Biological Control of Postharvest Diseases of Grape, Peach, and Apple with the Yeasts *Kloeckera apiculata* and *Candida guilliermondii*

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ABSTRACT

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A yeast, strain 138 of *Kloeckera apiculata*, isolated from the surface of grapes, was evaluated for its activity in reducing postharvest decay of grape, peach, and apple fruits. In an artificial infection assay in which detached grapes were immersed in an aqueous suspension of yeast cells (5×10^8 cfu/ml) and spray-inoculated with 10^3 sporangiospores per milliliter of *Rhizopus stolonifer*, strain 138 reduced decay, whereas strain 87 (= US-7) of *Candida guilliermondii* did not. In an assay in which naturally infected fruits were immersed in yeast suspensions, both strains were effective in reducing postharvest decay of grapes caused by *R. stolonifer*; however, neither yeast was effective in reducing decay caused by *Aspergillus niger*. Three strains of *K. apiculata* obtained from other sources were compared to strain 138 and strain 87 for activity against Rhizopus rot of peach and gray mold of apple. Fruit wounds were pretreated with water-suspended yeast cells (10^8 cfu/ml) and inoculated with 10^3 sporangiospores of *R. stolonifer* per milliliter or 10^5 conidia of *Botrytis cinerea* per milliliter. Two of three strains of *K. apiculata* were as effective as strains 138 and 87 for the control of Rhizopus rot of peach. All strains were equally effective in reducing gray mold of apple. Control of gray mold and blue mold of apples and Rhizopus rot of peaches was enhanced when strain 138 was applied as an aqueous suspension (10^8 cfu/ml) in 2% $CaCl_2$. This method did not reduce the incidence of brown rot of peach, caused by *Monilinia fructicola*; however, decreased lesion diameter was observed in fruit treated with yeast and $CaCl_2$.

Additional keywords: calcium chloride, *Debaryomyces hansenii, Hanseniaspora uvarum, Penicillium expansum*

Postharvest losses in fruit caused by fungal decay can be extensive. Losses can be reduced by postharvest treatment of fruit with fungicides or microbial antagonists. Fungal and bacterial antagonists, investigated as alternatives to fungicides, have been found effective for the control of various postharvest diseases in peach (20,22,23), apple (13,14,15,21,24), pear (15), citrus (5,6,11,27), cherry (26), and grape (3,9). Specifically, the biocontrol agents that have been studied for the control of postharvest diseases of peach have included *Bacillus subtilis* (Ehrenberg) Cohn for the control of brown rot, caused by *Monilinia fructicola* (G. Wint.) Honey (22,23), and *Enterobacter cloacae* (Jordan) Hormaeche and Edwards for the control of Rhizopus rot, caused by *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill. (26). For the control of apple decay caused by *Penicillium expansum* Link and *Botrytis cinerea* Pers.:Fr., the antagonistic microorganisms have included the filamentous fungus *Acremonium brevae* (Sukapure & Thirumalachar) W. Gams (13), the bacterium *Pseudomonas cepacia* Palleroni and Holmes (15) and several species of yeasts (14,21).

Yeast strains of *Cryptococcus laurentii* (Kuff.) C. E. Skinner and *Candida guilliermondii* (Castellani) Langeron & Guerra (erroneously identified as *Debaryomyces hansenii* (Zopf) Lodder & Kreger van-Rij [16]), have been studied for the biological control of gray and blue molds of apple (18,19,21,24). A proposed means by which strains of *C. guilliermondii* elicit control is nutrient competition (8). Data from our laboratory indicate that *C. guilliermondii* strains also may effect partial degradation of the mycelium of *B. cinerea* (4,30).

Other species of yeast could be more effective for reducing postharvest diseases than those that have been reported to date. This report focused on a yeast strain isolated from the fruit surface of grape, strain 138 of *Kloeckera apiculata* (Reess emend. Klöcker) Janke. Yeast strains identified as *K. apiculata* or its teleomorphic state, *Hanseniaspora uvarum* (Niehaus) Shehata et al, are frequently isolated from the fruit surface of grapes (2,17,25). This research evaluates the effectiveness of this strain, additional strains of *K. apiculata* from various sources, and one strain of *C. guilliermondii* for their relative activity against postharvest diseases of grape, peach, and apple fruits. This report also evaluates biological control activity in the presence and absence of calcium chloride, a method that has been shown to enhance the biocontrol effectiveness of strains of *C. guilliermondii* (18,21). Earlier findings of this research were reported elsewhere (20).

MATERIALS AND METHODS

Source of fruit. Grapes of the cultivar Thompson Seedless were harvested at commercial maturity (14–16 °Brix) and used immediately in experiments. Apples (cv. Golden Delicious) were harvested at commercial maturity and stored at 2 C for 3 mo or less before use. Peaches (cv. Loring) were picked at the firm ripe stage (5.5–8.5 kg of pressure) and stored at 2 C for 1 wk or less prior to use.

Yeast and pathogen strains. *C. guilliermondii* strains 87 (= US-7) and 101 were isolated from the surface of lemon fruit as previously reported (27). Strains 143 (ATCC 32369), 144 (ATCC 34436), and 145 (ATCC 34537) of *K. apiculata* were obtained from the American Type Culture Collection (Rockville, MD). Strain 138 of *K. apiculata* was isolated from the surface of Thompson Seedless grapes as follows. Grapes were picked from clusters, and three berries were added to each of several 125-ml Erlenmeyer flasks containing 50 ml of sterile distilled water. The flasks were shaken in a reciprocating shaker at 250 rpm for 30 min, and aliquots of serial dilutions were plated onto potato-dextrose agar (PDA) or nutrient-yeast extract-dextrose agar (NYDA) (21). Single colonies were isolated and restreaked onto NYDA for reisolation. The culture was identified by the Centraalbureau Voor Schimmelcultures (Baarn, Netherlands). Cultures were routinely stored on silica gel and recovered by plating onto NYDA.

Isolates of *R. stolonifer, P. expansum*, and *B. cinerea* were obtained from de-

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cayed fruit and stored as lyophilized cultures or on PDA.

Culture conditions and preparation of yeast and pathogen spore suspensions. Yeast strains were streaked on NYDA plates and incubated for 48 hr at 27 C. Cultures in nutrient-yeast dextrose broth (NYDB), 50 ml in a 250-ml Erlenmeyer flask, were started with a concentration of approximately 10^8 cfu of yeast. For experiments on peach and apple fruits, cultures were incubated for 18–24 hr at 26 C on a rotary shaker at 200 rpm until the culture reached late log phase. Tests on grapes utilized 48-hr cultures of the yeast strains. Cells were then pelleted by centrifugation, resuspended in sterile distilled water, and adjusted to a concentration of 5×10^8 cfu/ml (grape tests) or 1×10^8 cfu/ml (peach and apple tests) (21).

Conidia of *B. cinerea* were obtained from 2-wk-old PDA 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 cultures incubated at 27 C in the dark (21). Sporangiospores of *R. stolonifer* were obtained from 3-day-old PDA cultures grown at 27 C in the dark. Conidial suspensions of *M. fructicola* were prepared from sporulating lesions on inoculated peach fruit. Conidial suspensions of the pathogens were prepared as previously described (21).

Biocontrol assays on grape. In natural infection assays, individual berries (cv. Thompson Seedless) were removed from clusters at the stem so as to provide a wound to facilitate infection. Twenty berries were placed in a plastic mesh basket ($20 \times 20 \times 15$ cm) and immersed for 1 min in an aqueous or culture broth suspension containing 5×10^8 cfu/ml yeast. In artificial infection assays, berries were treated with yeast cells as previously described, dried, and then sprayed to runoff with 10^4 sporangiospores per milliliter of *R. stolonifer* using a thin-layer chromatography sprayer. Berries were air-dried and incubated at 22 C under high humidity in enclosed plastic tubs ($47 \times 35 \times 12$ cm) containing water (27). There were three replicates of 20 berries per treatment, with one replicate of each treatment per container. Treatments were randomized within each container. Percent fruit infection was measured at 48 hr after inoculation. This test was conducted four times.

Biocontrol assays on apple and peach fruit. Fruits were surface-disinfested with 10% commercial bleach and air-dried 4 hr prior to wounding (21). They were then placed, 20 per tray, on sterile styrofoam packing trays inserted in plastic tubs. Single wounds (5 mm deep and 3 mm wide) were made on each fruit as previously described (27). Immediately after wounding, 50 μl of an aqueous yeast suspension (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 temperature (24–26 C) before challenge with 20 μl of pathogen spore suspension. The challenge inoculum was 10^3 sporangiospores per milliliter of *R. stolonifer* or 10^5 conidia per milliliter of *B. cinerea*. Additional tests were conducted with peach and apple fruits to determine biocontrol activity against brown rot and blue mold, using 20-μl inoculations with suspensions containing 10^4 conidia per milliliter of *M. fructicola* or *P. expansum*. There were three replicate trials of 10 fruits per treatment. Treatments were randomized in each test, with five fruits per treatment in each tray. Fruits were incubated at 24 C at high humidity and observed for percent infection at 4 (brown rot) or 7 (blue mold) days after challenge inoculation.

Effect of calcium chloride on biocontrol activity. Calcium chloride was included in cell suspensions of strains 87 and 138 in one set of experiments. Yeast cell suspensions were prepared with washed cells from 18-hr NYDB cultures at 10^8 cfu/ml in either distilled water or 2% $CaCl_2 \cdot 2H_2O$. Fruits were treated with these suspensions and challenged as in the previous assay. There were six trials consisting of 10 replicate fruits per treatment in the apple biocontrol tests, and there were three trials of 20 replicate fruits per treatment in the peach biocontrol tests. Treatments were randomized as in the previous test. Fruits were incubated at 24 C at high humidity and observed for percent infection at 7 days after challenge inoculation.

Statistical analyses. Data analysis was performed with the SAS statistical package (1). Analysis of variance was performed on arcsine square root-transformed data by the general linear models procedure. Residuals analyses from the analysis of variance were done to evaluate the need for data transformation. Factorial analysis of variance was performed on tests that included $CaCl_2$ in yeast treatments. Mean separations were performed using the least significant difference method.

RESULTS

Biological control of postharvest decay in grape. In fruit inoculated with 10^4 sporangiospores per milliliter of *R. stolonifer*, 63% of the check fruit (H_2O control) were decayed after 48 hr (Table 1). Decay was significantly ($P < 0.05$) reduced in treatments with NYDB- and H_2O-suspended cells of strain 138. Cells of strain 138 suspended in H_2O were the most effective treatment for reducing Rhizopus rot. Water-suspended cells of strain 87 and cell-free culture filtrate of strain 138 did not significantly reduce decay relative to the H_2O control treatment.

Natural infection of grapes was significantly reduced by strains 138 and 87 as compared to the water control (Table 1). There was no decay caused by *R. stolonifer* in fruit treated with either yeast strain, but there was substantial rot in the check. Decay caused by *Aspergillus niger* Tiegh. was evident in each of the treatments, and there was no evidence of control by either yeast strain.

Biological control of postharvest decay of apples and peaches. Strains 138, 87, and 101 and several strains (143, 144 and 145) of *K. apiculata* obtained from the American Type Culture Collection were compared for their relative activity against Rhizopus rot of peach and gray mold of apple (Table 2). In peach fruit inoculated with *R. stolonifer*, percent rot measured 4 days after challenge was

Table 1. Effect of strain 138 of *Kloeckera apiculata* and strain 87 of *Candida guilliermondii* on biological control of postharvest rots in Thompson Seedless grapes[x]

	Infected fruit (%)[y]		
		Natural infection	
Strain and treatment[z]	Inoculated with *Rhizopus stolonifer*	*R. stolonifer*	*Aspergillus niger*
K. apiculata			
138, H_2O	12.0 c	0.0 b	5.0 a
138, NYDB	30.0 bc	NT	NT
138, culture filtrate	65.0 a	NT	NT
C. guilliermondii			
87, H_2O	40.0 ab	0.0 b	18.0 a
H_2O control	63.0 a	70.0 a	10.0 a

[x] Twenty fruit were placed in a plastic mesh basket ($20 \times 20 \times 15$ cm) and immersed in yeast suspensions for 1 min. They were then air-dried and sprayed with 10^4 sporangiospores per milliliter of *R. stolonifer* in the artificial infection assay. They were incubated at 22 C in a moisture chamber in both assays.

[y] Average percent infection measured after 48 hr (fruit inoculation assay) or 5 days (natural infection assay) of incubation. There were three replicates of 20 fruit per treatment. Treatments were completely randomized, and each test was conducted three times. NT = not tested. Treatment means with different letters are significantly different ($P \leq 0.05$), according to LSD mean separation.

[z] Yeast suspensions were harvested from 48-hr nutrient-yeast dextrose broth (NYDB) cultures, washed, and suspended in either sterile distilled water at 5×10^8 cfu/ml (138, H_2O and 87, H_2O) or in sterile NYDB at 5×10^8 cfu/ml (138, NYDB). Cell-free culture broth from a 48-hr culture of yeast strain 138 (138, culture filtrate) was prepared for one of the treatments.

lowest when pretreated with strains 138 (23.3%), 101 (34.4%), 87 (35.2%), and 144 (36.7%). In control treatments in which peach fruits were not pretreated with yeast cells, 100% of the fruit were infected at 4 days. Strain 143 of *K. apiculata* was ineffective. All of the yeast strains reduced gray mold of Golden Delicious apples to 7–18%, compared to 75% in the nonyeast check (Table 2). Assays were also conducted to determine the activity of yeast strain 138 against brown rot of peach, caused by *M. fructicola*, and blue mold of apple, caused by *P. expansum*. Four days after inoculation of Loring peach with *M. fructicola*, all the fruits in the check treatment and the yeast pretreatment were infected. However, yeast strains 138 and 87 significantly reduced lesion diameters on fruit (34.5 and 31.5 mm, respectively) as compared to nonyeast check fruit (43.6 mm diameter). In assays in which Golden Delicious apples were inoculated with conidia of *P. expansum*, 75–80% of the fruit treated with the yeast strains were infected at 7 days, compared with 100% infection in the nonyeast check.

Effect of calcium chloride on biocontrol activity. Strains 87 and 138 were also compared for activity against Rhizopus decay of peach and gray mold of apple when applied in 2% aqueous solutions of $CaCl_2$ (Table 3). In each test, there was a significant reduction of disease by $CaCl_2$-yeast treatments, as compared to yeast treatments without $CaCl_2$ ($P < 0.01$ in gray mold test on Golden Delicious apple and $P < 0.05$ in the Rhizopus rot test on Loring peach). Factorial analysis did not show a yeast and $CaCl_2$ interaction. Calcium chloride applied without yeast cells did not significantly reduce the amount of disease. In yeast and $CaCl_2$-yeast treatments for activity against Rhizopus rot of peach, levels of statistical significance were $P = 0.01$ with strain 138 treatments and $P = 0.05$ for strain 87 treatments as compared to the checks.

In other tests for activity of $CaCl_2$-yeast combinations, control of brown rot of peach and blue mold of apple was evaluated. Calcium chloride–yeast cell suspensions did not enhance control of brown rot of peach. Seven days after inoculation, percent infection in fruits treated with $CaCl_2$ treatments plus yeast strains 138 and 87 was 97 and 100%, respectively.

In the test for control of blue mold of apple, calcium chloride amendment significantly enhanced control with both yeast strains. Seven days after inoculation, percent infection in control (minus $CaCl_2$) fruit treated with 138, 87, and H_20 was 96.7–100%, whereas in treatments with $CaCl_2$, percent infection was 77.5% ($P < 0.05$), 24.0% ($P < 0.01$), and 100%, respectively.

Table 2. Activity of *Kloeckera apiculata* and *Candida guilliermondii* strains against Rhizopus rot of Loring peach and gray mold of Golden Delicious apple[y]

	Infected fruit (%)[z]	
Yeast strain	**Rhizopus rot of peach**	**Gray mold of apple**
C. guilliermondii		
87	35.2 a	6.7 a
101	34.4 a	NT
K. apiculata		
138	23.3 a	15.0 a
143	86.7 bc	12.5 a
144	36.7 a	12.5 a
145	50.0 ab	17.5 a
None	100.0 c	75.0 b

[y] Individual fruit wounds were treated with 50-µl aliquots of aqueous cell suspensions (10^8 cfu/ml) of each yeast strain. Control treatments without yeast consisted of fruit treated with 50 µl of sterile distilled water. Fruit were challenged 2 hr afterwards with 20 µl of a 10^3 cfu/ml sporangiospore suspension of *Rhizopus stolonifer* (peach) or a 10^5 cfu/ml conidial suspension of *Botrytis cinerea* (apple). There were three trials consisting of 10 replicate fruits per treatment.

[z] Percent infection recorded at 4 (peach) or 7 (apple) days after inoculation. Treatment means within a column that are followed by different letters are significantly different ($P \leq 0.05$) according to the least significant difference method. NT = not tested.

Table 3. Effect of calcium chloride on biological control of Rhizopus rot of Loring peach and gray mold of Golden Delicious apple with strain 87 of *Candida guilliermondii* and strain 138 of *Kloeckera apiculata*[y]

	Fruit rot 7 days after inoculation (%)[z]			
	Gray mold of apple		Rhizopus rot of peach	
Yeast strain	**−$CaCl_2$**	**+ $CaCl_2$**	**− $CaCl_2$**	**+ $CaCl_2$**
C. guilliermondii strain 87	18.0**	2.0**	50.0*	24.0*
K. apiculata strain 138	27.9**	4.0**	33.7*	13.3**
None	100.0	100.0	92.7	79.3

[y] Individual fruit wounds were treated with 50-µl aliquots of yeast strains 138 or 87 (10^8 cfu/ml) suspended in sterile distilled water (−) or in 2% (w/v) $CaCl_2$ (+). Control treatments consisted of fruit pretreated with 50 µl of sterile distilled water or 2% $CaCl_2$. Fruit were challenged 2 hr later with 20 µl of a 10^3 sporangiospore suspension of *Rhizopus stolonifer* (peach) or a 10^5 cfu/ml conidial suspension of *Botrytis cinerea* (apple). There were three to six trials consisting of 10–20 replicates per treatment.

[z] Treatment means within a column followed by asterisks are significantly different from the water check (* = $P \leq 0.05$, ** = $P \leq 0.01$). Data were analyzed using the least significant difference method.

DISCUSSION

This study demonstrates that strain 138 of *K. apiculata* significantly reduces diseases in grape, peach, and apple caused by *R. stolonifer, B. cinerea*, and *Penicillium digitatum* (Pers.:Fr.) Sacc. The degree of control conferred by this strain was equivalent to that conferred by strain 87 of *C. guilliermondii* in tests for activity against gray mold and blue mold on apple and Rhizopus decay on peach. Strain 138 resulted in better control of Rhizopus rot on grape when spore levels of *R. stolonifer* were high (Table 1). Ben-Arie et al (3) have also shown that strain 138 will significantly reduce Rhizopus rot of grape when infected berries are nested among healthy berries.

Strains of *K. apiculata* that were compared with strain 138 were equal in their ability to reduce gray mold of apple. In tests of these strains for activity against Rhizopus decay of peach, a greater range of effectiveness was observed. One of the strains was totally ineffective for controlling Rhizopus decay.

Further tests of yeast strain 138 are needed to determine its suitability for biological control under conditions of low temperature and controlled atmosphere conditions. Experience with strain 87 has shown that results obtained under 24 C incubation conditions are likely to predict results obtained at lower temperatures (5). Regardless, additional tests are needed to determine if these yeasts can reduce postharvest decays, such as Aspergillus rot of grape and brown rot of peach, that are not controlled by these yeasts at room temperature. Conditions of cold storage may also improve control of blue mold of apple, in which strains 138 and 87 have been observed to be marginally effective for reducing decay.

Results indicated that control of Rhizopus decay of grape may be improved under conditions of natural infection, as compared to the assays in which fruit were artificially inoculated. The inoculum level of *R. stolonifer* in the artificial infection assay may overwhelm or substantially reduce the yeast's ability to

effect control. This has been observed in similar tests on peach (*data not shown*). Control of postharvest diseases by yeast strain 87 is directly related to the spore concentration of the pathogen and the cell concentration of the yeast (8,21). This relationship underscores the need to conduct evaluations of these yeasts under conditions that simulate probable infection conditions in the postharvest environment.

Calcium chloride improved the activity of strain 138 against gray and blue molds of apple and Rhizopus rot of peach. Enhancement of control of the apple postharvest decays has been observed in similar tests with strain 87 of *C. guilliermondii* (21). Lack of enhanced control of brown rot is disappointing; however, the ability of this treatment to slow disease progress, as evidenced by reduced lesion diameter, suggests that additional tests conducted under conditions of cold storage may be a productive line of research. Yeast applications also could be combined with reduced levels of fungicides or with a biocontrol agent that is effective against brown rot (20,21).

Additional research is needed to determine if yeast strains 138 and 87 have similar mechanisms by which they confer disease control. Tests with cell-free culture filtrates (Table 1) indicate that strain 138 does not produce antibiotics that could effect biological control. Similar tests also have shown that strain 87 does not produce antibiotics in culture (8). Other studies have indicated that strain 87 may control postharvest diseases by both nutrient competition to limit spore germination and by production of extracellular substances in the wound site that cause collapse and degradation of fungal hyphae (4,8,28,29). The mechanism of $CaCl_2$-enhancement of disease control by strain 87 is poorly understood; however, one study indicates that yeast cells do not have to be present in order for this salt to inhibit germination of *B. cinerea* spores (18).

K. apiculata, or its teleomorphic state *Hanseniaspora uvarum*, is a naturally occurring epiphyte on the surface of various tree and vine fruits (2,17,24). This yeast is the predominant component of the epiphytic microflora on ripening or ripened grapes (17). Some reports have shown that certain strains of *K. apiculata* are associated with decay of strawberries (7) and sour rot of grapes (10). In contrast, our studies with strain 138 and strains of *K. apiculata* used for comparison (Table 2) did not show any evidence of decay by these yeasts. Decay or sour rot of grapes has not been observed in tests with strain 138.

Strain 138 of *K. apiculata* appears to be well suited for the control of several postharvest diseases of fruit. Since this yeast has been shown to be as effective as strain 87 of *C. guilliermondii* in some circumstances, it is now possible to consider it as an alternative treatment for the control of postharvest decays. This is an attractive possibility, since future use of strain 87 for biocontrol may be limited by concerns about potential residues on treated fruit. Strains of *C. guilliermondii* have been isolated from humans and have been determined to be opportunistic pathogens (12). In contrast, *K. apiculata* does not have this potential, since it cannot grow at 37 C. Preliminary tests on the toxicology of strain 87 have been negative (*data not shown*). Variation within strains of *C. guilliermondii* for potential human pathogenicity is poorly understood. Strains of this species occur in a wide variety of habitats, including fruit surfaces, and they exhibit considerable heterogeneity in their physiological characteristics (16,17). At this stage, however, rigorous toxicological testing is needed to establish whether strain 87 of *C. guilliermondii* poses a human health risk.

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