Improved Control of Apple and Citrus Fruit Decay with a Combination of *Candida saitoana* and 2-Deoxy-D-Glucose

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ABSTRACT

El-Ghaouth, A., Smilanick, J. L., Wisniewski, M., and Wilson, C. L. 2000. Improved control of apple and citrus fruit decay with a combination of *Candida saitoana* and 2-deoxy-D-glucose. Plant Dis. 84:249-253.

A combination of *Candida saitoana* with 0.2% 2-deoxy-D-glucose to control decay of apple, lemon, and orange fruit was evaluated. Growth of *C. saitoana* in vitro was reduced by 2-deoxy-D-glucose; however, in apple wounds, the yeast grew as well in the presence of 2-deoxy-D-glucose as in its absence. When applied to fruit wounds before inoculation, the combination of *C. saitoana* with 0.2% 2-deoxy-D-glucose was more effective in controlling decay of apple, orange, and lemon caused by *Botrytis cinerea*, *Penicillium expansum*, and *P. digitatum* than either *C. saitoana* or the application of a 0.2% solution of 2-deoxy-D-glucose alone. Increasing the concentration of 2-deoxy-D-glucose from 0.2 to 0.5% did not improve control significantly. The combination of *C. saitoana* with 0.2% 2-deoxy-D-glucose was also effective against infections established up to 24 h before treatment. When applied within 24 h after inoculation, the combination of *C. saitoana* with 0.2% 2-deoxy-D-glucose was very effective in controlling blue mold of apple and green mold of orange and lemon. The level of control of green mold was equivalent to imazalil treatment. When either *C. saitoana* or 0.2% 2-deoxy-D-glucose was applied within 24 h after inoculation, neither had an effect on disease development on apple, orange, or lemon, and the incidence of decay was similar to the water-treated control.

Additional keywords: analog, citrus, gray mold, microbial antagonist

In recent years, there has been an increased interest in the development of microbial biocontrol agents as an alternative to synthetic fungicides for the control of postharvest diseases of fruit (11,20). Antagonistic bacteria and yeasts have been isolated and shown to reduce decay of pome and citrus fruit (2,3,9,11,13,15). Presently, three antagonistic microorganisms, a yeast (*Candida oleophila* Montrocher) and two strains of a bacterium (*Pseudomonas syringae* van Hall) are commercially available under the trade names ASPIRE, BIOSAVE-100, and BIOSAVE-110, respectively.

Although antagonistic microorganisms have been shown to protect a variety of fruit, their efficacy under semi-commercial conditions is often lower than chemical control (5,9). In large-scale tests, antagonists often provide a level of control

equivalent to synthetic fungicides only when combined with low doses of synthetic fungicides (1,3,5,6). Furthermore, currently available antagonistic microorganisms do not appear to be able to control previously established infections and are most effective when applied prior to infection by the pathogen (9,16,21). Most infections on citrus and pome fruit occur through wounds inflicted during harvest and subsequent handling. Eradication of these incipient infections, which often occur within 24 to 48 h after harvest, is required to achieve an economically sufficient level of disease control. Thus, it has become apparent that alternative methods are needed to overcome the variability in efficacy and augment the effectiveness of microbial antagonists. The biocontrol activity of antagonists can be enhanced by naturally occurring organic and inorganic additives. The addition of CaCl_2 was shown to increase the protective effect of some antagonists and greatly reduced the populations of yeasts required to give effective control (12,14,22). Enhancement of the biocontrol activity of antagonists by nitrogenous (L-asparagine and L-proline) and carbohydrate (2-deoxy-D-glucose) compounds was also reported in apple and pear fruit, with a lesser effect on pear fruit (10,13). When applied before inoculation with the pathogen, 2-deoxy-D-glucose was

shown to improve biocontrol activity of two antagonists, *P. syringae* and *Sporobolomyces roseus* (10). The use of 2-deoxy-D-glucose, however, was not evaluated for its ability to control incipient infections that originate 24 h prior to postharvest treatment with biocontrol products.

The sugar analog 2-deoxy-D-glucose could be a useful additive to antagonistic microorganisms, provided that the antagonist is resistant to its fungicidal action. This analog is known to interfere with the growth of major postharvest pathogens and controls decay of apple and peach fruit, with a marked effect at higher concentrations (7,8,15). Recently, 2-deoxy-D-glucose was found to be compatible with the antagonistic yeast *Candida saitoana* Nakase et Suzuki (19). Preliminary studies indicated that the efficacy of *C. saitoana* against apple decay was enhanced markedly by combining the antagonist with 0.2% (wt/vol) 2-deoxy-D-glucose.

This investigation was undertaken to assess the potential of 2-deoxy-D-glucose as an additive to enhance the efficacy of *C. saitoana* and to determine whether or not this compound by itself, or in combination with *C. saitoana*, could have an impact on previously established infections (curative activity). The lack of a curative activity has been identified as a major limitation of biocontrol antagonists for postharvest diseases (21).

MATERIALS AND METHODS

Reagents, microorganisms, and fruit material. The sugar analog 2-deoxy-D-glucose was purchased from Sigma Chemical Co. (St. Louis). Imazalil (44% a.i., Bromozil 50) was obtained from Janssen Pharmaceutical (Titusville, NJ). Isolates of *Penicillium expansum* Link, *P. digitatum* (Pers.:Fr.) Sacc., and *Botrytis cinerea* Pers.:Fr. were obtained from infected fruit and maintained on potato dextrose agar (PDA). A spore suspension was obtained by flooding 2-week-old PDA cultures of *B. cinerea*, *P. digitatum*, and *P. expansum* with sterile distilled water containing 0.1% (vol/vol) Tween 80. Spores were counted with a hemacytometer, and spore concentrations of the pathogens were adjusted with sterile distilled water to obtain 10^6 spores/ml of *B. cinerea* and 10^4 spores/ml of *P. digitatum* and *P. expansum*. *C. saitoana* was grown at 24°C for 48 h in

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Accepted for publication 12 November 1999.

Publication no. D-1999-1222-02R
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shake-flask cultures of nutrient-yeast broth (NYB). Yeast cells were pelleted by centrifugation with a Sorval RC-58 centrifuge (Dupont Instruments, Wilmington, DE) at $3,000 \times g$ for 20 min, resuspended in sterile distilled water, and centrifuged again. The resulting pellets were dispersed in sterile distilled water containing different concentrations of 2-deoxy-D-glucose and the concentration of the yeast was adjusted to 10^8 CFU ml^{-1} using a hemacytometer.

Tree-ripe apple (*Malus domestica* Borkh.) cv. Red Delicious fruit were hand-harvested at commercial maturity at the United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Appalachian Fruit Research Station, Kearneysville, West Virginia. The fruit were sorted on the basis of size and the absence of physical injuries or infections and stored at 4°C under regular air for 4 months before their use in biocontrol tests. Navel orange cv. Washington (*Citrus sinens* (L.) Osbeck) and lemon cv. Eureka (*Citrus lemon* (L.) Burm), grown in the San Joaquin Valley of California, were harvested within 2 days before treatment, washed with water on a processing line, and randomly assigned to treatments.

Effect of 2-deoxy-D-glucose on *C. saitoana*. The effect of different concentrations of 2-deoxy-D-glucose on the survival of *C. saitoana* was determined in 50-ml Erlenmeyer flasks containing 20 ml of 5.0% (wt/vol) yeast-maltose broth (YMB). Autoclaved YMB was amended with membrane-sterilized solutions of 2-deoxy-D-glucose to obtain concentrations of 0, 0.2, and 0.6% (wt/vol) 2-deoxy-D-glucose and dispensed immediately into sterile 50-ml Erlenmeyer flasks. Shake-flasks of nutrient-yeast broth medium supplemented with 2-deoxy-D-glucose were

inoculated with approximately 3.5×10^5 CFU ml^{-1} of yeast cells and incubated on a rotary shaker at 24°C for 5 days. Individual flasks served as one replicate in a randomized complete block design, and four replicates were sampled daily from each treatment. Samples were individually collected and dilution-plated in triplicate on yeast-maltose agar medium. Plates were incubated at 24°C and colonies were counted after 48 h and the results expressed as the mean number of CFU ml^{-1} .

The effect of 2-deoxy-D-glucose on the survival of *C. saitoana* was also determined in apple cv. Red Delicious wounds. Apple fruit were wounded (3 mm by 5 mm deep) once as described previously (14) and fruit wounds were treated with 35 μl of a yeast-cell suspension (10^7 CFU ml^{-1}) containing 0 or 0.2% (wt/vol) 2-deoxy-D-glucose. Treated fruit were stored at 24°C under high (95%) relative humidity (RH) in enclosed plastic trays. Individual fruit served as one replicate in a randomized complete block design, and three replicates were sampled from each treatment at each sampling time. Wound tissue was sampled 0, 1, 3, and 7 days after treatment. From each replicate fruit, tissue samples containing the wounds were removed with a number 7 cork borer (6 mm in diameter), individually homogenized in 5 ml of sterile water, vortexed, dilution-plated in triplicate on a yeast-maltose agar medium, and the plates incubated at 24°C. Colonies were counted after 48 h and the results are expressed as the mean number of CFU per wound.

Biocontrol activity of the combination of 2-deoxy-D-glucose with *C. saitoana*. *Experiment 1.* The effect of different concentrations of 2-deoxy-D-glucose on the

biocontrol activity of *C. saitoana* was investigated. Apple fruit were wounded as described above. A 35- μl drop of a cell suspension of *C. saitoana* at 10^8 CFU ml^{-1} containing 0, 0.2, or 0.6% (wt/vol) 2-deoxy-D-glucose was placed in each wound and allowed to dry. Within 30 min of treatment, wounds were challenge-inoculated with 30 μl of a conidial suspension of *B. cinerea* or *P. expansum*. Inoculated control fruit were treated with sterile distilled water. Treated fruit were stored at 24°C as described above. Each treatment was applied to four replicates of 18 fruit each within each experiment. The entire experiment was repeated twice. Fruit were evaluated daily for symptoms over a period of 14 days. An arcsine transformation was applied to the data prior to analyses of variance. Data from the two experiments were combined because statistical analysis determined there was homogeneity of variances between them. Treatment means were separated by Duncan's multiple-range test ($P = 0.05$).

Experiment 2. The protective and curative effects of the combination of *C. saitoana* with 0.2% (wt/vol) 2-deoxy-D-glucose on apple cv. Red Delicious inoculated with *P. expansum*, and lemon cv. Eureka and navel orange cv. Washington inoculated with *P. digitatum*, were determined. Apple fruit were wounded once as described above. Lemon and orange fruit were wounded by puncturing each fruit once with a stainless-steel probe with a tip 2 mm long and 1 mm wide (17). Wounded fruit were treated with a yeast-cell suspension containing 0 or 0.2% (wt/vol) 2-deoxy-D-glucose, 0.2% (wt/vol) 2-deoxy-D-glucose alone, sterile water, or 1,000 $\mu\text{g/ml}$ of imazalil. Only citrus fruit were treated with aqueous solutions of imazalil. Fruit wounds were treated either with the different treatments and then inoculated with a spore suspension of the postharvest pathogen, or inoculated and incubated at 24°C for 24 h before the treatments were applied. Initial observation of hand sections from inoculated controls, collected 24 h after inoculation with *B. cinerea*, showed that the spores had germinated and that hyphae had ramified throughout the wounded tissue (data not shown).

The different treatments, including the inoculum, were either pipetted directly into apple wounds as described above or applied using a handheld sprayer on citrus fruit. Approximately 0.5 ml was applied to each fruit. Each treatment was applied to four replicates of 18 apples, 60 oranges, or 80 lemons each. The entire experiment was repeated three times. Treated fruit was stored 14 days under high humidity (95% RH) in enclosed plastic trays at 24°C, then evaluated for percentage of fruit with lesions. An arcsine transformation was applied to the data prior to analyses of variance. Data from the three experiments

Fig. 1. Population dynamics of *Candida saitoana* grown in 2-deoxy-D-glucose solutions supplemented with 5.0% yeast maltose broth. Bars represent standard deviations.

were combined because statistical analysis determined that there was homogeneity of variance among them. Treatment means were separated by Duncan's multiple-range test ($P = 0.05$).

RESULTS

Effect of 2-deoxy-D-glucose on *C. saitoana*. The growth of *C. saitoana* in vitro was affected slightly by the addition of 2-deoxy-D-glucose (Fig. 1). Within 24 h of incubation, the yeast population grown in the absence of 2-deoxy-D-glucose increased by approximately twofold over the initial population levels and remained elevated for 5 days. In the presence of 2-deoxy-D-glucose, however, no marked increase in yeast population size was observed after 24 h at 24°C. By the third day of incubation, the population size of *C. saitoana* in 2-deoxy-D-glucose solutions started to decline slightly below the initial populations levels. Increasing the concentration of 2-deoxy-D-glucose from 0.2 to 0.6% did not influence yeast growth.

In contrast, in apple wounds, yeast growth did not appear to be affected adversely by 2-deoxy-D-glucose (Fig. 2). *C. saitoana* multiplied similarly in the presence or absence of 2-deoxy-D-glucose. Yeast populations recovered from apple wounds within 1 h after treatment were slightly lower than the initial population applied on the fruit (*data not shown*). However, within 24 h, the *C. saitoana* populations increased nearly 10-fold and remained over 6.2 log CFU/wound for 7 days.

Biocontrol activity of the combination of 2-deoxy-D-glucose with *C. saitoana*. When combined with *C. saitoana*, 2-deoxy-D-glucose enhanced significantly the biocontrol activity of *C. saitoana* against decay of apple (Table 1). In apple fruit, the combination of *C. saitoana* and 0.2% 2-deoxy-D-glucose, when applied before inoculation, was significantly more effective in controlling gray and blue mold than either *C. saitoana* or 0.2% 2-deoxy-D-glucose alone. Increasing the concentration of 2-deoxy-D-glucose from 0.2 to 0.6% resulted in no significant difference in the efficacy of the combination of *C. saitoana* and 2-deoxy-D-glucose. Apple fruit treated with the combination of 0.2 to 0.6% 2-deoxy-D-glucose and *C. saitoana*, and then inoculated with either pathogen, showed symptoms of infection only after 10 days of storage at 24°C while, among control fruit, lesions were visible by the third day of storage (*data not shown*).

A similar pattern in the control of decay was observed in lemon and orange fruit challenge inoculated with *P. digitatum* (Table 2). The level of control obtained from the combination of *C. saitoana* and 0.2% 2-deoxy-D-glucose on lemon and orange was significantly greater than either *C. saitoana* or 0.2% 2-deoxy-D-glucose alone. After 14 days of storage, decay inci-

dence among orange and lemon fruit treated with the combination of *C. saitoana* and 0.2% 2-deoxy-D-glucose in comparison to the *C. saitoana* treatment was threefold and twofold lower, respectively.

The combination of *C. saitoana* with 0.2% 2-deoxy-D-glucose, when applied 24 h after inoculation, was also effective in controlling decay of apple caused by *P. expansum* (Table 3); whereas, the applica-

Fig. 2. Population dynamics of *Candida saitoana* in cv. Red Delicious apple wounds in presence of 2-deoxy-D-glucose. Bars represent standard deviations.

Table 1. Effect of the combination of *Candida saitoana* with different concentrations of 2-deoxy-D-glucose on decay of apple cv. Red Delicious caused by *Botrytis cinerea* and *Penicillium expansum*

Treatment ^z	Infected fruit (%) ^y	
	<i>B. cinerea</i>	<i>P. expansum</i>
Control	100 a	100 a
0.2% 2-deoxy-D-glucose	71 b	65 b
0.6% 2-deoxy-D-glucose	41 c	47 c
<i>C. saitoana</i>	19 d	25 d
0.2% 2-deoxy-D-glucose + <i>C. saitoana</i>	7 e	12 e
0.6% 2-deoxy-D-glucose + <i>C. saitoana</i>	5 e	8 e

^y Means are averaged values of two experiments, each containing four replicates of 18 fruit each per treatment. Values followed by the same letter are not significantly different at $P = 0.05$, according to Duncan's multiple-range test.

^z Apple wounds were treated with a yeast-cell suspension (10^8 CFU ml⁻¹) containing 0, 0.2, or 0.6% (wt/vol) 2-deoxy-D-glucose, 0.2 and 0.6% (wt/vol) 2-deoxy-D-glucose, or sterile water. Treated wounds were then challenge inoculated with 20-μl spore suspensions of *B. cinerea* or *P. expansum*. Fruit were evaluated for symptoms of decay after 14 days of storage at 24°C.

Table 2. Effect of the combination of *Candida saitoana* with 2-deoxy-D-glucose on decay of navel orange cv. Washington and lemon cv. Eureka fruit caused by *Penicillium digitatum*

Treatment ^z	Infected fruit (%) ^y	
	Orange	Lemon
Control	100 a	100 a
2-deoxy-D-glucose	60 b	62 b
<i>C. saitoana</i>	45 c	55 c
2-deoxy-D-glucose + <i>C. saitoana</i>	12 d	28 d

^y Means are averaged values of three experiments, each containing four replicates of 60 oranges and 80 lemons each per treatment. Values followed by the same letter are not significantly different at $P = 0.05$, according to Duncan's multiple-range test.

^z Orange and lemon fruit were wounded, treated with a yeast-cell suspension (10^8 CFU ml⁻¹) containing 0 or 0.2% (wt/vol) 2-deoxy-D-glucose, 0.2% (wt/vol) 2-deoxy-D-glucose alone, or sterile water, and then challenge inoculated with 20 μl of *Penicillium digitatum* at 10^4 conidia ml⁻¹. Fruit were evaluated for symptoms of decay after 14 days of storage at 20°C.

tion of *C. saitoana* or 0.2% 2-deoxy-D-glucose alone, 24 h after inoculation, had no effect on disease development. After 1 week of storage at 24°C, the incidence of decay among apple fruit treated with *C. saitoana*, 2-deoxy-D-glucose, and sterile water was above 75%, while only 12% of the apples treated with the combination of *C. saitoana* and 0.2% 2-deoxy-D-glucose were diseased.

Similar control of decay by the combination of *C. saitoana* and 0.2% 2-deoxy-D-glucose was also observed in lemons and oranges challenged with *P. digitatum*. The application of the combination of *C. saitoana* and 0.2% 2-deoxy-D-glucose 24 h after inoculation with *P. digitatum* significantly controlled green mold of lemon and orange. The incidence of green mold among orange and lemon fruit treated with this combination was comparable to that observed after treatment with 1,000 mg/ml of imazalil (Table 3). The level of decay among fruit treated with either *C. saitoana* or 0.2% 2-deoxy-D-glucose alone was not significantly different from the water control.

DISCUSSION

The biocontrol of postharvest decay of apple by the antagonistic yeast *C. saitoana* and 2-deoxy-D-glucose was reported previously (7,8). In the present study, we demonstrated that the combination of *C. saitoana* with a low dose of 0.2% 2-deoxy-D-glucose has both protective and curative effects against gray and blue mold of apple and green mold of lemon and orange fruit. The effectiveness on citrus fruit was similar to that of imazalil, a common fungicide with worldwide usage. When applied prior to inoculation with a pathogen, the combination of *C. saitoana* with 0.2% 2-deoxy-D-glucose was more effective in controlling postharvest diseases of apple, lemon, and orange than either *C. saitoana* or 0.2% 2-deoxy-D-glucose when used alone. A similar protective effect was also reported in apple and pear fruit treated with a combination of *P. syringae* or *Sporobomyces roseus* and 2-deoxy-D-glucose when applied before inoculation (10).

Importantly, the combination of *C. saitoana* with 0.2% 2-deoxy-D-glucose, in addition to having a protective effect, had curative activity against blue and green mold of apple and citrus fruit, respectively, which was comparable to that observed with imazalil. Conversely, *C. saitoana* and 0.2% 2-deoxy-D-glucose, when applied alone 24 h after inoculation, did not inhibit disease development. The curative activity of a combination of *C. saitoana* with 2-deoxy-D-glucose represents a substantial improvement over existing microbial biocontrol products. Currently available microbial antagonists confer only a protective effect that diminishes with ripening and provides no control of previously established infections (16,18,21). The inability of antagonists to offer the level of control comparable to synthetic fungicides has been attributed in part to their inability to eradicate incipient infections that occur during and after harvest (1,6,21). Most wound infections of citrus and apple fruit occur during harvesting and subsequent handling; therefore, biological products, in order to be commercially acceptable, must protect wounds and control incipient infections in a manner similar to synthetic fungicides.

Although 2-deoxy-D-glucose is known to interfere with the growth of several filamentous fungi, including major postharvest pathogens (7,8,15), it showed no apparent effect on the growth of *C. saitoana* in wounds. This made it possible to exploit both the antifungal activity of 2-deoxy-D-glucose and the antagonistic activity of *C. saitoana*. The enhanced biocontrol activity of the combination of *C. saitoana* and 0.2% 2-deoxy-D-glucose observed strongly suggests a synergistic effect between *C. saitoana* and 2-deoxy-D-glucose. This was well illustrated by the superior performance of the combination of *C. saitoana* with 0.2% 2-deoxy-D-glucose in comparison to *C. saitoana* and 0.2% 2-deoxy-D-glucose alone. By itself, 2-deoxy-D-glucose at a low concentration (0.2% wt/vol) slightly reduced decay. When combined with *C. saitoana*, how-

ever, the inhibitory effect of 2-deoxy-D-glucose was amplified. The antifungal activity of 2-deoxy-D-glucose increases in the absence of carbohydrates (15). The ability of an antagonist to outcompete the pathogen for nutrients (4,9) could result in an increase in the ratio of 2-deoxy-D-glucose to utilizable sugar in the wound site to a level that is inhibitory to the pathogen. This may explain, in part, the observed curative effect of the combination of *C. saitoana* with 0.2% 2-deoxy-D-glucose.

In conclusion, combining antagonistic yeasts with 2-deoxy-D-glucose can be expected to provide a greater reliability and effectiveness than the approach utilizing biological agents alone.

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Table 3. Curative effect of the combination of *Candida saitoana* with 2-deoxy-D-glucose on decay of apple cv. Red Delicious, navel orange cv. Washington, and lemon cv. Eureka fruit

Treatment ^a	Infected fruit (%) ^b		
	Apple	Orange	Lemon
Control	98 a	100 a	83 a
2-deoxy-D-glucose	93 a	87 b	81 a
<i>C. saitoana</i>	88 a	83 b	77 a
2-deoxy-D-glucose + <i>C. saitoana</i>	12 b	14 c	11 b
Imazalil (1,000 µg/ml)	...	9 c	7 b

^a Means are averaged values of three experiments, each containing four replicates of 18 apples, 60 oranges, or 80 lemons each per treatment. Values followed by the same letter are not significantly different at $P = 0.05$, according to Duncan's multiple range test.

^b Fruit were challenge inoculated with spore suspension of *P. expansum* (apple) or *P. digitatum* (orange and lemon) at 10^4 conidia ml⁻¹ and, 24 h later, treated with a yeast-cell suspension (10^8 CFU ml⁻¹) containing 0 or 0.2% (wt/vol) 2-deoxy-D-glucose, 0.2% (wt/vol) 2-deoxy-D-glucose alone, imazalil (1,000 µg/ml; orange and lemon only), or sterile water. Decay was evaluated after 7 days of storage at 20°C.

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