Application of *Candida saitoana* and Glycolchitosan for the Control of Postharvest Diseases of Apple and Citrus Fruit Under Semi-Commercial Conditions

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ABSTRACT

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The efficacy of the combination of *Candida saitoana* with 0.2% glycolchitosan (the bioactive coating) as a biocontrol treatment of postharvest diseases of apple and citrus fruit was evaluated in tests with natural inoculations that simulated commercial packinghouse conditions. The growth of *C. saitoana* in apple wounds and on fruit surfaces was not affected by glycolchitosan. The bioactive coating was more effective in controlling decay of several cultivars of apples (Red Delicious, Rome, Golden Delicious, and Empire) than either *C. saitoana* or 0.2% glycolchitosan alone. Depending on the apple cultivar used, the bioactive coating was comparable or superior to thiabendazole in reducing decay. The bioactive coating was also superior to *C. saitoana* in controlling decay of oranges (cvs. Washington navel, Valencia, Pineapple, and Hamlin) and cv. Eureka lemons, and the control level was equivalent to that with imazalil. The bioactive coating and imazalil treatments offered consistent control of decay on Washington navel oranges and Eureka lemons in early and late seasons, while *C. saitoana* or 0.2% glycolchitosan were most effective on early-season fruit. The combination of *C. saitoana* with 0.2% glycolchitosan also reduced the incidence of stem-end rot of cv. Valencia oranges, but control was less effective than treatment with imazalil.

Additional keywords: *Botrytis cinerea*, chitosan, *Penicillium digitatum*, *Penicillium expansum*

In recent years, considerable laboratory success has been reported with antagonistic microorganisms to control postharvest diseases, and a large body of information regarding postharvest biocontrol antagonists is now available (14,19). Antagonistic yeasts and bacteria isolated from fruit surfaces were shown to have a broad spectrum of activity against a number of postharvest pathogens on a variety of fruit (3,4,11–13,15–17). Presently, the yeast *Candida oleophila* Montrocher and two strains of the bacterium *Pseudomonas syringae* van Hall are available commercially under the trade names Aspire, Biosave-100, and Biosave-110, respectively. Acceptance of microbial biocontrol products as an economically viable alternative to synthetic fungicides will depend on their commercial performance. So far, available antagonistic microorganisms, when used alone, have not shown levels of control comparable to synthetic fungicides (2,5,6,20). This has led to the search for additives that will enhance the effectiveness of microbial antagonists (8,19,20). In semi-commercial trials, the addition of a low dose of the synthetic fungicide thiabendazole was found to increase the efficacy of antagonistic yeasts to levels equivalent to recommended doses of synthetic fungicides (2,4,5). Similarly, but in laboratory studies, the combination of microbial antagonists with $CaCl_2$, nitrogenous compounds, or the sugar analog 2-deoxy-D-glucose was shown to enhance the effectiveness of certain antagonists and greatly reduced the populations of yeasts and bacteria required to give effective control (13,16,21).

Recently, we developed a biocontrol product called "a bioactive coating" consisting of a unique combination of an antagonistic yeast with chemically-modified chitosan (10). In laboratory studies, the combination of *C. saitoana* Nakase et Suzuki with glycolchitosan was more effective in controlling decay of apple and citrus fruit than *C. saitoana* or the glycolchitosan treatment alone (10). The bioactive coating made it possible to exploit the antifungal property of glycolchitosan and the biological activity of the antagonist.

The present investigation was undertaken to determine the efficacy of the bioactive coating for the control of postharvest diseases of apple and citrus fruit under semi-commercial conditions over several seasons. It is only by testing over several seasons with naturally inoculated fruit that the performance of the combination can be accurately determined. We also report the effect of citrus packinghouse practices, such as soda ash and degreening, on the performance of the combination of *C. saitoana* with glycolchitosan.

MATERIALS AND METHODS

Reagents, yeast preparations, and fruit materials. Glycolchitosan was obtained from Sigma Chemical Co. (St. Louis). Imazalil (Fungaflor 500EC, 44.6% a.i.) was purchased from Janssen Pharmaceutical (Titusville, NJ). Thiabendazole (2-4-thiazolylbenzimidazole) was obtained from GreenChemicals (Winchester, VA). *C. saitoana* was grown at 24°C for 48 h in 2-liter shake-flask cultures containing nutrient-yeast broth (NYB). Yeast cells were pelleted by centrifugation with a Sorval RC-58 centrifuge (Dupont Instruments, Wilmington, DE) at 3,000 × *g* for 20 min, resuspended in sterile distilled water, and centrifuged again; the pelleted material was used immediately. *C. saitoana* was also grown in NYB in IF-15 fermentor (volume, 15 liters; New Brunswick Scientific Co., Edison, NJ). Fermentation conditions were: aeration, 0.666 vol air/vol medium/min; agitation, 250 rpm; temperature, 24°C. After 72 h, the culture**,** which consisted of 6.5% solids**,** was harvested using a Sharples AS-16V Super centrifuge, and the resulting wet paste was stored at 4°C. Yeast paste (1 g) was approximately equivalent to 2.1×10^{10} CFU.

Tree-ripe apple (*Malus domestica* Borkh.) cvs. Red Delicious, Rome, Golden Delicious, and Empire were hand-harvested at commercial maturity at the Appalachian Fruit Research Station, Kearneysville, West Virginia. Early- and late-season cv. Washington navel oranges

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(*Citrus sinens* (L.) Osbeck) and cv. Eureka lemons (*C. lemon* (L.) Burm), grown in the San Joaquin Valley of California, were harvested within 2 days before treatment. Orange cvs. Valencia, Pineapple, and Hamlin grown in Lake Alfred, Florida were hand-harvested 24 h before treatment. Apples, oranges, and lemons were sorted to remove any with apparent injuries or infections, washed with water on a processing line, and randomly assigned to different treatments.

Semi-commercial tests. Experiments were conducted from 1995 to 1997 on simulated packing lines at Kearneysville, West Virginia; Lindcove, California; and Lake Alfred, Florida. At Kearneysville, apple fruit (cvs. Red Delicious, Rome, Golden Delicious, and Empire) were wounded (3 mm by 5 mm deep) once, placed on a dry-dump conveyor belt, and passed over rotating brushes through a washer operating at 5.8×10^3 N m^{-2} to an air drier operating at 32°C.

The dried fruit were treated with a yeast-cell suspension (10^8 CFU/ml) containing 0.2% (wt/vol) glycolchitosan at approximately 15 ml/kg of fruit, sterile water, or 600 µg/ml of thiabendazole using 6 flat-fan nozzles, at a pressure of 9.2×10^2 N m^{-2}, installed before the drier. Treated fruit were passed through an air drier to a moving-belt sorting table, where decayed or damaged fruit were removed. Within each experiment, treatments were applied to five replicates of 56 apples and the tests were conducted during three successive seasons. Before the application of each treatment, the rollers and nozzles were washed extensively. Treated fruits were packed in cardboard boxes and stored for 2 to 5 weeks at 18°C, to mimic consumers' display conditions and to get sufficient disease level. Fruit were evaluated periodically for disease development.

At the packinghouse in Lindcove, orange and lemon fruit were placed on a dry-dump conveyor belt and passed over rotating brushes through a high-pressure washer operating at 4.3×10^4 N m^{-2}, where they were washed extensively with water containing 50 µg/ml of sodium hypochlorite at pH 7.2. After being pressure-washed, the fruit passed over foam-rubber rollers (to dry them) to a moving-belt sorting table, where decayed or damaged fruit were removed. Lemon fruit were submerged for approximately 1 min into a 2,400-liter-capacity tank containing a solution of 3% (wt/vol) sodium carbonate heated to 37°C, then lifted on rollers to a washer with a series of overhead nozzles and to foam-rubber rollers for drying. Orange fruit by-passed the sodium carbonate treatment and were moved to the washing unit, which consisted of nylon brushes for washing and foam-rubber rollers for drying.

Dried fruit were then treated with a yeast-cell suspension (10^8 CFU/ml) containing 0.2% (wt/vol) glycolchitosan at the same rate described above, water, or 2,000 µg/ml of imazalil. After treatment, the fruit was passed through a high-velocity air drier operating at 32°C, waxed, and dried again by passing through a high-velocity air drier. All treatments, except imazalil, were applied using an on-line overhead spray system installed before the waxer. Rollers and nozzles were washed extensively between application of different treatments. The imazalil treatment was applied in an emulsion storage wax (2% solids content) using the waxer with an overhead single nozzle that continually moved from side to side (set-up number for ¼ J round spray, Spray Systems Co). Within each experiment, treatments were applied to 12 replicates of 60 to 75 navel oranges and 10 to 12 replicates of 110 to 140 lemons. Tests were conducted during three successive seasons. Treated fruit were packed in cardboard boxes, stored at for 25 days at 10°C, and the incidence of decay was determined.

At the packing line in Lake Alfred, Florida, orange cvs. Valencia, Pineapple, and Hamlin were placed on a dry-dump conveyor belt, where decayed or damaged fruit were removed, then passed over rotating brushes through a washer and dryer unit similar to the one described above. Fruit were treated with a yeast-cell suspension (10^8 CFU/ml) containing 0.2% (wt/vol) glycolchitosan, water, or 1,000 µg/ml of imazalil using a controlled-drip applicator that delivered 350 ml/min of the suspension to fruit rotating on brushes saturated with the treating material. The controlled-drip applicator was installed before the drier. Before the application of each treatment, the rollers and nozzles were washed extensively. After treatment, the fruit were dried by passing through a high-velocity air drier operating at 32°C. Early-season cv. Valencia oranges were processed on line, treated with different treatments, and degreened with ethylene (10 ppm) for 3 days. Within each experiment, treatments were applied to eight replicates of 50 oranges (cvs. Hamlin, Pineapple, and Valencia). Tests were conducted during three successive seasons. Fruit were packed in cardboard boxes, stored for 21 to 28 days at 18°C, and evaluated periodically for disease development.

Statistics. An arcsine-square root transformation was applied to the percentage of infected fruit from the different trials prior to analyses of variance. Homogeneity of variance in each experiment was evaluated by Hartley's F-Max test at $P = 0.05$ of the arcsine-square root transformation of the percentage of infected fruit. Data from separate experiments were combined when statistical analyses determined that variances were homogeneous. Duncan's new multiple range test ($P = 0.05$) to separate means was applied to compare treatments.

Fruit colonization by *C. saitoana*. The effect of glycolchitosan on the population of *C. saitoana* in cv. Golden Delicious apple wounds and on fruit surfaces was determined. Apples were individually wounded, processed on the packing line, and treated with a yeast-cell suspension (10^8 CFU/ml) containing 0 or 0.2% (wt/vol) glycolchitosan as described above. Treated fruit were stored at 18°C under high relative humidity (RH) in enclosed plastic containers. For each treatment, 20 fruit were arranged in a randomized complete block.

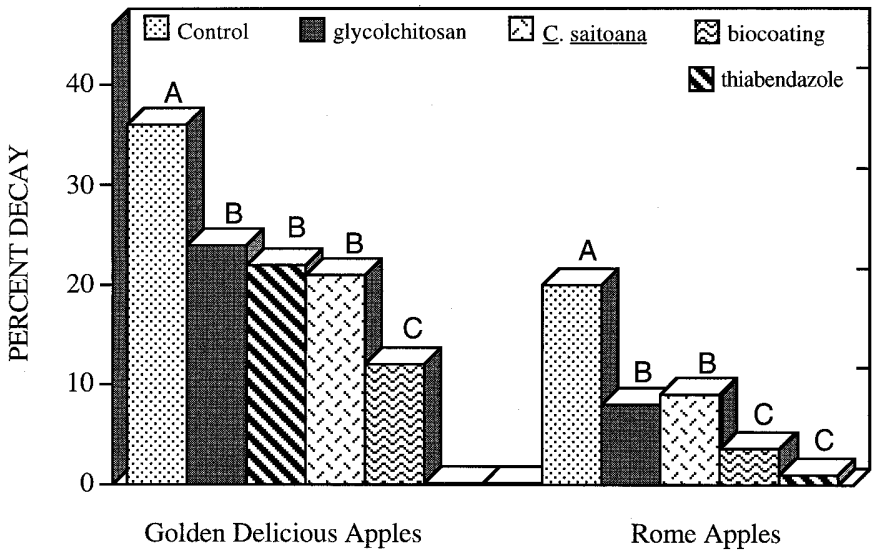

Fig. 1. Effect of the combination of *Candida saitoana* with 0.2% (wt/vol) glycolchitosan on decay of apple cvs. Golden Delicious and Rome. For each cultivar, the percentage of decay was based on five replicates of 56 fruit each. Incidence of decay on Rome and Golden Delicious was determined after 14 and 56 days of storage at 18°C. Bars within each cultivar with the same letter are not significantly different according to Duncan's multiple range test, $P = 0$ .05.

Samples from the different treatments were collected 0 and 42 days after treatment. At each sampling time, tissue samples containing the wounds were removed with a number 7 cork borer (6 mm in diameter) from four apples selected randomly from each treatment. Tissue samples were homogenized in 5 ml of sterile water and vortexed. The selected fruit with removed wounds were individually placed in sterile plastic bags with 200 ml of sterile water and mixed vigorously for 5 min (18). Tissue homogenate and fruit wash were dilution-plated in triplicate on yeast-maltose agar medium and the plates were incubated at 24°C. Colonies were counted after 48 h and the results were expressed as the mean number of CFU per square centimeter or per wound.

RESULTS

Semi-commercial trials on control of apple decay. The bioactive coating was effective in controlling natural infection of apple cvs. Golden Delicious, Red Delicious, Rome, and Empire. The predominant decay pathogens were *Botrytis cinerea* and *Penicillium expansum*. On cvs. Golden Delicious and Rome, the bioactive coating reduced decay significantly more than *C. saitoana* or 0.2% glycolchitosan alone (Fig. 1). In all apple cultivars tested, except Rome, the bioactive coating gave significantly better control than thiabendazole (Figs. 1 and 2). Thiabendazole was ineffective against decay on cvs. Red Delicious and Empire and, in some cases, the level of decay was slightly higher than the water-treated control (Fig. 2). On Rome apples, however, thiabendazole reduced decay to a level similar to the bioactive coating. Regardless of the apple cultivar used, decay incidence among fruit treated with *C. saitoana* or 0.2% glycolchitosan alone was significantly lower than in the control (Fig. 1). At the end of the storage period, the bioactive coating reduced decay of apple by 49 to 82%, depending on the cultivar used, whereas a reduction of 42 and 55% was observed among cvs. Golden Delicious and Rome treated with *C. saitoana* alone (Figs. 1 and 2)

Effect of glycolchitosan on population dynamics of *C. saitoana.* *C. saitoana* multiplied similarly in apple wounds and on fruit surfaces in the presence or absence of glycolchitosan (Table 1). After 42 days, the population of *C. saitoana* in apple wounds increased by more than 20-fold; whereas, on fruit surfaces, the population of *C. saitoana* increased by approximately threefold.

Semi-commercial trials on control of citrus decay. At Lindcove, California, the incidence of decay on lemon cv. Eureka and navel orange cv. Washington fruit was reduced significantly by all treatments when compared to the water-treated control. Green mold was the most prevalent decay, and the incidence of blue and sour rot caused by *P. italicum* Wehmer and *Geotrichum candidum* Link:Pers., respectively, was very low. Decay of oranges and lemons was higher on late-season than on early-season fruit. On early-season oranges and lemons, the bioactive coating and imazalil were the most effective treatments (Figs. 3 and 4). The bioactive coating was superior to *C. saitoana* in controlling green mold on early-season oranges and lemons, and control was comparable to 2,000 g/ml of imazalil. After 24 days of storage, the bioactive coating reduced green mold on early-season oranges and lemons by approximately 70%, a level of control equivalent to that achieved by the imazalil treatment. *C. saitoana* reduced green mold of oranges and lemons by 44%. A reduction of 34 and 15%, respectively, was observed on early-season oranges and lemons treated with 0.2% glycolchitosan.

Effective control by the bioactive coating and imazalil was also observed on late-season oranges and lemons (Figs. 3 and 4). Control with the bioactive coating was equivalent to imazalil and significantly superior to *C. saitoana* or 0.2% glycolchitosan alone. Treatment of late-season oranges and lemons with the bioactive coating or 2,000 g/ml of imazalil resulted in over 70% less green mold on lemons and oranges than the water control. *C. saitoana*, when used alone, reduced green mold of lemons and oranges by 15 and 17%, respectively.

In semi-commercial tests conducted in Florida, the bioactive coating controlled green mold of orange cvs. Hamlin, Valencia, and Pineapple as effectively as 1,000 µg/ml of imazalil. The level of green mold varied from 10 to 21%, depending on the orange cultivar used, and was reduced by 3 to 8% with the bioactive coating and imazalil treatments (Fig. 5).

The bioactive coating also reduced the incidence of stem-end rot of oranges caused by either *Diplodia natalensis* or *Phomopsis citri* (Fig. 6). The level of control of stem-end rot obtained with the bioactive coating was significantly superior to

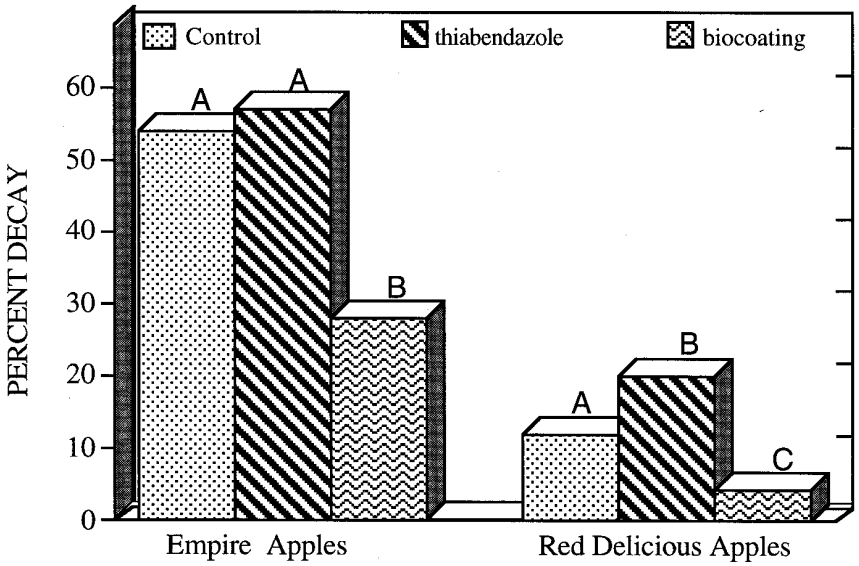

Fig. 2. Effect of the combination of *Candida saitoana* with 0.2% glycolchitosan on decay of apple cvs. Empire and Red Delicious. For each cultivar, the percentage of decay was based on five replicates of 56 fruit each. Incidence of decay on Empire and Red Delicious was determined after 15 and 28 days of storage at 18°C. Bars within each cultivar with the same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.

Table 1. Populations of *Candida saitoana* in cv. Golden Delicious apple wounds and surfaces in the presence or absence of glycolchitosan

	Yeast-cell counts (log CFU)[a]	
Treatment, storage period (days)[b]	**Surface**	**Wound**
C. saitoana		
0	4.47 ± 0.11	4.60 ± 0.13
42	4.90 ± 0.15	6.01 ± 0.24
C. saitoana + glycolchitosan		
0	4.62 ± 0.13	4.51 ± 0.12
42	4.81 ± 0.16	5.91 ± 0.20

[a] Population densities of *C. saitoana* on apple surface and wounds were expressed as $\log_{10}$ CFU per square centimeter or per wound, respectively. Values are mean ± SEM.

[b] Wounded apples were treated with a yeast-cell suspension (10^8 CFU/ml) containing 0 or 0.2% (wt/vol) glycolchitosan.

the water-treated control, but inferior to imazalil. After 28 days of storage, the bioactive coating and the imazalil treatment reduced the incidence of stem-end rot of degreened Valencia oranges by 48 and 78%, respectively.

DISCUSSION

The present study was undertaken to compare efficacy of the bioactive coating with synthetic fungicides for the control of postharvest decay of apple and citrus fruit. The semi-commercial trials were conducted under a worst-case scenario, where fruit were stored for a long period at high temperature, conducive to disease development, and highly susceptible late-season navel oranges and lemons were used. Under semi-commercial conditions, the bioactive coating was effective in controlling decay of apple, lemon, and orange fruit, and the level of control was usually comparable to the fungicides approved for these applications (thiabendazole and imazalil). On apple cvs. Golden Delicious, Red Delicious, and Empire, the poor performance of thiabendazole may be due to resistant strains of the pathogens. In the presence of resistant biotypes of *Penicillium expansum*, thiabendazole was shown to be ineffective against blue mold of pear (4).

Control levels equivalent to commercial fungicide treatments were also reported with other antagonistic yeasts when used in combination with low doses of fungicides (2,4,5). For instance, *C. oleophila* in combination with 200 µg/ml of thiabendazole controlled citrus decay at the level equivalent to the commercial fungicide treatment and reduced the variability often observed when using the antagonistic yeast alone (5,6). Similar results were reported on apple fruit treated with a combination of *Cryptococcus infirmo-miniatus* with 264 µg/ml of thiabendazole (4).

In semi-commercial tests, the level of control provided by antagonistic yeasts,

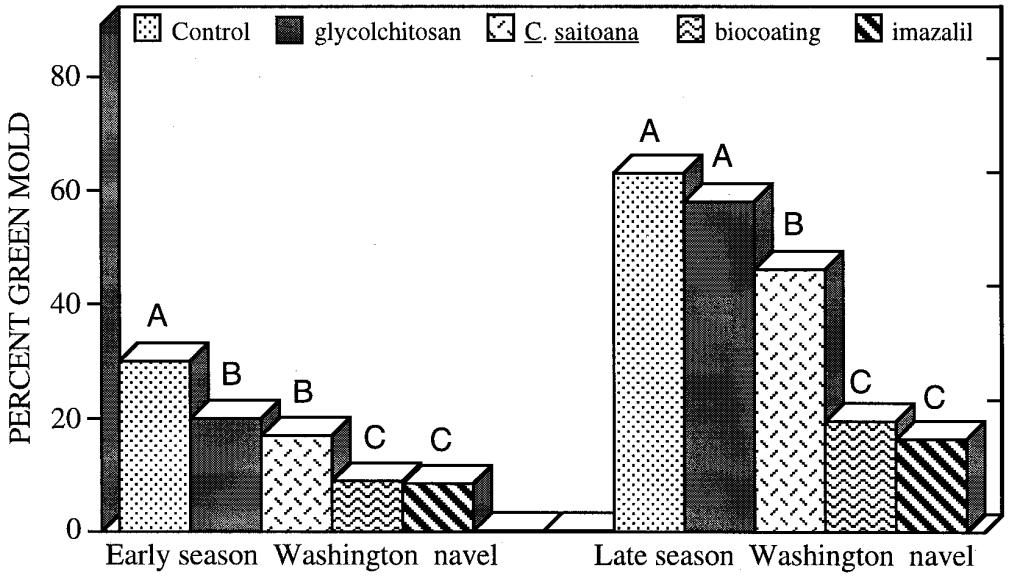

Fig. 3. Effect of the combination of *Candida saitoana* with 0.2% glycolchitosan on decay of early- and late-season navel oranges. The percentage of decay was based on 12 replicates of 60 to 75 fruit each. Incidence of decay was determined after 25 days of storage at 10°C. Bars within each season with the same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.

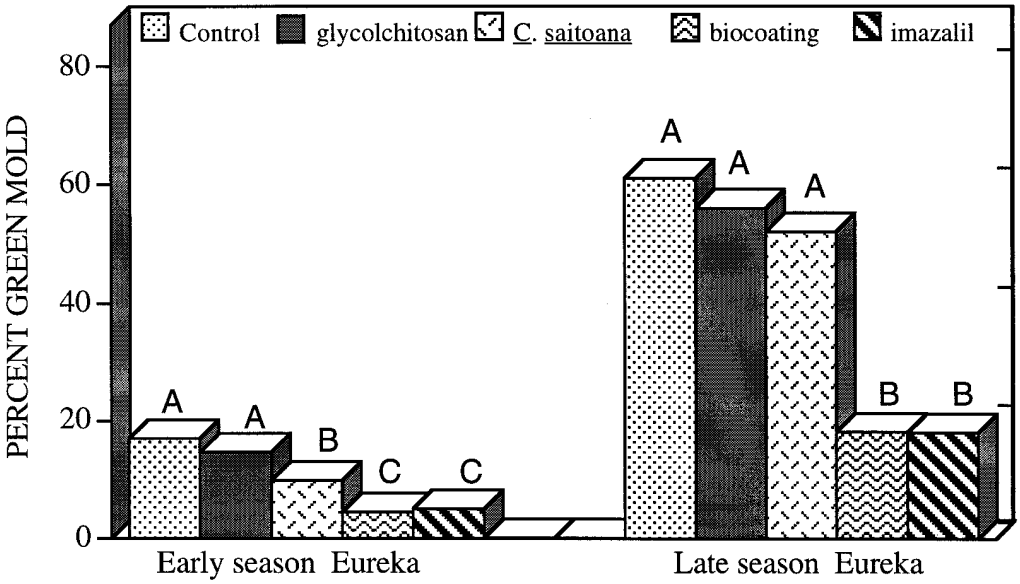

Fig. 4. Effect of the combination of *Candida saitoana* with 0.2% glycolchitosan on decay of early and late cv. Eureka lemons. The percentage of decay was based on 10 to 12 replicates of 110 to 140 fruit each. Incidence of decay was determined after 25 days of storage at 10°C. Bars within each season with the same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.

when used alone, is often inferior to control provided by commercially recommended fungicides (2,5,6). This was attributed to fruit quality, inoculum density, level of susceptibility of fruit to infection, and the time between initial infection and the application of the treatment (6). Similarly, in the present study, the level of control obtained with *C. saitoana* was significantly lower than control with imazalil and highly affected by fruit quality, as indicated by poor performance on late-season fruit. The addition of glycolchitosan to *C. saitoana* overcame some of the limitation of microbial biocontrol agents. This is supported, in part, by the improved efficacy of the bioactive coating and its higher performance on both early- and late-season oranges and lemons.

The efficacy of the bioactive coating is likely the result of the interplay of the antifungal property of glycolchitosan and the antagonistic activity of *C. saitoana*. Chitosan and its derivatives are known to affect adversely the growth of filamentous fungi, including postharvest pathogens (1,9). When tested in vitro, glycolchitosan was shown to inhibit spore germination of *B. cinerea* and *P. expansum*, but had no adverse effect on the growth of *C. saitoana* (10). On surfaces and wounds of apple, *C. saitoana* grew equally well in the presence and absence of glycolchitosan, thus making it possible to exploit the additive and synergistic effects of both biocontrol protectants. Such a combination can be expected to have greater stability and effectiveness than the use of a single biocontrol agent. This was confirmed, in part, by the observed superior performance of the bioactive coating in comparison to *C. saitoana* used alone.

In conclusion, this study demonstrates that the biocontrol activity of *C. saitoana* against decay of apple, lemon, and orange was significantly enhanced by the addition of glycolchitosan. The level of control conferred by the bioactive coating was superior to *C. saitoana* alone and equivalent to thiabendazole and imazalil. Combining antagonistic yeasts with glycolchitosan can be expected to provide more effective disease control and management of fungicide-resistant isolates of *P. digitatum* and *P. expansum* (4,7). Commercial trials are now warranted to compare the efficacy of the bioactive coating to registered fungicides.

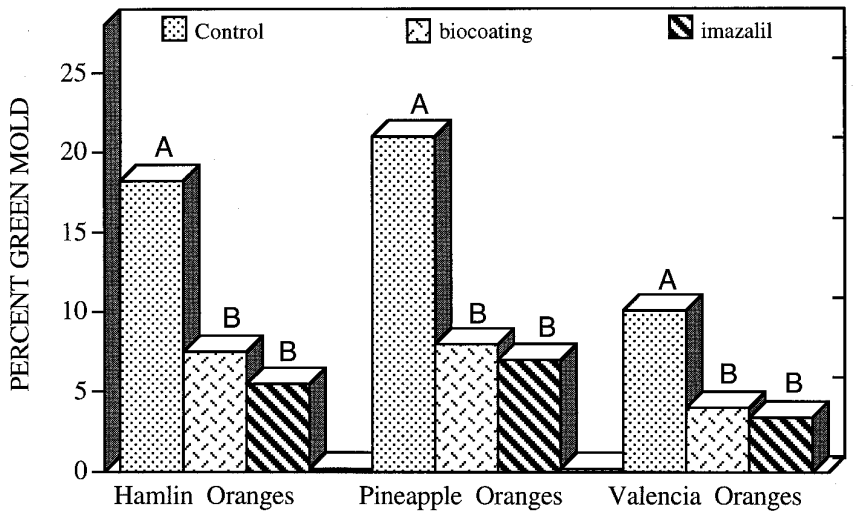

Fig. 5. Effect of the combination of *Candida saitoana* with 0.2% glycolchitosan on decay of orange cvs. Hamlin, Pineapple, and Valencia. For each cultivar, the percentage of decay was based on eight replicates of 50 fruit each. Incidence of decay on Hamlin, Pineapple, and Valencia oranges was determined after 21, 28, and 21 days of storage at 18°C, respectively. Bars within each cultivar with the same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.

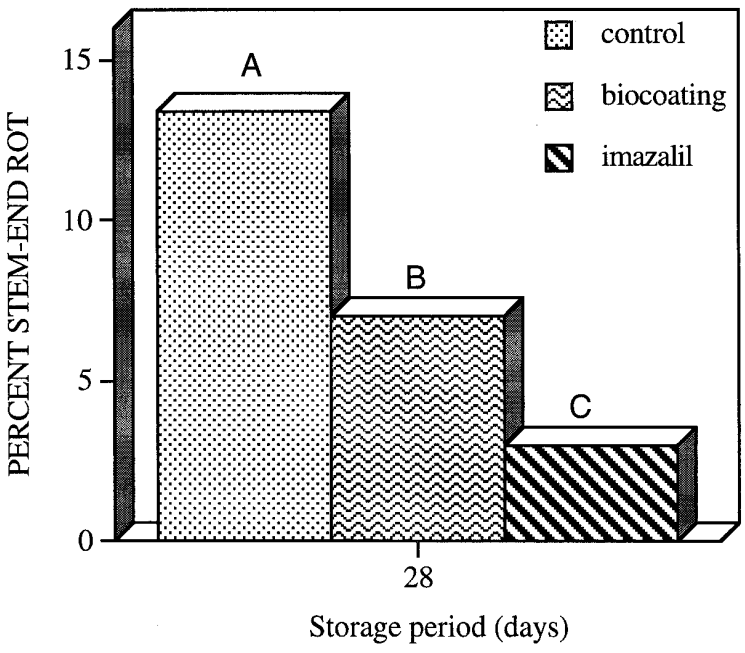

Fig. 6. Effect of the combination of *Candida saitoana* with 0.2% glycolchitosan on stem-end rot of degreened cv. Valencia oranges. The percentage of decay was based on eight replicates of 50 fruit each. Incidence of stem-end rot was determined after 28 days of storage at 18°C. Bars with the same letter are not significantly different according to Duncan's multiple range test, $P = 0.05$.

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