Postharvest Pathology and Mycotoxins

Postharvest Biological Control of Gray Mold of Apple by Cryptococcus laurentii

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ABSTRACT


Cryptococcus laurentii is a basidiomycetous yeast that occurs naturally on apple leaves, buds, and fruit. Puncture wounds in surface-disinfested cultivar Golden Delicious apple fruit were treated with phosphate buffer, cell suspensions of C. laurentii, or benomyl, then inoculated with 2 × 10⁶ conidia per milliliter of Botrytis cinerea and incubated 12 days at 5, 10, 15, or 20°C. Treatment of wounds with washed cells of C. laurentii at 10⁵–10⁶ cells per wound effectively reduced or prevented development of decay by B. cinerea at all temperatures compared with controls and was comparable in effectiveness to preinoculation application of benomyl at the postharvest label rate. Treatment of wounds with cell-free culture filtrates of C. laurentii were not effective in preventing decay and resulted in greater lesion diameters than in inoculated, buffer-treated wounds. As the interval between wounding and inoculation with B. cinerea increased from 0 to 72 hr, susceptibility of wounds to decay by B. cinerea decreased. Population densities of C. laurentii in wounds increased rapidly, even at 5°C, and were never associated with necrosis or discoloration of host tissue.

During recent years, a combination of biological and regulatory factors has increased interest and effort in the development of agricultural production, storage, and marketing systems that utilize reduced chemical inputs. Public awareness of pesticide residues on food products has increased, and government regulatory agencies have responded by reevaluating the use of many pesticides. Concomitant development of fungicide resistance by postharvest decay fungi (17,19) has stimulated efforts to develop alternate systems of disease control for agricultural products. Biological control of biotic postharvest diseases of stored food products is a logical area of study, because commodities such as fruits and vegetables can be a source of direct ingestion of fungicides. Although postharvest biological control systems for fruit have been actively investigated for the past decade with notable progress (8,10–12,15), there are no postharvest biocontrol systems in widespread commercial use.

A screening program was initiated in 1986 to isolate and identify from plant surfaces naturally occurring bacteria and yeasts having potential as biocontrol agents effective against postharvest disease fungi found in the Pacific Northwest, including Penicillium expansum Link ex Gray, Mucor piriformis Fischer, Alternaria alternata (Fr.) Keissler, Botrytis cinerea Pers. ex Pers., and

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**Penicillium viridicatum** Westling. One yeast strain isolated from apple (*Malus sylvestris* Mill.) leaf tissue was effective in inhibiting gray mold disease of cultivar Golden Delicious apple fruit caused by *B. cinerea* and subsequently was identified as the obligately aerobic, basidiozymecyte psychrophile *Cryptococcus laurentii* (Kufferath) Skinner. This paper reports the first use of *C. laurentii* for control of postharvest disease in apple fruit and the evaluation of physiological and ecological factors that may determine its commercial usefulness as a biological control agent.

**MATERIALS AND METHODS**

**Source of fruit.** Apple fruit (cultivar Golden Delicious) used for bioassays of potential biocontrol agents and subsequent tests were harvested from trees at the Washington State University Tree Fruit Research and Extension Center, Wenatchee, or were purchased from commercial growers. Fruit were harvested at commercial maturity for controlled-atmosphere storage (starch index >2, firmness = 80-84.5 N) and run through a small-scale packing line. Packing operations included: immersion in dump tank water (pH 6.6-6.9) containing 3-5 mg/L of chlorine dioxide (Rio Linda Chemical Co., Inc., Sacramento, CA) and 200 mg/L of Neodol 91-6, a non-ionic surfactant (North Coast Chemical Co., Inc., Seattle, WA); potable water rinse; hot drying at about 60 C; and sizing. Sized fruit were packed in new 35-L (1 bushel) cardboard boxes and placed until use in a cold (0 C) room or in a commercially operated room with controlled atmosphere (O2 = 1.5%, CO2 = 2%, 0 C).

**Isolation and screening of antagonists.** Orchards with minimal or no pesticide spray programs were preferentially, but not exclusively, selected for sampling. Leaves, flowers, stem tissue, and fruit collected from local apple, cherry, and pear orchards were placed in plastic bags on ice until returned to the laboratory. One-gram samples of leaf, stem, flowers, or fruit peels were placed in 10 ml of sterile deionized water, vortexed briefly, evacuated by water aspiration for 20 min, then vortexed again. Serial dilutions of the water were plated onto nutrient-yeast extract-dextrose agar (NYDA; 20 g of agar, 8 g of nutrient broth, 4 g of yeast extract, 1.5 g of dextrose, and 1 L of deionized water) plates and incubated at 26 C. Colonies that differed in appearance on isolation plates were streaked onto nutrient agar to ensure purity, then stored in sterile deionized water at 4 C until screened for biocontrol potential.

Antagonists were evaluated by a modification of the final screening method used by Janiszewicz (10). Bacteria and yeasts were grown for 48 hr in nutrient broth in an environmental shaker at 26 C and 200 rpm. Cultures then were diluted with sterile deionized water to give suspensions of 0.5 A950nm (about 10^7 colony-forming units [cfu]/ml). Golden Delicious apple fruit were puncture-wounded (about 6.0 mm deep and 2.4 mm in diameter) three times each with a sterile, blunted, no. 13 stainless steel cannula, and 10 μL of a bacterial or yeast suspension was introduced into the wounds on each of five apples. After 30-60 min, 10 μL of sporangiospore suspensions of *Mucor sp.*, or conidial suspensions of *A. alternata, P. expansum, P. viridicatum*, or *B. cinerea* at 2 x 10^7 spores per milliliter (as determined by hemacytometer) were introduced into the wounds of one fruit per pathogen. Positive controls consisted of wounded fruit that received pathogen spores but were not treated with potential antagonists. Wounded, water-treated fruit served as negative controls. Agent-alone controls were not used because of limitations on availability of fruit, and because production of lesions by a potential antagonist in screenings would itself indicate nontoxicity. Inoculated fruit were held on metal or plastic rings on wet paper towel in closed polyethylene bins at 18 C for 10 days, when lesion diameters were measured.

In all subsequent tests, fruit from controlled-atmosphere storage were sorted by YID (yellow color values) determined by three averaged readings per fruit on a diode-array, tristimulus colorimeter (Pacific Scientific Co., Silver Spring, MD). Fruit with YID values from 68-74 were equatorially wounded once as described, and 10 μL of 0.5 A950nm (about 1 x 10^7 cfu/ml) suspensions of washed cells of *C. laurentii* (RR87-108) and conidial suspensions of *B. cinerea* (RR88-89) at 2 x 10^7 conidia per milliliter were used. Both antagonist and pathogen suspensions were prepared in 0.05 M phosphate buffer, pH 7.0. After 12 days of incubation, the percentage of fruit that developed lesions was recorded or maximum lesion diameters were measured after each fruit was sliced longitudinally through the wound. Slicing fruit before measurement permitted detection of occasional internal lesions that were not apparent by superficial examination, and prevented recording false negative data. Wounds inoculated with *C. laurentii* alone never developed lesions, so agent-alone controls were not used.

**Relative efficacy of *C. laurentii* and benomyl for gray mold control.** Sets of 20 fruit were wounded, and either yeast suspensions in buffer (five fruit) or buffer alone (10 fruit) were introduced into the wounds. Five fruit per set were wounded, dipped in benomyl at the postharvest label rate (0.6 g/L, 50% a.i.), and allowed to air dry. After 30 min, conidia of *B. cinerea* were introduced into all wounds, except for the five that received only buffer and served as negative controls. Each treatment consisted of five single-fruit replicates in a randomized complete block design. Sets of 20 treated fruit were incubated 12 days in polyethylene moist chambers at 5, 10, 15, or 20 C, then evaluated for percentage of fruit with lesions.

**Effect of interval between wounding and inoculation with *B. cinerea*.** Incubation temperature and the interval between wounding and inoculation with *B. cinerea* were varied to determine the effect on biocontrol with *C. laurentii*. Fifty fruit were wounded, then each wound was either treated with buffer or inoculated with *C. laurentii*. Inoculations with *B. cinerea* were made at 0, 1, 24, 48, or 72 hr after wounding. After inoculation with *B. cinerea*, fruit were incubated in polyethylene moist chambers at 10 and 20 C. A single fruit served as one replicate in a randomized complete block design, with five replicates per set for each time and temperature combination.

**Population studies of *C. laurentii* in wounds.** The ability of cells of *C. laurentii* to survive and multiply in wounds was studied to determine if *C. laurentii* is an effective colonizer of apple fruit wounds. Fruit were wounded, inoculated with washed cells of *C. laurentii* in buffer, and held in moist chambers at 5, 10, 15, or 20 C. Viable cell concentrations of the inoculum were determined by dilution plating onto NYDA. Individual fruit wounds served as one replicate in a randomized complete block design, and three replicates were sampled at each sampling time and temperature. Wounds were sampled 0 and 4 hr after inoculation, then every 24 hr until 96 hr had elapsed. Samples were taken by excising the entire wound from the apple with an ethanol-flamed, 6.7 mm (internal diameter) cork borer, trimming the resultant tissue cylinder to about 8 mm in length, and placing the sample in 10 ml of sterile 0.05 M phosphate buffer, pH 7.0. The tissue plug then was macerated with a glass rod, vortexed, dilution-plated in triplicate on NYDA, and incubated at 26 C for 3 days when colonies were counted. Population densities of *C. laurentii* were expressed as log_{10} cfu per wound.

**Effect of temperature and concentration of *C. laurentii* on biocontrol efficacy.** To determine the effect of cell concentration of *C. laurentii* on biocontrol efficacy, 0.5 A950nm suspensions were serially diluted to 10^{-3}, then 10 μL of each dilution was introduced into each of 10 wounded fruit, replicated four times per yeast concentration and temperature in a randomized complete block design. Wounds in control fruit received sterile buffer instead of yeast suspensions. Wounds were immediately challenged by inoculation with conidia of *B. cinerea*, then the fruit were sealed in polyethylene-lined 35-L (1-bu) apple boxes and incubated at 5, 10, 15, or 20 C for 12 days.

**Mode of action.** To determine if extracellular metabolites in culture broth were responsible for antagonism of *C. laurentii* against *B. cinerea*, *C. laurentii* was grown for 48 hr as described, and cell and culture broth were separated by centrifugation. The supernatant was decanted, filtered through a 0.2-μm polycarbonate membrane filter, and reserved. Yeast pellets were washed three times with 45 ml of 0.05 M phosphate buffer, pH 7.0. Ten
micrometers of cell-free culture supernatant, suspensions of washed cells of *C. laurentii* in buffer, or buffer was introduced into each of five apple wounds in a randomized complete block design. After 30 min, conidia of *B. cinerea* were introduced into each wound, and the fruits were incubated for 12 days in polyethylene moist chambers at 20°C.

**Statistical analysis.** All experiments were repeated at least once. Homogeneity of variance for different trials of each experiment was evaluated by Hartley's F-Max test at \( P = 0.05 \) of the arcsine-square root transformation of the percentage of fruit that developed lesions. If variances did not differ significantly, data from separate trials were pooled, except for the effect of yeast concentration and temperature on the biocontrol efficacy experiment. Pooled data was analyzed by analysis of variance, then means were separated by Waller-Duncan K-ratio \( t \) test (\( \alpha = 0.05 \)), or regression analysis in SAS (18) was performed as appropriate.

**RESULTS**

Variances of trials from all experiments did not differ significantly, so data from all trials were pooled for each experiment before analysis. The larger sample size for the effect of concentration and temperature on biocontrol efficacy experiment precluded the need to pool data, so trials were analyzed separately.

**Screening of antagonists.** Of 125 isolates of bacteria and yeasts assayed for antagonistic activity, two yeast and nine bacterial isolates caused a 47–98% inhibition in lesion development compared to appropriate controls. A yeast isolate (RR87-108) from an apple leaf that exhibited the greatest inhibition of lesion development by *B. cinerea* in initial screenings was identified by the Centraalbureau voor Schimmelmilities, Baarn, The Netherlands, as *C. laurentii*, and was selected for further study.

**Relative efficacy of *C. laurentii* and benomyl for gray mold control.** A positive relationship between temperature and the percentage of fruit that developed lesions was observed for fruit treated with *C. laurentii* \( (r^2 = 0.89) \) and for the inoculated control \( (r^2 = 0.97) \), but was absent in the benomyl-treated fruit \( (r^2 = 0.10, \text{Table 1}) \). Uninoculated control fruit did not develop lesions at any temperature. The percentage of wounds treated with *C. laurentii* that developed lesions did not differ from benomyl-treated wounds at 5, 15, and 20°C, but was significantly less than the benomyl treatment at 10°C. The percentage of fruit treated with *C. laurentii* that developed lesions was significantly less than in the inoculated control buffer at all incubation temperatures. Similar reductions in percentage of fruit with lesions were observed in benomyl-treated fruit except at 10°C, where the percentage of fruit with lesions did not differ from the inoculated control.

**Effect of interval between wounding and inoculation with *B. cinerea***. The percentages of fruit that developed lesions after inoculation with *B. cinerea* 0, 1, 24, and 72 hr after wounding were 80.0 ± 24.5, 50.0 ± 10.0, 25.0 ± 8.7, 5.0 ± 8.7, and 0.0 ± 0.0, respectively. The relationship of the percentage of fruit that developed lesions \( (Y) \) and the interval between wounding and inoculation \( (X) \) was described by \( Y = 60.93 + 0.95X \), \( r^2 = 0.72 \). Wounds treated with *C. laurentii* at 10⁶ cfu per wound did not develop lesions after inoculation with *B. cinerea* after any interval between wounding and inoculation with *B. cinerea*.

**Population dynamics in wounds.** Population densities of *C. laurentii* in wounds increased with incubation time at all temperatures (Fig. 1). Although growth was more rapid at warmer temperatures, population densities of *C. laurentii* in wounds increased by approximately 2 log units over the initial population levels at all temperatures by 96 hr after inoculation. Regression analysis showed the relationship of the log of the mean number of cells per wound \( (Y) \) and hours of incubation \( (X) \) was: at 5°C, \( \log Y = 4.62 + 0.0204(X) \), \( r^2 = 0.99 \); at 10°C, \( \log Y = 4.77 + 0.0246(X) + 0.212(X^2) \), \( r^2 = 0.98 \); at 15°C, \( \log Y = 4.94 + 0.0234(X) + 0.409(X^2) \), \( r^2 = 1.00 \); at 20°C, \( \log Y = 5.08 + 0.0232(X) + 0.554(X^2) \), \( r^2 = 0.97 \). Repeated trials gave similar results.

**TABLE 1.** Control Botrytis rot after 12 days of incubation at different temperatures following treatment of wounds of apple fruit (cultivar Golden Delicious) with cell suspensions of *Cryptococcus laurentii*, benomyl suspensions, or buffer

<table>
<thead>
<tr>
<th>Temp (C)</th>
<th>C. laurentii</th>
<th>Benomyl</th>
<th>Inoculated buffer control</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.0 ± 0.0 d</td>
<td>0.0 ± 0.0 d</td>
<td>30.0 ± 30.0 c</td>
</tr>
<tr>
<td>10</td>
<td>0.0 ± 0.0 d</td>
<td>40.0 ± 0.0 bc</td>
<td>40.0 ± 0.0 bc</td>
</tr>
<tr>
<td>15</td>
<td>20.0 ± 20.0 d</td>
<td>20.0 ± 20.0 d</td>
<td>70.0 ± 10.0 b</td>
</tr>
<tr>
<td>20</td>
<td>30.0 ± 10.0 c</td>
<td>40.0 ± 0.0 bc</td>
<td>100.0 ± 0.0 a</td>
</tr>
<tr>
<td></td>
<td>0.89</td>
<td>0.10</td>
<td>0.97</td>
</tr>
</tbody>
</table>

7 Means are averages of two trials ± the standard deviation. Values followed by the same letter are not significantly different at \( P = 0.05 \) according to analysis by Waller-Duncan K-ratio \( t \) test of the arcsine transformation of the percentage of fruit that developed lesions.

8 Values are from linear regression analysis of the arcsine-square root transformation of the percentage of fruit that developed lesions.

**TABLE 2.** Effect of temperature and concentration of *Cryptococcus laurentii* on lesion development by *Botrytis cinerea* in cultivar Golden Delicious apple fruit after incubation for 12 days

<table>
<thead>
<tr>
<th>Temperature (C)</th>
<th>Log₁₀ C. laurentii per wound</th>
<th>Percentage of fruit with lesions*</th>
<th>( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.00 ± 0.00</td>
<td>5.0 ± 5.0</td>
<td>0.83</td>
</tr>
<tr>
<td>10</td>
<td>12.5 ± 1.0</td>
<td>22.5 ± 2.5</td>
<td>1.00</td>
</tr>
<tr>
<td>15</td>
<td>30.0 ± 2.0</td>
<td>42.5 ± 0.0</td>
<td>0.98</td>
</tr>
<tr>
<td>20</td>
<td>50.0 ± 5.0</td>
<td>62.5 ± 5.0</td>
<td>0.99</td>
</tr>
<tr>
<td>2.03</td>
<td>105.0 ± 0.0</td>
<td>75.0 ± 10.0</td>
<td>0.98</td>
</tr>
</tbody>
</table>

*Percentages are actual values; \( r^2 \) values are from linear regression analysis of the arcsine-square root transformation of the percentage of fruit that developed lesions.
Effect of temperature and concentration of C. laurentii on biocontrol efficacy. As the incubation temperature increased, the concentration of suspensions of C. laurentii necessary to prevent lesion development increased (Table 2). The percentage of fruit that developed lesions increased as either the incubation temperature increased at each yeast concentration or the concentration of C. laurentii decreased at each temperature. The relationship of the percentage of fruit that developed lesions (Y) and the concentration of C. laurentii applied to the wounds (X) and incubated at different temperatures was described by the equations: at 5 °C, Y = 10.0 - 2.51 × 10^(-X) + 2.0 × 10^(-2X), r^2 = 0.84; at 10 °C, Y = 6.40 - 2.43 × 10^(-X) + 2.0 × 10^(-2X), r^2 = 0.79; at 15 °C, Y = 78.8 - 2.74 × 10^(-X) + 2.0 × 10^(-2X), r^2 = 0.77, and at 20 °C, Y = 91.1 - 2.81 × 10^(-X) + 2.0 × 10^(-2X), r^2 = 0.93. The relationship of the percentage of fruits that developed lesions (Y) to incubation temperature (X) at differing wound densities of C. laurentii was: controls, Y = -16.9 + 14.3X - 0.425X^2, r^2 = 0.92; 1.11 × 10^4 cfu/wound, Y = 1.9 + 11.4X - 0.325X^2, r^2 = 0.80; 1.11 × 10^5 cfu/wound, Y = 7.5 + 4.6X, r^2 = 0.87; 1.11 × 10^6 cfu/wound, Y = -5.0 + 4.3X, r^2 = 0.84, and 1.11 × 10^7 cfu/wound, Y = -20.0 + 3.05X, r^2 = 0.75.

Mode of action. Wounds treated with triple-washed cells of C. laurentii did not develop lesions after inoculation with B. cinerea. Wounds treated with cell-free culture broth of C. laurentii developed rapidly expanding lesions (62.5 ± 11.9 mm in diameter) after inoculation with B. cinerea. Lesions that developed from wounds treated only with buffer before inoculation with B. cinerea were significantly (P = 0.05) smaller (33.8 ± 20.8 mm in diameter) than those from wounds treated with culture broth. All culture broth- or buffer-treated fruit developed lesions after inoculation with B. cinerea.

DISCUSSION

When wounds on Golden Delicious apple fruit were pretreated with C. laurentii, development of lesions of B. cinerea was effectively reduced or prevented. No necrosis or darkening of fruit tissue was associated with any concentration of C. laurentii in wounds. The level of control of lesion development of B. cinerea by C. laurentii compared very favorably with levels observed in benomyl-treated fruit and was accomplished without apparent antibiotic by extracellular metabolites characteristic of certain postharvest biocontrol agents (8,12,14,15). Fokkema et al. (5,7) demonstrated that epiphytic populations of Cryptococcus spp. on rye and wheat leaves were not affected by application of benomyl. Hislop and Cox (9) showed that phylloplane populations of “white yeasts,” including Cryptococcus spp., were essentially unaffected by application of captan to apple leaves. Growth of C. laurentii (RR87-108) was not inhibited in preliminary tests when benomyl-impregnated filter paper disks were placed on agar medium on which C. laurentii was grown (unpublished data). Simultaneous application of benzimidazole fungicides and C. laurentii is, therefore, a possibility, and may prevent or slow the development of benomyl-resistant strains of B. cinerea.

Competition for carbon or nitrogen sources between cells of C. laurentii and B. cinerea could be responsible for control, as conidial germination of some strains of B. cinerea are known to be affected by the carboxylate and nitrogen status of their immediate environment (2,3). The ability of C. laurentii to multiply rapidly in wounds, and, perhaps, preemptively utilize available nutrients, may facilitate biocontrol by nutrient competition. The quadratic growth curve of C. laurentii in apple wounds at 20 °C (Fig. 1) suggests that cells multiply rapidly, then decline in a pattern typical of microbial populations whose nutrients become limiting. The demonstrated effect of incubation temperature and initial concentration of C. laurentii on biocontrol efficacy and the effect of temperature on population densities of C. laurentii at the wound site provides presumptive evidence that biocontrol is achieved when actively multiplying populations of C. laurentii are present in wounds. C. laurentii is nonfermentative and, since tests were conducted under aerobic conditions, production of ethanol by C. laurentii in wounds was unlikely. Increased lesion diameters in culture broth-treated wounds compared with pathogen control wounds probably resulted from accelerated growth of B. cinerea caused by residual nutrients in the culture broth. Although antifungal compounds were presumed absent from the cell-free culture broth, additional studies are needed to determine if antifungal metabolites are produced by C. laurentii in situ in the wound site.

A wound healing response in fruit has been demonstrated (13) or suggested (20) in other studies, but usually has been observed at higher temperatures and has developed much more slowly than the development of resistance to infection after wounding observed during this study. Wounds in some less susceptible to infection by B. cinerea, as reflected in the lower degree of inoculation in fruit that developed lesions, as the interval between wounding and inoculation increased. Wound healing does not appear to be a significant factor affecting control of B. cinerea by C. laurentii, however, because the yeast gave effective control in fresh (0 hr) wounds and in older (72 hr) wounds. However, prolonged delays between challenge of wounds by a pathogen after treatment with an antagonist may introduce a favorable bias towards interpretation of an antagonist’s effectiveness. Although higher percentages of fruit sealed in plastic bags developed lesions (Table 2) than those held in the polyethylene bins (Table 1), desiccation of the wounds is an unlikely explanation for this response because all fruit held in the polyethylene bins were incubated over free water in moist chambers. The mechanism for this apparent resistance to infection is unknown. No microorganisms other than C. laurentii colonized the wounds during the incubation period. Cryptococcus spp. have been identified as common components of phylloplane mycflora of apples and other plants (1,4,6,7,9), and have been implicated in biological control of several foliar diseases, including leaf spots of wheat and rye caused by Septoria nodorum and Cochliobolus sativus (5,7), Botrytis blight of roses (16), and leaf spot of maize caused by Colletotrichum graminicola (21). Although Cryptococcus spp. reduced disease severity or incidence from 26-50% as foliar antagonists of these diseases, reduction in percentage of fruit that developed lesions by postharvest application of C. laurentii to wounded apple fruit was much greater (55-100%) in experiments reported herein. The rapid growth of C. laurentii in wounds over a wide range of temperatures, especially cold temperatures, and its ability to control lesion development by Botrytis provides evidence that C. laurentii is well adapted to the wound environment in apple fruit and has considerable potential as a biological control agent. Although the growth of C. laurentii in wounds was more rapid at warmer temperatures, its ability to multiply in wounds at 5 °C is significant because of the low temperature at which fruit commonly are stored. It is probable that initial wound populations of C. laurentii that are suboptimal for immediate control of B. cinerea could increase to effective levels within a few hours or days. As a naturally occurring member of resident mycoflora of apple buds, leaves, and fruit (1,4,9), C. laurentii would be expected to survive and flourish on apple fruit after application. Although no data are available on survival and growth on intact apple fruit surfaces in storage, populations of C. laurentii prosper in fruit wounds under aerobic conditions over a wide range of temperatures. The psychrophilic nature of C. laurentii may give it a considerable competitive advantage over other wound inhabitants not well adapted to growth at low temperatures. Evaluations of growth and survival of C. laurentii on intact fruit and under less aerobic conditions, such as those found in controlled atmosphere storage, would be useful to further characterize the potential for C. laurentii as a commercially useful biological control agent.

LITERATURE CITED