Postharvest Biological Control of Blue Mold on Apples

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ABSTRACT

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Blue mold, a major postharvest disease of apples caused by *Penicillium expansum* was controlled with an antagonistic bacterium (L-22-64) and a yeast (F-43-31). Potential antagonists were isolated from apple plants throughout the year and from stored fruit. Golden Delicious apples were wound-inoculated with varying concentrations of antagonists and *P. expansum*. Lesion development was related to pathogen spore concentration and the concentration of the antagonist. The highest spore concentration of the pathogen (1×10^7 spores per milliliter) was totally controlled by higher concentrations of the antagonist. This protection was ongoing because subsequent reinoculation of wounds did not result in lesion development.

Biological control of postharvest diseases is a newly emerging area of research. *Trichoderma viride* Pers. & S. F. Gray applied to strawberry plants in the field partially controlled grey mold caused by *Botrytis cinerea* Pers. & Fr. on strawberry fruit after harvest (14). Also, partial control of rot of apples caused by *B. cinerea* was obtained by applying conidia of *T. harzianum* Rifai to apple trees during bloom (16). Brown rot of peach caused by *Monilinia fructicola* (Wint.) Honey was controlled with *Bacillus subtilis* (Cohn) Prazmowski in the first successful example of biological control of a major postharvest disease (9,18). Control was comparable to fungicidal treatment (10).

An increasing number of fungicide-tolerant strains of pathogens associated with fruit and vegetables in storage (1,11–13,17) has emphasized the need to develop alternative methods to control postharvest diseases. The research presented herein was initiated to develop biological control of major postharvest diseases of pome fruits. At the present time the work is concentrated on one of the important diseases of pome fruits: blue mold caused by *Penicillium expansum* Lk. & Thom. A preliminary report has been published (6).

MATERIALS AND METHODS

Antagonist isolation and screening. Organisms to be tested for antagonistic activity were isolated at weekly intervals from apple buds, leaves, and fruit in the field throughout the growing season and from apples in storage. In vitro screening was conducted in two stages: First, the previously mentioned plant organs were washed in 0.5 M phosphate buffer (5) in a beaker by shaking (150 rpm) for 10 min. After serial 0.10 dilutions, washings were plated on nutrient yeast dextrose agar (NYDA) medium in petri plates. After 48 hr of incubation at 24 C, plates were seeded with 0.05% Tween 80 aqueous suspension of spores of *P. expansum* at a concentration of 10^5 spores per milliliter. After further incubation for 72 hr, plates were examined for inhibition zones around the original colonies. Colonies with clear zones were isolated, purified, and retested for antagonistic activity. A second type of in vitro screening was conducted in 16-mm-diameter wells of tissue culture plastic plates. A 0.125-ml phosphate buffer (0.5 M) suspension of the potential antagonist (obtained from previous screening on petri plates or picked randomly from 0.10 dilution washing plates not seeded with *P. expansum*) was mixed with 0.125-ml 0.05% Tween 80 aqueous suspension of spores of *P. expansum* to produce a final spore concentration of 5×10^3 per milliliter. Water suspensions of the potential antagonists were prepared by transferring a loopful of a 24- to 36-hr-old culture to 4.5 ml of 0.05 M phosphate buffer followed by agitation. The plates were incubated for 48 hr at 24 C, then the wells were evaluated for spore germination.

The final screening was carried out in vivo on apples (*Malus sylvestris* Mill.) (Fig. 1). Surface sterilized Golden Delicious apples were wounded, at the stem (top) and calyx (bottom) end. The wounds were 3 mm in diameter and 3 mm deep. Each apple constituted a single replicate and each treatment was replicated three times. Twenty microliters of water suspension of an antagonist (three loopfuls of 24- to 36-hr-old NYDA grown cultures per 5 ml of water) was pipetted into the wound and followed by inoculation with 20 μl of 10^5 spores per milliliter suspension of *P. expansum*. Lesion diameters were measured 6 days after incubation in plastic trays at 24 C. The most effective organisms from bacterial and yeast antagonists were selected for further study.

Dilution end point determination. To determine the dilution end point at which selected antagonists (L-22-64 and F-43-31) would still prevent decay development, experiments were conducted with very mature (most susceptible stage) Golden Delicious apples previously stored at 1 C. Fruits, wounded at the lower or at the upper side or in both places, were dipped in a water suspension of the antagonist and each wound was immediately challenged with a 20 μl 0.05% Tween 80 aqueous suspension of spores of *P. expansum* at a concentration of 10^5/ml. In the first experiment, apples were dipped for 0.5 min in the antagonist suspensions, but in all others the time was 5 min. Antagonist suspensions were prepared by growing cultures in nutrient yeast dextrose broth (NYDB) for 24 hr at 24 C, after which the medium was centrifuged at 10,000 *g* and the culture was washed twice for 5 min by shaking with sterile, distilled water. Final dilutions were adjusted according to a standard curve using a spectrophotometer. The lesion diameter was measured after incubation on plastic trays at 24 C for 6 days.

The same experiment with 5-min dips in antagonist suspensions was conducted on less mature apples taken from controlled atmosphere storage in late spring. After 10 days, all wounds that did not develop lesions, including water checks, were reinoculated with spores of *P. expansum* as described previously and, after an additional week of incubation, the diameter of old and new lesions was measured. Each apple constituted a single replicate and each treatment was replicated three times. This experiment was repeated.

Effect of pathogen spore concentrations on biocontrol effectiveness. Factorial experiments were conducted with Golden Delicious apples from controlled atmosphere storage. These fruit

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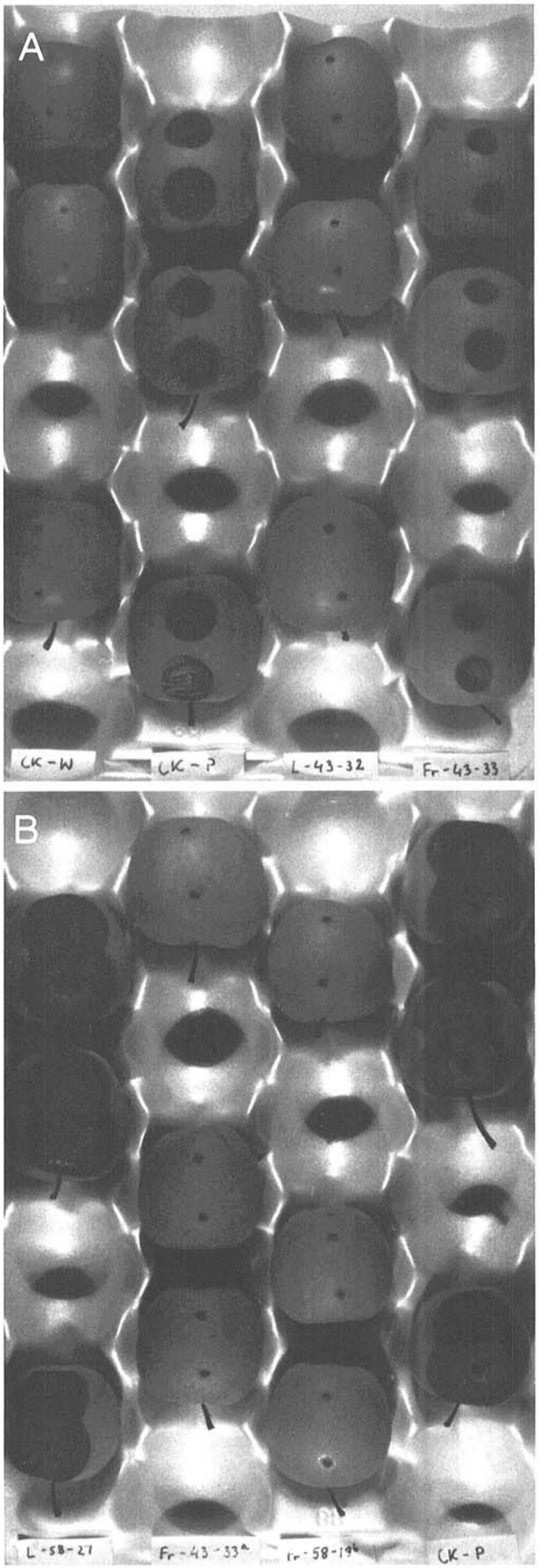

were wounded in the center and dipped (5 min) in a range of concentrations of water suspensions of bacterium L-22-64 or yeast F-43-31. Each apple constituted a single replicate and each treatment was replicated three times. The experiments were repeated. Treated apples were immediately inoculated at the wound site with a range of spore concentrations of *P. expansum* in a 0.05% Tween 80 water spore suspension, 20 μl per wound. Lesion diameter was measured after 6 days of incubation (24 C) in plastic trays. All wounds that did not develop lesions, including water checks, were reinoculated with *P. expansum* after 10 days with L-22-64 and after 7 days with F-43-31. After an additional week of incubation, all old and new lesions were measured.

RESULTS

Antagonist isolation and screening. More than 800 potential antagonists were isolated and tested in vitro. Only 97 of these were screened in vivo. Of these, only a few prevented fruit decay. Bacterial isolate L-22-64 identified as a *Pseudomonas* spp. and white yeast isolate F-43-31 were selected for further study. Many antagonists that performed well in vitro did not inhibit decay development on fruit (Fig. 1). In all cases where total protection was observed, tissue that did not develop lesions at the time of the first examination (6 days after inoculation with *P. expansum*) remained lesion free for the next 7 days or more, whereas lesions on apples inoculated with *P. expansum* as a control and other treatments that did not give total protection enlarged.

Dilution end point determination. Apples dipped for 0.5 min in an

TABLE 1. Effect of dipping Golden Delicious apples for 0.5 or 5 min in a suspension of bacterial strain L-22-64 and subsequent inoculation with spores (1×10^5/ml) of *Penicillium expansum* on blue mold lesion development

Bacterial concentration (cfu/ml)	Lesion diameter (mm) when fruit inoculated at:[a]			
			Both	
	Lower	Upper	Lower	Upper
0.5-min dip				
9×10^7	22.0	23.7	22.3	23.3
2×10^8	17.0	26.0	22.7	25.0
4×10^8	25.3	21.7	27.3	24.0
6×10^8	23.7	24.0	21.3	21.3
O-CK-P[b]	31.3	38.3	30.3	33.3
O-CK-W[c]	0.0	0.0	0.0	0.0
d'[d]	4.2	7.8	8.9	5.3
5-min dip				
9×10^7	10.3	15.7	9.0	7.0
2×10^8	7.7	10.7	3.0	2.7
4×10^8	12.7	5.3	5.7	7.0
6×10^8	13.7	0.0	5.0	14.7
O-CK-P[b]	31.0	33.0	28.7	31.3
O-CK-W[c]	0.0	0.0	0.0	0.0
d'[d]	18.8	9.7	16.8	12.9

[a] Lower portion of fruit, calyx end; upper portion of fruit, stem end.
[b] Apple inoculated with *P. expansum* as a control.
[c] Apple treated with sterile water only as a control.
[d] Means within a column of the antagonist-treated apples are compared with the control treated with *P. expansum* according to Dunnett's procedure ($P = 0.05$).

Fig. 1. Development of blue mold lesions during in vivo screening on Golden Delicious apples after wounding, protection with water suspension of potential antagonist (performing well in vitro), and challenging with 20 μl per wound of 0.05% Tween 80 water-spore suspension of *Penicillium expansum* (10^5 spores per milliliter). **A,** Six days after inoculation with isolates L-43-32 and Fr-43-33. **B,** Fourteen days after inoculation with isolates L-58-27, Fr-43-33[a], and Fr-58-19[b]. CK-W = apple treated with sterile water only as a control, CK-P = apple inoculated with *P. expansum* as a control.

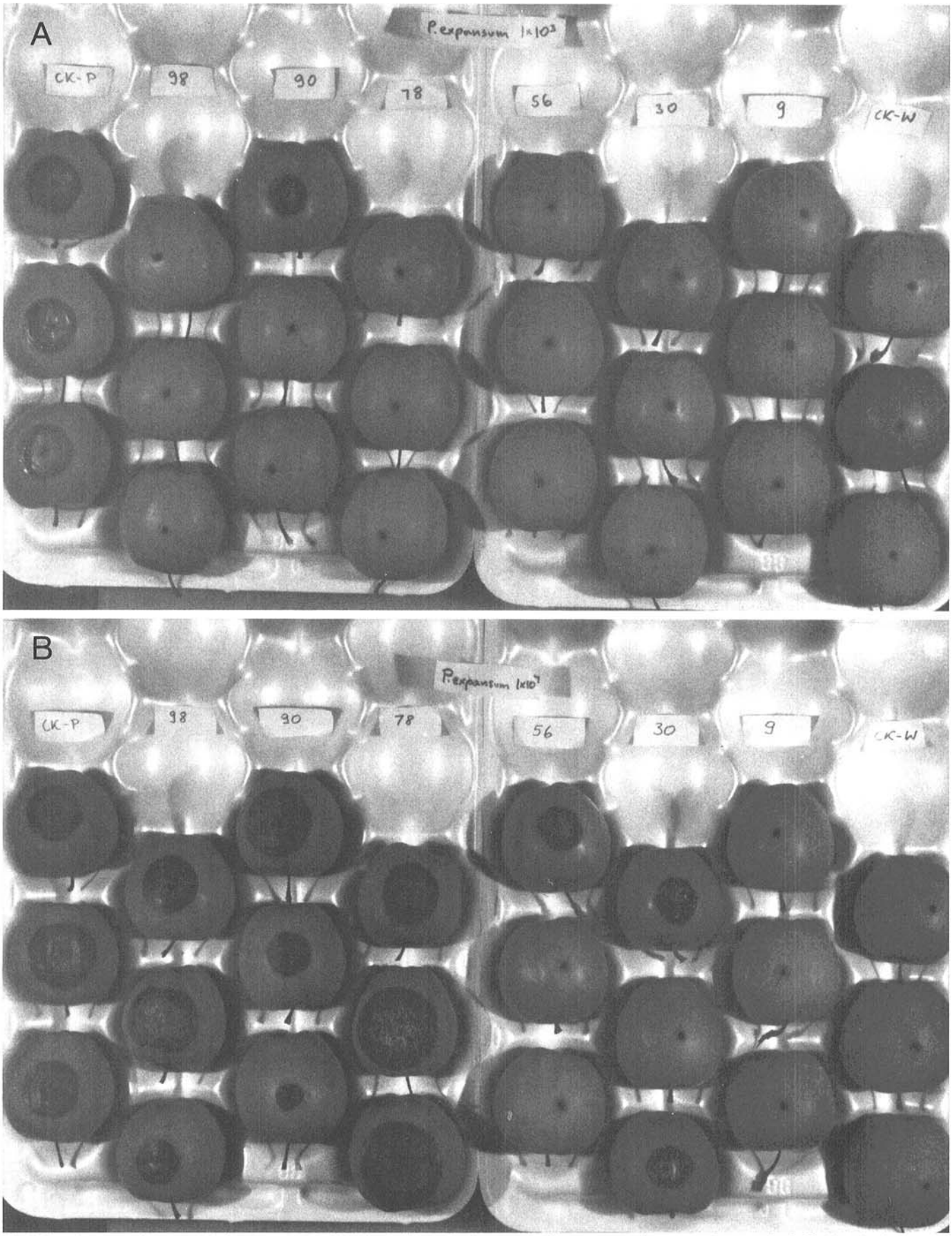

Fig. 2. Development of blue mold lesions on wounded Golden Delicious apples treated with a concentration range of antagonist L-22-64 and subsequently challenged with 20 μl per wound of 0.05% Tween 80 water suspension of spores of *Penicillium expansum*. **A,** Challenged with spore concentration 10^7 per milliliter. **B,** Challenged with spore concentration 10^3 per milliliter. Numbers represent percentage of transmittance in spectrophotometer of antagonist water suspensions; thus, the higher the number the lower the concentration of antagonist. CK-P = apple inoculated with *P. expansum* as a control, CK-W = apple treated with sterile water only as a control.

TABLE 2. Effect of bacterial strain L-22-64 (5-min dip) on blue mold lesion development on Golden Delicious apples (from control atmosphere storage) inoculated with *Penicillium expansum* spores (1×10^5/ml) and reinoculated 10 days later

Bacterial concentration (cfu/ml)	Lesion diameter (mm) when fruit inoculated at:[a]			
			Both	
	Lower	Upper	Lower	Upper
Initial inoculation only				
9×10^7	0.0	3.0	0.0	0.0
2×10^8	0.0	0.0	0.0	0.0
4×10^8	0.0	0.0	0.0	0.0
6×10^8	0.0	0.0	0.0	0.0
O-CK-P[b]	22.7	27.3	15.0	23.0
O-CK-W[c]	0.0	0.0	0.0	0.0
d'[d]		4.9		
1 wk after reinoculation				
9×10^7	0.0	22.3	0.0	0.0
2×10^8	0.0	0.0	0.0	0.0
4×10^8	0.0	0.0	0.0	0.0
6×10^8	0.0	0.0	0.0	0.0
O-CK-P[b]	83.3	90.7	69.3	80.0
O-CK-W-P[e]	0.0	3.7	0.0	2.7
d'[d]		35.6		

[a] Lower portion of fruit, calyx end; upper portion of fruit, stem end.
[b] Apple inoculated with *P. expansum* as a control.
[c] Apple treated with sterile water only as a control.
[d] Means within a column of the antagonist-treated apples are compared with the control treated with *P. expansum* according to Dunnett's procedure ($P = 0.05$).
[e] Apple water control treated with *P. expansum* only during reinoculation procedure.

TABLE 3. Lesion development (mm) on Golden Delicious apples after protection with different concentrations of bacterial strain L-22-64 and inoculation with various concentrations of spore suspensions of *Penicillium expansum* and reinoculation after 10 days

Bacterial concentration (cfu/ml)	Pathogen spore concentration				
	10^7	10^6	10^5	10^4	10^3
Initial inoculation only					
8×10^7	19.3	6.3	0.0	0.0	0.0
9×10^7	8.7	0.0	0.0	0.0	0.0
2×10^8	7.7	0.0	0.0	0.0	0.0
4×10^8	0.0	0.0	0.0	0.0	0.0
6×10^8	0.0	0.0	0.0	0.0	0.0
8×10^8	0.0	0.0	0.0	0.0	0.0
O-CK-P[a]	21.7	22.7	27.7	20.7	20.0
O-CK-W[b]	0.0	0.0	0.0	0.0	0.0
d'[c]	9.5	8.9			
1 wk after reinoculation					
8×10^7	62.7	26.7	0.0	0.0	0.0
9×10^7	46.3	0.0	0.0	0.0	3.0
2×10^8	42.7	0.0	0.0	0.0	0.0
4×10^8	0.0	0.0	0.0	0.0	0.0
6×10^8	0.0	0.0	0.0	0.0	0.0
8×10^8	0.0	5.3	0.0	0.0	0.0
O-CK-P[a]	89.3	74.3	82.3	82.0	69.3
O-CK-W-P[d]	0.0	0.0	0.0	0.0	0.0
d'[c]	46.9	37.0			11.8

[a] Apple inoculated with *P. expansum* as a control.
[b] Apple treated with sterile water only as a control.
[c] Means within a column of the antagonist-treated apples are compared with the control treated with *P. expansum* according to Dunnett's procedure ($P = 0.05$).
[d] Apple water control treated with *P. expansum* only during reinoculation procedure.

antagonistic bacterial suspension developed decay, and frequently there was no difference between the apples inoculated with *P. expansum* as a control and bacterial treated apples (Table 1). However, a 5-min dip resulted in good protection (Table 1). All antagonist-treated apples had lesions significantly smaller than the control with *P. expansum*. Treatments of apples from controlled atmosphere storage with strain L-22-64 resulted in total protection in all treatments except in the treatment with the higher dilution of the antagonist (Table 2). No new lesions developed after healthy wounds were reinoculated whereas old lesions enlarged and few small lesions developed on water controls (which were inoculated with *P. expansum* during reinoculation 10 days later) (Table 2).

Effect of pathogen spore concentration on biocontrol effectiveness. The pathogen spore concentration had a significant impact on biocontrol effectiveness (Fig. 2). As concentrations of spores in the inoculum decreased, lower concentrations of antagonists were required to totally control the disease (Tables 3 and 4). However, even the highest pathogen spore concentration (10^7/ml) could be controlled with antagonist concentrations of 4×10^8 cfu per milliliter in the case of bacteria L-22-64 or 1×10^8 cells per milliliter in the case of yeast F-43-31.

Control of blue mold persisted for at least 7–10 days, and reinoculation of all wounds, which at the first examination did not have lesions, resulted in only two new small lesions out of the 93 that were reinoculated with strain L-22-64 and three new lesions out of 51 reinoculations in the case of yeast strain F-43-31 (Tables 3 and 4).

DISCUSSION

It was not surprising that most potential antagonists that inhibit pathogen growth on media or spore germination in the well test did not inhibit lesion development in vivo on apples. Discrepancies between tests in vitro and in vivo in other systems have been

TABLE 4. Lesion development (mm) on Golden Delicious apples after protection with different concentrations of yeast strain F-43-31 and inoculation with various concentrations of spore suspensions of *Penicillium expansum* and reinoculation after 7 days

Yeast concentration (cells per milliliter)	Pathogen spore concentration				
	10^7	10^6	10^5	10^4	10^3
Initial inoculation only					
1×10^6	23.0	23.3	23.0	13.3	0.0
2.5×10^6	18.0	21.7	23.3	3.3	4.0
5×10^6	26.0	21.7	15.3	0.0	0.0
1×10^7	6.7	8.7	3.0	0.0	0.0
6×10^7	9.3	2.0	2.6	0.0	0.0
1×10^8	0.0	0.0	0.0	0.0	0.0
O-CK-P[a]	26.3	29.0	24.0	21.3	24.0
O-CK-W[b]	0.0	0.0	0.0	0.0	0.0
d'[c]	14.4	8.7	6.9	5.6	5.5
1 wk after reinoculation					
1×10^6	44.0	40.0	49.0	32.0	0.0
2.5×10^6	36.0	40.0	58.7	8.0	12.7
5×10^6	56.7	48.3	41.7	0.0	0.0
1×10^7	13.7	22.3	5.0	0.0	0.0
6×10^7	23.3	8.7	13.3	0.0	0.0
1×10^8	0.0	0.0	0.0	12.3	0.0
O-CK-P[a]	41.7	56.3	46.7	36.7	40.7
O-CK-W-P[d]	10.0	0.0	0.0	0.0	0.0
d'[c]	27.7	23.0	22.4	20.6	17.3

[a] Apple inoculated with *P. expansum* as a control.
[b] Apple treated with sterile water only as a control.
[c] Means within a column of the antagonist-treated apples are compared with the control treated with *P. expansum* according to Dunnett's procedure ($P = 0.05$).
[d] Apple water control treated with *P. expansum* only during reinoculation procedure.

frequently reported (3). In the present system few isolates that worked in vivo could totally inhibit lesion development. Several of these isolates (including isolate L-22-64) indicate that the actual mode of action on apple differs from the one in vitro and probably involves apple tissue response. The mode of action of these antagonists is being investigated.

The period of time apples are submerged in antagonist suspensions is an important factor influencing biocontrol effectiveness. Nonselective attachment of bacteria to different substrates is a widely known phenomenon (7) and was well documented in *Pseudomonas lachrymans* Smith & Bryan by Leben and Whitmoyer (8), who showed adherence rate to leaves of host and nonhost plants to be a straight-line, log-log function. Attachment of the antagonist, L-22-64, to its target may explain differences observed in the 0.5 min and 5-min apple dip.

Effectiveness of biocontrol agents is closely related to pathogen spore concentrations; however, in the case of bacterial strain L-22-64 and yeast strain F-43-31, total control was obtained even at the highest pathogen spore concentration by increasing antagonist concentration. For both antagonists, concentrations used were reasonable for possible further commercial development. The highest spore concentration per milliliter of *P. expansum* in the experiment was higher than those occurring in commercial drench tanks (2,4) by almost 1,000. Protection with either antagonist is ongoing because wound reinoculation with the pathogen after 7–10 days did not produce new lesions.

An experiment with survival of bacterium L-22-64 showed excellent persistence at 1 C over a 2-mo period—the duration of the experiment (*unpublished*). The feasibility of using these biological control agents to control postharvest diseases on commodities stored at low temperatures (15,16) needs future study.

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