Biological Control of Blue Mold and Gray Mold on Apple and Pear with *Pseudomonas cepacia*

W. J. Janisiewicz and J. Roitman

ABSTRACT

Janisiewicz, W. J., and Roitman, J. 1988. Biological control of blue mold and gray mold on apple and pear with *Pseudomonas cepacia*. Phytopathology 78:1697-1700.

Control of gray mold, caused by *Botrytis cinerea*, and reduction in blue mold, caused by *Penicillium expansum*, was obtained on Golden Delicious apples and Bosc pears protected with *Pseudomonas cepacia* isolated from apple leaves. The bacterium strongly inhibited fungal growth during in vitro screening on nutrient yeast dextrose agar medium. An effective antifungal compound was isolated from the bacterial cells and culture medium. This compound, identified as a pyrrolnitrin, inhibited growth of both fungi at a concentration of 1 mg/L during an agar diffusion test in vitro. Complete control of gray mold was obtained on apples and pears protected with a pyrrolnitrin concentration of 10 mg/L at a pathogen inoculum level of 10^3-10^5 conidia/ml. Blue mold was controlled at the same concentration of pyrrolnitrin at inoculum concentrations of 10^3 conidia/ml for pears and 10^3 and 10^4 conidia/ml for apples. At concentrations of 50 mg/L or higher, complete control was obtained of both diseases on both fruits at all tested inoculum levels.

Additional keywords: antagonist, *Malus domestica*, *Pyrus communis*.

Pathogen resistance to fungicides (21) and concern for public safety (2) have combined to increase interest in alternative methods to fungicidal treatment in controlling fruit diseases. This has led to rapid development of biological control research (11,27). Antagonists have been reported for a few major field and postharvest pathogens (1,6,7,10,17,19,25,26).

The soil-isolated, antibiotic-producing *Bacillus subtillis* (18) has been tested on a semicommercial scale for postharvest control of brown rot in peach. Natural antagonists to postharvest pathogens isolated from fruit or leaves of pome fruit trees completely controlled blue mold (incited by *Penicillium expansum* Lk. & Thom.) and gray mold (incited by *Botrytis cinerea* Pers. & Fr.) diseases of apple (9,10,12). The antagonists were bacteria, yeast, or filamentous fungi. Some antagonists, when combined, controlled both diseases simultaneously (12). Pathogen and antagonist concentration were important to effective biocontrol on fruit of apple (9,10,12). More antagonist was required to obtain disease control on fruit challenged with higher concentrations of a pathogen. The existence of a quantitative relationship later was confirmed in biocontrol of Rhizopus rot in peach (26).

In our research, screening for antagonistic activity of organisms isolated from plant material was designed to include both antibiotic-producing and nonproducing organisms (12,13). Previously described antagonists for control of postharvest diseases of apples did not exhibit antibiotic activity in vitro, and the possibility exists that the mode of action is through stimulation of host (fruit) resistance (10,12).

The current study describes the control of blue mold and gray mold of apples and pears by *Pseudomonas cepacia* Burkh., and by the antifungal compound it produces, pyrrolnitrin. A preliminary report was published (13).

MATERIALS AND METHODS

Isolation of microorganisms and screening for antagonistic activity in vitro. Potential antagonists were isolated from apple

leaves and fruit according to previously described procedures (12). Leaves and fruit collected in the orchard were washed in 200 ml of phosphate buffer, pH 6.8, on a rotary shaker for 10 min at 100 rpm. The washings were discarded, and leaves or fruit were washed a second time for 10 min with sonication for 30 sec in a Branosomic 521 sonicator (Branson Co., Shelton, CT) at the beginning of the wash. The sonication step was used to facilitate detachment of organisms from the plant surface.

Washings from sonicated samples were plated on nutrient yeast dextrose agar (NYDA) (0.1 ml/plate) and incubated for 24 hr at 23 ± 2 C. After incubation, in part of the screening designated for isolation of antibiotic-producing microorganisms, plates were seeded with a conidial suspension of a pathogen (1×10^6 conidia/ml) and incubated for an additional 24–48 hr. Colonies producing zones of inhibition in the fungal growth were isolated and purified by single-colony isolations after triple restreaking on NYDA.

Antagonistic activity on fruit. The organism that produced the largest zone of inhibition during in vitro screening was selected for further study. This organism was identified as *P. cepacia* by means of fatty acid profiles on the Hewlett Packard 5898A gas chromatography Microbial Identification System (Microbial ID., Inc., Newark, DE) and by bacteriocin typing. The bacterial suspension was prepared by growing cultures in nutrient yeast dextrose broth (NYDB) for 24 hr at 24 C with shaking at 150 rpm. The medium then was centrifuged at 10,000 rpm (12,100 g) for 10 min and cells were resuspended in water. Desired concentrations were obtained by adjusting the suspension according to a standard curve with a spectrophotometer. Golden Delicious apples were wounded at the equator (one wound 3 mm in diameter and 3 mm deep per apple) and 20 μl of appropriate concentration of aqueous suspension of *P. cepacia* was applied to each wound. This was followed by inoculation with 20 μl of aqueous conidial suspensions of *P. expansum* or *B. cinerea*. The concentration of the conidial suspensions was determined with a hemacytometer. Treated apples were incubated at 23 ± 1 C and $76 \pm 4\%$ relative humidity for 7 days, after which the diameter of lesions was measured. The experiment was arranged in a randomized block design. Each apple constituted a single replicate, and each treatment was replicated six times. The separation of means was conducted with appropriate least significant difference analysis at $P = 0.05$. The

experiment was repeated.

Bosc pears were treated similarly, except that two wounds were made on each fruit, one closer to the stem end and the other closer to the calyx end. The diameter of the lesion was measured after 5 days. Each pear constituted a single replicate, and each treatment was replicated six times. The experimental design and statistical analysis were conducted similarly to the experiment with apples. The experiment was repeated with Anjou pears.

Isolation of the antifungal compound. P. cepacia was grown in 250 ml of NYDB in 2,800-ml flasks for 3 days at ambient temperature on a rotary shaker at 250 rpm. The culture was centrifuged at 10,000 rpm (16,300 g) for 10 min. The pellet of bacterial cells was suspended in water, sonicated for 5 min at 350 watts with a Branson Sonnifier 350 equipped with 1.3-cm horn, and centrifuged again at 10,000 rpm for 10 min. The pellet was discarded, and the supernatant was stirred with Amberlite XAD-7 (Rohm & Haas, Philadelphia, PA) resin for 3 hr (150 ml of resin per liter of solution). The resin was collected by filtration on a coarse sintered glass funnel and washed with deionized water until clear; the wash was discarded, and the material retained on the resin was washed off with methanol. The methanol was evaporated on a rotary evaporator, and the residue was resuspended in methanol and filtered (Whatman No. 50). The filtrate was dried by rotary evaporation, redissolved in methanol, filtered through a 0.45-μm filter, and separated by preparative high-pressure liquid chromatography (HPLC). The HPLC was equipped with a reverse phase (C18) column (21.4 $\times$ 250 mm) and a 50-mm guard column. Isocratic elution was used with 60% acetonitrile and 40% water at a flow rate of 2 ml/min. The detector was set at 254 nm. Twenty fractions were collected at 1-min intervals. The fractions were dried

Fig. 1. High-pressure liquid chromatography (HPLC) separation of methanol wash from Amberlite XAD-7 resin. Fraction #14 contained antifungal compound.

by rotary evaporation and assayed for antifungal activity with the agar diffusion test: Fractions were dissolved in methanol, diluted with water, and placed in a well (1 cm diameter) made in the center of petri plates containing 15 ml of NYDA. After incubation for 24 hr at 24 C, plates were seeded with an aqueous spore suspension (1 $\times$ 10^6 conidia/ml) of three fungi (B. cinerea, P. expansum, or Mucor piriformis Fisher), and again incubated for 48 hr at 24 C, after which the plates were evaluated for zones of fungal growth inhibition. Antifungal activity was observed in fraction #14, which eluted at about 14 min (Fig. 1). For further purification, fraction #14 was dried on a rotary evaporator and rechromatographed by HPLC through an IBM cyano column (10 $\times$ 250 mm) by isocratic elution with 50% chloroform and 50% hexane at a flow rate of 5 ml/min. Activity was observed in the fraction collected 6 min after sample injection. The fraction was evaporated and recrystallized from hexane. The melting point, UV spectrum, and proton NMR spectrum were in good agreement with data reported in the literature (3) for pyrrolnitrin. Mass and carbon NMR spectra were obtained, further confirming the structure of the antifungal substance as pyrrolnitrin. By the same procedure, starting with Amebelite XAD-7 mixing, pyrrolnitrin also was isolated from the supernatant of a centrifuged culture of P. cepacia.

Pyrrolnitrin activity on apples and pears. Pyrrolnitrin, purified from bacterial cells or from the medium in which the bacterium grew, was dissolved first in methanol (1 mg of pyrrolnitrin per 1 ml of methanol) and then diluted with water to the desired concentrations. Apples and pears were wounded as described above, and 20 μl of pyrrolnitrin solution was placed into each wound. Fruit inoculation with pathogens, incubation, lesion measurements, and experimental design were all similar to those described for treatment with cells of P. cepacia. The experiment was repeated.

RESULTS

Antagonistic activity of P. cepacia on apple and pear. The organism producing the largest zone of fungal growth inhibition during screening in vitro was identified as P. cepacia. The antagonist strongly inhibited development of gray mold and blue mold lesions on apple and pear. Most activity was observed on apples (Table 1), where no lesion developed on fruit treated with the two highest concentrations of the antagonist and 10^3 or 10^4 conidia/ml of B. cinerea. At 10^5 conidia/ml, only small lesions developed. In the case of P. expansum, although significant reduction in lesion size occurred in almost all treatments, no treatment resulted in complete lesion suppression. On Bosc pears (Table 1), the best protection occurred on fruit treated with the
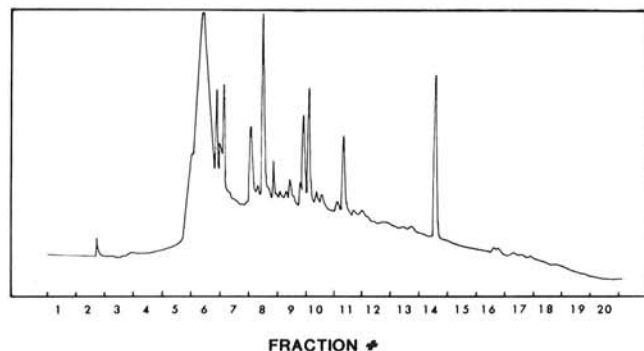
TABLE 1. Lesion diameter (mm)[a] on Golden Delicious apples and Bosc pears protected with *Pseudomonas cepacia* and wound-inoculated with conidial suspensions of *Penicillium expansum* or *Botrytis cinerea*

Fruit	*P. cepacia* concn. (cfu/ml $\times$ 10^7)	*Penicillium expansum* (conidia/ml)			*Botrytis cinerea* (conidia/ml)		
		10^3	10^4	10^5	10^3	10^4	10^5
Apples							
	0	41.5	44.8	43.8	57.5	35.8	61.7
	1.4	26.5	23.0	33.7	14.3	0	11.0
	2.2	9.8	30.7	32.7	22.6	7.3	12.0
	4.0	4.7	31.5	33.0	0	0	12.0
	11.0	23.7	30.8	39.2	0	0	4.3
	LSD$_{0.05}$	13.1	11.9	7.2	16.9	15.9	15.6
Pears							
	0	20.1	23.9	27.5	27.7	33.3	35.3
	1.4	10.3	10.2	19.5	11.5	20.7	30.3
	2.2	8.3	12.6	19.1	9.8	20.3	26.9
	4.0	11.0	12.8	22.4	11.2	18.4	31.2
	11.1	6.8	12.6	23.3	1.0	30.3	30.3
	LSD$_{0.05}$	6.1	6.8	3.0	5.3	2.4	3.3

[a] Means of six lesions on apples (one lesion per fruit) and 12 lesions on pears (two lesions per fruit) measured 7 and 5 days after inoculation, respectively. Data from the first experiment.

highest concentration of the antagonist and the lowest concentration of *B. cinerea*. At the two highest pathogen inoculum levels, and at all antagonist concentrations, lesions were large and only slightly different from the control. Significant reduction in lesion size was observed on pears inoculated with conidia of *P. expansum*; however, complete inhibition did not occur.

Purification and antifungal activity of pyrrolnitrin on apple and pear. Zones of growth inhibition of *B. cinerea* and *P. expansum* were observed on plates with wells containing fraction #14. Only one peak was observed on the chart in this fraction (Fig. 1). Further purification on an IBM cyano column resulted in a single, pure, crystalline substance identified as pyrrolnitrin. It produced strong zones of inhibition to both fungi in an agar diffusion test (Fig. 2). At a concentration of 0.1 mg/ml, no growth occurred on plates seeded with either fungus, and, at 0.01 mg/ml, growth occurred only around the edges of the plates seeded with conidia of *P. expansum*. At 0.001 mg/ml, the lowest concentration tested, large zones of inhibition still were observed with both fungi.

Suppression of gray mold and blue mold on apple (Table 2, Fig. 3) and pear (Table 2) was obtained at all pyrrolnitrin concentrations tested. Lesions were observed only on fruit treated with the lowest concentration of the compound and inoculated with the two highest conidial concentrations of *P. expansum* for pears and the highest one for apples.

DISCUSSION

The strain of *P. cepacia* isolated in this study protected apples and pears from blue mold rot at concentrations sufficiently low to consider this bacterium for possible commercial development. *P. cepacia* is one of the most nutritionally versatile bacteria (5),

capable of using as a sole source of carbon and energy a number of carbohydrates and carbohydrate derivatives, fatty acids, dicarboxylic acids, other organic acids, primary alcohols, amino acids, other nitrogenous compounds, and nitrogen-free ring compounds, which makes it very attractive for biocontrol use.

Others have studied the biocontrol possibilities of *P. cepacia*. The bacterium has been reported to reduce southern maize leaf blight caused by *Bipolaris maydis* in greenhouse tests (20), decrease peanut Cercospora leaf spot (23,24) and tobacco Alternaria leaf spot in the field (22), and to control damping-off of onion seedlings by *Fusarium oxysporum* f. sp. *cepae* (14).

No studies have been directed to elucidate the mode of action of *P. cepacia*. *P. cepacia* was reported to be an important member of the rice rhizoplane by being responsible for converting nitrate to nitrite (4), and for securing nodulation of roots of *Alnus rubra* by *Frankia* sp. (16). The principal mode of action of the isolate of *P. cepacia* in our studies appeared to be antagonism by the production of pyrrolnitrin, a powerful antifungal compound. This compound inhibited *B. cinerea* and *P. expansum* in vitro at concentrations below 1 mg/L. Other antifungal compounds called altericidins (A, B, and C), isolated from different strains of *P. cepacia* (15), still allowed in vitro mycelial growth of *Alternaria kikuchiana* at a concentration of 8 mg/L and sporidial growth of *Ustilago maydis* at 4 mg/L.

Apples and pears treated with pyrrolnitrin generally showed no lesion development from blue mold and gray mold fungi. This compound also has been involved in biocontrol of *Rhizoctonia solani* by *P. fluorescens* on cotton seedlings (8). *P. cepacia* isolated in our search for biocontrol agents against postharvest diseases of pome fruits is of great interest because the organism itself, or its antifungal compound with apparent broad spectrum activity, could be considered as a commercial control for various fruit diseases.
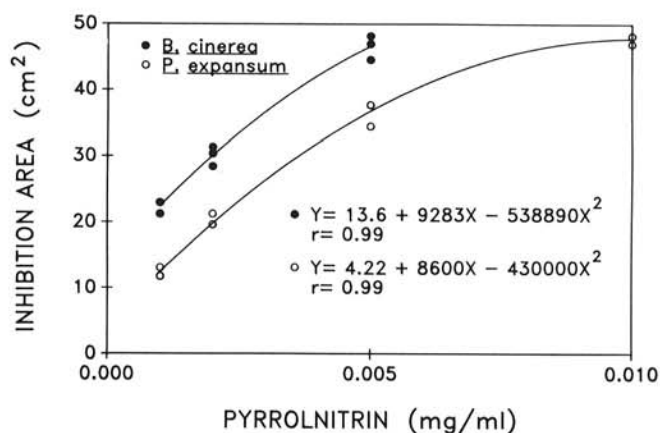
Fig. 2. Inhibition area of fungal growth on petri plates containing nutrient yeast dextrose agar medium with pyrrolnitrin applied in the center and seeded with pathogen conidial suspension (1×10^6 conidia/ml).

Equations in figure:
- B. cinerea: $Y = 13.6 + 9283X - 538890X^2$, $r = 0.99$
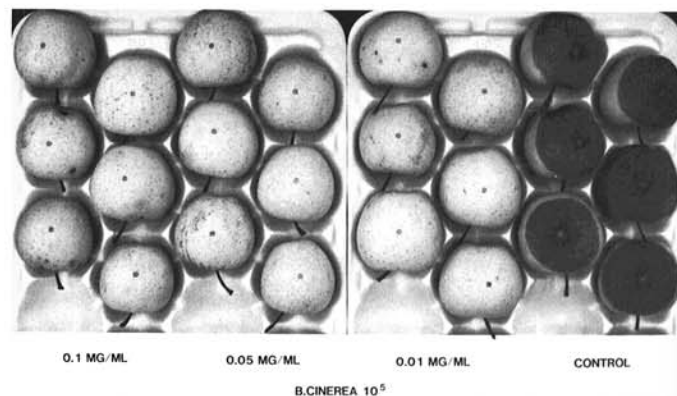- P. expansum: $Y = 4.22 + 8600X - 430000X^2$, $r = 0.99$

Fig. 3. Lesion development on wounded Golden Delicious apples protected with pyrrolnitrin and inoculated with *Botrytis cinerea* spores.

TABLE 2. Lesion diameter (mm)[a] on Golden Delicious apples and Bosc pears protected with pyrrolnitrin and wound-inoculated with conidial suspensions of *Penicillium expansum* or *Botrytis cinerea*

Fruit	Pyrrolnitrin concentration (mg/ml)	*Penicillium expansum* (conidia/ml)			*Botrytis cinerea* (conidia/ml)		
		10^3	10^4	10^5	10^3	10^4	10^5
Apples							
	0	39.3	40.3	41.7	18	30.6	41.5
	0.01	0	0	3.2	0	0	0
	0.05	0	0	0	0	0	0
	0.10	0	0	0	0	0	0
Pears							
	0	17.8	19.3	22.3	21.7	20.8	30.1
	0.01	0	2.8	7.6	0	0	0
	0.05	0	0	0	0	0	0
	0.10	0	0	0	0	0	0

[a] Means of six lesions on apples (one lesion per fruit) and 12 lesions on pears (two lesions per fruit) measured 7 and 5 days after inoculation, respectively. Data from the first experiment.

LITERATURE CITED

1. Andrews, J. H., Berbee, F. M., and Nordheim, E. V. 1983. Microbial antagonism to the imperfect stage of the apple scab pathogen, *Venturia inequalis.* Phytopathology 73:228-234.
2. Anonymous. 1987. Regulation Pesticides in Food—The Delaney Paradox. Board of Agriculture, National Research Council. National Academy Press. Washington, D.C. 272 pp.
3. Arima, K., Imanaka, H., Kousaka, M., Fukuda, A., and Tamura, G. 1965. Studies on pyrrolnitrin, a new antibiotic. J. Antibiot. A18:201.
4. Asanuma, S., Tanaka, H., and Yatazowa, M. 1980. *Pseudomonas cepacia*—Characteristic rhizoplane microorganisms in rice plant. Soil Sci. Plant Nutr. 26(1):71-78.
5. Ballard, R. W., Palleroni, N. J., Doudoroff, M., Stanier, R. Y., and Mandel, M. 1970. Taxonomy of the aerobic pseudomonads: *Pseudomonas cepacia, P. marginiata, P. alliicola* and *P. caryophylli.* J. Gen. Microbiol. 60:199-214.
6. Dubos, B. 1984. Biocontrol of *Botrytis cinerea* on grapevines by antagonistic strain of *Trichoderma harzianum.* Pages 370-374 in: Current Perspectives in Microbial Ecology. M. J. Klub and C. A. Reedy, eds. American Society for Microbiology, Washington, DC.
7. Gutter, Y., and Littauer, F. 1953. Antagonistic action of *Bacillus subtilis* against citrus fruit pathogens. Bull Res. Counc. Israel 3:192-197.
8. Howell, C. R., and Stipanovic, R. D. 1979. Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69:480-482.
9. Janisiewicz, W. J. 1985. Biological control of postharvest diseases of pome fruits. (Abstr.) Phytopathology 75:1301.
10. Janisiewicz, W. J. 1987. Postharvest biological control of blue mold on apples. Phytopathology 77:481-485.
11. Janisiewicz, W. J. 1987. Biological control of diseases of fruits. Pages 153-165 in: Biocontrol of Plant Diseases, Vol. II. R. J. Mukerji and K. L. Gary, eds. CRC Press, Boca Raton, FL. 198 pp.
12. Janisiewicz, W. J. 1988. Biocontrol of postharvest diseases of apples with antagonist mixtures. Phytopathology 78:194-198.
13. Janisiewicz, W. J., and Roitman, J. 1987. Postharvest mucor rot control on apples with *Pseudomonas cepacia.* (Abstr.) Phytopathology 77:1776.
14. Kawamoto, S. O., and Lorbeer, J. W. 1976. Protection of onion seedlings from *Fusarium oxysporum* f. sp. *cepae* by seed and soil infestation with *Pseudomonas cepacia.* Plant Dis. Rep. 60:189-191.
15. Kirinuki, T., Ichiba, T., and Katayama, K. 1984. General survey of action site of altericidins on metabolism of *Alternaria kikuchiana* and *Ustilago maydis.* Pestic. Sci. 9:601-610.
16. Knowlton, S., and Dawson, T. O. 1983. Effect of *Pseudomonas cepacia* and cultural factors on the nodulation of *Alnus rubra* roots by *Frankia.* Can. J. Bot. 61:2877-2882.
17. Lim, T.-K., and Rohrbach, K. G. 1980. Role of *Penicillium funiculosum* strains in the development of pineapple fruit diseases. Phytopathology 70:663-665.
18. Pusey, P. L., Hotchkiss, M. W., Baumgardner, R. A., Zehr, E. I., Dulmage, H. T., Reilly, C. C., and Wilson, C. L. 1987. Application and efficacy of *Bacillus subtilis* for brown rot control in commercial peach-packing operation. (Abstr.) Phytopathology 77:1776.
19. Pusey, P. L., and Wilson, C. L. 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis.* Plant Dis. 68:753-756.
20. Sleesman, J. P., and Leben, C. 1976. Microbial antagonists of *Bipolaris maydis.* Phytopathology 66:1214-1218.
21. Spotts, R. A., and Cervantes, L. A. 1986. Populations, pathogenicity, and benomyl resistance of *Botrytis* spp., *Penicillium* spp., and *Mucor piriformis* in packinghouses. Plant Dis. 70:106-108.
22. Spurr, H. W., Jr. 1978. Field biocontrol of two foliage diseases by a bacterial antagonist. (Abstr.) Phytopathology News 12:74.
23. Spurr, H. W., Jr. 1981. Experiments on foliage disease control using bacterial antagonists. Pages 369-381 in: Microbial Ecology of the Phylloplane. J. P. Blakeman, ed. Academic Press, New York. 503 pp.
24. Spurr, H. W., Jr., and Knudsen, G. R. 1985. Biological control of leaf diseases with bacteria. Pages 45-62 in: Biological Control on the Phylloplane. C. E. Windels and S. E. Lindow, eds. American Phytopathological Society, St. Paul, MN. 169 pp.
25. Tronsmo, A., and Raa, J. 1977. Antagonistic action of *Trichoderma pseudokoningii* against the apple pathogen *Botrytis cinerea.* Phytopathol. Z. 89:216-220.
26. Wilson, C. L., Franklin J. D., and Pusey, P. L. 1987. Biological control of Rhizopus rot of peach with *Enterobacter cloacae.* Phytopathology 77:303-305.
27. Wilson, C. L., and Pusey, P. L. 1985. Potential for biological control of postharvest plant diseases. Plant Dis. 69:375-378.