Effect of Inoculum Concentration and Salt Solutions on Biological Control of Postharvest Diseases of Apple with *Candida* sp.

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This research was supported by Grant No. US-1378-87 from BARD, the United States-Israel Binational Agricultural Development Fund.

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We gratefully acknowledge the technical assistance of Brian E. Otto and Wilbur Hershberger.

Accepted for publication 18 October 1989 (submitted for electronic processing).

ABSTRACT

McLaughlin, R. J., Wisniewski, M. E., Wilson, C. L., and Chalutz, E. 1990. Effect of inoculum concentration and salt solutions on biological control of postharvest diseases of apple with *Candida* sp. Phytopathology 80:456-461.

Two osmotolerant strains (87 and 101) of the yeast, *Candida* sp., were tested for ability to reduce lesion development in Golden Delicious apple after challenge with 10^5 or 10^4 conidia per milliliter of the postharvest pathogens *Botrytis cinerea* and *Penicillium expansum*. Lesion size and frequency of Botrytis rot were significantly less in fruit pretreated with aqueous suspensions of strains 87 and 101 (10^7 and 10^8 colony-forming units [cfu]/ml) as compared with controls pretreated with water. Significant, but marginal, reduction of Penicillium rot was observed in treatments with these yeasts at 10^8 cfu/ml. Biological control of Botrytis rot was enhanced when wounds were treated with strain 87 at 10^7 cfu/ml in 2% (w/v) aqueous solutions of $CaCl_2$, KCl, and $CaCO_3$, as compared with aqueous suspensions of the strain alone. Salt solutions applied to wounds without yeast cells did not reduce rot. The ability of the salt solutions to enhance control with yeasts was not related to the osmotic potential of the solutions. Calcium chloride was the most effective salt. Yeast strains differed slightly in their response to calcium chloride; a 1% concentration enhanced biocontrol with strain 87, whereas a 2% concentration was necessary to enhance control with strain 101. Calcium chloride facilitated control of Botrytis rot with yeast populations as low as 10^6 cfu/ml. Decay due to *Penicillium expansum* also was significantly reduced when fruit were treated with strains 87 and 101 in the presence of calcium chloride.

Additional keywords: blue mold, *Debaryomyces*, gray mold, *Malus domestica, Malus sylvestris*.

Postharvest losses in apple due to *Botrytis cinerea* Pers. ex Fr. (gray mold) and *Penicillium expansum* Link ex Thom. (blue mold) have been reduced by addition of various fungicidal compounds to flotation dump-tank water and by application of benomyl to fruit before cold storage (32). Benomyl applications to fruit, however, promote the selection of fungicide-tolerant strains of these pathogens (5,30). Also, reduced reliance on fungicide treatments after harvest may be necessary because a number of commonly used fungicides are under review by the Environmental Protection Agency due to health risk concerns (1).

As an alternative to fungicides, treatment of fruit with microbial agents has shown promise for the control of several postharvest fruit diseases. Fungi and bacteria are reported to effectively reduce postharvest diseases of peach (27,28,37), apple (19–21), pear (21), citrus (9,18,31,34), cherry (33), and grape (17). Specifically, the effective biocontrol agents for apple (*Malus sylvestris* Mill.) include the filamentous fungus *Acremonium brevae* (20), the bacterium *Pseudomonas cepacia* (21), and yeasts (19,36).

Two strains (87 and 101) of the yeast *Candida* sp. (previously identified as *Debaryomyces hansenii* Lodder et Krejer-Van Rij) have been found to effectively reduce fungal postharvest diseases of citrus (9,34). These strains were effective when applied to wounds at high concentrations (approximately 10^9 colony-forming units [cfu]/ml), but they did not inhibit mycelial growth or spore germination of the pathogen in vitro. In a preliminary report, Wisniewski et al (36) showed that one of the strains (87 = US-7) was effective for reducing Botrytis rot in apple.

The biocontrol capabilities of these yeast strains have not been fully characterized. To effectively utilize yeasts as a postharvest biocontrol method, detailed information is needed on the conditions that influence control and the mode of action by which the strains confer control.

The first objective of this research was to determine the ability of these strains to control postharvest rots of apple caused by *B. cinerea* and *P. expansum* and to characterize biocontrol activity of these strains as it is affected by inoculum concentration. Secondly, because preliminary tests showed that these strains of *Candida* were osmophilic, inorganic salts were evaluated for their potential as adjuvants for improving the efficacy of biocontrol. A preliminary report has been published (24).

MATERIALS AND METHODS

Yeast and pathogen strains. Yeast strains 87 and 101 were obtained from the surface of lemon fruit as previously reported (34). These strains do not exhibit antibiosis toward *B. cinerea* or *P. expansum* in vitro (34). These strains are osmotolerant, as demonstrated by their ability to grow on 50% glucose agar and 10% sodium chloride-5% glucose broth (23). Cultures were stored routinely on silica gel and recovered by plating on nutrient-yeast dextrose agar (NYDA, 0.8% nutrient broth, 0.5% yeast extract, 1.0% dextrose, and 1.5% agar, pH 7.0), with subsequent incubation at 27 C for 48 hr.

Isolates of the postharvest pathogens *B. cinerea* and *P. expansum* were obtained from diseased apples at the Appalachian Fruit Research Station, Kearneysville, WV, and routinely stored as lyophilized cultures.

Culture conditions and yeast preparation. Yeast strains were streaked on NYDA plates and incubated for 48 hr at 27 C. Nutrient-yeast dextrose broth (NYDA minus agar) cultures, 50 ml in a 125-ml Erlenmeyer flask, were started with approximately 10^8 cfu of yeast. Cultures were incubated at 24 C on a

rotary shaker set at 200 rpm. After cell growth reached late log phase (20 hr), cells were pelleted by centrifugation at 3,000 *g* for 15 min. Culture supernatant was discarded, and cells were resuspended in five volumes of sterile distilled water. Cells were repelleted and resuspended in sterile distilled water. Cell numbers were adjusted with sterile distilled water to the desired concentrations based on optical density at 610 nm (34).

Biocontrol ability of the yeast isolates. Fruit of Golden Delicious apple, harvested at the Appalachian Fruit Research Station, were used throughout the tests. Apples were stored at 2 C for 3 mo or less before use in biocontrol tests. Fruit were surface-disinfested by emersion in 10% commercial bleach for 5 min, air dried, and placed in plastic trays (34). Single wounds (5 mm deep by 3 mm wide) were made in each fruit as previously described (34). Immediately after wounding, 50 μl of an aqueous yeast (strains 87 and 101) suspension, at 0, 10^6, 10^7, or 10^8 cfu/ml, was pipetted into each wound site. The yeast suspensions were left to dry in the wound site for 2 hr at ambient temperatures (24–26 C) before challenge with pathogens.

Treated wounds were challenge-inoculated with conidia of either *B. cinerea* or *P. expansum*. Conidia of *B. cinerea* were obtained from 2-wk-old potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI) cultures incubated at 24 C under constant fluorescent light (24.0 ± 2.0 μE m^{-2} sec^{-1}). Conidia of *P. expansum* were obtained from 2-wk-old PDA (Difco) cultures incubated at 27 C in the dark. Conidial suspensions of the pathogens were prepared by flooding the culture dishes with 10 ml of sterile distilled water containing 0.1% Tween 20. Spore counts were determined with a hemacytometer, and concentrations were adjusted with sterile distilled water to obtain 10^5 conidia per milliliter for *B. cinerea* or 10^4 conidia per milliliter for *P. expansum*. Twenty microliters of spore suspension was applied to each wound site 2 hr after yeast application. There were 8–10 replicates per treatment with complete randomization. Fruit were incubated at 24 C in a moisture chamber after inoculation (34). Lesion diameters at the wound site were measured at 10 days after challenge. The test was repeated four times.

Effect of pathogen and yeast cell concentration on biocontrol effectiveness. Challenge spore levels of *B. cinerea* and treatment cell concentrations of strain 87 were varied. Fruit wounds were treated with 50 μl of 10^3, 10^5, 10^7, or 10^8 cfu/ml of washed cells of strain 87. Fruit were challenged 2 hr later with 20 μl of *B. cinerea* at 10^3, 10^4, 10^5, or 10^6 conidia per milliliter. There were 10 replicates per treatment with complete randomization. Fruit were incubated at 24 C, and lesion diameters were measured at 5, 7, 11, and 13 days after challenge. The test was repeated three times.

Effect of inorganic salts on biocontrol by yeast isolates. Cells from 20-hr-old cultures of strain 87 were pelleted by centrifugation and washed once in sterile distilled water. Pellets were resuspended in sterile, unbuffered salt solutions of $FeSO_4 \cdot 7H_2O$ (pH 5.7), KCl (pH 7.6), $MgCl_2 \cdot 6H_2O$ (pH 7.3), $MnCl_2 \cdot 4H_2O$ (pH 6.6), $CaCO_3$ (pH 8.8), $CaCl_2 \cdot 2H_2O$ (pH 6.9), NaCl (pH 7.0), or in sterile distilled water. All salt solutions were made 2% (w/v) except for $FeSO_4$, which was a 5 mM solution. Wounds were treated with 50 μl of yeast suspension at 10^7 cfu/ml, plus or minus salts, and challenged with 20 μl of a spore suspension of *B. cinerea* at 10^5 conidia per milliliter. There were 8–10 replicates (single fruit) per treatment with complete randomization. Fruit were incubated at 24 C, and percent infection was determined for each treatment at 14 days after challenge. The test was repeated four times.

Additional tests with strains 87 and 101 were done to determine the effect of different calcium chloride treatment levels on biocontrol. Fruit wounds were treated with 50 μl of yeast suspensions at 10^6, 10^7, and 10^8 cfu/ml in 0, 1, and 2% solutions of $CaCl_2$. Two hours later, wounds were challenged with 20 μl of either 10^5 (*B. cinerea*) or 10^4 (*P. expansum*) conidia per milliliter. Fruit were incubated at 24 C, and lesion diameter was measured at 10 days after challenge. There were two tests of five replicates per treatment. Replicates for each treatment were completely randomized in each test.

Statistical analyses. Factorial analyses of variance for the comparison of yeast isolates for biocontrol, the effect of inorganic salts, and the effect of $CaCl_2$ were performed with the general linear models procedure of SAS (2). Mean separations for the isolate comparison and $CaCl_2$ tests were calculated by the least significant difference (LSD; $P \leq 0.05$) method.

RESULTS

Comparison of strain effectiveness for the control of Botrytis and Penicillium rots. At 10 days after treatment and inoculation, control of *B. cinerea* was minimal for both yeast strains when applied to wounds at 10^6 cfu/ml (Fig. 1A). However, as yeast concentrations increased to 10^7 and 10^8 cfu/ml, strains 87 and 101 significantly inhibited lesion development. Analysis of variance showed a significant effect of inoculum concentration on lesion development. In fruit treated with strains 87 and 101 at 10^8 cfu/ml, only 20% of the inoculated fruit developed lesions.

The yeasts were less effective for the control of fruit rot caused by *P. expansum* (Fig. 1B). When strains 87 and 101 were applied to wounds at 10^8 cfu/ml, significantly smaller lesions developed; however, 100% of the inoculated fruit were infected at 10 days. Analysis of variance did not show a significant effect of inoculum concentration.

Effect of pathogen and yeast cell concentration on biocontrol effectiveness. Biocontrol of Botrytis rot with strain 87 was most effective at the application rate of 10^8 cfu/ml at all observed dates, and a typical dose-response relationship was observed (Fig. 2). Increased control was associated with increased yeast cell populations and decreasing challenge spore levels. Yeast cell concentrations of 10^3 and 10^5 cfu/ml were not effective in reducing disease progress at challenge levels greater than or equal to 10^4 conidia per milliliter. At challenge levels of 10^5 and 10^6 conidia per milliliter, infection and lesion development were merely delayed by treatment with 10^8 cfu/ml of strain 87, as 100% of the fruit were infected at 13 days after inoculation.

Effect of inorganic salts on biocontrol. Fruit treated with strain 87 minus salts had 59.5% infection, whereas fruit treated with water alone all became infected (Table 1). Fruit infection was

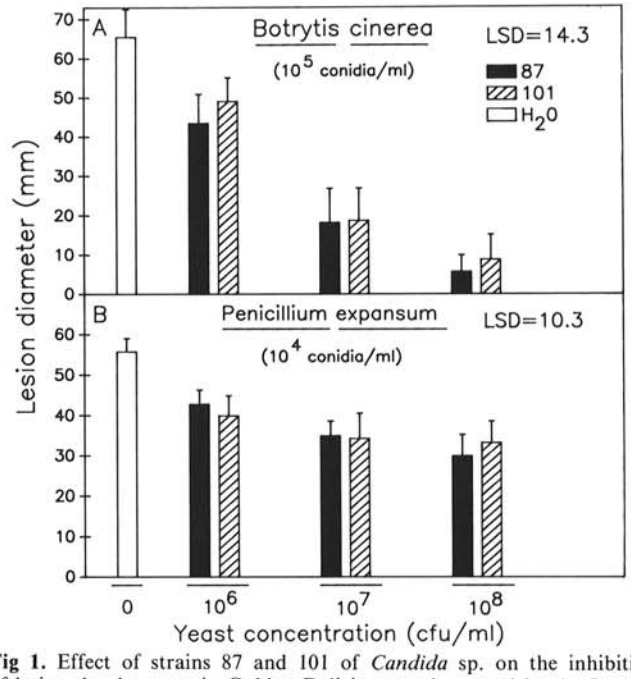

Fig 1. Effect of strains 87 and 101 of *Candida* sp. on the inhibition of lesion development in Golden Delicious apple caused by **A,** *Botrytis cinerea* and, **B,** *Penicillium expansum*. Data points represent average lesion diameter (mm) measured at 10 days after inoculation. Bars indicate standard errors of the mean. LSD = least significant difference ($P \leq 0.05$).

inhibited significantly when yeast cell suspensions were applied in 2% (w/v) solutions of $CaCl_2$, KCl, or $CaCO_3$. Fruit treated with strain 87 in $CaCl_2$ solution had the lowest infection (3.3%) of all treatments. There was no relationship between the osmotic potential of the salt solutions and the ability to facilitate control in the absence or presence of yeasts. With the exception of $CaCO_3$, inorganic salts in the absence of yeast cells did not significantly decrease fruit infection when compared with the water control. Percent infection in fruit treated with $CaCO_3$ (66.3%), however, did not differ significantly from treatments with water suspensions of strain 87.

Strains 87 and 101 were used in further tests with solutions of $CaCl_2$. Fruit were challenged with conidia of either *B. cinerea* or *P. expansum*, and lesion diameters were measured 10 days later. Analysis of variance of each test showed a significant interaction between strains, strain concentration, and $CaCl_2$ concentrations (Table 2). Significant effects of strain concentration and $CaCl_2$ were apparent in both tests, and strain differences were apparent in the test where conidia of *P. expansum* were used for challenge.

The data for these tests are shown in Figure 3 to demonstrate how the data may be interpreted. As expected, addition of $CaCl_2$ to yeast cell suspensions resulted in smaller average lesion diameters in fruit challenged with *B. cinerea* (Fig. 3A). Average lesion diameter was significantly less in fruit wounds treated with 1 and 2% $CaCl_2$ suspensions of strain 87 at 10^6, 10^7, and 10^8 cfu/ml, as compared with treatments with $CaCl_2$ alone and treatments with aqueous suspensions of yeast cells at 10^6 and 10^7 cfu/ml alone (= 0% $CaCl_2$). Treatment with 2% $CaCl_2$ did not significantly enhance control as compared with the 1% $CaCl_2$ treatments, regardless of yeast cell concentration. Strain 101 responded differently to increased $CaCl_2$ concentration. The best control was achieved with 2% $CaCl_2$ suspensions of this strain. As with strain 87, $CaCl_2$ enhanced control with strain 101 at the lowest cell concentrations of 10^6 and 10^7 cfu/ml. Control with strain 101 at 10^6 and 10^7 cfu/ml, in the absence of $CaCl_2$, was not significantly different from that in controls treated with $CaCl_2$ or water alone (= 0% $CaCl_2$).

Calcium chloride facilitated control of Penicillium rot with strains 87 and 101 (Fig. 3B). Cell suspensions of strain 87 at 10^7 and 10^8 cfu/ml were effective in inhibiting lesion development when applied in the presence of 1 and 2% $CaCl_2$. Strain 101 gave the best control at 10^8 cfu/ml with 1 and 2% $CaCl_2$; at 10^7 cfu/ml, significant enhancement of control was observed at 2% $CaCl_2$ only. At 0% $CaCl_2$, lesion development was not significantly inhibited with strain 87 at either cell concentration or with strain 101 at 10^7 cfu/ml.

DISCUSSION

In initial screening tests, we have shown that *Candida* sp., strains 87 and 101, reduced postharvest decay of apple caused by *B. cinerea* and *P. expansum*. Control of Penicillium rot by these strains, however, was marginal. In both cases, rot was best controlled at yeast cell population densities of 10^8 cfu/ml. Control of Botrytis rot with strain 87 was demonstrated to be a function of challenge spore concentration and yeast cell concentration. Control of rot was temporal, since, at low challenge levels of 10^3 conidia per milliliter, disease was eventually observed in fruit treated at the highest yeast cell population of 10^8 cfu/ml.

Calcium salts dramatically improved the efficacy of the biocontrol strains by lowering the number of yeast cells (10^6 to

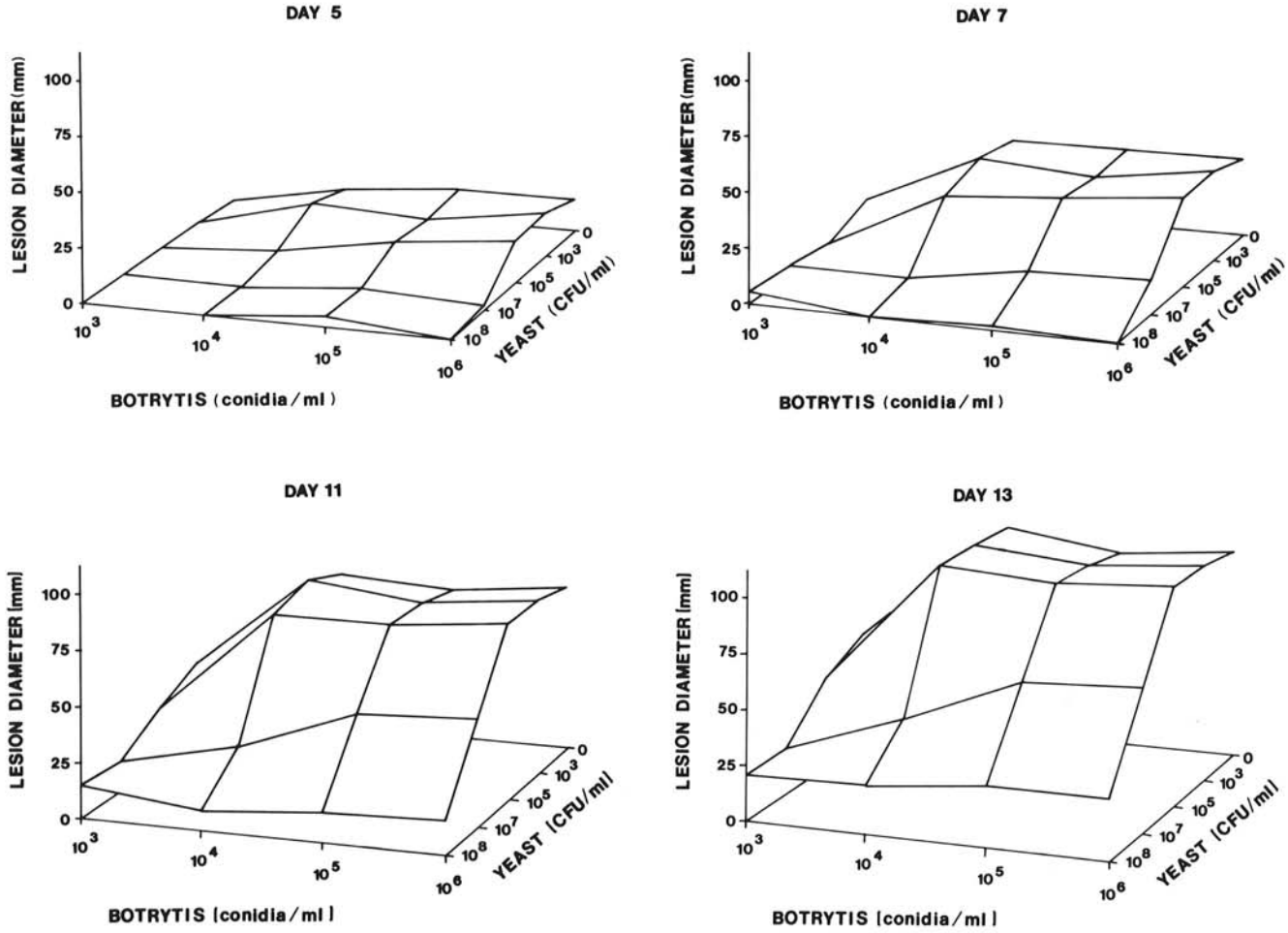

Fig. 2. Effect of cell concentration of yeast strain 87 and challenge concentration of conidia of *Botrytis cinerea* on lesion development in Golden Delicious apple. Data points represent average lesion diameter (mm) at 5, 7, 11, and 13 days after challenge inoculation.

10^7 cfu/ml) needed to elicit control of Botrytis rot. An additional benefit of calcium chloride in yeast cell suspensions was improved control of Penicillium rot, control that was marginal in treatments with strains 87 and 101 in the absence of salts. Application of calcium chloride/yeast suspensions as postharvest treatments of fruit, therefore, has potential for reducing the most common postharvest diseases in apple. In addition, this treatment would be effective at spore levels of *B. cinerea* and *Penicillium* sp. (to 10^4 spores per milliliter) that commonly have been observed in dump tanks (6,32).

Application of these yeasts in the presence of salts was hypothesized to have potential for increasing biocontrol effectiveness

TABLE 1. Effect of inorganic salts on biocontrol of Botrytis rot of Golden Delicious apple with strain 87 of *Candida* sp.[a]

Inorganic salt	Osmotic potential (10^5 Pascal)[c]	Percent infection[b]	
		No yeast	Strain 87
$CaCl_2$	−10.4	95.0 (± 5.0)	3.3 (± 3.2)*
$CaCO_3$	− 9.9	66.3 (± 14.6)	17.5 (± 14.4)*
$FeSO_4$	ND[d]	96.9 (± 3.1)	42.5 (± 14.4)
KCl	−13.4	100.0 (± 0.0)	31.3 (± 12.6)*
$MgCl_2$	− 7.4	100.0 (± 0.0)	40.0 (± 21.6)
$MnCl_2$	− 7.4	100.0 (± 0.0)	100.0 (± 0.0)
NaCl	−16.8	100.0 (± 0.0)	73.8 (± 15.5)
H_2O	0.0	100.0 (± 0.0)	59.5 (± 14.4)

[a] Strain 87 was applied in 50-μl aliquots to artificial wounds at 10^7 colony-forming units (cfu)/ml in sterile distilled water, or in 2% (w/v) or 5 mM ($FeSO_4$ only) salt solutions. Fruit were challenged with 20 μl of a conidial suspension of *Botrytis cinerea* at 10^5 conidia/ml.

[b] Average percent fruit infection of four trials recorded 10 days after inoculation. Values in parentheses are standard errors of the mean. Asterisk (*) indicates that means are significantly ($P \leq 0.05$) different from the water control (minus yeast) according to the least significant difference method. Analysis of variance performed on arcsin square root-transformed data.

[c] Osmotic potentials of the salt solutions were calculated from the molar concentrations by a modified van't Hoff equation (26).

[d] ND = not determined.

TABLE 2. Factorial analyses of variance on the effect of yeast strain, yeast concentration, and calcium chloride on the reduction of Botrytis and Penicillium rot in Golden Delicious apple[a]

Sources	Botrytis rot		Penicillium rot	
	df[b]	MS[c]	df	MS
Strain (S)	1	1,283	2	454*
Concentration (C)	2	16,644*	2	1,237**
$CaCl_2$	2	13,601**	2	4,151**
S × C	2	586	1	427
S × $CaCl_2$	2	12,664**	2	682**
C × $CaCl_2$	4	1,527**	2	51
S × C × $CaCl_2$	4	2,610**	1	762*
Error	234	349	155	133

[a] Data in Figure 3. Analysis of variance was performed with the Type III sums of squares method of the general linear models procedure of SAS.

[b] df = degrees of freedom.

[c] MS = mean squares. Probability levels for significant effects are $P \leq 0.05$ (*) and $P < 0.01$ (**).

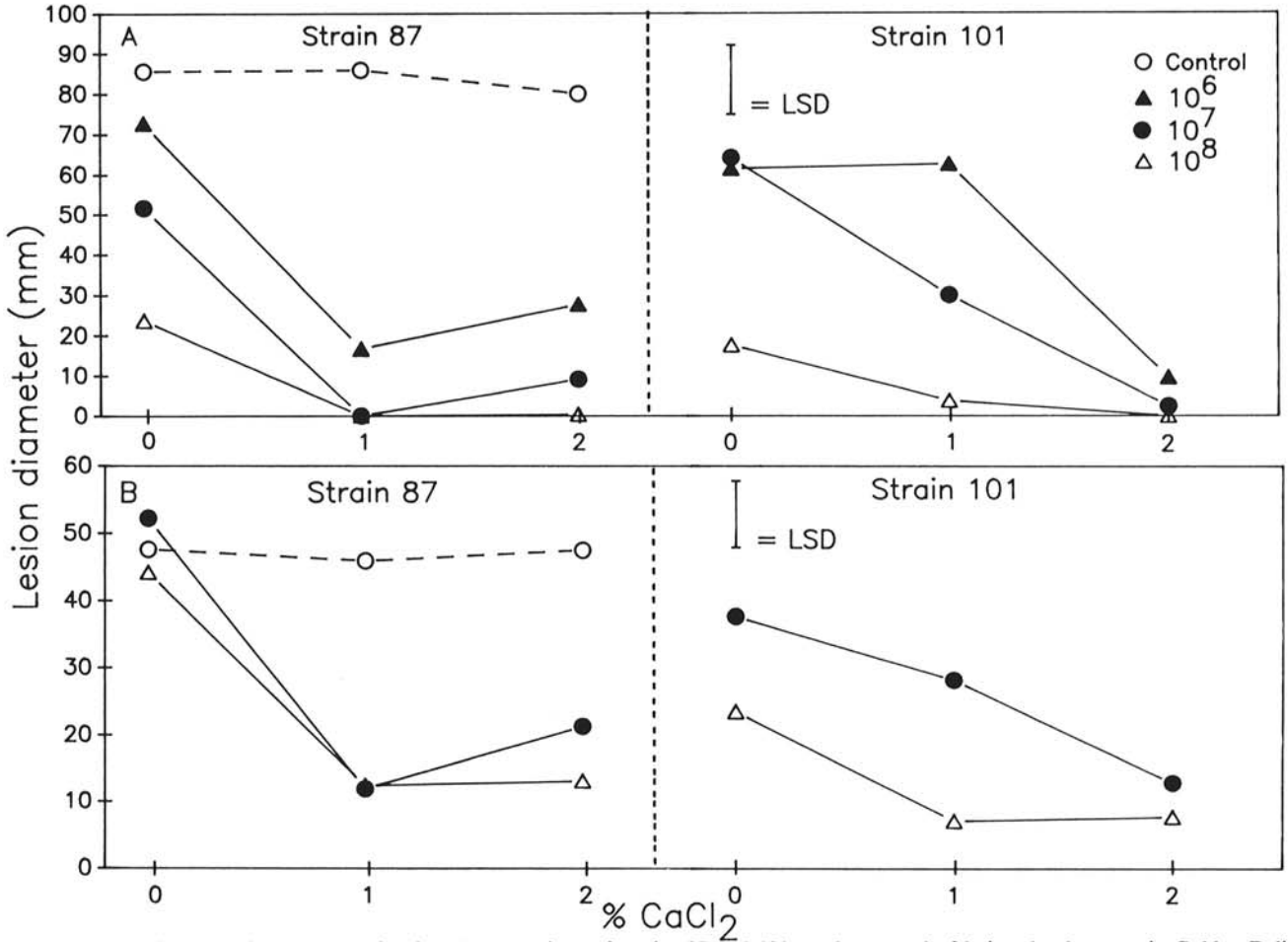

Fig. 3. Effect of calcium chloride concentration in cell suspensions of strains 87 and 101 on the control of lesion development in Golden Delicious apple caused by **A,** *Botrytis cinerea* (10^5 cfu/ml) and, **B,** *Penicillium expansum* (10^4 cfu/ml). Data points represent average lesion diameter measured at 10 days after inoculation. Dashed line represents control treatments (0, 1, and 2% $CaCl_2$) without yeasts, which applies to both yeast strains in each test. There was no effect on *P. expansum* with strain 101 at 10^6 cfu/ml at any concentration of $CaCl_2$, thus, these data points are not shown in B. LSD = least significant difference ($P \leq 0.05$). Note that treatment interactions invalidate strict use of the LSD mean separation procedure; however, this method was used to aid interpretation of the data where real differences occur.

due to the osmotolerant nature of the strains; however, the salt solution with the lowest osmotic potential (NaCl) did not facilitate control in the presence or absence of yeast cells. In addition, the osmotic potential of the two most effective salt solutions, $CaCl_2$ and $CaCO_3$, were relatively high. The ability of both calcium salts to facilitate control indicates that the Ca^{+2} cation may have an important role.

Other studies have shown that calcium chloride reduces postharvest soft rot in apple caused by *P. expansum*, presumably as a result of the interaction of the calcium cation with pectic substances in apple tissue (14–16). After vacuum infiltration of 8% $CaCl_2$, however, decay reduction was only 10% (14). Dipping of fruit in $CaCl_2$ at concentrations as high as 12% does not reduce decay. Therefore, it was expected that topical treatments with $CaCl_2$, in the absence of yeast cells, would not result in reduced decay. The effect of Ca^{+2} on biocontrol effectiveness of these strains of *Candida* sp. thus may be speculated to result from interaction with the yeast and/or its metabolic products in the wound site. Studies are under way to determine if presence of Ca^{+2} affects growth and survival of the yeast in the wound site, or if it stimulates physiological processes in the yeast that result in excretion of new or additional antifungal metabolites. Preliminary tests also have shown that strain 87 produces a copious extracellular matrix in wounded apple tissue (37) which may interact with Ca^{2+}. Interaction of these salts with fruit tissue compounds, such as exposed cell wall polysaccharides, may result in a small contribution toward resistance as well.

The ability of strains 87 and 101 to reduce decay in the absence of salts may be due in part to nutrient competition and/or site exclusion. Previous studies have shown that amending of inoculum of *B. cinerea* with nutrients can facilitate increased aggressiveness on different crops (3,10,11,22). Phylloplane-associated bacteria and fungi have been shown to decrease infection by *B. cinerea* and to cause lowered spore germination, perhaps, by nutrient competition (7,11,25). Depletion of nutrients also has been shown to inhibit germ tube elongation in *B. cinerea* (12). Biocontrol assays conducted in this lab also have shown that application of unwashed cells of these yeast strains, in culture medium, decreases their biocontrol effectiveness (data not shown). Thus, it seems probable that these yeast strains, in the absence of antibiosis, may be able to deplete the nutrient base required for germination of conidia of *B. cinerea* and, thus, reduce decay. Additional research is required to substantiate these factors as a part of the mechanism of control.

Preliminary investigations in this laboratory have shown that other *Candida* sp., including closely related species such as *Candida famata*, are less effective for the reduction of fruit decay (35). It is possible that yeasts isolated from fruit surfaces may be more effective biocontrol agents because they are phenotypically adapted to this niche and, thus, are able to more effectively colonize and compete for nutrients and space on fruit surfaces. Habitat specialization appears common in the hodgepodge, anamorphic species known as *Candida* (23). Other studies have established that yeasts, including *Candida* sp., are a major component of the epiphytic microbial community on mature fruits (4,8,12,13).

The speciation of strains 87 and 101 within the genus *Candida* is uncertain despite repeated phenotypic characterizations (American Type Culture Collection, Rockville, MD). These tests indicate that the strains do not share phenotypic similarity with any previously described species of *Candida*. The tests show, however, that the strains can be differentiated from species of *Candida* that are pathogenic toward humans, based on their ability to assimilate nitrate and key carbohydrates such as maltose, raffinose, melibiose, and erythritol. Additional tests need to be done, however, to corroborate these data to determine if these yeasts can be used safely as an alternative to fungicides for the control of fruit postharvest decay.

Yeast strains from other species, *Cryptococcus laurentii* (29) and *Hanseniaspora uvarum* (24, *unpublished results*) have been shown effective for reducing postharvest decays of apple and other fruits. Preliminary tests in our laboratory have shown that $CaCl_2$ enhances control with a strain of *H. uvarum*. Calcium chloride may facilitate improved control with *C. laurentii* as well. Since strains of yeasts have been shown to have the ability to control postharvest diseases of several fruits (9,24,34,36), it is likely that application of these biological control agents with calcium salt solutions will improve their effectiveness for biocontrol in different systems and facilitate their practical implementation.

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