

# Postharvest Control of Blue Mold and Gray Mold of Apples and Pears by Dip Treatment with Pyrrolnitrin, a Metabolite of *Pseudomonas cepacia*

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## ABSTRACT

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The antifungal compound pyrrolnitrin, isolated from *Pseudomonas cepacia*, an antagonist known to control gray mold (incited by *Botrytis cinerea*) and blue mold (incited by *Penicillium expansum*) of apples and pears, was assayed for its efficacy in controlling these diseases on wounded fruit at two temperatures (2 and 24 C). The compound was applied to wounded fruit after harvest at concentrations ranging from 6 to 200 µg/ml in dip solutions containing conidia of *P. expansum* or *B. cinerea* ( $1 \times 10^4$  conidia per milliliter). Pyrrolnitrin provided effective control of both diseases on apples and pears. The type of wound had a profound effect on control; infections at cut wounds were the easiest and those at "bruise" wounds were the most difficult to control. Higher concentrations of pyrrolnitrin were required for control at 24 C than at 2 C. Pyrrolnitrin at 200 µg/ml, when applied up to 34 hr after inoculation, eradicated infections by both pathogens on Golden Delicious apples with cut wounds. The pH of the pyrrolnitrin solution, throughout a range corresponding to the pH of the fruit juice, did not change its fungicidal activity in *in vitro* tests.

Additional keywords: natural compound

Blue mold and gray mold, incited by *Penicillium expansum* Link and *Botrytis cinerea* Pers.:Fr., respectively, are major postharvest diseases of pome fruits (1,9,17). Fungicides are routinely used to control these diseases after harvest (4,6,20,21,24). However, the development of resistance in the pathogens (3,18, 22,26) and the recent association of health hazards with some fungicides used for postharvest treatment of fruit (16) have necessitated the search for replacement fungicides and alternative non-chemical control methods.

Postharvest treatment of fruit with biocontrol agents has emerged recently as a potential alternative disease management tool (11,27). A number of antagonists, isolated from soil and plant material, have shown great promise in controlling postharvest diseases of pome, stone, and citrus fruits. These antagonists are diverse taxonomically and in their mechanisms of action (11). In our research, *Pseudomonas cepacia* Palleroni and Holmes (isolated from apple leaves) controlled blue mold and gray mold of apples and pears (13) and Mucor rot on apples (12) after harvest. This antagonist produces a strong antifungal compound,

which was isolated, purified, and identified as pyrrolnitrin (13).

Pyrrolnitrin was also isolated from a different antagonist, *P. fluorescens* Migula, which is effective in controlling cotton seedling pathogens (10). Pyrrolnitrin was isolated originally from *P. pyrociana* n. sp. by Arima et al (2) in Japan, where it is used in pharmaceutical preparations (23). Although most of the research on pyrrolnitrin has been directed toward its uses against dermatophytic fungi, the low mammalian toxicity of the compound (14,15) makes it attractive for some agricultural uses. Limited studies of the mechanism of action of pyrrolnitrin indicate that the compound may have systemic activity in mammals (5) and may inhibit fungal growth by inhibiting the respiratory electron transport (25).

In experiments designed to elucidate the mode of action of *P. cepacia*, drop application of pyrrolnitrin to wounds on apple and pear challenged with a conidial suspension of *B. cinerea* or *P. expansum* prevented rot development under laboratory conditions (13). The objective of this study was to determine the feasibility of using this metabolite alone as a dip treatment to control blue mold and gray mold of apple and pear.

## MATERIALS AND METHODS

**The pathogens.** *P. expansum* and *B. cinerea* were isolated from rotten apples stored in commercial storage for about 4 mo. Both isolates are highly pathogenic

to apples and pears. The organisms were maintained on potato-dextrose agar (PDA) and periodically transferred on PDA with 10% apple juice. To obtain inocula, *P. expansum* was grown on PDA in petri plates for 10 days at 24 C and *B. cinerea* for 14 days at 24 C with continuous light. Conidial suspensions were prepared by washing the colonies growing on PDA with 2 ml of sterile 0.05% Tween 80. Aliquots were then collected and diluted with sterile distilled water to the required concentrations as determined with a hemacytometer.

**The fruit.** Golden Delicious apples were harvested from an orchard at the U.S. Department of Agriculture Research Station in Kearneysville, WV, maintained with a minimal spray regime. The fruit were stored at  $1 \pm 1$  C and  $95 \pm 2\%$  relative humidity (RH) for at least 2 mo before use. The fruit used in the tests were selected for uniformity in size and ripeness. Bosc pears were harvested from a commercial orchard and stored under the same conditions and were used within 2 mo of harvest. At the time of the experiments, the firmness of the apples and pears was 11 and 14 lb of force, respectively, as determined by Effegi pressure tests.

**Wounding of fruit.** In earlier work (W. Janisiewicz, unpublished), the type of wound had affected rot control by *P. cepacia*. To determine whether this effect also held when the metabolite was used alone, fruit were wounded by one of three methods. Cut wounds were made by cutting a 3-mm-square incision 3 mm deep with a sharp instrument and removing the tissue. Two wounds were made approximately 2 cm apart on each fruit. Puncture wounds were made with two 6.1-mm-diam nails 2 cm apart and protruding 4 mm from a wooden block. "Bruise" wounds were made by pressing two screw heads (1 cm in diameter) against the fruit surface to a depth of 2-3 mm, which also broke the skin. The screw heads were mounted on a wooden block 2 cm apart. All wounds were made about halfway along the line between the calyx and stem end.

**Isolation of pyrrolnitrin.** Pyrrolnitrin was isolated from cells of *P. cepacia* by a modification of the procedure described earlier (13). The modification allowed more efficient extraction of

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pyrrolnitrin from bacterial cells. The bacterium was grown in minimal salt broth (8) in shake culture for 7 days at 27 C. Acetone was used to extract pyrrolnitrin from the cells of centrifuged pellets after sonication. The resulting mixture was recentrifuged, and the supernatant aqueous acetone solution was separated. The acetone was removed under reduced pressure, and the remaining aqueous residue was lyophilized. This preparation was stirred with methanol, concentrated, and filtrated through a 0.45- $\mu\text{m}$  Millipore filter. It was then purified by reverse-phase high-pressure liquid chromatography (21.4  $\times$  250 mm Rainin Dynamax C18 column eluted with acetonitrile and water [3:2]). The fraction that appeared at 9.5–12.5 min was collected and rechromatographed on a silica column (Rainin Dynamax 21.4  $\times$  250 mm) eluted with chloroform-hexane (1:1). The peak eluted at 13.5 min was collected, the solvent was removed, and the residue was recrystallized from chloroform-hexane. This gave pure pyrrolnitrin (melting point [mp] 124–125 C; literature mp 124.4 C [3]). Spectroscopic measurements (ultraviolet, mass spectrometry, and  $^1\text{H}$  and  $^{13}\text{C}$  nuclear magnetic resonance) agreed with reported data, confirming the identity of pyrrolnitrin. Purified pyrrolnitrin was stored in dark glass vials at  $-10\text{ C}$ .

**Fruit dip test.** Fruit were dipped in suspensions containing conidia of *B. cinerea* or *P. expansum* ( $1 \times 10^4$  conidia

per milliliter) alone or mixed with various concentrations of pyrrolnitrin. Pyrrolnitrin solutions were prepared by dissolving the required amounts of the compound in 5 ml of methanol. These solutions were added to 1.5 L of the conidial suspensions.

Wounded apple or pear fruit (two wounds per fruit) were dipped in the suspensions for 2 min with occasional agitation. The fruit were then placed on polystyrene fruit tray-packs with the wounded face up in 1-bu fruit boxes with polyethylene liners. Fruit from each treatment were placed on separate trays in separate boxes. There were five fruit per treatment, and each treatment was repeated three times. The boxes were placed in storage as randomized blocks.

One set of fruit was stored at 24 C and  $95 \pm 2\%$  RH for 7 days, and the other set was stored at 2 C for 30 days. Storage at 24 C created optimal conditions for development of the diseases, and lengths of storage periods were selected to make it possible to measure lesions before fruits were entirely rotted.

At the end of the storage period, diameters of lesions were measured perpendicular to the axis connecting the two wounds. Preliminary experiments conducted before those reported here gave similar results.

**Treatment of established infections.**

Each of two cut wounds on Golden Delicious apples was inoculated with  $20\ \mu\text{l}$  of a suspension containing  $1 \times 10^4$

conidia of *B. cinerea* or *P. expansum* per milliliter. Fruit were incubated on trays at 24 C. At various time intervals, pyrrolnitrin solutions at concentrations of 100 and 200  $\mu\text{g}/\text{ml}$  were applied to the wounds ( $20\ \mu\text{l}$  per wound). The number of wounds that were infected was recorded after 1 and 2 wk of incubation. Each treatment, consisting of three fruits, was replicated three times.

**pH stability.** Stability of pyrrolnitrin was tested at pHs ranging from 3.4 to 10.0. A concentrated solution of pyrrolnitrin (1 mg/ml) was prepared by dissolving the chemical in methanol. The solution was added to buffers with different pH values to obtain the final concentration of 10  $\mu\text{g}/\text{ml}$ . The control consisted of the appropriate concentration of methanol. The buffers were made following standard procedures (7) from the following stock solutions: pH 3.4, citric acid (0.1 M) and  $\text{K}_2\text{HPO}_4$  (0.1 M); pH 5.8, 7.0, and 8.0,  $\text{K}_2\text{HPO}_4$  (0.2 M) and  $\text{KH}_2\text{PO}_4$  (0.2 M); and pH 10.0, glycine (0.2 M) and NaOH (0.2 M). The buffered pyrrolnitrin solutions were stored at 18 C in the dark and were used in experiments after 2 and 5 days.

Buffered solutions of pyrrolnitrin (100  $\mu\text{l}$ ) were placed in wells made with a 1-cm cork borer in the center of petri plates containing 15 ml of nutrient yeast-dextrose agar. After incubation for 24 hr at 4 C, the plates were seeded with conidial suspensions of *B. cinerea* or *P. expansum* ( $1 \times 10^5$  conidia/ml) and incu-

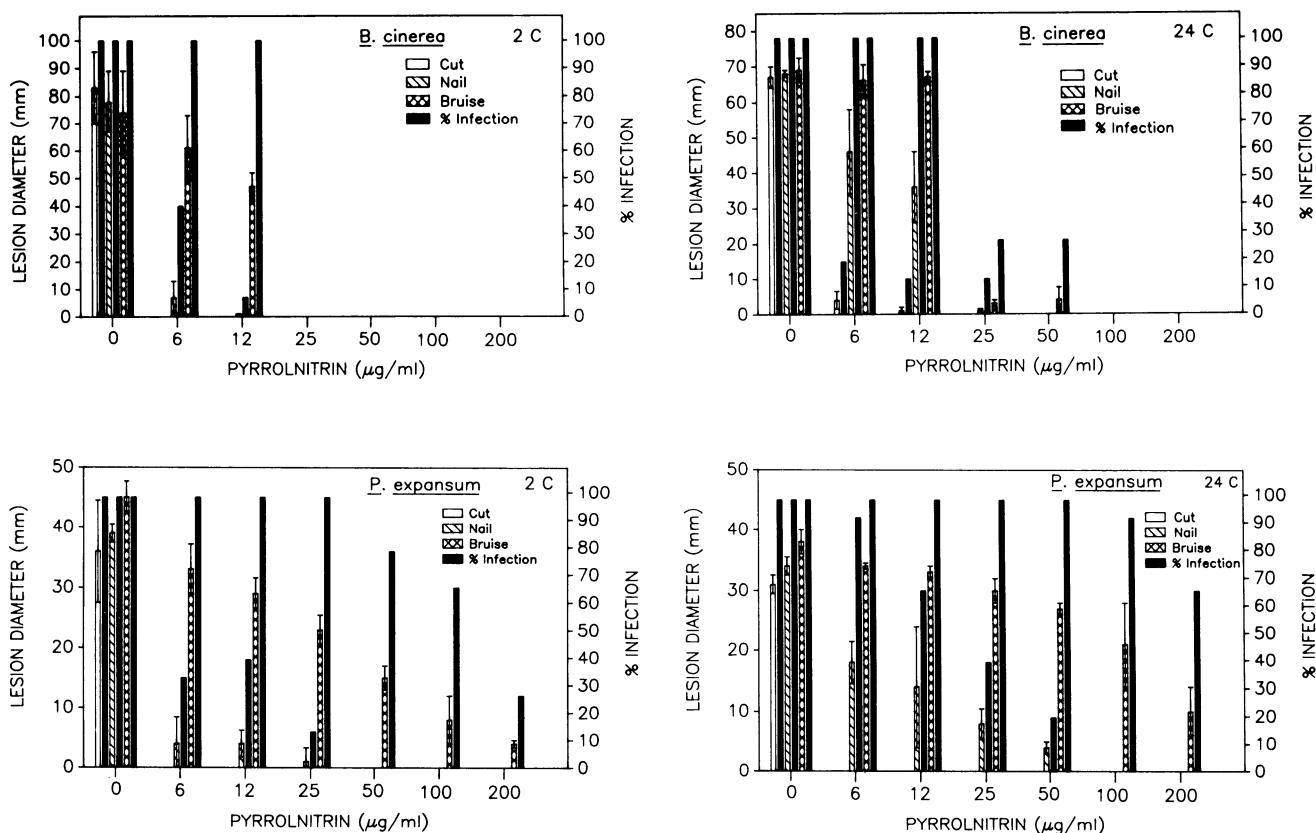


Fig. 1. Average lesion diameter and percentage infection of Bosc pears wounded in one of three ways, inoculated with *Botrytis cinerea* or *Penicillium expansum*, treated with various concentrations of pyrrolnitrin, and stored at 2 C or 24 C. Brackets indicate one standard deviation.

bated for 48 hr at 24 C. The diameters of the zones of inhibition of fungal growth were measured.

**Data analysis.** An analysis of variance was performed on the data from the fruit dip test on rot development. The general linear model procedure of the Statistical Analysis System (19) was used. Linear and quadratic effects of pyrrolnitrin concentration were included in the full model. The effect of pH on fungicidal activity of pyrrolnitrin was analyzed by regression analysis.

## RESULTS AND DISCUSSION

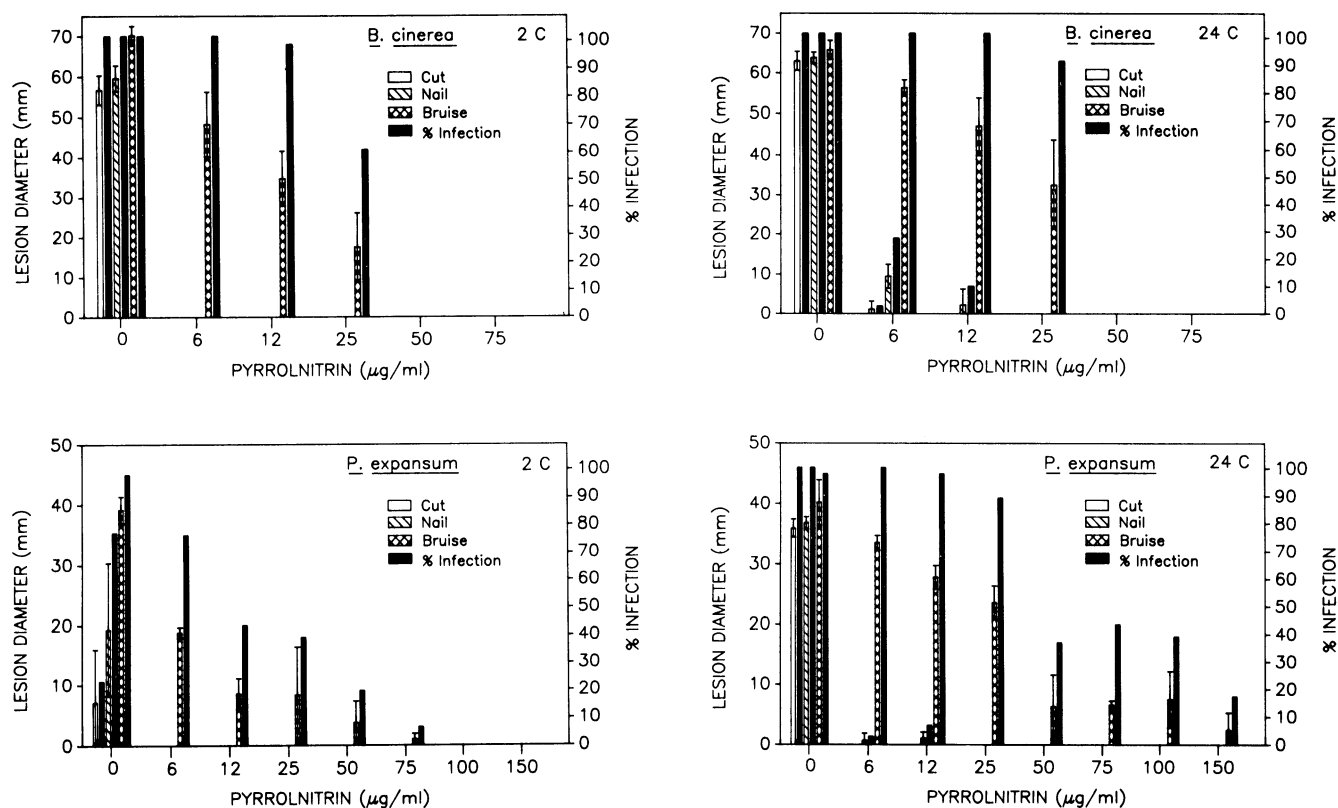
**Control of rots.** Pyrrolnitrin controlled blue mold and gray mold on pears (Fig. 1) and apples (Fig. 2). Type of wound and concentration of pyrrolnitrin

significantly ( $P = 0.001$ ) affected lesion development on both types of fruit inoculated with either pathogen (Table 1). Temperature affected apples and pears inoculated with *P. expansum* and apples inoculated with *B. cinerea*. Temperature and pyrrolnitrin concentration interacted with the pathogens on apple. Temperature interacted with wound type on both fruits inoculated with *P. expansum*. Pyrrolnitrin concentration interacted with wound type to affect lesion development ( $P = 0.001$ ) by each pathogen on both fruits. Rot caused by *B. cinerea* was controlled by a lower pyrrolnitrin concentration than that caused by *P. expansum* on both fruits. Pyrrolnitrin at 50  $\mu\text{g/ml}$  eliminated rot caused by *B. cinerea* on both fruits with

all three types of wounds in 11 of 12 treatments. In the 12th treatment, small lesions developed in bruise wounds on pears stored at 24 C.

*P. expansum* was easier to control on apples than on pears. Pyrrolnitrin at 100  $\mu\text{g/ml}$  eliminated the disease on apples under cold storage conditions. However, at 24 C, small lesions developed on bruise-wounded fruit treated with pyrrolnitrin at 150  $\mu\text{g/ml}$ , the highest concentration used. Small lesions developed on bruise-wounded pears treated with pyrrolnitrin at 200  $\mu\text{g/ml}$  at both storage temperatures. Pyrrolnitrin had no phytotoxic effect on fruits at the concentrations used.

In a similar experiment (*data not shown*), complete control of both



**Fig. 2.** Average lesion diameter and percentage infection of Golden Delicious apples wounded in one of three ways, inoculated with *Botrytis cinerea* or *Penicillium expansum*, treated with various concentrations of pyrrolnitrin, and stored at 2 C or 24 C. Brackets indicate one standard deviation.

**Table 1.** Summary analysis of variance for lesion development on apples and pears wounded by one of three methods, treated with varying concentrations of pyrrolnitrin (PN), inoculated with conidia of *Botrytis cinerea* or *Penicillium expansum*, and stored at 2 C or 24 C<sup>a</sup>

Source of variation	Apple				Pear			
	<i>B. cinerea</i>		<i>P. expansum</i>		<i>B. cinerea</i>		<i>P. expansum</i>	
	df	MS	df	MS	df	MS	df	MS
Wound type	2	66,261.2***	2	11,147.0***	2	40,022.6***	2	47,089.6***
PN concentration (PN conc.)	1	172,238.3***	1	28,325.0***	1	251,586.2***	1	56,692.6***
Temperature (temp.)	1	9,052.0***	1	7,393.1***	1	1,381.3	1	2,868.1***
Temp. × PN conc.	1	7,570.4***	1	2,628.5***	1	818.1	1	16.4
Temp. × wound type	2	53.9	2	1,707.7***	2	4,233.6	2	828.5***
PN conc. × wound type	2	29,747.8***	2	4,265.4***	2	16,172.1***	2	4,412.0***
Temp. × PN conc. × wound type	2	1,370.3***	2	91.2	2	1,916.6**	2	694.4***
Error	1,040	166.0	1,386	88.5	1,210	420.5	1,240	85.6
R <sup>2</sup>		0.72		0.43		0.56		0.65

<sup>a</sup>df = Degrees of freedom. MS = mean square. Mean squares were derived from type III sums of squares for unbalanced linear model and randomized block design at two temperatures. Three asterisks indicate significance at  $P = 0.001$ ; two asterisks indicate significance at  $P = 0.01$ .

diseases was achieved on wounded (cut and puncture wounds) Golden Delicious apples treated with pyrrolnitrin at a concentration of 6 µg/ml or higher and stored in perforated plastic bags for 60 days at 1 C. Attempts to isolate *B. cinerea* from lesions that did not develop rots failed. *P. expansum* was isolated from wounds treated with a concentration of 6 µg/ml but not from wounds treated with higher concentrations. However, all colonies were white, deformed, and no larger than 10 mm in diameter after growing on PDA for 14 days.

**Effect of wound type.** Gray mold and blue mold rot development was greatest on fruits with bruise wounds, less on fruits with puncture (nail) wounds, and least on fruits with cut wounds. On fruits with cut wounds, with the exception of apples and pears inoculated with *B. cinerea* and stored at 24 C, both fungi were controlled with pyrrolnitrin at 6 µg/ml, the lowest concentration tested.

Pyrrolnitrin at 6 and 25 µg/ml controlled both rots on apples with nail wounds stored at 2 and 24 C, respec-

tively. On pears with nail wounds, concentrations of 25 and 50 µg/ml were needed to control *B. cinerea* and *P. expansum*, respectively, at 2 C, and concentrations twice as high were effective at 24 C.

Pyrrolnitrin at 50 µg/ml controlled *B. cinerea* on apples with bruise wounds at both storage temperatures, and concentrations of 25 and 100 µg/ml controlled this pathogen on pears with bruise wounds at 2 and 24 C, respectively. Pyrrolnitrin at 100 µg/ml gave complete control of *P. expansum* on inoculated, bruise-wounded apples stored at 2 C. All other treatments greatly reduced but did not completely control lesions.

Spalding et al (21) observed that fungicides controlled blue mold more effectively in punctured than in bruised apples. Our results support this observation, although the lesions in our study were slightly different from and more severe than those noted by Spalding et al. The nail wounds in our study closely resemble the wounds that commonly result when fruit stems puncture fruit surfaces, and the bruise wounds with broken skin are the most severe of the three types of wounds. Our results further emphasize the importance of standardized wounding procedures in tests of the effectiveness of fungicides in controlling postharvest diseases of pome fruits.

**Effect of storage temperature.** In general, higher concentrations of pyrrolnitrin were necessary to control rots at 24 C than at 2 C. In a number of cases, rots on fruits with either cut or nail wounds were controlled by lower concentrations of pyrrolnitrin at 2 C than at 24 C, indicating greater severity of the test at 24 C.

**Eradicative effect.** Pyrrolnitrin applied at concentrations of 100 µg/ml up to 26 hr after inoculation and at 200 µg/ml up to 34 hr after inoculation prevented development of lesions caused by *B. cinerea* and *P. expansum* (Fig. 3). Lesions developed on only one of 18 wounds inoculated with the pathogens

on fruit treated with pyrrolnitrin at 100 µg/ml 34 hr after inoculation. Further delays in applying pyrrolnitrin resulted in higher percentages of wound infection, ranging from 15 to 45% with a 46-hr delay and from 79 to 100% with a 52-hr delay.

**pH stability.** Pyrrolnitrin activity remained stable over a range of pH tests (Table 2). Thus, the compound remained active under acidic conditions (14) corresponding to those occurring at the wound site.

In summary, our results indicate that postharvest dip of apples and pears in a pyrrolnitrin solution may be an effective way to control rots caused by *B. cinerea* and *P. expansum* after harvest. Because the compound also has strong inhibitory activity in vitro against other postharvest pathogens (W. Janisiewicz, unpublished), it may also reduce losses from these pathogens as well. The excellent disease control capabilities of pyrrolnitrin make this bacterial metabolite an attractive prospect for control of postharvest diseases on fruits, vegetables, and other agricultural products.

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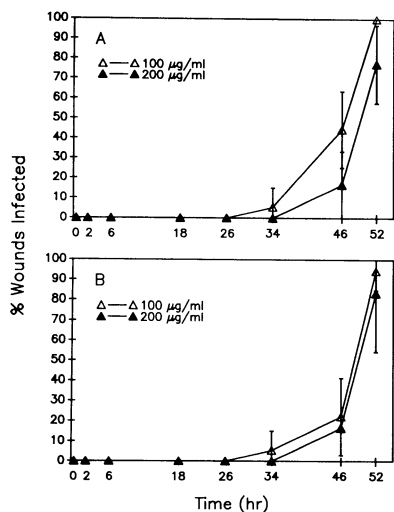


Fig. 3. Rot development on wounds of Golden Delicious apples inoculated with conidia of *Botrytis cinerea* (A) and *Penicillium expansum* (B) and treated with pyrrolnitrin at various times after inoculation. Brackets indicate one standard deviation.

Table 2. Zone of growth inhibition (mm) of *Botrytis cinerea* and *Penicillium expansum* by pyrrolnitrin solution (10 µg/ml) at various pH values over time in agar diffusion plate assay<sup>a</sup>

Pathogen	Time (hr)	pH				
		3.4	5.8	7.0	8.0	10
<i>B. cinerea</i>	0	60.7 ± 2.5	62.0 ± 4.4	62.0 ± 2.6	64.1 ± 1.5	63.3 ± 2.1
	48	63.0 ± 1.0	61.0 ± 1.0	61.7 ± 1.5	63.0 ± 2.0	65.7 ± 2.1
	120	56.3 ± 2.1	59.3 ± 1.2	53.7 ± 1.5	57.7 ± 0.6	56.0 ± 1.0
<i>P. expansum</i>	0	48.0 ± 2.6	51.0 ± 2.0	50.3 ± 6.0	53.3 ± 6.4	52.0 ± 1.7
	48	50.7 ± 2.5	49.7 ± 4.2	52.3 ± 2.1	51.2 ± 2.5	50.7 ± 3.2
	120	55.7 ± 1.5	53.0 ± 5.1	49.3 ± 6.0	50.7 ± 2.3	53.3 ± 1.2

<sup>a</sup>The stock solution of pyrrolnitrin dissolved in methanol (1 mg/ml) was added to buffers with different pH values to obtain the final concentration of 10 µg/ml. Buffered solution (100 µl) was added to wells made in the center of petri plates containing 15 ml of nutrient yeast-dextrose agar immediately after preparation of the solution (time 0) or after 48 or 120 hr. After incubation for 24 hr, the plates were seeded with conidial suspensions of the pathogens and incubated for 48 hr at 24 C. The diameter of the growth inhibition zone was then measured. Regression analysis showed no significant effect of pH on fungicidal activity of pyrrolnitrin.

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