Evaluation of Bacterial Antagonists for Biological Control of Broccoli Head Rot Caused by *Pseudomonas fluorescens*

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ABSTRACT

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Pectolytic strains of *Pseudomonas fluorescens* are opportunistic pathogens of broccoli, causing head rot in temperate regions of the world. In this study, we investigated the potential of two bacterial isolates, *P. fluorescens* m6418 and *Bacillus* sp. A24, for biological control of broccoli head rot caused by *P. fluorescens* 5064, isolated from diseased broccoli in Scotland, UK. *P. fluorescens* m6418, a Tn5 mutant of wild-type 5064, is nonpathogenic and overproduces an extracellular metabolite with strong antimicrobial activity. In this study, we identified the anti-

microbial metabolite produced by strain m6418 as pyrrolnitrin. *P. fluorescens* m6418 had significant inhibitory effects against strain 5064 both in culture and on broccoli leaves. In an excised broccoli head pathogenicity test, strain m6418, when coinoculated with *P. fluorescens* 5064, reduced disease by 41%. *Bacillus* sp. A24 produces an enzyme that can degrade *N*-acyl homoserine lactones, signaling molecules employed by bacteria for quorum sensing. *Bacillus* sp. A24 was capable of out-competing *P. fluorescens* 5064 when grown together in culture, and could degrade the quorum sensing signal of *P. fluorescens* 5064 (and thereby attenuate its virulence gene production). However, *Bacillus* sp. A24 had only a limited biocontrol effect on *P. fluorescens* 5064 in the excised broccoli head assay.

Broccoli (*Brassica oleracea* L. var. *italica* Plenck) head rot is a serious disease in broccoli production regions of Australia, Canada, the United States, Ireland, and the United Kingdom (6,25,38,51). Disease losses may occur from 30 to 100% in some fields in the United States (26). In the United Kingdom, crop losses of 29% average have been quantified in a wet year, resulting in a \$20M loss to the industry (23). This opportunistic bacterial disease is caused by *Pseudomonas fluorescens* GpIV (pectolytic *P. fluorescens*, sometimes referred to as *P. marginalis*) and *Erwinia carotovora* (currently *Pectobacterium carotovorum*), the former being the most frequently isolated (3,6,7,25,26,37, 42,51). There are very few bactericidal chemicals available for controlling this disease—most are copper formulations with limited effectiveness; therefore, alternative strategies such as biological control are desirable.

Pathogenic *P. fluorescens* produces biosurfactant that has been reported to play a major role in virulence (26). In a previous study, we have provided evidence that *P. fluorescens* 5064, a pathogenic broccoli isolate from the United Kingdom, regulates its biosurfactant production through quorum sensing (11).

In previous work in our laboratory, a library of wild-type *P. fluorescens* 5064 mutants deficient in biosurfactant production was generated by Tn5 mutagenesis (12). One of these mutants, m6418, overproduced a substance with strong antibiotic properties against wild-type *P. fluorescens* 5064 and *Escherichia coli* (unpublished data). Both *Pseudomonas* and *Bacillus* groups have drawn considerable attention as biological control agents since they are widely occurring, many nonpathogenic species are available, and they are highly effective at eliminating competing microbes (2,5,32,45,46,50). The antibiotic-producing strain m6418,

which has lost its pathogenicity, and the quorum sensing signal (QSS) degrading strain *Bacillus* sp. A24 might have potential as biocontrol agents.

P. fluorescens has been reported to produce a diverse array of antibiotics, which are largely responsible for the biocontrol effectiveness of this bacterium. The important antimicrobial metabolites are phenazines (Phz) (49), pyrrolnitrin (Prn) (27,41), pyoluteorin (Plt) (28), and 2,4-diacetylphloroglucinol (Phl) (40,48).

Quorum sensing is a bacterial communication system by which bacteria control the expression of certain genes in response to their population density via a small signal molecule, the QSS, the most widespread of which in gram-negative bacteria are the *N*-acyl homoserine lactones (AHLs) (20). Since many plant-pathogenic bacteria employ quorum sensing to control their virulence factor production, quorum sensing has become an attractive target for antimicrobial therapy (10,19). Dong et al. (16) discovered an enzyme from *Bacillus* sp. 240B1 that was capable of degrading the QSS of *E. carotovora*, thereby blocking its quorum sensing system and reducing virulence. Later studies discovered that this QSS degrading enzyme, designated as AiiA, and its homologues are widespread in many subspecies of *Bacillus* (15,35). Expression of aiiA in the transformed *E. carotovora* strain SCG, a pathogen that causes soft rot disease in many plants, significantly reduces release of QSS, decreases extracellular pectolytic enzyme activities, and attenuates pathogenicity in potato, eggplant, Chinese cabbage, carrot, celery, cauliflower, and tobacco (16). In a recent study, Dong et al. (17) discovered that *B. thuringiensis*, when coinoculated with *E. carotovora*, abolished its accumulation of AHL signal and significantly decreased the symptom development of potato soft rot caused by this pathogen.

The objectives of this study were to (i) identify and assay the antibiotic substance produced by *P. fluorescens* m6418; (ii) determine if *Bacillus* sp. A24 is capable of degrading the QSS, acyl-hydroxyoctanoyl-homoserine lactone, produced by the pathogenic *P. fluorescens* 5064; and (iii) evaluate *P. fluorescens* m6418 and *Bacillus* sp. A24 for their abilities to antagonize the pathogen *P. fluorescens* 5064 and control broccoli head rot disease.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Wild-type *P. fluorescens* 5064, isolated at Scottish Agricultural College from infected broccoli collected in SE Scotland (42), was grown at 26°C in Luria-Bertani (LB) medium. Mutant strain m6418 (derived from *P. fluorescens* 5064 by Tn5 mutagenesis [12]) was grown at 26°C in LB medium with kanamycin (50 mg/liter). *Pythium ultimum* was maintained, and the antifungal assay was carried out on potato dextrose agar (PDA) plates at 28°C. A spontaneous rifampicin-resistant (Rif^r) derivative of *P. fluorescens* 5064 was selected to facilitate monitoring the cell numbers of coinoculated bacteria in the pathogenicity tests. It was grown in LB medium with rifampicin (50 mg/liter). The AHL-degrading strain *Bacillus* sp. A24 was provided by G. Defago (ETH, Zurich, Switzerland). This bacterium was grown in LB medium at 30°C. The biosensor strain used in this study to detect QSS was *E. coli* (pSB401), which was provided by P. Williams (Nottingham University, UK). It was grown in LB medium at 37°C with tetracycline (20 mg/liter).

Antibiotic extraction. Cell-free supernatants from *P. fluorescens* strains 5064 (as negative control) and m6418 were prepared from 1 liter of LB broth grown at 26°C for 50 to 55 h (optical density = 2.1 at 500 nm). The supernatants were extracted twice with equal volumes of dichloromethane. The dichloromethane organic phases were combined and taken to dryness by rotary vacuum evaporation at 37°C. The resulting residue was redissolved in 1 ml of methanol (at a concentration of ×1,000 higher than that in the original culture) and sterilized with a 0.22 µl filter (Millex-GS, Millipore Co., Billerica, MA).

Thin layer chromatography. Twenty microliters of each antibiotic extract was spotted onto thin-layer plates coated with Silica Gel 60 F₂₅₄ (E. Merck, AG, Darmstadt, Germany). The plates were developed in chloroform/acetone (9:1, vol/vol). The developed plates were visualized under long- and short-wave UV light and then sprayed with Pauly's reagent (diazotized sulfanilic acid in which 5% sodium hydroxide in 50% ethanol is used as the base) or with Ehrlich's reagent (1% p-dimethyl aminobenzaldehyde in HCl followed by 0.1% sodium nitrite in water). Two-dimensional thin layer chromatography (TLC) was performed for the plates used for antibiotic preparation. The plates were developed in chloroform/acetone (9:1, vol/vol) first, turned through an angle of 90°, and placed into the second solvent: toluene/acetone (4:1, vol/vol). The developed plates were observed under UV light to detect fluorescing and adsorbing bands. Bands and blank areas were scraped separately from the plates and eluted with 40 µl of chloroform. The eluates were kept at 4°C for the antifungal assay. TLC of antibiotic extracts was performed three times, largely to obtain satisfactory images for photography.

Antifungal assay. An 8-mm-diameter hole was punched, using a sterile pipette tip, in the center of a PDA plate and filled with a pre-prepared sterile-water suspension of oospores (at a concentration of 10⁶ oospores per ml) of *Pythium ultimum*. A small piece of filter paper (Whatman International Ltd., Maidstone, Kent, England), which had been dipped to saturation into the eluted antibiotic solution (produced by *P. fluorescens* m6418), was placed on the plate about 2 or 3 cm from the middle hole. Another piece of filter paper, which had been dipped into the solvent (chloroform), was placed on the other side of the hole and served as the control. The plate was incubated upright at 28°C for 2 days before the inhibitory effect could be observed. The antifungal assay was performed three times.

QSS degradation assay. *Bacillus* sp. A24 was coinoculated with *P. fluorescens* 5064 in 300 ml of LB broth at the same time, 6 h earlier or 6 h later. The bacterial mixtures were grown for 48 h before proceeding to the following QSS extraction. Cell-free supernatants were prepared from the above bacterial mixtures and extracted twice with equal volumes of dichloromethane. The di-

chloromethane organic phases were combined and taken to dryness by rotary vacuum evaporation at 37°C. The resulting residue (QSS extract) was redissolved in 50 µl of 95% ethanol for further AHL bioassay. The AHL bioassay was carried out with biosensor *E. coli* (pSB401) that responds to exogenous QSS by emitting bioluminescence (11). Five milliliters of prewarmed semi-LB agar (0.5%, wt/vol), heated in a microwave until melted and then cooled to about 50°C, was seeded with 50 µl of bacterial biosensor from an overnight LB culture. In addition, 50 µl of QSS extract was added. The mixture was mixed well and immediately overlaid on the surface of a 35-mm petri dish containing LB agar. The plate was incubated at 37°C in an upright position for 4 h and the induced luminescence was visualized with a ChemiImager 4400 camera (Alpha Innotech Corporation, San Leandro, CA).

Quantification of viable bacterial cell number. Two microliters of each sample to be quantified was diluted in a series of 10-fold dilutions with sterile distilled water and 50 µl of each diluted bacterial solution was spread on LB plates with the appropriate antibiotic. Plates were then incubated at 30°C for 2 days to allow colonies to develop, which were then counted at suitable dilutions (using three replicates).

Effects of mutant strain m6418 on *P. fluorescens* 5064 growth in culture. Strain m6418 was cocultured with *P. fluorescens* 5064 Rif^r at the same time, 6 h earlier or 6 h later. The bacterial mixtures were incubated at 30°C. Six hours after the incubation and then every few hours after that, samples were taken for the quantification of viable bacterial cell number (as described previously). These experiments were performed twice.

Population dynamics of m6418 and *P. fluorescens* 5064 when coinoculated onto broccoli seedlings. Broccoli seeds (cv. Skiff FI) were sown into five separate pots (25 seeds per 10 by 18 cm pot) to germinate. After 4 weeks, when enough true leaves were present, 25 true leaves in each pot were selected and marked to distinguish them from leaves appearing later as the plants grew. The marked leaves were sprayed evenly with bacterial suspensions according to different treatments. One treatment was applied per pot. The bacterial concentrations used for both bacteria were 1 × 10⁸ CFU/ml. *P. fluorescens* m6418 was sprayed at the same time, 1 day earlier or 1 day later than *P. fluorescens* 5064 Rif^r. Strain m6418 and *P. fluorescens* 5064 Rif^r also were sprayed alone on the seedlings as controls. The seedlings were placed in a Fisons environmental cabinet (Fisher Scientific UK Ltd., Loughborough, England – formerly Fisons Ltd.) with a 16-h photoperiod and high humidity (100%) at 20°C (day)/10°C (night). The marked leaves were removed on the first, second, fourth, eighth, and sixteenth day after inoculation, weighed, and placed into sterile tubes containing 10 ml of sterile distilled water. The tubes were sonicated at 40 KHz in a water bath sonicator (Decon FS200, Decon Laboratories Ltd., Hove, East Sussex, England) for 7 min to remove the bacteria from the leaf surfaces and to distribute the cells evenly in the suspensions. The bacterial suspensions were serially diluted in sterile distilled water. A 100-µl aliquot of each dilution was plated onto appropriate LB plates (i.e., *P. fluorescens* 5064 onto LB plates with rifampicin; m6418 onto LB plates with kanamycin; coinoculated samples were spread onto both kinds of plates, simultaneously). All the plates were incubated at 30°C for 48 h to allow the total number of colonies to be recorded. The number of bacterial cells was related to the fresh weight of the leaf from which they were collected. Five replications were made for each treatment at each sampling time. This experiment was performed twice although only one data set was obtained due to growth cabinet failure in the first attempt.

Excised broccoli head pathogenicity test. The excised broccoli head pathogenicity test procedure was the same as described by Darling et al. (13). Mature broccoli heads (purchased from a supermarket) were inoculated by placing two pieces (2 × 2 cm) of cotton lint on the head (one upon the other), which had been soaked in a suspension of bacteria. Heads were kept upright with

sponge collars in closed Magenta GA7 vessels (Sigma Chemicals, Poole, Dorset, England) with 20 ml of sterile distilled water to maintain humidity. Heads were incubated for 5 days at 20°C (day)/10°C (night) with a 16-h photoperiod in a Fisons environmental cabinet. Four treatments were applied, namely coinoculation with strain m6418 and wild-type *P. fluorescens* 5064, *P. fluorescens* 5064 alone, strain m6418 alone, and a water control. The coinoculum ratio of strain m6418 to *P. fluorescens* 5064 was 1:1 and the final concentration of each strain was 1×10^8 CFU/ml. For *Bacillus* sp. A24, six treatments were applied, including different inoculum ratios of *Bacillus* sp. A24 and *P. fluorescens* 5064, i.e., ratios of A24/5064 of 1:0, 1:3, 1:1, 3:1, and 0:1 plus a water control. The concentration for each bacterium (strain m6418 and *Bacillus* sp. A24) was 1×10^8 CFU/ml and the experiments were performed again with a concentration of 1×10^6 CFU/ml for each strain. Ten replicates were performed for each treatment and the experiment was repeated twice. A five point scoring scale was used to assess the severity of disease symptoms (13): 0 = no symptoms, 1 = water soaking (loss of waxy bloom from florets), 2 = water soaking and tissue browning but no rot, 3 = tissue browning and softening (with rot), and 4 = extensive black soft rot. Data were subjected to percentile angular transformation using arcsine (44) before analysis; thus, the above categories 0 to 4 became 0 (=0), 33 (=1), 50 (=2), 67 (=3), and 100% (=4), respectively. Statistical analysis, i.e., analysis of variance and

Fisher's protected least significant difference test, were conducted using Genstat 5 (Rothamsted Experimental Station, Rothamsted, England).

RESULTS

Identification of antibiotics produced by strain m6418. The antibiotic extracts from strain m6418 and wild-type *P. fluorescens* 5064 were separated by TLC in chloroform/acetone (9:1, vol/vol). With strain m6418, a dark spot, corresponding to an R_f of 0.48 was observed under short-wave UV light. This spot, which could not be visualized under long-wave UV light, was impressively large compared with the other spots on the plate, reflecting a large amount of the compound. The corresponding spot was not present in the wild-type sample. As Prn typically shows a dark non-fluorescent spot under short-wave UV light and was reported to have an R_f value of 0.48 under the chromatographic conditions used in this study (4), we tentatively identified the antibiotic produced by strain m6418 as Prn. The color reaction results were further consistent with this conclusion. The antibiotic spot turned orange with Pauly's reagent and red with Ehrlich's reagent (Fig. 1), indicating the presence of phenolic and pyrrolic forms (1,39,47).

A bioassay using *Pythium ultimum* was performed to identify antibiotic activity of the Prn spot produced by strain m6418. *Pythium ultimum* is a fast-growing fungus, and after 2 days,

Fig. 1. Detection of pyrrolnitrin on thin layer chromatography plates. wt = *Pseudomonas fluorescens* 5064, pathogenic to broccoli; m6418 = *P. fluorescens* 5064 mutant 6418, nonpathogenic to broccoli. The pyrrolnitrin spot turned orange with Pauly's reagent (left) and red with Ehrlich's reagent (right).

Fig. 2. Agar plate bioassay of pyrrolnitrin, produced by *Pseudomonas fluorescens* m6418, against *Pythium ultimum*. Note the zone of inhibition around the pyrrolnitrin disk is not uniform because of overgrowth by this fast-growing pathogen from the control (solvent) disk.

colony growth was inhibited on the side of the agar plates containing the Prn filter paper disk (Fig. 2).

The TLC profile also enabled detection of other antibiotics such as Phl, Plt, and Phz in strain m6418 and wild-type *P. fluorescens* 5064 (Table 1). In addition to the major antibiotic Prn, strain m6418 also produced Phl, which was visible under long-wave UV light (blue fluorescence) with an Rf value of 0.41 (toluene/acetone, 4:1) (31). In contrast to strain m6418, wild-type *P. fluorescens* 5064 did not produce any detectable antibiotics listed above. Although a color spot also appeared at Rf 0.48 in the wild-type *P. fluorescens* 5064 chromatograms (Fig. 1), this spot, under short-wave UV light, had blue fluorescence instead of the dark nonfluorescent dot corresponding to Prn in the chromatogram for strain m6418; therefore, we concluded this was not Prn.

Degradation of *P. fluorescens* 5064 QSS by *Bacillus* sp. A24.

Biosensor *E. coli* (pSB401) produces luminescence in response to the presence of AHL produced by *P. fluorescens* 5064, as we showed in a previous study (11). No visible luminescence was observed when either *Bacillus* sp. A24 was inoculated in the medium before strain 5064 or when they were coinoculated (Fig. 3). The *Bacillus* sp. A24 thus has a strong ability to degrade the QSS produced by *P. fluorescens* 5064. When *P. fluorescens* 5064 was incubated 6 h before *Bacillus*, a faint but visible luminescence occurred (Fig. 3). This indicated that, although inoculated 6 h later than *P. fluorescens* 5064, *Bacillus* sp. A24 could still grow well and destroy most, but not all, of the QSS produced by *P. fluorescens* 5064.

Effects of *Bacillus* sp. A24 on growth of *P. fluorescens* 5064 in culture. When *P. fluorescens* 5064 and *Bacillus* sp. A24 were coinoculated simultaneously, the final cell number ratio of *P. fluorescens* to *Bacillus*, after 2 days incubation, was 1:25 (actual culturable cell numbers were 5.0×10^5 : 1.2×10^7 , *P. fluorescens*

5064/*Bacillus* sp. A24). Even when inoculated 6 h later than *P. fluorescens* 5064, *Bacillus* sp. A24 could still grow well and showed a ratio of *P. fluorescens* to *Bacillus* of 1:1.2 (culturable cell numbers were 9.0×10^5 : 1.1×10^6 , respectively). If *Bacillus* sp. A24 was inoculated 6 h earlier than *P. fluorescens*, then no *P. fluorescens* could be detected in the culture (cell numbers $0.8.0 \times 10^8$, *P. fluorescens*/*Bacillus*). Based on these results, *Bacillus* sp. A24 could out-compete *P. fluorescens* 5064.

Effects of strain m6418 on *P. fluorescens* 5064 growth in culture. Strain m6418 significantly inhibited the growth of *P. fluorescens* 5064 in liquid culture (Figs. 4 and 5). The greatest inhibitory effect resulted when strain m6418 was inoculated 6 h before *P. fluorescens* 5064, when the *P. fluorescens* 5064 cell number was five times less than when grown alone (Fig. 4). In the reciprocal experiment where the effect of *P. fluorescens* 5064 on growth of strain m6418 was examined, the m6418 population initially remained low when inoculated 6 h later than wild-type 5064, but it increased quickly after 20 h incubation (Fig. 5). In contrast, the 5064 population remained at a low level throughout the experiment when inoculated 6 h later than strain m6418 (Fig. 4).

Population dynamics of strain m6418 and *P. fluorescens* 5064 when coinoculated on broccoli seedlings. When inoculated 1 day later than the wild type, strain m6418 reduced the growth rate of the wild type, but not when inoculated 1 day before it (Fig. 6). When strains 5064 and m6418 were coinoculated simultaneously, m6418 inhibited 5064 growth on the first and second day; however, the inhibitory effect was eliminated after that (Fig. 6). Strain m6418 showed less fitness on the leaf surface compared with 5064, indicated by the faster decline in the population of m6418 (Figs. 6 and 7). These observations were derived from an unrepeatable experiment.

TABLE 1. Antibiotics produced by wild-type *Pseudomonas fluorescens* 5064 and *P. fluorescens* m6418 as detected by thin layer chromatography (TLC)

Antibiotics	Identity on TLC	Presence in	
		m6418	Wild type
2,4-Diacetyl-phloroglucinol (Phl)	Rf = 0.41 (toluene-acetone = 4:1) Fluorescence under long-wave UV light	Yes	No
Pyoluteorin (Plt)	Rf = 0.27 (chloroform-acetone = 9:1) Rf = 0.31 (toluene-acetone = 4:1) Fluorescence under short-wave UV light	No	No
Pyrrolnitrin (Prn)	Rf = 0.48 (chloroform-acetone = 9:1) Rf = 0.53 (toluene-acetone = 4:1) Dark dot under short-wave UV light Pauly's reagent (red or orange color) Ehrlich's reagent (red color)	Yes	No
Phenazine (Phz)	Colony pigmentation (dark zone surrounding colonies on agar)	No	No

Fig. 3. Quorum sensing signal degradation in *Pseudomonas fluorescens* 5064 by *Bacillus* sp. A24. Quorum sensing signal (*N*-3-hydroxyoctanoyl-homoserine lactone [11]) was extracted from culture filtrates of different bacterial co-inoculation mixtures after 48 h, added to semi-Luria-Bertani (LB) agar seeded with biosensor *Escherichia coli* (pSB401), and overlaid on LB agar plates. The biosensor produces light in the presence of quorum sensing signal. Left: photographed in white light. Right: photographed in darkness. Treatments were as follows: 1, negative control (biosensor *E. coli* [pSB 401] only); 2, co-inoculation of *Bacillus* sp. A24 and *P. fluorescens* 5064 at the same time; 3, positive control (*P. fluorescens* 5064); 4, *P. fluorescens* 5064 inoculated 6 h earlier than *Bacillus* sp. A24 (faint luminescence); and 5, *Bacillus* sp. A24 inoculated 6 h earlier than *P. fluorescens* 5064.

Biocontrol efficacy of *Bacillus* sp. A24 and *P. fluorescens* strain m6418 in excised broccoli head pathogenicity tests. Inoculation of broccoli heads with pathogenic *P. fluorescens* 5064 resulted in 80% disease after 5 days with extensive black soft rot (Fig. 8). Simultaneous coinoculation of the pathogen with the nonpathogenic mutant m6418 reduced disease to 47% (=41% reduction); tissue was brown but not soft-rotted. Heads inoculated with water alone had a disease score of 20% (heads were slightly darkened and showed a loss of the waxy bloom), which is normal for negative controls. Heads inoculated with strain m6418 had virtually no symptoms at all (3%). This suggested that some of the darkening of the water control treatment may have been due to residual nonpathogenic bacterial activity beneath the water-soaked cotton lints and this activity was abolished by the biocontrol activ-

ity of the mutant. The achieved level of significance in the *F* test prior to performing Fisher's protected LSD test was $P = 0.0382$.

To test the biocontrol activity of *Bacillus* sp. A24, we co-inoculated it with the pathogen at different ratios of *Bacillus* to *P. fluorescens* 5064 and at two different final inoculum concentrations (Fig. 9). When the pathogen was inoculated at a rate of 1×10^8 CFU/ml, the *Bacillus* was ineffective, but at a rate of 1×10^6 , the *Bacillus* gave a disease reduction of 26%. The *Bacillus* itself, when inoculated alone, induced greater disease symptoms (tissue darkening) compared with that of the water control, although these differences were not significant. The achieved levels of significance in the *F* tests for these experiments prior to performing Fisher's protected LSD test were $P = 0.0325$ and 0.0296 for the log 6 and log 8 CFU/ml tests, respectively.

Fig. 4. Effects of *Pseudomonas fluorescens* m6418 on *P. fluorescens* 5064 population dynamics in liquid culture. Bars = standard error of the mean.

Fig. 5. Effects of *Pseudomonas fluorescens* 5064 on *P. fluorescens* m6418 population dynamics in liquid culture. Bars = standard error of the mean.

DISCUSSION

In this study, we examined the potential for the biological control of broccoli head rot, caused by GpIV *P. fluorescens* strains, with two bacterial isolates: a Tn5 mutant derived from the pathogen, *P. fluorescens* m6418, and *Bacillus* sp. A24. We have shown that the two biocontrol isolates are inhibitory toward the pathogen in two different ways: strain m6418 produces large amounts of the antimicrobial substance Prn, and *Bacillus* sp. A24 is capable of degrading the 3-hydroxyoctanoyl homoserine lactone produced by the pathogen. *P. fluorescens* m6418 was able to reduce head rot in an excised broccoli head bioassay by 41%; *Bacillus* sp. A24 was less effective at 26%.

Prn has been reported to suppress a wide range of fungal and bacterial pathogens either in agar bioassay or tissue pathogenicity tests, such as *Rhizoctonia solani* (8), *Botrytis cinerea* (29,30),

Penicillium expansum (29), *Pyrenophora tritici-repentis* (41), *Mycobacterium tuberculosis* (14), and *Fusarium sambucinum* (4). El-Banna and Winkelmann (18) tested the antimicrobial effect of Prn on almost 40 bacterial and fungal strains. In their study, Prn was active against several filamentous fungi including *Pythium ultimum*, yeasts, and gram-positive bacteria (including some *Streptomyces* species, especially *S. antibioticus*). However, almost all gram-negative bacteria tested, including *E. coli* K12 and *P. fluorescens* ATCC 13525, were resistant to the doses tested except for *Proteus vulgaris* ATCC 13315. Levenfors and colleagues (36) provided data demonstrating that Prn gave an inhibitory effect against *Pythium ultimum* when applied at a high concentration (50 mg/liter). However, Howell and Stipanovic (27) concluded that Prn was ineffective against *Pythium ultimum*. *Pythium ultimum* is a widespread and important oomycete plant pathogen causing seed decay, pre- and postemergence damping off, and

Fig. 6. Effects of *Pseudomonas fluorescens* m6418 on *P. fluorescens* 5064 population dynamics on broccoli leaves. Bars = standard error of the mean.

Fig. 7. Effects of *Pseudomonas fluorescens* 5064 on *P. fluorescens* m6418 population dynamics on broccoli leaves. Bars = standard error of the mean.

root rot in many plant species (24). In our study, Prn produced by strain m6418 had strong antibiotic activity against *Pythium ultimum*, as well as wild-type *P. fluorescens* 5064 and *E. coli* JM109 (data for the latter two not shown). The inhibitory effects shown by Prn toward *Pythium ultimum* in our study might partly be due to the high concentration of Prn produced by strain m6418.

Because Prn production is an important mechanism of biological control against fungal plant pathogens, extensive work has been carried out to elucidate its gene expression and regulation. In addition to the identification of the Prn synthesis

gene operon (containing four genes) (22,33), Prn has also been reported to be under global genetic control by a two-component regulatory system composed of the sensor protein ApdA (also called LemA) (9) and the response regulator GacA (21,34). A mutation in *apdA* or *gacA* of *P. fluorescens* abolishes the production of Prn (9,34). A sigma factor encoded by *ropS* is also reported to be necessary for Prn production (41,43). In our study, the overexpression of Prn in the Tn5 mutant 6418 implies that the gene disrupted by Tn5 is a repressor within the Prn biosynthesis or regulation pathway.

Fig. 8. Biological control of head rot on excised broccoli heads using nonpathogenic *Pseudomonas fluorescens* mutant m6418. The pathogen (*P. fluorescens* 5064) and nonpathogenic mutant (*P. fluorescens* m6418) were simultaneously co-inoculated on broccoli heads in a 1:1 ratio (each at 1×10^8 CFU/ml). 0% = no disease symptoms; 100% = severe head rot. A five point scoring scale was used to assess the severity of disease symptoms: 0 = no symptoms, 1 = water soaking (loss of waxy bloom from florets), 2 = water soaking and tissue browning but no rot, 3 = tissue browning and softening (with rot), and 4 = extensive black soft rot. Data were subjected to percentile angular transformation using arcsine before analysis; thus, the above categories 0 to 4 became 0 (=0), 33 (=1), 50 (=2), 67 (=3), and 100% (=4), respectively. Bars = standard error of the mean. Different letters indicate treatments that were significantly different based on Fisher's protected least significant difference test, $P < 0.05$.

Fig. 9. Biological control of head rot on excised broccoli heads using *Bacillus* sp. A24. The pathogen (*Pseudomonas fluorescens* 5064) and biocontrol agent (*Bacillus* sp. A24) were simultaneously co-inoculated on broccoli heads in a 1:1 ratio in two separate experiments: (i) each at a concentration of 1×10^6 CFU/ml, and (ii) each at a concentration of 1×10^8 CFU/ml. 0% = no disease symptoms; 100% = severe head rot. A five point scoring scale was used to assess the severity of disease symptoms: 0 = no symptoms, 1 = water soaking (loss of waxy bloom from florets), 2 = water soaking and tissue browning but no rot, 3 = tissue browning and softening (with rot), and 4 = extensive black soft rot. Data were subjected to percentile angular transformation using arcsine before analysis; thus, the above categories 0 to 4 became 0 (=0), 33 (=1), 50 (=2), 67 (=3), and 100% (=4), respectively. Bars = standard error of the mean. Different letters when compared within each data set (i.e., when comparing treatments within either 1×10^6 CFU/ml or 1×10^8 CFU/ml) indicate treatments that were significantly different based on Fisher's protected least significant difference test, $P < 0.05$.

The research conducted in this study has provided evidence that the antibiotic-producing strain m6418 could be used as a biocontrol agent to control broccoli head rot disease. Strain m6418 had significant inhibitory effects against *P. fluorescens* 5064 in culture, on broccoli seedlings, and excised broccoli heads. In culture, the inhibitory effect of strain m6418 was greater when it was inoculated and allowed to establish before *P. fluorescens* 5064, although this was not the case on broccoli leaves, possibly because of its lower fitness on leaves.

With *Bacillus* sp. A24, no disease control effect was observed at the higher pathogen inoculum concentration of 1×10^8 CFU/ml. However, at a pathogen inoculum concentration of 1×10^6 CFU/ml, *Bacillus* sp. A24 did suppress head rot. The lower effectiveness of *Bacillus* sp. A24 as a biocontrol agent might be due to the following reasons. (i) A level of 1×10^8 CFU/ml for the pathogen is very high and represents a severe test for a biocontrol agent. In natural infections, disease symptoms are initiated at levels of 10^5 to 10^6 cells of *P. fluorescens* on the head (data not shown). (ii) As we mentioned before, when *P. fluorescens* 5064 reaches a threshold population, its quorum sensing system will be activated and its virulence factors start to be expressed. Therefore, it will not make much difference for *Bacillus* sp. A24 to degrade the AHL of *P. fluorescens* after the quorum sensing system has already been triggered and the virulence factors have been expressed. *Bacillus* sp. A24 might have a better disease control effect in the natural environment, where *P. fluorescens* concentrations are less than we created in the laboratory tests (3). The prospective disease control effect of *Bacillus* depends on its ability to degrade the QSS and therefore block biosurfactant production, one of the pathogen's virulence factors (controlled by quorum sensing). However, subsequent assays showed that *Bacillus* sp. A24 itself is a biosurfactant producer (data not shown). Given the role of biosurfactant in broccoli head rot (26), it is possible that the biosurfactant produced by *Bacillus* sp. A24 enhanced disease, either by direct damage to the head surface and/or by encouraging damage by pathogenic *P. fluorescens*.

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