7	33
	2	100.0 ± 11.5	22.6 ± 4.8	23
	4	129.0 ± 27.8	27.6 ± 8.6	21
SPB2137	1	184.0 ± 40.0	57.0 ± 10.2	31
	2	141.0 ± 28.1	25.3 ± 5.8	18
	4	134.0 ± 27.3	18.6 ± 7.6	14

Note: Numbers in the table are the means of five replications. The value of measurement errors is shown as standard deviation.

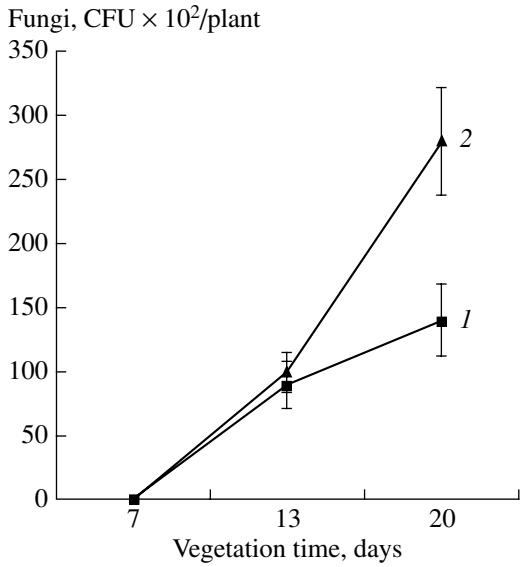
Table 3. Distribution of microorganisms in the layers of greenhouse substratum at inoculation of cucumber plants of Ventura variety with strains *P. chlororaphis* SPB1217 and *P. fluorescens* SPB2137

Vegetation time, weeks	Substratum layer, cm	*P. chlororaphis* SPB1217		*P. fluorescens* SPB2137	
		CFU × 10^6/cm^3	% of total number of bacteria	CFU × 10^6/cm^3	% of total number of bacteria
1	0–3	50.0 ± 9.4	33	47.5 ± 11.0	31
	3–4.5	0	–	0	–
	4.5–6	0	–	0	–
2	0–3	20.6 ± 3.0	28	17.0 ± 3.2	14
	3–4.5	18.6 ± 2.8	21	21.1 ± 3.8	19
	4.5–6	16.0 ± 2.7	16	24.2 ± 2.5	21
4	0–3	18.3 ± 4.1	20	11.4 ± 2.4	12
	3–4.5	23.7 ± 4.3	21	16.0 ± 2.2	14
	4.5–6	31.0 ± 5.8	23	23.0 ± 3.6	17

Note: Numbers in the table are the means of five replications. The value of measurement errors is shown as standard deviation.

well, retaining their abundance within limits sufficient for efficient biocontrol. Table 2 shows the results of analysis of the dynamics of bacterial numbers in the mineral substrate after the sowing of cucumber seeds inoculated with pseudomonad strains. A week after the sowing of the seeds, the abundance of SPB1217 and SPB2137 was, respectively, 33 and 31% of the total quantity of bacteria determined by plating (Table 2). Such high indices suggest their high competitive ability in comparison with the aboriginal microflora of the greenhouse substrate. Strains SPB1217 and SPB2137 are characterized by the ability of efficient utilization of plant root secretions [9]. Under the conditions of low quantities of organic matter in the mineral substratum, this may be a factor providing a high level of colonization activity already at the stage of seed germination. It should be noted that initially the almost sterile greenhouse substrata, such as mineral wool, used in the present study, contained as a rule insufficient biodiversity of microorganisms which led to a "biological vacuum." Under such conditions, even not very competitive phytopathogens may grow actively, be rapidly distributed over the greenhouse area, and cause a wide infestation of young plants. This is characteristic of the hydroponic systems where the system of recirculation of the nutrient solution enables easy distribution of *Pythium* zoospores [4]. However, the conditions that favor the active development of phytopathogens are also optimal for PGPR strains. During further vegetation, the abundance of the investigated strains decreased somewhat, although their quantity in relation to the total number of bacteria remained at a rather high level, constituting 21% for the strain SPB1217 and 14% for the strain SPB2137 after 4 weeks (Table 2).

As for the distribution of strains SPB1217 and SPB2137 in layers of the greenhouse substrate (Table 3), after a week the introduced bacteria were found only in the upper layer (at the depth of 0–3 cm). After two weeks, the maximum numbers of SPB1217 were also found in the upper layer of the substrate. However, in the course of root growth within the bloc



Dynamics of abundance of fungi in the rhizosphere of cucumber of the Ventura variety after treatment of the seeds with strain *P. fluorescens* SPB2137 (*1*) and in the control (*2*). Fourfold replication.

of the mineral substrate, strain SPB1217 spread further and became rather homogeneously distributed throughout the substrate after four weeks of vegetation. The numbers of SPB1217 in the upper layer of the substratum decreased 2.7-fold over four weeks of vegetation; the total numbers of bacteria also decreased 1.6-fold (from 159 to 91 CFU × 10^6/cm^3).

The abundance of strain SPB2137 relative to the total numbers of bacteria in the substrate layers was somewhat lower than that of strain SPB1217, although the distribution of bacteria in the layers of the greenhouse substrate was also rather homogeneous (Table 3). The absolute numbers of strain SPB2137 in the surface layer of the substrate decreased 4.2-fold after four weeks of vegetation, while the dynamics of the abundance of the aboriginal bacteria was similar to that in the variant with strain SPB 1217. The growth of strain SPB2137 might be more dependent on the nutrients supplied by root secretions, and its development was best in the zone of young roots characterized by high exudation activity [7].

The ecophysiological characteristics of the rhizosphere of the cucumbers grown on the mineral substrate demonstrated clear differences in the bacterial complex at different depths [10]. In the zone of young growing roots, rapidly growing bacteria dominated which actively utilized simple sources of carbon (such as monosaccharides and organic acids). The microorganisms which utilized more complex substrates (e.g., disaccharides) predominated in the upper layer of the substratum. Similar differences were revealed also for the different phases of vegetation of cucumber plants: the general profile of the metabolic activity of the rhizosphere bacterial community shifted towards the consumption of more complex substrates at a greater age of plants. The fact that the numbers of strains SPB1217 and SPB2137 in the deep layers of the substratum were rather stable indicated that they spread with the young actively exudating parts of roots and were able to compete successfully with the aboriginal microflora for the nutrients present in root secretions.

The effect of the introduced strains on the micromycetes of the greenhouse substrate was determined in the experiment in which the abundance of fungi in the control (without treatment of seeds with bacteria) and after inoculation with strain SPB2137 were compared. In the control and in the variant with inoculation, the abundance of fungi increased identically up to 13 days (figure). The significant difference in the abundance of fungi in the tested variants was observed after 20 days of vegetation of plants: the abundance of fungi in the control was twice as high as in the variant inoculated with strain SPB2137.

Introduction of a genetically modified strain *P. putida* WCS358r containing the constitutionally expressing gene of the biosynthesis of phenazine-1-carboxylic acid *phz* into the wheat rhizosphere resulted in structural changes of the complex of fungal soil microflora [11]. Similar results were obtained with *P. fluorescens* strain CHA0 introduced into the rhizosphere of cucumber [12]. The effect of strain SPB2137 on the dynamics of the abundance of micromycetes may be related to its ability to produce exometabolites possessing antifungal action [9].

The obtained data on the dynamics of abundance of strains SPB1217 and SPB2137 lead to the conclusion that they are able to survive in the rhizosphere of cucumber grown in the mineral substratum under the conditions of industrial greenhouses and to retain their abundance which constituted 19–28% of the total number of bacteria determined by inoculation for four weeks of vegetation. The investigated strains have a stable level of abundance in deep layers of the greenhouse substratum, spread with the young, actively exudating parts of the roots, and are able to successfully compete with the aboriginal microflora for the nutrients present in root secretions. The significant difference in the abundance of fungi in the tested variants was observed after 20 days of vegetation of plants: the abundance of fungi in the control was twice as high as that in the variant with inoculation with strain SPB2137.

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REFERENCES

1. Whipps, J.M., Microbial Interactions and Biocontrol in the Rhizosphere, *J. Experim. Botany*, 2001, vol. 52, pp. 487–511.
2. Lugtenberg, B.J.J. and Dekkers, L.C., What Makes *Pseudomonas* Bacteria Rhizosphere Competent?, *Environ. Microbiol,* 1999, vol. 1, pp. 9–13.
3. Thomashow, L.S and Weller, D.M., Current Concepts in the Use of Introduced Bacteria for Biological Disease Control: Mechanisms and Antifungal Metabolites, in *Plant Microbe Interactions*, Stacey, G. and Keen, N., Eds., New York: Chapman and Hall, 1996, pp. 187–235.
4. Paulitz, T.C. and Bélanger, R.R., Biological Control in Greenhouse Systems, *Annu. Rev. Phytopathol.,* 2001, vol. 39, pp. 103–133.
5. Lugtenberg, B.J.J., Dekkers, L., and Bloemberg, G.V., Molecular Determinants of the Rhizosphere Colonization by Pseudomonas, *Annu. Rev. Phytopathol.*, 2001, vol. 39, pp. 461–490.
6. Kravchenko, L.V., Makarova, N.M., Azarova, T.S., Provorov, N.A., and Tikhonovich, I.A., Isolation and Phenotypic Characterization of Plant Growth-Promoting Rhizobacteria with High Antiphytopathogenic Activity and Root-Colonizing Ability, *Mikrobiologiya,* 2002, vol. 71, no. 4, pp. 521–525 [*Microbiology* (Engl. Transl.), vol. 71, no. 4, pp. 444–448].
7. Simons, M., van der Bij, Brand, I., de Weger, L.A., and Wijffelman, C.A., Gnotobiotic System for Studying Rhizosphere Colonization by Plant Growth-Promoting *Pseudomonas* Bacteria, *Mol. Plant–Microbe Interact.*, 1996, vol. 9, no. 7, pp. 600–607.
8. Sambrook, J., Fritsch, E.F., and Maniatis, T.A., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, 1989.
9. Kravchenko, L.V., Azarova, T.S., Leonova-Erko, E.I., Shaposhnikov, A.I., Makarova, N.M., and Tikhonovich, I.A., Root Exudates of Tomato Plants and Their Effect on the Growth and Antifungal Activity of *Pseudomonas* Strains, *Mikrobiologiya,* 2003, vol. 72, no. 1, pp. 48–53 [*Microbiology* (Engl. Transl.), vol. 72, no. 1, pp. 37–41].
10. Folman, L.B., Postma, J., and Van Veen, J.A., Ecophysiological Characterization of Rhizosphere Bacterial Communities at Different Root Locations and Plant Developmental Stages of Cucumber Grown on Rockwool, *Microb. Ecol.,* 2001, vol. 42, pp. 586–597.
11. Glandorf, D.C.M., Verheggen, P., Jansen, T., Jorritsma, J.-W., Smit, E., Leeflang, P., Wernars, K., Thomashow, L.S., Laureijs, E., Thomas-Oates, J.E., Bakker, A.H.M., and van Loon, L.C., Effect of the Genetically Modified *Pseudomonas putida* WCS358r on the Fungal Rhizosphere Microflora of Field-Grown Wheat, *Appl. Environ. Microbiol.*, 2001, vol. 67, pp. 3371–3378.
12. Girlanda, M., Perotto, S., Moenne-Loccoz, Y., Bergero, R., Lazzari, A., Defago, G., Bonfante, P., and Luppi, A.M., Impact of Biocontrol *Pseudomonas fluorescen*s CHA0 and a Genetically Modified Derivative on the Diversity of Culturable Fungi in the Cucumber Rhizosphere, *Appl. Environ. Microbiol.*, 2001, vol. 67, no. 4, pp. 1851–1864.