Influences of Antagonist Population Levels, Blossom Development Stage, and Canopy Temperature on the Inhibition of *Sclerotinia sclerotiorum* on Dry Edible Bean by *Erwinia herbicola*

G. Y. Yuen, M. L. Craig, E. D. Kerr, and J. R. Steadman

Department of Plant Pathology, University of Nebraska, Lincoln 68583.
Journal paper 10371 of the Agricultural Research Division, University of Nebraska.
We thank S. Choobineh and L. Richardson for their fine technical assistance. This project was funded in part by a grant from the Nebraska Dry Bean Commission.
Accepted for publication 15 February 1994.

ABSTRACT

Yuen, G. Y., Craig, M. L., Kerr, E. D., and Steadman, J. R. 1994. Influences of antagonist population levels, blossom development stage, and canopy temperature on the inhibition of *Sclerotinia sclerotiorum* on dry edible bean by *Erwinia herbicola*. Phytopathology 84:495-501.

Three strains of *Erwinia herbicola*, applied to blossoms of dry edible bean (*Phaseolus vulgaris*) prior to inoculation with ascospores of *Sclerotinia sclerotiorum*, inhibited ascospore germination and subsequent development of white mold lesions in a bioassay. Although the strains exhibited similar multiplication rates on blossoms, increasing from initial levels of $<10^2$ cfu per blossom to stationary-phase populations of $>10^7$ cfu per blossom within 16 h at 25 C, they were effective in inhibiting *S. sclerotiorum* after different periods of multiplication and at different population levels. Strain B1 required incubation for at least 24 h, whereas strains B346 and B367 were inhibitory after only 6 h of incubation when population levels were $<10^5$ cfu per blossom. Multiplication of all strains was restricted to bean blossoms at the fully expanded, mature stage, which lasts for 1 day under field conditions. The strains differed in the duration of protection they provided to individual blossoms from colonization by *S. sclerotiorum* in the field. Following application of B346 and B367 to closed buds, antagonism against the pathogen first occurred when blossoms became fully expanded and continued as blossoms senesced and finally deteriorated. Pathogen inhibition by strain B1 did not occur, however, until blossoms had begun to senesce. In experiments conducted in western Nebraska, none of the strains was effective in reducing white mold disease severity by the end of the season; this was attributed to blossoms harboring insufficient population levels of applied bacteria. Multiplication and antagonism by *E. herbicola* strains on blossoms in the laboratory were highest at 28–30 C and were greatly reduced at 20 C. Leaf temperatures measured within the canopy of dry edible bean were unfavorable to *E. herbicola* growth (<20 C) an average of more than 16 h per day and favorable to *E. herbicola* growth (>25 C) for less than 6 h per day. Therefore, low temperatures within the canopy may have limited the ability of *E. herbicola* strains to multiply while blossoms were in the mature, fully expanded stage and thereby reduced the potential for protecting individual blossoms from infection by *S. sclerotiorum*.

White mold disease of bean (*Phaseolus vulgaris* L.) and other legumes, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is initiated upon the germination of ascospores on blossoms and the utilization of blossoms as a nutrient substrate by mycelia prior to invasion of green plant tissues (2). Biological control with blossom-colonizing antagonists has been explored as a strategy to inhibit this critical stage. Most of the research has involved fungi as control agents (6,7,29). In spite of the precedence for using bacteria to control diseases in which the blossom is the primary infection court (3,13,18,20,25), there are few reports relating to the application of bacteria against white mold disease of legumes (14,27). Huang et al (14) reported on the suppression of pod rot of dry peas by a strain of *Bacillus cereus* in the greenhouse. In a study by Yuen et al (27), *Erwinia herbicola* strain B1 controlled white mold on dry edible bean under greenhouse conditions but was inconsistent over repeated field experiments in western Nebraska. Following field application, strain B1 survived on leaves and colonized blossoms through the 1-mo-long blossoming period, and therefore long-term epiphytic survival and establishment did not appear to be a major limiting factor.

A predictive screening strategy is needed in order to identify microorganisms with greater potential for field efficacy. Development of a more effective model would be facilitated by knowledge of how strain B1 functions in antagonism under controlled environments and what conditions limit biocontrol activity in the field. Strain B1 was detected on blossoms of treated bean plants in field experiments at population levels ranging from 10^2 to 10^6 cfu per blossom (27), but the population threshold for inhibition of white mold and the time required for effective populations to be achieved were not determined. Considering that *S. sclerotiorum* can germinate, penetrate blossom tissues, and completely colonize blossoms within 3, 24, and 48 h, respectively (2), the ability of an antagonist to multiply to effective population levels and induce mechanisms of inhibition within these time frames would be crucial to its effectiveness as a biological control agent. Factors that may affect attainment of effective B1 populations on blossoms have not been identified. Major changes in blossom physiology occur during maturation and senescence (4,8,9,19) and may account for *S. sclerotiorum* infection of open bean blossoms but not of green buds (2). Bacterial antagonists to blossom-infecting pathogens have been reported to occupy specific blossom sites (13,14,25), suggesting that colonization by bacteria also may be influenced by blossom physiology. The temperature range for multiplication and antagonism by B1 and its relationship to temperatures occurring in the canopy of dry edible bean have not been established. Optimum temperatures for laboratory growth of *E. herbicola* strains were reported to be 28–30 C (11). These levels, however, represent the upper limits for blossom infection by *S. sclerotiorum*, which is most favored at 20–25 C (1).

The first objective of this study was to determine the relationship of antagonism by B1 and by two previously unreported *E. herbicola* strains against *S. sclerotiorum* to population levels and multiplication phase of the bacteria. The second was to assess the effects of blossom development stage and temperature on the ability of the strains to achieve pathogen-inhibitory populations. The final objective was to evaluate the bacterial antagonists for effectiveness in colonizing leaves and blossoms of dry edible bean

and in reducing the severity of white mold disease in the field. Preliminary aspects of this work have been published (26,28).

MATERIALS AND METHODS

General bacteriological methods. The isolation and selection of *E. herbicola* strain B1 were previously reported (27). Other bacterial strains, including *E. herbicola* B346 and B367, were isolated from healthy, mature blossoms of the dry edible bean cultivar Great Northern (GN) Harris, grown in Scottsbluff, Nebraska. Blossoms were washed with sterile 10 mM phosphate buffer, pH 7, and the liquid was cultured on 10% tryptic soy agar. Bacterial colonies were selected at random from the cultures. The strains were identified by standard physiological and nutritional tests (17,22). Nutrient utilization profiles, as determined on GN Microplates (Biolog, Inc., Hayward, CA), served as a supplemental identification method. All bacterial strains were stored in 10% glycerol at −70 C. Fresh subcultures were produced from frozen storage for each experiment and propagated on KG agar medium (27). Cells from 24- to 48-h-old cultures were collected and suspended in phosphate buffer for application onto plants. Initial cell concentrations in the suspensions were standardized by adjusting the turbidity of the suspensions to MacFarland Standard no. 1, which for B1 was approximately 10^8 cfu/ml. Unless stated otherwise, bacteria were applied in laboratory experiments to fully expanded, white blossoms of greenhouse-grown cultivar GN Spinel by pipetting 10-μl volumes of bacterial suspensions onto the standard or wing petals. Treated blossoms then were incubated for various time periods in petri dishes lined with moist filter paper prior to population and antagonism assays. Petri dishes were placed in incubators with temperature controls of ±1 C. Except in experiments in which various temperatures were tested, 25 C was the standard incubation temperature.

Rifampicin-resistant strains generated through spontaneous mutation were used in all experiments unless stated otherwise. They were enumerated on 10% tryptic soy agar amended with rifampicin and cycloheximide, each at 100 mg/L. The plating efficiency of rifampicin-resistant mutants on the amended medium was the same as that on unamended tryptic soy agar. When wild-type strains were tested, population numbers were determined by using maltose medium (27), which is semiselective and differential for *E. herbicola*. Population levels of applied bacteria on blossoms were determined by first placing individual blossoms in sterile plastic bags with 1 ml of sterile phosphate buffer and then crushing the blossoms by rolling a wooden dowel over the bags. Numbers of bacteria in the extracts were then measured through standard dilution plating and a plate-dilution frequency method as described by Yuen et al (27). In all experiments, nontreated blossoms served as controls. There were usually four to eight replicate blossoms per treatment. A population level of 1.0 cfu per blossom was assigned to samples with undetectable levels of applied bacteria. Population numbers were converted to $\log_{10}$ cfu before analysis of variance and the least significant difference (LSD) test were performed.

Relationship of population levels and multiplication phase to antagonism. Detached bean blossoms were treated with approximately 10^2, 10^4, or 10^6 cfu of strain B1 and incubated in petri dish moist chambers. Blossoms to which no bacteria were applied served as controls. Four blossoms per treatment were assayed for numbers of B1 every 4 h over a 24-h period and subsequently at 48 and 72 h. Another set of at least six blossoms was inoculated with *S. sclerotiorum* ascospores and evaluated for suppression of white mold disease in a bioassay. Ascospores of a Nebraska bean isolate of *S. sclerotiorum* (N1980) were produced and collected by methods described by Steadman and Cook (23) and then suspended in sterile distilled water. Spore concentrations were determined with a hemacytometer. Generally, 10-μl volumes of spore suspension were applied to give approximately 10^3 ascospores per blossom. The inoculated blossoms then were placed on detached trifoliolate leaves from GN Spinel plants grown in the greenhouse. The leaves were maintained in a green, turgid state by submerging the petioles in distilled water contained in 20-ml tubes. Parafilm was used to close the tube openings and to hold the leaves in place. Inoculated blossoms and leaves were kept at 23 C in plastic boxes, which were lined with wet paper towels and sealed to maintain relative humidity of >95%. These conditions were favorable to ascospore germination and colonization of blossoms by the fungus. After 3 days, leaves were inspected for the round, water-soaked lesions under the blossoms that are typical of infection by *S. sclerotiorum,* and lesion diameters were measured. There often was a high degree of variability in lesion diameters within treatments. Lesion diameters in the no-bacteria controls, in particular, varied from 2 to 6 cm. Therefore, the measurements were converted to a rating of 1–4 (1 = no lesion formed; 2 = lesion diameter of <1 cm; 3 = 1–3 cm; and 4 = >3 cm), which reduced treatment variances. Analysis of variance and the LSD test were conducted on the rating data. This experiment was performed twice.

The detached leaf assay was used to identify strains antagonistic to *S. sclerotiorum* from among bacteria isolated from bean blossoms. In the screening procedure, blossoms were treated with approximately 100 cfu of bacteria. After a 6-h incubation at 25 C, the blossoms were inoculated with ascospores and evaluated for white mold development in the detached leaf assay. Strains B346 and B367, which were effective in the screening assay, were compared with B1 for multiplication on bean blossoms and for efficacy of antagonism in the leaf assay after 0, 6, and 24 h of incubation on blossoms following application at 100 cfu per blossom. The bacterial strains also were tested for their effects on germination of *S. sclerotiorum* ascospores. In this assay, 10^5 ascospores were applied to the bacteria-treated blossoms following the appropriate incubation period. The inoculated blossoms then were maintained in the petri dish moist chambers at 25 C for an additional 4–6 h. Ascospores were recovered from the blossoms by washing and then were stained and examined for germination as described by Yuen et al (27). The experiment was repeated with starting bacterial populations of 100 and 10^6 cfu per blossom. Populations of the bacteria were not determined in the second experiment.

Multiplication and antagonism at different blossom development stages. In experiments involving blossoms at different stages of development, each stage was designated by a number: stage 0 = fully closed bud; 1 = partially opened blossom; 2 = fully expanded, white, mature blossom; 3 = turgid, yellow blossom entering senescence; and 4 = dried or deteriorated, senescent blossom, which may be attached to a developing bean pod. Once buds began to open, blossoms usually developed into each successive stage on a daily basis.

Multiplication of bacterial strains on blossoms at different stages of development was examined in the greenhouse on cultivar GN Spinel. Blossoms were treated while intact on the plants with 10 μl of the bacterial suspension, which equaled approximately 10^4 cfu per blossom. All nontreated blossoms and buds on the same raceme were removed. The plants were maintained in the greenhouse for 24 h, during which time temperatures were 20–32 C and relative humidity was 50–100%. Afterward, blossoms were assayed for numbers of applied bacteria.

Experiments were conducted under field conditions in Lincoln, Nebraska, to investigate the population dynamics of bacterial strains and the induction of antagonism against *S. sclerotiorum* on blossoms as they changed from the bud stage to senescence. Dry edible beans were planted in 5.6- × 9-m plots at a stand density of approximately 173,000 plants per hectare with 56-cm row spacing on 15 May 1992. Irrigation was applied weekly by sprinkler to supplement rain. Two genotypes, cultivar GN Tara and breeding line Pinto EP-1, were used. Because EP-1 blossoms earlier than does GN Tara, one experiment could to be conducted on EP-1 and then repeated on GN Tara over a 3-wk period that began on 2 July. Preliminary experiments indicated no significant differences among these two genotypes and GN Spinel in supporting populations of bacteria. In each experiment, 10 plants were flagged and treated with a suspension (10^4 cfu/ml) of a strain. A minimum of three nontreated plants separated treated plants within the same row, while one row of nontreated plants

separated treated plants across rows. From each treated plant, all but one unopened flower bud were removed from each raceme. The remaining bud was sprayed to run-off with a cell suspension by using a small-volume hand mister. Six to 10 racemes per plant were treated. One or two blossoms were sampled from each plant on a daily basis such that blossoms at development stages 2, 3, and 4 were picked 2, 3, and 4 days after treatment, respectively. Blossoms were put into separate collection bags, stored in a cooler, and processed within 4 h. Each blossom was cut aseptically into two; half was assayed for populations of applied bacteria, and the second half was evaluated for degree of antagonism to white mold in the detached leaf bioassay.

Temperature effects on growth and antagonism. The effects of temperature on the multiplication of B1, B346, and B367 were determined by first applying approximately 100 cfu to detached blossoms, which then were incubated in petri dishes at 14–38 C. Four blossoms per strain were assayed every 2–3 h for populations of the applied bacterium. Data from sampling periods in which populations were in multiplication phase, i.e., in numbers statistically higher than the applied level but $<10^7$ cfu per blossom, were used to calculate multiplication rates. This experiment was conducted at least twice for each strain.

To investigate effects of incubation temperature on antagonism against *S. sclerotiorum,* strains B1, B346, and B367 were applied to blossoms (10^3–10^4 cfu per blossom) and incubated at 20 and 28 C. B1-treated blossoms were incubated for 24 h, while those treated with B346 or B367 were incubated for 6 and 24 h. For each strain-temperature-time combination, six blossoms were tested for inhibition of *S. sclerotiorum* in the detached leaf assay, and four blossoms were used in determining bacterial numbers. Blossoms to which no bacteria were applied were the controls. In preliminary tests, nontreated blossoms sustained the same level of white mold development in the detached leaf assay regardless of the temperature at which the blossoms were held prior to inoculation with the pathogen. Therefore, the controls were incubated only at laboratory temperature (23 C). An experiment comparing B1 and B367 was performed twice. Because heterogeneity of error variance was not indicated, data from the two experiments were pooled for analysis of variance. Two experiments testing only B346 also were conducted.

Field efficacy experiments. Experiments were conducted near Scottsbluff, Nebraska, to evaluate bacterial antagonists for white mold control efficacy and to monitor strain populations. They were located at the Mitchell Experiment Station in 1991 (Mit91) and 1992 (Mit92) and in a commercial dry edible bean production field at the Harimon Farm in 1992 (HF92). Cultivar GN Harris, which is viny, was planted in Mitchell experiments, while cultivar GN Beryl, which has a semiupright growth habit, was planted at HF92. In all experiments, there were approximately 173,000 plants per hectare with 56-cm row spacing. Planting occurred between 1 and 10 June. Furrow irrigation was applied to supplement rain.

Experiments Mit91 and HF92 were designed as randomized complete blocks with six replicates. Each plot consisted of three rows each 6 m long. Wild-type strains of B1, B346, and B367 were applied in Mit91, whereas rifampicin-resistant mutants were tested in HF92. Cell suspensions (10^7–10^9 cfu/ml) were sprayed once onto bean plants at a rate of 50 ml/m of row at full bloom (22 July 1991 and 20 July 1992). In each trial, benomyl (methyl 1-[butylcarbamoyl]-2-benzimidazolecarbamate) was applied once at a rate of 1.1 kg (a.i.)/ha in 378 L of water as a separate treatment. Nontreated plots were the controls. Disease severity, measured as the percentage of the canopy affected by white mold, was determined 90 days after planting in the center row of each plot. Yield (as seed weight) from the same center rows also was determined. Analysis of variance and the LSD test were performed on the yield and arc sine-transformed disease severity data.

During the blossoming period, blossoms and leaves were sampled from Mit91 and HF92 at various intervals following bacterial treatment. Three yellow blossoms from outer rows of each plot were collected individually into sterile plastic bags and processed as separate samples. Three leaves from each plot were collected and bulked as a sample. All samples were transported under refrigeration to the laboratory where they were assayed for populations of the applied bacteria.

In experiment HF92, an additional set of 30 blossoms was sampled from B1- and B367-treated plots and from the nontreated controls 10 days after bacterial application. These were subjected to the detached leaf assay for antagonism against *S. sclerotiorum* as described above. This procedure was intended to provide a measure of the protection levels provided by the bacteria during the blossoming period. For this same purpose, strains B1 and B367 were applied in Mit92 to plots that were four rows wide and 6 m long. Blossoms were sampled from these plots 10 days after treatment and tested for both bacterial populations and inhibition of *S. sclerotiorum.*

Canopy temperature measurements. The numbers of hours each day during which leaf temperatures within a canopy of dry edible bean were near optimum (>25 C) or unfavorable (<20 C) to growth of *E. herbicola* were calculated from data reported by Deshpande (10) and provided by K. Hubbard, University of Nebraska, Lincoln. The data sets were collected during 2–28 August 1991 and 4–13 August 1992, respectively. The first 2 wk in August have been found to be peak periods for ascospore dispersal and blossom infection in western Nebraska (J. R. Steadman, *unpublished data*). Leaf temperatures at 10 cm above the soil surface within the canopy of GN Tara, a viny cultivar, were measured every 10 min with an infrared thermometer (10). The plots in which temperatures were recorded were located within 30 m of Mit91 and Mit92 experiments.

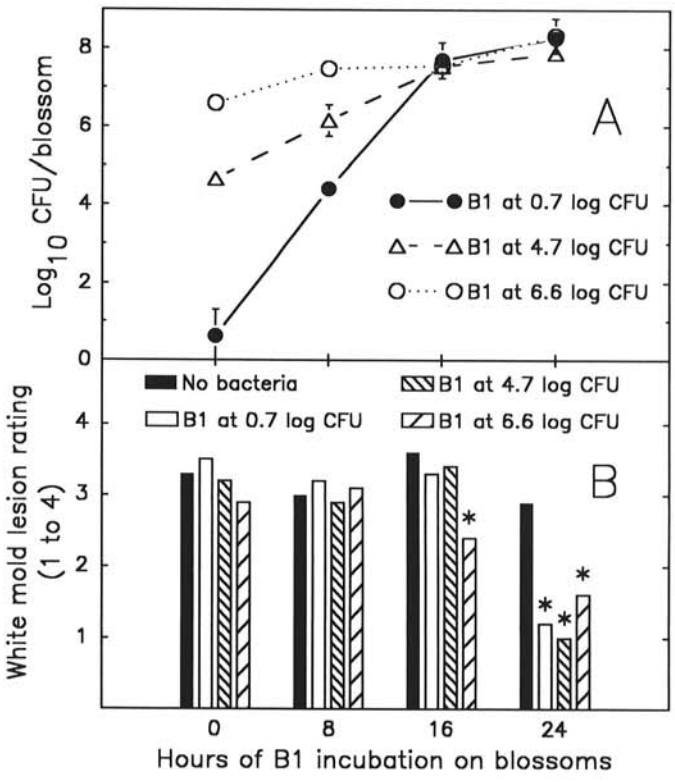

Fig. 1. Effects of incubation period on **A,** the numbers of *Erwinia herbicola* strain B1 and **B,** the extent of white mold lesion development from blossoms of dry edible bean (cultivar Great Northern Spinel) following treatment with B1 at three population levels. White mold lesion development was determined by inoculating blossoms with *Sclerotinia sclerotiorum* ascospores after each incubation period and then placing blossoms on detached bean leaves for 3 days. Lesions formed on leaves were rated according to procedures described in the text. All values represent a mean of six replicates. Error bars denote SE. * = Significant difference ($P \leq 0.05$) from the no-bacteria control for that sampling period according to the LSD test. Applied bacteria were not detected on control blossoms; the data are not presented.

RESULTS

Relationship of population levels and multiplication phase to antagonism. Inhibition of *S. sclerotiorum* blossom infection by *E. herbicola* strain B1 under laboratory conditions was dependent upon the B1 population on a blossom reaching stationary phase and remaining at that level for a minimum period. B1 populations reached stationary phase at >7 $\log_{10}$ cfu per blossom in 8 h when applied at 6.6 $\log_{10}$ cfu per blossom and in 16 h when applied in lower numbers (0.7 and 4.7 $\log_{10}$ cfu per blossom) (Fig. 1A). Regardless of the initial numbers of B1 applied, antagonism against *S. sclerotiorum,* as determined in the detached leaf assay, was not evident until the B1 population had been in stationary phase for 8 h (Fig. 1B). B1 populations remained between 7 and 8 $\log_{10}$ cfu per blossom for 2 days after reaching stationary phase; ascospores of *S. sclerotiorum* applied during this period were prevented from infecting the blossoms (data not shown). Similar results were found when the experiment was repeated.

Of more than 300 strains of bacteria isolated from bean blossoms, 10 were found to be effective in inhibiting *S. sclerotiorum* after a 6-h growth period on blossoms. Herein referred to as "rapid" strains, all were identified as *E. herbicola.* In a comparison of two representative strains (B346 and B367) against B1, the rapid strains significantly reduced the frequency of *S. sclerotiorum* ascospore germination on blossoms (Fig. 2A) and reduced the size of white mold lesions relative to the control (Fig. 2B) when the bacteria were applied 6 h prior to ascospore inoculation. Strain B1, in contrast, did not inhibit ascospore germination or lesion development unless applied to blossoms 24 h prior to the ascospore inoculation. None of the bacterial strains had any effect on ascospore germination or lesion development when the pathogen and bacteria were applied to blossoms simultaneously. Earlier induction of antagonism by the rapid *E. herbicola* strains was not related to faster multiplication on blossoms, since these strains exhibited multiplication rates on detached blossoms similar to those of B1 (Fig. 2C). When antagonism was first evident, populations of the rapid strains were in exponential multiplication phase at 4–5 $\log_{10}$ cfu per blossom, whereas B1 populations were in stationary phase at approximately 8 $\log_{10}$ cfu per blossom. Similar results occurred in a second experiment in which bacteria were applied initially at 10^2 and

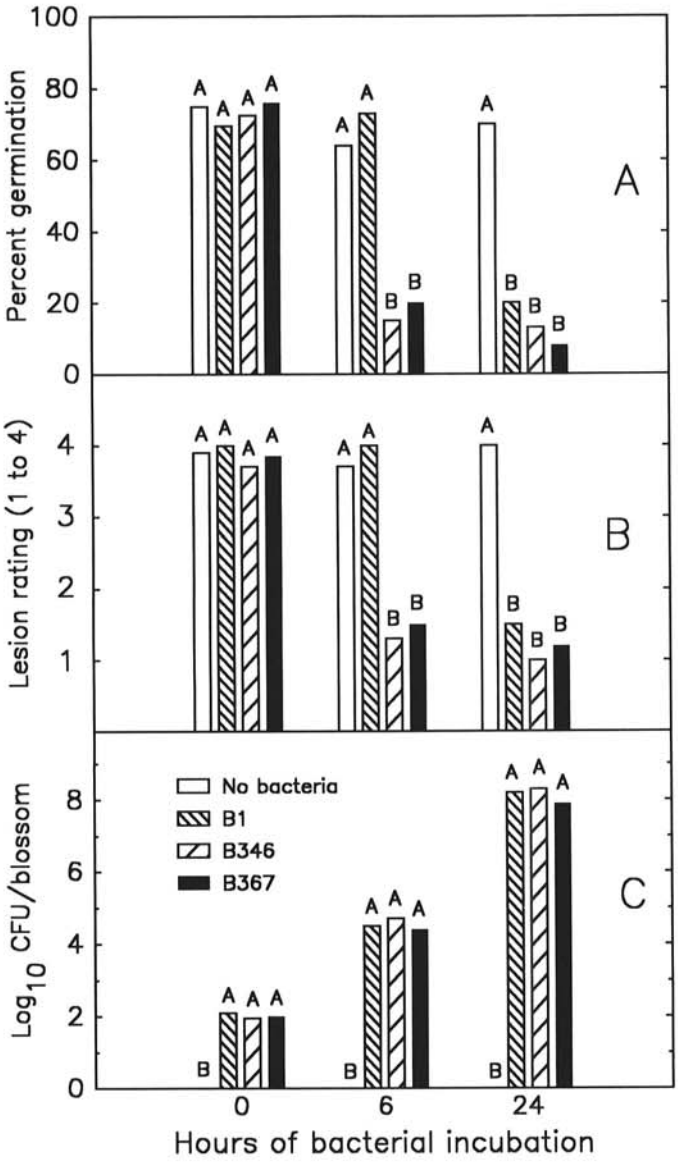

Fig. 2. Effects of *Erwinia herbicola* strains applied to dry edible bean (cultivar Great Northern Spinel) blossoms and durations of bacterial incubation on **A,** germination of *Sclerotinia sclerotiorum* ascospores, **B,** white mold lesion development, and **C,** population levels of *E. herbicola* strains. Blossoms were inoculated with ascospores after each incubation period. Spore germination was measured 4 h after inoculation. Lesion ratings were determined 3 days after ascospore-inoculated blossoms were placed on detached bean leaves. Procedures for rating lesions are described in the text. All values represent a mean of six replicates. Values in a sampling followed by the same letter are not significantly different ($P \leq 0.05$) according to the LSD test.

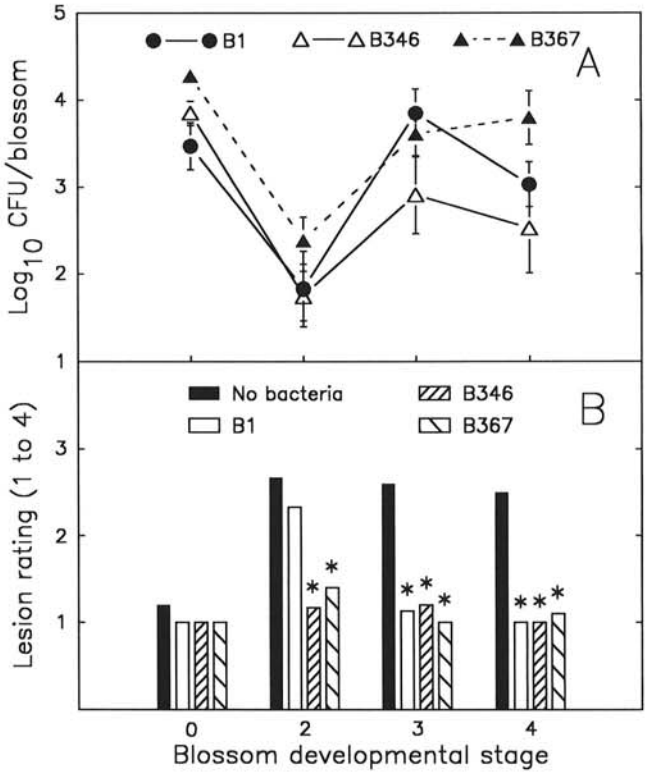

Fig. 3. A, Population levels of *Erwinia herbicola* strains B1, B346, and B367 on bean blossoms sampled at different stages of development following application of the bacteria to closed buds; and **B,** ratings of white mold lesions developing on detached bean leaves from the bacteria-treated blossoms. Buds of Pinto EP-1 were treated with the strains and maintained on plants in the field during blossom development. Blossoms at development stages 0 (closed bud), 2 (fully opened, white), 3 (yellow, senescing), and 4 (deteriorated) were sampled after 0, 2, 3, and 4 days, respectively. Nontreated blossoms sampled at the same development stages served as controls. Half of each blossom was assayed for bacterial populations. The other half was inoculated with ascospores of *Sclerotinia sclerotiorum* and placed on detached bean leaves in the laboratory. Lesion ratings were determined 3 days later following procedures described in the text. All values represent means of at least 10 replicates. Error bars denote SE. * = Significant difference ($P \leq 0.05$) from the control for that sampling period according to the LSD test. Applied bacteria were not detected on control blossoms; the data are not presented.

10^6 cfu per blossom (data not shown).

Effects of blossom development stage on multiplication of bacteria and antagonism against white mold. Multiplication of B1, B346, and B367 on blossoms in a greenhouse occurred only on blossoms at anthesis. When applied onto immature, partially expanded (stage 1) blossoms, the bacteria decreased in number by approximately 2.5 log units within 24 h (data not presented). In contrast, when the strains were applied to fully expanded, mature (stage 2) blossoms, population levels increased by >2 log units over the same period. Population numbers did not increase from applied levels on blossoms in early and advanced stages of senescence (stages 3 and 4).

Increase in *E. herbicola* population levels on blossoms under field conditions also was affected by blossom development stage. Following application of B1, B346, or B367 to closed buds of Pinto EP-1, population levels decreased during the first 2 days, during which the buds opened and approached maturity (stage 2) (Fig. 3A). As mature blossoms entered into senescence (stage 3), numbers of applied bacteria increased by 1.5–2 log units. Subsequently, populations either decreased or remained the same on deteriorating (stage 4) blossoms.

In the same experiment, antagonism against *S. sclerotiorum* occurred on different blossom stages depending upon the bacterial strain. The onset of white mold suppression was not always related to an apparent increase in numbers of applied bacteria (Fig. 3B). Rapid strains B346 and B367 reduced lesion development on stage 2 blossoms when bacterial population levels were at the lowest levels. Strain B1, on the other hand, was ineffective at this stage. All the *E. herbicola* strains controlled lesion development on blossoms entering senescence (stage 3), following a rise in bacterial numbers, and continued to control *S. sclerotiorum* on deteriorating blossoms (stage 4) when the bacterial numbers were no longer increasing. No lesions developed from inoculation of closed buds (stage 0) regardless of the bacterial treatment. Similar results were obtained when the experiment was repeated on cultivar GN Tara (data not shown).

Effects of temperature on bacterial multiplication and antagonism. Strains B1, B346, and B367 were similar in growth response on detached blossoms when subjected to various temperatures in the laboratory. Maximum multiplication occurred when the bacteria were incubated on blossoms placed at 30 C, increasing by roughly 2.5 log units over 5 h (i.e., generation time of 35 min; data not presented). As incubation temperatures were reduced to 22 and 14 C, bacterial multiplication rates declined such that populations increased in 5 h by only about 1.3 and 0.2 log units, respectively. Calculated generation times at 22 and 14 C were 70 min and 7 h, respectively.

Inhibition of multiplication of *E. herbicola* strains by lower incubation temperature was associated with reduced levels of antagonism against *S. sclerotiorum*. When B367-treated blossoms were incubated for 6 h at 20 and 28 C and then inoculated with *S. sclerotiorum* in the leaf bioassay, the mean lesion rating of blossoms incubated at 28 C was significantly lower than those of blossoms incubated at 20 C and the no-bacteria control (Table 1). Population levels of B367 did not increase during the 6-h incubation at 20 C, whereas multiplication was apparent at 28 C. When the incubation period was extended to 24 h, B367 population levels reached 7.2 $\log_{10}$ cfu per blossom under both temperature regimes and resulted in no lesion development. When strain B346 was used, results were similar to those obtained with B367 (data not presented). Antagonism by strain B1 appeared to be more sensitive to lower temperature than was cell growth. White mold lesion development was inhibited on B1-treated blossoms incubated for 24 h at 28 C but not on those incubated at 20 C. This occurred even though B1 populations at both incubation temperatures increased from the initial level of <4.0 $\log_{10}$ cfu per blossom to stationary-phase levels of >7.5 $\log_{10}$ cfu per blossom.

Colonization and biocontrol efficacy in the field. Strains B1, B346, and B367 colonized bean blossoms and leaves to similar levels. In experiment Mit91, which involved wild-type strains, mean populations of *E. herbicola* on yellow blossoms from bacteria-treated plots decreased in 8 days from initial populations of approximately 7 $\log_{10}$ cfu per blossom to levels of 4.2–5 $\log_{10}$ cfu per blossom and remained at the same levels in samples taken between day 8 and day 22 (Fig. 4A). By day 22, numbers of *E. herbicola* on blossoms from bacteria-treated plots were still >2 $\log_{10}$ units higher than the control. The bacterial strains maintained average populations on leaves of >4 $\log_{10}$ cfu per leaf through the last sampling at 3 wk posttreatment (Fig. 4B). Data from experiment HF92 are not presented, because bacterial population levels in this experiment were very similar to those found in Mit91, with the exception that mean populations on blossoms continued to decline to <2 $\log_{10}$ cfu per blossom by the third week after treatment.

In experiment HF92 at 10 days posttreatment, 37 and 23% of the blossoms from B1- and B367-treated plots, respectively, contained applied *E. herbicola* strains at population levels of 4 $\log_{10}$ cfu per blossom, and none had populations >7 $\log_{10}$ cfu per blossom. In a test of blossoms collected from the same plots for antagonism in the leaf bioassay, the proportion of blossoms from bacteria-treated plots that initiated white mold lesions was the same as that from the no-bacteria control (26 of 30). Mean size of lesions that developed from B367-treated blossoms (lesion rating of 2.8) was reduced ($P = 0.10$) compared with the control (lesion rating of 3.4), whereas B1 had no effect on lesion size. In a similar assessment of bacterial populations and antagonism levels on blossoms collected from Mit92 on day 10, there were no significant differences in lesion development between treatments; less than 20% of blossoms from B1- or B367-treated plot contained >4 $\log_{10}$ cfu of applied bacteria.

Applications of *E. herbicola* strains were ineffective in controlling white mold disease measured at the end of the growing season (data not presented). In Mit91, in which 34% white mold severity occurred in the control, bacterial treatments had no significant effects on disease levels or yield of seed. Benomyl, on the other hand, significantly decreased disease severity to 7% but did not affect yields. There was no white mold development in the HF92 experiment, and therefore disease severity was not

TABLE 1. Effects of temperature on antagonism of *Erwinia herbicola* strains B1 and B367 against *Sclerotinia sclerotiorum* on blossoms of dry edible bean (cultivar Great Northern Spinel) as determined by inhibition of lesion development on detached bean leaves following various periods of preincubation of bacteria on blossoms

Treatment[v]	Duration[w] (h)	Temperature[w] (C)	Lesion rating[x]	Population level[y] (log cfu/blossom)
B367	6	20	3.2 a[z]	3.58 d
		28	1.8 b	4.30 c
	24	20	1.0 c	7.18 b
		28	1.0 c	7.26 b
B1	24	20	2.7 a	7.54 ab
		28	1.3 bc	8.26 a
No bacteria	6	23	3.3 a	0.00 e
	24	23	3.0 a	0.00 e

[v] B1 and B367 were applied as cell suspensions to blossoms at 3.5 $\log_{10}$ cfu per blossom.

[w] Durations and temperatures at which bacteria-treated blossoms were incubated prior to inoculation with the pathogen. No-bacteria controls were incubated only at 23 C because preliminary experiments had indicated that incubation temperature had no effect on lesion development from nontreated blossoms.

[x] Each blossom was treated with 10^3 ascospores following the preincubation period and then placed on a detached bean leaf kept in a moist chamber at 23 C. After 3 days, white mold lesions were rated on a scale of 1–4: 1 = no lesion; 2 = lesion diameter <1 cm; 3 = 1–3 cm; 4 = >3 cm. Each value represents a mean of two experiments each with six replicate blossoms.

[y] Population levels determined on a separate subsample of four replicate blossoms at the time of inoculation with the pathogen. Each value represents a mean from two experiments.

[z] Values within a column followed by the same letter are not significantly different ($P = 0.05$) according to the LSD test.

measured at the end of the experiment. None of the treatments in the experiment affected seed yield.

Bean canopy temperatures during field experiments. Leaf temperatures in 1991 at or near the optimum for *E. herbicola* growth (>25 C) occurred an average of only 6 h each day (Fig. 5). The longest duration with these temperatures was 9 h. Temperatures considered to be unfavorable to *E. herbicola* growth (<20 C) were detected an average of 13 h per day. In 1992, durations of favorable and unfavorable temperatures averaged 6 and 16 h per day, respectively (data not shown).

DISCUSSION

Antagonism against *S. sclerotiorum* by *E. herbicola* strains under controlled conditions required not only that the bacteria multiply on blossoms to threshold levels but also that they reach particular growth phases. For strain B1, the required growth phase also had to be maintained over a minimum period. Inhibition by B1 did not occur until it had multiplied to stationary phase at >7 $\log_{10}$ cfu per blossom and had remained at this level for 8 h. Its ineffectiveness in controlling white mold disease in the field could be attributed to the fact that few blossoms harbored such a population. In comparison to B1, the rapid *E. herbicola* strains were not as limited temporally. B346 and B367 were inhibitory in early exponential multiplication phase at populations as low as 4 $\log_{10}$ cfu per blossom. Although mechanisms of antagonism have not been determined for any of the strains in this study, the pattern of antagonism by the rapid strains is parallel to that reported for in vitro production of herbicolin A by *E. herbicola* strain A111 (12), suggesting that herbicolin A may have been involved. Production of the antibiotic was initiated upon bacterial growth rather than occurring in stationary phase (12), as do most secondary metabolites. Herbicolin A also has been reported to be involved in the inhibition of fungal pathogens by *E. herbicola* strain B247 on the phylloplane and in the rhizosphere (15,16).

The association of antagonism by the rapid strains with multiplication and population levels was not as evident in the field as in the laboratory. Therefore, efficacy of the rapid strains could not be predicted solely on the basis of population levels found in the field. Antagonism in the leaf bioassay was manifested in one field experiment before numerical increases in B346 and B367 were detected (Fig. 3). Conversely, these strains were not effective in controlling white mold disease in efficacy experiments, although mean populations were found to be at or above the threshold level. An explanation for the first discrepancy is that the daily measurements of population numbers in the field were not as sensitive to population changes as the measurements repeated over hours that were performed in laboratory experiments. The second discrepancy can be attributed to mean population figures not accurately reflecting the percentage of sampled blossoms with B346 and B367 populations above the threshold. The poor relationship between mean populations and frequencies of sample units harboring threshold populations has been reported for phylloplane-colonizing bacterial pathogens (21).

Populations of applied bacteria and levels of protection against *S. sclerotiorum* detected in field experiments were not as high as those consistently observed in the laboratory. Field conditions may have been less favorable to multiplication and expression of antagonism by *E. herbicola* strains. Different cultivars of dry edible bean were used among laboratory and field experiments. On the basis of preliminary experiments, however, cultivars did not differ significantly in their ability to support populations of *E. herbicola* or in white mold susceptibility, and therefore host genotype probably was not an important factor. We did find a close relationship between bacterial multiplication and blossom development. Major biochemical changes that occur during blossom maturation and senescence may have been responsible for the restriction of *E. herbicola* multiplication to blossoms in the fully open stage. Cellulase activity has been found to increase in stigmata and anthers of *P. vulgaris* prior to anthesis (9). Concomitantly, there is increased exudation of materials from the stigma, including proteins, amino acids, glucose, and lipids (19), which may be utilized by microorganisms. *E. herbicola* strains active in antagonism against *Erwinia amylovora* multiplied preferentially on stigmatic tissues of apple blossoms rather than on the calyx (13), perhaps in response to stigmatic exudates. Studies on ornamental plant species have shown an increase in membrane permeability and a loss of sugars and proteins from petals with the onset of senescence (4,8). Carbon and nitrogen compounds were thought to be redistributed to the enlarging ovary (8), and thus these materials may be unavailable to microorganisms. This may account for the lack of multiplication by *E. herbicola* strains on bean blossoms past anthesis. Regardless of the controlling mechanism, the restriction of multiplication to a specific blossom stage, in conjunction with the prerequisite of multiplication to

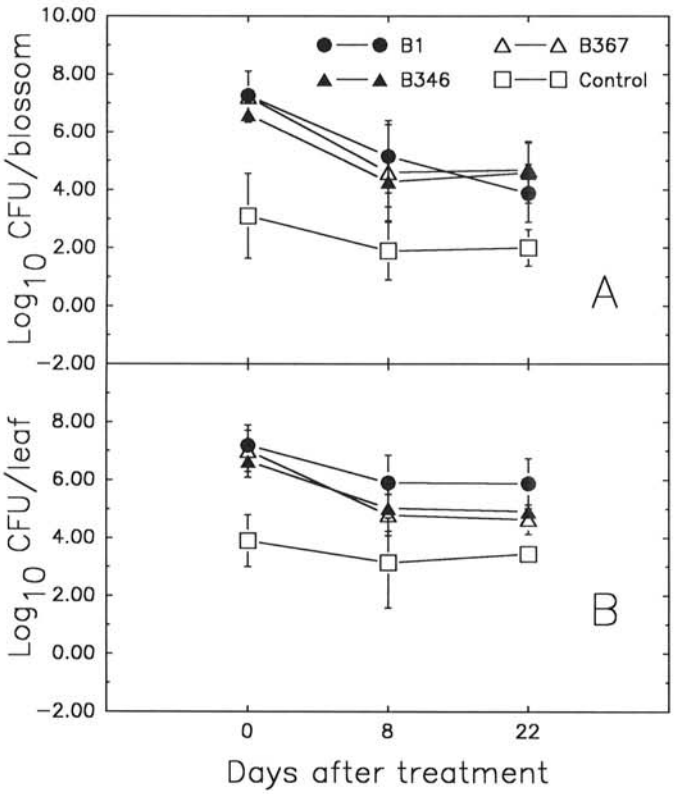

Fig. 4. Population levels of *Erwinia herbicola* detected on **A,** blossoms and **B,** leaves with maltose medium following application of strains B1, B346, and B367 to dry edible bean cultivar Great Northern Harris in the Mitchell 1991 experiment. Vertical bars denote SE.

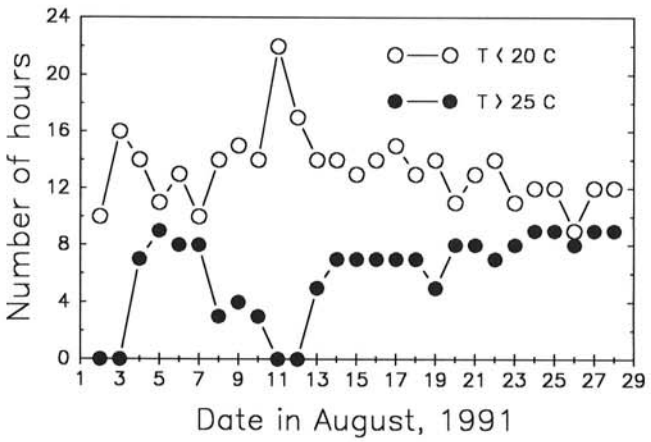

Fig. 5. Duration of time in which leaf temperatures within the canopy of dry edible bean cultivar Great Northern Tara were near optimal (>25 C) or unfavorable (<20 C) for growth of *Erwinia herbicola.* Values were calculated from data reported by Deshpande (10).

effective populations for induction of antagonism, may limit the potential of *E. herbicola* strains for controlling white mold disease. On each blossom, the bacteria have only a 1-day period during which multiplication can occur. Should unfavorable conditions hinder bacterial growth during the 1-day window of protection, the blossom in all subsequent stages is left vulnerable to infection by *S. sclerotiorum*.

Canopy temperature may be an important limiting factor acting during this critical window. While the macroclimate, particularly temperature, in western Nebraska is normally favorable to *E. herbicola* but unfavorable for white mold development, the microclimate within bean canopies, particularly those of cultivars with a viny, dense growth habit, allows development of white mold (5,10,24). Canopy temperatures, particularly at night, during the peak period of ascospore discharge and infection in early August can fall to levels that are unfavorable to *E. herbicola* growth. Considering the short period each day when temperatures are favorable to *E. herbicola* growth, the probability that strain B1 will multiply to effective numbers is very low. The rapid strains, requiring shorter periods of multiplication, have greater potential to achieve effective population status during the 6 h of favorable temperatures. In reality, the favorable temperatures occur during the day when other conditions, e.g., ultraviolet light and humidity, may limit growth.

Bacterial antagonists were tested for inhibition of *S. sclerotiorum* in this study through in planta assays with blossoms as substrates for bacterial growth and as sites for competition. By modifying one parameter in the system, namely shortening the bacterial incubation period prior to challenge with the pathogen, strains were found with more efficient antagonism against *S. sclerotiorum* compared with strain B1. Although the procedure represented an improvement over in vitro screening methods, it was not sufficiently rigorous to identify organisms effective in biological control in the field. Having found blossom development and temperature to be important ecological limiting factors, we will incorporate these two conditions into the testing of future strains. This is not to suggest that screening for strains insensitive to blossom development or tolerant to wider temperature ranges will automatically yield field-effective biocontrol agents, since a host of unidentified factors also may control efficacy. Nevertheless, continued study of the antagonistic and ecological attributes of less successful organisms may result in identification of greater numbers of limiting factors, which when combined in laboratory screening assays would create more field-predictive systems.

LITERATURE CITED

1. Abawi, G. S., and Grogan, R. G. 1975. Source of primary inoculum and effects of temperature and moisture on infection of beans by *Whetzelinia sclerotiorum*. Phytopathology 65:300-309.
2. Abawi, G. S., Polach, F. J., and Molin, W. T. 1975. Infection of bean by ascospores of *Whetzelinia sclerotiorum*. Phytopathology 65:673-678.
3. Beer, S. V., Rundle, J. R., and Norelli, J. L. 1984. Recent progress in the development of biological control for fire blight—A review. Acta Hortic. 151:195-201.
4. Bieleski, R. L., and Reid, M. S. 1992. Physiological changes accompanying senescence in the ephemeral daylily flower. Plant Physiol. 98:1042-1049.
5. Blad, B. L., Steadman, J. R., and Weiss, A. 1978. Canopy structure and irrigation influence white mold disease and microclimate of dry edible beans. Phytopathology 68:1431-1437.
6. Boland, G. J., and Hunter, J. E. 1988. Influence of *Alternaria alternata* and *Cladosporium cladosporioides* on white mold of bean caused by *Sclerotinia sclerotiorum*. Can. J. Plant Pathol. 10:172-177.
7. Boland, G. J., and Inglis, G. D. 1989. Antagonism of white mold (*Sclerotinia sclerotiorum*) of bean by fungi from bean and rapeseed flowers. Can. J. Bot. 67:1775-1781.
8. Borochov, A., and Woodson, W. R. 1989. Physiology and biochemistry of flower petal senescence. Hortic. Rev. 11:15-43.
9. del Campillo, E., and Lewis, L. N. 1992. Occurrence of 9.5 cellulase and other hydrolases in flower reproductive organs undergoing major cell wall disruption. Plant Physiol. 99:1015-1020.
10. Deshpande, R. Y. 1992. Effects of plant architecture microclimate, white mold and yield of dry beans (*Phaseolus vulgaris* L.) and implications for disease management. Ph.D. diss. University of Nebraska, Lincoln.
11. Gibbins, L. N. 1978. *Erwinia herbicola:* A review and perspective. Pages 401-431 in: Proc. Int. Conf. Plant Pathog. Bact., 4th. Vol. 2. Station de Pathologie Vegetale et Phytobacteriologie, I.N.R.A., Angers, France.
12. Greiner, M., and Winkelmann, G. 1991. Fermentation and isolation of herbicolin A, a peptide antibiotic produced by *Erwinia herbicola* strain A111. Appl. Microbiol. Biotechnol. 34:565-569.
13. Hattingh, M. J., Beer, S. V., and Lawson, E. W. 1986. Scanning electron microscopy of apple blossoms colonized by *Erwinia amylovora* and *E. herbicola*. Phytopathology 76:900-904.
14. Huang, H. C., Kokko, E. C., Yanke, L. J., and Phillippe, R. C. 1993. Bacterial suppression of basal pod rot and end rot of dry peas caused by *Sclerotinia sclerotiorum*. Can. J. Microbiol. 39:227-233.
15. Kempf, H.-J., Bauer, P. H., and Schroth, M. N. 1993. Herbicolin A associated with crown and roots of wheat after seed treatment with *Erwinia herbicola* B247. Phytopathology 83:213-216.
16. Kempf, H.-J., and Wolf, G. 1989. *Erwinia herbicola* as a biocontrol agent of *Fusarium culmorum* and *Puccinia recondita* f. sp. *tritici* on wheat. Phytopathology 79:990-994.
17. Lelliott, R. A., and Dickey, R. S. 1984. Genus VII. *Erwinia*. Pages 469-476 in: Bergey's Manual of Systematic Bacteriology, vol. 1. N. R. Krieg and J. G. Holt, eds. Williams & Wilkins, Baltimore.
18. Lindow, S. E., Arny, D. C., and Upper, C. D. 1983. Biological control of frost injury: An isolate of *Erwinia herbicola* antagonistic to ice nucleation active bacteria. Phytopathology 73:1097-1102.
19. Lord, E. M., and Webster, B. D. 1979. The stigmatic exudate of *Phaseolus vulgaris* L. Bot. Gaz. (Chicago) 140:266-271.
20. Redmond, J. C., Marois, J. J., and MacDonald, J. D. 1987. Biological control of *Botrytis cinerea* on roses with epiphytic microorganisms. Plant Dis. 71:799-802.
21. Rouse, D. I., Nordheim, E. V., Hirano, S. S., and Upper, C. D. 1985. A model relating the probability of foliar disease incidence to the population frequencies of bacterial plant pathogens. Phytopathology 75:505-509.
22. Schroth, M. N., and Hildebrand, D. C. 1988. 'Amylovora' or non-soft rot group. Pages 37-43 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria, 2nd ed. N. W. Schaad, ed. American Phytopathological Society, St. Paul, MN.
23. Steadman, J. R., and Cook, G. E. 1974. A simple method for collecting ascospores of *Whetzelinia sclerotiorum*. Plant Dis. Rep. 58:190.
24. Weiss, A., Hipps, L. E., Blad, B. L., and Steadman, J. R. 1980. Comparisons of within-canopy microclimate and white mold disease (*Sclerotinia sclerotiorum*) development in dry edible beans as influenced by canopy structure and irrigation. Agric. Meterol. 22:11-21.
25. Wilson, M., Epton, H. A. S., and Sigee, D. C. 1992. Interactions between *Erwinia herbicola* and *E. amylovora* on the stigma of hawthorn blossoms. Phytopathology 82:914-918.
26. Yuen, G. Y., Craig, M. L., Kerr, E. D., and Steadman, J. R. 1993. Bean blossom developmental stage and canopy temperature affect antagonism of *Erwinia herbicola* strains against *Sclerotinia sclerotiorum* (Abstr.) Phytopathology 83:1385.
27. Yuen, G. Y., Godoy, G., Steadman, J. R., Kerr, E. D., and Craig, M. L. 1991. Epiphytic colonization of dry edible bean by bacteria antagonistic to *Sclerotinia sclerotiorum* and potential for biological control of white mold disease. Biol. Control 1:293-301.
28. Yuen, G. Y., Kerr, E. D., Steadman, J. R., and Craig, M. L. 1992. Bacterial biocontrol of white mold disease (*Sclerotinia sclerotiorum*). Annu. Rep. Bean Improv. Coop. 35:54-55.
29. Zhou, T., and Reeleder, R. D. 1989. Application of *Epicoccum purpurascens* spores to control white mold of snap bean. Plant Dis. 73:639-642.