Biological Control of *Pseudomonas syringae* pv. *syringae*, the Causal Agent of Basal Kernel Blight of Barley, by Antagonistic *Pantoea agglomerans*

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ABSTRACT

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Strains of *Pantoea agglomerans* (synanamorph *Erwinia herbicola*) suppressed the development of basal kernel blight of barley, caused by *Pseudomonas syringae* pv. *syringae*, when applied to heads prior to the *Pseudomonas syringae* pv. *syringae* infection window at the soft dough stage of kernel development. Field experiments in 1994 and 1995 revealed 45 to 74% kernel blight disease reduction, whereas glasshouse studies resulted in 50 to 100% disease control depending on the isolate used and barley cultivar screened. The efficacy of biocontrol strains was affected by time and rate of application. Percentage of kernels infected decreased significantly when *P. agglomerans* was applied before pathogen inoculation, but not when coinoculated. A single *P. agglomerans* application 3 days prior to the pathogen inoculation was sufficient to provide control since popu-

lations of about 10^7 CFU per kernel were established consistently, while *Pseudomonas syringae* pv. *syringae* populations dropped 100-fold to 2.0×10^4 CFU per kernel. An application to the flag leaf at EC 49 (before heading) also reduced kernel infection percentages significantly. Basal blight decreased with increasing concentrations (10^3 to 10^7 CFU/ml) of *P. agglomerans*, with 10^7 CFU/ml providing the best control. For long-term preservation and marketability, the survival of bacterial antagonists in several wettable powder formulations was tested. Over all formulations tested, the survival declined between 10- to >100-fold over a period of 1.5 years ($r = -0.7$; $P = 0.000$). Although not significant, storage of most formulations at 4°C was better for viability (90 to 93% survival) than was storage at 22°C (73 to 79%). However, long-term preservation had no adverse effect on biocontrol efficacy.

Additional keywords: barley bacterial diseases, *Hordeum vulgare*, integrated pest management.

We previously reported about the etiology and significance of basal kernel blight of barley caused by *Pseudomonas syringae* pv. *syringae* (29,30). The critical period of infection is from the late milk to soft dough stages of kernel development, and high moisture is necessary during this period for infection and disease development (28). Discolored barley has a reduced market value due to weight, yield, and quality loss (3,5) and can be discounted or rejected by the malting and brewing industries when blight percentages exceed 4% in 100-g samples (30). Since no bactericides are available on small grains that might provide control of bacterial pathogens, the only mitigating measure to date is the avoidance of irrigation during the most susceptible kernel developmental stage at soft dough and the use of less-susceptible cultivars (30). Over the last 3 years, we developed an effective biocontrol system based on the application of the common phyllosphere colonist *Pantoea agglomerans* prior to the *Pseudomonas syringae* pv. *syringae* infection window (10,12,13). *P. agglomerans*, synanamorph *Erwinia herbicola* (15,17), has been used as a biological control agent (BCA) against several fungal and bacterial diseases. Seed treatment with *P. agglomerans* protected wheat from infection by *Fusarium culmorum* (22), *F. nivale*, and *Pythium ultimum* (1,32). Seed bacterization with an *P. agglomerans* isolate also inhibited *Rhizoctonia solani*, thus providing sheath blight protection (35). A strain of *P. agglomerans* isolated from sclerotia of *Verticillium dahliae* was found to inhibit this pathogen in vitro (8). In the phyllosphere, *P. agglomerans* was reported to control *Puccinia recondita* f. sp. *tritici* on wheat (22), *Alternaria solani* on tomato (39),

and *Sclerotinia sclerotiorum* on dry edible beans (41). Plant growth-promoting effects of *P. agglomerans* strains from the winter wheat phyllosphere were noted by Ruppel et al. (36) and Scholz-Seidel and Ruppel (37). The authors detected wheat and barley yield increases of up to 500 kg/ha in field experiments with prebacterized seeds. Furthermore, Amellal et al. (2) described a positive effect of *P. agglomerans* in the regulation of the water content of the wheat rhizosphere by improving soil aggregation. Control of bacterial diseases by *P. agglomerans* has involved antagonism against ice nucleation active (INA-plus) bacteria (26,27) and several other bacterial pathogens. These include protection of cotton seeds from *Xanthomonas campestris* pv. *malvacearum* (34); rice seedlings from *X. oryzae* (18); mungbeans from *X. campestris* pv. *vingnaeradiatae* (9); and the control of the fire blight pathogen *E. amylovora* on apples (6,7,21,38), pear (20,38), and hawthorn (40). The objectives of the current study were to develop an effective and practical biocontrol system using *P. agglomerans* to suppress *Pseudomonas syringae* pv. *syringae* and the development of basal kernel blight. In addition to field and glasshouse studies, we present data on the time of BCA application and the concentration needed for successful disease prevention. Furthermore, we investigated the population dynamics of an antibiotic-resistant pathogen and antagonist population and evaluated the effectiveness of several formulations for long-term preservation of these bacterial antagonists.

MATERIALS AND METHODS

Bacterial cultures and inoculum preparation. *P. agglomerans* strains were isolated from asymptomatic barley kernels in fields that had endemic basal kernel blight in Fairfield, MT, in 1990, 1993, and 1994. *Pseudomonas syringae* pv. *syringae* strains (Pss) were isolated from barley kernels showing symptoms of basal blight in

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1993 to 1995. Isolates were identified according to standard tests (16) and based on the BIOLOG GN (Biolog, Inc., Hayward, CA) and MIDI (Microbial ID Inc., Newark, DE) identification systems. They were stored at -70°C in nutrient broth containing 15% glycerol. To produce cells for barley head inoculation, bacteria were retrieved from storage, streaked onto King's medium B agar (*Pseudomonas syringae* pv. *syringae*) (23) or tryptic soy agar (TSA) (Difco Laboratories, Detroit) (*P. agglomerans*), and incubated at 28°C for 48 or 24 h, respectively. Cells were suspended in 10 ml of phosphate-buffered saline (PBS) buffer (0.85% NaCl, 0.03 M NaOH, and 0.05 M KH_2PO_4 , pH 7.0) and the density of the suspension adjusted to 10^8 CFU/ml. The suspension was diluted 1:10 in 90 ml of sterile water containing 0.025% Tween 20 (polyoxyethylene-sorbitan monolaurate) as surfactant to a final concentration of 10^7 CFU/ml. In the glasshouse, *Pseudomonas syringae* pv. *syringae* strains were inoculated on barley heads at the soft dough stage (EC 85). In field experiments, natural infection by *Pseudomonas syringae* pv. *syringae* was enhanced by brief periods of daily overhead sprinkler irrigation for 5 to 10 days during the window of infection (30).

Application of *P. agglomerans* in field experiments. In 1994, four *P. agglomerans* strains (Eh 236, Eh 239, Eh 454, and Eh 460) were sprayed on barley cvs. B 2601 and B 1202 at Fairfield, MT, prior to the expected infection window for *Pseudomonas syringae* pv. *syringae* attack at the late milk stage (EC 77; 10 July). Natural infection was favored by providing overhead sprinkler irrigation for 4 h in the morning and 4 h in the evening for 5 days during the dough stages (EC 83 to EC 85) from 14 to 18 July, delivering an average of 15 mm of water per 4 h of irrigation (30). Traces of precipitation (0.6 mm) fell from 14 to 23 July in addition to the 150 mm provided by irrigation. Fifty milliliters of biocontrol bacterial suspensions (1×10^9 CFU/ml) were sprayed per 1.5-m row (0.4 liter/3.5 m^2) on barley heads until run-off using a compressed air sprayer (KGRO all purpose plant sprayer; K-mart Corp., Troy, MI). Assuming an average of 100 plants with 153 spikes per row and about 30 kernels per spike, the application rate was 1.0×10^7 CFU per kernel. A water/Tween 20 check; heat-killed Eh 236, Eh 239, Eh 454, and Eh 460; and the fungicide Tilt (propiconazole; Novartis Crop Protection, Greensboro, NC) applied at 0.3 liter/ha on 17 June were used as controls. The effect of Tilt on the disease was tested due to the observation of increased blight levels by farmers in their barley fields in the Fairfield area. The experiment was conducted as a two-factorial split plot design with cultivars as main plots (two levels) and treatments as subplots (10 levels) with four blocks. Each treatment was applied to a 1.5-m row within a plot consisting of six rows, 30 cm apart. The two border rows remained untreated and 60-cm alleys separated plots. Fifteen barley heads randomly selected at the soft dough stage (EC 85) represented one sample per replication and were picked at harvest and evaluated for basal kernel blight infection. Percentage of infection was calculated after harvest (2 August) according to the following formula: % kernel blight = $(a/b) \times 100$, in which a = number of blighted kernels per sample, and b = total number of kernels per sample.

All treatments were compared with nontreated water/Tween 20 controls of each cultivar to derive blight reduction data. In 1995, the same four *P. agglomerans* strains used in 1994 were sprayed on the four barley cvs. B 2601, B 2912, B 5133, and B 1202 prior to the infection window at the early milk stage (EC 73). In 1995 field experiments, 200 ml of bacterial suspensions at a concentration of 4.0×10^8 CFU/ml were sprayed until run-off per 1.5-m row (3.0 liters/5 m^2) on barley heads on 13 July. Assuming an average of 100 plants with 153 spikes per row and about 30 kernels per spike, the application rate was 1.0×10^7 CFU per kernel. Control treatments included the water/Tween 20 check and autoclaved bacterial antagonists. Additional treatments consisted of a 50 mM L-tartaric acid (pH 3.5) treatment, biocontrol strain combinations, and the Tilt application on 20 June. The tartaric acid treatment was included in the 1995 field study as a means of bacterial disease con-

trol because of its consistent reduction of the fire blight disease in Montana apple orchards (D. C. Sands, *unpublished data*). Sprinkler irrigation provided 300 mm of precipitation from the early milk to hard dough stages (15 to 25 July). Additionally, 16 mm of rain fell during the irrigation period. Disease evaluation was completed after harvest (22 August) in the same way as in 1994. The experiment was conducted as a two-factorial split plot design with cultivars as main plots (four levels) and treatments as subplots (16 levels) with four blocks.

***P. agglomerans* application in glasshouse experiments.** In 1994, two *P. agglomerans* strains (Eh 454 and Eh 239) were sprayed on cvs. B 1202 and B 2601, respectively, at the soft dough stage 3 days prior to inoculation with *Pseudomonas syringae* pv. *syringae* strain Pss 552. A water/Tween 20 application, the positive pathogen control (Pss 552), and the heat-killed strain Eh 239 were included as controls. One pot (19 cm in diameter) with three barley spikes per replication ($n = 5$) was inoculated and BCAs sprayed on heads until run-off (approximately 5 ml per spike) using a hand air brush sprayer (model Paaschi D500 1/10 H.P.; Paaschi Inc., New York). Plants were incubated in a mist chamber (95% relative humidity) for 72 h after BCA application. Thereafter, the pathogen Pss 552 was inoculated and plants incubated again for 72 h before being transferred to the glasshouse bench. The glasshouse was maintained at $22/18 \pm 2^{\circ}\text{C}$ (day/night) with a 12-h photoperiod. Basal kernel blight percentages were calculated after harvest by the formula described above and blight reduction data obtained by comparing treatments with the positive control. Each experiment (cultivar) was conducted as a one-factorial completely randomized design (CRD). The same experiment was performed in 1995 using strains Eh 236 and Eh 460 instead of strains Eh 454 and Eh 239 to see differences in the efficacy of different antagonistic bacteria. To investigate the performance of biocontrol strains against several pathogenic *Pseudomonas syringae* pv. *syringae* strains, a glasshouse experiment was conducted in 1997 as a one-factorial CRD with eight treatments and four replications. The biocontrol strains Eh 460, Eh 237, and Eh 234 were sprayed on cv. B 2601 heads 3 days prior to pathogen Pss 552 or Pss 793 inoculation at the soft dough stage. Controls included the Pss 552 and Pss 793 positive checks. Plant incubation and disease evaluation was carried out as described above.

Time and rate of BCA application. In most experiments, bacterial antagonists were applied 3 days prior to the pathogen inoculation or enhanced natural infection. To determine whether time of biocontrol application was critical, a glasshouse experiment was conducted in 1996 as a one-factorial CRD with four treatments and five replications. Treatments were a chloramphenicol-resistant *P. agglomerans* strain Eh 460 chl^R, which was applied to barley heads (cv. B 2601) at the soft dough stage 3 days prior to a rifampicin-resistant *Pseudomonas syringae* pv. *syringae* strain Pss 552 rif^R inoculation (= 3d, conventional application); the application of strain Eh 460 chl^R immediately before inoculation of Pss 552 rif^R (= 0d, coinoculation); and control applications of Pss 552 rif^R and water/Tween 20. Eh 460 chl^R was applied at a concentration of 7.9×10^7 CFU/ml and Pss 552 rif^R at 6.3×10^7 CFU/ml. The spontaneous antibiotic-resistant mutations had no effect on pathogenicity, survival, or biocontrol activity of either strain Pss 552 rif^R or strain Eh 460 chl^R as determined by in vitro and in vivo tests (data not shown). Plant inoculation, incubation, and disease evaluation after harvest were done as described above. The experiment also included a population dynamics study to investigate the development of the pathogen population after introduction of biocontrol bacteria in the barley phyllosphere under controlled conditions. The experiment used a CRD with four treatments, eight reisolation dates, and four replications (four treatments $\times$ eight dates $\times$ four replications = 128 heads = 44 pots with three plants per pot). Treatments consisted of Eh 460 chl^R application 3 days prior to Pss 552 rif^R inoculation at the soft dough stage (Eh 460 chl^R 3d); coinoculation of Eh 460 chl^R and Pss 552 rif^R (Eh 460 chl^R 0d); inocula-

tion of Pss 552 rif^R alone (Pss 552); and inoculation of Eh 460 chl^R alone (Eh 460 chl^R). Thirty-five kernels from each of four barley heads (replications) were randomly collected per treatment and shaken (150 rpm) in 50 ml of PBS buffer containing 15 5-mm glass beads for 30 min at 22°C. The suspensions were serially diluted and plated on semiselective King's medium B plus boric acid and cephalixin (31) amended with 100 µg of rifampicin per ml for Pss 552 rif^R reisolation and on TSA amended with 60 µg of chloramphenicol per ml for Eh 239 chl^R reisolation. Epiphytic bacterial population sizes were expressed as log CFU per kernel. The mean log-transformed population size of each strain was estimated from four heads (35 kernels) and six plate replicates per treatment.

The question of whether the BCA could be introduced earlier than the soft dough stage was examined in a separate glasshouse experiment in 1997 by applying *P. agglomerans* strain Eh 239 at EC 49, the last stage of booting before seedhead emergence. Pathogen *Pseudomonas syringae* pv. *syringae* strain Pss 552 was inoculated at the soft dough stage (EC 85). Other treatments included the conventional Eh 239 application at EC 85 3 days prior to inoculation of the pathogen, the water control, and the Pss 552 positive control. The experiment was conducted as a CRD with four treatments and five replications. Plant inoculation, incubation, and disease evaluation were performed in the same way as described above.

To estimate the rate (CFU/ml) of BCAs needed for disease control, a glasshouse experiment was conducted in 1996. *P. agglomerans* strain Eh 460 was sprayed on cv. B 2601 barley heads at EC 85 at concentrations of 10³, 10⁵, and 10⁷ CFU/ml 3 days prior to Pss 552 inoculation. The water/Tween 20 and the Pss 552 treatment without *P. agglomerans* antagonists served as controls. The experiment was performed as a CRD, with five treatments and six replications. Plant inoculation, incubation, and disease evaluation were done as previously described.

Formulation of *P. agglomerans*. Formulations of BCAs were prepared using the oil/starch/sugar encapsulation method developed by Quimby et al. (33). Briefly, this involved growing the BCAs on TSA plates overnight at 28°C. Plates were overlaid with 10 ml of 1 M sucrose solution and bacteria scraped off the plates into 100-ml sterile beakers using a sterile cotton swab. Five grams of the water-absorbent starch Water-lock (Grain Processing Corporation, Muscatine, IA) and 5 ml of unrefined corn oil (Spectrum Naturals Inc., Petaluma, CA) were added to each beaker. These

primary components were blended into a doughlike mass in a mixer (Sigma Chemical Co., St. Louis). A total of 17.5 g of the silica-based granulating agent Hi-Sil (Cabot Corp., Tuscola, IL) was added and blending continued until the dough crumbed, thereby producing a granular product. The wettable powder granules were spread in foil pans and air-dried in a laminar flow cabinet for 48 h at room temperature. The dried formulation was then sieved in the size range of 250 to 710 µm and viable bacterial colony counts recorded over a period of 1.5 years for *P. agglomerans* strains Eh 239 and EhL13 stored at 22 and 4°C, respectively. For lyophilization, strains Eh 239 and Eh 460 were grown in 200 ml of 2% potato dextrose broth (Difco Laboratories) for 20 h, centrifuged at 6,000 × g (DuPont Sorvall RC 5-B; DuPont Instruments Inc., Newtown, CT) for 15 min, and the cell pellet resuspended in 10 ml of PBS buffer. Ten percent dry milk powder (pasteurized nonfat dry milk, fortified with vitamins A and D, but no preservatives; Carnation Company, Los Angeles, CA) was added as a food base and the suspension vortexed. Samples were freeze-dried (freeze dry system/Lyphlock 4.5; Labconco, Kansas City, MO) for 24 to 48 h following freezing in liquid nitrogen for 10 min. Strains were kept at 4°C and viable colony counts recorded over time. For dry milk formulation of strain EhL13, the oil/starch/sugar encapsulation method was slightly modified by replacing starch with 10 g of dry milk powder. The powder was stored at room temperature over a storage period of 1.5 years. Viable colony counts were performed by adding 0.1 g of powdered granules to 9.9 ml of PBS buffer and vortexing for 1 min. Serial dilutions were prepared from this stock solution to 10⁻⁷ and spiral plated on TSA plates. Bacterial numbers were calculated as log CFU/g of powdered formulation and isolations were done 1 day, 2 weeks, 4 weeks, 3 months, 6 months, 1 year, and 1.5 years after formulation.

Statistical analyses. All statistical analyses were performed as appropriate to the experimental design using the MSU STAT statistical analysis package (Microcomputer version 5.2; Montana

TABLE 1. Percentage of basal blight disease incidence over all cultivars and percentage of kernel blight reduction on barley cvs. B 2601 and B 1202 obtained in a field experiment at Fairfield, MT, in 1994

Treatment ^w	Disease incidence all cultivars (%)	% Blight reduction	
		B 1202	B 2601
H ₂ O	2.3 a ^x	...	...
Eh 236	1.1 c	33	59 ^y *
Eh 239	0.9 c	50	68*
Eh 454	1.4 bc	30	45*
Eh 460	0.9 c	58	65*
Eh 236 D ^z	2.2 a	0	26
Eh 239 D ^z	2.5 a	0	17
Eh 454 D ^z	2.7 a	0	27
Eh 460 D ^z	2.0 ab	0	27
Tilt	2.3 a	0	25
LSD (0.05)	0.82	...	...

^w The water/Tween 20 check; biocontrol *Pantoea agglomerans* strains Eh 236, Eh 239, Eh 454, and Eh 460; heat-killed bacteria (D); or Tilt were applied to plant heads at the late milk stage prior to the *Pseudomonas syringae* pv. *syringae* infection window.

^x Values are means of four replicates and two cultivars, and values followed by the same letter are not significantly different at *P* ≤ 0.05 according to Fisher's protected least significant difference (LSD) test.

^y * = Significant reduction values of treatments compared with those of non-treated controls within a cultivar at *P* ≤ 0.05 using single-degree-of-freedom contrasts.

^z D = Heat-killed bacteria.

TABLE 2. Percentage of basal kernel blight disease incidence over all cultivars and percentage of blight reduction on barley cvs. B 2601, B 2912, B 1202, and B 5133 obtained in a field experiment at Fairfield, MT, in 1995

Treatment ^w	Disease incidence all cultivars (%)	% Blight reduction			
		B 2601	B 2912	B 1202	B 5133
H ₂ O	7.7 a ^x	...	...	...	...
Eh 236	3.4 g-i	48.9 ^y *	66.5*	52.86*	51.16
Eh 239	2.7 hi	60.3*	73.8*	67.14*	48.83
Eh 454	2.6 i	68.9*	64.2*	68.57*	61.39*
Eh 460	3.5 g-i	60.3*	60.0*	64.3*	36.7
Eh 236 D ^z	6.5 a-d	23.9	0.0	28.6	12.1
Eh 239 D ^z	7.2 ab	0.0	0.0	11.4	20.5
Eh 454 D ^z	5.9 b-d	0.0	45.6	52.9*	18.1
Eh 460 D ^z	6.6 a-c	0.0	13.8	38.6*	26.5
Eh 239+454	5.2 c-f	48.4*	40.7	64.3*	33.5
Eh 460+236	5.1 d-f	44.2*	46.8	34.3*	58.1*
Eh 239+460	4.1 f-h	42.1*	26.9	42.9*	10.7
Eh 236+239	4.2 fg	42.1*	25.9	62.9*	20.0
All Eh	4.4 e-g	45.5*	52.3*	45.7*	16.3
Tartaric acid	6.5 a-d	26.0	20.3	35.7*	20.5
Tilt	5.7 c-e	40.3*	0.0	25.7	43.3
LSD (0.05)	1.4	...	...	...	...

^w The water/Tween 20 check; biocontrol *Pantoea agglomerans* strains Eh 236, Eh 239, Eh 454, and Eh 460; heat-killed bacteria (D); biocontrol strain combinations; a 50 mM L-tartaric acid treatment; and Tilt were applied to barley heads at the early milk stage prior to the *Pseudomonas syringae* pv. *syringae* infection window.

^x Values are means of four replicates and four cultivars; data were log-transformed prior to analysis and values followed by the same letter are not significantly different at *P* ≤ 0.05 according to Fisher's protected least significant difference (LSD) test.

^y * = Significant reduction values of treatments compared with those of the non-treated controls within a cultivar at *P* ≤ 0.05 using single-degree-of-freedom contrasts.

^z D = Heat-killed bacteria.

State University, Bozeman). Data were analyzed by analysis of variance (ANOVA), and the sources and amount of variation were compared using an *F*-ratio test. When variances were not homogeneous in the Bartlett's test for homogeneity, a probability of significance was calculated using the ANOVA *F*-ratio test performed on the linearized data after transformation. To compare differences between two treatment means, Student's *t* tests were performed. To compare multiple treatment means, least significant differences were calculated by Fisher's least significant difference test at $\alpha = 5\%$ unless stated otherwise. Means followed by different letters demonstrated a statistical significant difference at $P \leq 0.05$. When quantitative factors (e.g., application rate, time) were studied, regressions were calculated instead of ANOVA. The experiments were repeated at least once. All calculations involving bacterial population densities were log-transformed prior to analysis, and means were presented as CFU per unit.

RESULTS

Suppression of basal kernel blight by *P. agglomerans* in field experiments. In 1994, both the two-row cv. B 1202 and the six-row cv. B 2601 had less than 4% of kernels exhibiting symptoms of basal kernel blight, presumably due to hot and dry weather conditions (0.6 mm of rainfall) and limited levels of overhead sprinkler irrigation (5 days = 150 mm of precipitation) during the window of infection from heading to soft dough (30). However, the two-factorial variance analysis revealed a significantly lower disease incidence (*F* was significant at $P = 0.0001$) over both cultivars in the treatments in which *P. agglomerans* strains were applied prior to the expected infection window (Table 1). Over all treatments, the disease incidence of cv. B 2601 was 2.1% and that of cv. B 1202 was 1.6%, although this difference was not significant ($P = 0.1186$). Furthermore, the interaction between the two main factors (cultivar $\times$ treatment) was not significant ($P = 0.3835$). The *P. agglomerans* strains significantly reduced the basal kernel blight percentage by 45 to 68% on cv. B 2601 when compared with the nontreated controls (Table 1). On cv. B 1202, the blight reduction ranged between 30 to 58% depending on the bacterial strain used. The Tilt treatment neither increased nor decreased the basal kernel blight disease incidence under field conditions (Table 1). Heat-killed biocontrol strains did not significantly reduce basal blight com-

pared with the nontreated water control (Table 1). In 1995, a year with average rainfall (16 mm) and extended overhead irrigation (10 days = 300 mm of precipitation) during the infection window, significant disease developed with infection percentages in the water controls of 9.5 and 8.9% for the six-row cvs. B 2601 and B 2912, respectively, and of 7.1 and 5.5% for the two-row cvs. B 1202 and B 5133, respectively (data not shown). Due to variance in homogeneity, data were log-transformed prior to analysis, and the two-factorial variance analysis revealed significant differences between cultivar means (*F* was significant at $P = 0.0000$) and treatment means ($P = 0.0000$). The interaction between cultivar $\times$ treatment was not significant ($P = 0.4949$). Over all treatments, blight percentages were significantly higher in the six-row cvs. B 2601 (6.3%) and B 2912 (6.1%) compared with those in the two-row cvs. B 1202 (4.1%) and B 5133 (3.8%) (data not shown). The disease incidence over all cultivars was lowest (2.6 to 3.5%) in the *P. agglomerans*-treated plots, with blight reductions ranging from 36 to 74% when compared with those in the nontreated controls (Table 2). Disease reductions by strain combinations also were significant depending on the cultivar. Blight reduction due to strain combinations was 42 to 48% in cv. B 2601 and 34 to 64% in cv. B 1202. However, strain combinations were inferior compared with the application of individual strains (Table 2). In 1995 field experiments, there was a significantly decreased disease incidence over all cultivars in the Tilt-treated plots (5.7%). Reductions due to Tilt application ranged between 0 to 43%, which were only significant on cv. B 2601 (Table 2). The tartaric acid treatment resulted in a disease incidence of 6.5% over all cultivars, which was not significantly different from that in the nontreated water control. However, on cv. B 1202, the blight reduction was 35.7%, which was significant compared with that in the nontreated control. Except for isolate Eh 454D (5.9%), heat-killed *P. agglomerans* did not significantly reduce the disease incidence over all cultivars (Table 2). Blight reductions were significant only in Eh 454D- and Eh 460D-treated plots on cv. B 1202 (Table 2).

Performance of *P. agglomerans* in glasshouse tests. Eh 454 and Eh 239 applied to cv. B 1202 3 days prior to *Pseudomonas syringae* pv. *syringae* pathogen inoculation reduced the basal kernel blight infection significantly ($P = 0.0026$) by 91 to 100% compared with the positive control (Pss 552), which displayed 11% basal kernel blight disease (Table 3). The heat-killed bacterial suspension Eh 239D, however, also reduced basal kernel blight infection levels significantly by 52% (Table 3). On cv. B 2601, the infection level due to pathogen application was 34%. Eh 454 significantly reduced the infection by 48% and Eh 239 by 64% ($P = 0.0364$). The heat-killed strain Eh 239D displayed a disease inci-

TABLE 3. Incidence of basal kernel blight disease (%) on cvs. B 2601 and B 1202 after application of *Pantoea agglomerans* Eh 239 and Eh 454 in a glasshouse experiment

Treatment ^u	% Basal kernel blight			
	B 2601		B 1202	
	Mean ^v	SD	Mean ^w	SD
Water control	0 ^x	...	0 ^x	...
Pss 552 control	34.06	±21.25	11.00	±4.14
Eh 239 D ^y	26.88	±7.99	5.22**	±3.88
Eh 239	12.40***	±4.87	0 ^x	...
Eh 454	17.58**z	±5.79	1.04**	±2.33
<i>P</i>	0.0364		0.0026	

^u Barley heads were inoculated with *Pseudomonas syringae* pv. *syringae* (Pss 552) at the soft dough stage. The water/Tween 20 control, *Pantoea agglomerans* strains Eh 239 and Eh 454, and the heat-killed strain Eh 239 (D) were applied to barley heads 3 days prior to the *Pseudomonas syringae* pv. *syringae* inoculation.

^v Values are means of five replicates ($n = 5$) and data were square root-transformed prior to analysis of variance.

^w Values are means of five replicates ($n = 5$).

^x 0 = No disease detected.

^y D = Heat-killed bacteria.

^z ** = Significant disease reduction compared with that of the Pss 552 control at $P \leq 0.05$ using single-degree-of-freedom contrasts. * = Significant disease reduction compared with that of the Pss 552 control at $P \leq 0.1$ using single-degree-of-freedom contrasts.

TABLE 4. Incidence of basal kernel blight disease (%) on cvs. B 2601 and B 1202 after application of *Pantoea agglomerans* Eh 236 and Eh 460 in a glasshouse experiment

Treatment ^v	% Basal kernel blight			
	B 2601		B 1202	
	Mean ^w	SD	Mean ^x	SD
Water control	0 ^y	...	0 ^y	...
Pss 552 control	45.47	±16.37	27.78	±9.16
Eh 236	8.71***z	±7.32	0.63***	±0.73
Eh 460	12.78***	±8.76	0.07***	±0.06
<i>P</i>	0.0005		0.0000	

^v Barley heads were inoculated with *Pseudomonas syringae* pv. *syringae* (Pss 552) at the soft dough stage. The water/Tween 20 control and *Pantoea agglomerans* strains Eh 236 and Eh 460 were applied to barley heads 3 days prior to the *Pseudomonas syringae* pv. *syringae* inoculation.

^w Values are means of five replicates ($n = 5$).

^x Values are means of five replicates ($n = 5$) and data were log-transformed prior to analysis of variance.

^y 0 = No disease detected.

^z *** = Significant disease reduction compared with that of the Pss 552 control at $P \leq 0.01$ using single-degree-of-freedom contrasts.

dence of 27%, which was statistically not different from that in the positive control.

Eh 236 and Eh 460 sprayed on cv. B 1202 3 days prior to Pss 552 inoculation reduced the kernel blight disease significantly by 98 and 100%, respectively, as compared with the inoculated control (F was significant at $P = 0.0000$) (Table 4). On cv. B 2601, Eh 236 reduced the blight percentage significantly by 81%, while Eh 460 reduced it by 72% ($P = 0.0005$) (Table 4).

To investigate the question whether the performance of *P. agglomerans* is consistent over more than one *Pseudomonas syringae* pv. *syringae* strain, several BCAs were applied to cv. B 2601 at the soft dough stage prior to inoculation of either Pss 552 or Pss 793. *P. agglomerans* Eh 460, Eh 234, and Eh 237 significantly

Fig. 1. Effect of application of *Pantoaea agglomerans* strains Eh 460, Eh 237, and Eh 234 on the percentage of basal kernel blight caused by *Pseudomonas syringae* pv. *syringae* Pss 552 and Pss 793 on barley cv. B 2601. Antagonistic *P. agglomerans* was applied to spikes in the glasshouse 3 days prior to pathogen inoculation at the soft dough stage. The percentage of basal blight was determined after harvest by counting infected versus noninfected kernels. Error bars represent standard deviations ($n = 4$). n.d. = No disease detected.

Fig. 2. Effect of time of *Pantoaea agglomerans* Eh 460 chl^R application on the percentage of basal kernel blight caused by *Pseudomonas syringae* pv. *syringae* Pss 552 rif^R on cv. B 2601. Eh 460 chl^R was applied 3 days prior to (3 days) or coinoculated (0 days) with Pss 552 rif^R in a glasshouse experiment at the soft dough stage. The percentage of basal blight was determined after harvest by counting infected versus noninfected kernels. Error bars represent standard deviations ($n = 5$). n.d. = No disease detected. Check is the water/Tween 20 control.

reduced basal kernel blight infection levels ($P = 0.0000$) (Fig. 1). Although the kernel infection levels were higher for Pss 793 (48%) than for Pss 552 (37%), disease reductions ranged between 96 to 100% in the Pss 793 system, while they ranged between 72 to 91% in the Pss 552 system depending on the biocontrol strain tested (Fig. 1).

Time and rate of *P. agglomerans* application. An important factor in the development of an effective biocontrol system is to determine the time and rate of BCA application needed for control. In several glasshouse experiments, we detected that time of application is indeed an aspect to consider when using *P. agglomerans* to protect barley heads from *Pseudomonas syringae* pv. *syringae* attack. For instance, when Eh 460 chl^R was applied 3 days prior to Pss 552 rif^R inoculation, inoculation infection levels were reduced significantly by 75%, while no significant disease reduction was noticed when Eh 460 chl^R and Pss 552 rif^R were coinoculated ($P = 0.0106$) (Fig. 2). The treatment of barley heads with Eh

Fig. 3. Population dynamics of *Pseudomonas syringae* pv. *syringae* Pss 552 rif^R alone (○), coinoculated with *Pantoaea agglomerans* Eh 460 chl^R (□), and inoculated 3 days after the introduction of *P. agglomerans* Eh 460 chl^R (Δ) in the glasshouse at the soft dough stage of kernel development on barley cv. B 2601. The population dynamics of *P. agglomerans* Eh 460 chl^R alone (●), coinoculated with Pss 552 rif^R (■), and applied 3 days prior to Pss 552 rif^R inoculation (▲) is shown for comparative purposes.

Fig. 4. Effect of *Pantoaea agglomerans* strain Eh 239 applied 3 days prior (3d) to pathogen inoculation at the soft dough stage or at the booting stage (EC 49) in the glasshouse on the percentage of basal kernel blight caused by *Pseudomonas syringae* pv. *syringae* Pss 552 on barley cv. B 2601. Error bars represent standard deviations ($n = 4$). n.d. = No disease detected. Check is the water/Tween 20 control.

460 chl^R alone (data not shown) or the water/Tween control did not result in any basal kernel blight disease symptoms, while the Pss 552 rif^R inoculation revealed 29.3% infected kernels (Fig. 2), with the pathogen population varying between 3.2×10^5 to 6.3×10^6 CFU per kernel over a period of 12 days (Fig. 3). The coinoculation of Eh 460 chl^R and Pss 552 rif^R demonstrated no significantly reduced infection levels (30.6%) (Fig. 2) compared with that in the Pss 552 rif^R control. In this treatment, both the pathogen population and the *P. agglomerans* population remained between 4.0×10^6 to 2.5×10^7 CFU per kernel for Eh 460 chl^R and between 1.0×10^6 to 1.6×10^7 for Pss 552 rif^R (Fig. 3). However, the application of Eh 460 chl^R prior to Pss 552 rif^R inoculation resulted in only 7.2% infected kernels (Fig. 2) and provided statistically significant disease control (75%). Additionally, the pathogen population decreased 100-fold over time from 2.0×10^6 to 2.0×10^4 CFU per kernel (Fig. 3).

For a commercial barley grower, application of BCAs 3 days prior to pathogen attack at the soft dough stage may be too narrow an application window to be practical. Therefore, we investigated whether an earlier BCA application, for instance at the time recommended for the last fungicide application (EC 49), could also lead to a successful biological control of basal kernel blight. Indeed, when Eh 239 was applied to barley heads 3 days prior to Pss 552 inoculation, the infection levels were significantly decreased by 87% (*F* was significant at $P = 0.0000$) (Fig. 4). When it was applied at EC 49, it decreased infection significantly by 82%, which was not different from that of the application 3 days prior to Pss 552 inoculation. Thus, the window for effective application of the BCA is at least between EC 49 and EC 85.

The concentration of the antagonist Eh 460 needed to successfully suppress the occurrence of basal kernel blight was tested at

rates of 10^3 , 10^5 , and 10^7 CFU/ml. When barley heads were inoculated with the pathogen Pss 552 without prior protection by *P. agglomerans*, 26% of the kernels were infected. Since concentration is a quantitative factor, means were separated by orthogonal contrasts comparing every Eh 460 dose rate with the nontreated positive control. When Eh 460 was applied 3 days prior to the pathogen inoculation at a low rate of 10^3 CFU/ml, the percentage of infected kernels was significantly reduced to 7.1% ($P = 0.006$) (Table 5). Application of Eh 460 at a rate of 10^5 CFU/ml also demonstrated significantly reduced blight levels (7.5%) ($P = 0.002$). However, the best disease control was reached when Eh 460 was applied at a concentration of 10^7 CFU/ml. Only 3% ($P = 0.000$) infected kernels were recorded, which is under the critical threshold level of 4% in a 100-g sample.

Formulation of *P. agglomerans*. The development of BCAs into commercial products is dependent on stable, efficacious, and economical formulations (42). Over all formulations tested, the oil/starch/sugar (USDA) formulation stored at 4°C was better for bacterial survival than was storage at 22°C, although this difference was not significant when calculated over time (Table 6). However, after 547 days (1.5 years) of storage at 4°C, 94% of Eh 239 bacteria and 95% of EhL13 bacteria survived in the USDA formulation. Survival rates dropped to 77% for Eh 239 and 74% for EhL13 when stored at 22°C. Despite storage at 4°C, lyophilization (FD) was significantly less efficient for bacterial survival than was the USDA formulation and resulted in only 75% survival of Eh 239. Dry milk formulation (DM) of EhL13 stored at 22°C was better in its survival rate (82%) than was the USDA formulation stored at 22°C (74%), although this difference was not significant. Comparing bacterial survival in the formulations from day 1 to the end of the storage period (day 1 versus day 547), we noted no significant differences in bacterial survival only with Eh 239 in the USDA formulation stored at 4°C ($P = 0.3654$). Second best was EhL13 in the USDA formulation stored at 4°C. Here, the reduction in bacterial survival was only significant when tested at the 10% α level. In all other cases, the difference in bacterial survival at the beginning and end of the storage period was moderately ($P \leq 0.05$) to highly ($P \leq 0.01$) reduced. Over all treatments, the regression coefficient over time was $r = -0.7005$ ($P < 0.001$, with the regression curve of $y = -0.297x + 10.3$).

DISCUSSION

Field experiments in 1994 and 1995 demonstrated 45 to 74% reductions in basal kernel blight-infected seeds of plants sprayed with *P. agglomerans* prior to the *Pseudomonas syringae* pv. *syringae* infection window. Glasshouse experiments resulted in 50 to 100% disease reduction depending on the barley cultivar used and biocontrol strain screened. A difference in susceptibility of culti-

TABLE 5. Effect of increasing dosages (10^3 , 10^5 , and 10^7 CFU/ml) of *Pantoea agglomerans* Eh 460 application in the glasshouse on the percentage of basal kernel blight caused by *Pseudomonas syringae* pv. *syringae* Pss 552 on cv. B 2601 and on the percentage of disease reduction

Treatment ^w	Dose (CFU/ml)	% Basal kernel blight			% Disease reduction
		Mean ^x	SD	<i>P</i> value	
Water control	0	0 ^y	...	...	...
Pss 552 control	0	26.3	±10.84	...	...
Eh 460	10^3	7.1*** ^z	±5.66	0.006	73.0
Eh 460	10^5	7.5***	±8.21	0.002	71.5
Eh 460	10^7	3.0***	±1.81	0.000	89.0

^w Eh 460 was applied to barley spikes 3 days prior to pathogen inoculation at the soft dough stage.

^x Values are means of six replicates per treatment and data were log-transformed prior to analysis.

^y 0 = No disease detected.

^z *** = Significant disease reduction compared with the Pss 552 control at $P \leq 0.01$ using orthogonal contrasts.

TABLE 6. Long-term survival of *Pantoea agglomerans* strains Eh 239, EhL13, and Eh 460 after 1, 14, 28, 91, 182, 365, and 547 days of storage in the oil/starch/sugar formulation (USDA), and lyophilized in dry milk powder (FD) and in the oil/starch/dry milk formulation (DM) at 4 or 22°C, respectively

Formulation	Strain/storage temperature	Days past formulation							Average formulation ^u	LSD _(0.05) time	% Survival ^v	<i>P</i> value ^w <i>t</i> test
		1	14	28	91	182	365	547				
USDA	Eh 239/22°C	10.0 ^x	9.8	9.3	9.0	8.8	7.7	7.7	8.89 BC	...	77	0.0388*** ^y
USDA	Eh 239/4°C	10.0	9.3	9.7	9.5	10.0	8.6	9.4	9.50 AB	...	94	0.3654 ns
USDA	EhL13/22°C	10.3	9.9	9.7	9.0	8.9	7.8	7.6	9.03 AB	...	74	0.0005***
USDA	EhL13/4°C	10.3	9.8	9.6	9.3	9.5	8.7	9.8	9.56 A	...	95	0.0723*
FD	Eh 239/4°C	9.9	8.5	8.3	8.4	8.5	7.6	7.4	8.37 C	...	75	0.0008***
DM	EhL13/22°C	10.3	10.0	9.6	9.5	9.0	8.5	8.4	9.33 AB	...	82	0.0136**
LSD _(0.05) formulation	...	...	...	...	...	...	...	...	0.6203	...	...	...
Average time ^z	...	10.13 A	9.56 AB	9.35 B	9.10 B	9.13 B	8.15 C	8.37 C	...	0.6203	...	...

^u Values (log CFU/g) are mean of formulation treatments over time.

^v Values represent the percentage of surviving bacteria comparing formulation treatments 1 day versus 547 days.

^w Values are *P* values of a *t* test comparing formulation treatments 1 day versus 547 days.

^x Values (log CFU/g of formulation) are mean of three plate replicates ($n = 3$).

^y Significant differences between treatment means at $P \leq 0.1$ (*), $P \leq 0.05$ (**), and $P \leq 0.01$ ***; ns = not significant; and ... = not applicable.

^z Values (log CFU/g) are mean of time over all formulation treatments.

vars to *Pseudomonas syringae* pv. *syringae* has been demonstrated in previous experiments (30). These results were confirmed here. A tendency was found that the efficacy of *P. agglomerans* to decrease the infection percentages depends on the susceptibility of the cultivars to the pathogen. Since the susceptibility of six-row cultivars in general is higher, the efficacy of *P. agglomerans* is greater on the six-row cultivars such as B 2601 and B 2912 than on the two-row cvs. B 1202 and B 5133. However, no significant interactions were observed in field experiments in 1994 and 1995. Reduction due to a Tilt application in the 1995 field trials could either be related to decreasing kernel blight-inducing fungi or an effect of Tilt on *Pseudomonas syringae* pv. *syringae*. An effect of Tilt on *Pseudomonas syringae* pv. *syringae* was revealed in a batch culture bioassay, when Tilt levels were above 100 µg/ml, which is usually exceeded in field applications (A. Braun-Kiewnick and B. J. Jacobsen, unpublished data). Similar good levels of disease control by *P. agglomerans* in field trials have been demonstrated in other systems. Lindow et al. (27) obtained 50% less frost damage on field-grown corn plants treated with INA-minus *P. agglomerans* (lacking ice nucleation activity). Under controlled environmental conditions, they achieved 88 to 100% frost damage (26). Protection from fire blight (42 to 51%) was demonstrated in research orchards by spraying apple blossoms with *P. agglomerans* 1 day prior to *E. amylovora* inoculation (6). Isenbeck and Schulz (19) saw 20 to 40% fire blight disease reduction using *P. agglomerans* under field conditions following natural infection with *E. amylovora*. More recently, Wilson et al. (40) observed the reduction of fire blight of hawthorn with *P. agglomerans* isolates under controlled environmental conditions. One isolate provided over 80% control of blossom blight and 100% control of shoot blight when applied at a BCA-to-pathogen ratio of 10:1. Field trials conducted with *P. agglomerans* isolates in Oregon in 1991 and 1992 and in Washington in 1992 revealed a 88% fire blight disease reduction in pear blossoms in Oregon in 1991 compared that in with the water controls. In 1992, the disease was reduced by 50% in Oregon and by 56% in Washington (20).

Glasshouse and field recovery data demonstrated that rifampicin-resistant *P. agglomerans* strains were established at stable levels in the barley phyllosphere after their initial introduction (10,12). They could be recovered in high numbers (3.0×10^6 to 3.0×10^7 CFU per kernel) over a period of time known to be critical for *Pseudomonas syringae* pv. *syringae* attack, suggesting one-time foliar spray application may be sufficient for controlling basal kernel blight. Many bacterial antagonists effectively colonize emerging and mature plant tissues for relatively long periods of time (1 to 3 months) following a single foliar application (25). However, time of BCA application was critical with respect to disease control. In the glasshouse, the rifampicin-resistant pathogen population was reduced 100-fold after the introduction of the chloramphenicol-resistant Eh 460 chl^R population 3 days prior to the Pss 552 rif^R inoculation, while no reduction was observed when Eh 460 chl^R and Pss 552 rif^R were coinoculated. Disease control data correlated well with population dynamics. Kernel blight infection was significantly reduced when plants were treated with the BCA Eh 460 chl^R before inoculation with the pathogen, while no disease control was observed when *P. agglomerans* and the pathogen were coinoculated. *P. agglomerans* could even be applied as early as before heading (EC 49), the stage relevant for the last fungicide application against foliar pathogens. This offers the possibility of combining fungicide applications with BCAs in commercial barley fields in an integrated disease control program. Similar population dynamics with respect to frost damage prevention have been found by Lindow et al. (27). An INA-minus *P. agglomerans* isolate decreased the population of epiphytic INA-plus bacteria 10- to 100-fold in growth chamber and field experiments. More recently, Johnson et al. (20) reported a direct relationship between a lower *E. amylovora* population on pear blossoms treated with bacterial antagonists and fire blight reduction. Lindow et al. (26) also demon-

strated that the percentage of leaves damaged by frost decreased significantly when the INA-minus antagonists were applied any time before, and up to 12 h after, application of the INA-plus pathogen. This was different from our studies, in which the antagonists had to be present prior to the pathogen and a coinoculation of antagonist and pathogen did not lead to successful disease prevention. Increasing rates of Eh 460 (10^3 to 10^7 CFU/ml) applied 3 days prior to pathogen inoculation resulted in better disease control. This indicated a desirable rate of *P. agglomerans* application at 10^7 CFU/ml for disease control below a critical 4% threshold. However, it should be noted that concentrations as low as 10^3 to 10^5 CFU/ml still reduced the disease significantly. Similarly, frost damage on corn decreased with increasing densities of an INA-minus *P. agglomerans* isolate applied 24 h before inoculation with an INA-plus isolate (26). Best control was achieved by spray application of 10^8 CFU/ml 24 h prior to inoculation of INA-plus bacteria (26). In addition, the reduced potential of *P. agglomerans* to protect individual bean blossoms from infection by *Sclerotinia sclerotiorum* suggested that low temperatures within the canopy may have limited the ability of *P. agglomerans* to multiply to levels needed for disease control (41).

A central issue in developing BCAs into commercial products is the large-scale, cost-efficient production and delivery of bacterial antagonists to the plant. Advances here are likely to be the single most important contribution to biological control in the coming decade (4). Dry preparations seem to be best for commercial formulations of bacteria. Therefore, screening potential antagonists for survival and activity as early as possible seems warranted (24). Since there has been little progress with formulations in phylloplane microbiology, we addressed the question of long-term viability of *P. agglomerans* in storage (at least 6 months, preferably 1 to 2 years). The USDA wettable powder formulation (33,42) preserved the viability of the tested Gram-negative *P. agglomerans* strains efficiently over a period of 1.5 years. While differences between treatments were not significant in most cases because of variability over time, survival of formulated bacteria stored at 4°C was generally better than that with storage at 22°C. An effect of temperature on survival of *Pseudomonas syringae* pv. *tabaci* in a sucrose/oil granular formulation has been reported by Quimby et al. (33). Bacterial survival was 63% when stored at 22°C for 365 days compared with 94% when stored at 2°C. Replacement of starch by dry skim milk in the oil/starch/sugar encapsulation method (USDA) of EhL13 stored at 22°C did not reveal any differences between the two formulations as to viability, suggesting that dry milk encapsulates the active bacterial cells as efficiently as starch, and promotes bacterial biofilm formation and adherence of the encapsulated bacteria to treated foliage (33). While differences in disease reduction between Eh 239 formulations applied prior to pathogen attack and the unprotected Pss 552 control were significant, we detected no statistical differences between the different Eh 239 formulations and the storage at different temperatures in a glasshouse test (10). Thus, the biocontrol efficacy of formulated *P. agglomerans* Eh 239 after 1.5 years of storage was not negatively influenced by formulation or by storage temperature.

In conclusion, *P. agglomerans* isolates indigenous to healthy barley kernels were selected for their ability to reduce kernel blight induced by *Pseudomonas syringae* pv. *syringae*. Our data indicated that the above-described organisms, when applied prior to the infection window, reduced the epiphytic population size of *Pseudomonas syringae* pv. *syringae* (10,12) and, consequently, basal kernel blight infection of barley (10,13). Thus, given the unavailability of synthetic bactericides and the known restrictions and regulations of using antibiotics in field applications, *P. agglomerans* bacteria may represent promising BCAs. They could be used in addition to less-susceptible cultivars and reduced irrigation during the infection window to prevent the occurrence of this disease. The mechanisms by which the antagonists interact with the pathogen to control basal kernel blight have been investigated (10,11,14) but will be discussed elsewhere.

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