BIOLOGICAL CONTROL OF *PSEUDOMONAS AVENAE* WITH EPIPHYTIC BACTERIA ISOLATED FROM CORN.

II. SELECTION AND EVALUATION OF ANTAGONISTS[1]

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ABSTRACT – Of 179 bacteria collected from corn plants in three locations, 34 were antagonistic in vitro to *Pseudomonas avenae*. Among these, 29 were fluorescent pseudomonads. Various levels of control of bacterial leaf blight and stalk rot (BLBSR) on sweet corn were obtained, but no relationship was found between antagonism in vitro and disease control. In greenhouse, strains F-11 and F-24 of *P. fluorescens* and strain U-46 of *Bacillus* sp. consistently controlled BLBSR on sweet corn. Control achieved with F-11 was equivalent to reducing the inoculum dose (ED_{50}) of *P. avenae* by at least 1000 times, as determined by infectivity titrations. In the field, F-11 controlled BLBSR as effectively as 100 ppm streptomycin when treatments were applied immediately before inoculation with *P. avenae*. Streptomycin applied 12 or 24 hr before inoculation did not control the disease, whereas F-11 reduced BLBSR severity by more than 50%. Antagonist U-46 did not affect disease development at any time of application. Antagonist F-11 was recovered from corn whorls for eight days after application to plants, but U-46 was not recovered 84 hr after application.

Index terms: biocontrol, *Zea mays saccharata*, *Pseudomonas fluorescens*, bacterial leaf blight, stalk rot.

CONTROLE BIOLÓGICO DE *PSEUDOMONAS AVENAE* COM BACTÉRIAS EPÍFITAS ISOLADAS DE PLANTAS DE MILHO: II.SELEÇÃO E AVALIAÇÃO DE BACTÉRIAS ANTAGÔNICAS

RESUMO – De 179 bactérias coletadas de folhas de milho em três localidades (29 do grupo das pseudomonas fluorescentes) foram antagônicas a *Pseudomonas avenae*. Em casa de vegetação, vários níveis de controle à queima-bacteriana-da-folha, causada por *P. avenae*, foram obtidos, não havendo correlação entre antagonismo *in vitro* e controle da doença. Os isolados F-11 e F-24 de *P. fluorescens* e o isolado U-46 de *Bacillus* sp. controlaram consistentemente a doença em casa de vegetação. Testes de titulação de infectividade indicaram que F-11 proporcionou um controle equivalente à redução do nível de inóculo de, pelo menos, 1000 vezes. Em campo, F-11 foi tão eficiente no controle da doença quanto 100 ppm de estreptomicina, quando ambas foram aplicadas imediatamente antes da inoculação. Estreptomicina aplicada 12 ou 24 horas antes da inoculação não foi eficiente, enquanto F-11 reduziu a severidade da doença em mais de 50%. O antagonista U-46 não afetou o desenvolvimento da doença. Quanto à persistência dos antagonistas, F-11 pode ser recuperado de cartuchos de plantas até oito dias após sua aplicação, enquanto U-46 não foi recuperado além de 84 horas.

Termos para indexação: controle biológico, biocontrole, *Zea mays saccharata*, *Pseudomonas fluorescens*, queima-bacteriana-da-folha, podridão do caule.

INTRODUCTION

Bacterial leaf blight and stalk rot (BLBSR) of corn (*Zea mays* L.) incited by *Pseudomonas avenae* Manns, can cause significant economic losses if some sweet corn cultivars are grown under subtropical weather conditions.

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Although resistance to BLBSR has been reported for some cultivars (Summer & Schaad 1977), the level of resistance is not enough to be a sole method of control.

Gitaitis (1979) observed that areas where water collected in sweet corn plants corresponded to areas with symptoms of BLBSR and that lesions developed mostly on leaves emerging from or in whorls at the time of inoculation. Since rapid growth of a corn plant in the vegetative phase results in a constant supply of susceptible tissue, any protective control measure must have the property of redistribution on the plant surface, or be applied frequently.

The influence of leachates on plant-surface micro-organisms is very complex. It is known, however, that they affect microbial populations either by stimulating or inhibiting their growth (Godfrey 1976, Tuckey 1971). Inhibition of microbial growth has been related to phenolic or terpenoid substances secreted by leaves (Blakeman & Fokkema 1982). Therefore, it is conceivable that temporal changes of these inhibitory substances, as well as the concentration of nutrient exudates, may play an important role in the succession of micro-organisms on the plant surface.

Gitaitis (1979) analyzed the fluid collected from the whorl of sweet-corn plants and found that it contained essential compounds for microbial growth, such as sugars, organic acids, and amino acids. The filtrate of this fluid was not inhibitory to one culture of *P. avenae*.

The objectives of this study were (1) to isolate and to evaluate epiphytic bacteria from corn plants that prevent or reduce severity of BLBSR, and (2) to determine the persistence of components some of the components of the bacterial population in the whorls of sweet corn plants in relation to the biological control of the disease.

MATERIALS AND METHODS

Collection of epiphytic bacteria

Fluid that accumulated during the night in the top funnel of furled leaves was collected before 8:00 a.m. from plants of different varieties and inbred Lines of field and sweet corn in Florida. Samples were randomly collected (following a "W" pattern) in 1983, 1984, and 1985 in Zellwood (AREC-San ford Zellwood Farm), Belle Glade (Everglades Experimental Station), and Gainesville (Horticultural Unit and Agronomy experimental plots). Bacterial diseases were absent in all locations, except for Belle Glade, where BLBSR severity in specific plots ranged from slight to severe. In these plots, samples were collected from symptomless plants. Only naturally ocurring resident microorganisms which have become adapted for survival and growth on the phylloplane of corn plants were studied. Introduced organisms are less likely to survive as long and, therefore, whould need to be reapplied more frequently to the plants (Blakeman & Fokkema 1982).

Ten to twenty plants were sampled per hectare. Sampling and isolation of bacteria was done according to Lopes & Stall (1990).

Individual colonies of the two to four most prevalent bacteria, based on colony appearance after growth for 2 days at 30°C, were streaked into TSA plates to determine purity and, if pure, were stored in sterile tap water at room temperature (22-24°C). In addition, dilution plates which yielded 20-100 colonies per plate were sprayed for 1 sec with about $5x10^6$ cfu/ml of *P. avenae* strain 83-1 grown on NA for 24 hr at 30°C and suspended in sterile tap water. A chromist spray unit (Gelman Instrument Company, Ann Arbor, Michigan) was used to deliver the the spray. Plates were incubated for 24 hr at 30°C and examined for zones of inhibition. Colonies which formed zones of inhibition were streaked twice on TSA plates for purification and, when pure, the bacteria were stored as previously described. Whenever the same colony type from samples collected in the same area appeared in many plates, only four to six of these bacteria were saved. Because pseudomonads are known to produce a diversity of secondary metabolites, including antibiotics and siderophores (Leisinger & Margraff 1979), sixty cultures of fluorescent bacteria were isolated from sweet corn cultivars Merit and Silver Queen in a 21-day period at the Horticultural Unit.

Antagonism in vitro

A wettable powder formulation of *Bacillus* sp. (ABG 4000) antagonistic in vitro to *P. avenae*, provided by Abbott Laboratories, North Chicago, Il,

was included as a control. Droplets of a turbid suspension of each of 179 bacterial cultures (about 10^8 cfu/ml) were spotted with a 1-mm loop on a TSA plate. Three equidistant droplets were placed on each of three plates, incubated at 30°C for 48 hr and then sprayed with about $5x10^6$ cfu/ml of *P. avenae* strain 83-1 with a chromist sprayer, as previously described. The plates were then reincubated at 30°C for 24 hr. Inhibition was measured as the diameter of the zone of inhibition minus the colony diameter of the antagonist.

To determine if strains of *P. avenae* were equally inhibited by diffusible substances from a given epiphyte, the antagonists known to produce zones of inhibition on TSA to *P. avenae* strain 83-1 were tested against six different cultures of the pathogen. The experiment was set up as a factorial design with three replications. Appl cation of the antagonists and the pathogen to the plates, and data collection were performed as previously described.

Preliminary screening

Sweet corn plants of cultivar Merit were grown in a greenhouse (26-40°C) in 10 cm plastic pots containing steam-sterilized Metromix 300 (Grace Horticultural and Agricultural Products, Cambridge, MA). Eight experiments (20-25 antagonists/ experiment) were carried out in a randomized block design with two replicates. Each treatment ("antagonist") consisted of a row of eight pots with three plants per pot. Each of the 179 potential antagnists was grown in Trypticase soy broth (TSB) at 30°C for 48 hr, centrifuged at 2000 rpm for 10 min, and resuspended in sterile tap water. A suspension of approximately 10^8 cfu/ml was obtained by adjusting turbidity to 0.3 A at 600 nm. About 120 µl of the bacterial suspension was pipeted into whorls of each 10-15 day-old seedlings, when the fourth leaf was one-half to two-thirds unfurled. *Bacillus* sp (ABG 4000) and water were always used as controls. Care was taken to not disturb the plants so liquid would remain in the whorl.

Inoculations with *P. avenae* strain 83.1 also consisted of pipeting 120 µl of a suspension of about $5x10^8$ cfu/ml into the whorl, 30-60 min after application of the antagonist.

Tween 20 at 0.01% was added to the all suspension before inoculation, since it noticeably enhanced revelopment of BLBSR in preliminary experiments.

Disease was assessed 4-6 days after inculation as the percentage of plants with BLBSR symptoms or by using a scale of 0 to 5, with 0 representing no symptoms and 5 representing dying or dead plants.

Mean values for each antagonist in each experiment was transformed to represent the percentage of disease in relation to the water control. Analysis of variance and Duncan's multiple range tests were performed using a SAS program for each data set.

Unless otherwise specified, inoculum concentration and preparation, antagonist concentration and preparation, incubation conditions and disease assessment were done as previously described.

Final screening

The ten best antagonists detected in the preliminary screening were tested further, along with the five best antagonists detected in petri dish tests, for control of BLBSR on seedlings of sweet corn cv. Merit. They also were retested for antagonism in vitro against *P. avenae* on TSA medium as described previously. A randomized block design with three replications of eight pots each, three plants per pot, was used. Tween 20 at 0.01% was added to the suspension of *P. avenae* at $5x10^8$ cfu/ml, immediately before inoculation. Inoculum was applied 12 hr after the application of a suspension of 10^8 cfu/ml of each antagonist.

Streptomycin at 100 ppm and water were applied as controls at the same volume and time as the antagonists.

Identification of the two strains of the fluorescent pseudomonads (F11 and F24) were acording to Gilardi (1985), Misaghi & Grogan (1969), Palleroni (1984), and Sands et al. (1980). Antagonist U-46 was identified using tests proposed by Gibson & Gordon (1974).

Infectivity titration for quantifying the antagonism of F-11

Antagonist F-11 was applied to one of two rows of corn plants in the greenhouse. Each row had seven pots and each pot had three plants. Treatments were serial dilutions of *P. avenae* ranging from 10^8 to 10^2 cfu/ml. Inoculation consisted of placing 120 µl of the pathogen suspensions in the whorls 12 hr after application of 120 µl of F-11. Each level of the pathogen was applied to both rows.

The experimental design was a randomized block design with three replications of 21 plants. Linear regression of disease severity and incidence values vs. log dose of *P. avenae* were used to determine an Ed_{50} value.

Disease control in the field

Ten 100 m rows of sweet corn, cv. Merit were planted on September, 1985 at the Horticultural Unit, University of Florida, Gainesville. Row width was 0.8 m and a planter delivered one or two seeds per 20 cm of row. Soil preparation, fertilization, and herbicide applications were performed according to recommendations for the crop in Florida (Showalter 1984). The plants were sprayed every three or four days after emergence with a carbaryl insecticide for control of leaf-damaging insects.

A factorial experiment consisting of a split plot design with three blocks was used. The two best antaonists based on greenhouse experiments, F-11 and U-46, and water and streptomycin (100 ppm) control were applied to the main plots which consisted of two 30 m rows. the subplots were 10 m long and consisted of the time (0, 12, and 48 hr) between application of antagonist (or controls) and inoculation with *P. avenae* strain 83.1.

Approximately 2 ml of a suspension containing $5x10^7$ cfu/ml of the antagonists and the controls were applied per plant. Antagonists and controls were sprayed in the afternoon with a hand sprayer for about 1 sec per plant, with the jet directed toward the whorl. Sprayers were washed between applications with 0.5% sodium hypochlorite, and thoroughly rinsed with tap water two times. Plants were inoculated at the seven-leaf stage.

About 1 ml of *P. avenae* (10 cfu/ml in 0,1% tween 20) was applied per plant with a hand sprayer directed at the plant whorl. Disease intensity was assessed on leaves that were one-half to two-thirds unfurled at time of inoculation. Thirty to 40 leaves were harvested from the center section of both rows of each treatment in each block. Disease severity was assessed according to a 0-5 scale.

Survival of antagonists in the field

Survival of rifampin-resistant (rif^+) mutants of strains F-11, F-24, and U-46 was determined on corn plants in the field. To obtain rifampin-resistant mutants, parent cultures were grown in 5 ml of TSB for 24 hr at 30°C in test tubes constantly shaken at 100 rpm. Spontaneous mutants were isolated by plating 0.5 ml of the cultures onto TSA containing 100 μg/ml of rifampin (Sigma Chemical Company, St. Louis, MO). Stability of each mutant was tested by growing single colonies in four subcultures in antibiotic-free TSB and then counting the number of cells by dilution plating on TSA with and without 100 μg/ml of rifampin. Stable mutants were those that were identical in colony numbers in plates with and without the antibiotic.

Each rif^+ antagonist was sprayed on each of five randomly selected plants (six-leaf stage) of sweet corn, cv. Merit in the late afternoon in a 20x100 m field. Approximately 1.5 ml of a suspension of 10^8 cfu/ml was applied to the whorls of plants with a Chromist spray unit. Samples of approximately 0.5 ml of fluid were withdrawn from the whorls of the same plants with a graduated disposable pipet at 0, 12, 36, 84, and 204 hr after application of antagonists. Pipets were sealed and transported to the laboratory on ice and processed the same day. Populations of applied and total bacteria were determined through the dilution-plate method in KMB medium containing 100 μg/ml rifampin and on TSA, respectively. Average daily temperature and rainfall data were obtained from a weather station located near the experimental plot.

RESULTS

Antagonism in vitro

Thirty-four of 179 bacteria collected from whorls of corn plants inhibited the growth of *P. avenae* on TSA plates. Among the 34 antagonists, 29 were fluorescent pseudomonads, four were *Bacillus* spp., and one was of an unidentified genus. When zones of hibition produced by the nine selected antagonist against the six strains of *P. avenae* were compared statistically, an interaction was noticed between antagonists and strains. Antagonists which produced the largest inhibition zones were effective against all the cultures of *P. avenae*. Only antagonist U-44 did not inhibit all cultures of *P. avenae*.

Preliminary screening

Eleven antagonists significantly prevented BLBSR as determined by statistical analyses of each experiment (Table 1). All disease indices were not statistically compared among themselves because they were obtained from different experiments which were completed

under different environmental conditions. Nine of the 11 antagonists were fluorescent pseudomonads. None of the cultures tested seemed to synergistically interact with *P. avenae* to increase disease development.

Final screening

Some antagonists that reduced disease in the preliminary screening were not as efficient in the final screening. This difference might be attributed to variation in the conditions under which the experiments were conducted. However, antagonist F-11, identified as *Pseudomonas fluorescens* bv V was the best in all experiments in which it was included. Furthermore, it was effective against four out of six isolates of *P. avenae* (Lopes & Stall 1990). The other two best antagonists, U-46 (*Bacillus* sp.) and F-24 (*P. fluorescens* bv. V) were also selected for further testing

No correlation was found between the ranking of the antagonists according to their ability to control BLBSR on sweet-corn plants and to produce a zone of inhibition against *P. avenae* in vitro (Table 2).

TABLE 1. Control of bacterial leaf blight and stalk rot of corn in the greenhouse by bacterial antagonists (preliminary screening).

Antagonist[1]	Fluorescence[2]	Disease Index[3]
F-4	+	0.57
F-11	+	0.19
F-13	+	0.58
F-24	+	0.64
F-27	+	0.55
F-41	+	0.43
F-43	+	0.79
F-59	+	0.60
U-23	+	0.49
U-49	-	0.64
ABG 4000[4]	-	0.66
CONTROL (water)		1.00

[1] Best antagonists from 179 cultures tested.

[2] Fluorescence on King's medium B (KMB).

[3] Disease index is the amount of disease developed as compared with the water control value of 1.00 (100%). The indexes were not statistically compared because data were taken from different experiments.

[4] ABG 4000 is a *Bacillus* species used as control.

TABLE 2. Relationship between the efficiency of control of bacterial leaf blight and stalk rot on sweet corn cv. Merit in the greenhouse and the diameter of zones of inhibition produced by selected bacteria antagonistic to *Pseudomonas avenae*.

Antagonist	Test in planta		Test in vitro	
	Efficiency[1]	rank	Inhib zone[2]	rank
F-11	68a	1	0.5	15
U-46	55ab	2	6.0	9
F-24	51b	3	1.0	14
STR[3]	45bc	4	-	-
F-41	33cd	5	3.0	11
F-13	33cd	5	3.0	11
F-27	33cd	5	9.0	6
F-43	32cd	8	3.0	11
F-59	28de	9	7.0	8
F-47	23def	10	4.5	10
U-23	21def	11	12.0	4
F-22	19def	12	16.5	2
F-4	13ef	13	9.5	7
W-1	12efg	14	12.0	4
ABG 4000[4]	07g	15	15.0	3
U-132	03g	16	25.0	1
CONTROL (water)	00g	-	-	-

[1] Efficiency based on percentage reduction of disease as compared with the water control.

[2] Inhibition zone (mm) on Trypticase soy agar. (TSA)

[3] STR = 100 ppm streptomycin.

[4] ABG 4000 is a *Bacillus* species used as control.

Results are average of three and two replicates for disease control and inhibition zones, respectively. Numbers followed by the same letter are not significantly different according to Ducan's multiple range test ($p = 0.05$).

Infectivity titrations of quantifying the antagonism of P. fluorescens F-11

Based on the regressions from the infectivity titrations, a high correlation existed between log dose and BLBSR incidence; however, a better correlation was found when disease severity was assessed with plants protected and nonprotected with F-11 (Fig. 1). Disease incidence was reduced by F-11 for all but the highest inoculum doses of *P. avenae*

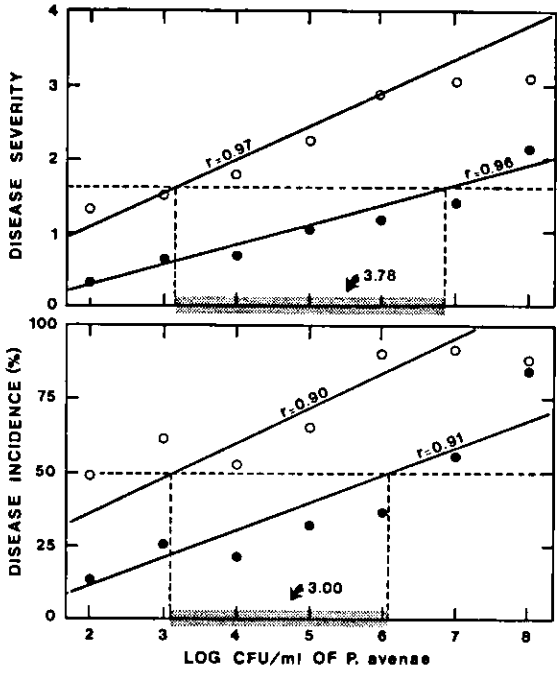

FIG. 1. Infectivity titration of *Pseudomonas avenae* on sweet corn cv. Merit protected (•) and not protected (o) with culture F-11 of *P. fluorescens*. Points represent average data from 54-63 plants in three replicates. Horizontal broken line represents 50 percent severity or incidence of disease. The vertical broken line represents the ED_{50} values. Nonprotection with F-11 had the effect of increasing the inoculum dose of *P. avenae* as much as Log_{10} 3.78 when disease severity was assessed and as much as Log_{10} 3.0 when disease incidence was assessed.

The ED_{50} values for the F-11 protected and nonprotected treatments were very distinct, regardless of the disease assessment method used (Fig. 1). The control factors for the antagonist, which were obtained by dividing the ED_{50} value of the protected treatments, were 1000 and 6025 when disease incidence and severity were assessed, respectively

Disease control in the field

Treatments were affected by the time between application of each treatment and the inoculation with *P. avenae* (Fig. 2). The best control of BLBSR was provided by the antagonist *P. fluorescens* F-11. this treatment was significantly different from the other treatments at all times, except for streptomycin applied at the time of inoculation (time 0). The antagonist *Bacillus* U-46 was not effective regardless of the time of application.

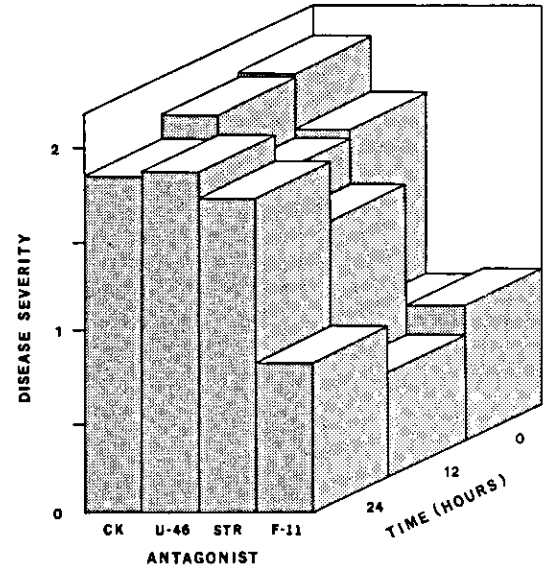

FIG. 2. Control of bacterial leaf blight and stalk rot on sweet corn cv. Merit in the field by application of antagonists *Bacillus* sp. U-46, *Pseudomonas fluorescens* F-11, 100 ppm streptomycin (STR), and water (CK) at 0, 12, and 24 hr before inoculation with *P. avenae*. Each value is an average of the disease severity of three replications.

Survival of antagonists in the field. Population of *Bacillus* sp. U-46 declined rapidly and were not detected at 84 hr after application in corn plant whorls. Conversely, *P. fluorescens* F-11 and F-24 persisted in significant numbers for up to eight days following application, although sampled populations did decline (Fig 3). The selected rifampin-resistant mutants of F-11, F-24, and U-46 were as effective as the wild types in controlling BLBSR in corn plants in the greenhouse.

DISCUSSION

Fluorescent pseudomonads were the most prevalent among the bacterial epiphytes of corn that produced substances antagonistic to *P. avenae* in vitro. The same trend was observed when these epiphytes were tested for their ability to control BLBSR on corn plants in the greenhouse. These results suggest that fluorescent pseudomonas be given special attention in future surveys for biological control agents against BLBSR and other plant diseases. In fact, Leisinger & Margraff (1979) have stated already that pseudomonads represent the major group of nondifferentiating microorganisms producing antibiotics.

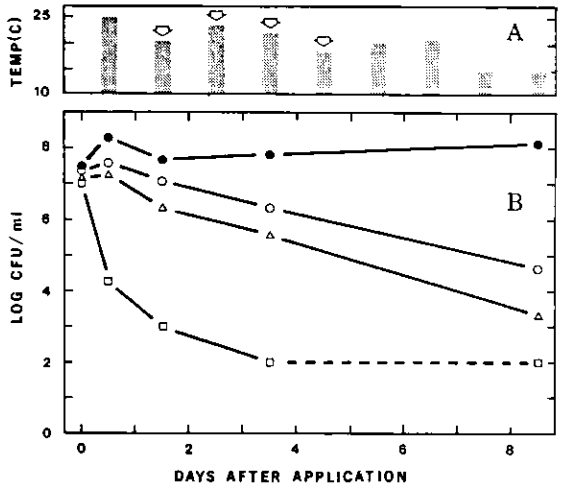

FIG. 3. A) daily temperature and rainfall (arrows) and B) Survival in the field of rifampin-resistant mutants of antagonists *Pseudomonas fluorescens* F-11 (○) and F-24 (△), and *Bacillus* sp. U-46 (□) in the whorl of sweet corn cv. Merit. Points represent averages of five plants, except for total bacteria (●), which represents an average of all plants sampled per day. The broken line represents the limit of detection for the three antagonists.

The study comparing presumptive antibiotic production on TSA plates and ability to control BLBSR on plants in the greenhouse by a selected group of antagonists indicated that there was no positive correlation between microbial antagonism in vitro and effectiveness of disease control in corn plants. This lack of correlation has previously been reported for bacteria colonizing the leaf surface of corn and other plants (Andrews 1985, Lindow 1985, Vidaver 1982). The implication of these results is that good biological control agents might be discarded if the agar plate assay is used as the sole criterion in an initial screening step, as in the scheme proposed by Blakeman (1982).

The sweet-corn cultivars Merit and Silver Queen, which are susceptible and resistant to BLBSR, respectively, harbored similar populations of epiphytic fluorescent pseudomonads which produced diffusible substances antagonistic to *P. avenae* in vitro. The number of antagonists in planta obtained from these two cultivars was also similar. Therefore, surveys for fluorescent pseudomonads antagonistic to *P. avenae* could be made on any corn cultivar, regardless of the level of resistance to BLBSR it carries.

Small differences in disease suppression by biocontrol agents are usually masked by severe disease. Similarly, we found no significant differences when plants were inoculated with a suspension of 10^6 cfu/ml of *P. avenae*, regardless of whether or not the antagonist F-11 was applied. Therefore, infectivity titrations can be a useful technique to detect small effects of applications of microbial antagonists as well as to compare the

effectiveness of different antagonists in controlling disease.

Application of streptomycin at 12 and 24 hr before inoculation did not control the disease in the field. This is in agreement with Citaitis (1979) who reported that antibiotics protect corn plants against *P. avenae* only for a short period. This is probably because the rapid growth of the corn plant causes a constant exposition of new and unprotected tissues to tissues to the pathogen. Conversely, *P. fluorescens* F-11 protected corn from BLBSR for a longer period. Redistribution of antag-onist F-11 was provided by its multiplication and survival in the corn whorl. Multiplication and survival data would be even more striking if it were possible to account, at sampling, for the dilution of populations of F-11 due to rain, mist, dew, and guttation water accumulated in the whorl. Furthermore, only the first two samplings were withdrawn from the same site in the whorl where the antagonist had been applied; subsequent samples were taken from the space between newly formed leaf blades. Therefore, the population of F-11 recovered was probably not constituted of cells originally applied, but of their progenies. It needs to be determined whether populations of the antagonist found in these new tissues at different times after applications are large enough to inhibit the infection by natural levels of incoming propagules of *P. avenae*. This would determine how frequently the antagonist needs to be applied in order to provide significant disease control.

The utilization of rifampin-resistant mutants allowed the detection of a small number of cells of the test species in relation to the total population on the plant.

Strain F-11 of *P. fluorescens* was shown to be a promising biocontrol agent. However, further tests are necessary before their application can be recommended for the control of BLBSR on corn at the commercial level.

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