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DIVERSITY OF *PSEUDOMONAS* SPECIES ASSOCIATED WITH SOFT ROT OF PLANTS IN POLAND

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Abstract. A total of 94 pectolytic and 60 nonpectolytic *Pseudomonas* isolates were obtained from 250 samples of rotted vegetable specimens representing various economically important vegetables. The isolates were identified on the basis of standard biochemical tests. *Pseudomonas fluorescens* biovar V and II and *Pseudomonas putida* were the most abundant species among pectolytic isolates and *Pseudomonas fluorescens* biovar I among nonpectolytic ones. Only 3 *Pseudomonas viridiflava* isolates were identified and all of them were obtained from potato. Isolates of pectolytic phenotype were scattered among nonpectolytic ones irrespective of their taxonomical status. Isolates identified biochemically, as *Pseudomonas marginalis* were also present in nonpectolytic group. PCR method is unsuitable for identification and differentiation of bacteria belonging to pectolytic fluorescens *Pseudomonas* group due to great diversity of species. However, the results of PCR amplification of the genes encoding pectate lyase suggest that genes responsible for production of this enzyme may also be present in isolates of nonpectolytic phenotype.

Key words: *Pseudomonas* sp., occurrence, identification, heterogeneity, PCR

I. INTRODUCTION

Bacterial soft rot is generally considered as one of the most important plant diseases. Although pectolytic *Erwinia* species are commonly assumed to be the principal cause of this disease, there are several other genera, namely *Pseudomonas*, *Flavobacterium*, *Xanthomonas*, *Bacillus* and *Clostridium*, which are also able to cause extensive decays of plants (Liao and Wells 1986; Liao and Wells 1987a; Liao and Wells 1987b). Although soft rotting pectolytic, fluorescent *Pseudomonas* (PFP) are less extensively studied than *Erwinia* species, they may become serious particularly for fruits and vegetables stored in low temperatures and it is estimated that in the United States strains of *P. fluorescens* and *P. viridiflava*, account for over 40% of bacterial rots of fruits and vegetables in storage and during transit (Liao and Wells 1987a). The classification of PFP group is unclear. Generally it is assumed that PFP species that are negative for reactions of oxidase, arginine dihydrolase and levan should be classified as *P. viridiflava*, while oxidase-positive, arginine dihydrolase-positive and levan positive strains are frequently named *P. marginalis* (Lelliot and Stead 1987; Hildebrand et al. 1988). This approach however, seems not entirely appropriate. The physiological and nutritional heterogeneity of *P. marginalis* was reported by Sands and Hankin (1975) who after examination of 140 *P. marginalis* strains from various sources discovered that they fall within the phenotype from *P. fluorescens* to *P. putida*. Similar diversity of PFP species were also reported by Wang and Kelman (1982), Liao and Wells (1987a) and Janse et al. (1992). Using conventional physiological and biochemical tests, pathogenicity tests

and whole cell fatty acid analysis they found that soft rot *P. marginalis* isolates were scattered among saprophytic *P. fluorescens* strains and therefore suggested that the use of the name *P. marginalis* for a separate taxon is artificial and should be abandoned. The current status of *P.marginals* species is still unclear because although it is not mentioned in The Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) it is still present on the List of the Names of Plant Pathogenic Bacteria 1864-1995 (Young et al. 1996).

Our previous study on soft rot bacteria showed that PFP species are also relatively common in Poland (Żołobowska and Pospieszny 1998; Żołobowska 1998). They comprised over 20% of pectolytic isolates obtained from various rotted plant samples. The extensive study of this group of pathogens was as yet not performed in Poland. Therefore the objectives of this work were to examine the diversity of PFP species associated with various host plants in Poland, evaluation of importance of particular groups on different vegetables and evaluation of usefulness of Polymerase Chain Reaction (PCR) method in identification and differentiation of PFP.

II. MATERIALS AND METHODS

The bacterial isolates originated from about 250 rotted vegetable specimens obtained from markets and storages in different regions in Poland.

Pectolytic *Pseudomonas* species were isolated from this plant material using standard procedures (Lelliot and Stead 1987; Hildebrand et al. 1988) and identified as PFP on the basis of pectolytic activity on CVP medium (Cuppels and Kelman 1974) and ability to produce fluorescent pigment on King B medium. Identification of species and biovars was performed according to standard procedures as well (Palleroni 1984; Lelliot and Stead 1987; Hildebrand et al. 1988). The following tests were completed: production of oxidase, fermentation of glucose, formation of levan, production of arginine dihydrolase, utilization of trehalose, adonitol, sorbitol, L-arabinose, D-galactose, sucrose and hypersensitive reaction on tobacco plants (Tab. 1).

Nonpectolytic *Pseudomonas* species were isolated from the same plant material. Isolation and identification of nonpectolytic *Pseudomonas* was performed using standard methods mentioned above.

Bacteria were grown and maintained on Nutrient Agar (Difco) at 27°C and 4°C, respectively.

Bacterial DNA used in PCR as a template was obtained in the following manner: bacterial cells from the colonies grown overnight on the Nutrient Agar medium (Difco) were suspended in 100 µl of sterile, distilled water to final concentration of 109cfu/ml. The cell suspensions were then boiled for 10 min., kept at 4°C for 10 min. and finally centrifuged for 10 min. at 10,000 g at room temperature. The template DNA was stored at -20°C and then 1 µl of this DNA was used for one PCR reaction.

PCR primers used in this study were designed on the basis of published sequences of *Pseudomonas* genes. We used the data from the GenBank and computer program Oligo 5.0 (Rychlik and Rhoads 1989). We used also the published primer sequences designed for identification of *P. viridiflava* species (Gitaitis et al. 1998). The primers were synthesized by Ransom Hill Bioscience, Inc. (Ramona, CA, USA)

Table 1

Biochemical characteristics of soft rot associated *Pseudomonas* species. (Palleroni 1984; Holt et al. 1994)

Tests	*P. fluorescence*					*P. putida*		*P. varidiflava*
	*I	II	III	IV	V	*A	B	
Fluorescent pigment production on King B medium	+	+	+	+	+	+	+	+
Pectate gel pitting	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	–
Levan	+	+	–	+	–	–	–	–
Arginin dihydrolase	+	+	+	+	+	+	+	–
Utilization of								
– glucose	+	+	+	+	+	+	+	+
– trhalise	+	+	+	+	+	–	–	–
– adonitol	+	–	d	–	d	–	–	d
– sorbitol	+	+	d	+	d	–	d	+
– L-arabinose	+	+	d	+	d	d	+	d
– D-galactose	+	+	d	+	d	–	d	d
– sucrose	+	+	–	+	d	–	d	–

\+ 90% or more of strains positive
– 90% or more of strains negative
d 11-89% of strains positive
* biovars

PCR assay was performed in a total volume of 10 µl in GeNunc Tubes (size 0.2 ml, USA). The paraffin oil reaction overlay was not used. The final reaction mixture contained: 1 µl of the DNA template, 1xPCR Buffer (TaKaRa Shuzo Co., Ltd. Biomedical Group, Japan), 0.25U of Recombinant Taq DNA Polymerase (TaKaRa Shuzo Co., Ltd. Biomedical Group, Japan), 0.25 mM of each of the deoxynucleoside triphosphates (TaKaRa Shuzo Co., Ltd. Biomedical Group, Japan), 1.5-2.5 mM MgCl2 and 5-10 M of each of the oligonucleotide primers. The precise reaction conditions regarding MgCl2 and primer concentration for each primer set were designated empirically.

The PCR was performed on a Biometra thermocycler UNO-Thermoblock (Germany) equipped with the heating lid (100°C). For PMA-PMARev primer set the thermocycler was programmed as follows: 94°C for 4 min., 30 cycles at 94°C for 1 min., 55°C for 1 min., 72°C for 2 min. and finally, one cycle at 94°C for 1 min., 55°C for 1 min. and 72°C for 10 min. For other primers the annealing temperature was raised to 60°C while the rest of the parameters remained unchanged.

The PCR products were analyzed by gel electrophoresis in 1% w/v agarose supplemented with ethidium bromide (final conc. 0.8 (g/ml) using AgaGel Mini apparatus (Biometra, Germany). Electrophoresis was performed in TBE buffer (10.8 g Tris base, 5.5 g Boric acid, 0.93 g EDTA, 1,000 ml H_2O, pH 8.3). Before elecrophoresis 5 µl of each sample was mixed with 1 µl of the Gel Loading Solution (Sigma). 123 bp DNA Ladder, 5 µl/ well (Sigma) was used as a molecular weight markers.

The gels were evaluated using a Biometra TI 3 (Germany) transiluminator.

III. RESULTS

A total of 94 pectolytic and 60 nonpectolytic *Pseudomonas* isolates were obtained from 250 samples of rotted vegetables representing various plant species. The results of the identification and differentiation of these isolates are presented in Tables 2 and 3.

The isolates from pectolytic group belonged mostly to *P. fluorescent* species. The most abundant was biovar V, which comprised 30 isolates (31.9%). Biovar II was also relatively frequent (26.6%). There were 10 isolates (10.6%) of biovar III and only 1 isolate (1.1%) of biovar I. The biovar IV of *P. fluor*escens was totally absent. *P. putida* species comprised 25 isolates (27.8%) of pectolytic group with both biovars (A and B) present in almost equal numbers. There were 14 isolates of biovar A (14.8%) and 11 isolates of biovar B (11.7%). Only 3 isolates representing *P. viridifava* species were obtained from potato.

Exclusively *P. fluorescens* and *P. putida* species represented the 60 isolates of non-pectolytic *Pseudomonas* group. Only 2 isolates of *P. fluorescens* biov. II were present. The most numerous was *P. fluorescens* biov. I (15 isolates-25.0%). Other biovars were represented in relatively equal numbers: 12 isolates (20.0%), 13 isolates (21.7%), 7 isolates (11.7%) and 11 isolates (18.3%) for *P. fluorescens* biov. III, *P. fluorescens* biov.V, *P. putida* biov. A and *P. putida* biov. B, respectively.

The target bacterial species, molecular weights of expected products, sequences and optimal conditions for each primer set are given in Table 4. PCR performed with PSEUD-PSEUDRev primer set, designed for identification of *Pseudomonas* species with pectolytic phenotype irrespective of their taxonomical status gave generally one or two bands with large number of various bacterial isolates tested. There was no correlation between the

Table 2

The primer sets used to differentiate fluorescent, pectolytic *Pseudomonas* species associated with soft rot

Bacterial species	Primer sets	Sequences of primers	Expected product (bp)	Optimal conditions of reaction		
				T_A	C_p	C_{MgCl2}
P. marginalis	PMA PMARev	5'CGG ATA TTT GGC TGG ACG TC3' 5'GGC ACC ATC GTC CTT ATC AA3'	395	55°C	5 µM	2.5 mM
P. fluorescens	PFL PFLRev	5'GTG GGA CCC TAC TGA CGG CA3' 5'TTC ACG GGT GAC GGT GCT3'	702	60°C	5 µM	1.5 mM
P. viridiflava	PVI PVIRev	5'GCA CAG GCC GAT ATC GCT AC3' 5'CGC TGC CAT CGT TCG C3'	403	60°C	5 µM	2.5 mM
pectolytic	PSEUD	5'CAA ACT GGC CAG TGC CG3'	562	60°C	5 µM	1.5 mM
Pseudomonas	PSEUD Rev	5'GCC GTT CTG GTC ATC GGT CA3'				
P. viridiflava	*PLIT PLITRev	5'GTA TTG CTG GTG TTA CCC3' 5'GGT ATC CAG AAA CGA CAC3'	606	60°C	10 µM	2.5 mM

TA – annealing temperature
CP – primer concentration
C_{MgCl_2} – magnesium chloride concentration
* – published primer sequences (Gitaitis et al. 1998)

Table 3

Pectolytic, fluorescent *Pseudomonas* species isolated from naturally rotted vegetable species in Poland[1)]

Plant species	*P. fluorescens* biovars:				*P. putida* biovars:		*P. viridiflava*	Isolates (no.)
	I	II	III	V	A	B		
Potato	1	7		1		2	3	14
Celery			6	8	5	4		23
Parsley		5	2	11	4	2		24
Carrot				1				1
Onion		8		2	2	1		13
Leek			1	6	1	1		9
Cabbage		1		1	1			3
Chinese cabbage		1						1
Chicory		2	1		1			4
Lettuce		1						1
Pepper					1			1
Total	1	25	10	30	14	11	3	94
%:	1.1	26.6	10.6	31.9	14.8	11.7	3.3	100.0

[1)] Bacterial isolates were identified on the basis of standard biochemical and physiological tests

Table 4

Nonpectolytic, fluorescent *Pseudomonas* species isolated from naturally rotted vegetable species in Poland[1)]

Plant species	*P. fluorescens* biovars:				*P. putida* biovars:		Isolates (No.)
	I	II	III	V	A	B	
Potato			1	3		1	5
Celery	1		2		3		6
Parsley	1				1		2
Carrot			1				1
Onion	2		4	4	2	2	14
Leek	2	1	1				4
Cabbage	2						2
Chinese cabbage		1		1		1	3
Chicory	1		1			1	3
Lettuce	1		1	1	1	1	5
Pepper	1		1	2			4
Cucumber	2					2	4
Broccoli	1			1		3	5
Beet	1			1			2
Total	15	2	12	13	7	11	60
%:	25.0	3.3	20.0	21.7	11.7	18.3	100.0

[1)] Bacterial isolates were identified on the basis of standard biochemical and physiological tests

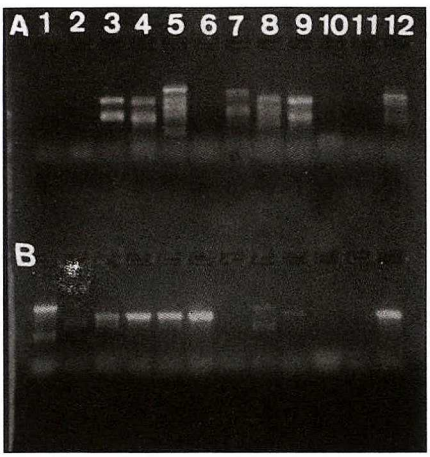

Fig. 1. PCR amplification of various pectolytic (line A) and nonpectolytic (line B) *Pseudomonas* species associated with soft rot of vegetables using PSEUD-PSEUDRev primer set. Lane A: 1-3-*Pseudomonas fluorescens* biovar II; 4-8-*Pseudomonas fluorescens* biovar V; 9-10-*Pseudomonas putida* biovar B; 11-12-*Pseudomonas putida* biovar A. Lane B: 1-4-*Pseudomonas fluorescens* biovar III; 5-6-*Pseudomonas putida* biovar B; 7-8-*Pseudomonas fluorescens* biovar I; 9-12-*Pseudomonas fluorescens* biovar V

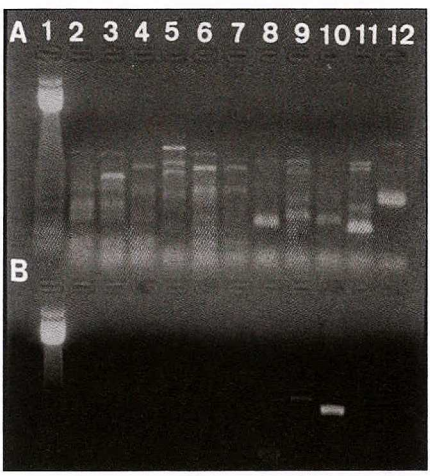

Fig. 2. PCR amplification of various pectolytic (lane A: 2-7) and nonpectolytic (lane B: 8-12) fluorescent *Pseudomonas* species using PVI-PVIRev primer set (line A) or PLIT-PLITRev primer set (lane B). Both lanes: 1-123 BP DNA Ladder (Sigma); 2-3-*Pseudomonas viridiflava*; 4-5-*Pseudomonas fluorescens* biovar V; 6-7-*Pseudomonas fluorescens* biovar II; 8-9-*Pseudomonas fluorescens* biovar V; 10-*Pseudomonas fluorescens* biovar III; 11-*Pseudomonas fluorescens* biovar I; 12-*Pseudomonas putida* biovar A

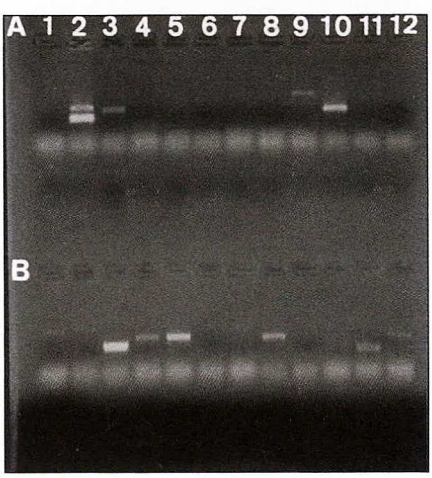

Fig. 3. PCR amplification of various pectolytic (line A) and nonpectolytic (line B) *Pseudomonas* species associated with soft rot of vegetables using PMA-PMARev primer set. Lane A: 1-3-*Pseudomonas fluorescens* biovar II; 4-6-*Pseudomonas fluorescens* biovar III; 7-10-*Pseudomonas fluorescens* biovar V; 11-12-*Pseudomonas putida* biovar A. Lane B: 1-2-*Pseudomonas fluorescens* biovar III; 3-4-*Pseudomonas fluorescens* biovar I; 5-7-*Pseudomonas fluorescens* biovar II; 8-10 *Pseudomonas putida* biovar A; 11-12-*Pseudomonas putida* biovar B

result of PCR amplification and taxonomical status or pectolytic properties of the isolate. However, the band patterns of some of the isolates were very similar although they belonged to various taxonomical groups (Fig.1). PFL-PFLRev primer set (for *P. fluorescens* species) gave several unspecific products with large number of various bacterial isolates including nonpectolytic ones. PVI-PVIRev and PLIT-PLITRev primer sets (both for *P. viridiflava*) also gave generally several unspecific products. The molecular weights of those bands were diverse and differed from expected 403 bp for PVI-PVIRev and 606 bp for PLIT-PLITRev primer sets. None of our isolates identified by biochemical and physiological tests as *P. viridiflava* was recognized by any of these primer sets, while some of the nonpectolytic *Pseudomonas* isolates gave one product of expected molecular weight after amplification with PLIT-PLITRev primers (Fig.2). PMA-PMARev (for *P. marginalis*) gave generally one product with some of the isolates. This product also differed among isolates regarding its molecular weight and there was also lack of correlation between identification based on biochemical tests and the presence of band (Fig. 3).

IV. DISCUSSION

In this study we established for the first time the spectrum of pectolytic, fluorescent *Pseudomonas* (PFP) species associated with soft rot of economically important vegetables in Poland. The results are particularly valuable because of the lack of any data on the subject for Polish conditions so far. The most important feature of the established spectrum is the domination of *P. fluorescens* species, which was represented by 70.2% of isolates and *P. putida* species represented by 26.5% of isolates. These results confirm the reports of Liao and Wells (1987a) and Janse et al. (1992), who established *P. fluorescens* and *P. putida* as the main PFP species associated with soft rot of vegetables in the United States and Netherlands respectively. Polish spectrum of PFP isolates is also very diverse nutritionally (data not shown). Representatives of one particular biovar of *P. fluorescens* or *P. putida* differed in respect of carbon source utilization however, within the limits allowed for classification (Tab.1). We decided against further grouping of the isolates, which would be based on carbon source utilization, in order to keep the results clear and only capability of trehalose utilization was used as the basis of differentiation between *P. fluorescens* or *P. putida* species. Our observations support the suggestion of others concerning heterogeneity of PFP species even within one biovar (Liao and Wells 1987a; Janse et al. 1992).

The taxonomical status of *P. marginalis* in Poland seems unclear. Although we identified relatively large group of bacteria (26.6%) with *P. marginalis* phenotype (*P. fluorescens* biovar II) among pectolytic isolates, we detected also 2 *P. marginalis* strains among nonpectolytic ones. This implies that in Poland *P. marginalis* strains are scattered among saprophytic ones similarly to situation presented by Janse et al. (1992) concerning Netherlands. Our results therefore, support the suggestion made by these scientists that *P. marginalis* name is artificial and that its use should be abandoned. Isolates representing other biovars of *P. fluorescens* and *P. putida* species also showed pectolytic or nonpectolytic activity.

This observation clearly implies that the lack of correlation between pectolytic phenotype of isolate and its taxonomical status concerns all *Pseudomonas* species associated with soft rot of vegetables and not only *P. marginalis*. To similar conclusions came Sands and Hankin (1975), Wang and Kelman (1982), Liao and Wells (1987) and Janse et al.(1992). However the comparison of numbers of isolates representing particular biovar of pectolytic and nonpectolytic group shows that different biovars are most abundant in both cases. This concerns mainly biovars I and II of *P. fluorescens* species. *P. fluorescens* biovar II, as it was mentioned earlier, comprises isolates of *P. marginalis* phenotype. It is more numerous among pectolytic *Pseudomonas* isolates in comparison to nonpectolytic ones. This in a way explains the long existence of separate *P. marginals* species. The situation is reversed for *P. fluorescens* biovar I. Only one isolate of this biovar was detected among pectolytic strains, while 15 nonpectolytic isolates was identified as biov. I of *P. fluorescens* species. The significance of this observation is unclear.

The results of this study also show the lack of correlation between the taxonomical status of bacterial strain irrespective of its pectolytic phenotype and plant species from which it was originally isolated. All biovars of *P. fluorescens* and *P. putida* species have broad host ranges in nature and may potentially occur on various host plants. However, because *P. fluorescens* is more numerous, this species and its biovars are in Poland of grater importance.

During our study we isolated only 3 representatives of *P. viridiflava* species. All of them were obtained from potato. *P. viridiflava* is potentially very important soft rot pathogen (Liao and Wells 1987a). In some regions, for instance in USA (Georgia), it may be responsible for severe looses in onion production both in the field and during storage (R.Gitaitis, pers. comm.). None of our 13 pectolytic onion isolates belonged to *P. viridiflava* species. The reason for this situation is most probably the different climatic conditions in Poland and in Georgia and different crop agriculture. We can assume that this bacterial species is at present rare in Poland and therefore of less economical importance.

In this study we attempted to use the PCR method to identify and differentiate PFP species. Because the extracellular pectate lyase is the enzyme responsible for the maceration of plant tissues and pectolytic phenotype of the PFP species, we based the sequences of our primers on published gene sequences encoding izoenzymes of pectate lyase: pel F of *P. fluorescens* (Liao et. al. 1993), pel V of *P. viridiflava* (Liao et al. 1974) and pel M of *P. marginalis* (Nikaidou et al. 1993). The primers prepared by others (Gitaitis et al. 1998) are also based on pel genes. Attempts to use PCR for identification and differentiation of PFP species associated with soft rot of vegetables were not reported so far with the only exception of *P. viridiflava* (Gitaitis et al. 1998), where the method proved useful particularly for isolates of onion origin. In our study, the PCR method using above mentioned primers proved to be unsuitable for identification and differentiation of Polish spectrum of bacteria with *P. fluorescens* and *P. putida* as predominant species. The possible explanation of this result is the large diversity of PFP also on biovar level mentioned above. The second reason is the high level of homology between known pel genes of various PFP species. Liao et al. (1996) demonstrated about 80% identity in amino acid sequence between proteins coded by pel genes of pectolytic *Pseudomonas*. Such high similarity reflected also on the DNA

level prevented the development of species-specific primers. However, our results imply other interesting feature of this group of bacteria, which is connected with the fact that the amplification of DNA of various isolates from both pectolytic and nonpectolytic group with PSEUD-PSEUDRev, PMA-PMARev or PLIT-PLITRev primer sets often resulted in obtaining one or two distinct bands. Although the molecular weights of this products varied with the isolates, it is still possible that the primers amplified the target pel sequence, which means that isolates of nonpectolytic phenotype carry the pel genes. Furthermore, the different molecular weights of the products may suggest that the structure of these genes is variable. This implies that the homologues of pel enzymes may be present in isolates that exhibit nonpectolytic phenotype and that this homologues underwent various types of modification or rearrangement during the evolution, as it was suggested by Liao (1991). Thus our results support the work of Liao (1991) who also demonstrated the presence of pel homologues in two *P. putida* strains that were originally isolated to be used for control of plant diseases and raised a concern about the safety of using *P. fluorescens* and *P. putida* as biocontrol agents.

The study on pathological properties of PFP isolates characterized here is currently carried out in our laboratory and will be reported elsewhere.

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ZRÓŻNICOWANIE BAKTERII Z RODZAJU *PSEUDOMONAS*, STOWARZYSZONYCH Z MOKRĄ ZGNILIZNĄ ROŚLIN W POLSCE

STRESZCZENIE

Z 250 prób, z różnych gatunków roślin warzywnych z objawami mokrej zgnilizny wyodrębniono 94 izolaty pektynolitycznych i 60 izolatów nie pektynolitycznych bakterii z rodzaju *Pseudomonas*. Identyfikację i różnicowanie izolatów przeprowadzono przy zastosowaniu standardowych testów fizjologiczno-biochemicznych. Spośród izolatów z grupy pektolitycznych *Pseudomonas* najliczniej występował gatunek *P. fluorescens*, który był reprezentowany przez 4 biowary (oprócz czwartego), przy czym dominowały biowary II i V. Mniej licznie występował gatunek *P. putida*, a *P. viridiflava* był reprezentowany jedynie przez 3 izolaty, pochodzące z ziemniaka. Podobna była struktura populacji izolatów z grupy niepektolitycznych *Pseudomonas*.

Metoda PCR okazała się być mało przydatna do wykrywania i różnicowania izolatów z grupy pektolitycznych *Pseudomonas* ze względu na duże ich zróżnicowanie. Amplifikacja DNA metodą PCR wykazała, że geny kodujące enzym liazy pektynowej występują także w izolatach niepektolitycznych rodzaju *Pseudomonas*.