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Selective isolation of bacterial antagonists of *Botrytis cinerea*

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Abstract

Most potential fungal antagonists have been identified only after exhaustive non-selective screening. Bacteria antagonistic to *Botrytis cinerea* were selected using a simple method based on techniques developed for trace enrichment studies, where a crude cell wall preparation from *B. cinerea* was used as a selective substrate and bacteria were isolated based on growth on cell wall agar media. Most of the 52 bacterial isolates that were obtained from the surface of 'organically grown' strawberry fruit and could grow on *B. cinerea* wall media in culture showed some ability to inhibit the growth of the fungus. Potato dextrose agar seeded with *B. cinerea* spores (10^4 ml^{-1}) were challenged with each of these bacterial isolates at concentrations of 10^9 and 10^6 colony forming units ml^{-1}. Eleven of the 52 isolates initially recovered, subsequently demonstrated strong antagonism *in vitro* and were selected for additional screening tests on strawberry fruit. All 11 isolates reduced grey mold rot incidence on fruit in storage. Three of the best isolates were tested in limited field trials, and also reduced grey mold rot on fruit under field conditions.

Introduction

Concerns about the use of chemical fungicide control measures on horticultural crops have increased interest in the use of antagonistic microorganisms to control plant pathogens in order to reduce postharvest decay in fruits (Campbell, 1986). Postharvest pathogens are attractive targets for biological control agents, since pathogen controlling treatments such as fungicides, must be applied to the fresh fruits and vegetables in order to be effective. Often chemical treatments are not allowed by regulatory agency policy unless applied well in advance of harvest, thus limiting their effectiveness for control of disease during postharvest storage. Other treatments, such as surface sterilization and high doses of irradiation, can damage fruit tissue or leave residues that adversely alter taste or other quality factors. Antagonistic microorganisms, however, can be used to alter the microflora of the harvested produce while minimizing problems of chemical residues, off flavors and/or physical damage. In addition, where effective chemical treatments do exist, microbial antagonists can often act synergistically so that application levels for chemicals can be reduced and control increased (Lorito et al., 1993). Finally, while chemical treatments can result in the selection of resistant pathogens (Chiba and Northover, 1988; Cooley, 1981; Staub, 1991), pathogen resistance is generally considered to be less of a problem when using microbial antagonists.

Chemical companies screen thousands of potential compounds for antifungal properties per year, but so far the search for effective biocontrol agents has been done on a modest scale (Campbell, 1986). Previous attempts to isolate biocontrol agents have utilized two basic approaches: (1) testing of microbes isolated from a target plant without selection (Peng and Sutton, 1991; Zhang et al., 1994), and (2) isolation based on some level of selection including (a) incidental observations or anecdotal evidence (Cooley, 1981), (b) isolation on media made with pasteurized or autoclaved homogenates of the pathogen (Valois et al., 1996),

observation of inhibition of pathogen growth on agar (Janisiewicz, 1988), and (c) use of purified polymers such as chitin to detect chitinase production (Lorito et al., 1993). Methods based on isolation without selection for properties potentially important for fungal control require a primary screen to identify potentially useful microbial antogonists. Isolation based on chitinase activity offers some level of selection, but fungal walls are complex, and effective microorganisms can be missed during the screening process. Similarly, pathogen homogenates may select for microorganisms that can live on cellular contents from the pathogen not normally available in the natural environment. Finally, isolation based on fungal growth inhibition on culture media without prior selection are time consuming, frequently are not predictive of a strain's performance under field conditions and often select for antibiotic producing organisms (Leifert et al., 1994) which may be contra indicated for use on consumed products.

We have developed a simple selection method for isolating bacterial antagonists to *B. cinerea*, a common pre- and postharvest pathogen of strawberry that accounts for substantial crop loss. This selection method is based on the ability of selected antagonistic bacteria to grow on insoluble material consisting primarily of cell wall material obtained following water and hexane extraction of freeze-dried mycelium. The method should, however, have general application to selection of antagonists to a variety of fungi. This method was designed based on techniques developed from enrichment studies (Krieg, 1981; Tomasek and Karns, 1989) that combine isolation and primary selection, thus reducing steps required for identification of potential bacterial antagonists. Bacteria were selected as the organisms we wished to isolate because they are less likely to be adversely impacted by preharvest control methods currently in use. Also, if antagonism is found to be related to a specific lytic activity, transfer of such a lytic activity to plants or antagonistic organisms would be facilitated by the relative ease of protein isolation and gene cloning from many bacteria (Chou et al., 1996; Tomasek and Karns, 1989).

Materials and methods

Preparation of fungal cell wall medium

Botrytis cinerea Pers. Fr. was isolated from decayed strawberry fruit and maintained on potato carrot agar slants. Transfers to potato dextrose (PD) agar (Difco[1], Detroit, MI USA) plates incubated at 20 °C under continuous light were made at regular intervals to provide conidia for inoculations. Conidia were removed from the PD agar plates with a sterile transfer loop and diluted in sterile distilled water to a concentration of 10^4 conidia ml^{-1} as determined by hemacytometer counts. One ml of this conidial suspension was placed into each of twenty 250 ml Erlenmeyer flasks containing 75 ml of sterilized PD broth. Cultures were placed on a shaker (1500 rpm; Lab-Line, Melrose Park, IL USA, Model 3520) at 20°C under continuous light (25 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps). These cultures were harvested after 4 days incubation, when the mycelial plug was of sufficient mass to indicate that nutrients might soon become a limiting factor in the culture.

Mycelial mats were collected on Miracloth (Calbiochem, La Jolla, CA) filters in a Buchner funnel, rinsed twice with distilled water, removed from the Miracloth with a spatula and placed in freeze-drying flasks. The samples were freeze-dried and then stored in covered 4 L jars at −20 °C. Sufficient cultures were grown and freeze-dried to prepare 200 g of freeze-dried mycelium for cell wall extractions. Freeze-dried mycelium was ground to a fine powder in liquid N_2 in a mortar and pestle that had been prechilled with liquid N_2. The ground powder was placed in a 100 ml sized Soxhlet thimble filled to within 2.5 cm from the top and the top was plugged with glass wool. The thimble was placed in the Soxhlet extractor and lipids removed by continuous extraction with hexane for 48 h. The extracted mycelium was rinsed with diethyl ether, air dried and then dried *in vacuo* in a desiccator over paraffin shavings for two days.

The dry hexane-extracted mycelium was suspended in glass distilled water (15 g in 500 ml) and stirred for 10 min. The suspension was filtered through Whatman (Hillsboro, OR) No. 1 paper and the residue rinsed with a small additional amount of water. The residue from the filter was collected and resuspended in 500 ml glass distilled water. Bactoagar (15 g L^{-1}, Difco, Detroit, MI) and cycloheximide (50 mg, Sigma, St. Louis, MO) were added, the *B. cinerea* medium (*Bc*M) was

[1]Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

autoclaved for 20 min, and then poured into plastic Petri dishes.

Isolation of potential bacterial antagonists

Fresh strawberries were obtained from a local 'organic' produce market and placed in a sterile 600 ml beaker. The berries (150 g) were covered with 400 ml of filter sterilized distilled water containing 0.05 ml of Tween 80 (Sigma) as a wetting agent. The beaker with berries and wash solution was placed in a water bath sonicator (Mettler Electronics, Anaheim, CA USA, Model ME11) for 10 min, the solution was passed through a sterile 10 micron nylon screen, and the filtered wash solution collected in a sterile container. One-ml of this wash solution was plated on each of 20 *Bc*M Petri dishes.

Bacteria from colonies that formed within 72 h of growth on *Bc*M at 25°C were isolated by streaking onto plates containing Luria Broth (LB) media (Gibco-BRL Life Technologies, Gaithersburg, MD) with 2% agar. This process of streaking on LB plates was repeated until single colonies of uniform morphology were isolated from each original colony. In this way, we obtained 52 pure isolates for further testing from the original 20 *Bc*M plates.

Only the eleven isolates that demonstrated strong antagonism to *B. cinerea* in our *in vitro* studies (see below) were identified using standard methods, including: microscopic observation under phase contrast; Gram staining; oxidase test; and tests of substrate utilization using Oxi/ferm tubes (Roche Diagnostic Systems, Nutley, NJ). More complete identity data was obtained using the MIDI system for lipid profiling with the TSBA database (Microbial ID Inc., Newark, DE) and the Biolog GN Microplate system for metabolic fingerprinting with the Microlog 3 database release 3.50 (Biolog, Hayward, CA), both according to the instructions provided by the manufacturers.

In vitro *screening for antagonism against* B. cinerea

PD agar plates were seeded by spreading 1 ml of a suspension of *B. cinerea* spores in sterile distilled water on each plate. Plates were incubated at 20 °C for 1 h and then challenged with 1 ml of a 1:1 dilution (in sterile distilled water) of a 24 h culture of each of 52 potential bacterial antagonists [10^9 CFU (colony forming units) ml^{-1}] which was spread over the germinating conidia. Plates were incubated at 20 °C and observations of fungal growth made daily for 2 weeks. Plates were rated as to the amount of fungal growth on a 1–3 scale, where 1 = normal mycelial growth and conidia production in the culture; 2 = mycelial growth, but no formation of conidia; and 3 = inhibition of mycelial growth. Three replications of the initial screening were made. If bacterial cultures showed promise at this level (with a score of 2 or better) they were screened again at two additional dilutions, approximately 10^9 and 10^6 CFU ml^{-1} obtained by diluting the bacterial suspensions with sterile distilled water. Only when the bacterial cultures showed evidence of inhibited fungal growth in all screens were they selected for additional bioassays. Eleven of the 52 original cultures that repeatedly gave indications of strongly inhibiting the growth of germinating *B. cinerea* by reducing mycelial growth and preventing sporulation were selected in this manner.

Screening for antagonism on strawberry fruit

The eleven cultures that inhibited fungal growth with the *in vitro* test were screened for antifungal activity on freshly harvested strawberry fruit that had been artificially inoculated with *B. cinerea*. Strawberries (cv. Delmarvel and Allstar) were harvested from local (Germantown, MD USA) commercial plots, sorted and transported to the laboratory. Five fruit were dipped in a suspension of 10^4 *B. cinerea* conidia ml^{-1}, prepared as indicated above. Fruit were allowed to dry for 1 h and were then inoculated with 24 h bacterial suspensions, prepared at different dilutions as indicated above. Control fruit dipped in conidia, dried and dipped in nutrient broth diluted 1 : 1 with sterile distilled water, were placed in the 1 L plastic retail produce container (with snap-on lid) as treated fruit. A plastic divider was used to separate treated from control fruit during storage. Fruit were incubated for 4 days at 20 °C, 7 days at 15 °C, or for 10 days at 10 °C before being scored for percent of fungal infected fruit by examining each fruit and recording the number of fruit showing visual signs of infection. All experiments were repeated 3 times.

A small scale field trial was conducted with three representative isolates (No. 3, 30 and 50) selected based on the postharvest study. Cultures were prepared in liquid LB media by inoculation of 120 ml of media with bacteria from a single colony and the cultures grown at 25 °C with orbital shaking for 72 h. Twenty ml of actively growing cultures were added to each of six flasks each containing 120 ml of medium and incubated with shaking at 1500 rpm for 24 h. The

bacterial cultures (840 ml) were then diluted to 3 L with sterile distilled water and applied to strawberry plants in the field using a plastic two gallon garden sprayer. Dilution plate assays of the spray solution indicated that they contained between 10^7 and 10^8 CFU ml^{-1} of each antagonist. Ten foot rows of eight strawberry cultivars were sprayed until leaf runoff with each antagonist or a control solution consisting of diluted medium. Spraying was done at the flowering stage and again 2 weeks later, when most primary berries were at the mature green stage.

Fruit were harvested when ripe and then transported to the laboratory where they were sorted and placed in plastic mesh half pint berry baskets. The baskets were wrapped with plastic film (Saran) to prevent fruit desiccation and stored at 15 °C for 4 days. At the end of the storage period the fruit were evaluated for decay. Four harvests were made over a 2 week period. Weather conditions were highly variable during the period of the experiment, thus harvests one and four were during dry periods, while field conditions were wet for harvests two and three.

Results and discussion

Petri plate challenges with bacteria isolated from strawberry fruit revealed that 11 of 52 isolates were capable of interfering with the conidial germination and/or growth of *B. cinerea*. This *in vitro* screening reduced by 80% the necessity of identifying the large number of bacterial isolates recovered from strawberries that were capable of utilizing *B. cinerea* cell walls as a carbon source, since they did not display significant antagonism with the *in vitro* assay. It also saved time by allowing preliminary evaluation to be done rapidly without utilizing inoculated produce, which could have introduced additional variables into the study.

All the bacteria isolated were oxidase positive, Gram negative rods, which fell into two classes based upon their motility (Table 1). Bacterial identification with both MIDI and the Biolog systems revealed that all the motile strains were *Pseudomonas putida*, a bacterium that has previously been shown to be antagonistic against postharvest soft rot diseases of potato (Colyer and Mount, 1984). Several other investigators

Table 1. Identification and properties of bacterial isolates that showed antifungal activity by *in vitro* screening

Isolate number	Morphology	Gram reaction	Oxidase reaction	Identification[1]
1	Nonmotile, short ovoid rods	Negative	Positive	*Chryseobacterium indologenes* (*Flavobacterium*)
3	Long, slender rods, very motile	Negative	Positive	*Pseudomonas putida* Biotype B
5	Long, slender rods, very motile	Negative	Positive	*Pseudomonas putida* Biotype B
12	Nonmotile, short ovoid rods	Negative	Positive	*Chryseobacterium indologenes* (*Flavobacterium*)
19	Motile rods	Negative	Positive	*Pseudomonas putida* Biotype B
27	Long, slender rods, very motile	Negative	Positive	*Pseudomonas putida* Biotype B
30	Long, slender rods, very motile	Negative	Positive	*Pseudomonas putida* Biotype A
40	Long, slender rods, very motile	Negative	Positive	*Pseudomonas putida* Biotype B
50	Nonmotile, short ovoid rods	Negative	Positive	*Chryseobacterium indologenes* (*Flavobacterium*)
51	Nonmotile, short ovoid rods	Negative	Positive	*Chryseobacterium indologenes* (*Flavobacterium*)
52	Nonmotile, short ovoid rods	Negative	Positive	*Chryseobacterium indologenes* (*Flavobacterium*)

[1]Identification of isolates was based on MIDI and Microlog tests.

have also successfully employed bacterization with pseudomonads to control plant diseases (Howell and Stipanovic, 1979; Kawamoto and Lorbeer, 1976; Nair and Fahy, 1972). All non-motile strains were identified as *Chryseobacterium indologenes* (*Flavobacterium*) (Weeks, 1981) by both systems. This bacterium has not previously been reported to have antagonistic properties, although it is a common soil inhabitant. There is a very strong possibility that the 11 isolates recovered may represent three strains of bacteria based on dendrograms of Biolog and MIDI results (data not shown).

Bacterial isolates that had been shown to inhibit *B. cinerea* growth *in vitro* were tested for their ability to reduce grey mold on strawberry fruit inoculated with *B. cinerea* conidia. These tests revealed that six of the eleven bacterial cultures (isolates #1, #12, #27, #40, #50 and #51) significantly reduced the level of subsequent grey mold rot in strawberries artificially inoculated with *B. cinerea* at three incubation temperatures (Table 2). Antagonist #3 was not evaluated at 10 °C, but reduced decay at 15 and 20 °C. Culture #40, when applied postharvest, caused softening and cell leakage on the underside of the fruit. This damage was not typical of grey mold rot, thus the injury appeared to be caused by the antagonist, perhaps related to a high concentration of nutrients. The three isolates tested in field trials (#'s 3, 30 and 50) were able to reduce the amount of grey mold rot on harvested fruit by more than 50% (Figure 1).

Since the strawberry fruit used in the *in vitro* assay and the field study were harvested under conditions where there was moderate to high *B. cinerea* levels in the field, some of the decay may have resulted from latent infections at the blossom stage. These infections may have thus been well established so that treatments with the antagonists had little effect on subsequent disease development (Peng and Sutton, 1991). Also, the potential for latent infection by *B. cinerea* is very high in soft fruited plants such as strawberries and fruit already so infected may not respond to post-infection treatment with an antagonist (Campbell, 1986). Such situations may limit the value of *B. cinerea*-colonized strawberry fruit as a model substrate for use in antagonism studies. Additional studies are thus planned using other fruits, including apples, where latent infection is not such a serious problem.

Table 2. Percent of antagonist-treated fruit exhibiting grey mold infection following storage at three temperatures. Data are the combined means from three experiments

Bacterial isolate	Temperature		
	10 °C	15 °C	20 °C
Non-treated Control	100	100	100
#1	13.3 c[a]	13.3 c	46.7 b
#3	—[b]	53.3 b	53.3 b
#5	80.0 a	46.7 b	53.3 b
#12	20.0 c	26.7 b	20.0 c
#19	13.3 c	13.3 c	46.7 b
#27	60.0 b	33.3 b	20.0 c
#30	80.0 a	40.0 b	46.7 b
#40	20.0 c	53.3 b	40.0 b
#50	40.0 b	33.3 b	40.0 b
#51	40.0 b	46.7 b	33.3 b
#52	86.7 a	26.7 b	40.0 b

[a]Mean separation by Duncan's multiple range test within columns, numbers followed by different letters are significantly different at $P = 0.05$.
[b] — indicates missing data.

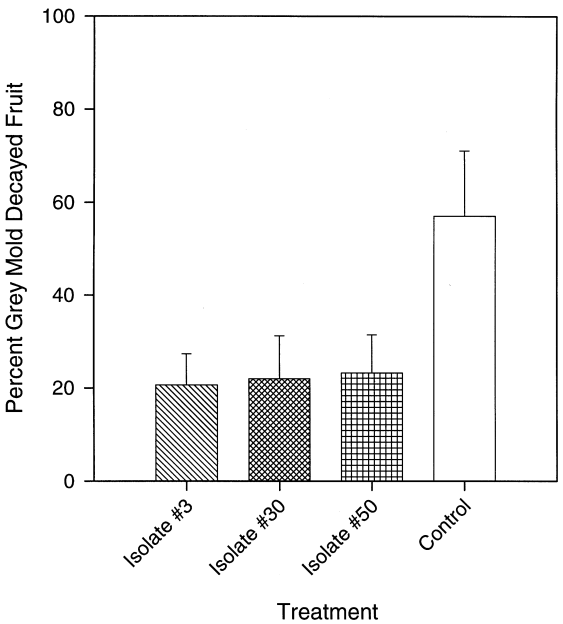

Figure 1. Average decay of strawberry fruit after harvest. Fruit were treated by spray application to run-off in the field prior to harvest with a suspension in diluted nutrient broth media containing 10^7 CFU ml^{-1} of isolates #3, #30, or #50. Control plants were treated with diluted nutrient broth media. Error bars indicate standard error. Based on paired *t*-test analysis (with data collected over several harvest dates), data are significantly different from controls, with $P = 0.034$, 0.024 and 0.018 for isolates #5, #30 and #50, respectively.

The fact that we were able to recover 11 isolates which displayed antagonism against *B. cinerea* from the 52 bacterial colonies initially picked from strawberry fruit reflects the relative simplicity and potential of the technique. All bacteria isolated were potential antagonists, since all utilized *B. cinerea* cell walls as a carbon source. We only selected those for additional studies that proved the most aggressive in subsequent tests. This screen is easy to conduct, has a high rate of success (better than 1 in 4 of those isolated passed the secondary screen) and because it selects for microorganisms with hydrolytic activity potentially deleterious to the pathogen, it is a valuable method for selecting candidate antagonists to postharvest pathogens.

Since *P. putida* has been reported to produce antibiotics (Cooley, 1981), we cannot rule out the possibility that some of its antagonistic behavior was a result of such activity. However, our isolation and assay techniques selected based on a bacterium's ability to grow on fungal mycelium wall material and the fact that these bacteria are able to grow on fungal cell walls infers that antibiotic activity was probably not solely responsible for the antagonism we observed. The basis for selection indicated that a component of the antagonism exhibited by these bacteria is that they are either strong competitors for complex substrates or use the fungi itself as a substrate for their growth. The isolation of a previously identified antagonist (Colyer and Mount, 1984; Cooley, 1981), *P. putida* confirms the potential of the selection method. In addition, isolation of a bacterial antagonist not previously reported, *C. indologenes* indicates that this method offers the potential for the isolation of bacterial strains not previously studied for fungal antagonism that may harbor hydrolytic activities useful for both biocontrol and for the molecular engineering of plant resistance.

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