Postharvest Pathology and Mycotoxins

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

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ABSTRACT


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 pyrolysintrin, 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 pyrolysintrin concentration of 10 mg/L at a pathogen inoculum level of 10^8 conidia/ml. Blue mold was controlled at the same concentration of pyrolysintrin at inoculum concentrations of 10^5 conidia/ml for pears and 10^4 and 10^5 conidia/ml for apples. At concentrations of 50 mg/L or higher, complete control was obtained on both fruits at all tested inoculum levels.


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 subtilis* (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 some fruit trees completely controlled blue mold (incited by *Penicillium expansum* Lk. & Th.) 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 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, pyrolysintrin. 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 Branson 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 x 10^8 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 75 ± 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 α = 0.05.
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 Sonifier 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 x 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 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 x 10^6 conidia/ml) of three fungi (*B. cinerea*, *P. expansum*, or *Mucor piriiformis* 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 cyan column (10 x 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 spectra 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 substrate as pyrrolnitrin. By the same procedure, starting with Amberlite 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 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 10^5 or 10^6 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...
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 314. Only one peak was observed on the chart in this fraction (Fig. 1). Further purification on an IBM cyanocobalt 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 were still 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 rhizosphere 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 sporidal growth of Ophiostoma mays in 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 x 10⁶ conidia/ml).](image)

![Fig. 3. Lesion development on wounded Golden Delicious apples protected with pyrrolnitrin and inoculated with Botrytis cinerea spores.](image)

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

<table>
<thead>
<tr>
<th>Fruit</th>
<th>Pyrrolnitrin concentration (mg/ml)</th>
<th>Penicillium expansum (conidia/ml)</th>
<th>Botrytis cinerea (conidia/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10³</td>
<td>10⁴</td>
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<tr>
<td>Apples</td>
<td>0</td>
<td>39.3</td>
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<tr>
<td></td>
<td>0.10</td>
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<td>0</td>
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<tr>
<td>Pears</td>
<td>0</td>
<td>17.8</td>
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<td></td>
<td>0.10</td>
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*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


