Inhibitory Effect of *Pseudomonas* spp. on the Development of *Botrytis cinerea* and *Penicillium expansum*

Hanna BRYK[1], Barbara DYKI[2] and Piotr SOBICZEWSKI[1]

[1]Research Institute of Pomology and Floriculture, Skierniewice, Poland; [2]Research Institute of Vegetable Crops, Skierniewice, Poland

Abstract

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The influence of antagonistic *Pseudomonas* spp. on the development of *Botrytis cinerea* and *Penicillium expansum* was studied in liquid cultures. Two strains of *Pseudomonas* spp. (B194 and B224), originally isolated from apple leaves and fruits, respectively, inhibited spore germination and germ tube elongation of *B. cinerea* or *P. expansum.* The inhibitory effect depended on the concentration of bacteria in the cultures. After a prolonged time of incubation (48–76 h) lysis and fragmentation of hyphae of both fungi was observed. In some cases the hyphae of *B. cinerea* developed abnormally if the bacterial strains were present – the hyphal tips were swollen and ball-shaped spore-like structures aggregated in chains were formed.

Keywords: biocontrol; blue mold; gray mold; microbial interaction; mechanism of biocontrol

Bacteria have lately been extensively studied as potential agents for biological control of plant pathogenic fungi. An effective protection of fruits against postharvest fungal diseases was obtained with bacteria belonging mainly to the genera *Pseudomonas* (Janisiewicz 1987; Janisiewicz & Roitman 1988; Smilanick *et al*. 1993; Bryk *et al*. 1999) and *Bacillus* (Pusey & Wilson 1984). However, representatives of other genera like *Pantoea agglomerans* (*Erwinia herbicola)* (Sobiczewski *et al*. 1996; Ritte *et al*. 2002) or *Enterobacter* spp. (Utkhede & Sholberg 1986; Wilson *et al*. 1987) are also promising.

The mechanisms of antagonistic activity of bacteria against pathogenic fungi are based mainly on antibiosis, direct parasitism and/or competition for nutrients and space (Whipps 1992; Janisiewicz & Korsten 2002). Of the Pseudomonads used to date in biocontrol of fruit diseases, those whose action was based on antibiosis (Janisiewicz & Roitman 1988; Mao & Camppellini 1989; Smilanick *et al*. 1993; Wilson & Chalutz 1989) or act by other mechanism (Janisiewicz 1987; Janisiewicz & Marchi 1992) have been selected.

The aim of the present work was to investigate *in vitro* the interaction of two strains of *Pseudomonas* spp. (B194 and B224) with *Botrytis cinerea* and *Penicillium expansum* to better understand their possible activity as biocontrol agents on apples. In our earlier study it was stated that these bacterial strains showed a high activity in protecting apples against grey mold (*B. cinerea)* and blue mold (*P. expansum)* during storage although they did not inhibit the growth of both fungi on potato dextrose agar (PDA) (Bryk *et al*. 1999).

MATERIALS AND METHODS

The bacteria. Two strains of *Pseudomonas* spp. isolated from apple fruit cv. Elstar (B194) and from apple leaves cv. Idared (B224) at the Research Institute of Pomology and Floriculture in Skierniewice were used. They were identified according

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to Bradbury's key (Bradbury 1988) as *Pseudomonas* spp. (atypical P. *viridiflava*), which do not produce pectinolytic enzymes (Bryk *et al*. 1999). The strains were maintained on Nutrient Agar (Difco) slants supplemented with 1.6% of glycerol under paraffin in the refrigerator. For preparation of a water suspension, bacteria were incubated at 24°C on Nutrient Agar supplemented with 1% glucose. After 24 h the cells were washed from the medium with sterile distilled water. The concentration of bacteria in the suspension was determined spectrophotometrically and adjusted to 10^{10} cfu/ml by serial dilution.

The fungi. *Botrytis cinerea* Pers. and *Penicillium expansum* (Link.) Thom were freshly isolated from apples and maintained on PDA at 20°C. A suspension of *B. cinerea* conidia was obtained by washing a 14-day-old culture with sterile water. Due to a very low germination of *P. expansum* conidia in water, their suspension was prepared by washing 7-day-old cultures with sterile apple juice, either undiluted or diluted (1:4, v/v) in water. The juice was obtained from Jonagold apples and autoclaved before use. The concentration of spores in all suspensions was determined under a microscope and adjusted to 10^5 per ml.

***Interaction of bacteria with** B. cinerea **and** P. expansum*. The studies were conducted according to the method described earlier (Bryk *et al*. 1998). The standardised suspensions of both bacterial strains (B194 and B224) were mixed in test tubes with suspension of conidia of the respective fungus in the following proportions: 1:5, 2:5, 3:5 and 4:5 (v/v). The mixtures were then incubated at room temperature. The control consisted of suspensions of *B. cinerea* spores in sterile water or *P. expansum* spores in undiluted or diluted apple juice. After 6, 12 and 24 h of co-cultivation, 30 µl aliquots were placed on sterile microscope slides and observed under a light microscope. Percent of spore germination and germ tube length were determined in 10 different microscopic fields. A total of 400 spores (100 spores per replicate) were observed at each sampling time. After a longer period of incubation (more than 48 h) the developed hyphae were observed under a light microscope and photographed.

Data analysis. Results were statistically evaluated with an analysis of variance. Percent values (germination of conidia) were transformed according to Bliss (y = arc sin$\sqrt{x/100}$) and values of germ tubes length were transformed according to $y = \log (x + 1)$. The significance of differences between means was determined with Duncan's multiple range test ($P \leq 0.05$).

RESULTS

The effect of bacteria on *B. cinerea*

Spores incubated in water (control) at room temperature swelled and after 3–4 h started to germinate, producing one and sometimes two germ tubes. After 6 h about 50% of the spores had germinated. However, spore swelling was strongly limited in co-cultivation with bacteria. They surrounded the spores and inhibited their germination (Table 1). After 6, 12 and 24 h of co-cultivation there was

Table 1. The effect of *Pseudomonas* spp. strains B194 and B224 on conidia germination and germ tube elongation of *Botrytis cinerea*

Incubation time (h)	B194					B224				
	C	1:5*	2:5*	3: 5*	4:5*	C	1:5*	2:5*	3:5*	4:5*
% of germinated conidia										
6	49.8 ef	8.0 bc	1.8 ab	0.9 ab	0.0 a	49.8 fg	30.4 def	12.0 bc	1.0 a	1.4 a
12	60.4 f	32.9 de	20.6 cd	6.0 b	4.7 b	60.4 g	60.0 g	46.2 f	23.6 cde	11.8 bc
24	81.2 g	35.9 de	34.5 de	8.5 bc	7.0 bc	81.2 h	64.4 g	35.5 ef	17.3 bcd	7.8 ab
Length of germ tube (µm)										
6	34.2 f	17.4 cde	13.0 bc	0.0 a	0.0 a	34.2 fg	17.6 cde	11.0 b	0.0 a	0.0 a
12	54.4 g	23.8 def	22.0 de	15.4 bcd	14.0 bc	54.4 hi	25.4 ef	24.2 def	16.8 bcde	16.2 bcd
24	137.2 h	50.6 g	26.8 ef	13.4 bc	9.8 b	137.2 j	67.6 i	42.6 gh	17.8 cde	14.8 bc

C – control (conidia of *B. cinerea* in water); *proportion of bacteria to fungal conidia in the mixture (1:5, 2:5, 3:5, 4:5)
Data followed by different letters (separately for B194 and B224) differ significantly at $P \leq 0.05$ (Duncan's *t*-test)

significant inhibition of germination in all mixtures with B194, and in mixtures 2:5, 3:5, and 4:5 with B224. Mixtures 3:5 and 4:5 of both strains gave the highest inhibition. After 24 h of co-cultivation, germination of spores in mixture 4:5 was inhibited by about 90% as compared to the control. After 64 h of co-cultivation the non-germinated spores shrunk and changed shape (data not presented).

The germ tubes elongated fast in water (control) – after 6 h their average length was 34.2 μm (Table 1). In the same time the germ tubes in mixtures 1:5 and 2:5 were significantly shorter than in the control. In mixtures 3:5 and 4:5 the spore germination was very low and only a few short germ tubes, with a length of approximately the spore size, were observed. In all mixtures the germ tubes were surrounded by bacteria and their further elongation was slower than in water (control). After 24 h of co-cultivation the inhibitory effect was related to the concentration of bacteria in the mixture. Germ tubes in mixture 4:5 were 93% (B194) and 89% (B224) shorter than those in water (Figure 1A, B).

Further development of hyphae in the presence of bacteria was also modified. After 48 h of incubation the control treatment (conidia in water) was opaque, with visible "strings" of mycelium, whereas the mixture of fungal conidia and bacteria (particularly 3:5 and 4:5) was still transparent. After 76 h of co-cultivation the germ tubes started to lyse, especially in mixtures 3:5 and 4:5 (Figure 1C, D). In the presence of both bacterial strains the hyphal tips (both long and short) were swollen after 76 h of co-cultivation and ball-shaped spore-like structures, aggregated in chains, were formed (Figure 2).

The effect of bacteria on *P. expansum*

After 6 h incubation of the spores without bacteria (control) their germination was significantly higher in diluted than in undiluted apple juice. After 24 h this difference disappeared (Tables 2 and 3).

In undiluted juice, strain B194 significantly inhibited spore germination only after 24 h of incubation in mixtures 3:5 and 4:5, whereas mixture 4:5 of strain B224 inhibited it after 6 and 12 h, and all its mixtures after 24 h (Table 2). After just 6 and 12 h of co-cultivation the spores and germ tubes were surrounded by bacteria of both strains (Figure 3B). Significant differences in the length of germ tubes appeared only after 24 h in mixture 4:5 of B194 and in mixtures 2:5, 3:5 and 4:5 of B224 (Table 2).

In diluted juice, strain B194 significantly inhibited spore germination after 6 and 12 h only in mixture 4:5, and strain B224 in mixtures 2:5, 3:5 and 4:5. Yet after 24 h, mixtures 2:5, 3:5, 4:5 of B194 and mixtures 3:5 and 4:5 of B224 were inhibitory. Significant differences in length of germ tubes

Table 2. The effect of *Pseudomonas* spp. strains B194 and B224 on conidia germination and germ tube elongation of *Penicillium expansum* in undiluted apple juice

Incubation time (h)	B194					B224				
	C	1:5*	2:5*	3: 5*	4:5*	C	1:5*	2:5*	3:5*	4:5*
% of germinated conidia										
6	42.3 abc	34.6 ab	34.8 ab	26.4 a	21.8 a	42.3 cde	28.4 abcd	27.4 abcd	24.9 abc	13.9 a
12	52.4 bc	54.2 bc	38.8 abc	34.6 ab	29.9 ab	52.4 e	46.4 de	44.1 de	40.5 bcde	22.6 ab
24	91.1 e	82.7 e	78.3 de	61.4 cd	34.5 ab	91.1 f	43.6 cde	38.4 bcde	29.4 abcd	19.7 a
Length of germ tube (μm)										
6	21.8 abc	15.2 a	17.0 ab	13.8 a	14.4 a	21.8 abcdef	20.6 abcde	16.6 abc	16.2 ab	15.2 a
12	34.6 cde	30.4 cde	26.6 bcd	28.4 bcd	21.4 abc	34.6 efg	30.4 def	26.0 abcdef	19.2 abcd	23.4 abcdef
24	58.2 f	48.4 ef	37.6 def	40.8 def	28.0 bcd	58.2 h	53.2 gh	35.8 fg	29.0 cdef	28.0 bcdef

C – control (conidia of *P. expansum* in undiluted apple juice); *proportion of bacteria to fungal conidia in the mixture (1:5, 2:5, 3:5, 4:5)

Data followed by different letters (separately for B194 and B224) differ significantly at $P \leq 0.05$ (Duncan's *t*-test)

Table 3. The effect of *Pseudomonas* spp. strains B194 and B224 on conidia germination and germ tube elongation of *Penicillium expansum* in diluted apple juice

Incubation time (h)	B194					B224				
	C	1:5*	2:5*	3: 5*	4:5*	C	1:5*	2:5*	3:5*	4:5*
% of germinated conidia										
6	60.1 cde	46.4 bcd	38.2 abc	41.3 abcd	29.6 ab	60.1 cde	44.2 abcd	36.5 ab	35.9 ab	26.6 a
12	62.4 def	50.7 bcd	57.1 cde	46.1 bcd	23.8 a	62.4 def	61.6 def	49.2 bcde	40.2 abc	36.8 ab
24	80.0 f	76.3 ef	41.4 abcd	44.8 abcd	32.5 ab	80.0 f	65.7 ef	63.3 def	38.9 a	34.6 ab
Length of germ tube (μm)										
6	17.2 a	15.8 a	15.0 a	14.2 a	16.2 a	17.2 ab	18.2 ab	17.4 ab	19.2 abc	13.2 a
12	38.4 cd	37.8 cd	27.6 bc	30.0 bc	22.4 ab	38.4 def	32.0 cdef	26.0 bcde	25.6 bcde	23.8 bcd
24	85.8 f	63.6 ef	51.2 de	43.0 cde	33.4 bcd	85.8 g	49.6 f	40.8 ef	32.4 cdef	34.6 def

C – control (conidia of *P. expansum* in diluted apple juice); *proportion of bacteria to fungal conidia in the mixture (1:5, 2:5, 3:5, 4:5).

Data followed by different letters (separately for B194 and B224) differ significantly at $P \leq 0.05$ (Duncan's *t*-test)

appeared only after 12 h in mixture 4:5 and after 24 h in mixtures 2:5, 3:5, 4:5 of B194, and after 24 h in all mixtures of B224 (Table 3).

After 48 h of incubation with both strains of bacteria the shape of ungerminated spores changed and lysis of germ tubes was observed (Figure 3C).

The inhibition of germination of *P. expansum* conidia and germ tubes elongation by bacteria was affected by juice dilution, proportion of bacteria to spores in the mixtures and duration of culture. The number of bacteria of either strain in mixture 1:5 was too low to significantly inhibit spore germination and germ tube elongation independently of juice dilution and duration of culture. In mixture 2:5 the antagonistic action by the bacteria was better, but only after 24 h of incubation. The best action of bacteria was shown in mixtures 3:5 and 4:5.

Dilution of apple juice had no influence on the activity of strain B224. In strain B194 a significant inhibition of spore germination was evident after just 6 h of incubation in diluted apple juice, whereas in undiluted apple juice this effect appeared only after 24 h (in mixture 4:5).

DISCUSSION

Strains B194 and B224 of *Pseudomonas* spp. inhibited spore germination and development of germ tubes of *B. cinerea* and *P. expansum*. The antagonistic activity of the bacteria was related to their concentration in the mixture; the greatest inhibition of spore germination was observed at the highest concentration of bacteria (4:5). The inhibitory effect of both strains on spore germination and germ tube elongation was stronger on *B. cinerea* than on *P. expansum*. For example, after 24 h of co-cultivation in mixture 4:5, germ tubes of *B. cinerea* were on average about 90% and those of *P. expansum* 60% shorter than the control.

The bacteria of strains B194 and B224 demonstrated a distinct positive taxis to spores and germ tubes of both *B. cinerea* and *P. expansum*. In our earlier study (Bryk *et al*. 1998), *Pantoea agglomerans (Erwinia herbicola)* showed a similar action with both fungi. It seems that this feature is very important in an antagonistic activity of bacteria as they can act on the pathogen in its closest vicinity. Wisniewski *et al.* (1991) pointed out that the antagonistic yeast *Pichia guilliermondii* attaches tightly to the hyphae of *B. cinerea* and acts as a mycoparasite. *Enterobacter cloacae* has also been reported to bind to cell walls of *Pythium ultimum* (Nelson *et al*. 1986).

Lysis of hyphae and non-germinated conidia of both fungi were observed in our experiments. It is supposed that such lysis could be an intermediate stage of decomposition of the fungus to obtain nutrients for the bacteria. Parasitism as a mode of action of potential biocontrol agents against fruit

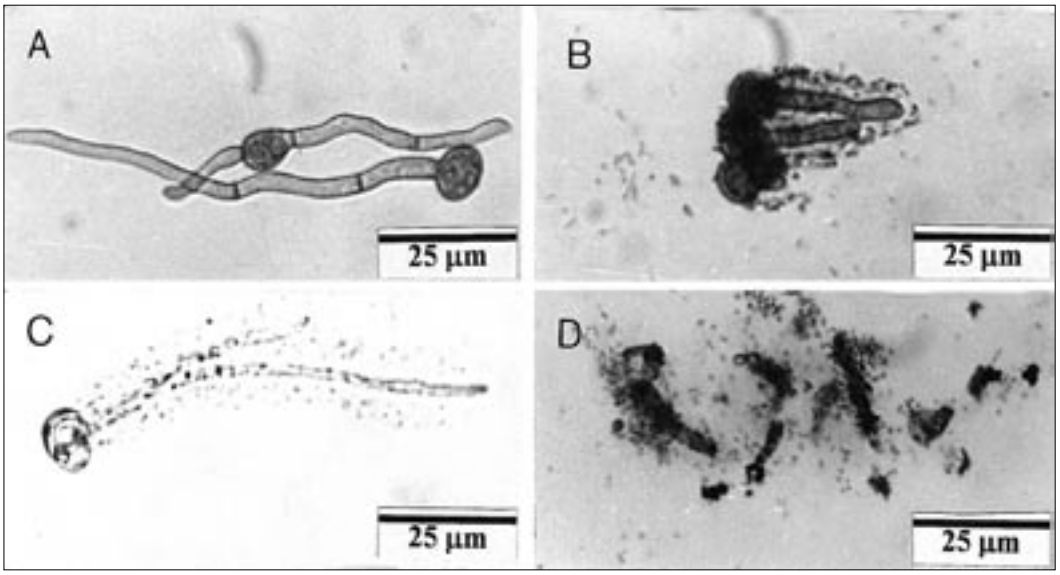

Figure 1. Interaction of *Pseudomonas* spp. with *Botrytis cinerea*. Germ tubes of *B. cinerea* after 12 h of incubation in water (A) and in the presence of B224 after 24 h of incubation (B); C, D – lysis and fragmentation of germ tubes of *B. cinerea* in mixtures with B194 (3:5) after 76 h of incubation

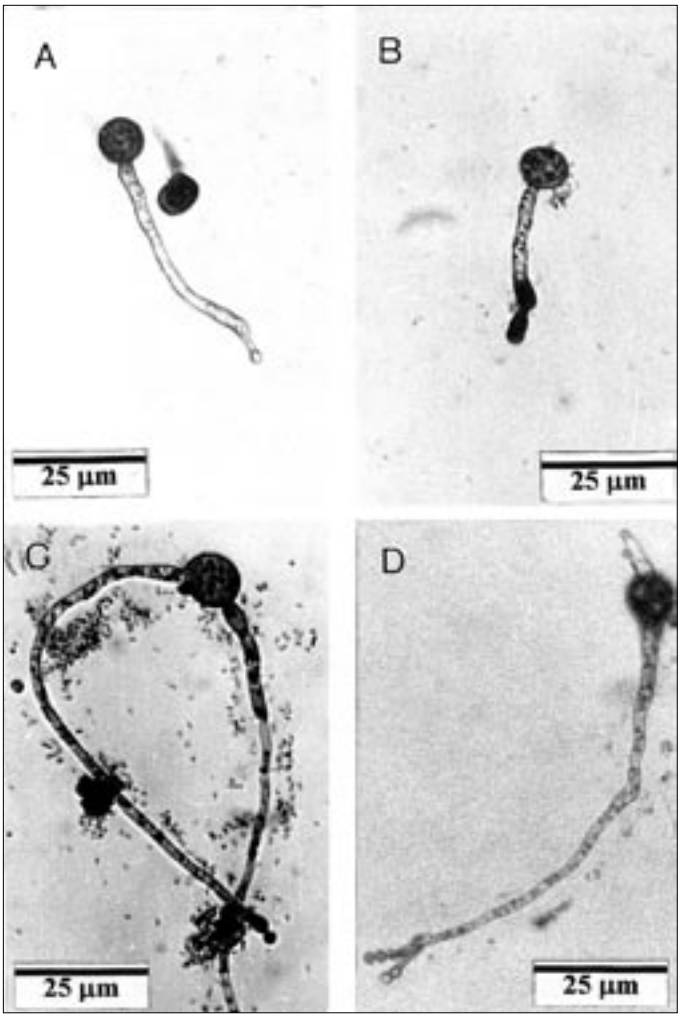

Figure 2. Development of ball-shaped structures on tips of *B. cinerea* hyphae under influence of *Pseudomonas* sp. strain B224, after 76 h of incubation. A, B – short hyphae; C, D – long hyphae

postharvest diseases was mentioned by other authors (Belanger & Deacon 1996; Bryk *et al*. 1998). Wisniewski *et al*. (1991) and El Ghaouth *et al*. (1998) suggested that yeast antagonistic to *B. cinerea* produces lytic enzymes, such as β-1,3-glucanase. In the case of *Penicillium digitatum*, the causal agent of green mold rot of orange, yeast rapidly colonised the fungal hyphae, which was followed by alterations in hyphal structure. This could be attributed to the action of lytic enzymes (β-1,3-glucanase and chitinase) (Arras 1996).

In the present study Pseudomonads bacteria caused fragmentation and lysis of hyphae of *B. cinerea*, and sometimes abnormal development of hyphae. Hyphal tips were swollen and ball-shaped spore-like structures, aggregated in chains, were formed. These structures had thin cell walls and their diameter was as small as microconidia. Similar structures were also observed in the study of interaction between strain B66 of *Pantoea agglomerans* and *B. cinerea* (Bryk *et al*. 1998). Abnormal development of *B. cinerea*

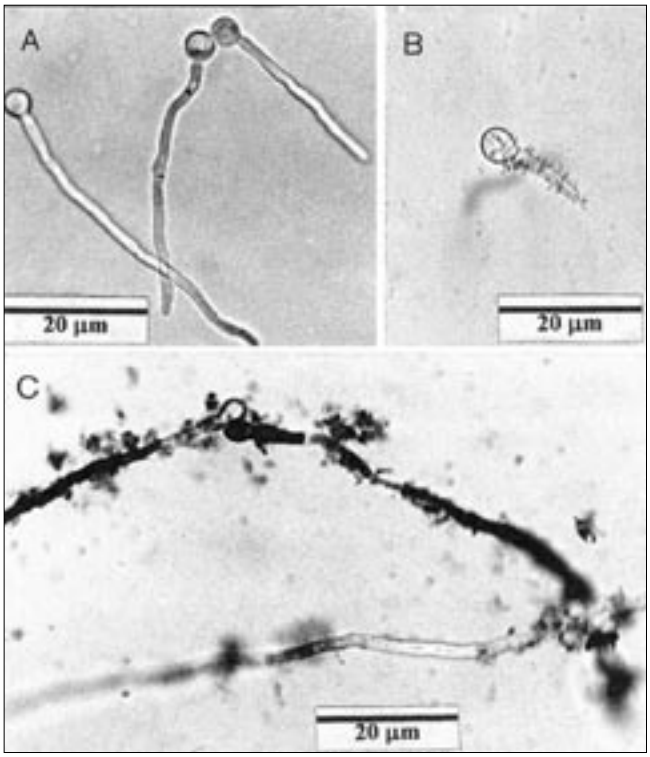

Figure 3. Interaction of *Pseudomonas* spp. with *Penicillium expansum*. Germ tubes of *P. expansum* after 24 h of incubation in apple juice without bacteria (A) and in the presence of B194 after 48 h of incubation (B); C – beginning lysis of germ tubes of *P. expansum* in mixtures with B224 (4:5) after 48 h of incubation

mycelium in the presence of the mycoparasite *Sporothrix flocculosa* was found by Belanger & Deacon (1996). However, in that case the reaction was characterised by intense and irregular branching accompanied by short and stubby septate segments. Hyphal tips were invariably shrunken, being either empty or containing granular and retracted remnants of cytoplasm.

Our earlier study had demonstrated that both strains of *Pseudomonas* spp. (B194 and B224) did not inhibit the growth of *B. cinerea* and *P. expansum* on PDA medium, which presumably indicates that they do not produce antibiotic substances against these fungi. However, the strains satisfactorily protected apples against blue and gray molds (Bryk *et al*. 1999). Janisiewicz (1987) and Janisiewicz and Marchi (1992), using similar methods to evaluate bacteria, also found that strain L59-66 of *Pseudomonas syringae* which is very effective in protecting apples and pears against the same diseases, did not produce antibiotics active on their causal agents.

The results of the present study suggest that the mechanism of antagonistic activity of *Pseudomonas* spp. (B194 and B224) to *B. cinerea* and *P. expansum* may be based on parasitism. On the other hand, a possible mechanism by which strain B194 may act as biocontrol agent against *P. expansum* is competition for nutrients. The activity of this bacterial strain was better in diluted than in undiluted apple juice. The diluted apple juice contained only 20% of the nutrients of undiluted juice. Nutrient competition has been identified as a factor in biological control systems (Wisniewski *et al*. 1989; Bryk *et al*. 1998; Janisiewicz *et al*. 2000).

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Souhrn

Bryk H., Dyki B., Sobiczewski P. (2004): **Inhibiční účinek druhů rodu** *Pseudomonas* **na vývoj hub** *Botrytis cinerea* **a** *Penicillium expansum***.** Plant Protect. Sci., **40**: 128–134.

V tekutých kulturách byl sledován vliv antagonistických druhů rodu *Pseudomonas* na vývoj hub *Botrytis cinerea* a *Penicillium expansum*. Dva kmeny rodu *Pseudomonas* (B194 a B224), původně izolované z listů a plodů jabloně, inhibovaly klíčení a prodlužování klíčního vlákna *B. cinerea* nebo *P. expansum*. Inhibiční účinek byl závislý na koncentraci bakterií v kultuře. Po prodloužené době inkubace (48–76 h) byla u obou hub zaznamenána lyze a fragmentace hyf. V některých případech se za přítomnosti obou bakteriálních kmenů vyvíjely hyfy *B. cinerea* abormálně – vrcholy hyf byly zduřelé a kulovité struktury podobné sporám se shlukovaly v řetězce.

Klíčová slova: biologická ochrana; plíseň štětičková; plíseň šedá; mikrobiální interakce; mechanismus biologické ochrany

Corresponding author

Prof. Dr. Piotr Sobiczewski, Research Institute of Pomology and Floriculture, Pomologiczna 18, 96-100 Skierniewice, Poland
tel.: + 48 46 833 20 21; fax: + 48 46 833 32 28, e-mail: psobicz @ insad.pl