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SELECTION OF BACTERIA FROM EPIPHYTIC POPULATIONS ON APPLE TREES AND SOIL ENVIRONMENT FOR ABILITY TO CONTROL FIRE BLIGHT (*ERWINIA AMYLOVORA*)[1]

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Abstract

From among 151 bacterial isolates obtained from apple leaves and soil environment 47 were able to protect pear fruitlets against fire blight to various extent. 21 isolates appeared highly effective and after their application the disease hardly developed on the fruitlets. Study on biotic relationship between these isolates and *Erwinia amylovora* on nutrient agar with sucrose and King B media indicate that only six isolates from this group and almost all from groups with low and very low efficacy inhibited pathogen growth on at least one of these media. This indicates that mechanisms other than antibiosis could be responsible for protective activity of bacteria and that medium composition influences production of secondary metabolites active against *E. amylovora* by the bacteria under study. Evaluation of selected isolates for ability to protect apple blossoms showed that results obtained from screening on pear fruitlets generally corresponded to that from blossom test.

Key words: bacteria, biocontrol, fire blight, pear fruitlet test, apple blossoms, *in vitro* activity

Introduction

Fire blight is the most devastating bacterial disease of apple, pear and many other plant species, especially of Rosaceae family (Vanneste 2000). Disease con-

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trol consists of integration of various methods to protect plants against infection, eliminate infection source and decrease plants' susceptibility. One of the possibilities of plant protection is the use of biopreparations based on bacteria and yeasts. Till now over 10 such products were developed and introduced into the market, particularly in United States and Europe (Werner and Aldwinckle 2006, Sobiczewski et al. 2007). Recently 'Blossom Protect' containing two strains of yeast *Aureobasidium pullulans* is being under process of registration in some countries of EU (Ertl et al. 2007).

Bacteria belonging to *Pantoea agglomerans* and *Pseudomonas* as well as *Bacillus* genera appeared very effective in protection of trees during bloom (Johnson and Stockwell 2000, Zeller 2006). However, the main problem considering their use on larger scale is stability in surviving and successive colonization of new developing blossoms and shoots as well as maintenance of high antagonistic activity against fire blight bacterium. At present, in several laboratories intensive research is conducted to improve the carrier for bacteria constituting active agents in already developed preparations and to increase the nutritional status so as to keep them alive and active. Also, the search for new effective isolates is still going on.

The aim of our study was isolation of epiphytic bacteria from apple trees and soil environment of different habitats and selection of effective isolates to control fire blight.

Materials and methods

Isolation of bacteria

Apparently healthy leaves from 'Idared' apple trees growing in the Pomological Orchard of the Research Institute of Pomology and Floriculture in Skierniewice were collected five times during 2005 and 2006 seasons. Each sample consisting of 10–20 leaves was placed into 250 ml of sterile distilled water in 500 ml flask. It was then shaken on a reciprocal shaker at 150 rev./min for 30 min. Afterwards 0.1 ml of aliquots was plated on 2.3% nutrient agar medium supplemented with 5% of sucrose (NAS). After three days of incubation at 26°C the bacterial colonies were transferred onto a new medium and purified. Selected isolates were stored in a mixture of PBS buffer (0.27% Na_2HPO_4, 0.04% NaH_2PO_4, 0.8% NaCl) and glycerol (4:1) at –76°C.

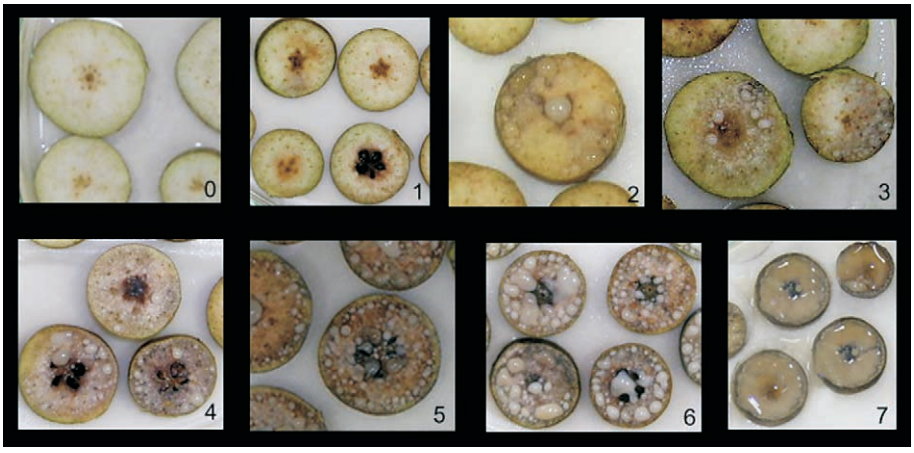
Soils samples of 10 g were collected several times during the seasons of 2007 and 2008 from apple orchard at Przybroda near Poznań (Experimental Station of The August Cieszkowski Agricultural University in Poznań). Each sample was placed in 90 ml of sterile water and shaken on reciprocal shaker similarly as in the case of leaf samples. Bacteria were isolated from the water suspension of soil in the same way as from leaves.

Preparation of bacterial water suspension

Bacteria of each isolate were cultivated on NAS medium for 24 h at 26°C and then washed out of the medium with sterile distilled water. The concentration of bacteria in suspension was adjusted with spectrocolorymeter Semco S91E at 630 nm and using serial dilution and plating method on NAS. For screening the bacteria biocontrol efficacy on pear fruitlets the suspension of 50% transmittance was used of each isolate, while for evaluation of selected isolates on apple blossoms, the suspension containing 10^8 cfu/ml was applied.

Test on pear fruitlets

The method described by Sobiczewski and Millikan (1985) was used. The pear fruitlet slides of cv. 'Conference' were momentarily dipped into water suspension of tested isolate and then placed on moist filter paper in Petri dishes. After 6 h the slices were inoculated with *Erwinia amylovora,* strain Ea 659 (from own collection), by spraying with its water suspension at 10^7 cfu/ml. The symptoms occurrence on slices was evaluated after seven days of incubation at room temperature. The following scale was used: 0 – no symptoms, 1 – one drop of bacterial ooze, 2 – several drops of ooze, 3 – presence of ooze drops on about half of the slice, 4 – about half of slice with ooze drops and necrosis, 5 – more than half of slice with ooze drops and necrosis, 6 – more than half of slice with necrosis and heavy ooze, 7 – ooze and necrosis of the entire slice (Phot. 1). Each isolate was tested on 40–50 fruitlet slices.



Phot. 1. Fire blight severity classes on pear fruitlet slices
(photo by A. Mikiciński)

Test on apple blossoms

The excised branches of apple cv. 'Linda' with blossoms at full blooming were used. Their basal end was put into beakers with water on the bottom (Phot. 2). The

Phot. 2. Apple branches of cv. 'Linda' with treated and inoculated blossoms were placed in humid chamber in the greenhouse (photo by A. Mikiciński)

branches were sprayed with water suspension of the selected bacterial isolate at the concentration of 10^8 cfu/ml and placed into humid chamber in the greenhouse. Six isolates were tested: 43M, 48M, 3M, L16, B90, 35M. The temperature in box was maintained at 22–26°C and air humidity of about 90–100%. Strains A506 of *Pseudomonas fluorescens* and C9-1 of *Pantoea agglomerans* as well as the chemical preparations Miedzian 50 WP (copper oxichloride) and Aliette 80 WP (fosetyl-Al) were used for comparison. After 24 h blossoms were spray inoculated with water suspension of Ea 659 at 10^7 cfu/ml. Six and nine days after inoculation the presence of blight symptoms on blossoms was recorded according to the slightly modified scale of Pusey (1999). Results were subjected to ANOVA analysis of variance. For separation of means the Newman-Keuls test at 5% significance was used.

Test on agar media

Evaluation of six selected isolates ability to inhibit the growth of *E. amylovora* was performed on two agar media containing various amount and C and N sources: NAS (nutrient agar 2.3%, sucrose 5%) and King B (Pseudomonas F agar 3.9%, glycerol 1.0%). Bacteria were seeded on each medium in the centre of Petri dishes and after three days of incubation at 26°C they were killed by vapors of chloroform

and then flooded with soft agar containing *E. amylovora*, strain Ea 659. The radius of inhibition zones around tested isolates was measured after 24 and 48 h.

Results

The screening of 151 bacterial isolates allowed determining their ability to protect pear fruitlets against fire blight. It was found that 40 isolates originating from apparently healthy apple leaves and seven isolates from soil showed such ability at various degrees. The lowest disease severity (0.0–1.0) scored seven days after inoculation was observed with protective application of 21 isolates (Table 1). This corresponded to the distribution of individuals (slices) among disease severity classes (Table 2). Almost 80 to 95% of slices protected with most efficient isolates have been classified into class 0 or 1 what means that during the experiment the disease did not develop on them at all or at a very low degree. Also, in the case of the second group of isolates (disease severity 1.1–2.0) showing high efficacy, the percentage of slices included to classes 1 and 2 was higher than 50%. Most of individual slices treated with isolates displaying the lowest protective activity (4.1–5.0) were placed into classes 4–7 and all of control slices – in classes 6 and 7.

Table 1

Distribution of individual pear fruitlet slices protected with each bacterial isolate among fire blight severity classes (%)

Range of disease severity	Isolate	Disease severity classes							
		0	1	2	3	4	5	6	7
1	2	3	4	5	6	7	8	9	10
0.0–1.0	B262	95.7	0	0	0	0	0	2.2	2.1
	B90	85.1	2.1	0	2.3	0	2.1	4.1	4.3
	L16**	86.0	0	0	0	0	0	14.0	0
	L6**	68.2	6.8	0	11.4	13.6	0	0	0
	3M*	79.5	4.6	2.3	2.2	2.3	4.6	4.5	0
	43M	70.5	25.0	0	0	0	0	0	0
	44M	50.0	38.1	0	11.9	0	0	0	0
	45M	50.0	38.1	0	9.5	2.4	0	0	0
	46M	85.7	11.9	0	2.4	0	0	0	0
	47M	69.8	23.2	0	7.0	0	0	0	0
	48M	86.0	4.7	0	2.3	0	7.0	0	0
	79M	80.0	10.0	0	7.5	0	2.5	0	0
	129M	90.0	5.0	0	5.0	0	0	0	0
	134M	95.3	4.7	0	0	0	0	0	0
	136M	70.8	18.7	0	10.5	0	0	0	0

Table 1 – cont.

1	2	3	4	5	6	7	8	9	10
	137M	45.0	30.0	0	25.0	0	0	0	0
	138M	65.0	30.0	0	5.0	0	0	0	0
	140M	45.0	40.0	0	15.0	0	0	0	0
	141M	85.0	15.0	0	0	0	0	0	0
	142M	76.0	16.0	0	8.0	0	0	0	0
	143M	85.0	0	0	5.0	0	10.0	0	0
1.1–2.0	49M	42.5	27.7	0	17.0	12.8	0	0	0
	75M	55.0	10.0	0	2.5	0	17.5	0	15.0
	78M	47.5	17.5	0	15.0	0	17.5	0	2.5
	135M	34.8	26.1	0	8.7	30.4	0	0	0
	139M	59.1	9.1	0	0	22.7	9.1	0	0
	145M	55.0	0	0	12.5	0	32.5	0	0
	147M	0	91.6	0	0	0	0	0	8.4
	150M	45.0	7.5	0	35.0	0	7.5	0	5.0
2.1–3.0	L13**	42.0	4.0	0	0	30.0	4.0	18.0	2.0
	35M	43.9	12.2	2.4	4.8	0	17.1	0	19.6
	42M	9.5	38.1	0	16.6	0	12.0	0	23.8
	80M	66.6	0	0	0	0	0	0	33.4
	151M	55.0	0	0	2.5	0	37.5	0	5.0
3.1–4.0	L21**	18.2	22.7	0	13.6	6.8	13.6	6.9	18.2
	19M	0	2.2	0	0	13.1	0	60.8	23.9
	22M	2.3	2.4	7.0	55.8	18.6	0	11.6	2.3
	28M	0	0	0	18.7	64.6	16.7	0	0
4.1–5.0	L18**	2.4	2.4	0	19.0	52.3	7.3	14.3	2.3
	21M	0	0	20.0	0	28.8	0	33.4	17.8
	29M	0	0	20.0	0	37.8	20.0	13.4	8.8
	34M	2.2	4.4	8.8	26.6	27.0	9.0	0	22.0
	37M	0	0	4.0	22.0	0	30.0	22.0	22.0
	50M*	0	0	12.5	7.5	25.0	30.0	25.0	0
	50MA	0	0	16.6	25.0	0	0	50.0	8.4
	51M	2.5	17.5	12.5	22.5	35.0	5.0	5.0	0
	144M	0	0	0	40.0	0	60.0	0	0
6.1–7.0	Control	0	0	0	0	0	0	14.0	86.0

Evaluation was made seven days after inoculation with *E. amylovora* (0 – no symptoms, 7 – ooze and necrosis of the entire slice, Phot. 1). Each isolate was tested on 40–50 pear fruitlet slices.

*Isolates obtained from apple orchard soil.

**Isolates obtained from forest soil (Mykoflor).

Other isolates originated from apple leaves.

Table 2

Inhibition zones of *Erwinia amylovora* growth on nutrient agar sucrose (NAS) and King B (KB) media by epiphytic and soil bacteria after 24 h and 48 h (mm) (means of two replications)

Isolate	NAS		KB	
	24 h	48 h	24 h	48 h
1	2	3	4	5
B262	0	0	10.5	8.5
B90	0	0	0	0
L16	16.5	10.5	7.5	5.5
L6	12	5	14.5	14.5
3M	4	4	13.5	6.5
43M	0	0	0	0
44M	0	0	0	0
45M	1	0	0	0
46M	0	0	0	0
47M	0	0	0	0
48M	0	0	0	0
79M	0	0	0	0
129M	0	0	1	0
134M	0	0	0	0
136M	0	0	0	0
137M	0	0	15.5	15.5
138M	0	0	0	0
140M	3.5	0	13.5	13.5
141M	0	0	0	0
142M	0	0	0	0
143M	0	0	13	13
49M	5	0	14	14
75M	0	0	15.5	15.5
78M	0	0	14.5	14.5
135M	0	0	13	13
139M	3.5	0	11.5	1
145M	0	0	14	14
147M	0	0	12	10.5
150M	5	0	14.5	14.5
L13	2	0	13	13
35M	1	1	13	1
42M	8	3.5	14.5	14.5
80M	10.5	0	0	0
151M	0	0	15	12

Table 2 – cont.

1	2	3	4	5
L21	3.5	3.5	5	0
19M	19	16.5	12	6
22M	12.5	6.5	6.5	2
28M	8.5	3.5	0	0
L18	2.5	2.5	5	0
21M	17	15.5	13	5
29M	5	4	0	0
34M	11	11	0	0
37M	19	14.5	11.5	3.5
50M	2	2	10	10
50MA	0	0	15.5	15.5
51M	1	1	10.5	9.5
144M	0	0	0	0

Table 3

Severity of fire blight on apple blossoms cv. 'Linda' after protective treatment with tested bacterial isolates (rating scale of severity: 0–4, 0 – no necrosis, 4 – total necrosis of ovary and peduncle; analyses were made separately for each day)

Treatment	Number of blossoms	Days after inoculation	
		6	9
Control	274	0.88 d	2.01 d
43M	260	0.11 ab	0.29 a (85.6)
48M	160	0.13 ab	0.38 a (82.1)
3M	232	0.18 ab	0.33 a (83.6)
L16	124	0.06 a	0.24 a (88.0)
B90	166	0.08 ab	0.42 a (79.1)
35M	149	0.14 ab	0.44 a (78.1)
C9-1	167	0.18 ab	0.35 a (82.6)
A506	204	0.30 bc	0.49 a (75.6)
Aliette 80 WP 0.25%	125	0.40 c	0.89 bc (55.7)
Miedzian 50 WP 0.3%	224	0.44 c	0.69 ab (65.7)
Miedzian 50 WP 0.15%	196	0.43 c	1.04 c (48.3)

Concentration of all isolates water suspension: 10^8 cfu/ml, *E. amylovora* used for inoculation: 10^7 cfu/ml.

Means followed by the same letter do not differ significantly at the level of 5% probability; numbers in brackets show efficacy (%).

The study on biotic relationship between tested isolates and *E. amylovora* on NAS and King B media indicates that only four isolates from the most efficient group inhibited the pathogen growth on both media and four other isolates on King B only (Table 2). Generally, the inhibition zones were larger on King B than on NAS medium. Almost all isolates with lower efficacy in protection of pear fruitlets showed the ability to inhibit fire blight bacterium on at least one of the media. More isolates demonstrated this activity on King B than on NAS.

Five isolates (3M, 43M, 48M, B90, L16) highly efficient on pear fruitlets, and the isolate 35M showing very high efficacy on fruitlets during the first five days of experiment, also appeared very effective on apple blossoms cv. 'Linda' (Table 3). Their efficacy determined nine days after inoculation with *E. amylovora* ranged from 78.1 to 88.0 which was very similar to that obtained with treatments with strains of A506 and C9-1, the biocontrol agents of Blight Ban A506 and Blight Ban C9-1, the preparations produced in USA (Table 3). It should be pointed out that the chemicals Miedzian 50 WP and Aliette 80 WP, used for comparison, protected blossoms at significantly lower level than the bacteria investigated. About 80% of blossoms protected with the evaluated isolates were apparently healthy while only 21% of those not protected did not show disease symptoms (Fig. 1).

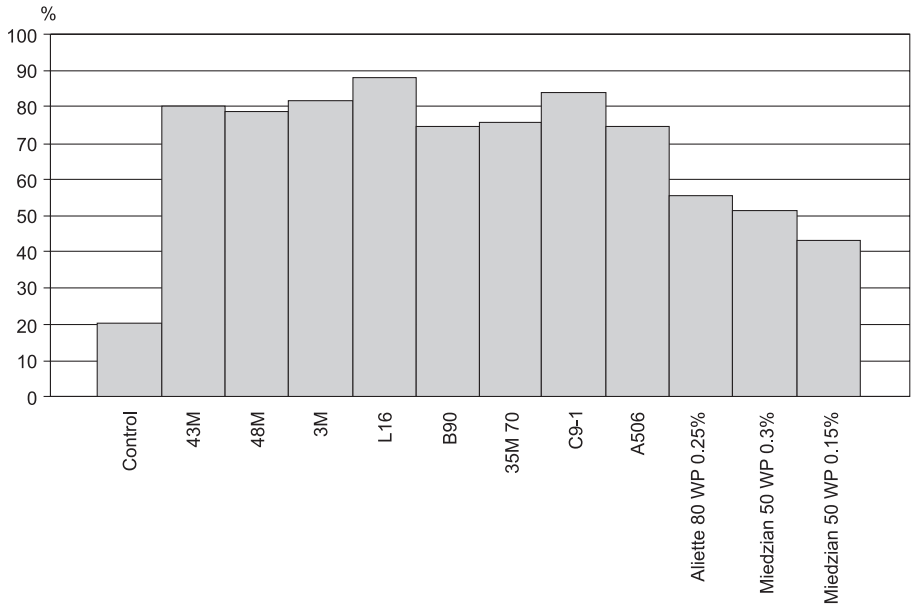


Fig. 1. Apparently healthy blossoms of cv. 'Linda' nine days after inoculation

Discussion

All six bacterial isolates tested on apple blossoms appeared very efficient and even better than commonly used in practice chemical preparations Miedzian 50 WP and Aliette 80 WP. However, because our study was performed under controlled conditions, with temperature and humidity ideal for bacterial survival and growth, it is assumed that in orchards their efficacy can be changeable. On the other hand, our experimental conditions were also very favourable for the pathogen development. Thus, using such a model it was possible to preliminary evaluate and select most efficient isolates. Our other study with those isolates allowed demonstrating some of their characteristics indicating possible mechanisms of action. Study on biotic relationship between these isolates and *E. amylovora* on five microbiological media (nutrient agar with sucrose, nutrient agar with glycerol, King B, LB and PYGA) revealed that L16, 3M and 35M inhibited growth of the pathogen on almost all of them while 43M, 48M and B90 did not show such activity. It was also found that isolates 3M, 35M and 48M produced lactones of homoserine (AHL) which indicated their ability to "communicate" with other bacteria. Phenotypic characterization and molecular analysis allowed classifying isolate 35M to *Pseudomonas syringae,* L16 to *P. fluorescens*, 43M to *Citrobacter farmer* (or *Pantoea agglomerans*), 48M and B90 to *P. agglomerans* and 3M to *Pseudomonas chlororaphis* (Sobiczewski et al. 2008).

The lack of ability to inhibit *E. amylovora in vitro* by some highly effective isolates indicates that mechanisms other than antibiosis may be responsible for their protective activity on pear fruitlets and apple blossoms. It is also possible that medium composition influenced production of secondary metabolites with activity against *E. amylovora*. One of the first methods of screening biotic relationship between bacteria and *E. amylovora* was the double layer technique (Beer et al. 1984 b, Johnson and Stockwell 2000). Various activities of bacteria on different media, including those we used, have been found by Knosel (1981), Isenbeck and Schultz (1985) and Psallidas et al. (1993). The problem was partially elucidated in model experiments with *E. herbicola* (present name *Pantoea agglomerans*) (Wodzinski et al. 1987 a, b, 1990, 1994). It was also found that some amino acids, e.g. casumino acid, cisteine or asparagines added to artificial media reduced production of substances antibiotic to *E. amylovora*. Thus, it can be concluded that composition of medium, either in plant tissue or in an artificial one, influences the production of some compounds playing crucial role in the establishment of quantitative and qualitative relationships between microorganisms living in the same biotope.

The comparison of our *in vitro* and *in vivo* study results showed that some of the isolates displayed activity under either condition. It should be pointed out, however, that on artificial media only the antibiotic activity of bacteria is demonstrated but on plant tissue also other mechanisms such as competition for nutrients and space and/or parasitism may take place (Johnson and Stockwell 2000). Thus, the lack of correlation between the bacteria ability to inhibit growth under *in vitro* conditions and their ability to protect plants in the field (Beer et al. 1984 b, Wilson et

al. 1990, 1992) was one of the main reasons to develop a method with application of plant tissue tests (Beer and Roundle 1983, Johnson and Stockwell 2000). Unripe pear fruits were used for the first time in England for testing *E. amylovora* pathogenicity (Billing et al. 1960). That was the basis for Sobiczewski and Millikan (1985) to work out the method for screening chemical preparations efficacy against fire blight. The finding of correlation between results of fruitlets test and efficacy of tested preparation under field conditions was very important (Norrelli and Gilpatrick 1982, Beer et al. 1984 a, Vanneste 1996). This conclusion is also partly of value in relation to our study. The most effective isolates will be a subject of further study in the field, aimed at development of an effective biopreparation.

We did not investigate to a greater extent the relationship between the ratio of tested bacteria and *E. amylovora* cells. In the case of some isolates it was only found that the protective activity of bacteria had not decreased even after diluting their water suspension 10 or 100 times (data not published). Various authors evaluated protective ability of bacteria using the qualitative criterion, determining presence of lack of disease (Beer and Roundle 1983, Vanneste et al. 1992, Kearns and Mahanty 1993) or the quantitative criterion, basing on the degree of fruitlets protection by bacteria (Isenbeck and Schultz 1985, Ishimaru et al. 1988, Wilson et al. 1990, Pusey 1996). In our study we used the latter criterion which allowed a more reliable evaluation of the protective activity of each isolate, but on the other hand, less rigorous. Vanneste (1996) pointed to the possibility of discrepancies in evaluation of bacteria tested on pear fruitlets varying with degree of ripening and physiological state which could greatly influence their susceptibility to fire blight. Our experiences confirm the observation, as it is important to use for screening uniform fruitlets collected at the same time and possibly early in the season, preferably in June.

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Streszczenie

SELEKCJA BAKTERII SPOŚRÓD EPIFITYCZNYCH POPULACJI WYSTĘPUJĄCYCH NA JABŁONIACH I W ŚRODOWISKU GLEBOWYM DO OCHRONY ROŚLIN PRZED ZARAZĄ OGNIOWĄ (*ERWINIA AMYLOVORA*)

Spośród 151 izolatów bakterii pochodzących z liści jabłoni i z gleby 47 w różnym stopniu wykazało zdolność ochrony zawiązków owoców gruszy przed zarazą ogniową. 21 izolatów okazało się bardzo efektywne i po ich zastosowaniu na

zawiązki choroba nie rozwijała się w ogóle lub w bardzo niewielkim stopniu. Badania nad biotycznymi relacjami między tymi izolatami a *Erwinia amylovora* na agarze odżywczym z sacharozą i pożywce King B wykazały, że tylko sześć izolatów z tej grupy i prawie wszystkie charakteryzujące się małą lub bardzo małą skutecznością ograniczały wzrost patogenu na co najmniej jednej z pożywek. Wskazuje to na występowanie raczej innego mechanizmu ochronnego działania bakterii niż antybioza oraz że skład pożywki, na której bakterie były hodowane, wpływał na wytwarzanie przez nie toksycznych wobec *E. amylovora* metabolitów. Ocena efektywności działania wybranych bakterii w ochronie kwiatów jabłoni przed chorobą wykazała, że wyniki testu na zawiązkach owoców gruszy korespondowały z tymi uzyskanymi na kwiatach.

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