Control of Storage Rots on Various Pear Cultivars with a Saprophytic Strain of *Pseudomonas syringae*

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ABSTRACT

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Dip treatment of wounded pear (*Pyrus communis*) fruit (cvs. Anjou, Bosc, Bartlett, and Red Bartlett) with a saprophytic strain of *Pseudomonas syringae* (L-59-66) provided complete or partial control of gray mold (*Botrytis cinerea*) and blue mold (*Penicillium expansum*) during storage at 18 C for 5 days or 1 C for 30 days. Cultivar, wound type, and storage temperature significantly affected the efficacy of the treatment. Control of both diseases as high as 100% was achieved in many tests with the addition of L-59-66 to a final concentration of 5.4×10^8 cfu/ml in the inocula (10^4 conidia per milliliter) of the pathogens. Disease control was best on Anjou, where frequently no rot developed, and worst on Bosc, where occasionally more than 90% of wounds developed disease. Nail wounds, which contained macerated tissue, were more difficult to protect than were clean cuts. Antagonist population in wound sites increased from 6.86 to 9.51 log cfu/ml per site during storage of fruit at 1 C for 30 days. Disease symptoms were not observed at any wound site inoculated with antagonist alone. Larger populations of antagonist were recovered from nail wounds than from cut wounds and from Bosc than from the other cultivars. Yet, the antagonist treatment was least effective with Bosc. A higher concentration of the antagonist must be used to achieve satisfactory control under these circumstances.

The development of resistance to many fungicides by major postharvest pathogens (1,3,18,22,23) and public concerns over synthetic pesticides in foods and the environment (14) have created interest in alternative methods of disease control. Biological control of postharvest diseases of fruit and vegetables has emerged as a promising option (7,9,25,26). The control of major postharvest pathogens through application of biological agents was reported for stone fruits (15,16), apples (5–13,17), citrus, and other fruit (7,9,24). The microbes used to treat fruits and vegetables have been isolated from soil and fruit or leaves of fruit trees (5,12,16,17,24). The microbes belong to various taxonomical groups including gram-positive (16) and gram-negative bacteria (5,12,13,24), yeasts (5,17,24), and filamentous fungi (8) and probably operate by various mechanisms. They have a somewhat restricted spectrum of activity. Some are effective against a few pathogens on various commodities, and others are more specific. A pilot test for commercialization of the biocontrol agent *Bacillus subtilis* (Ehrenberg) Cohn for control of brown rot on peach has been conducted (15). The results were variable and depended on the method of antagonist growth and formulation. Better fermentation, formulation, and application methods are needed.

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Biocontrol of blue mold (*Penicillium expansum* Link) and gray mold (*Botrytis cinerea* Pers.:Fr.) of apples has been reported (5,8,12,13). Although the same pathogens attack pears (*Pyrus communis* L.), biocontrol efforts have not been successful. The biocontrol of postharvest diseases of fruits is a complex phenomenon, and numerous interactions affect the efficacy of the treatment (5,6,8,10). The efficacy of these treatments depended on the pathogen antagonist/antagonist ratio, fruit maturity, wound severity, and environmental conditions (5,10,13). These factors appear to affect most biocontrol systems on fruit and should be considered in evaluating effectiveness of potential antagonists. Recently, we found that the cultivar also affects the outcome of biocontrol (W. J. Janisiewicz, *unpublished*). The objective of this study was to determine effectiveness of a new biocontrol agent, *Pseudomonas syringae* van Hall (strain L-59-66), against blue mold and gray mold of fruit from four common pear cultivars. Other factors, including concentration of the antagonist, type of wounding, and storage temperatures also were considered. Preliminary reports have been issued (11,13).

MATERIALS AND METHODS

Fruit of four pear cultivars (Bartlett, Red Bartlett, Anjou, and Bosc) were harvested from adjacent blocks in an orchard maintained under uniform cultural practices and stored at 1 C and 95% relative humidity (RH). The fruit were used in the experiments within 2 mo after harvest. The firmness of the Bartlett, Red Bartlett, Anjou, and Bosc fruits was 80.1, 77.8, 68.9, and 66.7 N, respectively, as determined by the Effegi pressure test (Effegi, 4801 Alfonsine, Italy). One day before treatment, the fruit were removed from cold storage and allowed to warm to 24 C. Just before treatment, two wounds were made on each fruit midway along the calyx stem end axis about 2 cm apart. Two types of wounds were used. Nail wounds simulated stem punctures, the most common wound in harvested pears. A wooden block that had been pierced with two sixpenny nails positioned 2 cm apart and protruding 3 mm was pressed against the side of each fruit. Cut wounds provided an open wound free of cellular debris. A sharp instrument was used to remove two $3 \times 3 \times 3$ mm blocks of fruit tissue. The fruit were treated within 30 min after being wounded.

Pathogens. *P. expansum* and *B. cinerea*, which had been isolated from decaying apples that had been in storage for 5 mo, were grown on potato-dextrose agar (PDA) with periodical transfer on PDA + 10% apple juice. When inoculated to Golden Delicious apple, both isolates produced the fastest expanding lesions among our collection of these pathogens. For inoculation, 2 ml of sterile distilled water with 0.05% Tween 80 was added to a 10- or 14-day-old culture of *P. expansum* or *B. cinerea*, respectively, that had been grown in PDA under continuous tungsten light. The dishes were shaken, and the resulting suspension of conidia was collected and then adjusted to the desired concentration with the aid of a hemacytometer. This stock conidial suspension then was added to a 15-L dip tank containing 12 L of tap water to obtain a final concentration of 10^4 conidia per milliliter.

Antagonistic bacterium. Procedures described earlier were followed for isolating microorganism from fruit and leaves and for screening for antagonistic activity (5,10). One promising antagonist was identified as *P. syringae* pv. *lachrymans* (Smith and Bryan) Young et al based on its fatty acid profile (FAMES test) (Microbial ID, Inc., Newark, DE). The identification was confirmed with physiological tests. The FAMES match of the unknown with the library for *P. s. lachrymans* was 0.836. However, the unknown appears to be saprophytic because it did not cause lesions in cucumber plants brush-inoculated with 5×10^6, 5×10^7, or 5×10^8 cfu/ml. Also, the organism did not cause hypersensitivity in tobacco leaves, lesions in wound-inoculated pear seedlings, or oozing on wound-inoculated small pear fruit. The antagonist, strain L-59-66, was grown in 50 ml of nutrient yeast dextrose broth medium in 250-ml Erlenmeyer flasks on a rotary shaker at 150 rpm. After 24 hr, the cells were pelleted by centrifugation at 7,000 rpm for 10 min. The pellets were resuspended in tap water and the concentration was determined with the aid of a spectrophotometer. The stock suspensions were added to 12 L of conidia suspension in 15-L tanks to achieve 2.2, 3.2, and 5.4×10^8 cfu/ml.

Fruit treatment. Wounded pears held in plastic mesh baskets were submerged in the suspension for 2 min and agitated occasionally. The treatments were 10^4 conidia of *B. cinerea* or *P. expansum* alone, or each mixed with 2.2, 3.2, or 5.4×10^8 cfu/ml of L-59-66. The fruit then were placed on polystyrene fruit tray packs in 1-bu fruit boxes with a polyethylene liner. Fruit from each treatment were placed on separate trays and the trays were placed in boxes. There were four trays per box, one containing a pathogen control, and the remaining containing a treatment with three concentrations of the antagonist. One set of fruit was stored at 18 C and 95% RH for 5 days and the other at 1 C and 95% RH for 30 days. The 18-C storage was selected because it was optimal for disease development, whereas the cold storage stimulated commercial conditions, and the storage periods made lesion measurements possible before rot encompassed the entire fruit. After storage, lesion diameters were measured perpendicular to the axis connecting the two wounds. There were 15 fruit per treatment and each was repeated three times. The boxes were placed in storage as randomized blocks. The entire experiment was repeated with nine fruit per treatment and a preliminary test was done with storage at 18 C only.

Antagonist recovery. Pears of the four cultivars were either nail or cut wounded and then dipped into suspensions containing 2.2, 3.2, and 5.4×10^8 cfu/ml of the L-59-66 for 2 min, placed on fruit trays, and stored at 1 C. Within 30 min after treatment and after 30 days of storage, the wounds were removed from fruit with a cork borer (1 cm diameter $\times$ 1 cm deep) and ground in a mortar with 1 ml of phosphate buffer (4). Each wound was handled separately. Serial 10-fold dilutions were made in a phosphate buffer, and 0.1-ml samples of each dilution were spread in triplicates in petri plates with nutrient yeast dextrose agar medium. The plates were incubated at 24 C for approximately 48 hr and the colonies were counted. There were three replications of three fruit per treatment. The boxes containing the treated fruit were placed in storage as randomized blocks. The experiment was repeated.

Data analysis. ANOVA was performed on rot development data from the fruit dip test and on recovery of the antagonist from the wounds before and after fruit storage with the general linear models (GLM) procedure of the Statistical Analysis System (19). The analysis of disease incidence (percent wounds infected) was performed on arcsine-transformed data. A Waller-Duncan multiple range test was performed for separation of means of lesion diameters, percentage of wounds infected, and antagonist population with the different cultivars separately on data from storage at 18 and 1 C. A Fisher *t* test was used to compare effects of the wound type on lesion development and antagonist population.

RESULTS

Effect of cultivar, wound type, and storage temperature on efficacy of biocontrol. Cultivar, wound type, antagonist concentration, and temperature significantly ($P = 0.0001$) affected the severity (lesion diameter) of gray mold and blue mold (Table 1).

The severity and incidence (percent wounds infected) of the disease differ among cultivars. On fruit inoculated only with *B. cinerea* and stored at 18 C, the severity of the disease was similar for Bosc and Anjou but was less on Red Bartlett and least on Bartlett ($F = 44.66$, $P = 0.0001$). The incidence of the disease has a similar pattern. On fruit stored at 1 C, disease severity was highest on Bosc (75.9 mm), followed by Anjou (72.5 mm), and Bartlett (43.8 mm) and Red Bartlett (43.0 mm), between which there was no difference ($F = 209.2$, $P = 0.0001$). There was no difference between these cultivars in incidence of the disease because more then 95% of the wounds were infected ($F = 2.41$). The incidence and severity of the disease was higher on fruit with nail wounds than on fruit with cut wounds at both storage temperatures ($P = 0.05$).

The addition of the antagonist to the conidia suspension reduced both the se-

Table 1. Summary of ANOVA for lesion diameter on four pear cultivars wounded by one of two methods, treated with varying concentrations of the antagonist *Pseudomas syringae* (L-59-66), inoculated with *Botrytis cinerea* or *Penicillium expansum*, and stored at 1 or 18 C

		Botrytis cinerea			*Penicillium expansum*		
Source of variation	**df[a]**	**Mean square[b]**	***F* value**	***P* > *F***	**Mean square**	***F* value**	***P* > *F***
Cultivar	3	51,436.37	325.59	0.0001	6,202.67	59.61	0.0001
Temperature	1	83,403.14	527.93	0.0001	9,021.34	86.70	0.0001
Wound	1	13,829.57	87.54	0.0001	3,379.26	32.48	0.0001
Cultivar × temperature	3	10,835.46	68.59	0.0001	11,039.79	106.10	0.0001
Wound × cultivar	3	724.44	4.59	0.0008	145.28	1.40	0.2618
Wound × temperature	1	3,705.67	23.46	0.0001	53.77	0.52	0.4775
Wound × cultivar × temperature	3	136.93	0.87	0.4684	164.79	1.58	0.2125
Rep(wound × cultivar × temperature)	32	157.98			104.05		
Concentration[c]	1	917,203.00	2,196.42	0.0001	287,114.28	1,275.66	0.0001
Concentration × cultivar	3	11,836.52	28.34	0.0001	1,913.75	8.50	0.0002
Concentration × temperature	1	6,740.28	16.14	0.0002	4,878.29	21.67	0.0001
Concentration × wound	1	4,562.11	10.92	0.0019	8,341.56	37.06	0.0001
Concentration × rep(wound × cultivar × temperature)	42	417.59			225.07		
$R^2 = 0.69$							

[a] Degrees of freedom.
[b] Mean squares were derived from type III sums of squares for unbalanced linear model and randomized block design at the two temperatures.
[c] The source of variation concentration has been treated as a covariate in analysis of covariance.

verity and the incidence of the disease. Complete reductions in gray mold on fruit with cut or nail wounds was achieved in many cases at the highest concentration of the antagonist at both storage temperatures (Fig. 1). Taking into account both types of wounds, on Anjou pears inoculated with a suspension containing *B. cinerea* conidia and the highest concentration of the antagonist, the reduction was from 47.7 to 0 mm and from 100 to 0% of the controls for severity and incidence of the disease, respectively, at 18-C storage. Under the same conditions, complete reduction in incidence and severity also was achieved on Bartlett and Red Bartlett. However, the reduction on Bosc was from 47.9 to 3.9 mm and from 100 to 21.9% of the controls for severity and incidence of the disease, respectively. Thus, at 18 C, disease severity ($F = 13.9$, $P = 0.0001$) and incidence ($F = 13.26$, $P = 0.0001$) was higher on Bosc than on the other cultivars, which had similar means.

On fruit subjected to the same treatment but stored at 1 C, reduction in severity on Anjou was from 72.5 to 5.7 mm and incidence from 100 to 6.7% of the controls. On Red Bartlett and Bartlett, the disease severity was reduced from 43.0 and 43.8 mm to 2.1 and 1.7 mm, respectively, and incidence from 97.4 and 96.7% to 30.8 and 13.8%, respectively. However, on Bosc, the reduction in severity was from 75.9 to 33.8 mm and in incidence from 100 to 85% of the controls. Thus, at 1 C, the highest severity of the disease was on Bosc, followed by Anjou, Red Bartlett, and Bartlett ($F = 11.66$, $P = 0.0001$). The incidence of the disease also was highest on Bosc, then on Red Bartlett, and then on Bartlett and Anjou, which had similar means ($F = 133.5$, $P = 0.0001$). The incidence and severity of the disease were higher on fruit with nail wounds than on fruit with cut wounds ($P = 0.05$) at both temperatures, except in a few instances where average lesions were small (less than 4.5 mm). In those cases, there was no difference or it was higher on cut than on nail wounds. As the concentration of the antagonist increased, the degree of reduction in severity of the disease was greater on cut than on nail wounds, at 18 than at 1 C, and on Bosc than the other cultivars.

On fruit inoculated only with *P. expansum* and stored at 18 C, severity of the disease was highest on Red Bartlett, next on Bartlett, and least on Bosc and Anjou, which had similar means ($F = 55.81$, $P = 0.0001$). However, there was no difference in incidence of the disease, because almost 100% of wounds were infected ($F = 1.00$). On fruit stored at 1 C, severity was highest on Bosc, next on Anjou, and least on Bartlett and Red Bartlett, which had similar means ($F = 55.81$, $P = 0.0001$). There was no difference among these cultivars in incidence of the disease, except on Anjou (93.3%), where it was significantly lower ($F = 4.31$, $P = 0.05$). The incidence and severity of the disease was higher on fruit with nail wounds than on fruit with cut wounds at both storage temperatures ($P = 0.05$), except in incidence of the disease at 18 C, where most fruit were infected and means were similar.

The addition of the antagonist to conidia suspension reduced both the severity and incidence of the disease. The highest concentration of the antagonist, in many cases, greatly reduced disease severity on fruit with cut and nail wounds at both storage temperatures (Fig. 2A and B). However, disease incidence was reduced less, particularly on fruit with nail

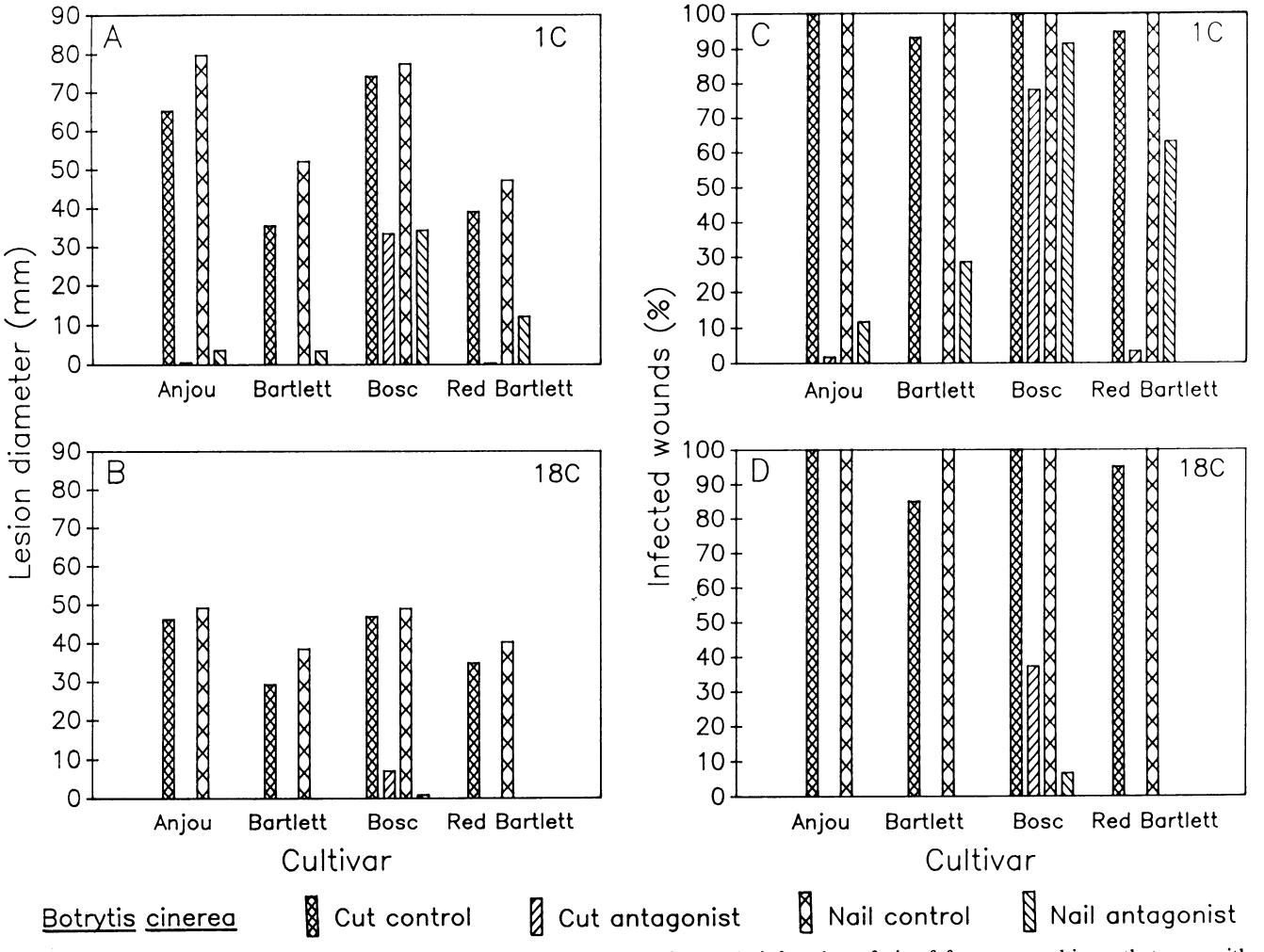

Fig. 1. (A and B) Average lesion diameter and **(C and D)** percentage of wounds infected on fruit of four pear cultivars that were either nail or cut wounded, dipped in a suspension containing antagonist *Pseudomonas syringae* (L-59-66) at a concentration of 5.4×10^8 cfu/ml and 10^4 conidia of *Botrytis cinerea* per milliliter, and stored at 1 or 18 C. LSD 0.05 = **(A)** 6.9; **(B)** 3.8; **(C)** 8.7; and **(D)** 6.0.

wounds (Fig. 2C and D). Taking into account both types of wounds, on Anjou pears inoculated with a suspension containing *P. expansum* conidia and the highest concentration of the antagonist, the reduction was from 25.8 to 1.3 mm and from 100 to 15.8% of the controls for severity and the incidence of the disease, respectively, at 18-C storage. Under the same conditions, severity was reduced from 26.0 to 5.7 mm, 31.6 to 10.5 mm, and 33.9 to 12.7 mm and incidence from 100 to 60.8%, 100 to 54.2%, and 99.4 to 59.2% of controls for Bosc, Bartlett, and Red Bartlett, respectively. Thus, at 18 C, highest severity of the disease was on Red Bartlett, then on Bartlett, Bosc, and Anjou ($F = 28.2$. $P = 0.0001$). The incidence of the disease was lower on Anjou than on remaining cultivars, between which there was no difference ($F = 34.84$, $P = 0.0001$).

On fruit subjected to the same treatment but stored at 1 C, the reduction in severity on Anjou was from 36.1 to 0.8 mm and incidence from 93.3 to 6.7% of the controls. Under the same conditions, severity was reduced from 26.8 to 3.9 mm, 28.8 to 7.4 mm, and 46.6 to 14.3 mm and incidence from 98.3 to 30.8%, 99.2 to 50.0%, and 100 to 59.2% of controls for Bartlett, Red Bartlett, and Bosc, respectively. Thus, at 1 C, severity was highest on Bosc, then on Bartlett and Red Bartlett, and the lowest on Anjou ($F = 15.9$, $P = 0.0001$). The incidence of the disease was greatest on Bosc and Red Bartlett, then on Bartlett, and the least on Anjou ($F = 17.96$, $P = 0.0001$). As the concentration of the antagonist increased, the degree of reduction in severity of the disease was greater on fruit with cut wounds than on fruit with nail wounds, on fruit stored at 18 C than on fruit stored at 1 C, and on Bosc than on other cultivars.

Effect of wound type, cultivar, and storage time on population of the antagonist. Cultivar, time, and wound type effected antagonist population on fruit (Table 2). Taking into account all concentrations and wound types tested, higher populations of the antagonist were recovered from Bosc than from any of the other cultivars. Populations of the antagonist increased after 30 days of storage at 1 C (6.3 vs. 7.85 log cfu/wound), and more antagonist was recovered from nail wounds than from cut wounds (7.57 vs. 6.66 log cfu/wound). There were significant interactions between wound type and cultivar ($F = 6.45$, $P = 0.01$) and wound type, cultivar, and time ($F = 4.04$, $P = 0.05$). The concentration of the antagonist had a significant effect on its recovery ($F = 38.02$, $P = 0.0001$). On fruit treated with the highest concentration of the antagonist, populations increased on all cultivars in both types of wounds, except on Anjou with cut wounds that had a slightly lower population (Table 3). Larger populations of the antagonist were recovered from nail wounds than from cut wounds.

DISCUSSION

Gray mold and blue mold of pears can be controlled by the application of the antagonistic bacterium *P. syringae* (L-59-66) under the given conditions using a fruit dip treatment. Control often approached 100%. In general, the best control was achieved on Anjou and the worst on Bosc pears. The severity and incidence of both diseases did not differ between these cultivars in control treatments at 18 C, but when the

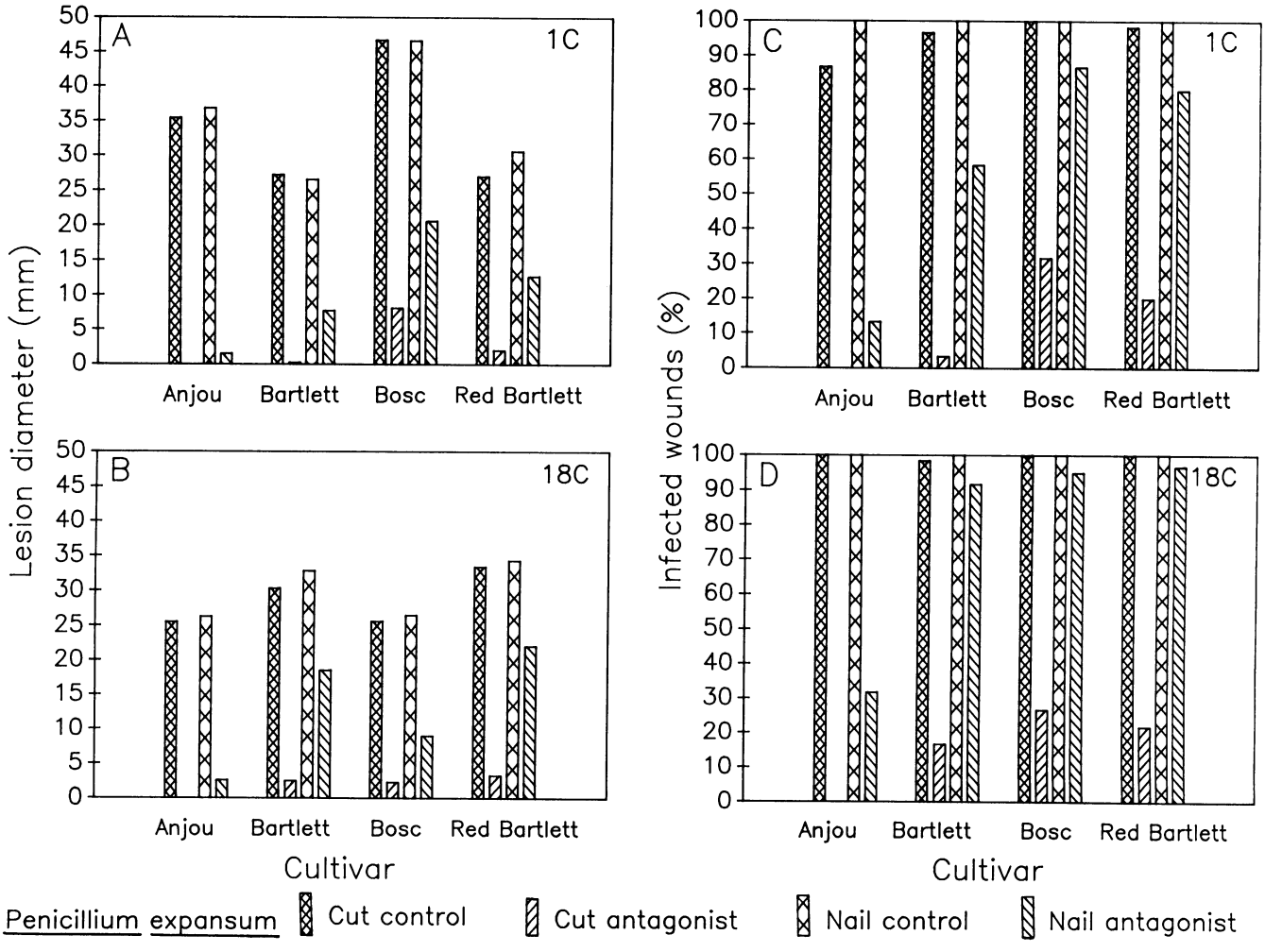

Fig. 2. (A and B) Average lesion diameter and **(C and D)** percentage of wounds infected on fruit of four pear cultivars that were either nail or cut wounded, dipped in a suspension containing antagonist *Pseudomonas syringae* (L-59-66) at a concentration of 5.4×10^8 cfu/ml and 10^4 conidia of *Penicillium expansum* per milliliter, and stored at 1 or 18 C. LSD 0.05 = **(A)** 5.1; **(B)** 1.5; **(C)** 9.7; and **(D)** 8.5.

antagonist was added, severity and incidence were consistently higher on Bosc than on any of the other cultivars. At 1 C, in control treatments, the severity of both diseases was greater on Bosc then on Anjou, but the differences were marginal. However, when the antagonist was added, the severity and incidence of both diseases was again greatest on Bosc. After 30 days of storage at 1 C, when all applied concentrations of the antagonist were considered, more antagonist was recovered from wounds on Bosc than from any other cultivar. Thus, reduced levels of control on Bosc were not attributable to a negative effect of this cultivar on the antagonist population but rather to its effect on the physiological processes governing antagonism.

So far, in all cases of biocontrol of postharvest diseases of fruits (7,9,26), a quantitative relationship exists between population of the antagonist and effectiveness of biocontrol. Perhaps the antagonist population did not reach the threshold concentration necessary for satisfactory control. In additional tests, decays on Bosc were completely controlled when higher concentrations of the antagonist (1.8×10^9 cfu/ml) were used (W. J. Janisiewicz, *unpublished*). However, these concentrations were above the limit for practical biocontrol development. Research is underway to stimulate populations of the antagonist on fruit to increase biocontrol effectiveness. Alternatively, if no antagonist is found that can control the diseases on all cultivars, it may be practical to use different antagonists with cultivars harvested at different times in the season. The future commercial development of microbial antagonists as biocontrol agents most likely will depend on relatively small niche markets where different antagonists can be used to control specific fruit diseases or the same diseases under various conditions (2).

Rots were more difficult to control on nail puncture wounds, where macerated tissue remains at the wound site, than on cut wounds. In many cases, where lesion development was prevented at cut wounds, small lesions developed on fruit with nail wounds subjected to the same treatment. This was not surprising, because the severity of fruit wounding decreases rot control by fungicides on pome fruits (20,21). The nail puncture wounds resemble stem punctures, which are the most common wounds occurring during postharvest handling. Earlier work (13) demonstrated increased levels of biological control of decay on fruit with cut wounds. Cut wounds were included in the experiments to detect levels of control that the antagonist could provide under more favorable conditions. Control levels on fruit with this wound were high and frequently 100%, except on Bosc. Although higher concentrations of the antagonist were recovered from nail wounds than from cut wounds, the environment created by nail wounds apparently favors growth of the pathogens more than the antagonist. Thus, higher populations of the antagonist must be applied to nail wounds to achieve satisfactory control. Results also underscore the fact that various treatments, with different levels, must be tested in evaluating potential antagonists to avoid improper conclusions concerning their effectiveness.

Although this bacterium is readily isolated from apple, health authorities must be satisfied that the treatment possesses no threat to human health before they will permit registration and commercial use. Regulations are being developed to address the safety issue of biocontrol agents.

Table 2. Summary of ANOVA for the recovery of antagonist *Pseudomonas syringae* (L-59-66) from four pear cultivars wounded by one of two methods, treated with varying concentrations of the antagonist, and stored at 1 C for 30 days

Source of variation	df[a]	Mean square[b]	*F* value	*P* > *F*
Cultivar	3	0.62	3.87	0.0182
Time	1	18.54	84.89	0.0001
Wound	1	2.62	16.36	0.0003
Cultivar × time	3	0.33	2.07	0.1233
Wound × cultivar	3	1.03	6.45	0.0015
Wound × time	1	0.07	0.47	0.4982
Wound × cultivar × time	3	0.65	4.04	0.0153
Rep(wound × cultivar × time)	32	0.16		
Concentration[c]	1	7.14	38.02	0.0001
Concentration × cultivar	3	0.60	3.17	0.0338
Concentration × time	1	0.73	3.86	0.0560
Concentration × wound	1	0.03	0.16	0.6882
Concentration × rep(wound × cultivar × time)	42	0.19		
$R^2 = 0.96$				

[a] Degrees of freedom.
[b] Mean squares were derived from type III sums of squares for unbalanced linear model and randomized block design.
[c] The source of variation concentration has been treated as a covariate in analysis of covariance.

Table 3. Effect of cultivar and wound type on population of the antagonist *Pseudomonas syringae* (L-59-66) on fruit[a] stored for 30 days at 1 C

	Antagonist recovery (log cfu/wound)			
	Day 0		Day 30	
Cultivar	Cut	Nail	Cut	Nail
Anjou	6.46	6.86	6.19	9.51
Bartlett	6.62	7.07	7.16	7.53
Red Bartlett	6.52	7.01	8.37	8.71
Bosc	6.42	7.29	7.90	9.03
LSD 0.05 = 0.15				

[a] The fruit were wounded in one of two ways (cut or nail), dipped in a suspension containing *P. syringae* (L-59-66) at the concentration of 5.4×10^8 cfu/ml, and stored at 1 C for 30 days. Within 30 min after treatment (day 0) and after 30 days of storage, the wounds were removed from fruit, grinded, and plated on the media, and after 48 hr in incubation, the colonies were counted.

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