Biological Control of *Botrytis cinerea* on Roses with Epiphytic Microorganisms

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ABSTRACT

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Fungi and bacteria isolated from rose petals were evaluated for their potential as biological control agents of Botrytis blight, a serious disease of greenhouse-grown roses. Preliminary evaluations identified four microorganisms with the ability to reduce the number of lesions caused by *Botrytis cinerea* on rose. Biological control by these antagonists, *Exophiala jeanselmei, Cryptococcus albidus,* an *Erwinia* sp., and a coryneform bacterium, was demonstrated by applying them to cut roses 1 day before inoculation with a suspension of 1,000 conidia of *B. cinerea* per milliliter. The most effective antagonist was a yeast, *E. jeanselmei*, which reduced the number of lesions by 63%. This level of control was not significantly different from control achieved by the fungicide iprodione (74%).

Botrytis cinerea Pers. causes many common diseases of flowers, fruit, and other soft plant parts. Under cool, wet conditions, it can be a limiting factor in the production, marketing, and storage of roses, gerberas, chrysanthemums, lettuce, grapes, strawberries, and tomatoes (12). Botrytis blight is one of the most serious diseases affecting the production and postharvest quality of cut roses. The disease first appears as small flecks or blisters on petals. Infections are not always visible at harvest, but they develop rapidly in transit to market under the conditions of high humidity maintained in shipping containers.

The current management program for Botrytis blight of rose is often unsuccessful. Control recommendations in California include frequent fungicide sprays in the greenhouse during the winter, with a postharvest dip of cut flowers in a fungicide solution year-round. This program is hampered by unsightly residues on the flowers and by the development of populations of *B. cinerea* resistant to the benzimidazoles (benomyl) and dicarboximides (vinclozolin and iprodione) (5,13,15). Fungicide resistance is widespread and can develop within one season (10,16), and the use of mixtures of fungicides may not delay the development of resistance (16). Sanitation practices involving removal of organic debris from greenhouse pathways and under rose canes to reduce the rapid buildup of *B. cinerea* inoculum could be helpful but are not effectively practiced in many greenhouses. Rose cultivars with resistance to *B. cinerea* are not available, and there are insufficient data to design reliable manipulations of the greenhouse environment to break the disease cycle.

Consideration of these problems has resulted in interest in biological control of *B. cinerea*, which if available, could introduce needed flexibility into the management program. A number of plant diseases, including several caused by *B. cinerea*, have been reduced by preinoculation of the phylloplane with epiphytic bacteria or fungi (3). Dubos (7), using the biological control agent *Trichoderma harzianum*, reduced the severity of bunch rot of grapes caused by *B. cinerea* by 60–70% during several years of field trials. *T. harzianum* also was reported to control dry eye rot of apple (21) and strawberry fruit rot (20) caused by *B. cinerea*. Bhatt and Vaughan (1) reduced rot caused by *B. cinerea* on strawberries in both greenhouse and field experiments using applications of the fungi *Cladosporium herbarum* or *Aureobasidium pullulans* on the flowers. Fokkema and Lorbeer (8) reported up to 59% reduction of lesions of *B. cinerea* on onion leaves when *A. pullulans* was applied with pathogen inoculum.

The purpose of this work was to investigate the potential of epiphytic microorganisms as biological control agents for Botrytis blight on rose. A portion of this research was reported previously (18).

MATERIALS AND METHODS

Survey of phylloplane microorganisms and their potential to control Botrytis blight. Isolations of filamentous fungi, yeasts, and bacteria were made from 10 g of petals taken from 12 unblemished rosebuds (17). The petals were ground in 100 ml of sterile distilled water, and this solution was diluted by factors of 10^1, 10^2, and 10^3. The isolations were made on the following selective media: acidified yeast malt extract agar (acid YM) containing 3 g of yeast extract, 3 g of malt extract, 5 g of peptone, 10 g of glucose, and 15 g of agar per 1 L of water and HCl added after autoclaving to adjust the pH to 4.5–5.0; acidified potato-dextrose agar (acid PDA, Difco, Detroit, MI, with 3 g/L oxgall and HCl added to acidify to pH 4.5–5.0); and nutrient agar (NA,

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Difco). Ten 0.1-ml aliquots of each of the 10^1 and 10^2 dilutions were spread onto plates of both the acid YM and acid PDA, and 10 aliquots of each of the 10^2 and 10^3 dilutions were spread onto plates of the NA. The isolation procedure was conducted four times, using a different rose cultivar each time. The cultivars used were an unreleased Jackson and Perkins Co. hybrid, Don Juan (both field-grown), Golden Wave, and Emblem (both greenhouse-grown). Two hundred subcultures of the most common isolates were maintained on nonacidified YM (yeasts), nonacidified PDA (filamentous fungi), and NA (bacteria).

Fifty-six bacterial isolates and 16 yeast isolates were evaluated to determine their abilities to reduce lesions of *B. cinerea* on detached petals of the rose cultivar Golden Wave. Bacteria were grown on NA and yeasts were grown on YM for 3 days at 25 C. The yeasts and bacteria were removed from the agar with sterile distilled water, washed once by centrifugation, and resuspended. An isolate of *B. cinerea* originally collected from a rose flower was grown on PDA at room temperature in continuous darkness for 11–15 days. Conidia were collected from sporulating colonies by dislodging them from the conidiophores with a rubber spatula into sterile distilled water that had been added to the plate. A drop of Tween 20 was added to the suspension to disperse the spores evenly, and the concentration of the suspension was determined with a hemacytometer. Inoculated rose petals were maintained at 95% RH (measured with a relative humidity probe, Campbell Scientific Inc.. Logan, UT) by placing them on wire screens over water in closed, clear plastic boxes (31 × 23 × 10 cm). The boxes were maintained at 25 C.

To evaluate the biological control potential of each of the bacteria and yeasts, one surface of 20 petals was sprayed first with a suspension of a potential antagonist and a day later with a suspension of 6×10^4 conidia of *B. cinerea* per milliliter. Two days after inoculation with the pathogen, the diseased area on each petal was estimated visually and a score was assigned to each petal according to the percentage of the surface area covered with lesions on a scale of 0–7, where 0 = 0%, 1 = >0–≤1%, 2 = >1–≤5%, 3 = >5–≤10%, 4 = >10–≤15%, 5 = >15–≤ 25%; 6 = >25–≤50%; and 7 = >50%.

Evaluation of all isolates required five separate tests. Several controls were included in each test. Twenty petals sprayed with sterile distilled water only served to detect background levels of infection, and 20 petals inoculated with *B. cinerea* alone indicated the maximum infection. Also, three of the isolates were included in all tests. If they were ranked in the same order each time they were evaluated, it indicated that the tests were consistent over time.

After all of the isolates had been evaluated and ranked, a final comparison was conducted of the most promising isolates identified in previous tests. The two bacteria and two yeast isolates that reduced disease the most in this last test were characterized with conventional microbiological techniques and were used in subsequent experiments.

Biological control of *B. cinerea* on whole cut flowers. The four most promising antagonists were further tested for their ability to control *B. cinerea* on whole cut roses. The bacteria were grown on NA and the yeasts were grown on YM for 2 days. The antagonists were washed from the agar, suspended in 0.1 M phosphate buffer at pH 7.1, washed by centrifugation, and resuspended. The culture of *B. cinerea* was handled as described previously.

The cultivar Golden Wave was used in all experiments. Rosebuds were sprayed with 10^7 colony-forming units (cfu/ml) of each of the yeasts or 10^8 cfu/ml of each of the bacteria. A fifth treatment combined all four test organisms in one spray, with each at a concentration of 10^7 cfu/ml. The fungicide iprodione (Chipco 26019), which is used for the control of *B. cinerea* in greenhouses in California, was applied at 1.8 g a.i./L as a sixth treatment. To determine the highest level of disease attainable under experimental conditions, a seventh group of roses was sprayed with buffer alone. All of the treatments were sprayed to runoff. Twenty-four hours after treatment with the antagonists, fungicide, or buffer, the roses were sprayed with a solution containing 10^3 conidia of *B. cinerea* per milliliter. As a final control treatment, uninoculated roses were sprayed only with phosphate buffer to detect possible effects of the buffer in which the antagonists were suspended and to quantify infections by *B. cinerea* not resulting from the inoculations. The roses were maintained in closed, clear plastic boxes with their stems submerged in water and the flowers supported on wire screens above the water so that they did not touch each other. Total lesions per rose were counted 2 days after inoculation.

Each box was treated as a block, containing one rose from each treatment randomly arranged within the box. The experiment was conducted three times, with 11 roses per treatment. The treatment combining all four antagonists was only included in two of the three replicates. Results from all of the replicates were combined and analyzed with standard analysis of variance procedures.

Population dynamics of *Exophiala jeanselmei*. The most effective biocontrol agent was a black yeast, *E. jeanselmei* (Langer) McGinnis & Padhye var. *lecanii-corni* (Benedek & Specht) de Hoog. To monitor changes in populations of *E. jeanselmei* on roses, the upper surfaces of similar sized petals of Golden Wave were sprayed to runoff with 10^5 cfu/ml of the yeast. Inoculated petals were maintained on wire screens in four plastic boxes as in previous experiments, and populations of *E. jeanselmei* were monitored for 4 days. Each day, one petal was removed from each of the boxes and washed separately in phosphate buffer on a reciprocal shaker for 15 min. Four 0.5-ml samples of the wash solution from each petal were spread onto plates of acid YM agar. *E. jeanselmei* colonies on each plate were counted after 4 days. The yeast colonies were distinguished from other microorganisms by their colony morphology and black pigmentation.

Effect of *E. jeanselmei* on inoculum density-disease incidence (ID-DI) relationships of *B. cinerea* on rose. The ability of *E. jeanselmei* to control *B. cinerea* at several inoculum densities was determined by spraying 10 cfu/ml of *E. jeanselmei* on whole cut roses 1 day before inoculation with *B. cinerea* with solutions containing 10^2, 5×10^2, 10^3, 5×10^3, or 10^4 conidia per milliliter. In control treatments, *B. cinerea* was sprayed at these rates on roses that had not been treated with the yeast. There were eight roses per treatment, and they were maintained in plastic boxes as in other experiments. Each box contained one rose of each treatment randomly arranged within the box. Lesions caused by *B. cinerea* in the presence of *E. jeanselmei* were counted after 2 days of incubation and compared with the numbers of lesions observed when *E. jeanselmei* was not applied.

RESULTS

Biological control potential of phylloplane yeasts and bacteria. The potential of 72 microorganisms isolated from rose petals to control *B. cinerea* was evaluated in a series of five tests. In two of these tests, the negative control (sprayed only with sterile distilled water) and the positive control (sprayed only with *B. cinerea*) defined the lower and upper limits in range of disease. For example, in test 1, the score of the negative control was 2.06 and the score of the positive control was 4.89. In three of the tests, the negative control continued to have the least disease, but in the presence of some of the bacterial isolates, infection by *B. cinerea* was greater than it was in the positive control. For example, in test 2, the score of the negative control was 1.2, the score of the positive control was 4.5, and the score of isolate B18 was 5.0. Three isolates included in all five tests were ranked in the same order each time, indicating consistency among tests.

The isolates that gave the best control in the five tests were compared with each other to choose the best ones for additional research. In this final comparison, the negative control scored 0.76, the positive control scored 3.6, and

the most disease was observed in the treatment with a bacterial isolate, B2, which scored 4.6. The four most promising isolates were *Cryptococcus albidus* (Saito) Skinner (score 2.2), an *Erwinia* sp. (score 2.7), *E. jeanselmei* (score 2.9), and a coryneform species (score 3.0).

Characterization of four antagonists. The four best antagonists were characterized with conventional microbiological tests. The *Erwinia* sp. was gram-negative, motilem facultatively anaerobic, catalase-positive, and oxidase-negative. It produced acid from the fermentation of glucose and did not reduce nitrates to nitrites. The coryneform bacterium had a bright yellow pigment and showed irregular morphology and a granular Gram stain. It was indole- and methyl red-negative but nitrate reductase-positive.

The *C. albidus* isolate assimilated nitrate and used inositol, sucrose, and maltose as its sole carbon source. Budding cells from young colonies on YM agar were hyaline, and colonies were cream-colored.

The black yeast, *E. jeanselmei* var. *lecanii-corni*, showed reproduction by ellipsoidal budding cells as well as by conidia from intercalary conidiophores. Repeated formation of conidia produced annellated zones. The yeast occurs worldwide and is often present on woody plant tissue. Identification of the isolate was confirmed by M. McGinnis, North Carolina Memorial Hospital Clinical Microbiology Department, who assigned the isolate number NCMH 2732 (*personal communication*).

Biocontrol on whole roses. The average number of lesions caused by *B. cinerea* on roses inoculated with *E. jeanselmei* was not significantly different from the number of lesions on roses treated with iprodione (Table 1). Treatment with *E. jeanselmei* and iprodione reduced lesions by 63 and 74%, respectively. All four microorganisms applied together provided no better control than *E. jeanselmei* alone. Treatment with the coryneform bacterium gave significantly less control (48%) than iprodione; however, control was not significantly less than that given by *E. jeanselmei*.

Population dynamics of *E. jeanselmei*. Populations of *E. jeanselmei* on rose petals were monitored over 4 days. When the yeast was applied to detached rose petals, its population multiplied almost 10-fold within 2 days, from 3.2×10^5 to 2.9×10^6 cfu per petal. Populations remained above 2.1×10^6 cfu per petal until the experiment ended (Fig. 1).

ID-DI relationship. Disease reduction by *E. jeanselmei* was only statistically significant at the three lowest inoculum levels of *B. cinerea*, 10^3, 5×10^2, and 10^2 conidia per milliliter ($P = 0.0001$, $P = 0.03$, and $P = 0.005$). Reduction in the number of lesions increased from 13% at the highest pathogen inoculum level to 68% at the lowest inoculum level (Fig. 2). The number of lesions was linearly correlated with inoculum density of *B. cinerea* when the pathogen was inoculated alone ($r = 0.98$, $P = 0.003$) and when the biological control agent was added ($r = 0.96$, $P = 0.001$). The slopes of the curves were 0.014 and 0.013 in the presence or absence of *E. jeanselmei*, respectively, and did not differ significantly from each other ($P = 0.7$).

Table 1. Reduction in *Botrytis cinerea* lesions on Golden Wave roses by treatments with four antagonists

Treatment	Mean number of lesions per rose[z]	Disease reduction (%)
No *B. cinerea*, no control agent	7.5 a	...
Irpodione (1.8 g a.i./L)	10.4 ab	74
Exophiala jeanselmei (10^7 cfu/ml)	19.7 bc	63
All antagonists together	21.2 bc	56
Coryneform bacterium (10^8 cfu/ml)	24.0 cd	48
Cryptococcus albidus (10^7 cfu/ml)	34.1 de	26
Erwinia sp. (10^8 cfu/ml)	34.9 de	25
B. cinerea only, no control agent	44.7 e	NA

[z] Means followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05\%$).

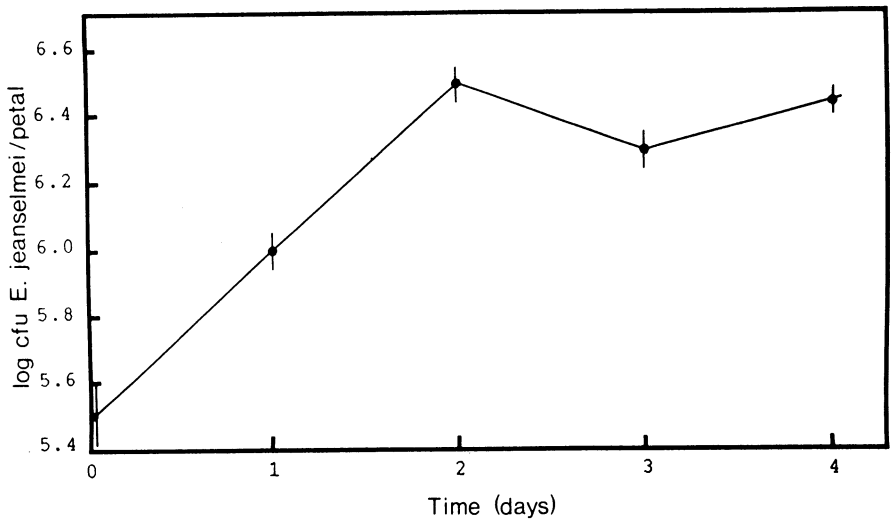

Fig. 1. Populations of *Exophiala jeanselmei* on detached rose petals after inoculation with a suspension of 10^5 cfu/ml (confidence bars = 95%).

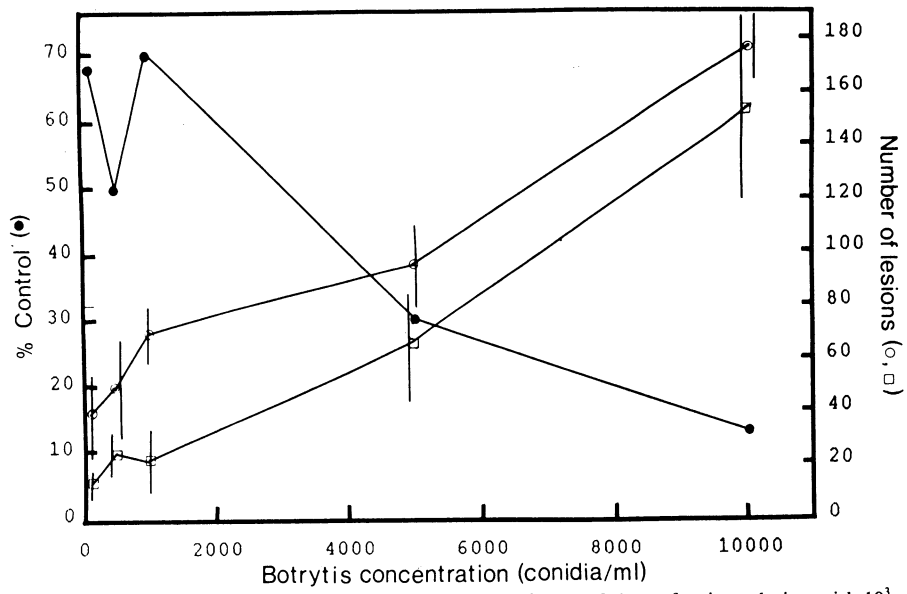

Fig. 2. Number of lesions developing on whole cut rose flowers 2 days after inoculation with 10^3 conidia of *Botrytis cinerea* per milliliter (○) or inoculated with 10^7 cfu/ml of *Exophiala jeanselmei* 24 hr before inoculation with *B. cinerea* (□). Disease control is expressed as a percent reduction in lesion numbers (•) (confidence bars = 95%).

DISCUSSION

E. jeanselmei and the coryneform bacterium have potential as biological control agents for *B. cinerea* on greenhouse-grown roses. Botrytis blight was controlled as effectively by *E. jeanselmei* as it was by the fungicide iprodione. Greenhouse trials are needed to further evaluate the two microorganisms and to establish optimum application concentration and timing.

The reduction of disease observed in these experiments was consistent with that found by other researchers when populations of phylloplane residents were augmented before applications of a pathogen. For example, when the common phylloplane yeasts *Sporobolomyces roseus* and *Cryptococcus laurentii* var. *flavescens* were sprayed as a mixture onto maize plants 2 or 3 days before inoculation with *Colletotrichum graminicola*, lesion density and necrosis were both reduced by about 50% (22). Mixed inoculations of *A. pullulans, S. roseus,* and *C. laurentii* var. *flavescens* reduced infection of wheat by *Septoria nodorum* by 50% or more (9).

The capacity of phylloplane yeasts to reduce the numbers of lesions caused by *C. graminicola* was attributed to their ability to reduce both conidial germination and prepenetration growth of the pathogen (22). Control of *B. cinerea* through a similar mechanism is plausible, because it has been shown to depend on exogenous nutrients for both conidial germination and growth before infection (6,14,23). A possible mechanism by which *E. jeanselmei* or the coryneform bacterium might achieve biological control is through competition for exogenous nutrients. Such competition has been demonstrated between other epiphytic microorganisms and *B. cinerea* (2,4). Further research is needed, however, to determine the mechanism of action in the rose pathosystem.

Analyses of ID-DI relationships as influenced by the presence of biological control agents may be useful for describing mechanisms of biological control of plant diseases (11,19). The slopes of the ID-DI curves observed in this experiment in the presence and absence of *E. jeanselmei* were parallel. This implied that infection was directly correlated with inoculum density of the pathogen regardless of the presence or absence of the biological control treatment (19). *E. jeanselmei* allowed only a constant proportion of *B. cinerea* lesions to develop, although disease reduction was statistically significant only at the lower inoculum densities. It was these lower inoculum densities that produced disease symptoms similar to those observed in the greenhouse. Inoculum density in the greenhouse does not appear to reach the high levels used in these experiments, and thus, control at the lower inoculum levels of *B. cinerea* is the most important.

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