Postharvest Biocontrol of Green and Blue Mold and Sour Rot of Citrus Fruit by *Debaryomyces hansenii*

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ABSTRACT

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The yeast *Debaryomyces hansenii,* isolated from the surface of lemon fruit, inhibited incidence of green and blue mold and sour rot of several citrus fruit cultivars. It was more effective against green mold than blue mold or sour rot. The lemon isolate of *D. hansenii* inhibited green mold on grapefruit more effectively than did any of eight other isolates of the same species. Efficacy of *D. hansenii* was maintained when applied simultaneously or prior to inoculation with *Penicillium digitatum.* Efficacy was reduced when *D. hansenii* was applied after inoculation. Control of green mold of grapefruit was maintained for 21 days at 11 or 22 C. The yeast antagonist also reduced the incidence of green mold decay of injured, naturally infected grapefruit stored at 11 C for 21 days. *D. hansenii* did not inhibit the growth in culture of *P. digitatum, P. italicum,* or *Geotrichum candidum.* A culture filtrate of the yeast antagonist failed to provide any protection against green mold of grapefruit. It may be that *D. hansenii* inhibits the pathogens by a mode of action other than antibiotic production.

The presence of pesticide residue in food is an issue of increasing public concern. In recent years, the possible adverse effect of chemical pesticides on human health (1,7) and the development of tolerance by pathogens to major fungicides have focused considerable attention on biological control of postharvest diseases of fruits (6,12).

An early observation of a potential microbial biocontrol agent of postharvest diseases of citrus was reported by Gutter and Littauer (5), who isolated from citrus fruit a strain of *Bacillus subtilis* (Ehrenberg) Cohn that inhibited the growth in culture of 10 citrus fruit pathogens. More recently, the efficacy of this bacterium as an antagonist of postharvest diseases was studied with citrus (10) and with other fruits (8,9,11,12). *B. subtilis* inhibited the pathogens by producing antibiotics (5,6,12), some of which have recently been characterized (4).

Our study was aimed at isolating non-antibiotic-producing antagonists of citrus postharvest pathogens from the surface of citrus fruits and testing their efficacy against these pathogens under laboratory conditions.

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MATERIALS AND METHODS

We assumed that naturally occurring microbial antagonists are present on the surface of citrus fruit in the orchard. This assumption was based on the low incidence of mold rots developing on nontreated control fruit that we frequently included in storage trials. In a recent semicommercial storage test of grapefruit stored at 11 C for 14 wk, for example, the incidence of green and blue mold on the nontreated control fruit was only 4.5%, compared to 37.0% in similar fruit that had been carefully rinsed with water and dried prior to storage. Presumably, rinsing the fruit removed the antagonists, in addition to removing part of the protective wax coating of the fruit. Furthermore, if we agitated freshly harvested grapefruits in sterile water and then plated the water on nutrient yeast-dextrose agar (NYDA), the plates yielded a dense bacterial and yeast population almost without any growth of filamentous fungi. If the liquid was diluted before plating, however, filamentous fungal colonies developed along with a few bacteria and yeast colonies (Fig. 1). Thus, the inhibition in culture of filamentous fungi, possibly including citrus fruit pathogens, may reflect the

natural conditions on the fruit surface. For these reasons, we selected potential antagonists either from nontreated control fruit that did not decay during extended storage or from freshly harvested citrus fruits from orchards that had not been exposed to chemical sprays for several months prior to picking. One of these antagonists, the yeast *Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij, isolated from lemon fruit and identified by the American Type Culture Collection (ATCC), was used in our present study.

Isolates of microorganisms present on the surface of the fruit were obtained by placing individual fruits in 600-ml beakers containing 200 ml of sterile water. Each beaker was placed on a rotary shaker at 100 rpm for 10 min. Wash water (0.1 ml) was then spread on a NYDA plate and allowed to incubate for 24 hr before colonies of isolates were selected. Each fruit received three consecutive washings using the same procedure. Selected colonies were then streaked three times across NYDA plates in order to obtain colonies likely to arise from a single cell. If all colonies on the plate at the final streaking appeared uniform, they were assumed to be pure; if not, they were streaked an additional three times. All cultures were stored on silica gel in a freezer until they could be tested on the fruit.

We obtained 200 isolates of yeasts and bacteria from citrus fruit surfaces using this procedure. A number of isolates appeared to be the same organism, but each was nonetheless tested on fruit for decay control. Each isolate was inoculated in 100 ml of nutrient yeast-dextrose broth (NYDB) in a 250-ml Erlenmeyer flask on a reciprocal shaker at 27 C for 48 hr.

Freshly harvested fruits were wiped with 95% ethanol and placed on moist paper in 50 × 100 × 15 cm plastic trays. Two to four conical wounds (3 mm deep and 3 mm wide) were cut in the fruit peel using the tip of a dissecting needle protruding from a rubber stopper. A few drops of the antagonist-NYDB mixture were then applied to each wound; sterile NYDB was applied to controls. Twenty μl of a conidial suspension was pipetted into the wound at the same time as the antagonist or at 1 hr, 3 hr, 7 hr, or 24 hr before or after the antagonist. The concentration of conidia was adjusted to one that resulted in 90–100% infection of control fruit after 6 days of incubation at 22–24 C. This conidia concentration varied from 5×10^3 to 5×10^5 conidia per milliliter, according to the season and the citrus cultivar. After inoculation, the tray was covered with a high-density polyethylene sleeve (to retain high humidity) and then incubated at 22–24 C for 24–48 hr, unless otherwise indicated. The number of inoculation sites in which decay developed was determined daily. Each treatment in each experiment consisted of at least three replicates of six fruits (36–72 inoculations per treatment). Each experiment was repeated at least three times.

The efficacy of the antagonist in reducing green mold infections of naturally infested grapefruit was evaluated on wounded fruit. Each fruit was wounded at four locations equally separated from each other, as described above. The fruit was then dipped momentarily in a cell suspension (10^9 cells per milliliter) of *D. hansenii*. Control fruit was dipped in water. The dipped fruit was dried at ambient temperature and then packed in commercial fruit cartons. There were two cartons per treatment, with 40 fruits in each one (for a total of 320 wounds per treatment). The packed cartons were stored at 11 C with 88–90% relative humidity and adequate fresh air introduction—the recommended storage conditions for Israeli grapefruit. The percentage of infection was determined weekly during a 3-wk storage period.

The antagonistic activity of *D. hansenii* was tested against *Penicillium digitatum* (Pers.:Fr.) Sacc., *P. italicum* Wehmer, and *Geotrichum candidum* Link, the wound pathogens responsible for the most important postharvest diseases of citrus fruit (green mold, blue mold, and sour rot, respectively) (3). Efficacy of the antagonist was evaluated on seven citrus cultivars, including grapefruit (*Citrus paradisi* Macf. 'Marsh Seedless'), oranges (*C. sinensis* (L.) Osbeck 'Shamouti' and 'Valencia'), lemon (*C. limon* (L.) Burm. 'Eureka'), Temple orange (tangor *C. reticulata* 'Blanco' × *C. sinensis*), kumquat (*Fortunella margarita* (L.) Osbeck), and pummelo (*C. grandis* (L.) Osbeck).

To evaluate the interactions between the antagonist and the pathogens in culture, we cut 15-mm-diameter disks from 5-day-old NYDA cultures of *D. hansenii* and then placed the disks on potato-dextrose agar (PDA) plates seeded with 0.5 ml of the conidial suspension. The effect of the yeast antagonist on the growth of the pathogens was compared with that of *B. subtilis*, a known bacterial antagonist.

We also evaluated the effect of two fungicides on the growth of *D. hansenii* and various pathogens in culture. In this case, 4-mm-diameter agar disks of the antagonist were placed on NYDA plates or 4-mm-diameter disks of the pathogen were placed on PDA plates. Each plate contained various concentrations of thiabendazole (TBZ) or imazalil.

RESULTS

Our screening of yeasts and bacteria obtained from fruit surfaces yielded several isolates that markedly inhibited

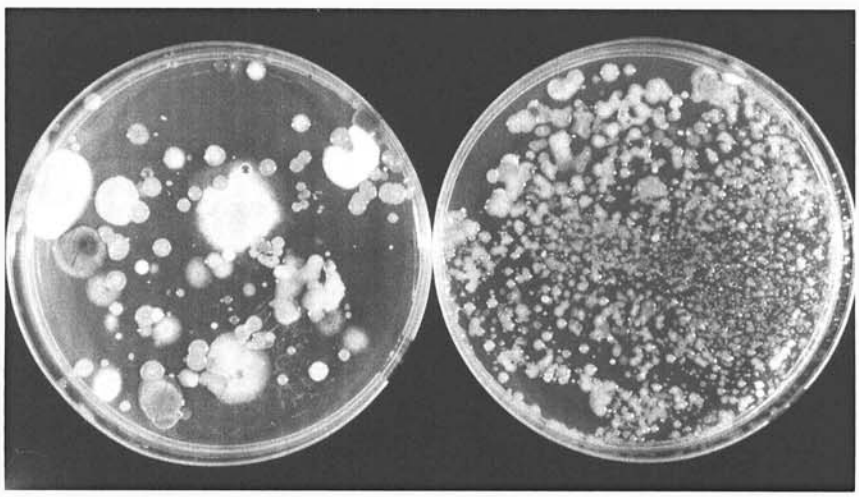

Fig. 1. Representative microorganisms present on the surface of citrus fruit in the orchard: (Right) Direct plating of the rinsing water, (left) plating after 10-fold dilution with sterile water.

Table 1. Inhibition of green mold, blue mold, and sour rot of several citrus fruit cultivars by the lemon isolate of *Debaryomyces hansenii* (US-7)

Citrus cultivar	Infection rate (%)[y]		
	Green mold	Blue mold	Sour rot
Grapefruit	6	6	9
Shamouti orange	10	15	...[z]
Valencia orange	8	19	...
Lemon	10	...	18
Temple orange	10	...	...
Pummelo	2	4	...

[y] Infection rate of control fruit was 96% or higher for green or blue mold and 77% or higher for sour rot. All values in the table are significantly different ($P = 0.05$) from controls according to Duncan's multiple range test.

[z] No data.

green and blue mold decay of grapefruit. One of these isolates, the yeast *D. hansenii*, was used in our present study.

The yeast's inhibitory activity against the green mold disease was highest after incubating for 48 hr in NYDB, as oppposed to shorter or longer incubation periods (*data not shown*). Cultures contained 1×10^9 cells per milliliter; these cultures were used in all our tests.

D. hansenii inhibited green mold, blue mold, and sour rot of several citrus fruit cultivars (Table 1). Although the antagonist reduced decay on all cultivars tested, its efficacy varied between cultivars; it was more effective on pummelo and less effective on the other cultivars. The antagonist was also more effective in reducing green mold than in reducing blue mold or sour rot. In kumquats, which were injured during picking and then dipped in the antagonist preparation, natural infections were reduced from 37% in the control to 12% in the treated fruit after 6 days of incubation (*data not shown*).

We compared the efficacy of the yeast isolated from lemon with that of eight different *D. hansenii* isolates obtained from the ATCC. While all isolates tested inhibited green mold infections, none was as effective as the lemon isolate (Table 2).

Table 2. Inhibition of green mold of grapefruit by the lemon isolate of *Debaryomyces hansenii* (US-7) and by eight isolates of the same organism obtained from the American Type Culture Collection (ATCC)

Isolate number	Source	Infection rate (%) after 6 days of incubation
18858	ATCC	63.1 b[z]
20220	ATCC	52.8 bc
10619	ATCC	52.0 bcd
34022	ATCC	47.1 bcd
36239	ATCC	39.8 bcd
18107	ATCC	39.0 bcd
36767	ATCC	22.0 cde
9367	ATCC	21.4 de
US-7	Lemon fruit	5.2 e
Control		98.5 a

[z] Values followed by different letters are significantly different ($P = 0.05$) according to Duncan's multiple range test.

The time of application of the antagonist (before, simultaneous with, or after inoculation) clearly affected disease inhibition (Table 3). Infection percentages were similar when *D. hansenii* was applied 1–24 hr before inoculation with pathogen conidia and when application and inoculation were simultaneous. Its efficacy was reduced if application was delayed until 3 hr or longer after inoculation.

We evaluated the persistence of the antagonistic activity of *D. hansenii* against green mold of grapefruit in inoculated fruit during incubation periods of up to 21 days. When fruit treated with the antagonist was incubated at 22 C, the incidence of infection normally did not increase after the first 7 days; incidence remained at 9–11% during the remainder of the incubation period (Table 4). In very susceptible fruit, such as grapefruit picked late in the season, incidence of infection reached 17–20% after 21 days of incubation. When the conidia concentration used to inoculate very susceptible fruit was reduced to 1×10^4 conidia per milliliter (which caused 80–90% infection of control fruit), the infection incidence among treated fruit was only 8% after 7 days of incubation. Incidence did not increase during the subsequent 2 wk of incubation (*data not shown*). At lower incubation temperatures, infection incidence gradually increased during the 3-wk incubation period. Under these conditions, infection incidence among treated fruit was 1% after 14 days and less than 7% after 21 days (Table 4).

Efficacy of *D. hansenii* was also maintained in naturally infected fruit stored at 11 C for 21 days (Table 5). While infection incidence increased to 22% after 14 days and to 33% after 21 days of storage, dipping the injured fruit in the antagonist preparation reduced the incidence of natural infections by 90% or more. Under these conditions, the TBZ-treated fruit maintained a low incidence of infection throughout the storage period.

Agar disks of *D. hansenii* NYDA cultures placed on PDA plates seeded with pathogens did not inhibit the growth of *P. digitatum, P. italicum,* or *G. candidum*. Under similar conditions, however, *B. subtilis* inhibited the growth of all pathogens by forming an inhibition zone (3- to 15-mm wide) around the disks. In addition, a filter-sterilized culture filtrate of a 48-hr-old culture of *D. hansenii* failed to provide any protection against green mold decay when applied to grapefruit wounds 1 hr before inoculation (*data not shown*).

The growth rate of *D. hansenii* on NYDA plates was determined also in the presence of various concentrations of TBZ and imazalil, two fungicides used to control postharvest rots of citrus fruit (3). The concentrations of these chemicals that inhibited 50% of the growth of the yeast antagonist were 3,000 ppm for TBZ and 5 ppm for imazalil. The corresponding concentrations for *P. digitatum* inhibition were 0.2 ppm for TBZ and 0.06 ppm for imazalil.

DISCUSSION

Our results show that *D. hansenii* isolated from lemon (isolate US-7) effectively reduced infection of the three major postharvest diseases of citrus. Disease control by the antagonist was not limited to specific citrus cultivars (Table 1). This nonspecific effect makes *D. hansenii* different from most biocontrol agents of fruits (6) and similar to *B. subtilis,* which antagonizes several postharvest pathogens (5,6,8,11). Unlike *B. subtilis,* however, *D. hansenii* did not inhibit the pathogens by producing antibiotics; it had no effect on the growth of the pathogens in culture and its culture filtrate had no antagonistic activity on pathogens inoculated onto fruit.

When compared with other isolates of the same yeast species, the US-7 isolate of *D. hansenii* exhibited the highest antagonistic activity against *P. digitatum* decay of grapefruit (Table 2). The relatively high efficacy of the isolate may be related to its being indigenous to the citrus fruit environment and, in particular, to nutritional and other conditions prevailing at the wound site. During our screening of other potential antagonists, we obtained several unidentified microbial isolates which inhibited infections by 50–80%, similar to the degree of inhibition of some of the *D. hansenii* isolates supplied by ATCC (Table 2). We considered this level of antagonism insufficient to warrant further testing.

The yeast antagonist exhibited its biocontrol activity most strongly against

Table 3. Inhibition of green mold of grapefruit by the lemon isolate of *Debaryomyces hansenii* (US-7) as affected by inoculation times of antagonist and pathogen

Treatment	Elapsed time between treatments (hr)	Infection rate (%)
Antagonist followed by pathogen	1	7.2 b[y]
	3	6.0 b
	7	5.2 b
	24	5.0 b
Antagonist and pathogen applied simultaneously	....	8.7 bc
Pathogen followed by antagonist	1	10.8 bc
	3	18.8 c
	7	72.0 d
	24	100.0 a
Control[z]	...	100.0 a

[y] Values followed by different letters are significantly different ($P = 0.05$) acording to Duncan's multiple range test.

[z] Delaying incubation of control fruit for up to 24 hr did not significantly affect the infection rate.

Table 4. Persistence of inhibition of green mold of grapefruit by the lemon isolate of *Debaryomyces hansenii* (US-7) at two incubation temperatures

Treatment	Infection rate (%) after incubation period		
	7 days	14 days	21 days
Incubation at 22 C			
Control	90.0 a[z]	100.0 a	100.0 a
D. hansenii	8.8 c	8.8 b	11.1 b
Incubation at 11 C			
Control	62.4 b	95.5 a	100.0 a
D. hansenii	0.0	1.1 c	6.7 b

[z] Within columns, values followed by different letters are significantly different ($P = 0.05$) according to Duncan's multiple range test.

Table 5. Inhibition of green mold of grapefruit by the lemon isolate of *Debaryomyces hansenii* (US-7) in injured, naturally infected fruit stored at 11 C

Treatment[y]	Infection rate (%) after storage period		
	7 days	14 days	21 days
Control	4.7 a[z]	21.8 a	33.3 a
D. hansenii	1.0 ab	2.6 b	2.6 b
Thiabendazole (2,000 ppm)	0.5 b	0.5 c	0.5 c

[y] Treatments were applied immediately after injury, before fruit was placed in storage.
[z] Within columns, values followed by different letters are significantly different ($P = 0.05$) according to Duncan's multiple range test.

infections that followed treatment of the injured fruit, not against infections already established in the fruit peel (Table 3). Nevertheless, the persistence of the antagonist's effect at normal and low temperatures (Table 4) and its relatively high efficacy in protecting naturally infected fruit that was packed and stored under conditions normally practiced by the industry (Table 5) suggest that biocontrol using *D. hansenii* might be achievable under commercial conditions. Moreover, isolate US-7 of *D. hansenii* was found to be more resistant than *P. digitatum* to two commonly used postharvest fungicides for citrus (TBZ and imazalil). Therefore, it might be possible to use *D. hansenii* and the chemical fungicides in combination, making it possible to reduce fungicide concentrations while maintaining adequate decay control. In addition, the very wide distribution of *D. hansenii* in food products (2), the lack of any known related species that cause plant diseases, and, particularly, the finding that no antibiotics are produced by *D. hansenii,* all increase the potential applicability of this biological control agent for combating postharvest diseases of fruits.

Our results do not clarify the mode of action of *D. hansenii* in the inhibition of citrus postharvest decay nor do they fully assess its efficacy under commercial practices. These areas require further study before the full potential of *D. hansenii* can be assessed.

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