Combinations of Fungicides with Phylloplane Yeasts for Improved Control of *Botrytis cinerea* on Geranium Seedlings

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Accepted for publication 24 September 2003.

ABSTRACT

Buck, J. W. 2004. Combinations of fungicides with phylloplane yeasts for improved control of *Botrytis cinerea* on geranium seedlings. *Phytopathology* 94:196-202.

Control of *Botrytis cinerea* on geranium seedlings was evaluated in treatments with phylloplane yeasts in combination with 10 fungicides used to manage Botrytis blight of ornamental plants. *Rhodotorula glutinis* PM4 significantly reduced the development of lesions caused by *B. cinerea* on geranium cotyledons; however, yeast biocontrol efficacy was highly variable between trials. Treatment with the yeast in combination with azoxystrobin or trifloxystrobin at one tenth the labeled rate (7.5 $\mu\text{g a.i. ml}^{-1}$) or the full labeled rate (7.5 $\mu\text{g a.i. ml}^{-1}$) reduced lesion development, compared to treatment with the yeast or the fungicide alone. Vinclozolin at half the labeled rate or the full labeled rate (250 or 500 $\mu\text{g a.i. ml}^{-1}$), in combination with *R. glutinis* PM4, significantly reduced the development of lesions caused by an isolate of *B. cinerea* resistant to vin-

clozolin. Copper hydroxide and iprodione at one-tenth the labeled rates, with or without yeast, were highly effective in limiting lesion development. Mancozeb did not increase the biocontrol efficacy of the yeast, and thiophanate-methyl negatively affected the yeast efficacy. Improved disease control was observed in treatments with vinclozolin at the labeled rate and *R. glutinis* PM4 at cell densities of 5×10^5 and 1×10^6 cells ml^{-1} , but not 1×10^5 cells ml^{-1} , on seedlings co-inoculated with *B. cinerea* in a suspension containing 1×10^5 conidia ml^{-1} . Disease control improved in treatments with combinations of vinclozolin and eight other isolates of *R. glutinis*, two isolates of *R. graminis*, and two isolates of *R. mucilaginosa*. Biocontrol was not observed in treatments with two isolates of *R. minuta*. The combination of yeast and vinclozolin significantly reduced the germination of conidia of *B. cinerea* and the growth of *R. glutinis* PM4 in vitro. All combinations of *R. glutinis* PM4 with azoxystrobin, trifloxystrobin, or vinclozolin provided highly effective and consistent disease control not observed in treatments with the fungicides alone or the yeast alone.

Saprophytic yeasts have been studied as potential biological control agents for the control of various fungal pathogens, including *Botrytis cinerea*, *Penicillium* spp., and *Monilinia fructicola* (6,14,21). Biological control yeasts have been used to manage postharvest losses due to these fungi in apples, grapes, pears, peach, sweet cherries, and citrus (6,10,11,15,17,18,21,22). The yeast *Candida oleophila* is the active ingredient in the commercial biocontrol product Aspire, which is marketed for the control of postharvest diseases of citrus (10). Antagonistic yeasts have also been used to manage Botrytis blight of bean, cucumber, and tomato, caused by *B. cinerea* (9,13).

One difficulty in using biological control agents is the need to integrate biological control into existing management plans and the requirement for consistent disease control comparable to that obtained by fungicides (12). Biological control can be enhanced by the use of mixtures of microorganisms, manipulation of the environment, or manipulation of formulations (17). In several postharvest systems, the addition of fungicides at reduced rates in combination with biocontrol yeasts has significantly enhanced disease control, compared to treatments with the yeasts alone. The combination of thiabendazole at 200 $\mu\text{g ml}^{-1}$ with either *C. oleophila* or *Pichia guilliermondii* was as effective as conventional fungicide treatments in reducing citrus fruit decay caused by *Penicillium* spp. (10,11). *Pichia membranefaciens* mixed with iprodione at 100 $\mu\text{g a.i. ml}^{-1}$ significantly reduced the diameter of lesions caused by *Rhizopus stolonifer* on nectarine fruits, compared to treatment with the yeast alone (22). In two of three trials

in commercial packinghouses, a combination of thiabendazole at 0.1 g liter⁻¹ and *P. guilliermondii* reduced the percentage of oranges infected by *Penicillium* spp., compared to treatment with the yeast alone (2). Combinations of yeasts with iprodione or thiabendazole at reduced rates (20 and 15 $\mu\text{g a.i. ml}^{-1}$, respectively) significantly enhanced postharvest control of *Penicillium expansum* on apple and pear and *M. fructicola* on sweet cherry, compared to treatment with the fungicides alone at high rates (6–8). In all cases, the disease was significantly reduced in fruit treated with the fungicide alone at the standard high dose, compared to untreated checks (2,6–8,10,11,22).

Botrytis blight of stems, leaves, and flowers of geranium is a limiting factor in commercial greenhouse production (16). Phylloplane yeasts isolated from various ornamental plants have been shown to be antagonistic to *B. cinerea* on geranium leaf disks (3). Yeasts initially identified as poor competitors have been found to significantly reduce lesion formation by *B. cinerea* when the ratio of yeast cells to conidia is 50:1 or 100:1 and nutrients are supplied at reduced levels. However, under these nutrient-limiting conditions, the biocontrol activity of some of yeast isolates varied widely in different trials (3). Phylloplane yeasts are resistant to a wide range of fungicides (5), but it is unknown whether any of these fungicides enhance biological control of *B. cinerea* on geranium by yeasts. The objectives of this work were (i) to determine whether fungicides used for managing *B. cinerea* on ornamental plants enhance disease control by the biocontrol yeast *Rhodotorula glutinis* PM4; (ii) to determine whether vinclozolin or thiophanate-methyl plus yeast enhance control against an isolate of *B. cinerea* resistant to both fungicides; (iii) to determine whether vinclozolin enhances control in combination with other *Rhodotorula* spp.; and (iv) to determine the effects of thiophanate-methyl and vinclozolin on the growth of the yeast and the germination of *B. cinerea*.

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Publication no. P-2003-1120-01R

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MATERIALS AND METHODS

Culture maintenance. Nine isolates of *R. glutinis* (PM4, 1C, 1D, 1F, SF1, SF2, SF3, R3, and R4) and a *Ustilago* sp. (isolate A5) from various ornamental plants and turfgrass were studied, having previously been screened for biocontrol activity against *B. cinerea* (3). *R. glutinis* PM4 and *Ustilago* sp. A5 were classified as “good” and “poor” antagonists of *B. cinerea*, respectively (3). Eight additional yeast isolates were obtained from the Agricultural Research Service Culture Collection, Peoria, Illinois: *R. glutinis* NRRL Y-2502 (type strain) and Y-2504, *R. minuta* Y-1589 (type strain) and Y-21, *R. graminis* Y-2474 (type strain) and Y-5791, and *R. mucilaginosa* Y-2510 (type strain) and Y-27053. All yeast isolates were stored in 15% glycerol at -70°C . Working cultures were started on potato dextrose agar (PDA) (Difco Laboratories, Detroit) and incubated for 3 days at 26°C in the dark. A loopful of test yeast was suspended in distilled water, yeast cells were enumerated by hemacytometer, and the suspension was diluted with water to a cell density of 1×10^6 cells ml^{-1} , unless stated otherwise.

B. cinerea was isolated from a symptomatic geranium plant (3). A conidial suspension was stored in 15% glycerol at -70°C . Working cultures were started on PDA and incubated beneath two 34-W cool white fluorescent light bulbs (8 h of light, approximately $50 \mu\text{mol s}^{-1} \text{m}^{-2}$, followed by 16 h of darkness) for 2 to 3 weeks at 22 to 24°C . Conidia were dislodged with a glass rod from cultures flooded with 8 to 10 ml of sterile 0.05% Tween 20 (J. T. Baker, Phillipsburg, NJ). The resulting suspension was filtered through five layers of sterile cheesecloth. The conidial suspension was centrifuged ($1,500 \times g$), the conidia were resuspended in sterile distilled water and enumerated by hemacytometer, and the suspension was diluted with water to a conidial density of 1×10^5 conidia ml^{-1} .

Botrytis seedling assay. Seeds of the geranium cultivar White Elite (*Pelargonium xhortorum*) were sown in Metro-Mix 360 (Scotts, Marysville, OH) in 72-square plug flats (TLC Polyform, Plymouth, MN), in which each square was $5.7 \times 4.0 \times 2.5$ cm. The flats were incubated under ambient light on a greenhouse bench with average day and night temperatures of 26 and 20°C until both cotyledons of the seedlings had fully expanded (about 12 to 14 days). Each 72-square flat was separated into 12 six-cell sections, with a single seedling per cell. Both cotyledons of each seedling were inoculated with a single 10- μl drop of a test suspension of *B. cinerea* conidia (1×10^5 conidia ml^{-1}) in various combinations with fungicides and biocontrol yeasts. All treatments included 25 mM glucose and 20-fold-diluted yeast nitrogen base (Difco) (3). Three replicate 6-cell sections per treatment were incubated in separate plastic containers ($59 \times 43 \times 15$ cm) (Sterlite, Townsend, MA) with sealed lids. An experimental unit (replicate) consisted of 12 cotyledons. Each container held 14 six-cell sections. Approximately 1 liter of tap water was added to each container to help maintain high relative humidity. Relative humidity in the containers was monitored by Watchdog data loggers (Model 150, Spectrum Technologies, Plainfield, IL) and ranged from 95 to 100%. After inoculation the sealed containers were placed in an incubator at 21°C in the dark for 72 h. The diameter (in millimeters) of lesions on both cotyledons of each seedling (36 cotyledons per treatment) was recorded. The seedlings were then moved to a greenhouse bench under ambient light, and seedling mortality (percent survival) was recorded after an additional 7 days (10 days after inoculation).

Fungicide screening. The following 10 fungicides registered for management of *B. cinerea* on geranium or other ornamental plants were tested to determine their effect on the biological control activity of yeasts: azoxystrobin (Heritage 50 WG, Syngenta Crop Protection, Greensboro, NC), chlorothalonil (Daconil Ultrex, Syngenta), copper hydroxide (Kocide 2000 T/N/O, Griffin, Valdosta, GA), fenhexamid (Decree, SePRO, Carmel, IN),

fludioxonil (Medallion, Syngenta), iprodione (Chipco 26019, Aventis Environmental Science, Montvale, NJ), mancozeb (Dithane T/O, Dow Agrosiences, Indianapolis), thiophanate-methyl (Cleary's 3336, Cleary Chemical, Dayton, NJ), trifloxystrobin (Compass O, Olympic Horticultural Products, Bradenton, FL), and vinclozolin (Touché EG, Lescro, Strongsville, OH). Flutolanil (Contrast 70 WSP, Scotts), which is not used to manage *B. cinerea* on ornamental plants, was included as a negative control, because some of the yeast isolates are sensitive to this fungicide (5).

Each fungicide was suspended in sterile distilled water, agitated, and added to a conidial suspension (1×10^5 conidia ml^{-1}) with or without a test yeast (1×10^6 cells ml^{-1}). Six of the fungicides were diluted to final concentrations of one-tenth the labeled rate and the full labeled rate: azoxystrobin, 7.5 and $75.0 \mu\text{g a.i. ml}^{-1}$; chlorothalonil, 139 and $1,390 \mu\text{g a.i. ml}^{-1}$; fenhexamid, 60 and $600 \mu\text{g a.i. ml}^{-1}$; fludioxonil, 7.5 and $75.0 \mu\text{g a.i. ml}^{-1}$; iprodione, 60 and $600 \mu\text{g a.i. ml}^{-1}$; and trifloxystrobin, 7.5 and $75.0 \mu\text{g a.i. ml}^{-1}$. Copper hydroxide ($64 \mu\text{g a.i. ml}^{-1}$) and mancozeb ($144 \mu\text{g a.i. ml}^{-1}$) were tested at one-tenth their labeled rates. Thiophanate-methyl and vinclozolin were tested at one-tenth and one-half their labeled rates and at the full labeled rates (50, 250, and $500 \mu\text{g a.i. ml}^{-1}$). Flutolanil was tested at a concentration of $25 \mu\text{g a.i. ml}^{-1}$ (5). The seedlings were treated with *B. cinerea* alone, *B. cinerea* with *R. glutinis* PM4 (no fungicide), *B. cinerea* with *R. glutinis* PM4 and fungicide, and *B. cinerea* with fungicide. *Ustilago* sp. A5 was tested with thiophanate-methyl and vinclozolin. Each fungicide was tested at least two times at each concentration. Azoxystrobin and trifloxystrobin were tested three times.

Effect of yeast concentration and yeast isolate on fungicide-biocontrol interaction. Geranium seedlings were inoculated with *R. glutinis* PM4 or *Ustilago* sp. A5, at cell densities of 1×10^5 , 5×10^5 , and 1×10^6 cells ml^{-1} , in combination with *B. cinerea* in a suspension containing 1×10^5 conidia ml^{-1} (3), with or without vinclozolin ($500 \mu\text{g a.i. ml}^{-1}$). The ratio of yeast cells to *B. cinerea* conidia at these concentrations was 1:1, 5:1, and 10:1. The experiment was conducted three times. Each of 16 isolates from four species of *Rhodotorula* was also tested for biological control activity against *B. cinerea* on geranium seedlings. The ratio of cells of each yeast isolate to *B. cinerea* conidia was 10:1. Seedlings were inoculated with *B. cinerea* alone or in combination with yeast, vinclozolin ($500 \mu\text{g a.i. ml}^{-1}$), or both yeast and vinclozolin. The experiment was conducted two times.

Effect of vinclozolin and thiophanate-methyl on *B. cinerea* conidial germination and yeast growth. Stock plants of the geranium cultivar White Elite were grown in Metro-Mix 360 in 11.4-liter pots and fertilized every 2 weeks with Peters 15-15-15 Geranium Special (Scotts). Ten- to 12-week-old leaves were removed and surface-sterilized by immersion in 0.53% NaOCl for 60 s and then in 70% ethanol for 20 s, followed by three rinses in distilled water. Disks (12 mm in diameter) were cut and placed on sterilized filter paper moistened with sterile water in glass petri dishes. The disks were inoculated with 25- μl drops containing *R. glutinis* PM4 alone or in combination with conidia of *B. cinerea*, with conidia of *B. cinerea* and vinclozolin or thiophanate-methyl ($500 \mu\text{g a.i. ml}^{-1}$), or with a fungicide but without *B. cinerea* and incubated at 22°C in the dark. The inoculum densities were 1×10^6 yeast cells ml^{-1} and 1×10^5 conidia ml^{-1} in 25 mM glucose and 20-fold-diluted yeast nitrogen base. Yeast populations on five individual leaf disks (one inoculum droplet per disk) were determined for each treatment by leaf washing and dilution plating (4) at the start of each experiment and after 24 h of incubation. Each disk was placed in 1 ml of sterile 0.05% Tween 20 solution in a 1.5-ml microcentrifuge tube and agitated on a rotary shaker at 250 rpm for 60 min. Yeast colony-forming units (CFU) were quantified by serial dilution, in which each leaf disk wash solution was diluted 50-, 500-, and 1,000-fold, and four 50- μl drops

of each dilution were placed on PDA amended with chloramphenicol (100 mg liter⁻¹). Colonies were counted after 48 to 72 h of incubation at 26°C in the dark. The experiment was repeated.

Germination of *B. cinerea* conidia was determined in four separate 100-μl aliquots of all pairwise combinations of conidia, *R. glutinis* PM4, and thiophanate-methyl or vinclozolin (each at 50 μg a.i. ml⁻¹). All treatments included 25 mM glucose and 20-fold-diluted yeast nitrogen base. The tested isolate of *B. cinerea* does not germinate in distilled water without the addition of nutrients (3). Each aliquot was dispensed into a separate well in a 96-well microtiter plate. The plates were incubated at 21°C for 24 h, and at least 200 conidia per well were examined microscopically to assess germination. A conidium was considered germinated if the length of the germ tube was greater than the width of the conidium. Data were converted to percent germination. The experiment was repeated.

Statistical analysis. If there were no significant trial-by-treatment interactions, data from repeated experiments were pooled for analysis. Data were subjected to analysis of variance using the general linear model procedure (PROC GLM) of SAS (SAS Institute, Cary, NC). Means were separated by Fisher's protected least significant differences at *P* = 0.05. The relationship between seedling mortality and lesion diameter was determined by the Pearson correlation procedure (PROC CORR) of SAS.

RESULTS

Screening of fungicides and yeasts in vitro and on seedlings. No lesion development was observed on seedlings inoculated

with *B. cinerea* conidia and treated with chlorothalonil, fenhexamid, or fludioxonil at either one-tenth the labeled rate or the full rate, regardless of whether *R. glutinis* PM4 was added. The average lesion diameter was 9.8 mm in trial 1 and 10.1 mm in trial 2. With the addition of *R. glutinis* PM4, lesion size was significantly reduced, to 6.1 mm in trial 1 (LSD = 1.9) and 1.2 mm in trial 2 (LSD = 1.1). Small lesions (less than 1 mm in diameter) were observed in treatments with iprodione at one-tenth the labeled rate (60 μg a.i. ml⁻¹) (average lesion diameter of 0.8 mm in trial 1 and 0.6 mm in trial 2) and copper hydroxide at one-tenth the labeled rate (average lesion diameter of 0.1 mm in trial 1 and 0.3 mm in trial 2). No lesions were observed when *R. glutinis* PM4 was included in treatment with copper hydroxide or iprodione. Treatment with mancozeb at one-tenth the labeled rate (144 μg a.i. ml⁻¹) resulted in an average lesion diameter of 5.2 mm in trial 1 and 9.0 mm in trial 2. Treatment with mancozeb combined with *R. glutinis* PM4 resulted in an average lesion diameter of 5.8 mm, which was not significantly different from the lesion development in treatments with mancozeb only or with yeast only in trial 1. In trial 2, lesion diameter in the treatment with both yeast and mancozeb (2.9 mm) was significantly larger than in the treatment with yeast alone (1.2 mm). Lesions were not observed on cotyledons that were not inoculated with *B. cinerea*.

In separate experiments, flutolanil (25 μg a.i. ml⁻¹) eliminated the biocontrol activity of *R. glutinis* PM4 (in two trials, the average lesion diameters were 15.0 and 11.0 mm, not significantly different from lesion diameters of 15.0 and 11.0 mm in the controls inoculated with *B. cinerea* alone). The average lesion diameter in the flutolanil treatment without yeast was 11.7 mm in trial 1 (LSD = 2.0) and 15.0 mm in trial 2 (LSD = 1.2).

Treatment with *R. glutinis* in combination with azoxystrobin or trifloxystrobin at 7.5 or 75.0 μg a.i. ml⁻¹ significantly reduced lesion formation caused by *B. cinerea*, compared to treatment with the fungicide alone or the yeast alone in trial 1 (Fig. 1A). Lesion diameters were less than 2 mm in all azoxystrobin-yeast and trifloxystrobin-yeast combinations. A significant trial-by-treatment interaction (*P* = 0.001) was observed in the first two trials, which was due to the difference in average lesion sizes in the yeast-*B. cinerea* control treatment (6.1 mm in trial 1 and 1.6 mm in trial 2). In the second and third trials (combined data shown in Fig. 1B), all yeast-fungicide treatments resulted in significantly smaller lesions than the fungicide treatments alone. No lesions were observed in treatment with *R. glutinis* plus trifloxystrobin at the full labeled rate (Fig. 1B); lesion development was significantly reduced in comparison to treatment with the yeast alone.

The isolate of *B. cinerea* used in these studies is resistant to thiophanate-methyl and vinclozolin (Table 1 and Fig. 2). In two seedling assays, vinclozolin at one-tenth the labeled rate (50 μg a.i. ml⁻¹) did not significantly affect the development of lesions

Fig. 1. Lesion development on geranium cotyledons inoculated with *Botrytis cinerea* alone or in combination with *Rhodotorula glutinis* PM4 and azoxystrobin or trifloxystrobin at rates of 7.5 and 75.0 μg a.i. ml⁻¹. Data are the average lesion diameters in three replicates of six seedlings for each treatment, in **A**, a single trial and **B**, combined results of two trials.

TABLE 1. In vitro germination of conidia of *Botrytis cinerea* alone or in combinations with *Rhodotorula glutinis* PM4, thiophanate-methyl, and vinclozolin

Treatment ^a	Germination (%) ^b	
	Trial 1	Trial 2
None (<i>B. cinerea</i> conidia alone)	72.0	82.2
Thiophanate-methyl	72.0	88.2
Vinclozolin	73.8	83.4
<i>R. glutinis</i> PM4	51.3	46.1
<i>R. glutinis</i> PM4 and thiophanate-methyl	46.0	53.4
<i>R. glutinis</i> PM4 and vinclozolin	24.3	34.6
LSD (<i>P</i> = 0.05)	8.5	6.0

^a Fungicides were added at a concentration of 50 μg a.i. ml⁻¹. All treatments included 25 mM glucose and 20-fold-diluted yeast nitrogen base.

^b At least 200 conidia were examined microscopically to determine germination in each of four replicate wells after 24 h at 22°C.

caused by *B. cinerea*, with average lesion diameters of 10.6 and 10.8 mm, respectively. Lesion diameters in treatment with *B. cinerea* alone were 10.1 mm in trial 1 (LSD = 1.1) and 11.0 mm in trial 2 (LSD = 2.0). Treatment with vinclozolin at 500 µg a.i. ml⁻¹ together with *R. glutinis* PM4 resulted in lesion diameters of 0.3 and 0.0 mm, compared to 1.2 and 1.6 mm in treatment with *R. glutinis* PM4 alone in trials 1 and 2, respectively. Data from the two trials could not be combined, because of a significant trial-by-treatment interaction ($P = 0.001$). Thiophanate-methyl at one-tenth the labeled rate reduced biocontrol efficacy in both trials, with average lesion diameters of 3.7 and 10.0 mm.

In two trials, vinclozolin at either half the labeled rate (250 µg a.i. ml⁻¹) or the full labeled rate (500 µg a.i. ml⁻¹) in combination with *R. glutinis* PM4 significantly reduced lesion development, compared to inoculation with *B. cinerea* alone or treatment with *B. cinerea* and *R. glutinis* PM4 (Fig. 2). However, thiophanate-methyl (250 and 500 µg a.i. ml⁻¹) and a mixture of thiophanate-methyl and vinclozolin (each at 250 µg a.i. ml⁻¹) did not reduce lesion development, with or without *R. glutinis* PM4, and significantly increased lesion size, compared to treatment with *R. glutinis* PM4 alone. Seedling survival was negatively correlated with lesion diameter ($r = -0.717$, $P = 0.0001$). Seedling survival and lesion size were 15.0% and 10.6 mm in treatment with *B. cinerea* alone, 9.5% and 11.5 mm in treatment with *B. cinerea* and vinclozolin at 250 µg a.i. ml⁻¹, and 23.3% and 10.5 mm in treatment with *B. cinerea* and vinclozolin at 500 µg a.i. ml⁻¹; seedling survival increased and lesion diameter decreased to 48.3% and 5.2 mm, 91.1% and 1.8 mm, and 88.3% and 1.6 mm, respectively, in these three treatments with the addition of *R. glutinis* PM4. Treatment with vinclozolin or thiophanate-methyl at three concentrations (50, 250, and 500 µg a.i. ml⁻¹) in combination with *Ustilago* sp. A5 did not significantly reduce lesion formation caused by *B. cinerea* (data not shown).

Effect of yeast concentration and vinclozolin on biocontrol efficacy. Data from the three trials could not be combined for analysis, because of significant trial-by-treatment interactions. The two trials with different results in treatments with 5×10^5 CFU ml⁻¹ are shown in Figure 3. *R. glutinis* PM4 alone reduced lesion development at cell densities of 5×10^5 CFU ml⁻¹ in one of three trials and 1×10^6 CFU ml⁻¹ in all three trials. No biocontrol was observed with a yeast concentration of 1×10^5 CFU ml⁻¹ in the three trials (Fig. 3). The addition of vinclozolin at 500 µg a.i. ml⁻¹

Fig. 2. Lesion development on geranium cotyledons inoculated with *Botrytis cinerea* alone or in combination with *Rhodotorula glutinis* PM4 and vinclozolin or thiophanate-methyl at half the labeled rate (250 µg a.i. ml⁻¹) and the full labeled rate (500 µg a.i. ml⁻¹). Data are the average lesion diameters in three replicates of six seedlings for each treatment, in the combined results of two trials. Vin/Thio = a mixture of vinclozolin and thiophanate-methyl, each at 250 µg a.i. ml⁻¹.

significantly improved control by *R. glutinis* PM4 at cell densities of 5×10^5 in two of the three trials and 1×10^6 CFU ml⁻¹ in all three trials (Fig. 3). Cell densities of 1×10^6 and 5×10^5 CFU ml⁻¹ were used to attain, respectively, 10:1 and 5:1 ratios of yeast to *B. cinerea*. Vinclozolin did not enhance biocontrol at a 1:1 ratio of yeast (1×10^5 CFU ml⁻¹) to *B. cinerea* (Fig. 3). Vinclozolin did not enhance the biocontrol activity of *Ustilago* sp. A5 at any fungicide concentration (data not shown).

Effect of vinclozolin on biocontrol efficacy of different yeast isolates. Eleven additional isolates of four species of *Rhodotorula* (*R. glutinis*, *R. mucilaginosa*, *R. graminis*, and *R. minuta*) were tested alone (at 1×10^6 CFU ml⁻¹) and in combination with vinclozolin at 500 µg a.i. ml⁻¹. In one trial (Fig. 4A), none of these yeast isolates reduced lesion development when used alone, but in combination with vinclozolin all but four of them (*R. glutinis* Y2504, *R. mucilaginosa* Y27053, and *R. minuta* Y1589 and Y21) significantly reduced lesion development, compared to treatments with no fungicide. In the second trial (Fig. 4B), all but five yeast isolates (*R. glutinis* Y2504, *R. mucilaginosa* Y2510 and Y27053, and *R. minuta* Y1589 and Y21) significantly reduced lesion development, compared to treatments with no fungicide and treatment with vinclozolin alone. The *R. minuta* isolates were not effective, either alone or in combination with vinclozolin, in any tests relative to the fungicide and no-fungicide controls. For the other isolates (except *R. graminis* Y2474), the addition of vinclozolin also resulted in reduced lesion development, compared to treatment with the yeast alone, in at least one of the two trials. Treatments with vinclozolin in combination with eight isolates of *R. glutinis* obtained from turfgrass and ornamental plants—isolates 1C, 1D, and 1F (Fig. 4) and isolates SF1, SF2, SF3, R3, and R4 (data not shown)—significantly reduced lesion development, compared to treatments with yeast alone.

Fig. 3. Effect of the concentration of *Rhodotorula glutinis* PM4 and vinclozolin (500 µg a.i. ml⁻¹) on lesion development on geranium cotyledons inoculated with *Botrytis cinerea* at 1×10^5 conidia ml⁻¹. Data are the average lesion diameters in three replicates of six seedlings for each treatment in A, trial 1 and B, trial 2.

Effect of fungicide and *R. glutinis* PM4 on germination of *B. cinerea* in vitro. Thiophanate-methyl and vinclozolin at a concentration of 50 µg a.i. ml⁻¹ did not reduce the germination of *B. cinerea* conidia (Table 1). Germination of *B. cinerea* was significantly reduced in both trials by the presence of *R. glutinis* PM4 at a 10:1 ratio of yeast to conidia, and germination was further reduced when vinclozolin was added to the yeast (Table 1). The germination of *B. cinerea* was not tested at the two higher concentrations of the fungicide (250 and 500 µg a.i. ml⁻¹), because of the difficulty of seeing conidia among the fungicide particles.

Effect of fungicide and *B. cinerea* on growth of *R. glutinis* PM4 in vitro. The growth of *R. glutinis* PM4, as determined by the increase in colony-forming units, was significantly reduced by the presence of thiophanate at 500 µg a.i. ml⁻¹ in one trial and by vinclozolin at 500 µg a.i. ml⁻¹ in both trials (Table 2). The combination of *B. cinerea* conidia and vinclozolin at 500 µg a.i. ml⁻¹ resulted in the lowest increase in yeast CFU in either trial, only a 16.1- and a 32.9-fold increase from the start of trials 1 and 2, respectively. For comparison, CFU of *R. glutinis* PM4 washed from leaf disks in the control treatments increased 92.6- and 131.5-fold over the 24-h incubation.

DISCUSSION

Improved control of *B. cinerea* on geranium seedlings by *R. glutinis* PM4 was observed when the yeast was combined with azoxystrobin, trifloxystrobin, or vinclozolin. Of the remaining fungicides, mancozeb did not enhance biological control, and flutolanil and thiophanate-methyl negatively affected it; chlorothalonil, fenhexamid, and fludioxonil eliminated lesion formation; and copper hydroxide and iprodione reduced lesion formation by *B. cinerea* so greatly that any potential enhancement in combination with the yeast could not be observed in this system.

Improved control by vinclozolin in combination with different *Rhodotorula* species was somewhat unexpected, because the *B. cinerea* isolate used in this study is resistant to that fungicide. While fungicide enhancement of biological control has been observed in postharvest systems (8,11,22), improved biocontrol on geranium leaves by yeast combined with fungicides has not been previously reported.

One of the primary mechanisms of the antagonism of yeasts to *B. cinerea* is nutrient competition (13,14,19), and under conditions of low nutrient availability many yeasts will antagonize *B. cinerea* (3). The exact mechanism of vinclozolin-mediated enhancement of biocontrol is not known. Germination of the vinclozolin-resistant *B. cinerea* conidia was significantly reduced in the presence of vinclozolin and *R. glutinis* PM4 together,

TABLE 2. Growth of *Rhodotorula glutinis* PM4 on geranium leaf disks alone and in combinations with conidia of *Botrytis cinerea*, thiophanate-methyl, and vinclozolin

Treatment ^a	<i>R. glutinis</i> PM4 (10 ⁷ CFU ml ⁻¹) ^b	
	Trial 1	Trial 2
None (<i>R. glutinis</i> alone)	10.43	12.54
Thiophanate-methyl	7.42	13.43
Vinclozolin	2.48	7.90
<i>B. cinerea</i>	7.73	9.49
<i>B. cinerea</i> and thiophanate-methyl	4.11	9.01
<i>B. cinerea</i> and vinclozolin	1.81	3.35
LSD (<i>P</i> = 0.05)	1.67	4.19

^a Fungicides were added at a concentration of 500 µg a.i. ml⁻¹. All treatments included 25 mM glucose and 20-fold-dilute yeast nitrogen base.

^b Inoculum at the start of the experiments was 1.13 × 10⁶ and 1.02 × 10⁶ CFU ml⁻¹ in trials 1 and 2, respectively, as determined by dilution plating. Colony-forming units on five leaf disks per treatment were determined by dilution plating after 24 h at 22°C.

Fig. 4. Effect of vinclozolin (500 µg a.i. ml⁻¹) on the biocontrol activity of 11 yeast isolates from four *Rhodotorula* species against *Botrytis cinerea*. Data are the average lesion diameters in three replicates of six seedlings for each treatment in **A**, trial 1 and **B**, trial 2. Check = no yeast, Rg = *Rhodotorula glutinis*, Rmu = *R. mucilaginosa*, Rgr = *R. graminis*, and Rm = *R. minuta*.

despite a decrease of more than 50% in yeast growth in this treatment, compared to treatments in which the fungicide was not added. The germ tubes and hyphae of the *B. cinerea* conidia appeared normal when the fungus was exposed to vinclozolin; similar observations of vinclozolin-resistant *B. cinerea* isolates were made by Yourman and Jeffers (25). Exposure to fungicides of the dicarboximide group, which includes vinclozolin, can result in a disorder of fungal cell structure and leakage of the cell contents (24). It is possible that exposure to vinclozolin caused minor damage to the conidia, which did not affect germination but reduced their ability to outcompete the yeasts for the limited nutrients available in the inoculum mixture. With fewer germinating conidia, lesion development on geranium cotyledons was significantly reduced.

Vinclozolin also could have improved biocontrol efficacy by negatively affecting other phylloplane microorganisms that compete with the yeast, and thus providing the yeast with a competitive advantage. However, the 2-week-old cotyledons used in the present study had an almost negligible phylloplane community, as determined by leaf washing, and yeast biocontrol was improved by vinclozolin on surface-sterilized geranium leaf disks (J. W. Buck, unpublished data). This mechanism could contribute to yeast biocontrol on the phylloplane of older geranium plants, which have a more established microflora. The ability of vinclozolin to enhance biocontrol could be widespread among phylloplane yeast populations, since many of these yeasts are resistant to vinclozolin (5), and in this study biocontrol enhancement was observed in isolates of three species of *Rhodotorula*.

Vinclozolin applied at the labeled rate did not affect the development of lesions caused by *B. cinerea* on geranium cotyledons but did enhance disease control when combined with phylloplane yeasts. This finding differs from the fungicide-enhanced biocontrol reported in several postharvest disease systems in which the target fungi were sensitive to the fungicides incorporated into treatments with biocontrol agents (2,6–8,10,11,22). Iprodione, another dicarboximide fungicide, has been found to enhance the efficacy of *Cryptococcus infirmo-minutus* YY6 and *C. laurentii* HRA5 in the biocontrol of *M. fructicola* on cherry (7) and the efficacy of *P. membranaefaciens* in the biocontrol of *R. stolonifer* on nectarine (22); however, iprodione alone, at both high and reduced rates, significantly reduced disease incidence, which indicates that the fungal isolates studied were sensitive to the fungicide (7,22). Chand-Goyal and Spotts (8) tested the biocontrol ability of *C. laurentii* HRA5 and *R. glutinis* HRB6 with and without thiabendazole, a benzimidazole fungicide, applied at half rates, against a sensitive and a resistant isolate of *P. expansum*. The presence of the fungicide enhanced biocontrol of the sensitive isolate by both yeasts, but it enhanced biocontrol of the thiabendazole-resistant isolate by *C. laurentii* HRA5 only (8). Thiabendazole alone significantly reduced the incidence of disease caused by the thiabendazole-resistant *P. expansum* to 45.5%, compared to 63.6% in a control group (8). The fungicide sensitivity of the target pathogen population could determine whether fungicide enhancement of yeast biocontrol is observed and to what degree.

One argument for incorporating fungicides at low concentrations (i.e., 10% of the labeled rate or less) into biocontrol mixtures is that disease control similar to that achieved by fungicides alone can be obtained with a significant reduction in fungicide use, lower levels of pesticide residues, and less potential impact on the environment (7,11). Application of at-risk fungicides at reduced rates could favor rapid selection for fungicide resistance or an increase in existing resistance in target fungal populations (20). While it is assumed that fungi will not develop resistance to yeast biocontrol agents (7,8), it is unknown whether a combination of yeast and fungicide will eliminate the development of fungicide resistance in a pathogen population. In the present study the combination of a yeast with several fungicides at one-

tenth their labeled rates was very effective in reducing, and in some cases eliminating, disease. This reduction in disease would minimize the risk of selection for fungicide resistance. Improved control by vinclozolin at labeled rates in combination with yeast could allow direct incorporation of the biocontrol agent into an existing disease management program. Spotts et al. (23) incorporated a postharvest dip in a suspension of *C. infirmo-minutus* with a preharvest application of iprodione at the labeled rate (1.13 kg a.i. h⁻¹) to obtain significant improvement in the control of *M. fructicola* on sweet cherry.

Many yeasts isolated from the phylloplane of turf or ornamental plants are antagonistic toward *B. cinerea* (3). However, a wide range of biocontrol efficacy was observed among these yeasts when screened at a 10:1 ratio of yeast cells to *Botrytis* conidia on geranium leaf disks (3). Increasing the concentration of yeast to 100:1 significantly reduced lesion development in treatments with yeast isolates initially designated as poor antagonists. Biocontrol of *B. cinerea* by *R. glutinis* PM4 and the other yeasts evaluated in these tests was highly variable in treatments with the yeasts alone, ranging from highly effective (lesions less than 2 mm in diameter) to ineffective (lesions more than 10 mm in diameter) in different tests. This has traditionally been one of the limitations of biocontrol (1). However, *R. glutinis* PM4 in combination with azoxystrobin, trifloxystrobin, or vinclozolin either eliminated lesion development or limited lesion diameter to less than 2 mm, a highly effective and consistent level of control not observed in treatments with the yeast alone. The combination of yeast at an increased concentration and the appropriate fungicide could result in more consistent biocontrol by different yeasts in the *B. cinerea*-geranium system. Combining fungicides with biocontrol yeasts in postharvest systems can provide consistency in performance and economically acceptable levels of disease control (10).

Recently it was suggested that recommendations for managing *Botrytis* blight of ornamental crops in greenhouses in the southeastern United States should de-emphasize the use of thiophanate-methyl, vinclozolin, and chemically related compounds, because of the low incidence of isolates still sensitive to these fungicides (25). Yeast biocontrol in conjunction with vinclozolin could allow continued use of this fungicide on ornamental crops. The enhancement of yeast biocontrol activity by vinclozolin needs to be further tested with other *B. cinerea* isolates, in scaled-up greenhouse trials with natural populations of the pathogen, and on different ornamental crops.

ACKNOWLEDGMENTS

I thank Goldsmith Seeds Inc. for providing geranium seed, R. Estes for technical assistance, and D. Mueller and T. Allen for helpful comments on drafts of the manuscript.

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