Fruit Surface Colonization and Biological Control of Postharvest Diseases of Pear by Preharvest Yeast Applications

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ABSTRACT

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The yeasts *Cryptococcus infirmo-miniatus*, *Cryptococcus laurentii*, and *Rhodotorula glutinis*, applied to Bosc and d'Anjou pear fruit in the field 3 weeks prior to harvest, maintained high population levels through harvest, while populations of *Candida oleophila* declined after 1 and 2 weeks, and by harvest were not significantly different from total yeast populations on untreated fruit. Yeasts were sprayed individually on fruit at concentrations of 1 to 3×10^8 CFU/ml, with approximately 2 ml applied per fruit. Initial population sizes for all four species averaged 5×10^6 CFU per fruit. *C. infirmo-miniatus* provided the most consistent decay control in fruit treated 3 weeks before harvest. *C. infirmo-miniatus* and *R. glutinis* also provided significant postharvest decay control in Bosc fruit treated 1 day before harvest.

Most postharvest disease in pear fruit (*Pyrus communis*) is initiated at wounds that occur during harvest or packing. Subsequent infection occurs at the wound by one of several pathogens (17,20). *Penicillium expansum* and *Botrytis cinerea* (causal agents of blue mold and gray mold, respectively) are relatively aggressive, fast-growing pathogens. *Phialophora malorum*, *Cladosporium herbarum*, and *Alternaria alternata* are less aggressive pathogens (causing side rots) that become problems in fruit stored for longer periods (11). *Phialophora malorum* and *A. alternata* are not sensitive to thiabendazole (TBZ) (9), the most commonly used postharvest fungicide for pome fruit in the United States. Most strains of *P. expansum* and *B. cinerea* are sensitive to TBZ, although resistant isolates are frequently encountered (15).

Several microorganisms have been discovered that are able to colonize wound sites and compete with and reduce pathogen establishment at those sites. *Cryptococcus infirmo-miniatus* strain YY6, *Rhodotorula glutinis* strain HRB6 (3), and *Cryptococcus laurentii* strain RR87-108 (12) are yeasts that were originally isolated from the surfaces of pear or apple fruit in the Pacific Northwest. When inoculated into wounds on pears and apples after harvest in combination with pathogens, these yeasts have provided good control of rots caused by *P. expansum*, *B. cinerea*, and

Phialophora malorum (3,12,19,21). *Candida oleophila* strain I-182 has also been effective in reducing postharvest decays (7) and is the active component of the biological fungicide Aspire (Ecogen Corp., Langhorne, PA), which is registered for postharvest use on pear.

While several studies have demonstrated the potential for biocontrol of postharvest disease when applied after harvest, few have addressed the practicality of applying biocontrol agents to the fruit while in the field with the purpose of controlling postharvest decays. Spotts and Chand-Goyal (16) found that *C. infirmo-miniatus* and *C. laurentii* reduced blue mold decay when applied to pear wounds 24 h after inoculation with *P. expansum*, but after 72 h only provided control when combined with a fungicide. This indicates the importance of prompt application of biocontrol agents and the potential value of applying biocontrol agents in the field prior to incidence of wounding during harvest (17).

An important consideration in preharvest application of biocontrol agents is the ability of the microorganisms to survive at sufficient populations on the fruit surface after application. The weather in pear growing regions is generally hot and dry in the preharvest period, which may have detrimental effects on yeast populations. Yeast may also be adversely affected by pesticide sprays (4) or washed off the fruit surface during irrigation or spraying. However, since these yeasts were originally isolated from fruit surfaces after or near harvest (3,12), they might be tolerant of these conditions. Some yeasts can colonize plant surfaces or wounds for long periods under dry conditions and produce extracellular polysaccharides that enhance their survival and restrict pathogen colonization sites (8,24).

The objectives of this work were to study the survival of the specific yeast biocontrol agents on fruit when applied prior to harvest, and to determine the ability of biocontrol agents applied prior to harvest to control postharvest decay on wounded pears during cold storage.

MATERIALS AND METHODS

Culture and preparation of biocontrol agents. The yeasts *C. infirmo-miniatus*, *R. glutinis*, and *C. laurentii* were used in 1996. These yeasts plus *Candida oleophila*, isolated from Aspire, were included in the experiment in 1997. Cultures of *C. infirmo-miniatus* and *R. glutinis* were supplied by R. A. Spotts (Mid-Columbia Agricultural Research and Extension Center, Hood River, OR). A culture of *C. laurentii* was provided by R. G. Roberts (USDA, Wenatchee, WA).

Yeast cultures stored at -20°C were activated by pouring 1 ml of thawed suspension into 75 ml of yeast malt dextrose broth (YMDB: 3 g of malt extract, 3 g of yeast extract, 5 g of peptone, and 10 g of dextrose per liter of medium). The suspensions were incubated on a shaker for 2 days at 20°C . After incubation, 0.1-ml aliquots of each yeast were spread on petri dishes containing yeast malt dextrose agar (YMDA: YMDB plus 18 g of agar per liter) and incubated for 2 to 3 days at room temperature. The colonies were then scraped from the plates with a sterile rubber spatula and suspended in sterile distilled water. Suspensions of *C. laurentii* were adjusted to a concentration of 2.8 to 3.3×10^8 CFU/ml by adjusting to 2% transmittance at 595 nm using a Spectronic 20 spectrophotometer. Suspensions of *C. infirmo-miniatus* and *R. glutinis* were adjusted to 1.0 to 1.5×10^8 CFU/ml by adjusting to 2% transmittance at 550 nm, and *Candida oleophila* was adjusted to approximately 1×10^8 CFU/ml by adjusting to 2% transmittance at 550 nm and diluting by one-half. Spectrophotometric determinations of yeast concentrations were confirmed by dilution plating of treatment suspensions.

Fruit. Pear fruit (cvs. Bosc and d'Anjou) were grown at the Southern Oregon Research and Extension Center in Medford, Oregon. In addition to being the principal winter pear cultivars of the Pacific Northwest, d'Anjou and Bosc present contrasting fruit surfaces, which could potentially impact the survival of microor-

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ganisms. d'Anjou pears have a smooth, waxy surface, while Bosc pears are coarsely russeted. Trees for treatment with each yeast species and untreated controls were selected at random throughout 0.5-ha blocks of each cultivar. The orchards were managed under a commercial spray program with under-tree sprinkler irrigation. Yeast suspensions were applied to fruit on the tree using a hand-held spray bottle, at a rate of approximately 2 ml per fruit. Ap-

proximately 30 fruit on each tree received the yeast application. Each treatment was replicated five times in 1996 and four times in 1997, with each tree as a replicate.

Yeast applications for population sampling were made 3 weeks prior to harvest (Bosc: 20 August 1996 and 13 August 1997; Anjou: 23 August 1996 and 13 August 1997). Yeast were also applied in the same manner to a separate set of fruit 1 day prior to harvest for postharvest patho-

gen inoculation tests. Climatic data were collected during the field experiments by an AgriMet weather station (U.S. Bureau of Reclamation) located approximately 250 m from the experimental orchard.

Population sampling. Three uniform-sized fruit from each replicate were sampled once per week to determine the number of yeast CFU on the fruit surface. The three fruit were combined in beakers containing 900 ml of 0.05 M sterile phosphate buffer (SPB) with 0.006% (vol/vol) Tween 20. The fruit and buffer were stirred for 5 min using a magnetic stirrer and then placed in a sonicating bath for 5 min (2). A 1-ml sample was removed from each beaker immediately after sonication, and 10-fold dilutions were made to 10^{-5} in SPB.

Samples of each dilution were then placed on petri dishes containing a selective, dilute YMDA medium (1.5 g of malt extract, 1.5 g of yeast extract, 2.5 g of peptone, 5 g of dextrose, and 18 g of agar per liter of medium, with 100 ppm chloramphenicol, 50 ppm ampicillin, and 2 ppm dichloran). Chloramphenicol and ampicillin were included to inhibit bacterial growth; dichloran was used to inhibit radial growth of filamentous fungi. The plates were incubated for 2 to 3 days at room temperature. After incubation, population levels were determined using the plate-dilution frequency technique of Harris and Sommers (6). The four yeast species used in this experiment exhibit distinct colony color and morphological characteristics, which were used to visually distinguish the different species. For a given treatment, only yeast species that were applied to that treatment were counted in the population samples. To determine population levels on the untreated controls, only colonies resembling those of the yeast species used in the treatments were counted.

Control of postharvest decay. Ten fruit from each replicate to be used for postharvest treatment were randomly selected from the harvested samples. Each fruit was wounded five times with the sterile head of a finishing nail (6 mm diameter $\times$ 3 mm deep), then placed into cold storage at -0.5°C for up to 4 months. In 1996, only fruit that were sprayed with biocontrol agents 1 day before harvest were wounded for postharvest experiments. In 1997, fruit sprayed both 3 weeks and 1 day before harvest were included.

Lesions on the fruit were counted, and the type of decay was determined visually after 2 and 4 months of storage at -0.5°C . The types of decay were divided into three categories and recorded as blue mold (*P. expansum*), gray mold (*B. cinerea*), or side rot (*Cladosporium herbarum*, *A. alternata*, or *Phialophora malorum*). The latter three were combined because they are indistinguishable on pear by symptomatology alone. These

Fig. 1. Populations of yeast biocontrol agents applied 3 weeks before harvest to (A) d'Anjou and (B) Bosc pear fruit in 1996. Yeasts were sprayed onto fruit at concentrations of 1 to 3×10^8 CFU/ml, with approximately 2 ml applied per fruit. d'Anjou: control: $Y = -0.065X + 3.19$, $R^2 = 0.06$, $P(b = 0) = 0.272$; *Cryptococcus laurentii*: $Y = -0.002X + 6.54$, $R^2 = 0.00$, $P(b = 0) = 0.825$; *Rhodotorula glutinis*: $Y = 0.001X + 6.66$, $R^2 = 0.00$, $P(b = 0) = 0.914$; *C. infirmo-miniatus*: $Y = 0.005X + 6.77$, $R^2 = 0.02$, $P(b = 0) = 0.649$. Bosc: control: $Y = -0.005X + 1.90$, $R^2 = 0.00$, $P(b = 0) = 0.931$; *C. laurentii*: $Y = -0.040X + 6.41$, $R^2 = 0.42$, $P(b = 0) = 0.002$; *R. glutinis*: $Y = -0.016X + 6.47$, $R^2 = 0.13$, $P(b = 0) = 0.122$; *C. infirmo-miniatus*: $Y = -0.031X + 6.73$, $R^2 = 0.00$, $P(b = 0) = 0.006$.

side rot fungi are relatively slow-growing wound pathogens, inciting lesions that appear after 3 or more months storage of pears at -0.5°C (9,18).

Statistical analysis. Populations were examined for significant differences among treatments using 95% confidence intervals, where nonoverlapping confidence intervals

indicate a significant difference. Postharvest decay data were analyzed by ANOVA; Fisher's least significant difference procedure was used to distinguish significant differences among treatment means, except that a t test was used to compare decay incidence between treatments applied 3 weeks and 1 day preharvest in 1997.

RESULTS

Colonization of fruit surfaces. In 1996, all three yeast species maintained populations at high levels throughout the duration of the experiment on both pear varieties (Fig. 1). There were no significant differences in population among the three yeasts, and all had significantly higher populations than the control. Initial populations on d'Anjou pears ranged from 2.9×10^6 CFU per fruit (*C. laurentii*) to 7.5×10^6 (*C. infirmo-miniatus*). Three weeks after application, population numbers were relatively unchanged, ranging from 3.3×10^6 CFU per fruit (*C. laurentii*) to 7.2×10^6 (*C. infirmo-miniatus*). On treated Bosc pears, initial population levels for the three yeasts ranged from 2.8×10^6 CFU per fruit (*R. glutinis*) to 6.8×10^6 (*C. infirmo-miniatus*). After 3 weeks, populations varied from 8.9×10^5 CFU per fruit (*C. laurentii*) to 1.8×10^6 (*R. glutinis*). Populations of yeast on untreated d'Anjou pears averaged 2.0×10^3 CFU per fruit initially and 32 CFU per fruit at the end of the experiment. On Bosc pears, initial populations averaged 2.0×10^2 CFU per fruit, ending at 3.2×10^2 CFU per fruit (Fig. 1).

In 1997, the three species used in 1996 behaved similarly to the previous year, with yeast populations on treated fruit remaining significantly higher than those on the control fruit throughout the experiment (Fig. 2). While populations of *C. laurentii*, *C. infirmo-miniatus*, and *R. glutinis* on d'Anjou fruit remained high (final populations from 9.2×10^5 to 2.9×10^6 CFU per fruit), populations of *Candida oleophila* declined rapidly. After 2 weeks, the population of *Candida oleophila* on fruit was not significantly different from the untreated control, and after 3 weeks the population averaged 2.6×10^4 CFU per fruit. On Bosc pears, the yeast populations showed similar patterns. Initial populations for *C. laurentii*, *C. infirmo-miniatus*, *R. glutinis*, and *Candida oleophila* were not significantly different and ranged from 3.2×10^6 CFU per fruit (*R. glutinis*) to 1.3×10^7 (*C. laurentii*). Throughout the 3-week study, populations of *C. laurentii*, *C. infirmo-miniatus*, and *R. glutinis* remained significantly higher than untreated fruit, and final populations ranged from 6.2×10^5 CFU per fruit (*C. laurentii*) to 1.5×10^6 (*R. glutinis*). However, the populations of *Candida oleophila* dropped quickly after application and at the end of the experiment were 8.8×10^3 CFU per fruit, not significantly different from the untreated fruit. Climatic data collected during the 3 weeks prior to harvest in each year are shown in Figure 3.

Storage decay. In Bosc pears in 1996, sprays with *C. laurentii* and *C. infirmo-miniatus* 1 day before harvest resulted in significantly lower levels of total decay than in the untreated control or in fruit treated with *R. glutinis*. There were no significant differences in total decay con-

Fig. 2. Populations of yeast biocontrol agents applied 3 weeks before harvest to (A) d'Anjou and (B) Bosc pear fruit in 1997. Yeasts were sprayed onto fruit at concentrations of 1 to 3×10^8 CFU/ml, with approximately 2 ml applied per fruit. d'Anjou: control: $Y = -0.001X + 3.90$, $R^2 = 0.10$, $P(b = 0) = 0.228$; *Cryptococcus laurentii*: $Y = -0.045X + 6.91$, $R^2 = 0.57$, $P(b = 0) = 0.005$; *Rhodotorula glutinis*: $Y = -0.038X + 7.07$, $R^2 = 0.34$, $P(b = 0) = 0.037$; *C. infirmo-miniatus*: $Y = -0.039X + 7.05$, $R^2 = 0.57$, $P(b = 0) = 0.001$; *Candida oleophila*: $Y = -0.138X + 6.64$, $R^2 = 0.73$, $P(b = 0) = 0.000$. Bosc: control: $Y = -0.062X + 4.14$, $R^2 = 0.01$, $P(b = 0) = 0.193$; *C. laurentii*: $Y = -0.064X + 6.95$, $R^2 = 0.72$, $P(b = 0) = 0.001$; *R. glutinis*: $Y = -0.016X + 6.42$, $R^2 = 0.24$, $P(b = 0) = 0.053$; *C. infirmo-miniatus*: $Y = -0.044X + 6.77$, $R^2 = 0.40$, $P(b = 0) = 0.009$; *Candida oleophila*: $Y = -0.190X + 6.94$, $R^2 = 0.93$, $P(b = 0) = 0.000$.

trol among treatments in d'Anjou fruit in 1996, but *C. infirmo-miniatus* significantly reduced incidence of gray mold and side rot (Table 1). In 1997, Bosc pears sprayed with *R. glutinis* or *C. infirmo-miniatus* 1 day before harvest had significantly lower levels of decay than did the unsprayed control fruit, while there were no significant differences in d'Anjou. *C. infirmo-miniatus* provided significant inhibition of total decay on Bosc and d'Anjou pears, and *R. glutinis* inhibited total decay on d'Anjou pears sprayed 3 weeks before harvest (Table 1). Decay incidence did not differ

between treatments of *R. glutinis*, *C. infirmo-miniatus*, or *C. laurentii* applied 3 weeks or 1 day before harvest. However, incidence of gray mold was significantly lower ($P < 0.05$) in both Bosc and d'Anjou fruit sprayed with *Candida oleophila* 1 day before harvest compared with fruit sprayed 3 weeks before harvest. Incidence of side rot in d'Anjou fruit sprayed with *Candida oleophila* 1 day preharvest was greater than in fruit sprayed 3 weeks preharvest.

Decay incidence and the types of decay observed varied between years and cultivars (Table 1). There were no apparent

trends among yeast biocontrol agents toward providing superior control of a particular type of decay.

DISCUSSION

The yeasts *C. infirmo-miniatus*, *C. laurentii*, and *R. glutinis* survived on the surface of pear fruit at high, relatively stable population levels for at least 3 weeks before harvest under typical hot and dry climatic conditions, while *Candida oleophila* was unable to maintain a stable population on the fruit surface during this period. However, *C. infirmo-miniatus* was the only yeast that provided significant decay control in both pear cultivars when applied 3 weeks before harvest. Since *C. infirmo-miniatus*, *C. laurentii*, and *R. glutinis* were recovered from fruit at similar population levels 3 weeks after they were applied, it may be that exposure to field conditions differentially affects their ability to colonize fruit wounds or to compete successfully with pathogens, even though all were able to grow on a rich culture medium. Significant biocontrol of total decay was observed in Bosc in 1996 and in both application timings in 1997, while control was only significant in d'Anjou in the 3-week preharvest application timing in 1997. It is possible that the russet surface of Bosc pears provides more protected locations for yeast colonists than the smooth surface of d'Anjou pears, allowing yeasts to better maintain their biocontrol capacity.

Improved control of gray mold by *Candida oleophila* applied 1 day before harvest compared to 3 weeks before harvest probably reflects the differences in yeast population levels expected to be present at harvest following each treatment (Fig. 2). Reasons for the higher incidence of side rot in d'Anjou following *Candida oleophila* treatment 1 day before harvest are not apparent.

The yeasts applied as treatments made up the majority of the yeast populations recovered from the untreated fruit. These yeasts may have been transported to the fruit by spray drift, insects, or splashing rain. They may also have been part of the original epiphytic community on the fruit. The fruit were not sampled prior to treatment to determine which species were common epiphytes in the orchard. However, in a previous survey, Chand-Goyal and Spotts (4) found that pear orchards in the Pacific Northwest typically support epiphytic yeast populations made up of from three to seven morphologically distinct species. Typical total yeast populations in their study ranged from 3.5×10^2 to 7.4×10^3 CFU per square centimeter of fruit surface, and varied depending on orchard location, degree of cultivation, and spray practices. *R. glutinis* was the only one of the four species used in our study that was found on fruit from the Medford area in their survey. Leibinger et al. (10)

Fig. 3. Daily maximum and minimum temperatures, mean dew point temperatures, and precipitation during the 3 weeks prior to harvest in 1996 and 1997 at the Southern Oregon Research and Extension Center, Medford.

found that a labeled strain of *R. glutinis* spread from inoculated trees to establish relatively low populations in nearby uninoculated trees and speculated that splashing rainfall was the dispersal mechanism.

Leibinger et al. (10) used various combinations of *R. glutinis* strain CF35, *Aureobasidium pullulans* strains CF10 and CF40, and the bacterium *Bacillus subtilis* strains AG704 and HG77 in preharvest applications on apples to control bulls-eye rot (caused by *Pezizula malicorticis*), as well as blue mold and gray mold. The microbial antagonists were able to colonize the fruit and provided significant reductions in average postharvest lesion sizes when compared with unsprayed fruit. The mixture of *R. glutinis* with *A. pullulans* suppressed decay in apple to the same extent as the fungicide Euparen, commonly used in Europe (9).

Teixidó et al. (22) reported reduction of blue mold incidence in apples treated 2 days before harvest with *Candida sake* strain CPA-1. Cells of this yeast strain modified for tolerance to low water activity by culture in high-glucose or high-glycerol media increased in number on the fruit surface, while numbers of unmodified cells remained relatively unchanged. Cells tolerant to low water activity also controlled decay better than did unmodified cells.

The mechanisms by which yeast biocontrol agents provide decay control are not fully understood. However, there is evidence that the mode of action of several yeast species used as biocontrol agents does not involve antibiosis but rather competition for nutrients at the wound site (3,5).

Because most decay originates at wounds caused during and immediately after harvest (17,20), application of biocontrol agents as close as possible to the

time of wounding should provide the agent with an opportunity to protect the fruit at the wound site (13,14,23). By applying biocontrol agents with the ability to survive on the fruit prior to harvest, the biocontrol agents may be available to colonize wounds quickly and exclude decay-causing organisms from a critical nutrient base. In addition, yeast on the fruit surface may be able to reduce incidence of latent infections by pathogens that do not require wounds for infection (1). Use of biocontrol agents with the ability to survive for extended periods on the fruit surface can also provide the grower with flexibility in scheduling treatment and harvest dates.

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Table 1. Postharvest decay in Bosc and d'Anjou pears following field treatments with yeast biocontrol agents and artificial wounding

Year	Application timing	Treatment ^a	Percent wounds infected ^b							
			Bosc				d'Anjou			
			Gray mold	Blue mold	Side rot	Total decay	Gray mold	Blue mold	Side rot	Total decay
1996	1 day preharvest	Control	29.0 a	21.5 b	33.9 a	84.4 a	11.4 a	11.7 a	11.3 ab	34.4 a
		<i>R. glutinis</i>	10.6 b	49.2 a	25.7 a	85.6 a	4.3 ab	22.4 a	18.1 ab	44.8 a
		<i>C. inf.-min.</i>	9.1 b	9.5 b	27.0 a	45.6 b	2.7 b	16.1 a	5.6 b	24.4 a
		<i>C. laurentii</i>	10.8 b	15.3 b	25.1 a	51.2 b	7.7 ab	18.9 a	19.8 a	46.4 a
1997	3 weeks preharvest	Control	13.1 a	3.9 a	14.6 ab	31.7 a	7.0 ab	10.5 a	8.1 a	25.6 a
		<i>R. glutinis</i>	5.5 ab	1.3 a	14.0 ab	20.8 ab	5.0 ab	2.3 ab	1.3 b	8.6 bc
		<i>C. inf.-min.</i>	4.0 b	1.0 a	1.7 b	6.7 b	1.0 b	1.0 b	4.3 ab	6.3 c
		<i>C. laurentii</i>	4.0 b	1.7 a	21.7 a	27.4 a	5.3 ab	0.0 b	9.7 a	15.0 abc
	1 day preharvest	<i>C. oleophila</i>	16.7 a*	1.7 a	24.5 a	44.9 a	15.0 a*	5.0 ab	1.3 b*	21.3 ab
		Control	16.0 a	1.0 a	25.0 a	42.0 a	2.0 a	3.0 a	11.7 a	16.7 a
		<i>R. glutinis</i>	3.0 b	1.3 a	11.3 a	15.5 b	1.0 a	4.0 a	13.2 a	18.2 a
		<i>C. inf.-min.</i>	3.0 b	0.0 a	8.5 a	11.5 b	0.0 a	0.0 a	10.0 a	10.0 a
		<i>C. laurentii</i>	5.0 b	0.0 a	14.8 a	19.8 ab	1.7 a	6.0 a	10.0 a	17.7 a
		<i>C. oleophila</i>	3.0 b*	1.3 a	12.5 a	16.8 ab	0.0 a*	5.3 a	14.7 a*	19.9 a

^a For each year and application timing, values within a column followed by the same letter are not significantly different ($P > 0.05$) according to Fisher's protected LSD procedure. Data were transformed to arcsine (square root) prior to analysis. Differences between corresponding values for 3 weeks preharvest and 1 day preharvest in 1997 were not significantly different except where noted by asterisk.

^b Fruit were sprayed with yeast suspensions containing *Rhodotorula glutinis* (1.0 to 1.5×10^8 CFU/ml), *Cryptococcus infirmo-minutus* (1.0 to 1.5×10^8 CFU/ml), *Cryptococcus laurentii* (2.8 to 3.3×10^8 CFU/ml), or *Candida oleophila* (1.0×10^8 CFU/ml).

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