Biocontrol of Postharvest Diseases of Apples with Antagonist Mixtures

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ABSTRACT

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Acremonium breve isolated from apple leaves showed strong antagonistic activity against *Botrytis cinerea* in laboratory fruit tests. Golden Delicious apples protected with an aqueous suspension of this antagonist (2.5×10^5 colony-forming units per milliliter) and subsequently challenged with *B. cinerea* spores (10^4 spores per milliliter) did not develop lesions. On fully mature apples, inoculation with the pathogen did not produce lesions when the antagonist was applied 4 hr before inoculation. This was in contrast to a *Pseudomonas* sp. antagonist for which 72 hr was required between its application and inoculation with *Penicillium expansum* spores to prevent lesion development for the first time. When the two antagonists were tested as mixtures on wounded apples challenged with mixtures of *B. cinerea* and *P. expansum* spores, inhibition of both types of lesions was observed. Total protection was obtained with the antagonists on apples challenged with pathogen spores in concentrations corresponding to the highest reported in commercial drench tanks.

Additional key words: blue mold, gray mold.

Biological control of fruit diseases has shown great promise in recent years. Antagonists have been found against major pathogens of grapes (5,7–9), strawberries (2,3,27), pineapples (19), citrus fruit (4,12,24), stone fruits (20–22), and apples (1,6,14–16, 26–28). Progress has been made particularly in the biocontrol of postharvest disease of fruit (15,16,22,23,29,30). The subject of biocontrol of fruit diseases was extensively reviewed recently (17). In research to date, a single biocontrol agent has been applied to combat a single pathogen, but little attention has been focused on diseases other than the targeted disease on those fruits. Considering that most fruits have a number of important pathogens, controlling only one may merely favor another. Neglecting this problem under commercial conditions can make biocontrol ineffective or short-lived and in the future may rapidly increase the incidence of previously less important diseases. Similar responses have occurred after chemical control (11,13).

Some of the most important postharvest diseases of apples are caused by *Penicillium expansum* Link ex Thom, *Botrytis cinerea* Pers. ex Fr., *Mucor* spp., *Gleosporium perennans* Zeller & Childs, and *Phialophora malorum* (Kidd & Beaum.) McColloch. Spores of these pathogens can be easily isolated from drench tanks in storage houses, and their concentration determined (25).

Biocontrol agents effective against *P. expansum* were isolated from apples (15,16), and a quantitative relation between antagonists and pathogen inoculum concentration was determined. This paper reports on biocontrol of *B. cinerea* with an antagonist isolated from apple leaves and its use in combination with an antagonist against *P. expansum* to control both diseases simultaneously.

MATERIALS AND METHODS

Isolation and screening for antagonists in vitro. Leaves and fruit were picked throughout the growing season from apple trees in four five-tree blocks, scattered over approximately a 4-acre area. Three apples and 10 leaves were randomly collected from different parts of each tree in each block. The samples were washed in phosphate buffer (10) by shaking them in a beaker on a rotary shaker for 10 min at 100 rpm. The buffer from the first washing was discarded, and leaves or fruit were washed for the second time with 30-sec sonication in a Bransonic 521 sonicator (Branson Co., Shelton, CT) at the beginning of the wash. Sonicated samples were plated directly on nutrient yeast-dextrose agar (NYDA) medium (0.1 ml per plate), or 10 ml of the washing was concentrated on a millipore membrane filter (0.2-μm pores). The membrane was removed and placed on NYDA medium. After 24 hr of incubation, half of the samples plated directly on NYDA medium were seeded with a spore suspension of the pathogen (1×10^6 spores per

milliliter), and the other half was incubated for an additional 24–48 hr. After the appearance of a mycelial mat on plates seeded with the pathogen, colonies exhibiting zones of mycelial inhibition were isolated. Colonies from unseeded plates were isolated arbitrarily on the basis of their different visual characteristics. Colonies developing on membrane filters were isolated at random after 24 to 72 hr of incubation, depending on colony frequency. After isolation, all colonies were purified by single-colony isolations after triple restreaking on NYDA medium.

Screening potential antagonists on apples. In vivo screening of apples was done following procedures described earlier (16), in which wounded apples were treated with aqueous suspensions of potential antagonists and an aqueous suspension of pathogen spores (1×10^4 spores per milliliter). Lesion diameter was measured after 7 days of incubation at 24 C. Each apple constituted a single replicate, and there were three replicates per treatment.

In vitro testing for inhibition of spore germination. In vitro testing for germination was conducted in wells (16 mm in diameter) of plastic tissue culture plates; 100 μl of an aqueous suspension of the antagonist and 100 μl of the aqueous suspension of the pathogen spores were mixed, and after incubation at 24 C, germination was determined according to arbitrary scales (Table 1) by examination under an inverted microscope. The antagonist concentrations in the wells were 8.5×10^8 colony-forming units (CFU) per milliliter for *Pseudomonas* sp. and 9.5×10^6 CFU/ml for *Acremonium breve* (Sukapure & Thirumalachar) W. Gams. The pathogen concentrations were from 10^2 to 10^7 spores per milliliter, in 10-fold increases, for *P. expansum* and from 10^3 to 10^5 spores per milliliter for *B. cinerea*. The plates were examined after 72 hr. At this point, equal volumes of freshly squeezed apple juice were added, and after an additional 48 hr incubation (120 hr from the beginning), the plates were examined a second time. Each well constituted a single replicate, and there were three replicates per treatment.

Antagonist mixture selection. The selection of antagonists from both the bacterial and the yeast groups was based on their good performance in screening on apples. Combinations of two antagonists, each known to inhibit the development of *B. cinerea* or *P. expansum* on fruit, were applied in mixtures as a water suspension to wounds (3 mm wide and 3 mm deep) on Golden Delicious apples. This was followed by a challenge with an aqueous mixture of *B. cinerea* and *P. expansum* spores. Antagonist mixtures consisted of either two bacteria, two yeastlike fungi, or organisms from these two groups; 18 such combinations were tested. The concentration of bacteria in the mixtures was approximately 3×10^8 CFU/ml, and that of the yeastlike antagonists was 3×10^6 cells per milliliter. For each combination, 20 μl of the mixture was applied to the wounds, and within 15 min, 20 μl of the suspension of *B. cinerea* and *P. expansum* (1×10^4 spores of each per milliliter) was added. The apples were then incubated for 7 days at 24 C, and the lesion diameters measured. Each apple constituted a single replicate, and each treatment was replicated six times. From the four treatments showing good inhibition of both pathogens, one combination of isolates was selected for further study.

The selected antagonist combination of *Pseudomonas* sp. and *A. breve*, in various concentrations, was tested against spore mixtures of *B. cinerea* and *P. expansum* on Golden Delicious apples in a factorial experiment. These spore mixtures consisted of a constant concentration of 1×10^4 *B. cinerea* spores per milliliter and concentrations of 0 to 1×10^7 *P. expansum* spores per milliliter, in 10-fold increases. The antagonist concentrations varied as shown in Figure 3A and B.

The apple inoculation method was identical to that described earlier for the initial screening of the organisms. The lesion diameter was measured 7 days after inoculation at 24 C. Each apple constituted a single replicate, and each treatment was replicated three times. The experiment was repeated once.

Effect of time of inoculation on control effectiveness. Wounded, fully mature Golden Delicious apples protected with an antagonist were inoculated with an aqueous spore suspension of the respective pathogen (for each wound, 20 μl of a suspension of 1×10^4 spores per milliliter) at intervals from 0 to 96 hr after treatment with the antagonist (Figs. 1 and 2). The antagonists were applied to the wounds as aqueous suspensions, 20 μl per wound, of 9×10^7 CFU/ml for *Pseudomonas* sp. and 2.5×10^5 CFU/ml for *A. breve*. The diameter of the lesions was measured 7 days after inoculation with the pathogen. The experiment was repeated once.

Pathogens. *B. cinerea* and *P. expansum* were isolated from decayed apples kept in cold storage for 5 mo. The cultures were maintained on potato-dextrose agar medium. Spores of *P. expansum* and *B. cinerea* were harvested from 10- and 14-day-old cultures, respectively.

Apples. The apple cultivar Golden Delicious was used in all experiments. For screening tests, the apples were used following harvest or after a short term (no longer than 3 mo) of regular storage at 2 C. On the maturity iodine test scale of 1 to 6 (with stage 6 being the most mature) the fruit were in stages 4 and 5 (18). For the factorial experiment evaluating the combination of antagonists, apples were taken from controlled-atmosphere storage after about 7 mo and were in the fifth stage of maturity. When the experiment was repeated, the apples were used just after harvest.

RESULTS

Isolation and screening of antagonists in vitro and in vivo. Washings from fruit and leaves contained numerous organisms exhibiting antagonistic action toward *B. cinerea* and *P. expansum*. Some organisms inhibited pathogen growth both on plates and on fruit; others inhibited growth on only one or the other. There seems

TABLE 1. Germination of *Penicillium expansum* and *Botrytis cinerea* spores in water suspension of antagonists at 72 hr and after an additional 48 hr following apple juice supplement

Pathogen	Antagonist	Hours	Pathogen spore concentration (spores per ml)					
			10^2	10^3	10^4	10^5	10^6	10^7
P. expansum	*Pseudomonas* sp.	72	+[a]	+	+	+	+	+
		120	+++	+++	+++	+++	+++	+++
	Acremonium breve	72	+	+	+	+	+	–
		120	+++	+++	+++	+++	+++	+++
	Control	72	+++	+++	+++	++	++	+
		120	+++	+++	+++	+++	+++	+++
B. cinerea	*Pseudomonas* sp.	72		+	+	–		
		120		+	+	+		
	A. breve	72		+	+	+		
		120		+	+	+		
	Control	72		++	++	+		
		120		+++	+++	+++		

[a] – = No germination; + = less than 1/3 germinated; ++ = 1/3 to 2/3 germinated; +++ = more than 2/3 germinated.

to be little relation between the antagonistic action on plates and that on fruit, although in a few cases strong inhibition on plates was paralleled by strong inhibition on fruit. Of 534 antagonists tested in vitro against both pathogens, 185 were tested in vivo; 63% of the latter were bacteria, and 27% yeastlike organisms. The organism antagonistic to *B. cinerea* and selected for further investigation did not inhibit the growth of *B. cinerea* on NYDA medium but completely inhibited fungal growth in tests on fruit. The organism was identified by the American Type Culture Collection as *A. breve*. It has a yeastlike growth in nutrient yeast-dextrose broth. The colonies have pink pigmentation. Sporulation occurs mainly in the middle of the colony. Conidia are oblong, formed in heads on unbranched phialides with no collarette. The culture was compared morphologically and macro- and microscopically to *A. breve* from the ATCC culture 14614. Growth rate, spore size, color change under light, and colonial morphology all were the same. Tests on Golden Delicious apples just after harvest (stage 4 on the maturity scale) showed that total protection from *B. cinerea* (in inoculations with 1×10^4 spores per milliliter) was obtained with 1.5×10^4 CFU/ml, the lowest concentration applied. The protection lasted for 10 wk (at 24 C), the duration of the experiment. When different combinations of antagonists were tested on fruit against the mixture of *B. cinerea* and *P. expansum* spores, the combination of *Pseudomonas* sp. (isolate L-22-64) and *A. breve* gave excellent protection and was selected for further studies. The *Pseudomonas* antagonist came from an earlier study and had already shown very good protection of fruit against *P. expansum* (16). *A. breve* did not inhibit the growth of *P. expansum* on NYDA medium and in vivo on Golden Delicious apples challenged with 1×10^4 spores of the pathogen per milliliter but only delayed the development of lesions, which with time enlarged to cover the whole fruit.

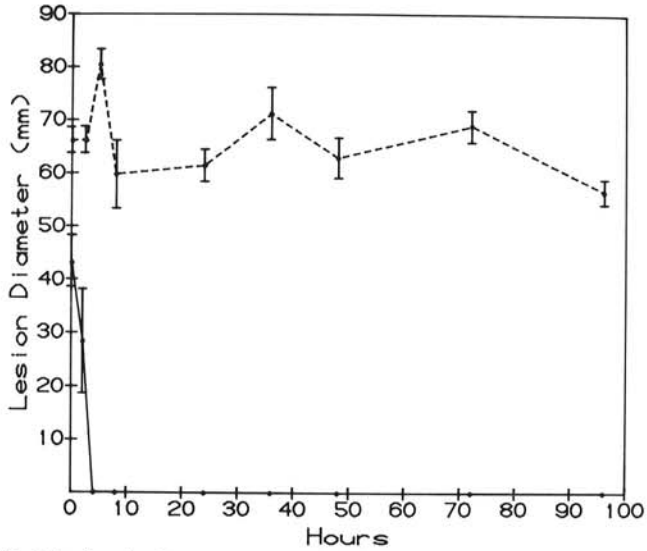

Fig. 1. Lesion development on wounded Golden Delicious apples protected with the antagonist *Acremonium breve* and inoculated with *Botrytis cinerea* spores at different times following antagonist application. Solid line: antagonist treatment; dashed line: control. Vertical bars indicate standard error.

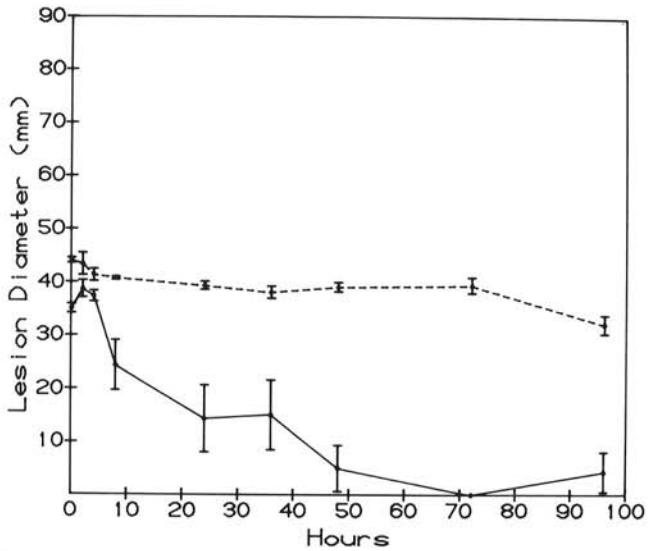

Fig. 2. Lesion development on wounded Golden Delicious apples protected with an antagonist *Pseudomonas* sp. and inoculated with *Penicillium expansum* spores at different times following antagonist application. Solid line: antagonist treatment; dashed line: control. Vertical bars indicate standard error.

Inhibition of spore germination. *A. breve* inhibited spore germination of *B. cinerea* and *P. expansum* in aqueous suspension in vitro (Table 1). The addition of apple juice eliminated the inhibitory effect partially in the case of *B. cinerea* and completely in the case of *P. expansum*. *Pseudomonas* sp. also inhibited the germination of *P. expansum* and *B. cinerea* spores. The inhibition was eliminated after the juice supplement in the case of *P. expansum* but not in the case of *B. cinerea*, in which a small increase in germination occurred only at a concentration of 10^5 spores per milliliter. Water controls for both fungi showed self-inhibition in spore germination at higher spore concentrations, but this was overcome with the addition of apple juice.

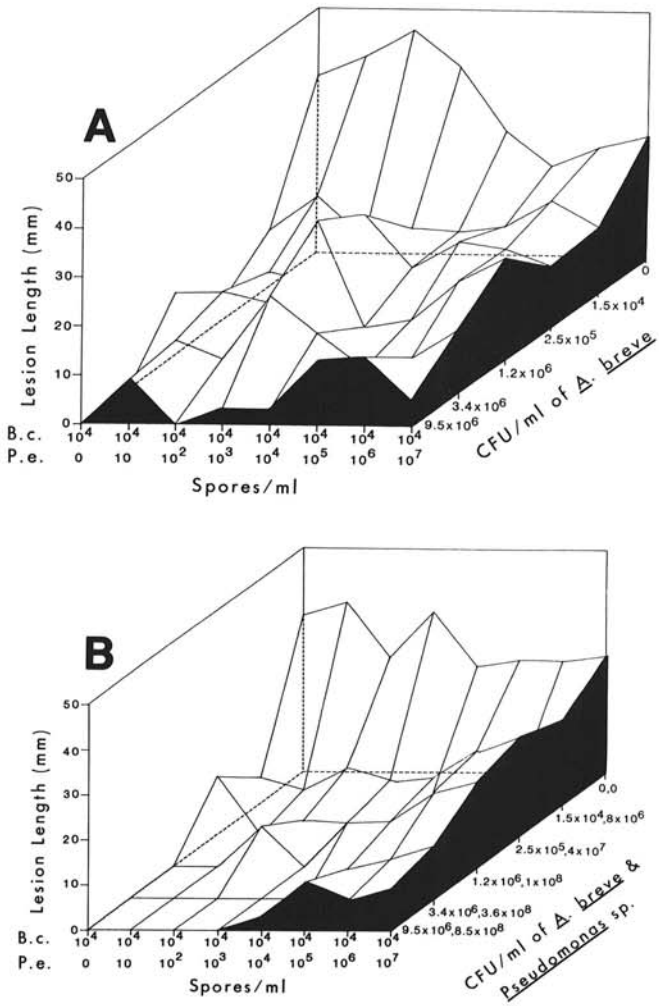

Fig. 3. Effect of the antagonist *Acremonium breve* (isolate LT-4-12) (**A**) and the antagonist mixture of *A. breve* and a *Pseudomonas* sp. (isolate L-22-64) (**B**) on lesion development resulting from inoculation of wounded Golden Delicious apples with mixtures of spores of *Botrytis cinerea* (B.c.) and *Penicillium expansum* (P.e.). CFU = colony-forming units.

Time-lapse experiments. The effectiveness of both antagonists increased with the amount of time between treatment of apples with the antagonist and inoculation with the pathogen (Figs. 1 and 2). In the case of *Pseudomonas* sp., this increase in effectiveness could be seen after an 8-hr delay in inoculation with *P. expansum*. Lesion diameter was proportionately smaller as the time between treatment with *Pseudomonas* sp. and inoculation with *P. expansum* increased. After 72 hr, no lesion development was observed on inoculated fruit. Protection with *A. breve* was rapid, since inoculations with *B. cinerea* 4 hr after antagonist application failed to develop into lesions.

Effect of antagonists on lesion development. Wounded apples inoculated with mixtures of *B. cinerea* and *P. expansum* spores produced a single wound lesion characteristic of both pathogens when the concentration of *P. expansum* spores in the mixtures was 10^2, 10^3, or 10^4 spores per milliliter and the concentration of *B. cinerea* remained constant at 10^4 spores per milliliter. Apples inoculated with spore mixtures containing lower or higher concentrations of *P. expansum* spores than those mentioned above developed lesions characteristic only of *B. cinerea* or *P. expansum*, respectively. The rate of decay of *B. cinerea* lesions is more rapid than that of *P. expansum* lesions, and this tendency can be seen in control treatments in both factorial experiments (Fig. 3). When the concentration of *B. cinerea* spores was higher than that of *P. expansum* spores in an inoculum spore mixture, the resulting lesions were large. However, if the concentration of *B. cinerea* spores was lower, smaller lesions appeared, characteristic of *P. expansum*.

When *A. breve* alone was challenged by mixtures of *B. cinerea* and *P. expansum* spores, protection occurred at the two highest concentrations of the antagonist (3.4×10^6 and 9.5×10^6 CFU/ml) challenged by mixtures containing, respectively, 0 and 10^2 *P. expansum* spores per milliliter (Fig. 3A). Lesions occurred in treatments with other antagonist concentrations; however, a trend toward reduced lesion size was visible, particularly where *B. cinerea* predominated in the mixture.

When both antagonists were used (Fig. 3B), and as the concentrations of the antagonists increased, there was a strong tendency toward smaller lesion size in inoculations with all spore mixtures of the pathogens. The antagonist mixtures with the two highest concentrations prevented lesion development on fruit inoculated with spore mixtures containing from 0 to 10^3 *P. expansum* spores per milliliter. This also occurred for the antagonist mixture with the third highest concentration, except in fruit inoculated with a mixture containing 10^2 *P. expansum* spores per milliliter, where a small lesion occurred. Also, no lesions developed on fruit treated with *A. breve* and *Pseudomonas* sp. at concentrations of 2.5×10^5 and 4×10^7 CFU/ml, respectively, and inoculated with a mixture containing 10 *P. expansum* spores per milliliter, and none developed on fruit treated with both antagonists at concentrations of 3.4×10^6 and 3.6×10^8 CFU/ml, respectively, and inoculated with the same spore mixture.

DISCUSSION

A. breve, found to be a very effective antagonist against *B. cinerea* in this study, showed a very rapid increase in effectiveness as time progressed from the moment of application on fully mature apples. This is in contrast to a gradual increase in the effectiveness of a *Pseudomonas* sp. antagonistic to *P. expansum*. The growth rate of *A. breve* in nutrient yeast-dextrose broth medium at 24 C was much slower, about half that of the *Pseudomonas* sp. As noted in this and in earlier work (16), apples produced a definite yellow ring around the wound treated with the *Pseudomonas* antagonist but a barely visible ring on wounded but untreated controls. Wound areas on fruit treated with *A. breve* showed distinct darkening. Studies are in progress to explain the modes of action of these antagonists.

The *Pseudomonas* sp. (15,16) and *A. breve* were very effective when tested separately against their respective pathogens. Since spores of both pathogens can occur in commercial drench tanks at the same time (25), simultaneous control is necessary. A comparison between the experiments with single antagonists and experiments with a mixture of the antagonists showed the effectiveness of the mixture in controlling both blue mold and gray mold fungi at the same time. The inoculum density of mixtures of *P. expansum* and *B. cinerea* spores was much greater than in the tests of a single pathogen versus a single antagonist.

Lesion development on apples depended on the quantitative relation between the antagonists and the pathogen spore concentration. Both diseases were controlled when apples were inoculated with pathogen spore concentrations corresponding to the highest recorded for commercial drench tanks (25). The concentration of the antagonists needed to obtain control were low enough to be considered for commercial use.

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