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Biological Control

*e-Xtra**

Biological Control of *Botrytis cinerea*: Interactions with Native Vineyard Yeasts from Washington State

Xuefei Wang, Dean A. Glawe, Elizabeth Kramer, David Weller,† and Patricia A. Okubara†

First, second, and third authors: Department of Plant Pathology, Washington State University, Pullman 99164-6430; and fourth and fifth authors: United States Department of Agriculture–Agricultural Research Service Wheat Health, Genetics and Quality Research Unit, Pullman, WA 99164-6430.

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ABSTRACT

Native yeasts are of increasing interest to researchers, grape growers, and vintners because of their potential for biocontrol activity and their contributions to the aroma, flavor, and mouthfeel qualities of wines. To assess biocontrol activity, we tested 11 yeasts from Washington vineyards, representing isolates of *Candida saitoana, Curvibasidium pallidicorallinum, Metschnikowia chrysoperlae, M. pulcherrima, Meyerozyma guilliermondii*, *Saccharomyces cerevisiae*, and *Wickerhamomyces anomalus*, for ability to colonize Thompson Seedless grape berries, inhibit the growth of *Botrytis cinerea* in vitro, and suppress disease symptoms on isolated berries. The yeast-like fungus *Aureobasidium pullulans* was also included based on its known biocontrol activity against *B. cinerea* in studies on apple and grape. All yeast strains multiplied rapidly in grape berries and reached densities of over log 6 cells per wound as early as 2 days after inoculation with 200 cells. One of the *Botrytis* isolates used in this study was much less virulent than the others and was provisionally identified as *B. prunorum* based on multilocus sequence analysis. Suppression of the growth of *B. cinerea* isolates 111bb, 207a, 207cb, and 407cb occurred on berries treated with *A. pullulans* P01A006, *Metschnikowia chrysoperlae* P34A004 and P40A002, *M. pulcherrima* P01A016 and P01C004, *Meyerozyma guilliermondii* P34D003, and *S. cerevisiae* HNN11516. Inhibition of *Botrytis* isolates by the yeast strains was more common on berries than in vitro, suggesting the possibility that niche competition was a more likely biocontrol mechanism than antibiosis in planta. Metabolic profiling of yeast strains and *B. cinerea* isolates using Biolog YT plates revealed seven distinct metabolic groups. Furthermore, the yeast strains showed partial to complete tolerance to the commonly used fungicides fluopyram, triflumizole, metrafenone, pyraclostrobin, and boscalid. Implications of these findings for field deployment of native Washington yeasts as biocontrol agents against *B. cinerea* are discussed.

Additional keywords: Botrytis bunch rot, gray mold, postharvest disease.

The state of Washington is the second largest producer of premium wines in the United States and is among the world's top wine grape-growing regions (WASS 2003). Most cultivars of grape are highly susceptible to Botrytis bunch rot (also called gray mold), which causes a destructive decay of vineyard grape and postharvest decay of table grape. The disease is most severe under cool, wet growing conditions but is a problem throughout the world (Elmer and Reglinski 2006; Steel et al. 2013). In 2010, a Botrytis bunch rot epidemic affected approximately 4,900 ha of wine grape in central Washington, leading to losses of $17 million (Moyer and Grove 2011).

The causal agent of Botrytis bunch rot, *Botrytis cinerea*, is a natural component of the vineyard microbiota (Steel et al. 2013); wounds or openings in host surfaces serve as infection courts. The pathogen can be very active during postharvest storage, even under strictly managed storage conditions (Keller et al. 2003). Latent infections were found to occur at a rate of approximately 0.5% in south-central Washington (Dugan et al. 2002). The fungus detracts from wine quality by producing laccases during fermentation, leading to tissue browning and discoloration of both white and red wines (Van Rensburg and Pretorius 2000).

†Corresponding authors: P. A. Okubara: E-mail: patricia.okubara@ars.usda.gov; and D. M. Weller: E-mail: david.weller@ars.usda.gov

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Management of Botrytis bunch rot continues to be a challenge for growers, winemakers, and table grape distributors. Grapevine canopy management, use of grape clones with loose cluster architecture, and application of fungicides are recommended management practices in Washington State (Moyer and Grove 2011). Because there are no highly resistant or tolerant commercial cultivars (Wilcox et al. 2015), application of synthetic fungicides has been the method of choice to manage Botrytis bunch rot for the last 60 years (Rosslenbroich and Stuebler 2000; Smilanick et al. 2010). Unfortunately, fungicides can cause problems such as bleaching injury, adulteration of wine flavor, sulfur allergies, environmental issues, and fungicide resistance among pathogens (Calvo-Garrido et al. 2014; Sanzani et al. 2012). In addition, fungicide residues on berries can influence the performance of yeasts during fermentation, and are regulated for use on table grape (Cabras and Angioni 2000).

Biological control is an attractive alternative to fungicides for the control of Botrytis bunch rot (Janisiewicz and Korsten 2002; Zhimo et al. 2014). Native (also called wild, "naturally occurring", or indigenous) grape-associated microbes are specifically adapted to the berry ecosystem (Janisiewicz and Korsten 2002; Parafati et al. 2015), and native yeasts have been shown to suppress the growth of *B. cinerea* from grape and apple in the laboratory (Dugan et al. 2002; Liu et al. 2010; Masih et al. 2000; Spadaro et al. 2002). Native yeasts are attractive as biocontrol agents of fruit disease due to their simple nutritional requirements, stability during storage, and ability to rapidly colonize pathogen infection courts (Chanchaichaovivat et al. 2007). Commercial biofungicides based on six species of native yeasts have been developed for postharvest control of *B. cinerea* of citrus and pome fruit (Spadaro and Droby 2016).

The objective of this study was to characterize *Botrytis* isolates obtained from grape berries grown in Washington vineyards, and

the biocontrol activities of native yeasts against these isolates. A previous survey of native yeasts on grape in a vineyard in central Washington identified over 50 species representing 16 genera (Bourret et al. 2013). Several of these yeast species are known to contribute to the aroma, flavor, and mouthfeel qualities of Washington wines (Jolly et al. 2006). These yeast species also inhibited *B. cinerea* in laboratory assays (Kramer 2015). For the present study, we selected 11 yeast strains with promising inhibitory activity against *B. cinerea*, and expanded our studies to Washington isolates of *B. cinerea*. As detailed herein, this study suggests that native yeasts exhibit biocontrol potential for use against *B. cinerea* in Washington but the amount of activity varies with the isolate of the pathogen.

MATERIALS AND METHODS

Native yeasts, *B. cinerea*, and grape cultivars used in this study. Eleven strains of native yeasts and nine isolates of *B. cinerea* were used in this study (Table 1). The yeast-like fungus *Aureobasidium pullulans* was included based on its well-documented biocontrol activity against *B. cinerea* (Castoria et al. 2001; Dugan et al. 2002; Vero et al. 2009). All of the non-*Saccharomyces* yeasts were isolated from wine grape in a research vineyard at the Washington State University Irrigated Agriculture Research and Extension Center, Prosser (Bourret et al. 2013). *Saccharomyces cerevisiae* HNN11516 was isolated from a native fermentation in the lab. Yeast strains were maintained on potato dextrose agar (PDA) (Becton Dickinson, Franklin Lakes, NJ) or Wallerstein Laboratory Nutrient Medium agar (WLNA) (Becton Dickinson). Yeast strains were stored in 30% glycerol at –80°C. Nine *B. cinerea* isolates, initially identified on the basis of cultural and morphological criteria, were collected from grapevine buds or berries at a vineyard in Prosser, WA (Dugan et al. 2002) and further identified in this study as described below. In 2015, the isolates were recovered from silica gel and single-spore isolates were established. The *Botrytis* isolates were routinely cultured on half-strength (1/2) V8 agar (10% V8 juice, 2% agar, and 0.15% $CaCO_3$) (Stevens 1981) and grown at 24°C under a 12-h photoperiod. Isolates were archived on 1.5-cm-diameter discs of sterile filter paper in autoclaved envelopes. Filter paper discs were placed on an agar plate of an isolate and the fungus was allowed to grow over the paper. After drying overnight under a stream of sterile air, the paper discs were placed in envelopes and stored at –20°C.

The growth rates of the *Botrytis* isolates were quantified on 1/2 V8 agar. For each isolate, a 4-mm-plug of inoculum was placed at the center of a Petri plate and incubated at 24°C. The colony radius was measured from the center of the plug to the leading edge of the colony every 12 h for up to 96 h. Each treatment (isolate) was replicated four times, with a single plate serving as a replicate. The experiment was conducted twice, the data were pooled, and the area under the growth curve (AUGC) was calculated. The colony morphology of each isolate was determined on acidified PDA (APDA), King's medium B (KMB), and pea agar medium (PAM) after 7 days of incubation at 20°C with a cycle of 12 h of light and 12 h of darkness, as described by Ferrada et al. (2016).

Grape berries of the cultivar Thompson Seedless (*Vitis vinifera* L.), purchased from a local grocery store, were used for *Botrytis* virulence tests and in vivo biological control studies.

Molecular characterization of *Botrytis* strains. Sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA and partial sequences of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and heat-shock protein60 (HSP60) genes were obtained to confirm generic assignment and species identity of the *Botrytis* isolates. *B. cinerea* isolates were cultured on 1/2 V8 agar for 48 h. Mycelia were collected and washed once in sterile water. Genomic DNA was extracted from fungal cultures grown for 48 h using a FastDNA SPIN KIT (MP Biomedicals, Solon, OH). Sequences encoding the 5.8S ribosomal RNA and the adjacent ITS1 and ITS2 regions were amplified using primers ITS1 and ITS4 (White et al. 1990). Polymerase chain reaction (PCR) primers to amplify the portions of the G3PDH and HSP60 genes were described previously by Staats et al. (2005). Amplification reactions (25 µl) contained 50 ng of DNA, 5× GoTaq Flexi Buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 4 pmol of each primer pair, and 1 U of GoTaq Taq Polymerase (Promega Corp., Madison, WI). Amplification conditions included an initial denaturation at 94°C for 3 min; followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 54.2°C, and extension for 1 min at 72°C; and a final extension for 10 min at 72°C. Excess primers and salts were removed from the amplification products using the Ultra Clean PCR Clean-Up Kit (MoBio Laboratories, Inc., Carlsbad, CA). Sequencing was carried

TABLE 1. Native Washington yeast strains and *Botrytis* isolates used in this study

Species	Isolate	GenBank accession numbers[x]			Cultivar[y]
		ITS/D1-D2	G3PDH	HSP60	
Aureobasidium pullulans	P01A006	JX188090	…	…	Riesling
Candida saitoana	P45A002	JX188109	…	…	Chardonnay
Curvibasidium pallidicorallinum	P45C001	JX188148	…	…	Chardonnay
Metschnikowia chrysoperlae	P34A004	JX188171	…	…	Chardonnay
M. chrysoperlae	P34B007	JX188169	…	…	Chardonnay
M. chrysoperlae	P40A002	JX188173	…	…	Riesling
M. pulcherrima	P01A016	JX188181	…	…	Riesling
M. pulcherrima	P01C004	JX188183	…	…	Riesling
Meyerozyma guilliermondii	P34D003	JX188191	…	…	Riesling
Saccharomyces cerevisiae	HNN11516	KX891350	…	…	Cabernet Sauvignon
Wickerhamomyces anomalus	P42B001	JX188245	…	…	Riesling
Botrytis cinerea	101V3Dd	KU173126	KY930930	KY930931	Chardonnay
B. cinerea	111bb	KU173125	KY930932	KY930933	Riesling
B. cinerea	207a	KU173124	KY930934	KY930935	Riesling
B. cinerea	207cb	KU173120	KY930936	KY930937	Riesling
B. cinerea	207db	KU173128	KY930938	KY930939	Riesling
B. cinerea	207e	KU173121	KY930940	KY930941	Riesling
B. cinerea	407cb	KU173122	KY930942	KY930943	Riesling
B. cinerea	407da	KU173123	KY930944	KY930945	Riesling
Botrytis sp.[z]	R1V55#13	KU173127	KY930946	KY930947	Chardonnay

[x] GenBank accession numbers were for internal transcribed spacer (ITS), partial glyceraldehyde 3-phosphate dehydrogenase (G3PDH), and heat-shock protein 60 (HSP60) sequences, except for the D1-D2 region of the 26S ribosomal RNA of *Saccharomyces cerevisiae* (Bourret et al. 2013; Dugan et al. 2002).
[y] Yeasts and *B. cinerea* were isolated from grape berries from a research vineyard at the Washington State University Irrigated Agriculture Research and Extension Center in Prosser, WA. The *S. cerevisiae* was isolated from a native fermentation of grape from Paterson, WA.
[z] Showed strong identity to *B. prunorum* by multilocus sequence analysis (Fig. 2).

out using the BigDye Terminator Cycle Sequencing Kit (v. 3.1; Applied Biosystems, Foster City, CA) at ELIM (Biopharmaceuticals, Inc., Hayward, CA). Sequence data were assembled and analyzed using Molecular Evolutionary Genetics Analysis (MEGA) software 7 (Kumar et al. 2016). Provisional taxonomic assignments were made from matches to GenBank accessions using Blasntn (Altschul et al. 1997). Nucleotide sequences were deposited in GenBank under accession numbers KU173120 through KU173128 for ITS; KY930930, 932, 934, 936, 938, 940, 942, 944, and 946 for G3PDH; and KY930931, 933, 935, 937, 939, 941, 942, 945, and 947 for HSP60 (Table 1).

Virulence of *B. cinerea* isolates on grape berries. *B. cinerea* isolates were cultured on 1/2 V8 agar at 25°C under a 12-h photoperiod for 4 days. Spores were collected, washed in sterile water, concentrated using centrifugation at 18,000 × *g* for 3 min, and resuspended in water. The concentration of the spores was determined using a hemocytometer and adjusted to give a concentration of 10^4 spores ml^{-1}. Each inoculation consisted of 1 µl (10 spores) of a spore suspension introduced into a wound site. Ten berries were inoculated for each *Botrytis* isolate. Inoculated berries were incubated at 25°C for 10 days, and disease symptoms were evaluated daily according to the following visual rating scale (0 to 7): 0 = healthy berry with no or minor browning around wound site; 1 = brown spots on the berry surface; 2 = browning over < 50% of the berry surface; 3 = browning over > 50% of the berry surface; 4 = browning over the entire berry surface; 5 = *Botrytis* spores covering the entire berry; 6 = as in 5, plus thinning and wrinkling of the berry skin; and 7 = as in 6, plus berry skin with visible shrinkage (Fig. 1C). The area under the disease progress curve (AUDPC) was calculated for disease severity ratings taken at 3 to 10 days postinoculation (dpi) according to the equation of Campbell and Madden (1990). The experiment was conducted three times.

Sensitivity of native yeasts to fungicides. Ten native yeast strains were tested for their sensitivity to the fungicides Luna Privilege (fluopyram; Bayer [Pty] Ltd., Port Elizabeth, South Africa), Pristine (pyraclostrobin and boscalid; BASF Corporation, Holly Springs, NC), Procure (triflumizole; Chemtura, Middlebury, CT), and Vivando (metrafenone; BASF Corporation) using a modified National Committee for Clinical Laboratory Standards (1984) procedure.

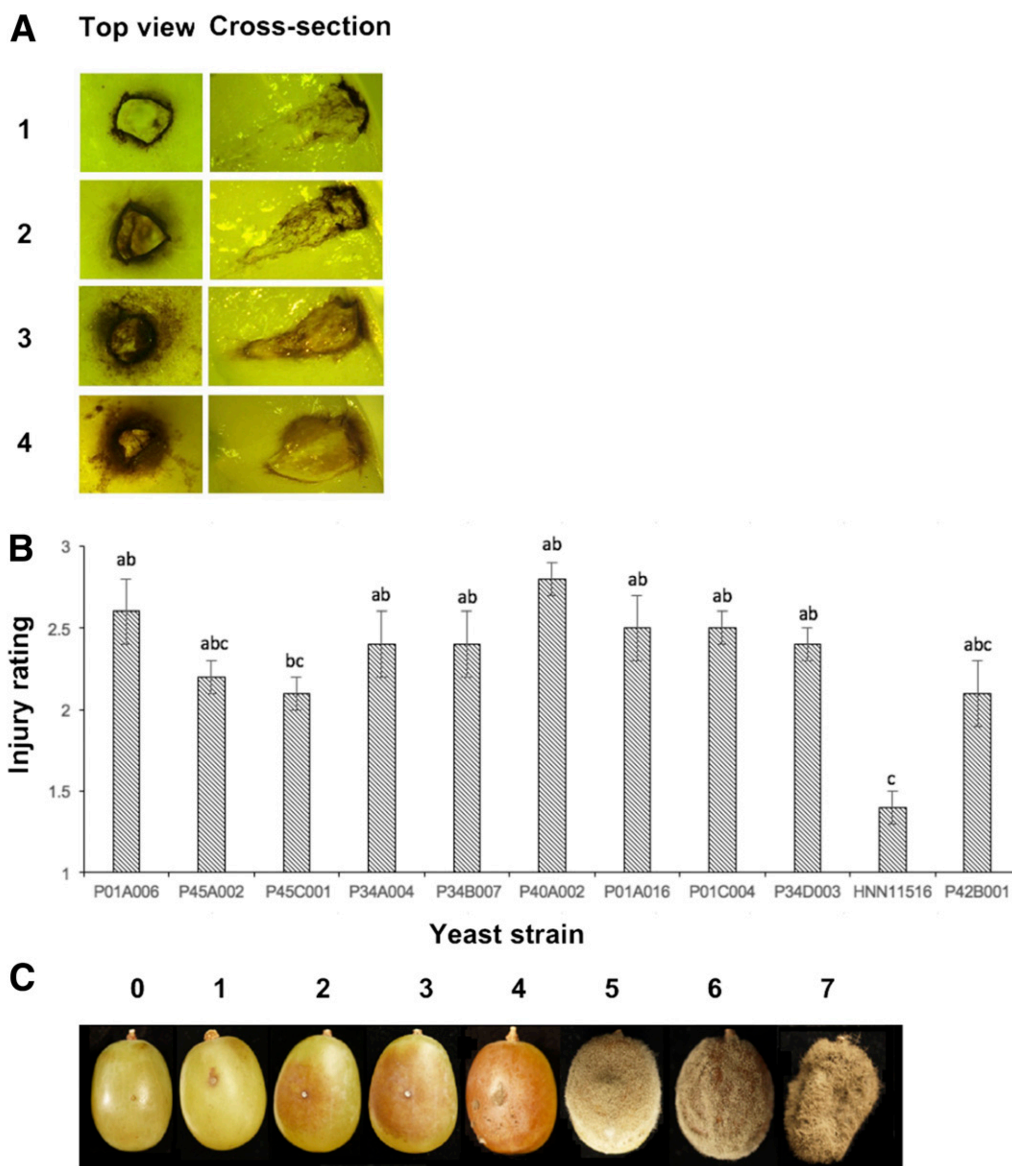

Fig. 1. A, Yeast-induced injury rating on a scale of 1 to 4, where 1 = light brown discoloration along the wound circumference edge or within the wound site, 2 = brown or black spots around the exterior surface of or within the wound site, 3 = brown or black necrosis around the perimeter of and within the wound site, and 4 = intense dark brown or black necrosis around the entire perimeter of and within the wound site. **B,** Extent of discoloration associated with yeast colonization of Thompson Seedless berries at 10 days postinoculation. Two hundred yeast cells were applied to wounds, and discoloration in and around the wound site was rated on a scale of 1 to 4, as in A. **C,** Botrytis disease severity rating on a scale of 0 (healthy) to 7 (grape skin thin and wrinkled; berry shrunken to one-half the size of healthy), based on Archbold et al. (1997).

These fungicides are used commonly in Washington vineyards to control Botrytis bunch rot. Yeast cells were cultured for 48 h on glucose yeast peptone agar (GYP agar; 0.5% glucose, 0.3% yeast extract, 0.5% peptone, and 1.5% agar), washed once, and suspended in 1 ml of sterile water. Population densities were adjusted to 10^6 cells ml^{-1} using a hemocytometer. Then, 100 µl (10^5 cells) was spread on a GYP agar plate. Each fungicide was diluted to 50, 500, and 1,000 µg ml^{-1} by weight of formulation. Fungicide dilutions (20 µl) were applied to 1-mm-diameter autoclaved disks of Whatman number 1 filter paper. Three fungicide-treated filter disks were laid over the yeast and assay plates were incubated for 72 h at 20°C under a 12-h photoperiod. Results were recorded as ++ when yeast colony growth was completely inhibited immediately adjacent to the filter paper disk, + when colonies were reduced in size relative to the nonfungicide control, and – when no yeast growth inhibition occurred around the filter paper disk.

Colonization of grape berries by native yeasts and identification of yeasts recovered from colonized berry tissue. Individual intact berries with pedicels were cut from clusters of Thompson Seedless grape. Berries were washed three times in water, treated once in 70% ethanol for 1 min and once in 0.5% sodium hypochlorite (NaClO) for 5 min, and rinsed twice with autoclaved distilled water. Berries were dried under a laminar flow hood for 1 h, and transferred to autoclaved, lidded plastic boxes (11.7 by 9 by 10 cm). Each box contained 12 berries that were separated by strips of clean, plastic-backed bench liner.

Yeast strains were cultured on yeast extract peptone dextrose (1% Bacto yeast extract, 2% Bacto peptone, and 2% dextrose) and incubated for 48 h at 25°C under a 12-h photoperiod. Cell suspensions were adjusted to 10^5 cells ml^{-1} using a hemocytometer. A single 3-mm-deep wound was made on each intact berry using a sterile wooden toothpick. Cell suspensions (2 µl each) at 10^5, 10^6, or 10^7 cells ml^{-1} (200, 2,000, or 20,000 cells/wound) were pipetted into wounds. Sterile water served as the inoculum for the control treatment. Berries were incubated at 25°C for 2, 4, 6, and 10 days. After 10 days, each berry was bisected through the wound center to evaluate yeast-associated injury on the basis of a scale of 1 to 4, where 1 = light brown discoloration along the wound circumference edge or within the wound site, 2 = brown or black spots around the exterior surface of or within the wound site, 3 = brown or black necrosis around the perimeter of and within the wound site, and 4 = intense dark brown or black necrosis around the entire perimeter of and within the wound site. Tissue from the wound site was excised from each berry using a sterile scalpel and macerated in 1 ml of sterile water in the presence of 0.64-cm ceramic sphere beads (MP Biomedicals) for 1 min on a vortex homogenizer. Yeast population densities were determined using a hemocytometer. For each treatment (yeast) and time point, 10 berries were inoculated and each berry served as a replicate. Colonization experiments were conducted three times and injury evaluations were conducted twice.

To determine whether the yeasts quantified from inoculated berries at day 10 were the same as those in the initial inoculum, 100 µl of yeast cells was diluted 10^3-fold and spread on WLNA (Hardy Diagnostics, Santa Maria, CA) supplemented with streptomycin at 50 µg ml^{-1}. Plates were incubated at 25°C for 3 days. Six representative colonies were selected from each yeast plating; when observed, colonies having visible morphological differences also were selected. ITS sequences were obtained and compared with accessions in GenBank (Altschul et al. 1997).

In vitro inhibition of *Botrytis* isolates by native yeasts. Yeast strains were grown on GYP agar for 48 h. Cells were washed once in sterile water and adjusted to 1×10^9 cells ml^{-1} using a hemocytometer. In an initial screen, 50 strains representing 16 genera were selected from a collection of 250 strains (Bourret et al. 2013) and assayed for activity against *B. cinerea* isolate 101V3Dd. A yeast cell suspension (100 µl) was introduced at the center of a GYP agar plate. A 5-mm plug from the leading edge of a 2-day-old culture of *B. cinerea* 101V3Dd was placed at the center of each Petri plate. Plates were incubated at 20°C under a 12-h photoperiod, and the diameter of the fungal colony was measured when the leading edge of the fungal colony on control plates reached the perimeter of the agar, usually at 5 days. Sterile water was used as the control. Yeast strains were considered to have inhibitory activity if the colony diameter of *B. cinerea* was <5 mm. Each treatment (yeast) was replicated three times, with a single plate serving as a replicate. The experiment was repeated three times.

In a quantitative assay of in vitro inhibition of *B. cinerea* isolates 101V3Dd, 111bb, 207cb, 207e, and 407cb, two 2-µl aliquots of yeast cell suspensions were placed 1 cm from the edge of the GYP agar at opposing spots on the plate and incubated at 24°C under a 12-h photoperiod. After 48 h, a 5-mm plug of a 2-day-old culture of *B. cinerea* was placed in the center of each plate. Control plates were inoculated with medium without yeast. When the fungus reached the perimeter of the plate (approximately 4 days), measurements were taken of the distance between the yeast and fungal colonies at the yeast–fungus interface, and the radius of the fungal colony where yeast was not present. The inhibition index was defined as $[y/(x + y)] \times 100$, where y is the distance between the leading edge of the fungus and the center of the yeast colony and x is distance between the leading edge of the fungus and the center of the agar plug (Mavrodi et al. 2012). Each treatment of a yeast and *Botrytis* isolate combination was replicated three times, with a single plate serving as a replicate. The experiment was conducted three times.

Biocontrol activity of native yeasts in vivo. For each yeast strain, 10 Thompson Seedless berries were wounded and inoculated with 200 yeast cells, as described above. Berries were incubated at 25°C for 48 h. Approximately 10 spores of each *B. cinerea* isolate were introduced to the wound site and the berries were incubated at 25°C for an additional 8 days. Disease symptoms were evaluated using the 0-to-7 visual rating scale (Fig. 1C). Each berry served as a replicate and the experiment was conducted three times.

Growth of native yeasts and *Botrytis* isolates on Biolog microplates. Eleven yeast strains and five *B. cinerea* isolates were grown on YT MicroPlates (Biolog Inc., Hayward, CA). Yeast cells were grown at 25°C for 48 h on BUY agar (Biolog Inc.). Suspensions in sterile water were adjusted to an absorbance at 630 nm (A_{630}) reading of 0.05 and 100 µl was added to each well of the YT microplate. *Botrytis* strains were cultured on BUY agar at 25°C for 48 to 96 h until spores were observed. Washed spores (100 µl) adjusted to 10^4 spores ml^{-1} in 0.2% water agar water were used for microplate inoculation. Microplates were incubated at 26°C. Readings at A_{590} nm were taken at 0 and 4 days using a BioTek ELx808 spectrophotometer (BioTek Instruments, Inc., Winooski, VT). To normalize absorbance values of each microplate, the reading from oxidation control well A1 was subtracted from wells A2 to C12, and that of assimilation control well D1 was subtracted from wells D2 to H12. For net A_{630} values, day 0 readings for each well were subtracted from those of day 4. Net A_{630} values > 0.10 were considered to be positive for growth. The experiment was repeated once.

Statistical analysis. Berries in colonization and biocontrol experiments were arranged by treatments in a partially balanced incomplete block design; other experiments were done in a completely randomized design. Differences in mean AUDPC, AUGC, yeast injury ratings, and *Botrytis* disease severity ratings were determined among treatments using a Kruskal-Wallis one-way analysis of variance (ANOVA) test followed by Dunn's test for multiple comparisons ($P = 0.05$), (Statistix 8.1; Analytical Software, St. Paul, MN). *Botrytis* in vitro and in vivo inhibition data and yeast population density comparisons were performed using the two-way ANOVA. Means were separated into letter classes using Tukey or Fisher's least significant difference pairwise comparisons ($P < 0.05$). Bartlett's test for homogeneity of variances was used to determine whether data from separate experiments could be combined ($P < 0.05$). The results from the YT microplates for each strain were subjected to two-way ANOVA with Minitab 17,

cluster analysis, and logistics regression (SAS Institute, Inc., Cary, NC).

RESULTS

Characterization of the *Botrytis* isolates. Traditional morphological classification of the *Botrytis* isolates was previously determined by Dugan and Lupien (Dugan et al. 2002). Eight of the nine isolates shared identical ITS sequences; however, isolate R1V55#13 harbored a single A polymorphism (Fig. 2). Partial sequences of G3PDH and HSP60 indicated that two main clades were distinguished in the genus *Botrytis*. Isolates 111bb, 101V3Dd, 2017a, 207cb, 207db, 207e, 407cb, and 407da clustered together, and their gene sequences were identical for G3PDH and HSP60. Isolate R1V55#13 varied at a few sites that distinguished it from other groups and it was closely related to *B. prunorum* (Fig. 2). The colony morphology of isolate R1V55#13 also differed from the other the *Botrytis* isolates on KMB, APDA, and PAM, and it was similar to the colonies of *B. prunorum* on the three media described by Ferrada et al. (2016) (Supplementary Fig. S1).

Virulence of *B. cinerea* isolates on grape berries. Because the *Botrytis* isolates had been in storage for several years, we completed Koch's postulates with them and showed that all nine isolates were pathogenic on grape berries. Eight of the nine *Botrytis* isolates caused substantial rot on Thompson Seedless berries over a 10-day interval. Relative virulence of the isolates was quantified by comparing the AUDPC for each pathogen isolate and disease severity at 10 days after inoculation (Table 2). Based on AUDPC, isolates 207a was significantly ($P < 0.05$) more virulent than isolates 111b, 207db, and R1V55#13. Although the pathogen isolates were not identical in virulence, generally they caused substantial disease in our tests and most caused severe damage by 6 dpi. The exception was isolate R1V55#13, which was significantly ($P < 0.05$) less virulent than the other eight strains based on disease ratings and AUDPC (Table 2). Isolate R1V55#13 was also the slowest growing of the *Botrytis* isolates (Table 2). The greatly reduced virulence of R1V55#13 is supported by the finding that it does not cluster with other *B. cinerea* and is more closely related to *B. prunorum.*

Sensitivity of yeasts strains to commercial fungicides. The native yeasts used in this study differed in their responses to three of four commercial fungicide formulations commonly used to control Botrytis bunch rot in Washington vineyards (Table 3). After 72 h, all yeast strains grew on Vivando to a similar degree; they were not different than the no-fungicide controls, and were considered to be tolerant to Vivando. In addition, *Curvibasidium pallidicorallinum* P45C001 was not affected by the other three fungicides. However, reduction in colony size was observed for most yeasts with Pristine or Luna Privilege. Growth of *Metschnikowia chrysoperlae*, *M. pulcherrima*, and *A. pullulans* was completely inhibited by Procure at 500 µg ml^{-1}.

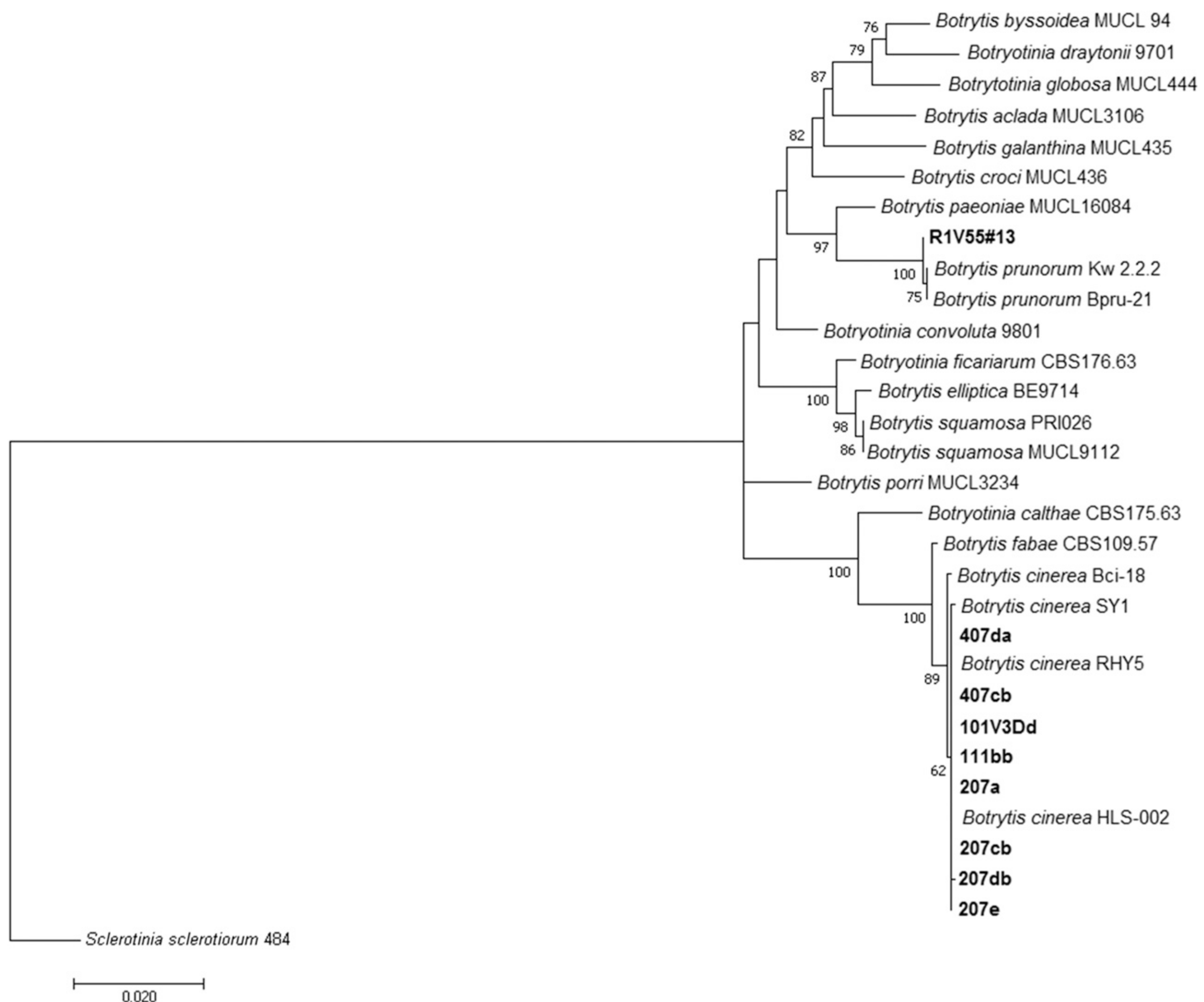

Fig. 2. Molecular phylogenetic analysis of *Botrytis* taxa and an outgroup species based on combined glyceraldehyde-3-phosphate dehydrogenase and heat-shock protein 60 data by maximum-likelihood method. The evolutionary history was inferred by using the maximum-likelihood method based on the Kimura two-parameter model (Kimura 1980). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values greater than 60% are shown at the nodes. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Grape berry colonization and injury by yeast strains. Populations sizes of the native yeasts and yeast-like fungus *A. pullulans* on grape berries increased 10,000-fold by 2 days after inoculation, increasing from 200 cells to approximately 1 million per wound, and then reached approximately 10 million cells per wound at 10 days (Table 4). The population density of *Wickerhamomyces anomalus* P42B001 at day 2 was significantly ($P < 0.05$) greater than all of the other strains, except for *Meyerozyma guilliermondii* P34D003 and *S. cerevisiae* HNN11516. The native yeasts caused mild browning of the berry wound site perimeter and internal wound tissue (ratings of 1.0 to 2.75) at 10 dpi, albeit when population densities were approximately 10^7 cells ml^{-1} (Fig. 1A). Observations of multiple berries indicated that *A. pullulans* caused the most consistent and pronounced browning (ratings of 1.6 to 2.5), even though the mean values of injury ratings for the strains did not differ significantly, except for *S. cerevisiae* HNN11516 (Fig. 1B). Among replicate berries inoculated with a given strain, there was variability in browning. *S. cerevisiae* caused the least amount of browning, and was most similar to the no-yeast control. The reisolated colonies of *A. pullulans*, *Candida saitoana, Metschnikowia chrysoperlae* P34A004, and *W. anomalus* were identical to those originally used. Mixed sequences were detected in *M. chrysoperlae* P34B007 and P40A002, *M. pulcherrima*, *Meyerozyma guilliermondii*, *Curvibasidium pallidicorallinum*, and *S. cerevisiae*.

TABLE 2. Virulence of nine *Botrytis* isolates on wounded Thompson Seedless grape berries

Isolate	Disease rating[x]	AUDPC[y]	AUGC[z]
101V3Dd	5.4 ± 0.1 a	25.2 ± 0.9 abc	8.0 a
111bb	5.2 ± 0.2 a	22.4 ± 0.9 c	7.9 a
207a	5.6 ± 0.2 a	27.9 ± 0.8 a	7.7 ab
207cb	5.7 ± 0.1 a	26.7 ± 0.8 ab	7.7 ab
207db	5.4 ± 0.2 a	23.9 ± 0.9 bc	7.7 ab
207e	5.4 ± 0.2 a	26.2 ± 0.8 ab	7.9 a
407cb	5.5 ± 0.2 a	26.9 ± 0.9 ab	7.7 ab
407da	5.1 ± 0.2 a	26.7 ± 0.7 ab	8.0 a
R1V55#13	2.1 ± 0.1 b	12.3 ± 0.5 d	7.4 b

[x] Disease severity was rated after 8 days on a scale of 0 (healthy) to 7 (grape skin thin and wrinkled; berry shrunken to one-half the size of healthy). Values are the average of 9 to 10 berries in three independent experiments (total $n = 27$ to 30), determined using Kruskal-Wallis analysis of variance followed by Dunn's test for multiple comparisons. Data from the experiments were pooled. Letters indicate significant ($P < 0.05$) differences among means within each column.

[y] Area under the disease progress curve (AUDPC) was calculated from a plot of disease severity ratings from day 3 to day 10. Values were calculated from average disease ratings of 9 to 10 berries from three experiments ($n = 27$ to 30). Data from the experiments were pooled.

[z] Area under the growth curve (AUGC) on half-strength V8 agar was calculated over 4 days. Values represent the means of four replicates in each of two experiments ($n = 8$). Data from the experiments were pooled.

Inhibition activity of the native yeasts in vitro. Eleven yeast strains showing consistent inhibition of *B. cinerea* in the in vitro qualitative assays (Kramer 2015) were selected from the yeast collection for further testing. In a preliminary in vitro inhibition experiment, we found that certain yeast strains were inhibitory only to certain *B. cinerea* isolates. These yeast–*Botrytis* pairs also showed inhibitory interactions in inhibition index studies (Table 5). *A. pullulans* P01A006 inhibited all five *Botrytis* isolates used in the inhibition index experiments. *C. pallidicorallinum* P45C001 inhibited three of the five isolates; *Metschnikowia pulcherrima* P01A016 and P01C004, *Meyerozyma guilliermondii* P34D003, *S. cerevisiae* HNN11516, and *W. anomalus* P42B001 inhibited two isolates. The other strains inhibited only one *Botrytis* isolate. *B. cinerea* isolate 207cb was the most sensitive to the native yeasts, whereas isolates 407cb and 101V3Dd were the least sensitive. The results demonstrated that yeast strains of the same species varied in their control of *B. cinerea*, and that the pathogen isolates differentially responded to each yeast in the in vitro assays.

Biocontrol activity of native yeasts on grape berries. All yeast strains significantly suppressed at least one of the four *Botrytis* isolates on the grape berries. However, the *Botrytis* isolates were differentially sensitive to the yeasts (Table 6). In general, the most effective strains were *A. pullulans* P01A006, *Metschnikowia chrysoperlae* P34A004 and P40A002, *M. pulcherrima* P01A016 and P01C004, *Meyerozyma guilliermondii* P34D003, and *S. cerevisiae* HNN11516. The disease suppressive capacities of *Candida saitoana* P45A002, *Curvibasidium pallidicorallinum* P45C001, *Metschnikowia chrysoperlae* P34B007, and *W. anomalus* P42B001 varied among the different *Botrytis* isolates relative to the no-yeast control.

A. pullulans P01A006 was the only strain that inhibited all of the *Botrytis* isolates both in vitro and on grape berries. With *Botrytis* isolates 111bb and 207cb, there was a general trend for yeasts that were inhibitory in vitro to be suppressive on the berries. In contrast, with *Botrytis* isolate 407cb, there was a trend for noninhibitory yeasts to be suppressive on the berries.

Growth of yeasts and *B. cinerea* on Biolog microplates. Metabolite oxidation (wells A2 to C12) and assimilate utilization (wells D2 to H12) by yeast and *B. cinerea* strains were profiled using Biolog YT microplates. The percent utilization of the 35 oxidation and 59 assimilation compounds by each yeast and *Botrytis* isolate was reproducible in two separate experiments. Visual inspection indicated that the compounds utilized in both experiments comprised seven distinct utilization groups among the yeast strains and *Botrytis* isolates (Table 7; Supplementary Fig. S2). Visual analysis was supported by principal component analysis (PCA) (Supplementary Fig. S3) and cluster analysis (data not shown). Group 1 comprised

TABLE 3. In vitro sensitivity of native yeast strains to four commonly used vineyard fungicides[y]

	Luna Privilege			Pristine			Procure			Vivando		
Yeast species, strain[z]	50	500	1,000	50	500	1,000	50	500	1,000	50	500	1,000
Ap P01A006	–	+	+	+	+	+	+	++	++	–	–	–
Cs P45A002	–	+	+	–	–	+	–	–	–	–	–	–
Cp P45C001	–	–	–	–	–	–	–	–	–	–	–	–
Mc P34A004	–	+	+	+	+	+	+	++	++	–	–	–
Mc P34B007	–	+	+	–	+	+	+	++	++	–	–	–
Mc P40A002	–	+	+	+	+	+	+	++	++	–	–	–
Mp P01A016	–	+	+	+	+	+	+	++	++	–	–	–
Mp P01C004	–	+	+	+	+	+	+	++	++	–	–	–
Mg P34D003	–	–	–	+	+	+	–	–	–	–	–	–
Wa P42B001	–	–	+	+	+	+	–	+	+	–	–	–

[y] Yeast strains were cultured on glucose yeast peptone agar containing Luna Privilege (fluopyram), Pristine (pyraclostrobin and boscalid), Procure (triflumizole), and Vivando (metrafenone) at 50, 500, and 1,000 μg ml^{-1} for 72 h at 20°C under a 12-h photoperiod. Experiments were repeated three times. Symbols: – indicates no inhibition of colony size relative to the no-fungicide control, + indicates visible inhibition of colony size relative to control, and ++ indicates complete inhibition of colony growth.

[z] Yeast or yeast-like species: Ap = *Aureobasidium pullulans*, Cs = *Candida saitoana*, Cp = *Curvibasidium pallidicorallinum*, Mc = *Metschnikowia chrysoperlae*, Mp = *M. pulcherrima*, Mg = *Meyerozyma guilliermondii*, Sc: *Saccharomyces cerevisiae*, and Wa = *Wickerhamomyces anomalus*.

three yeast strains that utilized 70.2 to 81.9% of the total Biolog metabolites. Groups 3, 4, and 5 included yeasts that were moderate utilizers, at 40.4 to 63.8% utilization. Groups 2, 5, and 6 comprised singletons of *A. pullulans*, *W. anomalus* (moderate utilizers), and *C. pallidicorallinum* (poor utilizer), respectively. The *Botrytis* isolates, also poor utilizers at 22.3 to 31.9% utilization, were placed in group 7.

Yeasts from all six utilization groups reduced the severity of disease caused by the *Botrytis* isolates. This was not unexpected because, as a group, the *Botrytis* isolates utilized 20 of the 59 assimilates (33.9%), whereas the yeasts in group 6 used 25 assimilates (42.3%), of which 9 (6.6%) were coutilized between the two groups. In contrast, the yeasts in group 1 used 42 assimilates (71.2%), of which 16 (27.1%) were also utilized by the *Botrytis* group. Twelve compounds produced by grape berries (Conde et al. 2007; Fugelsang and Edwards 2007; Gerós et al. 2012; Zhao et al. 2016) were present in the Biolog YT assimilate panel (Table 8). All yeast and *Botrytis* isolates utilized glucose, which is the most abundant sugar in the berry. However, yeast and *Botrytis* isolates varied considerably in their ability to utilize common grape carbon sources. For example, α-ketoglutaric acid was utilized only by group 1 isolates. D-mannitol was utilized by yeasts but not by *Botrytis* isolates, and D-xylose in combination with dextrin supported the growth of *Botrytis* but not of yeasts. Surprisingly, D-xylose by itself was utilized by both yeasts and *Botrytis* isolates. Sucrose was differentially utilized among both yeasts and *Botrytis* isolates in our experiments. The *Botrytis* group (group 7) utilized four of the berry assimilates, whereas yeasts in groups 1 and 6 used eight and four, respectively. The yeast strains and *Botrytis* isolates coutilized sucrose, D-xylose, or D-raffinose. The data also indicated that yeast groups 1 and 6 had the least degree of overlap in assimilate utilization, both using D-xylose, xylitol, and D-mannitol.

TABLE 4. Log population densities of yeast cells in artificial wounds on Thompson Seedless grape over a 10-day interval[y]

Yeast species, strain[z]	Day 2	Day 4	Day 6	Day 10
Ap P01A006	6.0 e	6.5 b	6.8 cdef	7.1 abcd
Cs P45A002	6.3 bcd	6.7 b	6.8 bcdef	7.0 bcd
Cp P45C001	6.1 de	6.4 b	6.6 f	6.8 d
Mc P34A004	6.1 cde	6.6 b	6.9 ef	7.2 abcd
Mc P34B007	6.1 cde	6.4 b	6.9 cdef	7.0 cd
Mc P40A002	6.3 bcde	6.5 b	7.1 ab	7.4 ab
Mp P01A016	6.2 bcde	6.6 b	7.1 abc	7.4 abc
Mp P01C004	6.2 bcde	6.7 b	7.1 abcd	7.5 a
Mg P34D003	6.5 ab	6.9 a	7.1 abcde	7.3 ab
Sc HNN11516	6.3 abc	6.7 b	6.8 def	7.0 bcd
Wa P42B001	6.6 a	7.1 a	7.3 a	7.5 ab

[y] Mean log cells per milliliter population densities of three combined experiments. Means in a column with a similar letter are not significantly different according to Tukey's test ($P = 0.05$).

[z] A suspension (2 µl) of 10^5 cells ml^{-1} (200 cells) was applied to wounded berries, 10 berries per strain per time point. Yeast and yeast-like species: Ap = *Aureobasidium pullulans*, Cs = *Candida saitoana*, Cp = *Curvibasidium pallidicorallinum*, Mc = *Metschnikowia chrysoperlae*, Mp = *M. pulcherrima*, Mg = *Meyerozyma guilliermondii*, Sc: *Saccharomyces cerevisiae*, and Wa = *Wickerhamomyces anomalus*.

TABLE 5. In vitro inhibition activity of native yeasts against *Botrytis cinerea* isolates 101V3Dd, 111bb, 207cb, 207e, and 407cb on glucose yeast peptone agar

	Isolates of *Botrytis cinerea*[y]				
Yeast species, strain[z]	101V3Dd	111bb	207cb	207e	407cb
Ap P01A006	20.6	26.4 a	24.9 a	25.8 a	21.5 a
Cs P45A002	na	na	na	12.9 b	na
Cp P45C001	na	na	12.1 b	12.1 bc	11.0 b
Mc P34A004	na	14.3 b	na	na	na
Mc P34B007	na	na	11.3 bc	na	na
Mc P40A002	na	na	na	11.3 bc	na
Mp P01A016	na	11.1 c	na	10.7 c	na
Mp P01C004	na	9.5 c	10.1 c	na	na
Mg P34D003	na	14.6 b	12.5 b	na	na
Sc HNN11516	na	7.7 d	11.0 bc	na	na
Wa P42B001	na	na	12.6 b	12.3 bc	na

[y] Inhibition index of *Botrytis cinerea* = $(y/x + y)(100)$, where y is the distance from the center of the yeast colony to the edge of the fungal colony at the yeast–fungus interface, and x is the distance from the center of the fungal colony plug to the leading edge of the fungus. Values represent the averages of three measurements from each of two combined experiments ($n = 6$). Means in the same column with a similar letter are not significantly different ($P < 0.05$) according to the Fisher's protected least significant difference test; na = no inhibitory activity, determined in a preliminary screen (data not shown).

[z] Yeast and yeast-like species: Ap = *Aureobasidium pullulans*, Cs = *Candida saitoana*, Cp = *Curvibasidium pallidicorallinum*, Mc = *Metschnikowia chrysoperlae*, Mp = *M. pulcherrima*, Mg = *Meyerozyma guilliermondii*, Sc: *Saccharomyces cerevisiae*, and Wa = *Wickerhamomyces anomalus*.

DISCUSSION

The high genetic variation and plasticity, abundant sporulation, and the polycyclic nature of the *B. cinerea* life cycle continue to render this pathogen problematic for the grape industry (Fernández-Ortuño et al. 2012). Grape growers throughout the world are seeking to use fewer chemical pesticides for control of pathogens because of concerns about environmental sustainability, food quality, and fungicide resistance (De Miccolis Angelini et al. 2014; Fernández-Ortuño et al. 2015). Isolates of native yeasts are known to exert biological control of fungal diseases such as Botrytis bunch rot (Fleet 2003).

Our collection of Washington *B. cinerea* isolates showed significant differences in the temporal dynamics of disease development on inoculated individual Thompson Seedless grape berries based on AUDPC; however, with the exception of isolate R1V55#13, all of the isolates tested caused the same level of disease by 10 days after inoculation. Our findings support those of Dugan et al. (2002), who previously compared the virulence of isolates R1V55#13 and 1101V3Dd and reported that R1V55#13 caused lesions on Chardonnay grape berries that were significantly smaller and only one-half

TABLE 6. Suppression of disease caused by *Botrytis cinerea* on Thompson Seedless grape by native yeast

	Isolates of *Botrytis cinerea*[y]			
Yeast species, strain[z]	111bb	207a	207cb	407cb
No-yeast control	3.8 a	4.5 a	5.5 a	4.0 a
Ap P01A006	2.1 bc	2.3 b	2.4 bcd	2.2 bc
Cs P45A002	3.0 ab	3.0 ab	3.3 bc	2.8 abc
Cp P45C001	2.8 abc	2.9 b	3.8 ab	2.8 abc
Mc P34A004	2.4 bc	2.4 b	2.1 cd	2.0 bc
Mc P34B007	2.6 bc	2.9 abc	3.4 abc	3.1 ab
Mc P40A002	2.2 bc	2.1 b	1.9 d	2.1 bc
Mp P01A016	2.0 c	2.1 b	2.2 cd	1.9 bc
Mp P01C004	1.9 c	2.1 b	2.0 d	1.8 c
Mg P34D003	2.0 c	2.0 b	3.1 bcd	2.4 bc
Sc HNN11516	2.5 bc	2.5 b	2.4 bcd	2.1 bc
Wa P42B001	2.7 abc	2.4 b	2.4 bcd	2.5 bc

[y] Approximately 10 spores of *B. cinerea* isolates 111bb, 207a, 207cb, or 407a were applied to a wound 48 h after inoculation with 200 yeast cells. Disease severity was rated on a scale of 0 (healthy) to 7 (grape covered with sporulating fungus, berry shrunken) 8 days postinoculation. Values are averages of 10 berries from each of two replicated pooled experiments ($n = 20$). Means in the same column with the same letter are not significantly different ($P < 0.05$).

[z] Yeast and yeast-like species: Ap = *Aureobasidium pullulans*, Cs = *Candida saitoana*, Cp = *Curvibasidium pallidicorallinum*, Mc = *Metschnikowia chrysoperlae*, Mp = *M. pulcherrima*, Mg = *Meyerozyma guilliermondii*, Sc: *Saccharomyces cerevisiae*, and Wa = *Wickerhamomyces anomalus*.

the size of lesions caused by 101V3Dd. Additionally, the significantly slower growth rate of R1V55#13 as compared with 101V3Dd in vitro prompted the hypothesis that the reduced virulence of R1V55#13 could be due to a double-stranded RNA virus of *B. cinerea* typically associated with reduced fungal growth and virulence (Castro et al. 2003; Wu et al. 2007). However, phylogenetic analysis revealed that R1V55#13 does not cluster with other *B. cinerea* isolates and is more closely related to *B. prunorum*, including *B. prunorum* strain Bpru-21 from plum in Chile (Ferrada et al. 2016), which explains the lower virulence of R1V55#13 on grape. Similarly, Ferrada et al. (2016) reported that *B. prunorum* isolates were less virulent than *B. cinerea* on plum flowers and fruit, apple, and kiwifruit. In addition, the colony morphology of isolate R1V55#13 on KMB, APDA, and PAM was similar to that of *B. prunorum* from Chile (Ferrada et al. 2016) and different from the Washington *B. cinerea* isolates. To the best of our knowledge, isolate R1V55#13 may be the first report of *B. prunorum* from grape and it exemplifies recent differences in host preference, disease phenotype, and morphology among the *B. cinerea* isolates that formed the bases for new species (Dugan 2016; Walker 2016). Overall, this diversity suggests that the Washington population of *B. cinerea* comprises several to many genotypes, and perhaps is part of a *Botrytis* complex. An expanded survey of Washington *Botrytis* isolates will be needed to characterize possible members of the complex, and population complexity should be considered when formulating a biocontrol plan.

TABLE 7. Seven metabolic groups of yeasts and yeast-like strains and *Botrytis cinerea* isolates based on percentage utilization of oxidation and assimilation compounds and visual patterns of utilization in Biolog YT microplate assays

Yeast or *Botrytis*[w]	Group	Oxidation (%)[x]	Assimilation (%)[y]	Total (%)[z]
Mg P34D003	1	85.7	79.7	81.9
Cs P45A002	1	71.4	81.4	77.7
Sc HNN11516	1	65.7	72.9	70.2
Ap P01A006	2	60.0	62.7	61.7
Mp P01A016	3	57.1	67.8	63.8
Mp P01C004	3	54.3	64.4	60.6
Mc P40A002	3	54.3	64.4	60.6
Mc P34B007	4	57.1	54.2	55.3
Mc P34A004	4	45.7	37.3	40.4
Wa P42B001	5	37.1	57.6	50.0
Cp P45C001	6	2.9	42.4	27.7
Bc 101V3Dd	7	2.9	49.2	31.9
Bc 407cb	7	0	50.8	31.9
Bc 207a	7	0	45.8	28.7
Bc 111bb	7	0	44.1	27.7
Bc 207cb	7	0	35.6	22.3

[w] Yeast and yeast-like species: Ap = *Aureobasidium pullulans*, Bc = *Botrytis cinerea*, Cs = *Candida saitoana*, Cp = *Curvibasidium pallidicorallinum*, Mc = *Metschnikowia chrysoperlae*, Mp = *M. pulcherrima*, Mg = *Meyerozyma guilliermondii*, Sc: *Saccharomyces cerevisiae*, and Wa = *Wickerhamomyces anomalus*.
[x] Percentage of the 35 oxidation compounds utilized in both of two independent experiments.
[y] Percentage of the 59 assimilation compounds utilized in both of two independent experiments.
[z] Percentage of 94 oxidation plus assimilation compounds utilized in both of two independent experiments.

TABLE 8. Utilization of assimilation compounds present in grape berries by native yeast and yeast-like strains and *Botrytis* isolates as determined from Biolog YT microplate assays

	Assimilate[y]												
Strain, isolate[z]	Dex	D-gl	Glu	Gly	KG	L-ma	Mal	D-mn	D-rf	Suc	Xyl	D-xy	D+D
Yeast group 1													
Cs P45A002	−	+	+	+	+	+	+	+	+	+	+	+	−
Mg P34D003	−	+	+	−	+	+	+	+	+	+	+	+	−
Sc HNN11516	+	−	+	−	+	+	+	+	+	+	+	+	−
Yeast group 2													
Ap P01A006	−	+	+	+	−	+	+	+	+	+	+	+	−
Yeast group 3													
Mp P01A016	−	+	+	+	−	+	+	+	−	+	+	+	−
Mp P01C004	−	+	+	+	−	+	+	+	−	−	+	+	−
Mc P40A002	−	+	+	+	−	+	+	+	−	+	+	+	−
Yeast group 4													
Mc P34A004	−	+	+	−	−	−	+	+	−	−	+	+	−
Mc P34B007	−	+	+	−	−	+	+	+	−	−	+	+	−
Yeast group 5													
Wa P42B001	−	−	+	+	−	+	+	+	+	+	−	+	−
Yeast group 6													
Cp P45C001	−	−	+	+	−	−	−	+	−	+	+	+	−
Botrytis group 7													
101V3Dd	+	−	+	−	−	−	−	−	+	+	−	+	+
111bb	+	−	+	−	−	−	−	−	+	+	−	+	+
207a	−	−	+	−	−	−	−	−	+	+	−	+	+
207cb	−	−	+	−	−	−	−	−	+	+	−	+	+
407cb	−	−	+	−	−	−	−	−	+	+	−	+	+

[y] Dex = dextrin, D-gl = D-gluconic acid, Glu = glucose, Gly = glycerol, KG = α-KG, L-ma = L-malic acid, Mal = maltitol, D-mn = D-mannitol, D-rf = D-raffinose, Suc = sucrose, Xyl = xylitol, D-xy = D-xylose, D+D = D-xylose + dextrin, + indicates utilization of assimilate, and − indicates no utilization.
[z] Yeast and yeast-like species: Ap = *Aureobasidium pullulans*, Cs = *Candida saitoana*, Cp = *Curvibasidium pallidicorallinum*, Mc = *Metschnikowia chrysoperlae*, Mp = *M. pulcherrima*, Mg = *Meyerozyma guilliermondii*, Sc: *Saccharomyces cerevisiae*, and Wa = *Wickerhamomyces anomalus*.

The yeasts used in this study showed potential as biocontrol microbes because they rapidly colonized grape berry wounds and reduced disease severity of one or more pathogen isolates on the berry without causing a significant wound response. The most promising biocontrol strains in our study were *Metschnikowia* spp., *Meyerozyma guilliermondii*, *S. cerevisiae, W. anomalus*, and the yeast-like fungus *A. pullulans*. *Metschnikowia pulcherrima* is well-known for biocontrol activity against *B. cinerea* on apple (Spadaro et al. 2002; Wisniewski et al. 1991). *S. cerevisiae* has been widely tested as a biocontrol agent of postharvest pathogens and is commonly added to fermentations to control undesirable microorganisms (Lopes et al. 2015). Our *S. cerevisiae* strain, isolated from a 2014 native fermentation (X. Wang and D. Glawe, unpublished data), was an effective antagonist of *B. cinerea* in laboratory assays; however, it is not clear whether this strain is part of the native vineyard microbiota or a commercial strain that escaped from the winery. Strains of *A. pullulans* have been tested as biocontrol agents of wheat and postharvest diseases of fruit (Calvo-Garrido et al. 2014; Mari et al. 2012; Vero et al. 2009; Zhang et al. 2012). However, this organism is also known to be an opportunistic phytopathogen, causing decay on apple fruit (Heidenreich et al. 1997) and skin discoloration, degradation, and abnormalities in sweet cherry (Kim 2014). On grape, it is a member of a complex causing melting decay of Red Globe grape (Morgan and Michailides 2004). None of our isolates caused a significant rotting and collapse of the grape berry as occurs with melting disease. Although our findings indicate that *A. pullulans* is an effective broad-range inhibitor of Washington isolates of *Botrytis*, its ability to cause browning on grape berries and off-target symptoms on other fruit crops justifies further study of its potential impact on the quality of Washington wines.

Given the genetic diversity that occurs within beneficial native yeasts such as *M. pulcherrima* (Janisiewicz and Korsten 2002) and *M. chrysoperlae*, we think it prudent to develop and deploy yeast strains that are indigenous on our locally adapted grape cultivars. The potential effects of introduced biocontrol yeast on fermentation and wine processing is especially important because Washington wine makers rely on native yeast in vineyards for their important contributions to the aroma, flavor, and mouthfeel qualities of local premium wines. For example, *M. pulcherrima* has a role in fermentation as an inhibitor of other yeasts that impart undesirable flavors and aromas (Jolly et al. 2006) and reducing ethanol content in musts with high sugar content (J. Aplin, C. Edwards, N. Rivera, and D. Glawe, unpublished data), thereby improving wine quality. Native yeast isolates also have other highly desirable metabolic capacities such as acetic acid reduction and malolactic fermentation (Vilanova et al. 2007) that need to be considered.

Fungicide tolerance in several Washington yeast strains suggests that they could be used in combination with chemical fungicides (Lima et al. 2003, 2006). Coapplication or staggered applications of yeasts and fungicides has potential as an integrated management tool but development will require studies on compatibility, formulation, and optimization of timing. Native yeasts might also be compatible with glycolchitosan to inhibit spore germination (El-Ghaouth et al. 2000) or with plant growth regulators such as indole-3-acetic acid (Yu and Zheng 2007).

Competition for nutrients and space (niche exclusion) are generally considered to be the major mechanisms of action of most antagonistic yeasts against postharvest pathogens (Bencheqroun et al. 2007; Droby et al. 1989; El-Ghaouth et al. 1998; Haidar et al. 2016; Parafati et al. 2015; Wisniewski et al. 1991). Wound-infecting necrotrophic fungi such as *B. cinerea* require nutrients for germination and initiation of infection and, thus, are especially sensitive to niche exclusion (Janisiewicz and Korsten 2002). Yeasts are most effective as biocontrol agents when they become established on the host prior to pathogen challenge, and when they are able to rapidly colonize host tissues, as demonstrated by commercial biocontrol yeast strains (Janisiewicz and Korsten 2002). Our berry-based inhibition assay was designed to find yeasts with these traits and they showed disease suppression in many of the yeast–*Botrytis* interactions, suggesting nutrient or niche competition as a mechanism of biocontrol.

Similarities in metabolite oxidation and nutrient utilization among native yeasts and *Botrytis* isolates were isolate-dependent, and sometimes differed among individuals of the same species. The three assimilates known to be present in the berry and utilized by both yeasts and *Botrytis* isolates suggested that these were major nutrients for *Botrytis* in situ, and were limiting to the pathogen when the yeasts were present. However, additional mechanisms such as competition for minerals, water, or niche occupancy could also be important on the berry. Our berry experiments also showed that the *B. cinerea* isolates were differentially sensitive to the yeasts, suggesting that other biocontrol mechanisms may be important (Janisiewicz and Korsten 2002). For example, induced host resistance by yeasts is known in various fruit (Droby et al. 2002; Fajardo et al. 1998; Porat et al. 1999), as in the case of *Candida saitoana* in apple (Ippolito et al. 2000) and *A. pullulans* in strawberry (Adikaram et al. 2002). Interestingly, Hadwiger et al. (2015) tested seven of the same isolates used in our study and demonstrated that all of them induced phytoalexin production in pea and some of the isolates suppressed potato late blight, possibly by induced resistance. Low molecular weight metabolites such as volatile organic compounds, extracellular proteases, cell-wall-degrading enzymes, iron competition, parasitism, biofilm formation, and oxidative stress can also contribute to pathogen suppression by yeast (Castoria et al. 2001; Grevesse et al. 2003; Mari et al. 2012; Parafati et al. 2015; Spadaro and Droby 2016; Wisniewski et al. 1991; Zhang et al. 2012). Our findings do not exclude antibiosis as a mechanism because antibiotic production can be dependent upon environmental and cultural conditions and interactions with the host and other microorganisms.

The differential sensitivity of our yeast suggests that Botrytis bunch rot control over a wide geographic area might require multiple yeast strains. Competition among biocontrol strains of yeast could restrict the use of yeast combinations for disease suppression (Xu and Jeger 2013; Xu et al. 2010). Improved efficacy and adaptability of a biocontrol formulation in the field (Janisiewicz and Korsten 2002) can be achieved using yeast isolates that do not compete for nutrient resources or do not antagonize each other (Woods and Bevan 1968; Young and Yagiu 1978). Therefore, we used Biolog plate studies as an initial comparison of the carbon and nitrogen utilization profiles among our yeast strains and *Botrytis* isolates. Both visual inspection and PCA suggested seven distinct metabolic groups. We noted that all yeasts and *Botrytis* isolates assimilated glucose, and observed substantial overlap in utilization among the yeast species. However, in the subset of assimilates present in grape berries, there were more differences in utilization. The yeasts have the potential to compete with each other if codeployed in the vineyard. In that regard, yeasts from groups 1 and 6 had the least degree of overlap in assimilate utilization. These data suggest that there is potential for combining specific yeast isolates in biocontrol formulations, especially if alternative biocontrol mechanisms such as iron sequestration and production of antimicrobial volatile organic compounds in *M. pulcherrima* (Parafati et al. 2015; Sipiczki 2006) are deployed.

This study used a popular table grape cultivar for biocontrol and colonization experiments because of its year-round availability; however, studies using wine grape cultivars such as Cabernet Sauvignon and Chardonnay, and selection and testing of single strains and strain combinations for improved biocontrol activity and wine quality, will be of interest in future studies.

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