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Testing Methods Affecting the Antagonistic Ability of *Pseudomonas* Biocontrol Strains

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Introduction

As fungicides may have disadvantageous effects on human health and the environment, alternative control procedures – biological control – are currently being developed. In the last decades therefore several micro-organisms have been tested to determine their abilities to suppress the soil-borne phytopathogens. Physical and chemical characteristics of the rhizosphere environment are determined by the interaction of soils, plants, and organisms associated with the root, including bacteria, fungi, protozoa, and nematodes (ELLIOT et al., 1984; YANG & CROWLEY, 2000). Root exudates provide micro-organisms with the necessary nutrients, rhizobacteria on the other hand can improve the crop yields (FEDI et al., 1997; BUYSENS et al., 1999). Some rhizobacteria, which are commonly called plant growth promoting rhizobacteria (PGPR), can protect the roots against pathogenic micro-organisms (KLOEPPER & SCHROT, 1978; LIGON et al., 2000). There are several mechanisms behind this beneficial behaviour, such as siderophore production, fast growing ability or release of some antibiotic compounds. The PGPR effect, therefore is the result of a complex interaction (BIRÓ et al., 1998). Under temperate climatic circumstances pseudomonads are common and ubiquitous members of the rhizobacterial microbiota in soils. These beneficial effects develop either in the form of suppression of diseases and deleterious effects caused by the soil-borne pathogens (WELLER, 1988; COOK, 1993) or in the form of better growth and fitness due to the secondary metabolite production. Fluorescent-putida type pseudomonads are the most commonly studied biopesticides (SCHWYN & NEILANDS, 1987).

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The aim of this work was to isolate and select antagonistic microbes (*Pseudomonas* strains) against the soil-borne pathogens of potato (*Solanum tuberosum* L.). The long-term crop rotation experiment of Westsik (Nyíregyháza, Hungary), provided unique possibilities for the appropriate strain isolation of the antagonists.

Materials and Methods

Twenty-six *Pseudomonas* strains were isolated from potato tubers (*Solanum tuberosum* L.) from different plots and treatments of the Westsik crop rotation experiment. The plots were treated by 3 types of organic fertilizer (straw, green manure and farmyard manure), enriched with different amounts of inorganic N, P and K fertilizers, which resulted in various fertile and natural conditions. Thirteen strains were identified as *Pseudomonas aeruginosa* by 16S rDNS (RAINEY et al., 1996; SAMBROOK et al., 1989; ALTSCHUL et al., 1997). Identification of the other tested *Pseudomonas* group is still in progress. Using selective media and some other biochemical tests and genotype analyses (ARDRA), they are preliminary characterized as members of the fluorescens-putida group. Strains involved in this study were previously tested and selected for their siderophore production on CAS agar plates by a modified procedure (SCHWYN & NEILANDS, 1987). The list and origin of the strains are given in Table 1.

The strains were individually tested for their ability to inhibit the mycelium growth of *Rhizoctonia solani* (Kühn) DSM No. 843 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) = ATCC 13289 (American Type Culture Collection) and *Fusarium solani* F.00715 (Collection of Agricultural and Industrial Micro-organisms, Szent István University, Buda-

Table 1
Code and origin of the investigated *Pseudomonas* sp. strains

Pseudomonas spp. types	Treatments in the Westsik experiment*			
	I. uncultivated	IV. straw (26.1 t/ha/3 years)	VIII. green manure (N.D.)	IX. farmyard manure (26.1 t/ha/3 years)
P. aeruginosa	A10; AX	A5/2; A35/2; A20	A6; A9; A34	A16; A23/1; A28a; A30/2; A36
Fluorescens-putida type *Pseudomonads*	F1; F2	F4; F8; F12	F15; F22; F38	F41; F44; F47; D65; D80

*Treatments were enriched with inorganic fertilizers, as follows: I = 32.5 kg/ha N, 25 kg/ha P and 20.5 kg/ha K; IV = 50 kg/ha N, 50 kg/ha P and 16.2 kg/ha K; VIII = 50 kg/ha P and 16.2 kg/ha K; IX = 50 kg/ha N. N.D. = not determined

pest, Hungary). Experiments were set up with malt-yeast extract solid agar medium (yeast extract 3.0 g, malt extract 3.0 g, proteose pepton 5.0 g, glucose 10.0 g, agar 20.0 g, dissolved in 1 litre of distilled water) by two different techniques:

a) Two loopful colonies of a bacterial strain (after a 24-hour incubation) were placed on opposing edges of the 90 mm Petri dish (spot transfer). One colony of a phytopathogenic fungus on a 5 mm agar disk was placed in the centre of the plate.

b) The bacterial suspensions were spread on the medium (spread plate). The pathogen on a 5 mm agar disk was placed in the centre of the plate.

Each combination of pathogens and antagonists was replicated three times and incubated at 26–28 °C. Eight days later, the fungal growth was measured. To estimate the inhibition activity of bacterial strains, the principal inhibition ratio of fungal colonies (PI%) was established. In case of spot transfer: PI% = (C–S)/C · 100; where C = diameter (mm) of the control colony of fungi tested, S = short diameter (mm) of tested fungal colony developed in an elliptic form. In case of spread plate: PI% = (C–T)/C · 100; where T = diameter (mm) of treated colony of the pathogen fungi tested, C = diameter (mm) of the control colony of pathogenic fungi. Data were subjected to analysis of variance (ANOVA). Means of the antagonistic ability are presented in the tables.

Results

Sensitivity of pathogens against Pseudomonas strains

All tested strains showed strong inhibition of the fungus *Rhizoctonia solani* ATCC 13289 and also of the fungus *Fusarium solani* F.00715 (Tables 2 and 3). *Rhizoctonia*, however was found to be more sensitive with both used techniques. The sensitivity range of *R. solani* ranged from 3.5 to 98.6 PI%; in the case of *F. solani* it was between 21.2 and 79.3 PI%. This behaviour, however can hardly be generalized, due to the fact that only one *Fusarium* and one *Rhizoctonia* strain was tested. The antagonistic activity of bacterial strains varied greatly with the two different testing methods. The strongest inhibition was recorded with the spread plate technique, where a direct contact could develop between the two microbes.

Antagonistic ability of the Pseudomonas strains

Pseudomonads were isolated from the various treatments of the Westsik long-term crop rotation experiment. All of them were selected for a positive siderophore production, which is one of the mechanisms involved in the antagonistic ability. Results are shown in Tables 2 and 3. In general, the highest

Table 2
Antagonistic effect of *Pseudomonas* sp. strains against *Rhizoctonia solani* ATCC 13289, in decreasing order (MI%)

Principal inhibition ratio of fungal colonies by *Pseudomonas* sp. strains using two different *in vitro* techniques (PI%)							
Spread plate				Spot transfer			
P. aeruginosa		*P.* sp.		*P. aeruginosa*		*P.* sp.	
Strain	MI%	Strain	MI%	Strain	MI%	Strain	MI%
A9	98.6	D80	98.6	A10	53.8	F4	41.2
A5/2	88.1	F2	81.8	A5/2	47.6	D80	21.7
A6	86.7	F8	76.2	A9	32.8	F12	16.1
A35/2	81.1	D65	46.9	A35/2	32.8	D65	11.9
A28a	76.9	F1	37.8	A23/1	24.5	F41	10.5
A34	76.9	F15	35.7	A16	22.4	F38	9.1
A16	76.9	F44	27.3	AX	21.7	F2	7.7
A10	76.9	F4	25.2	A28a	21.0	F8	7.0
A23/1	74.8	F12	16.1	A20	16.1	F1	6.3
AX	70.6	F41	9.8	A6	15.4	F47	5.6
A20	70.6	F38	6.3	A34	15.4	F22	5.6
A30/2	67.8	F47	5.6	A30/2	11.9	F15	4.2
A36	49.6	F22	5.6	A36	3.5	F44	3.5

Table 3
Antagonistic effect of *Pseudomonas* sp. strains against *Fusarium solani* F.00715, in decreasing order (MI%)

Principal inhibition ratio of fungal colonies by *Pseudomonas* sp. strains using two different *in vitro* techniques (PI%)							
Spread plate				Spot transfer			
P. aeruginosa		*P.* sp.		*P. aeruginosa*		*P.* sp.	
Strain	MI%	Strain	MI%	Strain	MI%	Strain	MI%
A9	79.3	F44	59.5	A9	53.1	F12	49.5
A5/2	56.8	D80	54.1	A5/2	52.7	F15	35.6
A35/2	50.9	F12	48.2	A10	52.2	F4	32.0
A10	50.9	F2	44.6	A35/2	50.0	F47	28.3
A20	41.9	F38	46.0	A6	41.0	D80	27.9
AX	44.6	F1	40.5	A20	37.8	F1	27.9
A28a	44.6	F15	40.5	AX	36.5	F44	24.3
A6	38.7	F47	41.0	A30/2	36.0	F22	23.0
A34	39.6	F4	32.4	A16	31.5	F41	22.0
A23/1	36.5	F8	32.4	A34	28.4	F2	22.0
A30/2	36.5	F41	22.5	A23/1	28.4	F8	21.6
A16	39.2	F22	24.3	A28a	27.5	F38	21.6
A36	26.1	FD65	21.2	A36	27.0	D65	21.2

P. sp.: fluorescens-putida type *Pseudomonas* strains

value of inhibition was observed in the case of *Pseudomonas aeruginosa* strains. The other group of fluorescens-putida type pseudomonads, however showed a weaker inhibition activity. Although no such difference is mentioned in literature, results suggest some well distinguishable mechanisms of the antagonistic ability, those are subjects of further studies.

Among the *Pseudomonas aeruginosa* group, two strains (A9 and A5/2) were the most efficient antagonists. From the other *Pseudomonas* sp. group, strains D80 and F44 were the best control agents.

Values of significant difference regarding the total average of inhibition levels presented in Tables 2 and 3 are shown in Table 4.

Comparison of in vitro techniques in the antagonistic ability

All of the tested pseudomonads but few strains significantly inhibited the growth of fungal strains with the two *in vitro* techniques used. Although the weaker inhibition was observed in the case of spot transfer technique, the strains were still able to inhibit the development of fungal sclerotia during the whole eight-day incubation period.

Table 5, on the other hand, shows the values of significant difference between the two *in vitro* techniques used for the sensitivity tests. Based on the data obtained, it seems to be clear that there were statistically reliable differences

Table 4
Significant difference values of simple ANOVA for total mean inhibitory activity of bacterial strains on the tested fungi with two *in vitro* techniques

In vitro techniques	Fungi	
	Rh. solani	*F. solani*
Spot transfer	3.55	2.99
Spread plate	3.34	2.11

Table 5
Significant difference values of multiple ANOVA for total mean inhibitory activity of bacterial strains on the fungi tested with two different *in vitro* techniques

In vitro techniques	Fungi			
	Rhizoctonia solani		*Fusarium solani*	
	Mean	SD value	Mean	SD value
Spot transfer	17.37	0.66	31.77	0.49
Spread plate	54.39		40.47	
Spot transfer and spread plate	54.39	0.60	40.47	0.49

SD value: significant difference value

between the techniques used for inoculation. There is an immanent property of these methods, namely spread plate is significantly more effective (causing larger inhibition zones) than spot transfer. Inhibition is stronger in cases when a direct contact generates between the biocontrol agents and pathogens.

Discussion

Two different *in vitro* techniques were used to study the inhibition potential of several beneficial rhizosphere bacteria against two strains of soil-borne plant pathogens. Spread plate technique produced an inhibition ratio of nearly 100%; the spot transfer, however, gave only 50%. This fact confirms the importance of direct contact between the biocontrol agents and target organisms. Either of the reported *in vitro* techniques can be used to screen the antagonistic behaviour of the strains; indicating a simple pre-selective method for biocontrol bacterial isolates.

The results infer a possible impact of siderophore production in the antagonism too, as all of the investigated bacterial strains were able to produce siderophore-like secondary metabolites, which may cause a nutrient competition effect for the other coexisting micro-organisms (ELLIOT et al., 1984; SCHWYN & NEILANDS, 1987; COOK, 1993).

The antifungal effects, however presumably depend on several other mechanisms differing from siderophore production; i.e. antibiotic production, growth rate, etc (BIRÓ et al., 1998; BUYSENS et al., 1999). The antagonistic behaviour of a single bacterial strain moreover depends greatly on the pathogens. There are complex interactions among the micro-organisms in the soil–plant system, where the antagonistic abilities are important for the control of soil-borne pathogens.

The antagonism of *P. aeruginosa* strains against phytopathogenic fungi is relatively poorly studied, due to the opportunistic human pathogenic feature of this species. They are however common, ubiquitous components in the soil, having a potential of interacting with the soil microbiota. In the present study, they proved to have significantly higher antagonistic effect against the tested fungi as compared to the other non-pathogen pseudomonads. The members of fluorescens-putida group on the other hand were also reported to be successfully used against soil-borne plant pathogens, such as the apple-replant-disease in pot experiments (WELLER, 1988; BIRÓ et al., 1998).

The use of *in vitro* selected antagonistic strains which produce the best performance in the real soil–plant systems, is highly encouraged therefore in this study.

Summary

The antagonistic effect of thirteen *Pseudomonas aeruginosa* and thirteen strains of other *Pseudomonas* species was studied on the soil-borne phytopathogenic *Rhizoctonia solani* and *Fusarium solani* fungi.

The inhibition of pathogen colony growth was tested with two different *in vitro* techniques using the same type of culture media. In case of the spread slant technique the antagonists induced a significantly stronger inhibition on the growth of pathogens than in case of spot transfer. Among the 26 investigated *Pseudomonas* strains, *P. aeruginosa* strains were generally more effective against the fungal pathogens. *Rhizoctonia solani* proved to be affected to a greater extent by the bacterial strains studied than the *Fusarium solani* representative.

The possibility of *in vitro* strain selection of biocontrol microbes is being further discussed.

Key words: *Pseudomonas, Fusarium, Rhizoctonia,* biocontrol, *in vitro* tehniques, comparison

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