Optimizing a Bacillus sp. for biological control of sugar beet Cercospora leaf spot
by Douglas Parker Collins

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology
Montana State University
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Abstract:
This study examines methods used to isolate and describe populations of the biocontrol agent (BCA) BacB, a Bacillus subtilis isolate, as well as its spatial and temporal population dynamics, in the field, during a Cercospora beticola epiphytotic. Populations of BacB fit a lognormal distribution and isolation of BacB was generally greater following pulverizing leaves than shaking leaves in buffer. The use of the selective BCA support substrate β-glucan, at up to 1.0% spray solution, did not influence differences in population numbers of a spontaneous rifampicin resistant isolate of BacB (Rif+) over a 14 day spray period. BacB Rif+ , applied as a spore formulation, declined predictably from 10000 CFU/cm^2 on day 0.5, to 100 CFU/cm^2 on day 14 at the three levels of β-glucan tested. BacB Rif+ populations applied with β-glucan did not have significantly fewer percent spores than those applied without β-glucan. The distributions of BacB Rif+ populations were modeled on a leaf scale, with and without beta;-glucan. Interpolating bacterial populations by nearest neighbor, or by kriging, did not lead to significantly different contour maps in most cases. Polyclonal antibodies, specific to BacB, were used to assist visualization of the BCA with fluorescent and scanning electron microscopy, providing spatial distribution information at another scale.

Examining the effects of varying β-glucan concentrations and levels of BacB at application demonstrated a complex interaction between β-glucan, BCA population, and disease severity. In the 1998 field season, disease severity was significantly decreased, as compared to the control, at an application rate of 1 X 10^6 CFU/ml, or higher, with 0 percent β-glucan. In 1999, there was less disease pressure, and no differences between treatments were discerned. Growth chamber experiments indicated that applying the bacteria as vegetative cells instead of spores, or applying the BCA before infection can significantly increase disease control. Laboratory experiments demonstrated the ability to induce germination and vegetative growth of BacB from a spore formulation, without shaking or fermentation equipment. This shows promise for optimizing Bacillus sp. for biological control. In field trials the vegetative cells did not perform better than the spore application, though the potential for β-glucan to increase disease was demonstrated.

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A thesis submitted in partial fulfillment
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of

Master of Science

in

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MONTANA STATE UNIVERSITY–BOZEMAN
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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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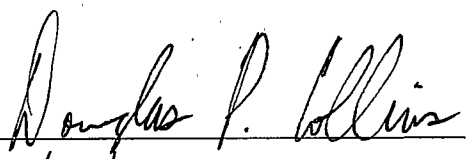
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ABSTRACT

This study examines methods used to isolate and describe populations of the biocontrol agent (BCA) BacB, a *Bacillus subtilis* isolate, as well as its spatial and temporal population dynamics, in the field, during a *Cercospora beticola* epiphytotic. Populations of BacB fit a lognormal distribution and isolation of BacB was generally greater following pulverizing leaves than shaking leaves in buffer. The use of the selective BCA support substrate β-glucan, at up to 1.0% spray solution, did not influence differences in population numbers of a spontaneous rifampicin resistant isolate of BacB (Rif+) over a 14 day spray period. BacB Rif+ , applied as a spore formulation, declined predictably from 10000 CFU/cm^2 on day 0.5, to 100 CFU/cm^2 on day 14 at the three levels of β-glucan tested. BacB Rif+ populations applied with β-glucan did not have significantly fewer percent spores than those applied without β-glucan. The distributions of BacB Rif+ populations were modeled on a leaf scale, with and without β-glucan. Interpolating bacterial populations by nearest neighbor, or by kriging, did not lead to significantly different contour maps in most cases. Polyclonal antibodies, specific to BacB, were used to assist visualization of the BCA with fluorescent and scanning electron microscopy, providing spatial distribution information at another scale.

Examining the effects of varying β-glucan concentrations and levels of BacB at application demonstrated a complex interaction between β-glucan, BCA population, and disease severity. In the 1998 field season, disease severity was significantly decreased, as compared to the control, at an application rate of 1 X 10^6 CFU/ml, or higher, with 0 percent β-glucan. In 1999, there was less disease pressure, and no differences between treatments were discerned. Growth chamber experiments indicated that applying the bacteria as vegetative cells instead of spores, or applying the BCA before infection can significantly increase disease control. Laboratory experiments demonstrated the ability to induce germination and vegetative growth of BacB from a spore formulation, without shaking or fermentation equipment. This shows promise for optimizing *Bacillus* sp. for biological control. In field trials the vegetative cells did not perform better than the spore application, though the potential for β-glucan to increase disease was demonstrated.

LITERATURE REVIEW

Project Background and Statement of Problem

Sugar beets are an important crop in Montana. In 1997 59,900 acres were planted to sugar beets in the state. In terms of total farm sale value, sugar beets accounted for 62,530,000 dollars in 1996, making this the fourth most valuable crop to the state, behind wheat, barley and hay (Stringer and Lund, 1998). Cercospora leaf spot (CLS), caused by the deuteromycete fungus, *Cercospora beticola* (Sacc.), is one of the most important diseases of sugar beets in Minnesota, North Dakota, Eastern Montana, and elsewhere in the world (Shane and Teng, 1992, Pundhir and Mukhopadhyay, 1987, Lamey *et al.*, 1996). Varieties resistant to CLS have not historically had the same yield potential as susceptible varieties. Therefore, susceptible varieties are most often planted, and fungicides are relied upon to control CLS (Shane and Teng, 1992, Lamey *et al.*, 1996). When environmental conditions favor disease development, plants not treated with a fungicide suffer a loss of photosynthetic area, decreased root tonnage, decreased sugar content, and increased impurities (Shane and Teng, 1992). Fungicide use to control *C. beticola* is intense in most areas where the crop is grown. Resistance in *C. beticola* to

benzimidazoles (benomyl) and tolerance to triphenyltin hydroxide (TPTH) has developed in most areas, making the search for new products or methods of control imperative.

Given this need for alternative methods of control, a biological control agent (BCA) screening program was initiated in Dr. Barry Jacobsen's lab. The screen was limited to bacteria in the genus *Bacillus*, due to their ability to form an endospore which makes these organisms relatively easy to commercialize and store. Potential Bacilli biocontrol agents, isolated from the sugar beet phyllosphere, were then screened for their ability to use chitin and β-glucan as substrates. An in-vitro antibiosis screen was used to screen chitinolytic and β-glucanalytic isolates against *C. beticola*. Candidates showing strong antibiosis were tested in a growth chamber assay. Two isolates, identified from this process, BacB (*Bacillus subtilis,* Ehrenberg) and BacJ (*Bacillus mycoides,* Flügge), were tested in the field in Sidney, MT in 1995, 1996, and 1997. Both isolates provided control significantly better than an untreated control, and did rival some fungicides (Kiewnick and Jacobsen, 1998). The current obstacle to commercialization is that the level of control is not acceptable.

This thesis addresses this obstacle through an ecological approach. The behavior of the BCA, BacB, on the leaf surface, through space and time, is described by isolation studies with a spontaneous rifampicin resistant mutant (Rif+). Polyclonal antibodies are also used to visualize the BCA on the leaf surface with fluorescent and scanning electron microscopy. Parallel studies examine the effect of different factors on the BCA's ability to control disease. Experiments specifically address the following: the effect of different levels of β-glucan (Nurture 1080, Nurture Inc., Missoula, MT) nutrient support for the

BCA, on disease control; the effect of applying log phase cells vs. spores; and the effect of timing of BCA application. An increased understanding of the behavior of the BCA in the environment in which it is expected to perform coupled with data reflecting the disease controlling ability of the BCA, when different behaviors are encouraged, will improve the ability to reliably and predictably employ the BCA.

Biololgy and Epidemiology of *Cercospora beticola*

Cercospora beticola conidia are borne on conidiophores in lesions that range from 3 to 5 mm in diameter. Conidia are spread by wind, splashing rain and insects. Upon contact with the leaf surface, conidia germinate to an appressoria, and penetrate the leaf through stomata (Whitney and Duffus, 1991). Necrosis in the plant leaf is induced in part by the many toxins produced by the pathogen, including cercosporin, Baylis and Payne's yellow toxin, and *Cercospora beticola* toxin (CBT) (Steinkamp, *et al.* 1981). Symptoms develop from 5 to 21 days after infection depending on environmental conditions. The pathogen overwinters in infected crop residue as conidia and stromata. Under humid conditions new conidiophores and conidia are formed, completing the disease cycle.

The epidemiology of *C. beticola* is well understood and a strong predictive model exists (Windels *et al.*, 1998). Ideal daytime temperatures for sporulation, germination, and infection are between 25 and 35°C. Night temperatures above 16°C and extended periods of high humidity (90 to 95%) or free moisture on leaves are also necessary for infection. The best economic control of the disease is effected by closely monitoring

fields for disease and environmental conditions for infection conditions, then executing a well timed spray program.

Fungicides currently used to control *C. beticola* are the systemics benomyl and thiophanate-methyl, and the protectants triphenyltin hydroxide (TPTH) and ethylenebisdithiocarbamate (maneb, mancozeb). Resistance in *C. beticola* to benomyl was confirmed in Minnesota in 1981 (Windels *et al.*, 1998). Isolates from eastern MT have been screened for resistance to benomyl and TPTH every year from 1994 to 1998. Benomyl resistant isolates and TPTH tolerant isolates from MT were confirmed in this lab in 1997 and 1998 (unpublished data). TPTH fungicides are not likely to be available in the near future as the US-EPA identified this chemical as a candidate for registration cancellation in 1995 (Jacobsen *et al.*, 1997). Current recommendations for the control of CLS, and resistant management include planting more resistant varieties and rotating the fungicides used during a growing season. In Montana, where benomyl resistance is not yet wide spread, it is recommended that benomyl or thiophanate-methyl be used as a first spray, then followed with applications of TPTH or mancozeb (Jacobsen, 1999). In Minnesota, where benomyl resistance is nearly ubiquitous, TPTH or thiophanate-methyl are recommended as early sprays, and mancozeb for later season applications (Lamey *et al.*, 1996).

Use of Bacilli With and Without Selective Nutrients in Biocontrol in the Phyllosphere

Bacillus sp. isolates have been demonstrated to control early leafspot of peanut (Kokalis-Burelle *et al.*, 1992), yam leaf spot (Michereff *et al.*, 1994), postharvest apple

diseases (Sholberg *et al.*, 1995), gray mold of strawberries (Swadling and Jeffries, 1996), and postbloom fruit drop of citrus (Sonoda et al, 1996). Of these studies, only Kokalis-Burelle *et al.* (1992) experimented with selective nutrient substrates in their applications.

Kokalis-Burelle *et al.* (1992), conducted a study examining the effects of an insoluble chitin formulation and a *Bacillus cereus* isolate on the incidence of early leafspot of peanuts (*Cercospora arachidicola*). The authors found that chitin alone decreased disease incidence as compared to untreated plants. There was an increase in chitinolytic organisms from <1% to >40%, and this was thought to explain the biological control from the substrate alone. The best disease control was observed with a combination of the *B. cereus* isolate plus 1% (w/v) chitin. In epiphytic microbial isolations, the authors found significantly more colony forming units (CFUs) of *B. cereus* from plants treated with chitin than without, 7 days after application.

The commercially available biocontrol agent "Kodiak", a *Bacillus subtilis* strain, was shown to reduce the incidence of postbloom fruit drop of citrus (causal organism *Colletotrichum acutatum*) on oranges (Sonoda *et al.*, 1996). The authors sprayed the product at a rate of 1 g/L or as a *B. subtilis* suspension grown in Luria-Bertani liquid medium mixed 1:1 with deionized water. Interestingly, the authors demonstrated significantly better control with the freshly grown culture than with the product formulation.

Bacillus subtilis has been identified in biocontrol agent screening programs for Curvularia leaf spot of yam by Michereff *et al.* (1994), and for control of postharvest diseases of apple by Sholberg *et al.* (1995). Michereff *et al.* (1994) screened for putative

biological control agents by isolating bacteria from the yam phylloplane then evaluating isolates with respect to inhibition of mycelial growth in vitro, inhibition of conidium germination, reduction of disease severity and persistence of antagonistic action on plants in the greenhouse. Of 162 bacterial isolates examined, a *B. subtilis* isolate that reduced disease by 75% in the greenhouse was deemed the best biocontrol agent for yam leaf spot. Sholberg *et al.* (1995) recovered 95 endophytic bacterial isolates from apples that had been stored at 1^0C for 6-7 months. These isolates were tested for biological control against the postharvest pathogens *Penicillium expansum*, *Botrytis cinerea*, and *Mucor piriformis* directly on apples. Fifteen isolates were effective in reducing decay caused by Penicillium expansum. Three of these were effective against *B. cinerea,* though none were effective against *M. piriformis*. All 15 of the effective biocontrol agents were identified as *Bacillus*, and seven of these were positively identified as *B. subtilis*.

Davis *et al.* (1992) demonstrated the possibility of using a selective substrate in combination with a fungal biological control agent. The authors demonstrated control of flyspeck on apples (caused by *Zygophiala jamaicensis*) and sooty blotch (caused by *Gleodes pomigena*) by the application of *Chaetomium globusum* ascospores with and without a formulation composed of colloidal cellulose. There was no significant difference in the degree of control provided by the BCA with cellulose and the BCA alone. *C. globusum* epiphytic growth was, however, enhanced by cellulose, as determined by SEM observations and dilution plating.

Phyllosphere Microbial Ecology

The following section examines the nature of the leaf surface as a habitat, the role of epiphytic microorganisms in disease control, and looks specifically at the dynamics of microbial communities on the phyllosphere of sugar beets. The concept of an autecological study of a biological control agent is introduced and the potential benefits of an increased understanding of the behavior of a BCA in increasing disease control ability is explained.

The surface of plant leaves is exposed to extreme fluctuations in temperature, relative humidity, and ultra-violet radiation. Saprophytic epiphytes are restricted to areas of the plant that provide a suitable physical and chemical habitat (Blakeman and Fokkema, 1982; Kinkle, et al., 1995). There is, however, evidence that epiphytes can enhance nutrient leakage from the host, and otherwise change the habitat by the production of a layer of extracellular polysaccharides (EPS) (Beattie and Lindow, 1999). Microorganisms are frequently found along veins, in the depressions between the anticlinal walls of epidermal cells, or at the bases of trichomes. Nutrient leakage is enhanced in these areas where there may also be higher relative humidities (Blakeman and Fokkema, 1982). If antibiosis or niche occupation are important for a BCA, then more general colonization of the leaf surface would be desirable.

The residential microbial community on leaf surfaces may effect the epidemiology of foliar pathogens. At least three mechanisms of biological control by communities or individual organisms are often cited. These include competition for niches and nutrients,

antibiosis, and parasitism (Marois and Coleman, 1995; Blakeman and Fokkema, 1982). A fourth mechanism involved in biological control, induced systemic resistance, is poorly understood and relatively sparsely explored. Induced systemic resistance involves the induction of pathogen-related (PR) proteins such as peroxidases, glucanases, or chitinases, generally as a reaction to an incompatible pathogen, or herbivorous injury (VanLoon, 1999; Chitoor *et al.*, 1999). Braun-Kiewnick *et al.* (1998) demonstrated the induction of glucanase, and peroxidase in sugar beets following the application of BacB and BacJ. Weltzien (1991) indicates that application of compost amendments, microbes, and microbial metabolites can induce resistance mechanisms in plants. Similarly, Smith *et al.* (1997) have recorded differences in biological control with different varieties of tomato, indicating the potential importance of host genotype contribution in successful biological control.

The structure of phyllosphere communities changes with leaf age and with the changing environment both daily and through the growing season. In the sugar beet phyllosphere, Thompson *et al.* (1993) found the greatest microbial numbers on senescing primary leaves and these numbers increased over most of the season. Colony forming units (CFUs) on senescent leaves were generally 10 to 100 times greater than populations on mature and immature leaves. Populations on mature leaves ranged from less than that of immature leaves to 100 times greater. Mature and immature leaves maintained a fairly constant microbial population throughout the season.

From the sugar beet phyllosphere, Thompson *et al.* (1993) isolated eight genera of filamentous fungi commonly, and *Cladosporium* and *Alternaria* were the most abundant.

Only three genera of yeast were commonly isolated and *Cryptococcus* and *Sporobolomyces* were the most abundant. *Pseudomonas* was the most commonly isolated bacterial genus and *Erwinia (Pantoea) herbicola* the most common species. It should be noted that the percentage of culturable microorgonisms has been estimated to be very low, usually below 3%, from many different environments (Amann *et al.*, 1995). Though not estimated by Thompson *et al.* (1993), the percent of culturable microorganisms is presumably minor in the sugar beet phyllosphere as well.

The selective and exposed nature of the leaf surface, coupled with the residential microbial community, create the environment in the phyllosphere for the biological control agent. More so than a chemical control strategy, successful biological control requires that researchers be cognizant of this environment and its effect on the BCA. The study of the behavior or dynamics of a population of a single organism in a particular habitat is known as autecology, while the study of a community of organisms and their interactions is referred to as synecology. Both types of studies will have important implications for biological control in the phyllosphere.

Microbial synecological studies in the phyllosphere, such as those conducted by Thompson *et al.* (1993), are less common than phyllosphere autecological studies (Beattie and Lindow, 1994; Beattie and Lindow, 1994b; Ji *et al.*, 1997; Wilson and Lindow, 1994b; Wilson and Lindow, 1995), probably because tracking and isolating one organism is infinitely easier than understanding the interactions of many microorganisms. Nevertheless, autecological studies have elucidated epidemiological information about bacterial pathogens (Wilson and Lindow, 1993; Wilson and Lindow, 1994a), behavioral

patterns of saprophytes (Beattie and Lindow, 1999) as well as behavioral patterns of biocontrol agents (Ji *et al.*, 1997). Few ecological studies have dealt with the behavior of *Bacillus* sp. BCAs (Kokalis-Burelle *et al.*, 1992). Given the desirable qualities of Bacilli for phyllosphere BCAs, coupled with the lack of ecological studies, the need for information about their behavior in the phyllosphere is evident.

The availability of cheap and effective fungicides and their ease of application to plant foliage has discouraged the use of biological control on aerial surfaces (Blakeman and Fokkema, 1982). Furthermore, many screens for biocontrol agents, including the screen for BacB, have relied on an *in-vitro* antibiosis screen. Upper (1991), cautions against this strategy, as this type of screen mimics the search for disease suppressing chemicals. The breadth of any search for biological control agents is immediately reduced to antibiosis, and limits the potential for elucidating BCA with complex control mechanisms. It is not surprising then that few successful phyllosphere BCAs have been identified by this method. Successful biological control in the phyllosphere will require a better understanding of how putative biocontrol agents behave in the environment, and how residential microbial communities can contribute to biological control. Such an understanding could perhaps lead to more elaborate screening methods.

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AUTECOLOGY OF A PHYLLOSPHERE COLONIZING *BACILLUS* SP. USED IN THE BIOLOGICAL CONTROL OF SUGAR BEET CERCOSPORA LEAF SPOT

Introduction

Biological control of foliar plant diseases is a promising method of reducing chemical inputs into agroecosystems. Public concern about pesticide residues in food and water and increasing resistance in fungal pathogens to commonly used chemicals necessitate research on alternative control mechanisms. Bacterial biological control agents have been used in the phyllosphere to control frost injury (Wilson and Lindow, 1994b), fire blight of pear (Wilson and Lindow, 1993); postbloom fruit drop of citrus (Sonoda et al., 1996), and early leafspot of peanut (Kokalis-Burelle et al., 1992) as well as other diseases. However, the number of commercialized biopesticides for use in the phyllosphere remains low. There are many challenges to the development of a biological control agent (BCA). To predictably employ a BCA it is necessary to demonstrate consistent disease control, understand the ecological mechanisms involved with disease control, and model the population dynamics of the BCA, all in the environment in which it is expected to perform.

Autecology is the evaluation of the behavior of the population of a single species within an ecosystem. Evaluating the behavior of a microbial population includes

modeling its numbers in time and space, and estimating its activity (Tate, 1986). Microbial biocontrol agents (BCAs) lend themselves to this type of study for many reasons. A BCA being used in the field is an organism for which its activity of interest, disease control, can easily be monitored. An understanding of the spatial and temporal population dynamics of the BCA will aid in predicting when the BCA will, or will not work. This study will examine the autecology of a *Bacillus subtilis* (Ehrenberg) isolate, BacB, in the sugar beet phyllosphere. Population dynamics were monitored through time and space to describe the BCA and disease interaction. The population was examined at different scales, including whole leaves, individual leaves, and on the microscopic scale.

Most BCAs are first tested in controlled conditions then later tested in field conditions. Given the exposed nature of the leaf surface, the importance of field studies of the BCA, both for monitoring population dynamics, and disease control, cannot be overestimated. Kinkel et al. (1996) demonstrated the necessity for field trials in predicting microbial survival on specific plants. Microcosm studies were of limited use in predicting field population sizes of *Pseudomonas syringae* strains inoculated onto 40 different plant species. Nevertheless, numerous autecological studies have been done in controlled conditions, often attempting to mimic field conditions (Wilson and Lindow, 1993; Beattie and Lindow, 1994; Wilson and Lindow, 1994a)

There is increasing evidence that aggregation of bacterial cells on leaf surfaces is a common behavior for phyllosphere bacteria. Costerton *et al.* (1995) describe how bacterial aggregation in an exopolysaccharide (EPS) matrix, or a bioflim, may concentrate nutrients from dilute sources, result in differential gene expression as

compared to planktonic cells, and provide protection against predators, lytic enzymes, antibiotics, and other inhibitory compounds. More recently, Morris et al. (1997) used epifluorescence microscopy, scanning electron microscopy, and confocal laser scanning microscopy to observe microbial biofilms directly on leaf surfaces. The biofilms contained exopolymeric matrices and diverse morphotypes of microorganisms. By isolating and plating phyllosphere biofilms, the authors found individual biofilms to be composed of a variety of gram-positive bacteria, fluorescent and nonfluorescent gram-negative bacteria, filamentous fungi and yeasts. The importance of aggregation in biological control in the phyllosphere is relatively unexplored.

Wilson and Lindow (1994a) also demonstrated the importance of microbial density. The authors used controlled conditions of high temperature and illumination, and low relative humidity to predict mortality rates for a specific microbe at different application rates. Higher inoculum levels of *P. syringae* resulted in lower bacterial mortality rates and a lower proportional population decline. Similarly, when a high level (10^9 CFU/ml) of dead cells were co-inoculated with a lower level (10^7CFU/ml) of live cells, the live cells maintained significantly higher populations than live cells inoculated without the dead cells. This indicated that bacterial extracellular polysacharides and protection from neighboring cells might increase survival.

Microbial ecology studies in general, and phyllosphere microbial ecology studies especially, must deal with the tremendous variation common in microbial populations on spatial and temporal scales (Kinkel et al, 1995; Kinkel et al., 1997; Beattie and Lindow, 1999). This fact indicates that to realistically model or understand a phyllosphere

microbial population, large numbers of relatively small sample units are necessary. Due to this variation, the probability distribution of epiphytic bacterial populations are often, though not always, better described by the lognormal than by the normal distribution (Hirano et al., 1982). The importance of the probability distribution of BCAs, in relation to their ability to effect disease control is likely to be important. For example, if a higher application density or application with a substrate, can affect distribution of the BCA on the leaf surface, and subsequently affect disease control, this would be important information.

BacB is a *Bacillus subtilis* isolate originating from the sugar beet phyllosphere that has been demonstrated to provide control of Cercospora leaf spot (CLS) of sugar beets (*C. beticola*) in three previous field trials (Kiewnick and Jacobsen, 1998). Critical factors in choosing this *Bacillus* sp. isolate as a BCA were its ability to form an endospore, its ability to use fungal cell wall components chitin and β-glucan as substrates, its ability to inhibit the growth of *C. beticola in vitro*, and its ability to effect disease control in glasshouse studies.

Endospores allow for relatively easy storage and commercialization. However, the BCA was originally applied in the field in the log phase, as washed vegetative cells. (The log phase is a rapid period of growth of a microbial culture, when the population increases logarithmically through time. For Bacilli, "log phase" indicates that the culture is composed primarily of vegetative cells and relatively few endospores, so the two terms are used interchangeably). Following successful disease control, with vegetative cells, BacB was fermented and dehydrated into a spore formulation by Chris Hansen

Biosystems, Inc. Endospores are an important ecological adaptation for Bacilli, allowing them to survive adverse environmental conditions. As an endospore, however, it is not likely that the microorganism is contributing to disease control. When considering the activity of a *Bacillus* sp. population, knowing what component of the population exists as endospores and vegetative cells would logically be critical.

Chitinalytic, β-glucanalytic, and antibiotic activity increase the chances for complex ecological mechanisms to operate in disease control. Fungal cell walls are composed of chitin, so the potential for a chitinalytic microorganism to use a fungal pathogen as a food source exists.

Mechanisms of control by BacB are thought to include antibiosis, induced systemic resistance (ISR), competition, and hyperparasitism. Antibiosis and hyperparasitism have been demonstrated in vitro (data not shown), while ISR has been demonstrated in growth chamber studies (Braun-Kiewnick et al., 1998). Competition for leaf micro-sites has not been demonstrated and its relative importance in disease control is not well understood.

This study examined several aspects of the autecology of BacB in the sugar beet phyllosphere. Most phyllosphere bacterial populations are best described by a lognormal distribution, and whether BacB populations follow this distribution was tested. Two different methods, leaf shaking and leaf pulverizing, were compared for their ability to dislodge viable BacB cells from sugar beet leaves. The ability of the selective nutrient substrate, β-glucan, to encourage growth of vegetative cells from a spore formulation was tested by pasteurizing isolated samples. BacB populations were described through time

following application with and without a selective nutrient substrate, β-glucan, by isolating bacteria from whole leaves. In addition to modeling bacterial populations through time, spatial statistics were used to interpolate and model populations on a leaf scale at different levels of a nutrient source. Two interpolation methods, nearest neighbor and kriging were compared. The spatial relationship between *C. beticola* and BacB applied as a spore formulation or as fresh cells was modeled with a growth chamber study. Finally, polyclonal antibodies were developed, tested for reactivity and specificity, and used to visualize bacterial cells on a smaller scale, with fluorescent and scanning electron microscopy.

Materials and Methods

Lognormal distribution of BacB in the phyllosphere

Most phyllosphere bacteria are best described by a lognormal distribution (Hirano *et al.*, 1982). This indicates that when data from a population is subjected to a frequency distribution analysis, the log transformed data will better fit a normal distribution than the untransformed data will. A population of a spontaneous rifampicin resistant isolate of BacB (Rif+) isolated from the sugar beet phyllosphere 7 days after uniform spray application was analyzed with histograms to determine if distribution of this phyllosphere bacterium is best described by a lognormal distribution.

Comparison of isolation methods

Isolation from leaf pieces. BacB Rif+ was applied to leaves (cultivar HH88) in the growth chamber in the log phase (vegetative cells) and as a spore application at 10^8 CFU/ml. Treatments were applied using about 10 ml per plant, allowing for runoff. Vegetative cells were prepared by inoculating 50 ml of TSA + 50ppm rifampicin with a loop of BacB Rif+ from an agar plate, and growing on a shaker (Lab-Line® Orbit environ-Shaker; 50rpms) for 24h at 28 °C. The culture was centrifuged for 12 minutes at 10,000 rpm, the broth was decanted, and the pellet was resuspended in 30 ml PBS (6.8g KH_2PO_4, 1.16g NaOH, 8.5g NaCl in 1 L distilled water). The concentration was adjusted to 2 X 10^8 CFU/ml using a standard curve and spectrophotometer at 600 nm. This was mixed with an equal amount of 0.2% methyl cellulose for a final bacterial concentration of 10^8 CFU/ml in 0.1% methyl cellulose. The spore treatment was prepared by adding the appropriate amount of spore formulation to PBS with 0.1% methyl cellulose for a final concentration of 10^8 CFU/ml. Bacterial concentrations were confirmed by spiral plating onto 5% TSA, incubating for 24 – 48 hours and counting.

Two leaves were picked from each treatment 3 days after application. Another isolation was done from the log phase treatment 9 days after application. Twelve 0.28cm^2 leaf sections were taken from each leaf. Alternating leaf sections were either shaken for 30 min in 5 ml sterile PBS, or pulverized at low speed with a hand held grinder (Omni International *TH*) for 5 seconds or until leaf tissue was completely macerated. Day 3 samples were spiral plated and a 0.5ml aliquot was drop plated. Day 9 treatments were drop plated using 1ml aliquots. Samples were plated onto a 5% TSA

plate amended with 100 ppm rifampicin and 50 ppm cyclohexamide. Drop plating was done by mixing the aliquot with 3ml of hot (50°C) 5% TSA + rifampicin and cyclohexamide, vortexing, and pouring onto an amended 5% TSA plate. Plates were counted after 3 days incubation at 28°C. Data were adjusted to $\log_{10}(CFU/cm^2)$ and analyzed with SAS proc ANOVA (SAS/STAT™, 1988).

Isolation from whole leaves. This experiment was done with cultivar KW2262, grown in the field at the Eastern Agricultural Research Center in Sidney, MT, in a Savage silt loam soil. Approximately six plants were sprayed with a hand held sprayer at a concentration of $1X10^8$ CFU/ml with a spore formulation of the Rif+ isolate with 0 or 1% β-glucan (Nurture 1080, Nurture, Inc., Missoula, MT) in tap water. Treatments were applied at a rate of about 140L per hectare, simulating the rate used to apply fungicides and biocontrol agents in the field with a CO_2 sprayer, and this does not allow for runoff. Treatments were applied on 18 August, 1999. Eight leaf samples were collected from plants from each treatment on 1 September, 1999, 14 days after application. Twenty leaf disks (13.5 cm^2) were taken from each leaf, and disks were alternatively placed in 50 ml of PBS for shaking or pulverizing, for a total of 10 disks/leaf. The sixteen samples for shaking (8 at 1% β-glucan, and 8 at 0% β-glucan) were shaken for 30min. The 16 samples for pulverizing were blended (kitchen type blender) for 30 seconds, or until completely macerated. An aliquot of each sample was pasteurized to determine percent spores (see below). Samples were plated by drop plating 1 ml of the sample, then incubated and the data analyzed as described above.

Percent spores

Percent spore data were collected in an attempt to monitor the percent of the population existing as vegetative cells or as endospores. The analysis was done on field samples from 1998 and 1999. Percent spores was determined for day 4 samples on 24 August, 1998. A 5ml aliquot was pasteurized for 20 min. at 80°C, then spiral plated. Percent spores was determined for samples from 21 July, 18 August, and 1 September, 1999, by pasteurizing a 2ml aliquot then drop plating. Data from 1998 (3 treatments) were analyzed with SAS proc ANOVA and data from 1999 (two treatments) were analyzed with SAS proc TTEST. Percent spores was determined by:

$$(CFU_{\text{after pasteurization}} / CFU_{\text{before pasteurization}}) * 100$$

BCA population through time with different nutrient concentrations

Field isolation experiment 1998. This experiment was conducted with the sugar beet cultivar HH88 at the Eastern Agriculture Research Center in Sidney, MT. Five treatments (Table 2-1) were applied in a randomized complete block design with four replications. A spontaneous rifampicin resistant mutant (Rif+) of BacB was applied at a concentration of $1X10^8$CFU/ml with three levels of β-glucan, and the wild type BacB isolate was applied with only 1% β-glucan. Treatments were applied with a backpack CO_2 sprayer (R&D Sprayers) at a rate of 140L per hectare, 4 times, at 14 day intervals during the season, beginning 9-July.

Table 2-1. Five Treatments in Isolation Study.

BacB @ 10^8 CFU/ml	Concentration (%) β-glucan
Wild Type	1.0
Rif+	0.0
Rif+	0.5
Rif+	1.0
none	0.0

Isolations of BacB Rif+ were made by picking 4 mature leaves from each plot of each treatment with the Rif+ isolate at 0.5, 4, 7,and 14 days after application. Leaves were processed separately for a total of 16 replications for each of the treatments. Approximately 20 leaf disks (5.067cm^2 per disk) were removed from each leaf, bulked, and shaken in 50 ml sterile PBS for 30min. A 35ml aliquot of the samples from days 7 and 14 was centrifuged at 12,000 rpm for 10 min, then resuspended in 5ml, for a 7.14 fold concentration. Samples were plated with a spiral plater (Spiral Systems, Inc. Model C) on 5% TSA with 100ppm rifampicin and 50ppm cyclohexamide, incubated for 24-48 hrs at 28°C, then counted. Two plates were done for each sample, and their results averaged. Samples with less than 10 CFU/plate were considered to be below the accurate detection level, and treated as missing data. Data were adjusted to $\log_{10}$(CFU/cm^2) and analyzed with SAS proc ANOVA. This experiment was repeated 3 times during the season.

Disease ratings were done in an attempt to monitor the Cercospora leaf spot epidemic. Ratings were done 6-Aug and 26-Aug, 1998 by rating 100 leaves per plot using the KWS-scale (Shane and Teng, 1992). Data were collected with a data logger and the percent disease severity was calculated. Data was analyzed with SAS proc ANOVA and means separated by LSD.

Spatial distribution of biocontrol agent

Field isolation 1999. A mini-plot was set up adjacent to the larger field experiments at the Eastern Agriculture Research Station at Sidney, MT. Approximately six plants (cultivar KW2262) were sprayed with a hand held sprayer at a concentration of $1X10^8$ CFU/ml with the Rif+ isolate at 0 and 1% β-glucan. Treatments were applied at a rate of about 140L per hectare. Treatments were applied on 8 July, 21 July, 4 August, and 18 August. One leaf was taken from each treatment on 21 July (13 days after application) and 18 August (14 days after application).

A plastic grid (pixel size 0.6cm X 0.6cm) was overlaid on each leaf, and digital pictures were taken to record the relationship of physical features on the leaf to each of the pixels made by the grid. A cork borer (0.28 cm^2) was used to punch a leaf disk of a constant area from each pixel. Leaf discs were suspended in 5ml PBS buffer and pulverized with a hand held grinder (Omni International *TH*), for 5 seconds, or until leaf tissue was pulverized. The grinder was sterilized between samples by rinsing well in 70% ethyl alcohol, followed by 2 rinses in sterile water. A negative control, used to ensure the effectiveness of this procedure, involved placing the grinder into PBS without any leaf material, grinding as if there was a sample, and then plating the PBS. No bacteria grew from the negative controls. The entire sample was drop plated, unless percent spores was to be determined. If percent spores was to be determined, a 2ml aliquot was pasteurized then plated (see above), and a 2 ml aliquot was plated without pasteurizing. Samples were drop plated by mixing the sample with 13 ml of hot agar (5% TSA + 100 ppm rifampicin and 50 ppm cyclohexamide, kept liquid in a 50 °C water

bath), vortexing and pouring into a sterile petri dish. Samples were incubated for 48-72h at 28°C and counted. Data were adjusted to $\log_{10}(CFU/cm^2)$

BCA in relation to *C. beticola* in growth chamber. BacB RIF+ was applied to plants (cultivar HH88) growing in a growth chamber as either vegetative cells from an overnight culture or in the spore formulation. These treatments were prepared and applied exactly as described above (see Isolation from leaf pieces) and both treatments adjusted to $1X10^8$ CFU/ml.

Cercospora beticola inoculum was prepared by growing a pure culture on V-8 agar for approximately 1 month at 24°C with 12 hours of light per day. Spores were harvested by depositing 10 ml of sterile 0.1% methyl cellulose on a plate, then scraping well with a rubber policeman. The spore and mycelium mix was moved to a sterile container and macerated with a hand held grinder (Omni International *TH*). Spore concentration was checked with a hemacytometer, and diluted to $1 X 10^4$ spores/ml.

Treatments and inoculum were applied at a volume of 10 ml per plant. The inoculum was applied immediately after the BCA treatment. Plants were placed in a mist tent inside a growth chamber (12 h at 28 °C and 12 h at 26 °C). Plants were exposed to 100% relative humidity by misting for 48 h continuously with two ultrasonic humidifiers, then at one hour intervals during the night for an additional 48 hours.

Leaves were picked 14 days after application. Grids were overlaid as described above, but before processing for bacterial density, a percent disease severity (estimation of the percent of the pixel damaged by *C. beticola* or its toxins) was recorded inside each pixel. Samples were then processed for BacB Rif+ $\log_{10}$ CFU/cm^2 as described above.

Statistical analysis and mapping. To collect data, for modeling the spatial distribution of the Rif+ populations, a grid was overlaid on leaves and approximately every other leaf piece was processed in a regular checkerboard pattern. The density of the Rif+ isolate (CFU/cm^2), was determined for each leaf piece sampled as described above. Each leaf piece, or pixel, was also given a location in space (x,y). Spatial statistics allows the researcher to gather density data (z) from only a portion of the pixels on the grid, and use relationships between the values of points and their position in space to interpolate values for pixels where no original data was gathered. The goal of such an analysis is to produce a map that provides the best representation of a set of density values in space.

When analyzing the spatial distribution of populations, the scale at which data are sampled can profoundly influence the final analysis. For this study, a scale, or size of sampling unit, was chosen that would allow for relationships between leaf veins and bacterial populations to be deciphered. As the size of a sampling unit is reduced, the ability to sample from every point in the area under consideration is compromised. If original data are collected from a surface in a well-spaced manner, then values for areas where no original data were collected can be interpolated. Most biological populations are spatially autocorrelated, meaning the realization of a variable at one location is dependent on its realization at nearby locations (Simard et al., 1992). Geostatistics takes advantage of this spatial autocorrelation when interpolating values. Nearby data points are given more weight in determining the value of an unknown point than points further away.

The statistical steps involved with moving from raw data on a coordinate system to a finished contour map are gridding or interpolating, and mapping. The gridding method employed will determine how the final map represents the data. Gridding is the process of using original observations in an XYZ data file to generate calculated data points on a regularly spaced grid. Two common, but very different gridding methods are kriging and nearest neighbor.

A gridding method should be chosen that best represents the data. The primary purpose of this spatial analysis was to look for bias between levels of the BCA and physical leaf features, position on the leaf, and disease incidence. A secondary objective was to probe the possibilities for mapping and modeling a phyllosphere bacterial population on a leaf scale. Therefore, a rigorous comparison of modeling methods and spatial statistical analysis was not attempted. Instead, the raw data are presented, and then two interpolations of this data, one done by kriging, and the other by nearest neighbor analysis will be shown. One of these representations is then overlaid on a leaf picture for analysis. From the field samples, I was particularly interested in deciphering bias from the physical features of the leaf, distance from the leaf petiole, or differences due to the level of nutrient present. From the growth chamber experiment, I was primarily interested in relationships between the level of the BCA and disease severity, and differences in this relationship due to applying the BCA as spores or as vegetative cells.

Kriging is a powerful method for predicting values of a spatially continuous variable. Bailey and Gatrell (1995) provide an exhaustive discussion of kriging, and

spatial statistics in general, though the various software manuals are also extremely helpful for the layperson (GS^{+TM} ; Surfer™ – see Keckler, 1997). Kriging provides an optimal interpolation estimate for a given coordinate location, as well as providing a variance estimate for the interpolation value. To interpolate unknown values, kriging uses knowledge about the underlying spatial relationships in a data set. This knowledge is provided by semivariograms. Semivariance analysis of the spatial distribution data was done with GS^{+TM} (Gamma Design Software, MI). GS^{+TM} defines semivariance as:

$$\gamma(h) = [1/2N(h)] \sum[z_i - z_{i+h}]^2$$

where

$\gamma(h)$ = Semivariance for interval distance class h;
z_i = measured sample value at point I;
z_{i+h} = measured sample value at point I+h; and
N(h) = total mumber of sample couples for the lag interval h.

The software evaluates semivariance by calculating $\gamma(h)$ for all possible pairs of points in the data set and assigns each pair to an interval class h. The semivariogram (or variogram) is a graph of all h's (lag intervals) vs. all semivariances for each interval class in the analysis. The integrity of a variogram is judged by an r^2 statistic, and, more importantly, by the reduced sums of squares (RSS). Developing a semivariogram is an iterative process and changing parameters of the model, such as lag distance, will effect the integrity of the model. Another important aspect of the semivariogram is the model chosen to fit the data. Five models are available: spherical, exponential, linear, linear to sill, and gaussian. GS^{+TM} chooses the model by default, on the bases of which model provides the lowest RSS value for the chosen lag distance.

Kriging is a robust gridding method because it looks for trends in the data. For example, a "ridge" may connect two "peaks" adjacent to each other. Another gridding method, such as nearest neighbor, might just show isolated peaks. A pitfall of kriging is the assumption that the variance and the mean are homogeneous throughout the space being modeled, and are independent of their absolute location within that space (Bailey and Gatrell, 1995). The proclivity for phyllosphere bacteria to aggregate around veins, cell wall junctions, and stomates, indicates that their distribution on the leaf surface may indeed be biased.

The nearest neighbor gridding method assigns the value of the nearest datum point to each grid node. This method is appropriate for data that is on a nearly complete grid with only a few missing values for filling in the holes in the data (Keckler, 1997). This situation describes the data sets being analyzed here, so this method was chosen for a comparison to the kriging grids.

Contour maps of BCA distribution were prepared with Surfer™ (Golden Software Incorporated, CO) from nearest neighbor grids or with kriging grids. Nearest neighbor grids were prepared with Surfer™ and kriging grids and semivariograms were prepared with GS^{+}™.

A post map shows the exact location where a datum was recorded. A classed post map shows the density of some parameter at the location where it was recorded. Post maps and classed post maps can be overlaid on a contour map to demonstrate some relationship. Classed post maps of *C. beticola* disease severity were overlaid on the contour maps of BacB density from the growth chamber experiment. Classed post maps

were also used to overlay vegetative cell density on the contour map of total cell density, where this data was available. Contour maps were overlaid on digital pictures of leaves and made partially transparent with Adobe® Photoshop® (Adobe Systems Inc., WA).

Immunocytochemical localization of BCA on leaf surface

Preparation of antigen. The bacterial antigen was prepared as described by McDermitt (1997). An overnight liquid culture of BacB, grown in TSB, was washed 3 times in 0.85% (wt/vol) sterile saline solution that had been filtered through a 0.22μm-pore-size filter. The washed cells were adjusted to 10^9 CFU/ml spectrophotometrically, placed in a stoppered test tube, heat killed by immersing in an 80^0C water bath and venting with a syringe for 10 min, then preserved with thimeresol (merthiolate) to a final concentration of 1:10,000. This solution was mixed 1:1 with Freund's complete adjuvant. Two white New Zealand rabbits were immunized with the antigen. A second injection was given 2 weeks later, and a booster was given 4 weeks after the initial injection. Twenty-five ml of serum (about 50ml blood) was harvested two weeks after the booster.

Testing of polyclonal antibodies. The polyclonal antibody serum was tested for reactivity against BacB and specificity with dot blots and by immunofluorscent microscopy.

Lazarovitis (1990) describes a method for the dot immunobinding assay that is specific for bacteria. This bacterial specific method follows closely to that described by Anonymous (1998). Cultures of BacB and all other isolates to be tested were grown

overnight on tryptic soy agar. A loopful of bacteria was suspended in PBST buffer (11.5g Na_2HPO_4 (80mM), 2.96g NaH_2PO_4 (20mM), 5.84g NaCL (100mM) diluted to 1000ml + 1ml Tween-20) and dilutions were made from this solution. Two µl of sample were blotted onto a piece of wetted and dried nitrocellulose paper. The dot was allowed to air dry, then the membrane was washed 2 times for 5 minutes in PBST, blocked for 1 hour in PBST + 3% dry milk powder, washed 2 times, incubated with the primary antibody (concentrations ranging from 1:1000 – 1:10000) for one hour, washed 2 times in PBST, then incubated with goat anti-rabbit horseradish peroxidase (HRP) tagged secondary antibody for one hour, then washed 2 times in PBST. Probing of the membrane was preformed by immersing the blot in SuperSignal® ULTRA Chemiluminescent Substrate (PIERCE Chemical Company) for 5 min. The blot was wrapped in plastic wrap, and used to expose autoradiographic film for 1-10 seconds. The film was developed and fixed, and the results read.

Immunofluorescence was also used to determine reactivity and specificity for the antisera. De Boer (1990) describes a method specifically designed for use with secondary tagged antibodies. Bacterial strains to be tested were grown overnight on TSA. A loopful was washed and suspended in distilled water, and dilutions were made from this solution. De Boer (1990) recommends using taxoplosmosis slides, but good success was obtained by marking a regular microscope slide with a grease pencil, after it had been warmed on a hot plate. Two parallel, vertical, lines were drawn, approximately 0.5 cm apart, to allow for good rinsing between incubations with the primary and secondary antibodies. Ten µl of the cell suspensions, and dilutions, were applied

between the grease pencil marks and allowed to air-dry on a hot plate. The slides were then fixed by flaming. Ten µl of primary antibody solution (1:40 mixed in 0.01 M PBS, pH 7.2 plus 0.2% milk powder blocker) was applied to each bacteria spot, and the entire slide incubated in a humidity chamber at 28°C for 30-60min. Slides were then rinsed with a gentle stream of distilled water for 30 seconds and allowed to air-dry on a hot plate. Ten µl of either fluoresceine (Fisher Scientific, FITC OB4010-02) or rhodomine (Fisher Scientific, TRITC, OB4010-03) tagged goat-anti rabbit, secondary antibody solution (1:20 dilution in PBS plus blocker) was applied to the bacterial cells and incubated in a dark humidity chamber for 30-60 min at 28°C. The slides were then rinsed and allowed to dry on the hot plate. Slides were mounted with fluorescent antibody mounting fluid (Difco Laboratories 3340-56), and viewed with a Leica Das Mikroskop Leitz DMR(Leica Microsystems, Nussloch, Germany), fluorescent microscope.

Fluorescent microscopy of leaf discs. Greenhouse and field inoculated leaf material was used for in-situ observation of BacB. Best results were obtained with FITC tagged secondary antibodies and blocking with a gelatin conjugated with RITC (Bohlool and Schmidt, 1968). Leaf disks were removed from leaves with a cork borer (0.28 cm^2). Disks were placed upside down on a 50µl drop of RITC gel for 15 min. Samples were moved directly to a 50µl drop of primary antibody solution and incubated in the dark for 1 hour at 28°C. Disks were then washed 3-4 times for 5 minutes each on 50 µl drops of buffer (0.01 M PBS, pH 7.2). Disks were moved to 50µl drops of secondary antibody solution at 4°C overnight. Disks were rinsed 2 times on buffer, then the underside dried with a cloth. Samples were prepared for viewing by mounting onto a slide with double

sided stick tape, immersed in fluorescent antibody mounting fluid, and covered with a cover slip. Samples were viewed with a Leica Das Mikroskop Leitz DMR, fluorescent microscope.

Spatial distribution of BacB wild type on microscopic scale. An experiment was done to evaluate the potential for gathering ecological data from field applied wild type BacB with immuno-fluorescence. This analysis provides data at yet another scale, though no attempt was made to document occurrence of cells in relation to physical features on the leaf surface. Leaves were gathered from an experiment in the field in 1999, inoculated with the wild type BacB (Table 3-3). The treatments sampled were vegetative + 0% β-glucan, vegetative +1% β-glucan and control plants with no application. One leaf was taken from each treatment, and six leaf pieces ($0.28cm^2$) were taken from each leaf. The pieces were taken in a line across the leaf about half way from the petiole. Leaf pieces were analyzed at 400X magnification, which allowed for 25 fields of view on each leaf piece, and a border of about 1 field of view on the edge of each leaf piece. Fluorescing bacteria were estimated in each field of view, and grid maps made with nearest neighbor interpolation.

Immuno-scanning electron microscopy of pure cultures and leaf discs. BacB was prepared for viewing by scanning electron microscopy by drying cells onto a silicon chip, and by taking treated leaf disks as described above. This method was adapted from Anonymous (1999) and Erlandesen et al. (1996). For viewing pure cultures on a silicon chip, bacteria were suspended in 1 ml sterile deionized water. The suspension was

centrifuged at 12,000 rpm for 1min, decanted, and resuspended for a final concentration of 10^8-10^9 CFU/ml. Twenty µl of this solution were blotted onto a stub, then dried on a hot pad. The cells were blocked in a humidity chamber for 20-30 min at 25°C by blotting 20µl of 0.1% BSA in Tris buffer onto the dried cells. The blocker was rinsed off with distilled water, and the stub dried on a hot plate. The blocked cells were incubated in a humidity chamber for 30-45 min. with the primary antibody solution (1:20 in Tris buffer; 20mM Tris, 225mM NaCl, pH 8.2). The cells were than rinsed well with distilled water and dried on a hot plate. The cells were than incubated with a 20µl drop of goat anti-rabbit 30nm gold conjugate (Ted Pella Inc. catalog #15729-4) solution (1:10 in Tris buffer) for 30-45 minutes at 28°C, or overnight at 4°C. The stubs were rinsed first in Tris buffer with elevated NaCl (2.5 M), followed by rinses in distilled water. After drying a thin layer of carbon was evaporated onto the stubs and the samples were viewed with a JEOL JSM 6100 Scanning Microscope. Controls included pre-immune primary serum, no secondary antibody, and reaction with a *Bacillus mycoides* isolate.

Leaf disks were prepared by inoculating plants with an overnight culture that was washed and resuspended in sterile distilled water to a final concentration of 10^9 CFU/ml. Incubation times, temperatures, and solutions used were the same as described above. More volume was used, as it was necessary to float the leaf disks on the drop. Leaf disks were punched out from leaves with a cork borer ($0.28cm^2$), and placed upside down on a 50µl drop Tris buffer + 0.1% BSA. Samples were incubated on a 50µl drop of primary antibody solution, washed 3-4 times for 5 minutes each on 50 µl drops of Tris buffer, then incubated on 50µl drops of secondary antibody solution. Disks were rinsed once on

Tris buffer with elevated NaCl, than 5 times for 5 minutes each on distilled water. The disks were allowed to air dry, then mounted on SEM stubs, dehydrated in a vacuum a thin layer of carbon was evaporated onto the sample.

Results

Lognormal distribution of BCA

Most bacterial populations are best described by a lognormal distribution. This indicates that the log transformation of the actual data will fit a normal curve better than the original data. Histograms are a useful way of viewing if a population is distributed normally. An untransformed population of BacB (Figure 2-1) is highly skewed while the same population log transformed (Figure 2-2) better fits a normal distribution.

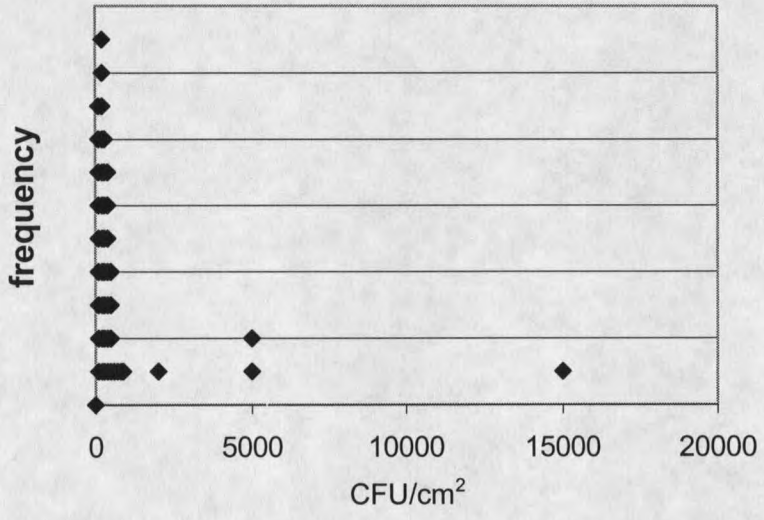


Figure 2-1. Distribution of Untransformed BacB Population. The distribution of a population of BacB Rif+ isolated from the field seven days after application is shown without log transformation.

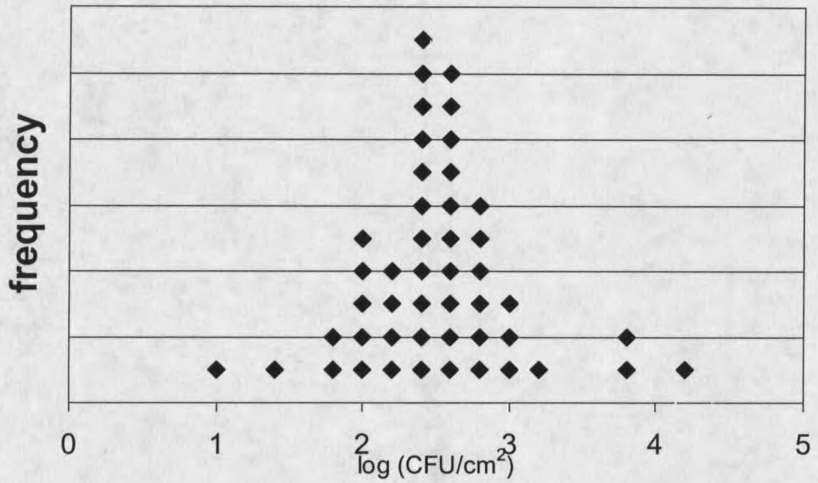


Figure 2-2. Distribution of Log-Transformed BacB Population. The distribution of a population of BacB Rif+ isolated from the field seven days after application is shown with log transformation.

Isolation methods: Shaking versus pulverizing

Isolation from whole leaves. BacB Rif+ was applied to leaves in the field with 1% or 0% β-glucan, then re-isolated 14 days later. For the isolation, leaf disks were taken from the leaf and alternatively pooled in jars for two different treatments. Ten leaf disks from each leaf were given the shaking treatment, while 10 leaf disks from the same leaf were given the pulverizing treatment. For the 1% β-glucan the shaking treatment yielded greater numbers of bacteria, while for the 0% β-glucan sample the grinding treatment yielded more bacteria. When the experiment was analyzed in one ANOVA, there was no significant nutrient effect ($p>0.75$), no significant treatment effect ($p>0.34$), but there was a significant nutrient by treatment interaction ($p>0.006$). Therefore, the data for the different levels of nutrient were analyzed separately (Table 2-2). The

treatment effect was significant for the 0% ($p>0.01$), but not significant for the 1% ($p>0.16$) levels of β-glucan.

Table 2-2. Effect of Isolation Method on Field Applied Populations of BacB Rif+.

Treatment	Level of β-glucan	N[1]	Mean [$\log_{10}$(CFU/cm^2)]	Standard Error	P>F
Pulverized	0%	8	2.54	0.08	
Shaken	0%	8	2.03	0.14	0.01
Pulverized	1%	8	2.19	0.08	
Shaken	1%	8	2.46	0.18	0.17

[1] One leaf was used as one sample.

Isolation from leaf pieces: BacB Rif+ was applied to leaves in the growth chamber as either vegetative cells (24 h culture), or a spore formulation. Isolations were made by taking 12 leaf pieces from each leaf and alternatively shaking or pulverizing each leaf section. Two leaves were done for the vegetative cell application and the spore application. An isolation done 3 days after application showed a significant leaf effect ($p>0.03$) and some leaf by treatment effect ($p>0.24$), but no treatment effect ($p>0.74$) for the spore application (Table 2-3). For the vegetative cell application (Table 2-3) there was no significant leaf effect ($p>0.51$), no significant leaf by treatment interaction (0.42), but there was a highly significant treatment effect ($p>0.01$). Similarly, 9 days after application, there was a significant treatment effect ($p>0.05$) for the vegetative cell application. (Table 2-4).

Table 2-3. Effect of Isolation Method on Growth Chamber Applied Populations of BacB Rif+ Spore Formulation and Vegetative Cells 3 Days Post Application.

Treatment	Type of Application	N[(1)]	Mean [$\log_{10}$(CFU/cm^2)]	Standard Error	P>F
Pulverized	Spore	12	5.82	0.04	
Shaken	Spore	12	5.84	0.06	0.82
Pulverized	Vegetative	12	2.82	0.11	
Shaken	Vegetative	12	2.35	0.11	0.01

[(1)]Six leaf pieces from two different leaves were used as individual samples.

Table 2-4. Effect of Isolation Method on Growth Chamber Applied Populations of BacB Rif+ Vegetative Cells 9 Days Post Application.

Treatment	Type of Application	N[(1)]	Mean [$\log_{10}$(CFU/cm^2)]	Standard Error	P>F
Pulverized	Vegetative	12	3.01	0.19	
Shaken	Vegetative	12	2.58	0.18	0.05

[(1)]Six leaf pieces from two different leaves were used as individual samples.

Vegetative growth at different nutrient levels

Percent spores was determined for whole leaf samples at 4 and14 days after application, and for leaflet samples 14 days after application (Table 2-5). There were no significant differences in percent spores in any of these trials, except for 18 August, 1999 ($p>0.12$). Except for this sample, percent spores are generally lower in the presence of a nutrient source. On the whole leaf scale there were fewer percent spores on day 14 (taken 1999) then there were on day 4 (taken 1998). However, these samples were taken in different seasons, so no statistical analysis was applied to test a day effect.

Table 2-5. Average Percent Spores at Different Levels of β-glucan.

Days since application (leaves or leaflets)	Level of β-glucan	Mean % spores	N	Standard Error	P>F
4 (leaves) 1998	0	86.8	7	4.89	
	0.5	89.0	7	2.66	0.22
	1	76.9	6	5.68	
14 (leaves) 1 Sept, 1999	0	18.1	8	11.8	
	1	7.9	8	2.2	0.41
14 (leaflets) 21 July, 1999	0	47.2	20	7.1	
	1	41.7	20	7.3	0.59
14 (leaflets) 18 Aug, 1999	0	41.5	25	7.5	
	1	56.0	25	6.34	0.12

High numbers of total cells is a strong indication of high numbers of vegetative cells, as these two parameters are highly correlated for populations of BacB in the presence of β-glucan (Figure 2-3, $r^2= 0.78$, $p>0.0001$) and in the absence of β-glucan (Figure 2-4, $r^2= 0.64$, $p>0.0001$).

There is a higher frequency of populations of dense vegetative growth in the presence of 1% β-glucan than with no supportive nutrient substrate on a leaf scale. This trend is more striking in the 21 July, 1999 sample (Figure 2-5) than in the 18 August, 1999 sample (Figure 2-6).

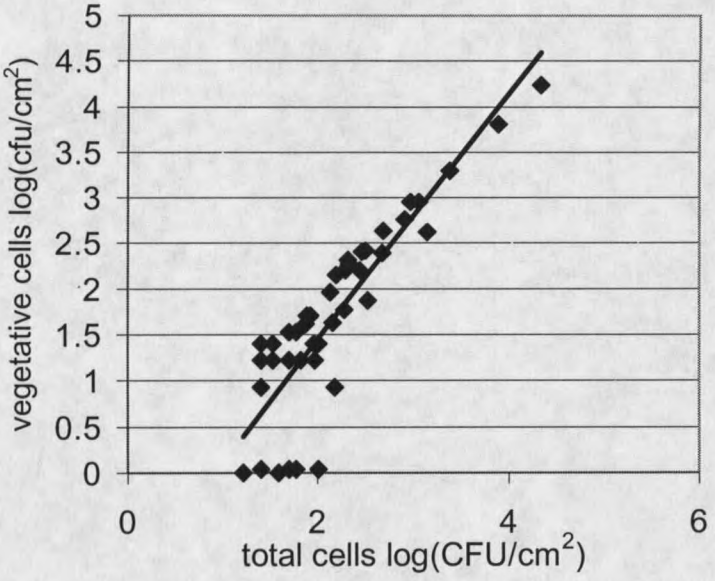


Figure 2-3. Total cells Vs. Vegetative Cells with 1% β-glucan. BacB populations applied with 1% β-glucan isolated from sugar beet leaf pieces on 21 July, 1999 and 18 August 1999, 14 days after application (r^2=0.78, p>0.0001).

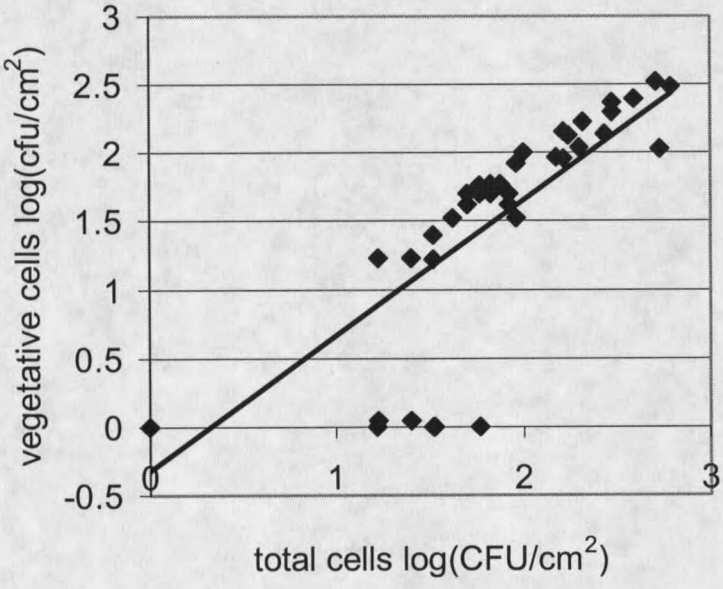


Figure 2-4. Total Cells Vs. Vegetative Cells with 0% β-glucan. BacB populations applied with 0% β-glucan isolated from sugar beet leaf pieces on 21 July, 1999 and 18 August 1999, 14 days after application. (r^2=0.64, p>0.0001).

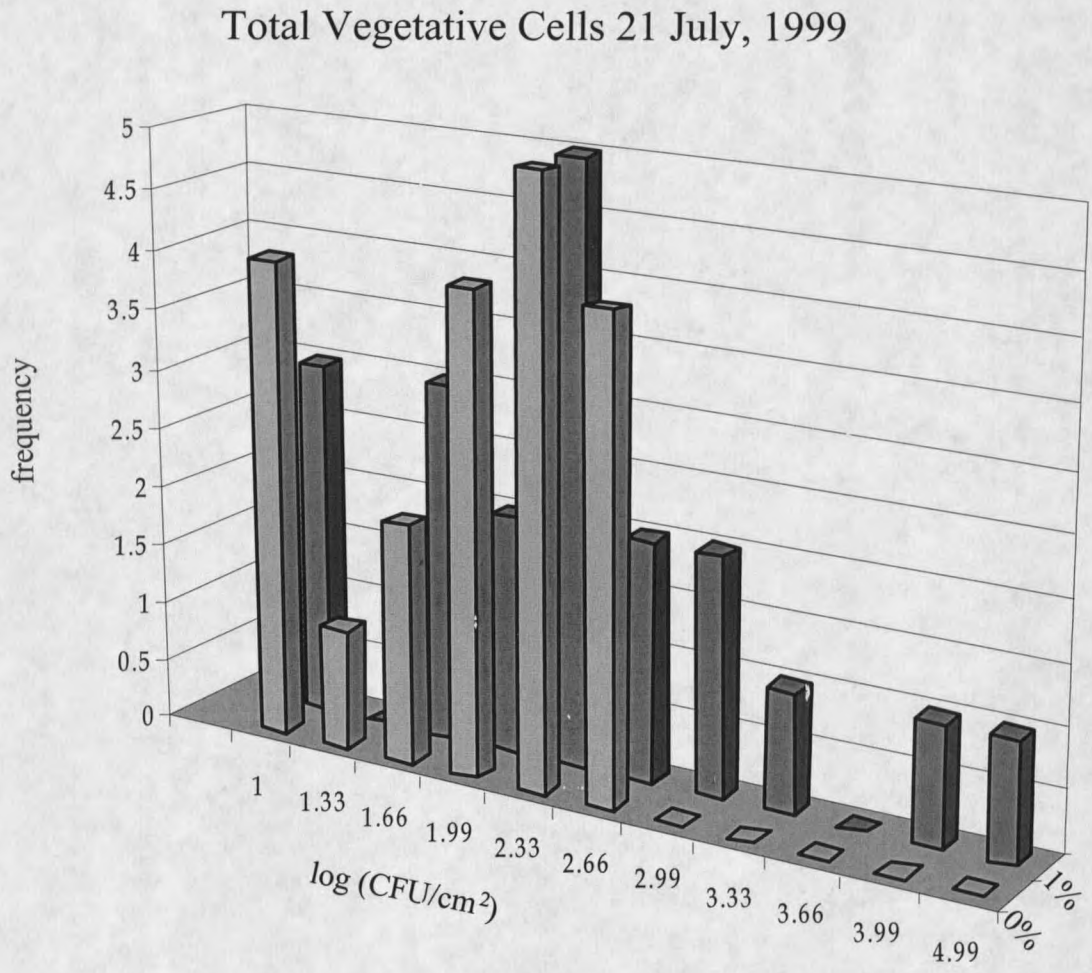


Figure 2-5. Frequency distribution of vegetative cells at two levels of β-glucan for leaf samples taken from the field 21 July, 1999, 14 days after application of BacB Rif+

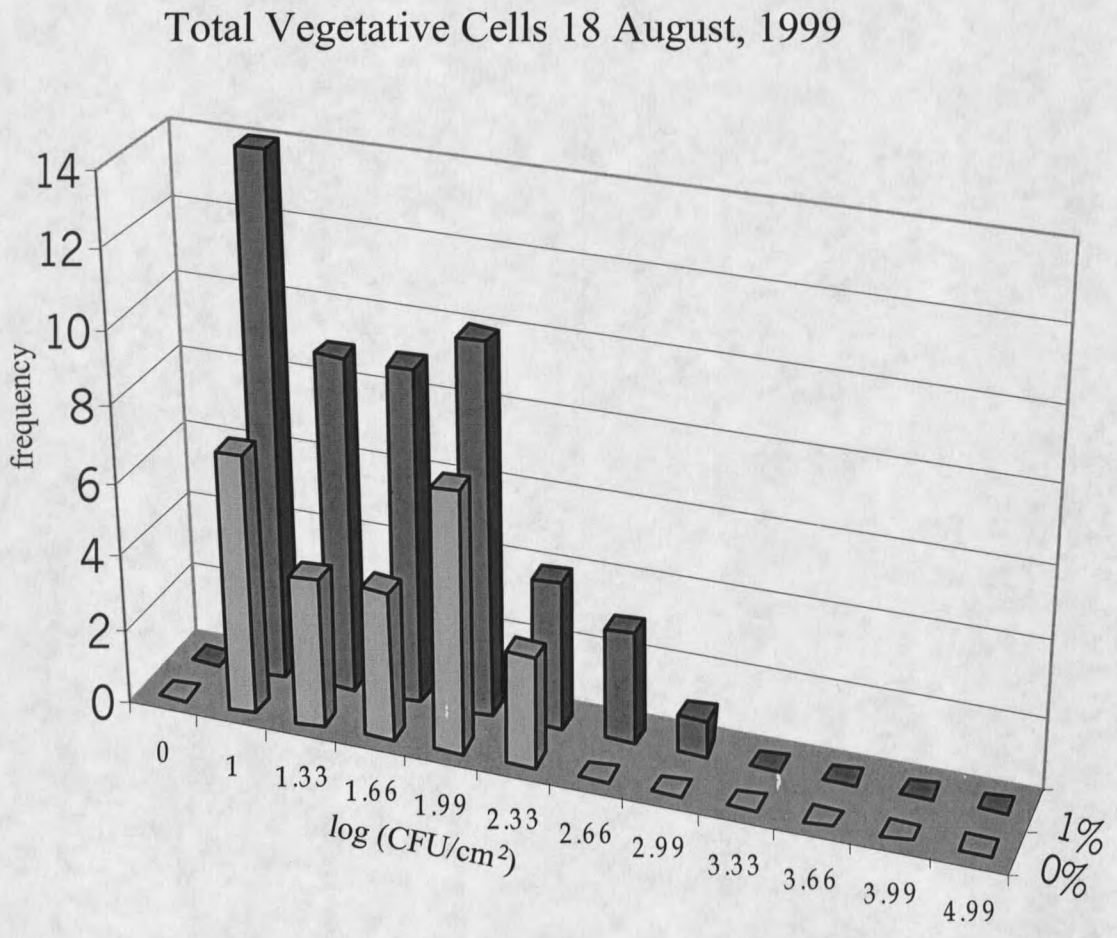


Figure 2-6. Frequency distribution of vegetative cells at two levels of β-glucan for leaf samples taken from the field 21 July, 1999, 14 days after application of BacB Rif+

BCA population through time with different nutrient concentrations

When applied in the field as a spore formulation, total colony forming units (CFUs) of BacB Rif+ declined over a 14 day period in three separate experiments (Figure 2-7). Though there were significant differences in populations isolated at the three different levels of β-glucan on some days, there was no consistent trend of increased survival due to increased nutrient concentration. Regressing bacterial populations against time (Figure 2-8), demonstrates that populations at all three levels of β-glucan declined predictably through time. The 3 regression lines are essentially superimposed, confirming that there is no clear trend of increased survival at higher levels of nutrient. The distribution of different population classes at the three levels of β-glucan on days 4, 7 and 14 shifts from a right skew on day 4 (Figure 2-9), to a more even distribution on day 7 (figure 2-10), to a left skew on day 14 (figure 2-11). This representation of the data shows that populations greater than 2.66 [$\log_{10}$(457 CFU/cm^2)] are more likely to persist at days 7 and 14 with the nutrient source.

Neither the Rif+ BacB isolate or the wild type provided significantly better disease control than the untreated control in the isolation experiment (Table 2-6).

Table 2-6. Average Percent Disease Severity (APDS) of Cercospora Leaf Spot with the Five Treatments in Field Isolation Study 1998.

Treatment	APDS[1]	
Wild Type BacB + 1% β-glucan	9.07	A
BacB Rif+ +0% β-glucan.	10.10	A
BacB Rif+ +0.5% β-glucan	9.35	A
BacB Rif+ + 1% β-glucan	10.31	A
Untreated control	11.51	A

[1]Means followed by the same letter are not significantly different at p>0.05.

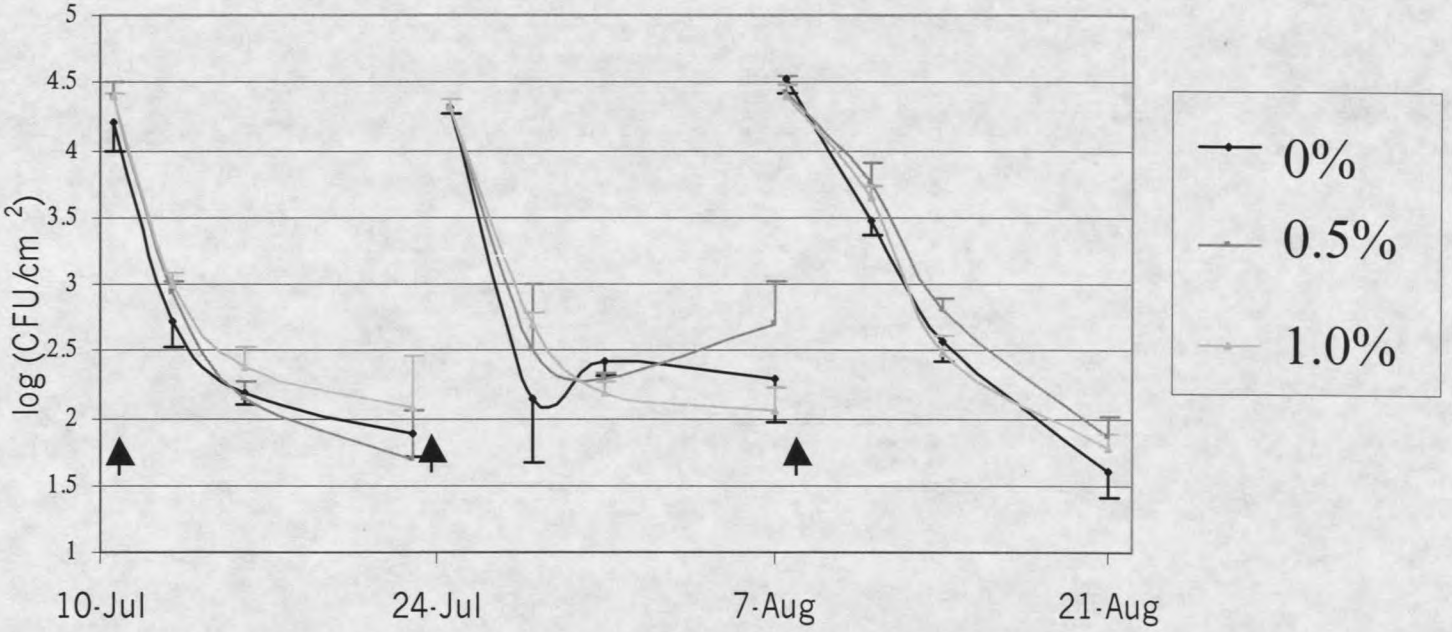


Figure 2-7. Isolation Experiments I, II, and III 10 July through 20 August 1998. BacB Rif+ population from sugar beet leaves through time. Samples were taken at days 0.5, 4, 7, and 14, after application. The date of each spray is shown with an arrow. Bars represent 1 standard error. Samples below detection level of 1.23 [$\log_{10}(17CFU/cm^2)$] were omitted.

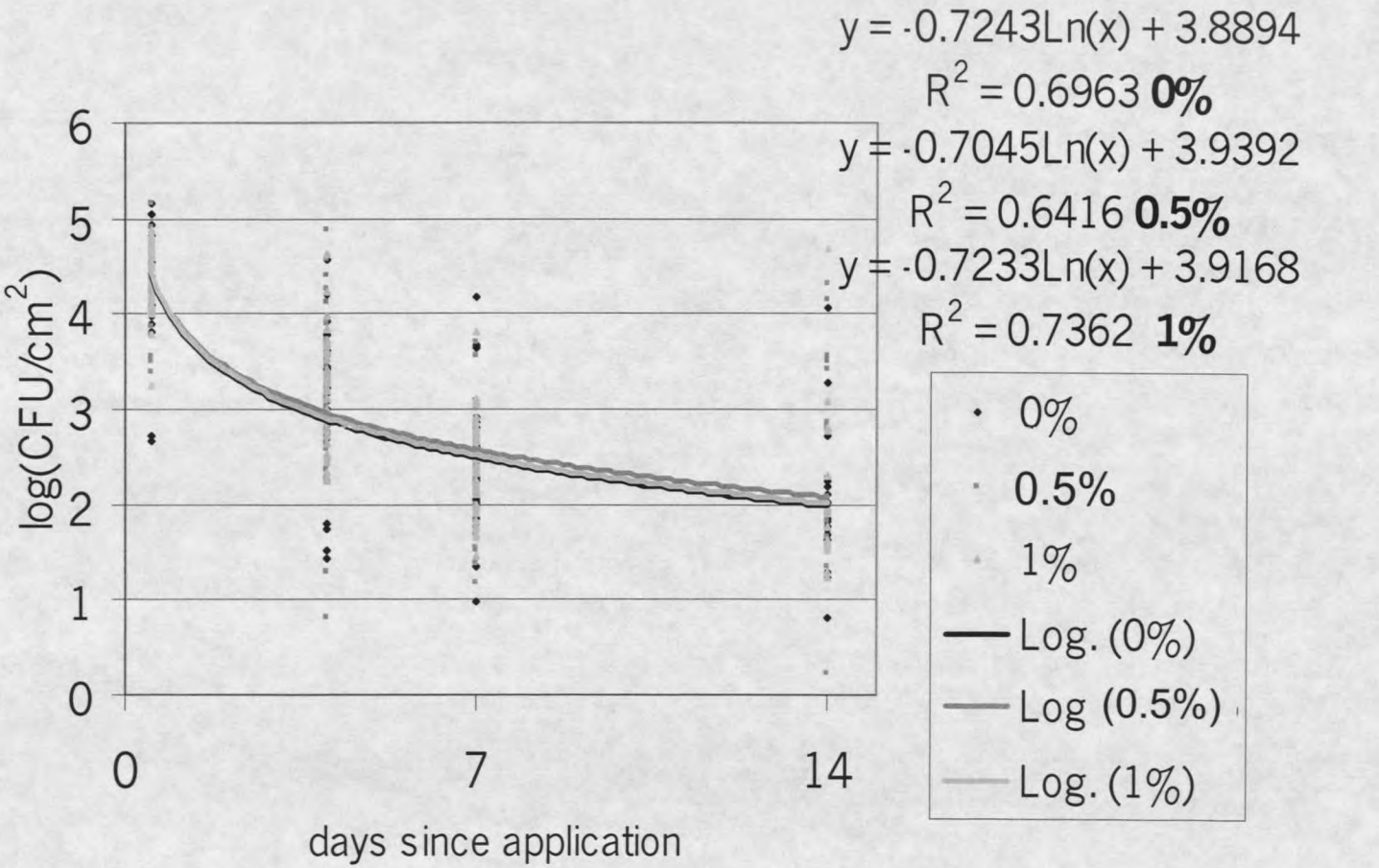


Figure 2-8. BacB Population through Time. Regression analysis of BacB Rif+ change through time. Data from 1998 experiments I, II, and III are combined.

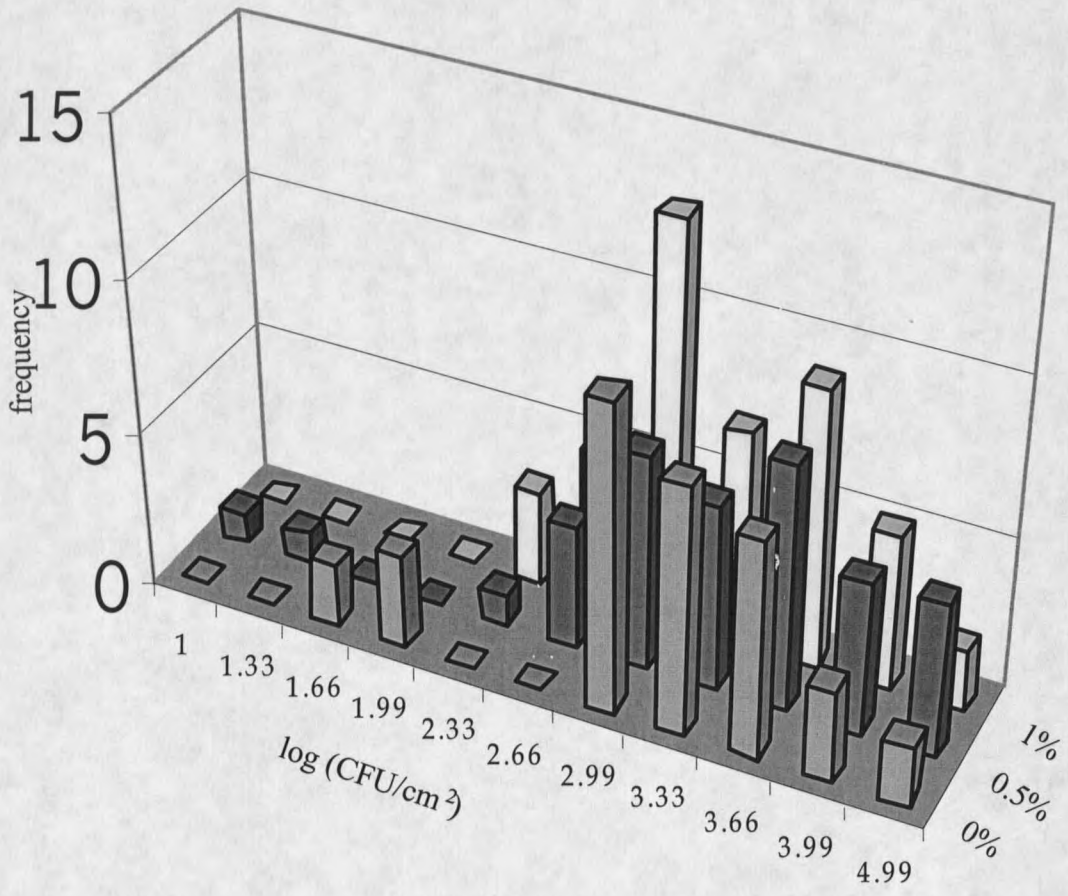


Figure 2-9. BacB Rif+ Distribution on Day 4. Data from 1998 experiments I, II, and III are combined.

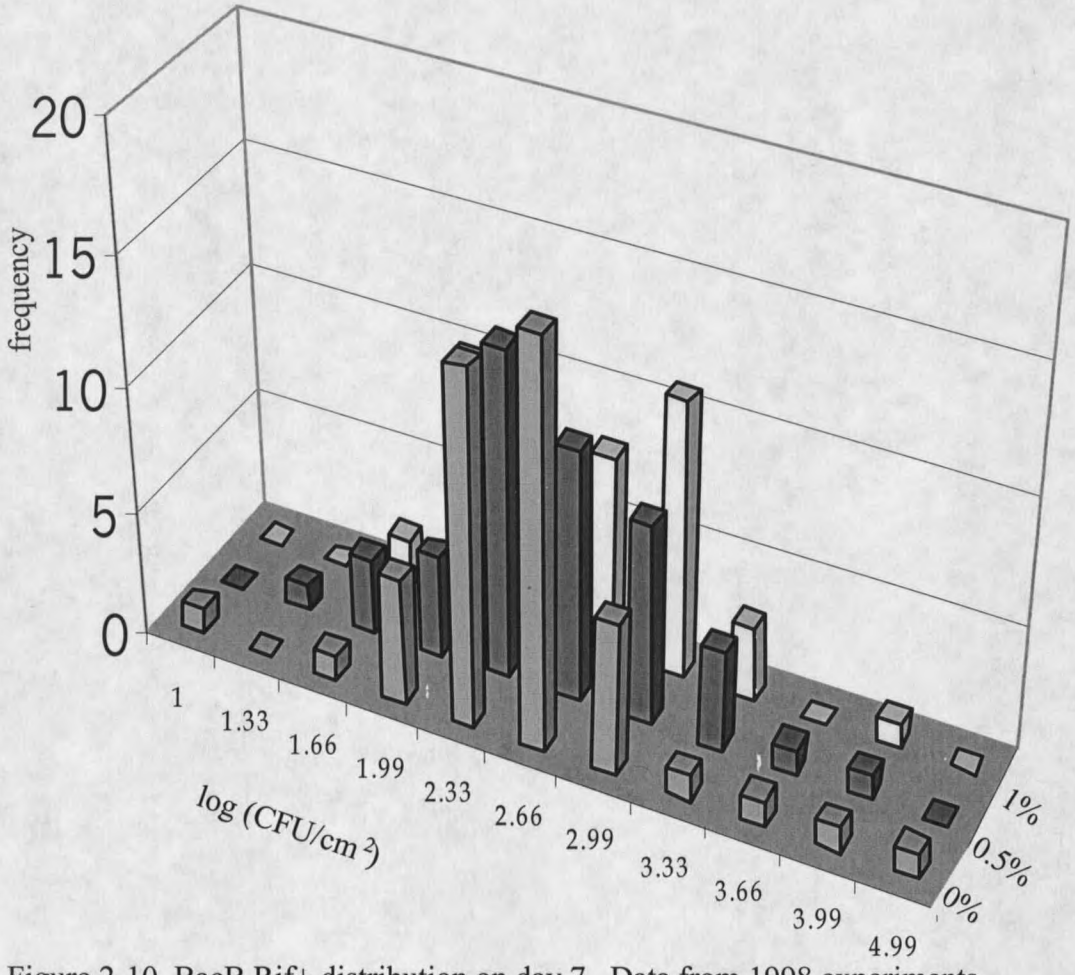


Figure 2-10. BacB Rif+ distribution on day 7. Data from 1998 experiments I, II, and III are combined.

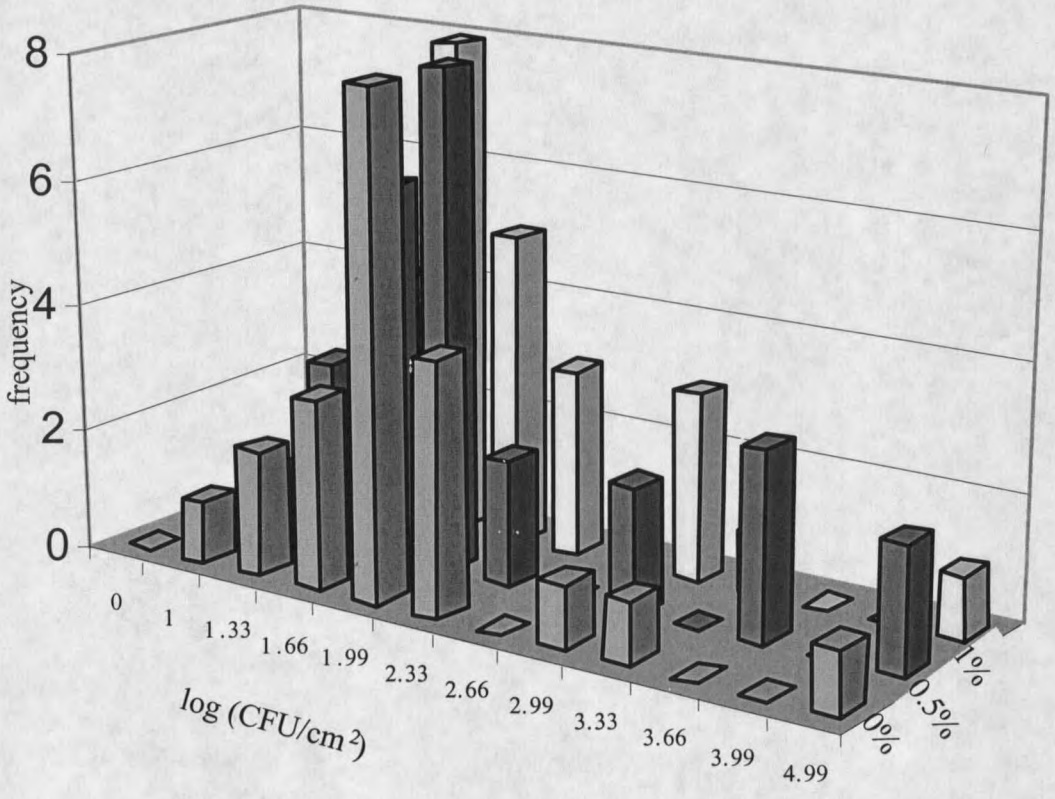


Figure 2-11. BacB Rif+ distribution on day 14. Data from 1998 experiments I, II, and III are combined.

Spatial distribution of BCA

Models describing spatial data. BacB populations were interpolated with nearest neighbor analysis and with kriging. The kriging interpolation is based on the underlying spatial relationships in a data set. These relationships are portrayed in a semivariogram. Semivariograms were computed for bacterial spatial data collected from the field on 21 July, 1999 with 0% β-glucan (Figure 2-12), 1% β-glucan (Figure 2-13), and samples collected on 18 August, 1999 with 0% β-glucan (Figure 2-14) and 1% β-glucan (Figure 2-15). Semivariograms were also computed to interpolate the distribution of BacB Rif+ applied to sugar beets in the growth chamber as a spore formulation (Figure 2-16) or as fresh cells (Figure 2-17), immediately prior to inoculation with *C. beticola*.

Calculating a semi-variogram model is an iterative process, and lag distance was adjusted to obtain the strongest model, or the one with the lowest RSS. The importance of lag distance is evident in Figure 2-16, where a much stronger model was developed with smaller lag distance. Most of the semivariograms demonstrate that strong models of spatial relationships were developed, with the exception of the model for the 0% (Figure 2-14) and especially the 1% β-glucan sample on 18 August, 1999 (Figure 2-15). Different lag distances were used to improve the model, but inspection and the low r^2 (0.35) demonstrate the weakness of the 1 % β-glucan model.

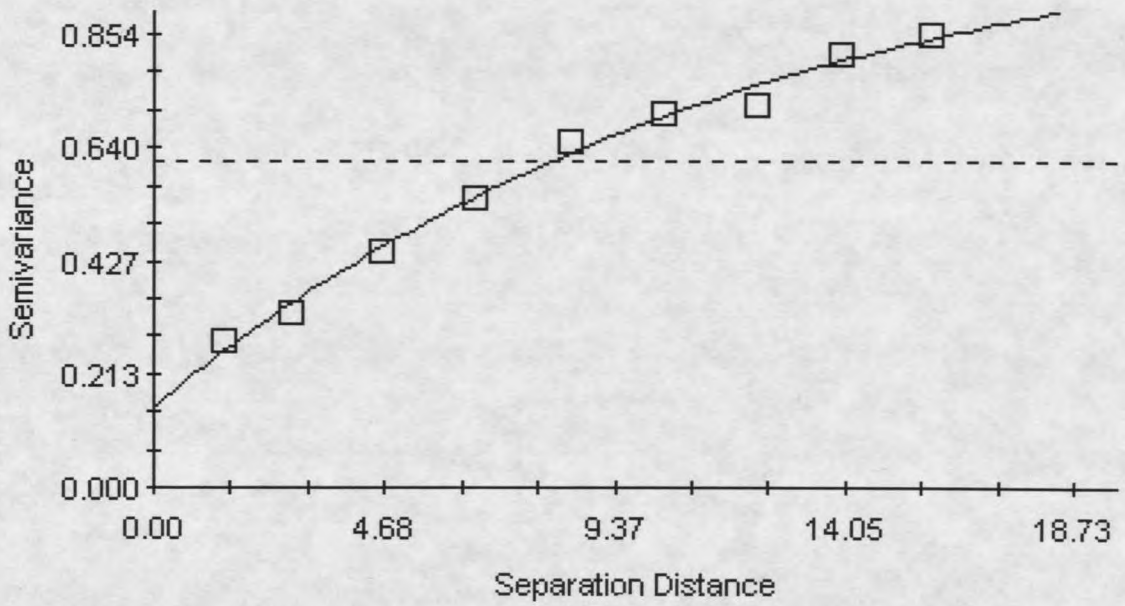


Figure 2-12. Semi-variogram for Kriging Interpolation of BacB Distribution for 21 July, 1999 Sample with 0% β-glucan. This is a linear model (N= 115, r^2=0.934, reduced sums of squares (RSS)=0.0299, lag distance =18.73).

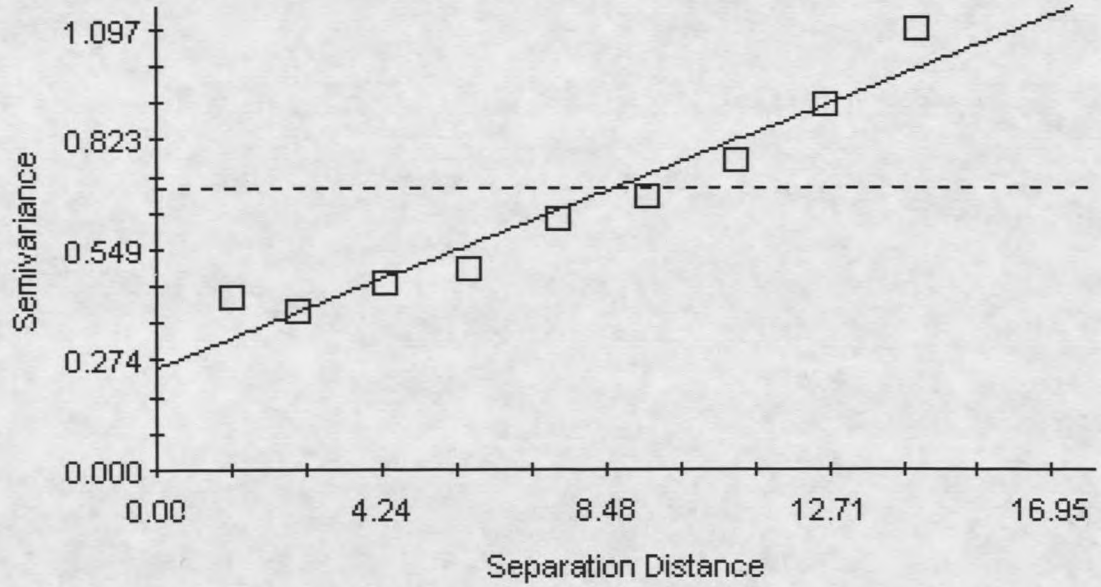


Figure 2-13. Semi-variogram for Kriging Interpolation of BacB Distribution for 21 July, 1999 1% β-glucan Sample. This is a linear model (N =97, r^2=0.934, RSS=0.0299, lag distance = 16.95).

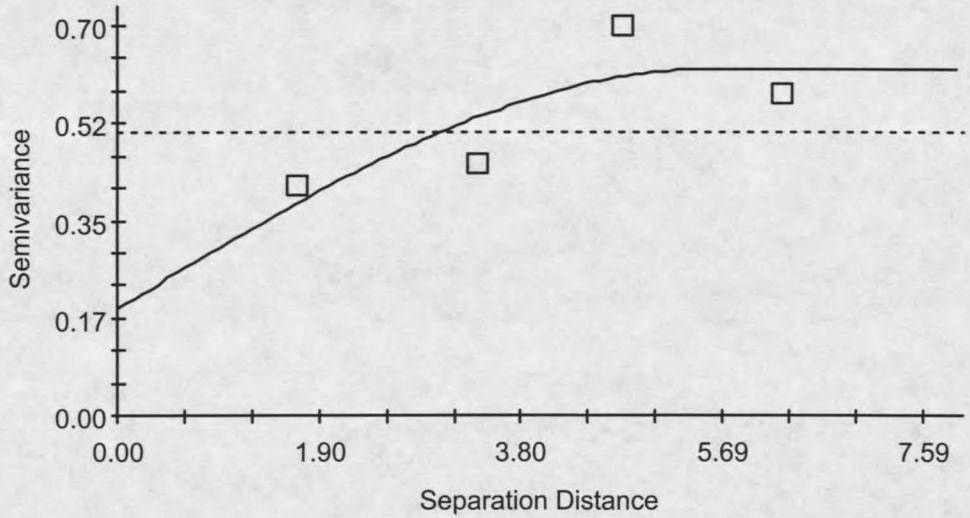
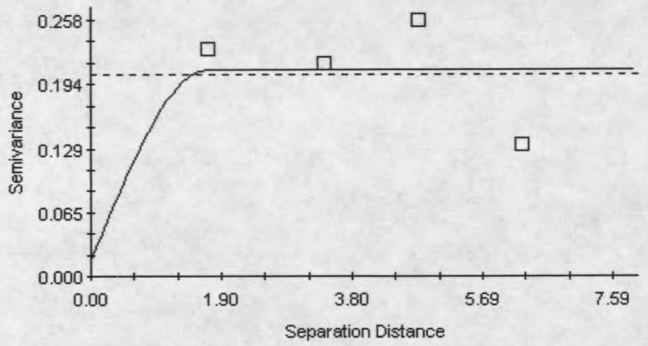


Figure 2-14. Semi-variogram for Kriging Interpolation of BacB Distribution for 18 August, 1999 0% β-glucan Sample. This is a spherical model (N= 25, r^2=0.645, RSS=0.0181, lag distance = 7.59).



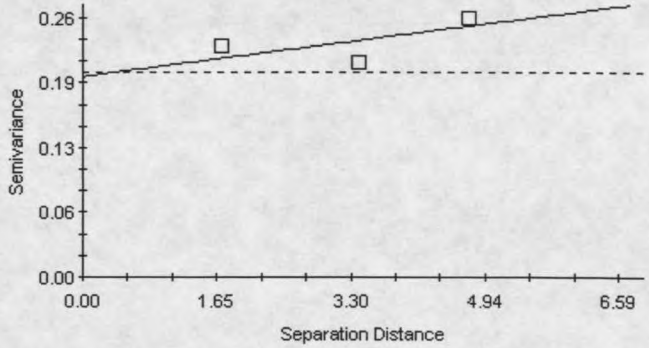


Figure 2-15. Semi-variograms with Different Lag Distances for Kriging Interpolation of BacB Distribution for 18 August, 1999 1% β-glucan Sample. The semi-variogram on the left is a spherical model with a lag distance of 7.59 (r^2=0.0, RSS=0.008714). The semi-variogram on the right is a linear model, with a lag distance of 6.59 (N = 25, r^2 =0.35, RSS =0.00064). The linear model was used for the kriging interpolation of the data.

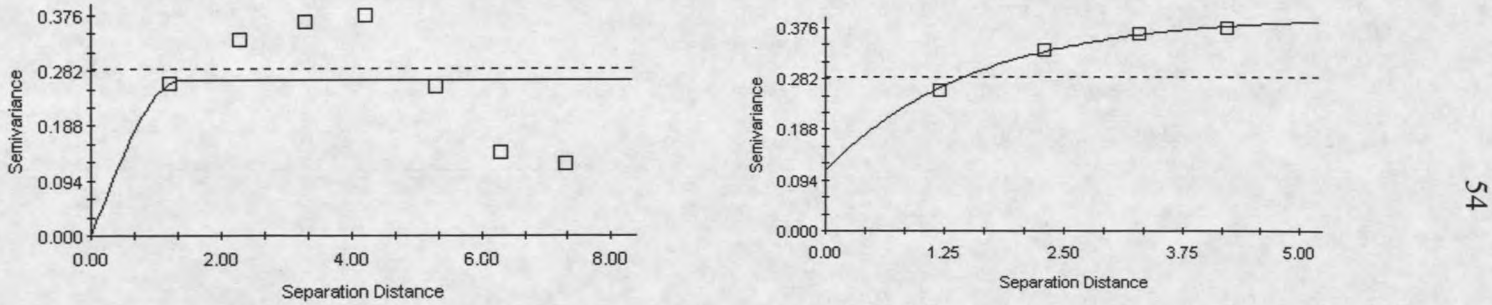


Figure 2-16. Semi-variograms with Different Lag Distances for Kriging Interpolation of BacB Distribution for Growth Chamber Spore Application. The semi-variogram on the left is a spherical model with a lag distance of 8 (r^2=0.0, RSS=0.06). The semi-variogram on the right is an exponential model with a lag distance of 5 (N= 60, r^2= 0.997, RSS=0.000000324). The exponential model was used for the kriging interpolation.b

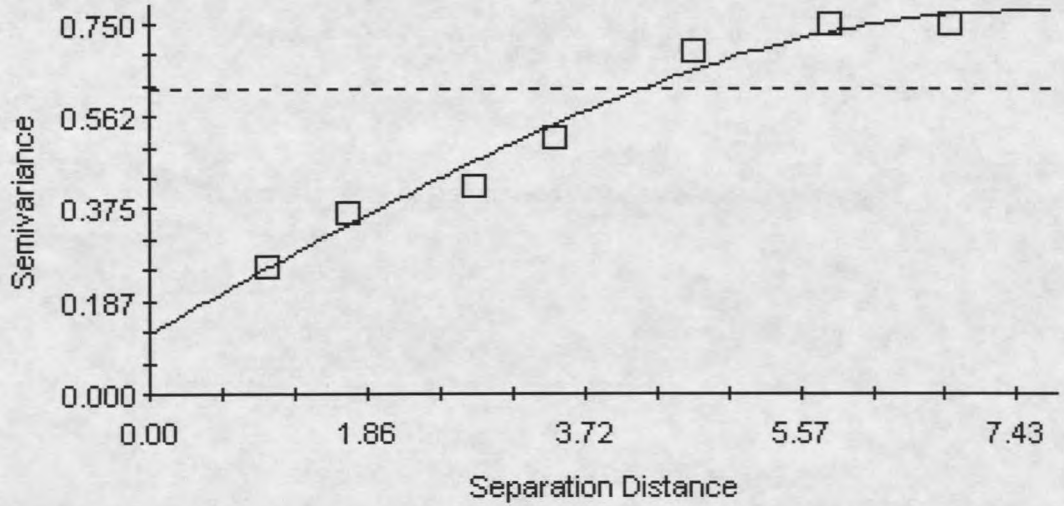


Figure 2-17. Semi-variogram for Kriging Interpolation of BacB Distribution for Growth Chamber Vegetative Cell Application. This is a spherical model. (N= 54, r^2=0.976, RSS=0.000569, lag distance =7.43).

BCA spatial distribution on leaf surface in the field. The benefit of interpolating spatial data is to see trends in the spatial distribution of some factor that were not readily apparent by inspection of the raw data. Figure 2-18 shows the original data, a nearest neighbor interpolation, and a kriging interpolation for the 21 July 1999 sample with 0% β-glucan. In this case, the different interpolation methods showed some different patterns in the data. The nearest neighbor interpolation shows a line of dense bacterial population along the center of the image, that is lost in the kriging interpolation. When the two interpolations are overlaid on a leaf, the nearest neighbor interpolation shows a line of more dense bacteria along the mid-vein, and along a secondary vein, that is not readily apparent in the kriging interpolation (Figure 2-19).

Figure 2-20 shows the original data and nearest neighbor and kriging interpolations for the data from 21 July, 1999 with 1% β-glucan. There is little difference between the two interpolations of this raw data. When the kriging interpolation is overlaid on the original leaf, there is no indication that dense areas of bacteria are associated with vein structures (Figure 2-21). Besides one datum that indicates a high level of bacteria at the leaf tip, there is a trend toward increased bacteria at the leaf base. Indeed, when bacterial density for the 1% β-glucan sample is regressed against distance from the leaf base there is a positive but weak inverse correlation ($p>0.0001$, $r^2=0.37$) with density decreasing toward the tip of the leaf. This correlation is strengthened when the outlying datum is removed ($p>0.0001$, $r^2=0.47$) (Figure 2-22).

Both the bacterial populations with 0% and 1% β-glucan demonstrate a high degree of spatial variability over a short distance. The 1% β-glucan sample is especially

characterized by aggregations of high numbers of BacB Rif+. The β- glucan sample apparently supported a higher number of bacteria over a broader area of the leaf.

A strong model could not be developed for either of the populations of vegetative cells (semi-variograms not shown) most likely due to the small sample of 20 for each level of β-glucan. However, the density of vegetative cells is shown in a classed post map (figure 2-23) overlaid over total cell populations for both levels of β-glucan. The occurrence of high levels of vegetative cells in regions of high total cell counts is especially apparent in the 1% β-glucan sample.

Though the models are not as strong as the 21 July, 1999 date, kriging was used to develop contour maps for the 0% (Figure 2-24) and 1% (Figure 2-25) leaf samples from 18 August, 1999. There is no indication of increased total bacterial numbers along the mid-vein. The 1% β-glucan sample has a generally higher population in this sample. When total vegetative cells were overlaid on the 18 August, 1999 interpolations of total bacterial populations, there is a trend toward higher levels of vegetative cells in the vicinity of high total bacterial populations (Figure 2-26). In the 1% β-glucan sample three samples with high vegetative cell populations occurred in the vicinity of the mid-vein.

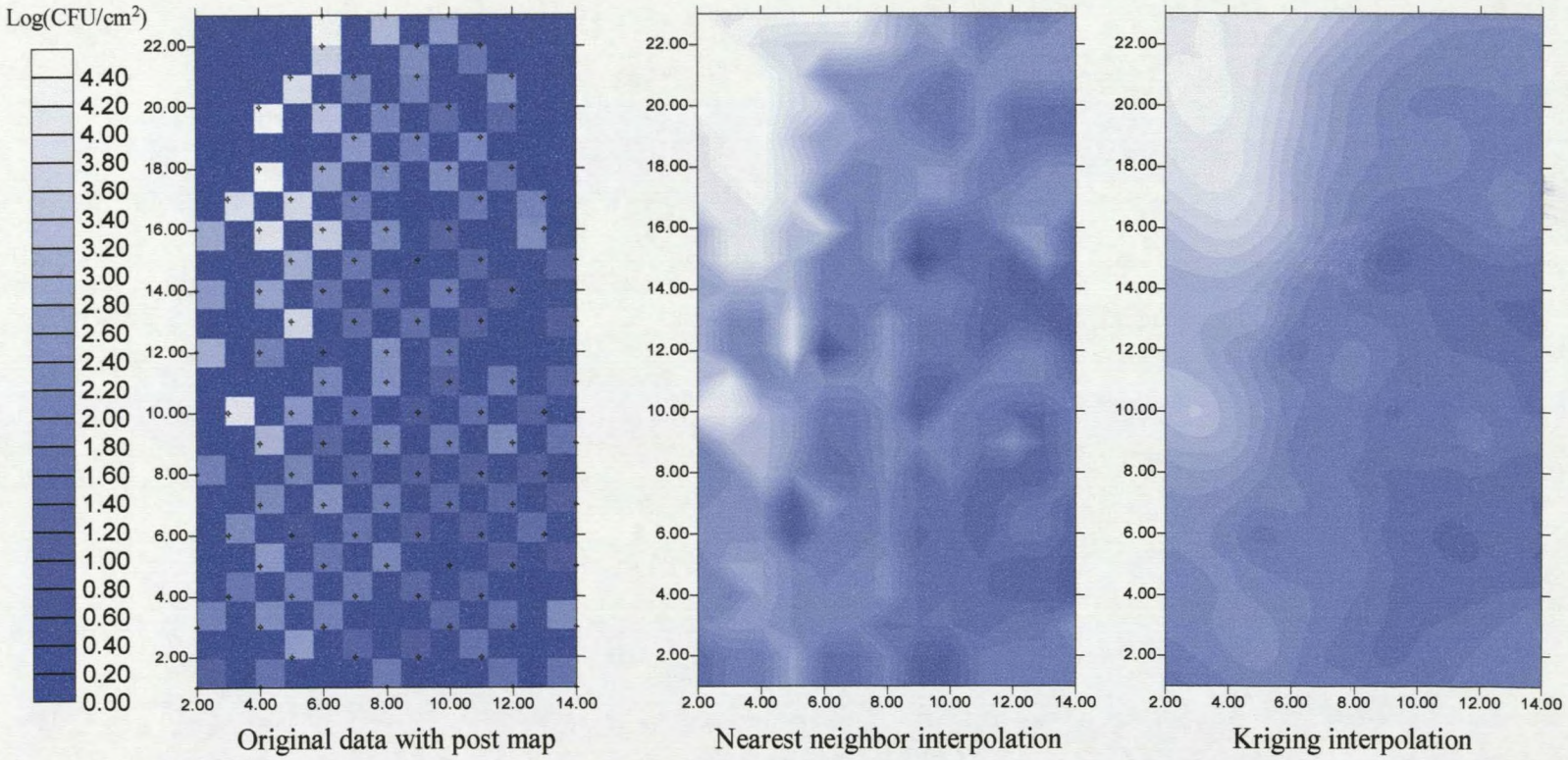


Figure 2-18. Original Data with Nearest Neighbor and Kriging Interpolations of BacB Rif+ Distribution for 21 July, 1999 Sample with 0% β-glucan.

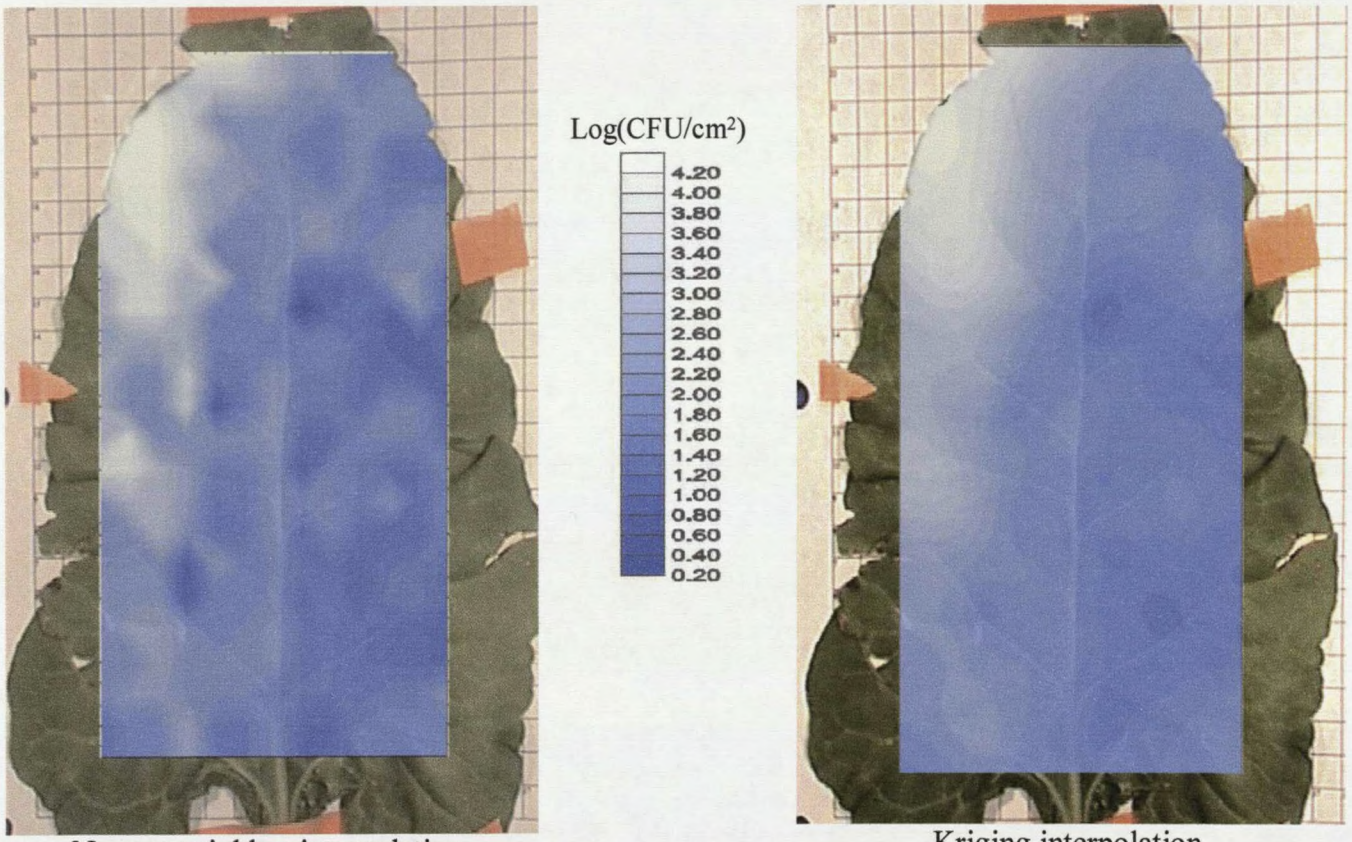


Figure 2-19. BacB Rif+ Distribution with Nearest Neighbor and Kriging Interpolations for 21 July, 1999 Sample with 0% β-glucan. Sample was taken from the field 14 days after application.

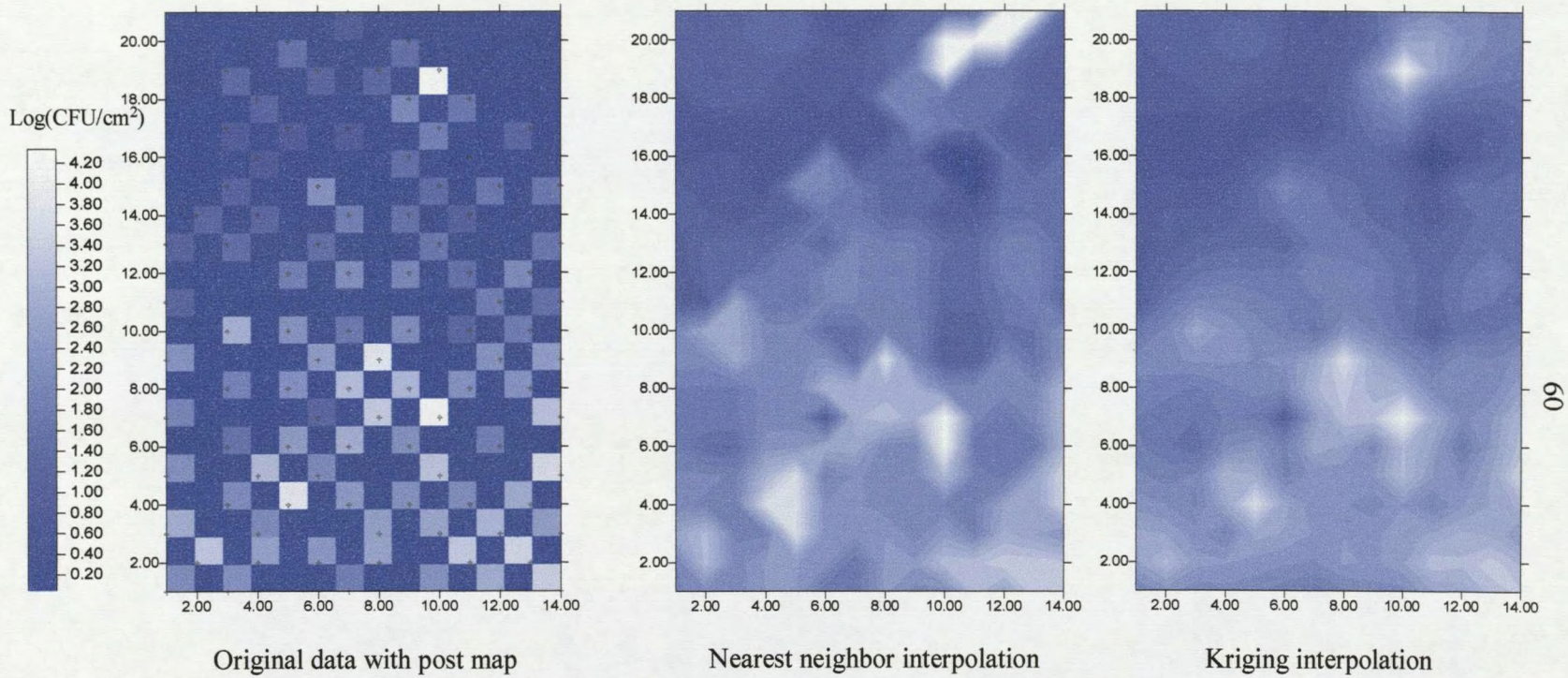


Figure 2-20. Original Data with Nearest Neighbor and Kriging Interpolations of BacB Rif+ Distribution for 21 July, 1999 Sample with 1% β-glucan.

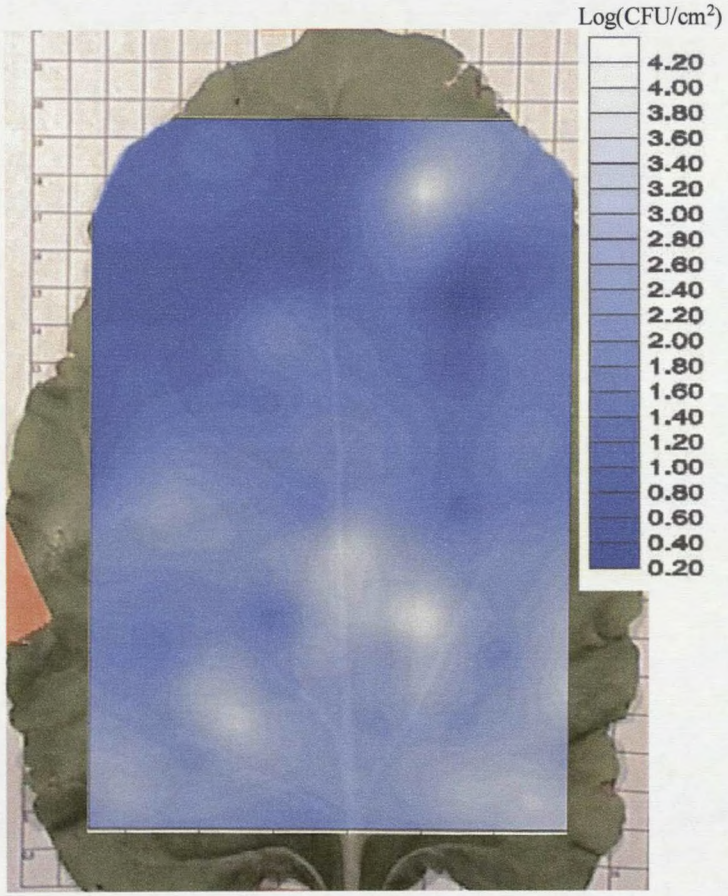


Figure 2-21. BacB Rif+Distribution with Kriging Interpolation for 21 July, 1999 Sample with 1% β-glucan. Sample was taken from the field 14 days after application.

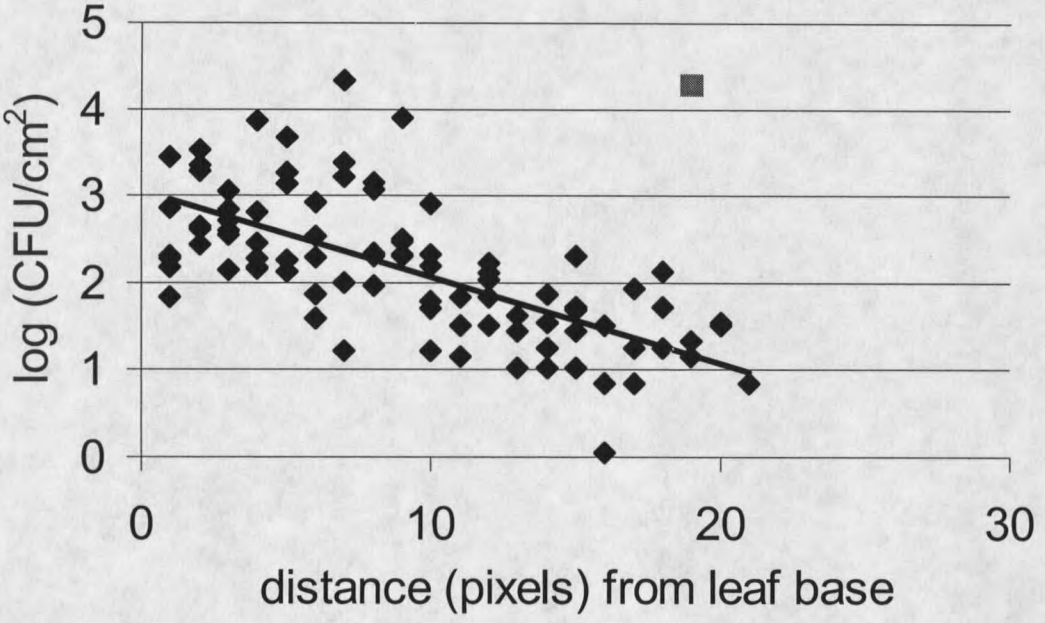


Figure 2-22. Distance from Base of Leaf Vs. Total BacB. These data are from the 21 July 1999 sample with 1% β-glucan. There is an inverse correlation between distance from the leaf base and number of bacteria. The one outlying point (shown as a square) is not included in the regression (r^2=0.47, p>0.0001).

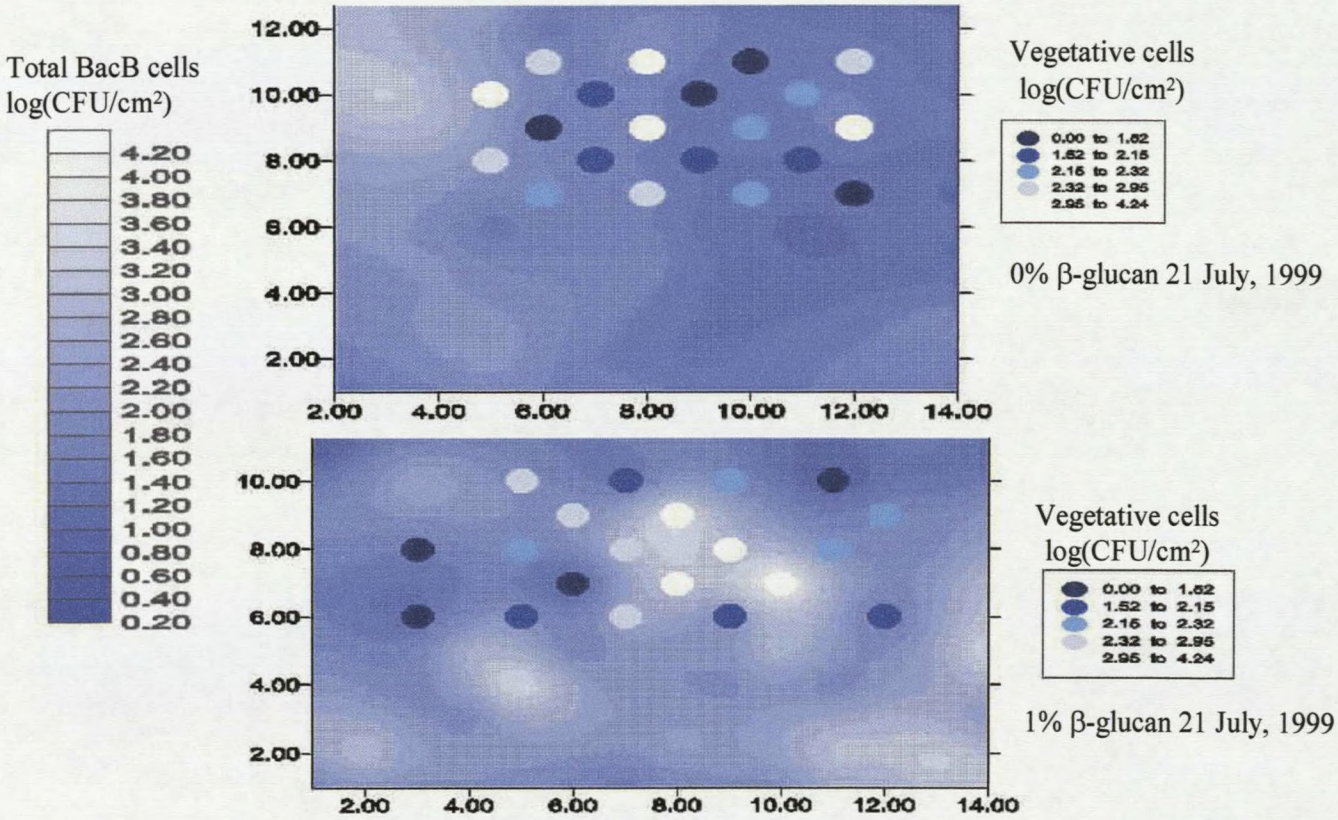


Figure 2-23. Vegetative Cell Density Overlaid on Total Cell Density for 0% and 1% β-glucan Samples from 21 July, 1999. Vegetative cell density is represented by a classed post map, with lighter circles representing more vegetative cells.

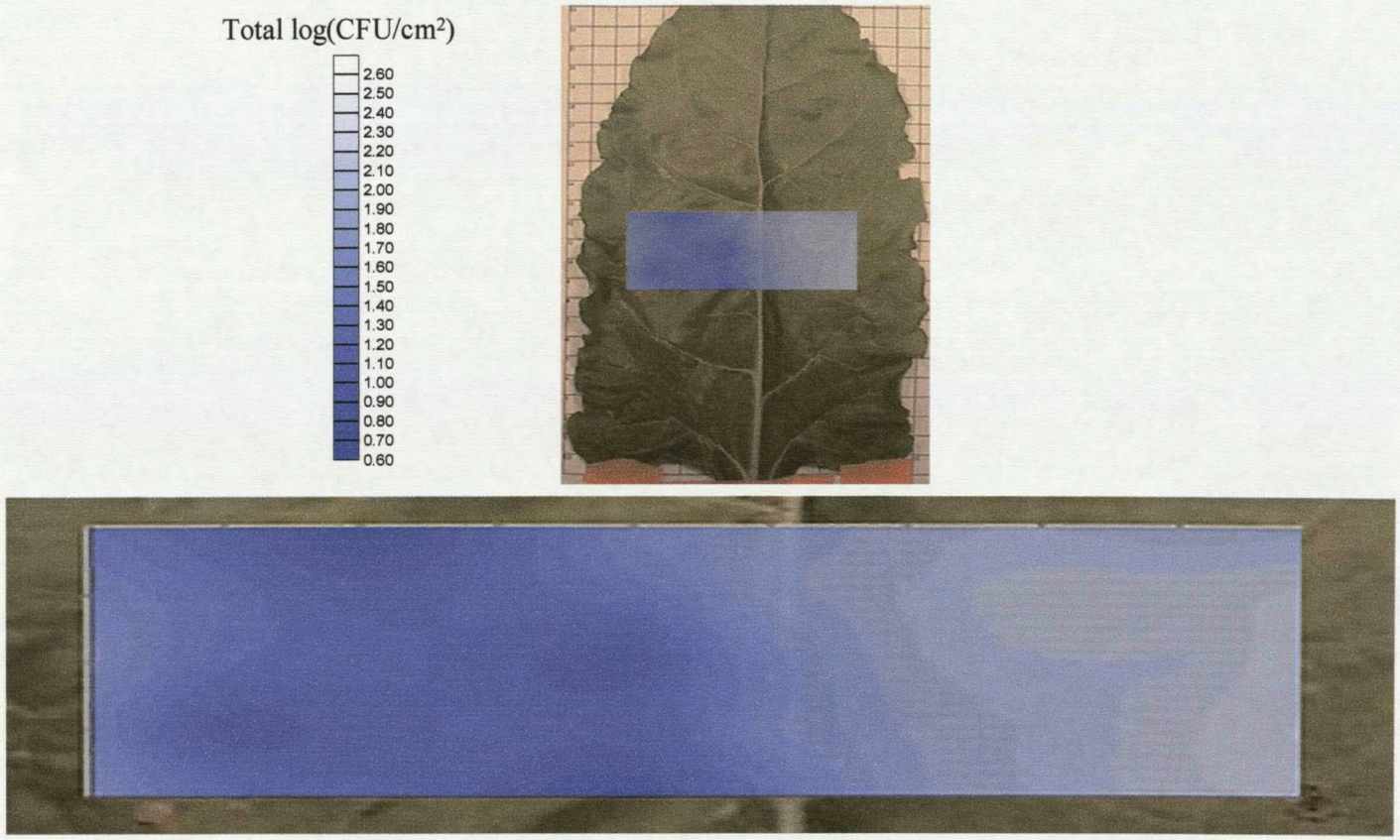


Figure 2-24. BacB Rif+ Distribution with Kriging Interpolation for 18 August, 1999 Sample with 0% β-glucan. This sample was taken from the field 14 days after application.

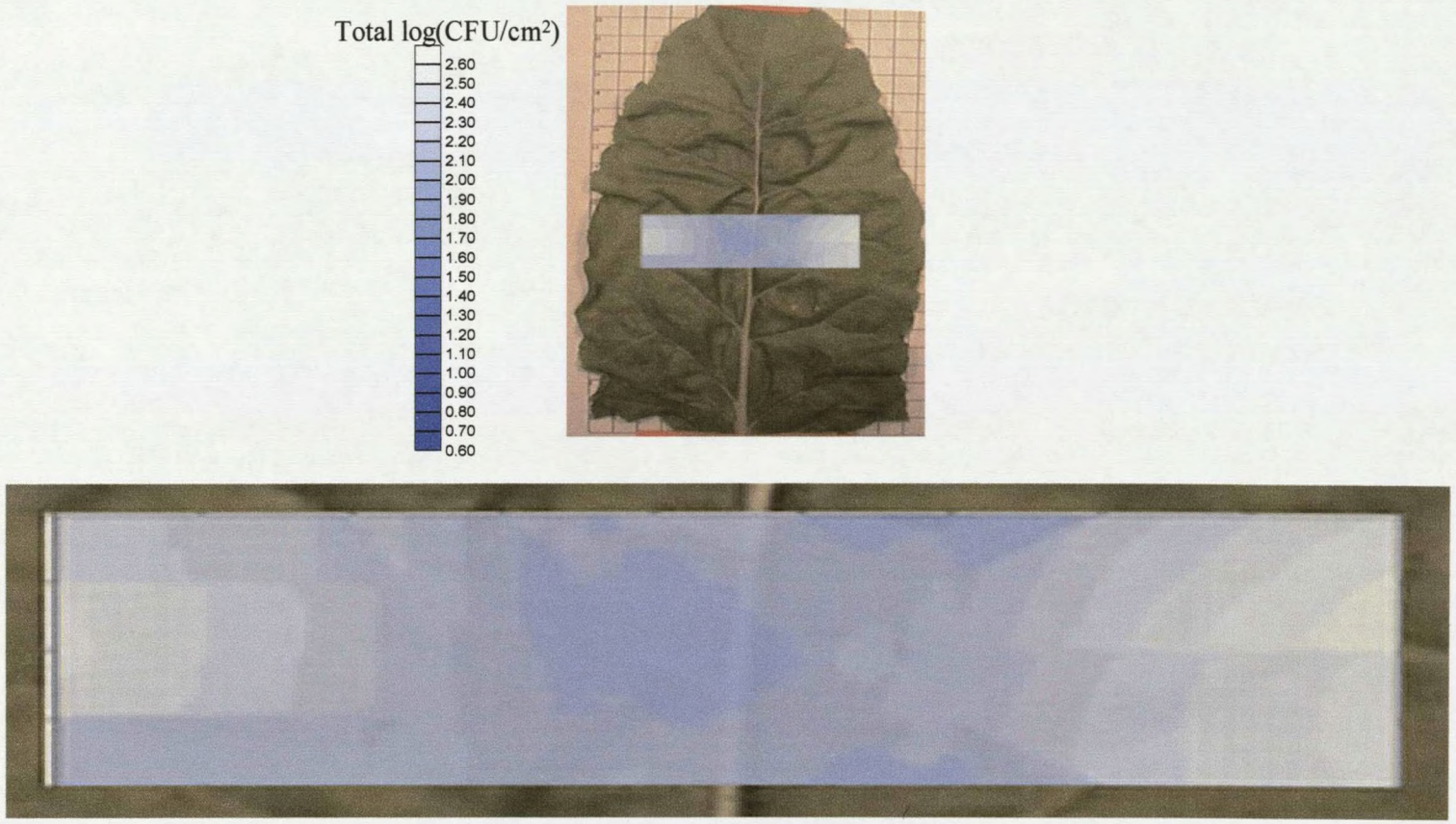


Figure 2-25. BacB Rif+ Distribution with Kriging Interpolation for 18 August, 1999 Sample with 1% β-glucan. This sample was taken from the field 14 days after application.

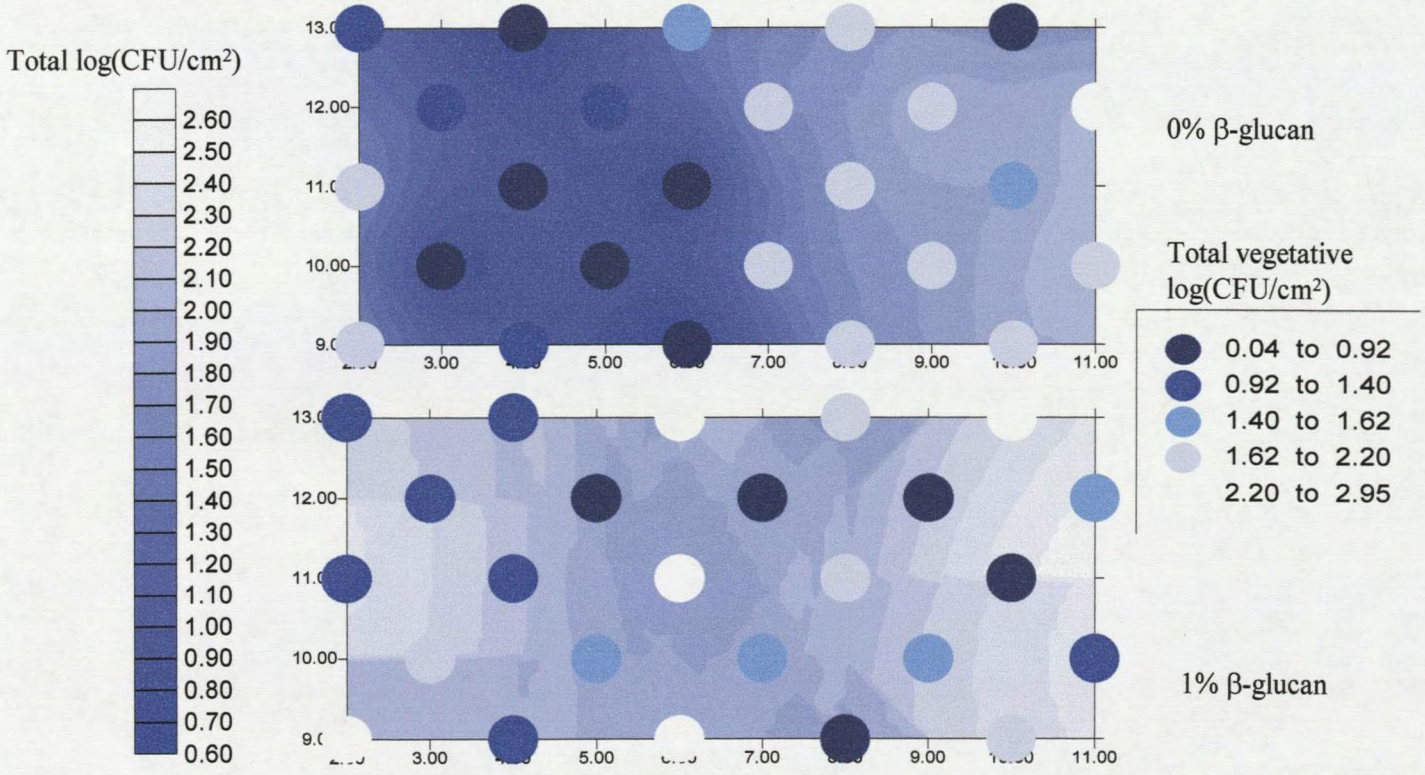


Figure 2-26. Vegetative Cell Density Overlaid on Total Cell Density for 0% and 1% β-glucan Samples From 18 August, 1999. Vegetative cell density is represented by a classed post map, with lighter circles representing more vegetative cells.

BCA in relation to *C. beticola* in growth chamber. Figure 2-27 shows the original data and nearest neighbor and kriging interpolations of the distribution of BacB Rif+ applied in the growth chamber as vegetative cells immediately before inoculation with *C. beticola.* There is little difference between the two interpolations of the original data. When the kriging interpolation is overlaid onto the original leaf, with a classed post map of *C. beticola* infection, it is seen that areas of high bacterial density tend to have little or no disease (Figure 2-28).

Figure 2-29 shows the original data and nearest neighbor and kriging interpolations of the distribution of BacB Rif+ applied in the growth chamber as a spore formulation immediately before inoculation with *C. beticola.* Again, there is little difference between the two interpolations of the original data. A similar trend as seen in the vegetative cell application, with areas of dense bacterial populations having less disease, is seen with the spore application (Figure 2-30).

When linear regression was attempted (scatter plots not shown) to determine if there was a significant correlation between level of bacteria and percent infection *with C. beticola*, the relationship was not significant for the vegetative cell application ($p>0.53$) or with the spore application ($p>0.31$).

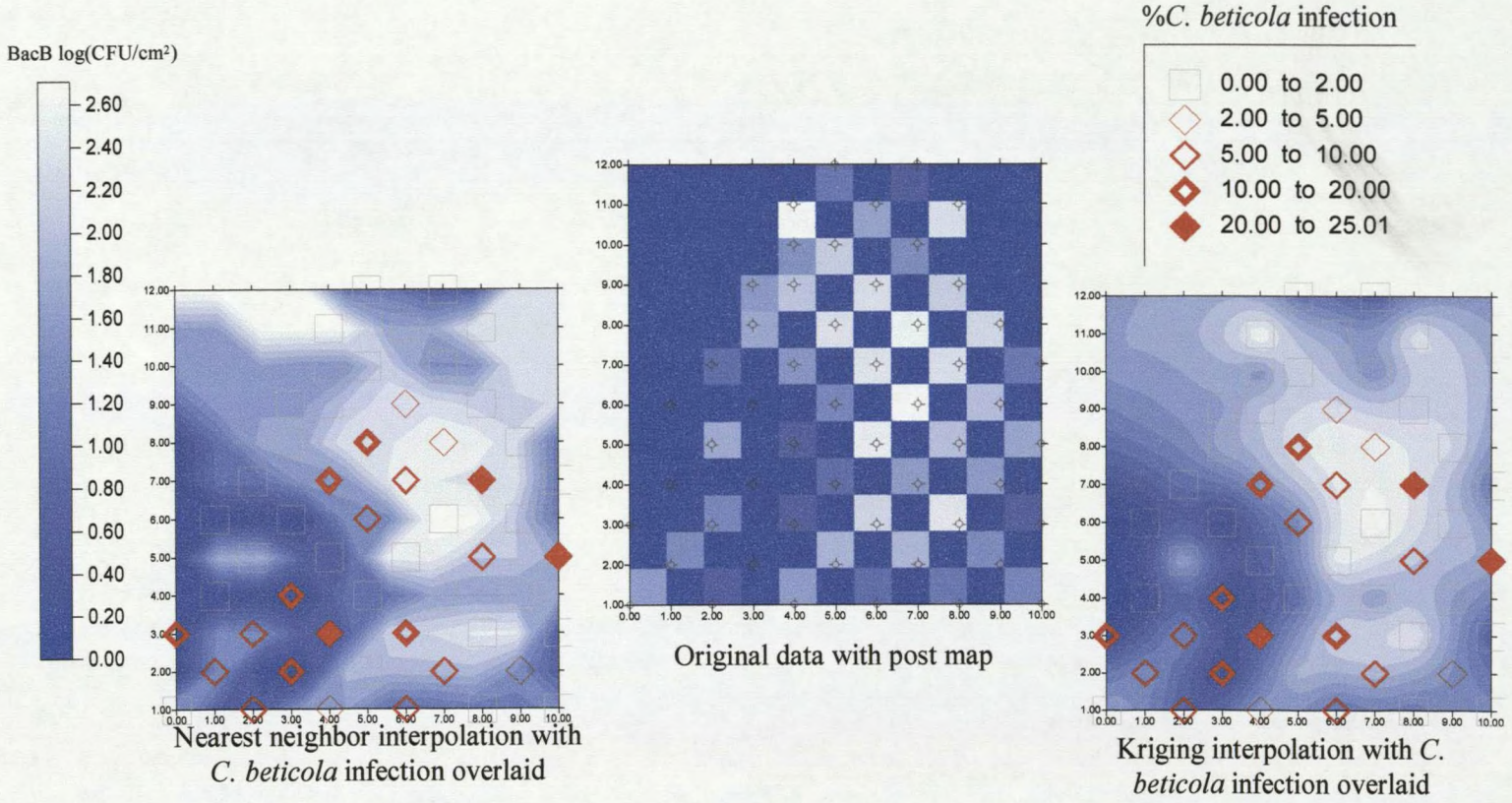


Figure 2-27. Original Data with Nearest Neighbor and Kriging Interpolations of BacB Rif+ Distribution for Growth Chamber Vegetative Cell Application. Increasing *C. beticola* disease severity is represented by increasing red in an overlaid classed post map.

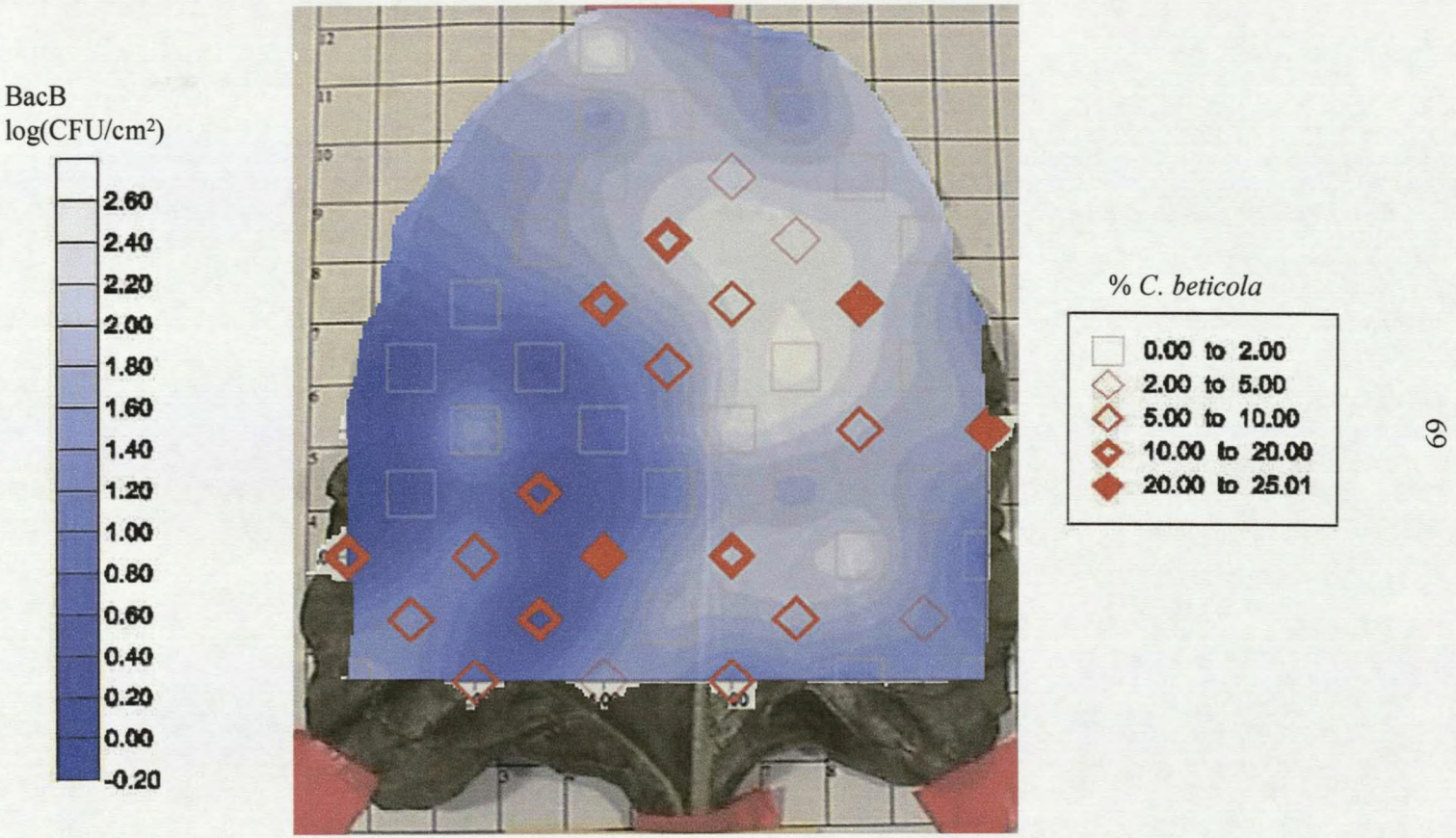


Figure 2-28. *C. beticola* Density Overlaid on Kriging Interpolation of BacB Vegetative Cell Application. Percent *C. beticola* infection is represented in an overlaid classed post map.

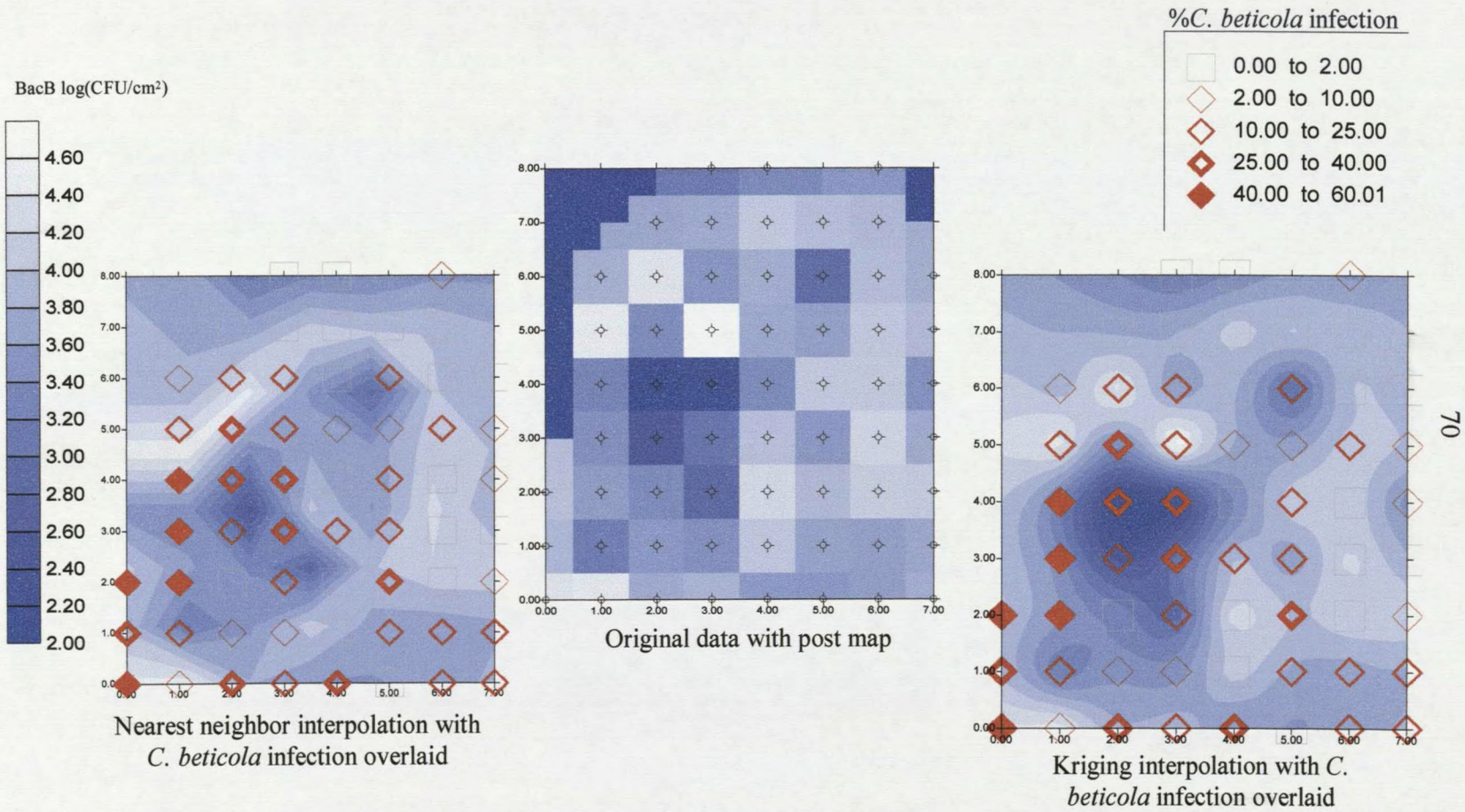


Figure 2-29. Original Data with Nearest Neighbor and Kriging Interpolations of BacB Rif+ Distribution for Growth Chamber Spore Application. Increasing *C. beticola* disease severity is represented by increasing red in an overlaid classed post map.

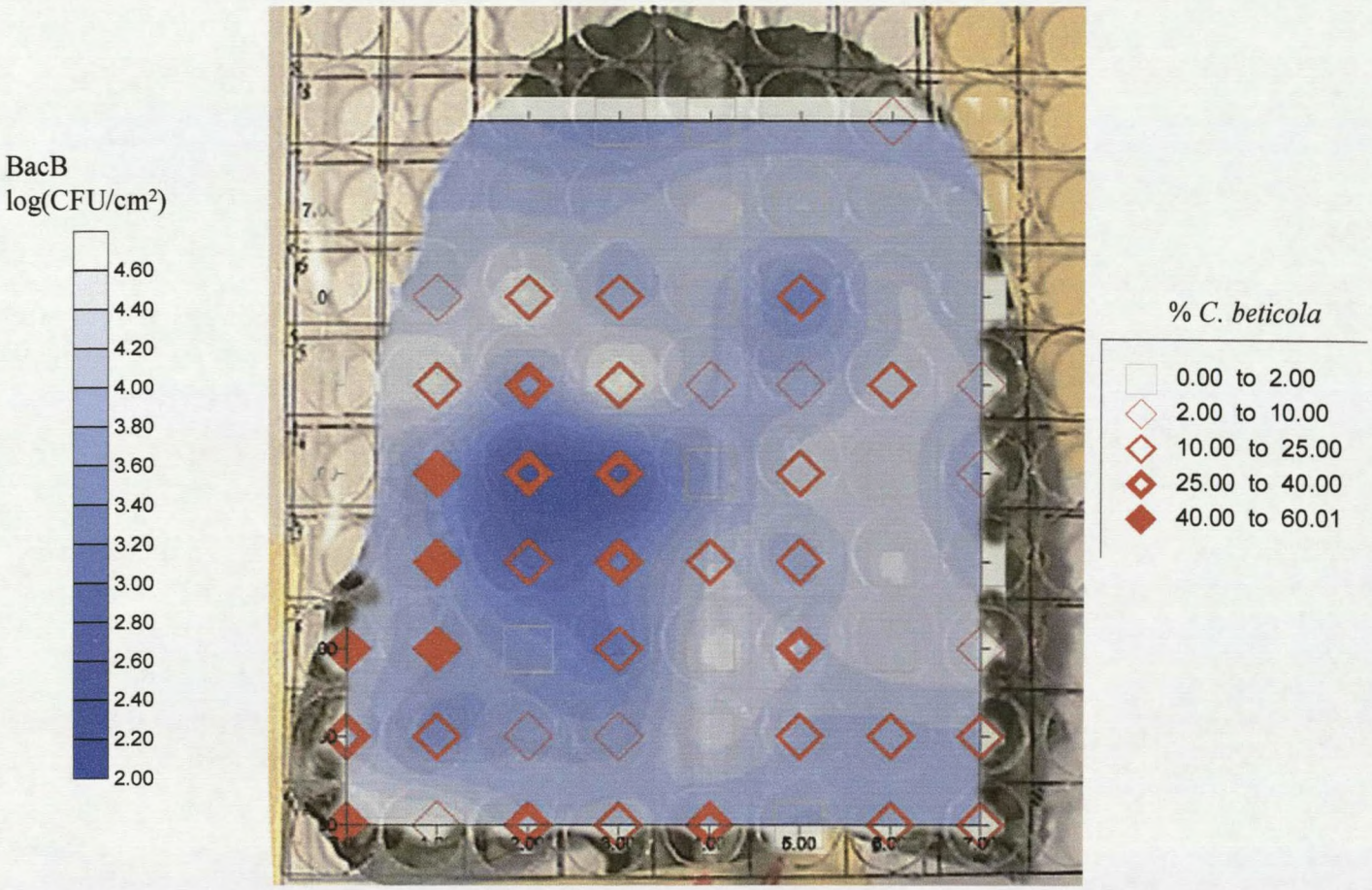


Figure 2-30. *C. beticola* Density Overlaid on Kriging Interpolation of BacB Rif+ Spore Formulation Application. Percent *C. beticola* infection is represented in an overlaid classed post map.

Immunocytochemical localization of BCA on leaf surface

Specificity and reactivity of polyclonal antibodies. Anti-sera against BacB was harvested from two different rabbits, 2263 and 2264. Preliminary dot blot data indicated that 2263 had a stronger reaction against BacB cells, so more thorough testing of specificity was done with the serum from this rabbit. Dot blots (Figure 2-31) demonstrated a strong specificity of this anti-serum for BacB. Fluorescent secondary anti-bodies were also used to test specificity. The assays were used to test reactivity against five *Bacillus* sp. isolated from the field in past years, 8 *Bacillus* sp. isolated in summer 1999, and 5 non-Bacilli from lab collections. In addition, BacB vegetative cells, BacB spore formulation, and BacB Rif+ were tested. As shown in Table 2-7, the two assays did not always agree, and the fluorescent antibody assay appears to be more sensitive. All of the Bacilli from the lab collection reacted negatively in dot blot assays, but three of these had weak reactions in the fluorescent antibody assay. Three Bacilli isolated in summer 1999 reacted negatively, one had a weak positive reaction, while 4 had a strong positive reaction. All of the non-Bacilli from the lab collection reacted negatively. BacB vegetative cells, spore formulation and Rif+ all reacted positively.

Table 2-7. *Bacillus* sp. and non-bacilli tested for reactivity against BacB antiserum (2263) with dot blot and fluorescent microscopy.

Isolates	Assay used to test[1]	
	Dot blot	Fluorescent microscopy
Bacillus sp.		
BacB vegetative cells	+	+
BacB spore formulation		+
BacB Rif+ (vegetative cells)	+	+
Bacillus mycoides	-	-
102-96	-	+/-
106-96	-	-
110-96	-	+/-
111-96	-	+/-
341-??		-
B1-99		+
B2-99		+
B3-99		+
B4-99		+
B5-99		-
B6-99		-
B7-99		+
B8-99		+/-
Non-*Bacillus* sp.		
Escherii coli	-	
Erwinia caratovora beta-vasculorum	-	-
Erwinia herbicola (EH239)	-	-
Pseudomonas fluorescence (AB245)	-	-
Pseudomonas syringae p.v *tabaci* (94-19)		-

[1] (+) = strong reaction, (-) = no reaction, (+/-) = weak reaction

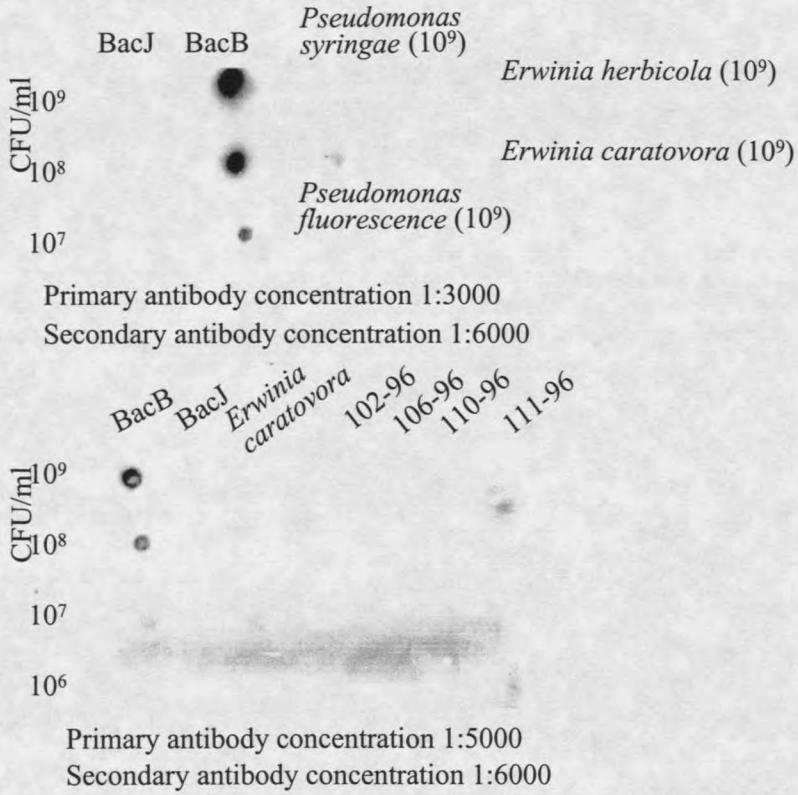
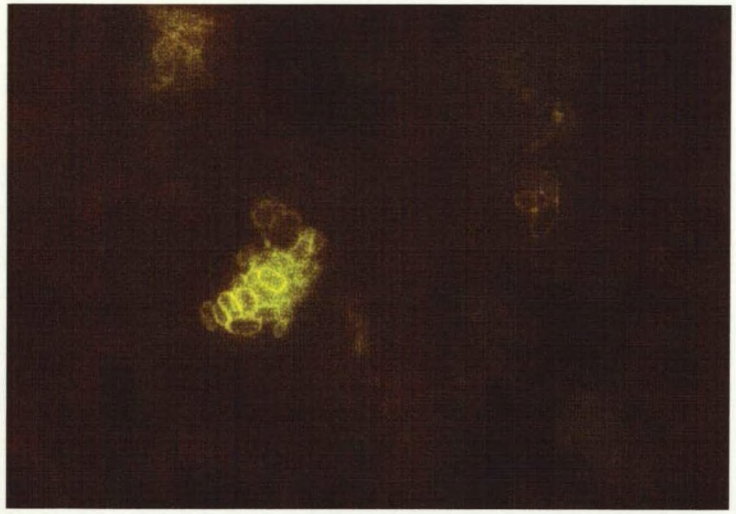


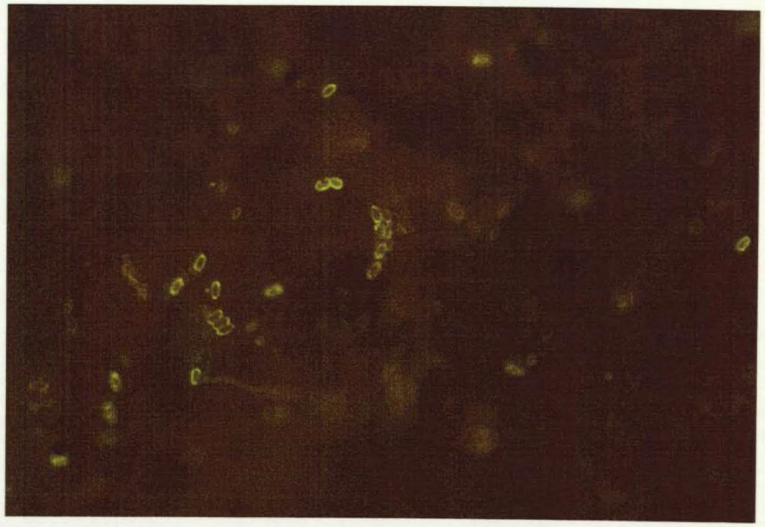
Figure 2-31. Sample Dot Blots Used to Evaluate Antibody Specificity.

Spatial distribution of BacB wild type on microscopic scale. BacB wild type cells were located on field leaves 14 days after isolation with fluorescein tagged antibodies (Figure 2-32). Auto-fluorescence was extreme in certain parts of the leaf, especially in areas heavily infected with *C. beticola*, or around stomates. Six leaf pieces from a leaf treated with BacB wild type and 0% β-glucan, and six leaf pieces from a leaf treated with BacB wild type and 1% β-glucan were prepared along with un-inoculated control leaf pieces for microscope analysis. Figure 2-33 shows the distribution of the populations in the six different leaf pieces at 0% β-glucan. Populations are extremely aggregated at this scale, and there are also few populations in the six leaf pieces examined. There is no indication that leaf pieces near the mid-vein supported higher populations. Figure 2-34 shows the distribution of bacteria in the six different leaf pieces at 1% β-glucan. Populations are also highly aggregated and there were generally more aggregates with the nutrient substrate than without.

Localization of BCA on the leaf surface with scanning electron microscopy. Pure cultures of BacB, and *Bacillus mycoides*, another Bacilli known to react negatively with the anti-serum, were prepared on silicon stubs for observation with SEM. Many gold particles could be seen clearly on and around BacB cells (Figure 2-35), while a few gold particles could be seen on *B. mycoides* cells. Growth chamber inoculated leaves were also prepared for microscopic observation (Figure 2-36) and BacB cells were located and a few gold particles seen. Attempts were made to locate BacB on leaves from the field that had been treated with the bacteria with and without β-glucan 14 days previously, but no gold could be seen on the few bacilliform cells found.



2400X



1200X

Figure 2-32. BacB with 1% β-glucan on Field Leaf 14 days After Application Reacted with Primary Antibody and Secondary Tagged Fluorosceine Antibody. The leaf pieces were blocked with RITC gelatin before reaction with antibodies. This method was used to locate and count BacB cells on a microscopic scale. The figure on the right shows one field of view.

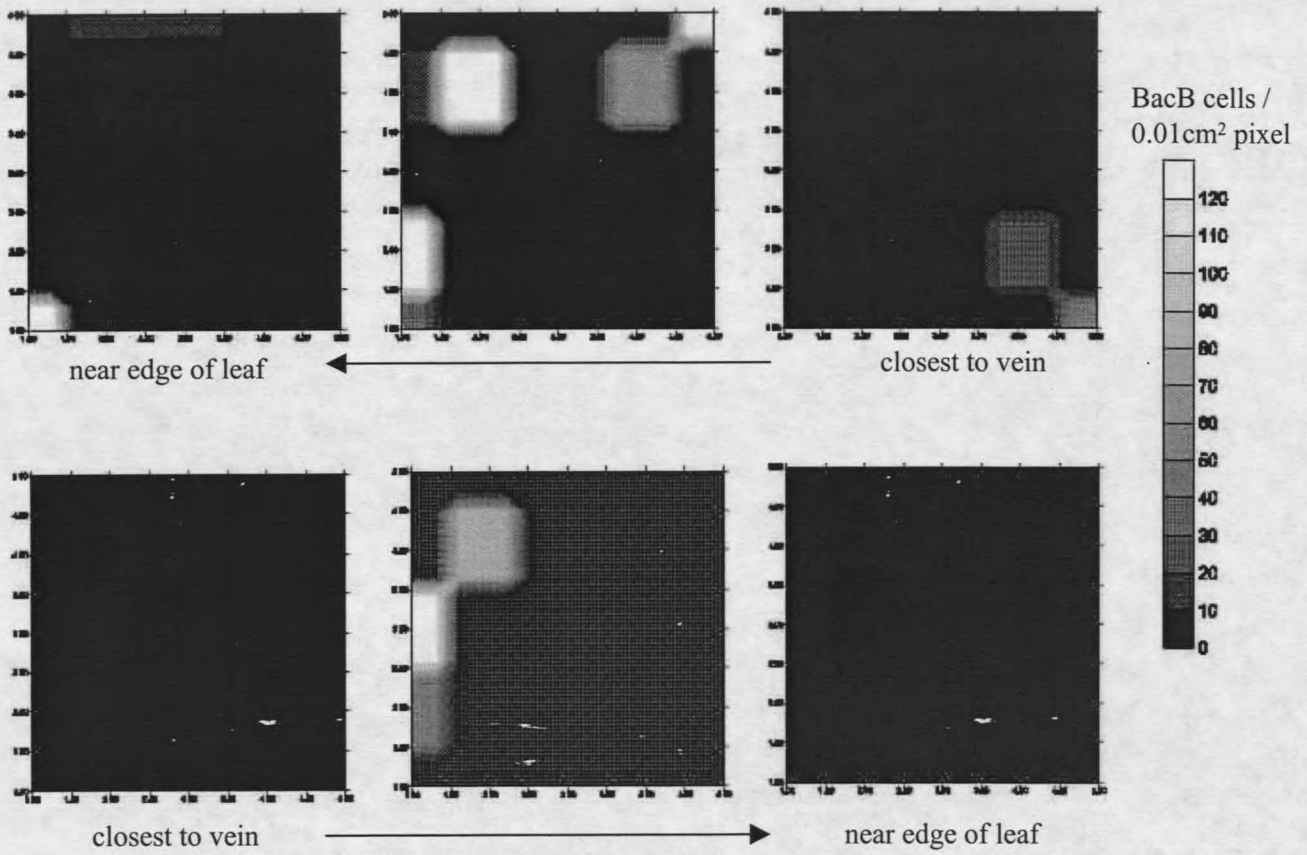


Figure 2-33. Distribution of BacB Cells on Microscopic Scale with 0% β-glucan. Each square represents one leaf pieceof $0.28cm^2$. Leaf pieces were taken in a line across the leaf, and their relative position is shown with the arrows and labels. Leaf pieces were divided into 25 fields of view of approximately $0.01cm^2$ and the number of fluorescing bacteria in each field of view counted.

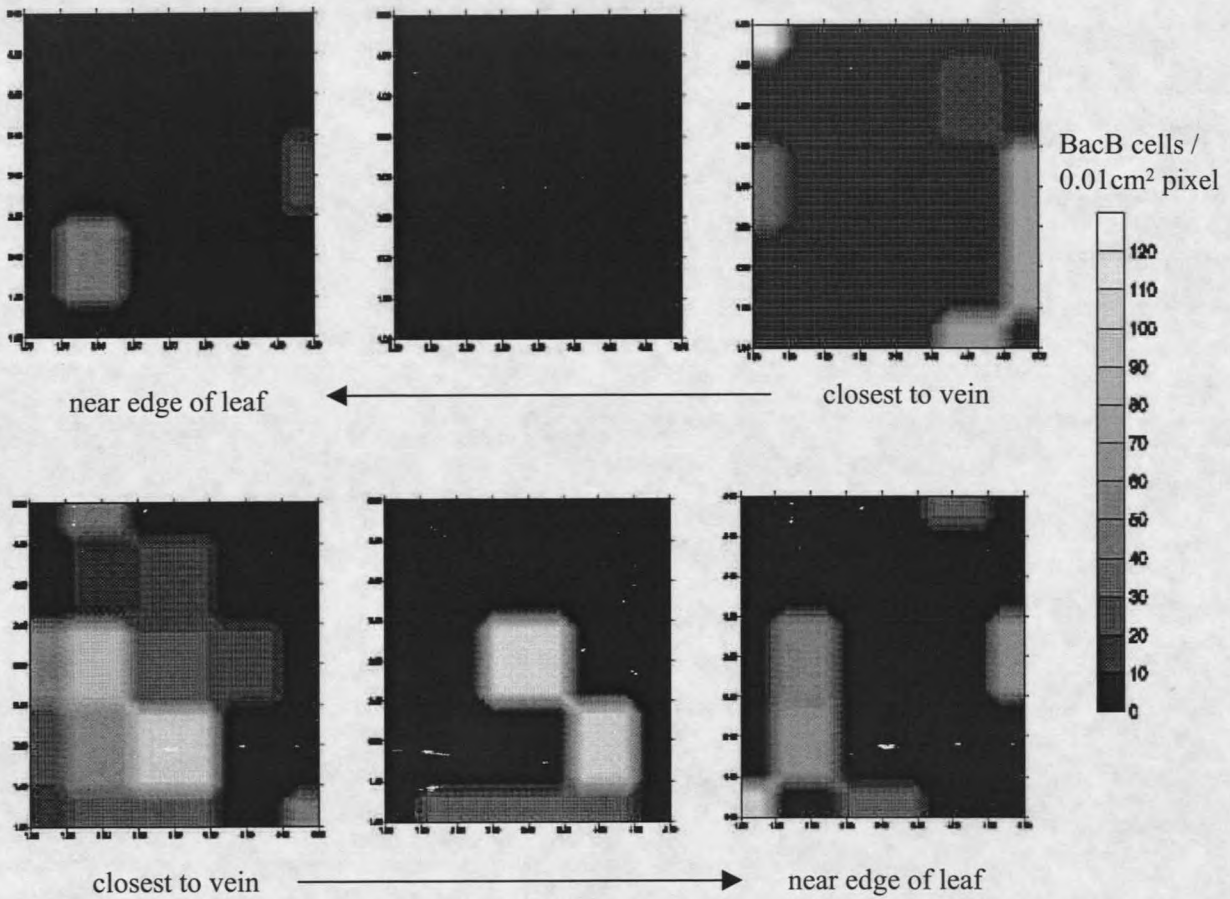


Figure 2-34. Distribution of BacB Cells on Microscopic Scale with 1% β-glucan. Each square represents one leaf pieceof $0.28cm^2$. Leaf pieces were taken in a line across the leaf, and their relative position is shown with the arrows and labels. Leaf pieces were divided into 25 fields of view of approximately $0.01cm^2$ and the number of fluorescing bacteria in each field of view counted.



BacB



BacJ

Figure 2-35. Scanning Electron and Back-Scatter Electron Micrographs of Pure Cultures of BacB and BacJ Reacted with Primary Antibody and Secondary Gold Tagged Antibody. Gold particles (arrows) cover the BacB cells, and bacterial debris adhering to the stub, while only a few gold particles are seen on the BacJ cells, or in their vicinity.

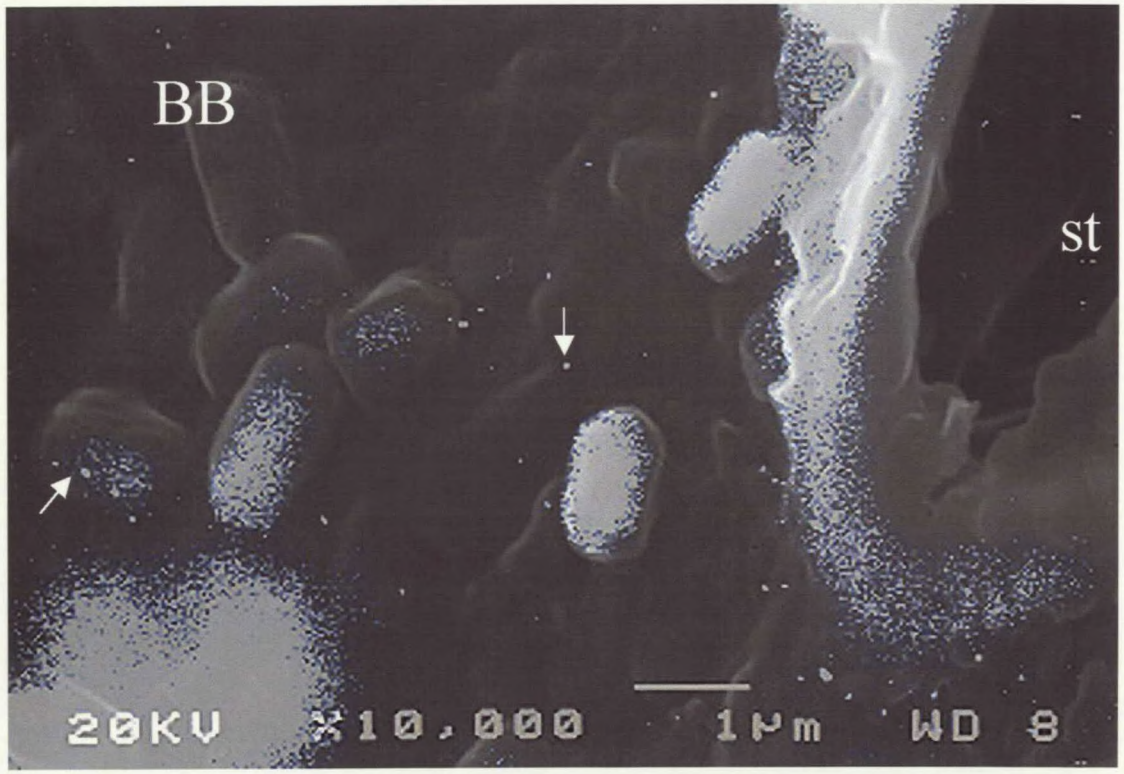


Figure 2-36. Scanning Electron and Back Scatter Electron Micrograph of BacB on a Sugar Beet Leaf Reacted with Primary Antibody and Secondary Gold Tagged Antibody. Only a few gold particles (arrows) are seen on BacB (BB) cells, and more in the vicinity of the cells. BacB cells are seen near a stomate (st).

Discussion

Log transformed data of BacB populations isolated from single leaves in the phyllosphere better fit a normal curve than do the same data presented without transformation (Figures 2-1 and 2-2). This result was expected, as most bacterial populations, especially phyllosphere populations, comply with this trend (Hirano *et al.*, 1982). Many studies, looking primarily at phytopathogenic bacteria, have demonstrated that bacteria are distributed lognormally on the scale of whole leaves (Hirano et al., 1982) as well as on the single leaf scale (Kinkel *et al.*, 1995; Morris *et al.*, 1997). This study demonstrated that populations of an epiphytic *Bacillus* sp. vary between leaves (Figure 2-1), and over the leaf surface (Figure 2-18), by as much as 10,000 fold. This variation leads to a highly skewed population if not log transformed.

To develop efficient methods of isolating BacB from whole leaves and leaf pieces, two different methods, leaf shaking and pulverizing, were compared for their ability to recover this *Bacillus* sp. BCA from the sugar beet phyllosphere. We were also interested in whether different preparations of the BCA would effect the yield of viable cells with different isolation methods.

The presence of the nutrient source significantly effected the yield of viable cells by the different methods. With 0% β-glucan, pulverizing recovered significantly more viable cells than shaking, while with 1% β-glucan, more viable cells were recovered by shaking, though the difference was not significant ($P>0.17$). There was no significant nutrient effect in this isolation ($p>0.75$), which is consistent with the experiments examining BacB distribution through time at different levels of β-glucan (Figures 2-7 and

2-8). The fact that the different isolation methods yielded different results with or without β-glucan may be evidence that BacB is colonizing the sugar beet phyllosphere in different ways with or without β-glucan. Shaking presumably isolates cells that are not as firmly attached to the leaf surface (Jacques and Morris, 1995). Perhaps there is a more intimate association between the BCA and the host plant in the absence of β-glucan. Without the supportive nutrient substrate, the BCA is required to be a prototypical saprophyte, surviving without assistance.

Alternatively, the opposed results of the two isolation methods with the two levels of β-glucan may be more a factor of the tremendous variation of the BCA over the leaf surface and between leaves, than due to the isolation method or colonization pattern at the different nutrient level. Kinkel and Lindow (1995) reported high variability in populations of *Pseudomonas syringae* applied to potato leaves at the leaflet, whole leaf and plant scales. Similar results were obtained in this study, with populations varying 10,000 fold on a per leaf or per leaflet scale. Therefore, the 12 leaf disks used for each treatment, was most likely not enough to deal with the inherent variation. More replication may provide significantly higher numbers from pulverizing, which would be consistent with other similar studies (Donegan *et al.*, 1991, Jacques and Morris, 1995). Pulverizing is highly labor intensive and time consuming relative to leaf shaking, and this must also be considered in determining a method for isolation.

Researchers tend to agree that the preferred method for reliable, efficient recovery of leaf-associated bacteria is with stomacher-blending (Donegan et al., 1991; Jacques and Morris, 1995). This equipment was not available, so shaking was chosen as the method

for isolating from whole leaves. Perhaps more important than the isolation method is to maintain a large number of leaves for replication. This is more easily accomplished with the relatively faster method of shaking.

On the leaflet scale total bacterial numbers will be less than on the leaf scale. Therefore, to obtain accurate counts at low numbers the method that could yield the highest numbers was essential. When applied as vegetative cells, pulverizing yielded significantly more bacteria ($p>0.01$), while the treatment effect was not significant for the spore formulation ($p>0.82$). Again, the fact that the different forms of bacteria application yielded different results with the isolation methods suggest that the bacterium is colonizing the beet phyllosphere differently when applied as a vegetative cell or spore formulation. Many more viable cells survived when the bacteria were applied as spores, but the results indicate that a significant portion of those cells surviving were not adhering tightly to the leaf surface. With the log phase cells, on the other hand, 1000 times fewer cells survived a three day period, but of those that did survive, their attachment to the leaf was not easily broken.

The grinding method is more easily performed on the leaflet scale than on the leaf scale. A hand held grinder is inserting into test tubes, each containing one leaf piece of known area. The grinder is easy to sterilize between samples and many samples can be processed rapidly. Therefore, this method was chosen for isolating the BCA at the leaflet scale.

Though endospores are undoubtedly an ecological advantage for the long-term persistence of Bacilli in natural environments, this trait may reduce their activity in the

phyllosphere. Given the importance of monitoring activity in autecology studies, as well as population size, we fractionated several populations for their spore and vegetative components.

The bacteria were applied to the field as spores, with different levels of β-glucan. A high percentage of spores were recorded for all levels of the nutrient source for a population isolated 24 August, 1998, 4 days after application (Table 2-5). There was no significant nutrient effect ($p>0.22$) though the 1% level of β-glucan had 76.9% spores, the lowest of the three levels of β-glucan. This trend was the same for a population isolated on 1 September, 1999, 4 days after application, though the level of spores was much lower for both levels of β-glucan. This lower level of spores is more consistent with what was reported by Kokalis-Burelle et al. (1992), who found 85-90% of a *B. cereus* population applied with chitin to be vegetative cells 7 days after application. The authors do not report what component of the population applied without chitin was vegetative cells.

At the leaflet level, 14 days after application, about half of the population monitored was spores. This experiment was repeated twice, and in one instance the population applied without β-glucan had significantly fewer percent spores ($p>0.12$). This was surprising as both isolations at the leaf scale indicated a lower percent spores with the nutrient source. However, histograms of the vegetative cell portion of the population indicate that on both days there are more areas of dense vegetative growth with the nutrient (Figures 2-5 and 2-6). The bulk of the data examining the fractions of this BCA population indicate that the nutrient source, β-glucan, can increase the activity

of the population, and lead to dense aggregates of the BCA. In terms of disease control, a more active population is likely to be advantageous. Microscopic observations of BacB (Figure 2-32), also from field samples from 14 days post application, revealed the occurrence of more than 100 cells in a $0.01cm^2$ area ($1X10^4$ CFU/cm^2, assuming they are all viable). These groups of cells were more common with β-glucan, however, they do not approach the concentration of aggregates of cells observed in natural biofilms by Morris *et al.* (1997).

The fact that total cells and total vegetative cells are highly correlated (Figures 2-5 and 2-6) is important for several reasons. Quorum sensing within an aggregate of cells may control the processes determining germination and vegetative cell growth of phyllospere Bacilli. Perhaps a critical mass of bacteria is necessary before germination and vegetative growth proceed. This also indicates that having only total CFU numbers, which are easier to collect than vegetative cell numbers, is a good estimation of total vegetative cells.

Figures 2-7 and 2-8 indicate that BacB populations do not persist at higher numbers in the presence of β-glucan. One leaf was used as one sample in these experiments, and the great variation between bacterial numbers from one leaf to another makes deciphering differences in population numbers difficult. Kokalis-Burelle *et al.*, (1992) reported higher populations of a *Bacillus cereus* BCA in the presence of a chitin formulation when sprayed onto peanut leaves in the field. We did expect to find similar results, and were surprised that the nutrient did not support higher levels of bacteria.

Another important result from this experiment is the rapid decrease in population numbers through time. Experiments I and II indicated approximately a 1000 fold decrease in the mean populations through the first 4 days, while experiment III showed a slower decline in the first few days, but a more rapid decline after day 4 (Figure 2-7). The highly predictable decline in viable cells also indicates BacB populations will persist on the leaf surface at around 100 CFU/cm^2 after a 14 day period (Figure 2-8). In terms of improved disease control, the rapid death of most of the cells sprayed onto the leaf suggests that a more frequent spray schedule should be considered.

Figures 2-9, 2-10, and 2-11 show more clearly some of the trends not readily deciphered in the scatter plot (figure 2-8). The population shifts from a right skew on day 4 to a more even distribution on day 7, to a left skew on day 14. There is also increased variation in the populations through time. This indicates that, though the chances for immigration were relatively equal on all leaves, only a few leaves are able to sustain high populations. Apparently the ability of BacB to successfully colonize and sustain in relatively large numbers is enhanced by β-glucan. By day 7, and more so by day 14, populations of BacB greater that 1000 CFU/cm^2 are more likely to occur in the presence of the nutrient source. However, the mode for the populations at the 3 levels of β-glucan remains about the same on all days, reflecting their approximately equal regression equation parameters (Figure 2-8).

None of the treatments in the Rif+ isolation experiment, including the wild type application provided disease control significantly better than the untreated control. This was surprising since a nearby experiment with the wild type did result in disease control,

though not the treatment with wild type + 1% β-glucan (Table 3-4). Disease pressure was severe during this season, and was more severe in this small plot than in the nearby experiment. The plot was located closer to the field edge and could have had a higher inoculum pressure. A final explanation for the lack of disease control in this experiment may be that leaves were harvested from nearly all of the plants at some point during the growing season, adding stress to the plants.

The spatial analysis of field and growth chamber applied BacB showed that the spatial distribution of this BCA could be modeled on the leaf scale. Interpolating bacterial populations by the relatively simple nearest neighbor method, or by the more involved kriging method, did not lead to significantly different contour maps in most cases. This is most likely because data was collected in a checkerboard fashion, and therefore the analysis began with an almost complete grid. Keckler (1996) indicates that nearest neighbor is a good gridding method for data collected in this fashion. However, this author also states that kriging with a linear model is a good method for most data sets.

A problem with using kriging for biological spatial data sets is the inherent assumption in the kriging methodology that the mean and the variance are homogeneous throughout the space being modeled, and are independent of their absolute location within that space (Bailey and Gatrell, 1995). Researchers in microbial phyllosphere ecology have consistently reported that bacteria have a preference for specific sites on the leaf surface, including trichomes, stomata, and epidermal cell wall junctions (see review by Beattie and Lindow, 1999). Indeed, the nearest neighbor interpolation of the 21 July,

1999 sample with 0% β-glucan indicates higher bacteria concentrations around the mid-vein and a secondary vein, while this pattern is lost with the kriging method. However, given past research indicating that phyllosphere bacteria have a bias for leaf veins (Kinkel et al., 1995), this nearest neighbor method is one I was more ready to accept, but is not the only way to model the data. Less arbitrary was the clear concentration of vegetative cells along the midvein seen in the 21 July, 1999 sample with 0% β-glucan (Figure 2-23).

In the 21 July, 1999 sample with 1% β-glucan, there was an inverse correlation between distance from the leaf base and number of viable cells (Figure 2-22). Trends like this one are violations of the assumptions of the kriging interpolation. With a more advanced analysis, however, it is possible to remove such a trend by a similar regression analysis, and strengthen the model.

The samples collected on 21 July, 1999 and 18 August, 1999 show more complete leaf coverage with BacB when the BCA was inoculated with 1% β-glucan. The nutrient substrate did not significantly increase the mean of whole leaf populations on most days (Figures 2-7, 2-8), though there was some indication that leaves with more than 1000 CFU/ml were more likely to occur with the nutrient. Similarly, leaf pieces with this level of density are apparently more common with the nutrient substrate. Of course, the draw back of decreasing the scale of an ecological observation to one leaf is that fewer leaves can be examined in the same amount of time. Nevertheless, with the few leaves examined here, those co-inoculated with the BacB and β-glucan showed better

colonization and survival by the BCA 14 days after application than those with no nutrient.

Modeling BacB density in the presence of *C. beticola* indicated that areas of dense BCA were less likely to have a high percent of disease. Again, the limits of reducing the scale of this ecological observation are troublesome. The trade-off for the detailed view of the BCA-pathogen interaction offered here is that only one leaf was examined. Given the great variation in microbial numbers between individual leaves reported here, and by others, the need for more similar maps is glaring. However, in that this method was experimental, using spatial statistics to model the BCA population, then assessing whether BCA density had a predictive value for percent disease, it was highly successful. Concerning mechanisms of disease control, these data indicate that competition or antibiosis might be important mechanisms in disease control, while hyperparasitism is probably not important.

The polyclonal antibodies developed against BacB did not react with any non-*Bacillus* sp. tested, though they did react with some other *Bacillus* sp. isolates. The fluorescent antibody technique was acceptable for locating BacB on the leaf surface, especially with the rhodomine gelatin blocking step. Auto-fluorescence from the leaf was severe in places, especially around stomates and within *C. beticola* lesions. Rhodomine tagged antibodies were also tried, and autofluorescence was reduced, but non-specific binding was more common without the gelatin blocking step. Finding and counting fluorescing bacteria on the leaf surface is time consuming, and will never generate as much data as quickly as culturing viable cells on a specific media. However,

the procedure does allow the researcher to bypass antibiotic resistant mutants and gather data on another scale.

When bacterial populations from leaves applied with BacB wild type 14 days previously were observed and counted with fluorescent microscopy, a high degree of bacterial aggregation was seen. Several of the leaf pieces examined had no bacteria, at all, while aggregates of 100 or more cells were found in some areas. These aggregates were more common with the nutrient substrate. This type of analysis could be improved with a band pass filter that allows only light within a narrow spectral range to pass through, eliminating much auto-fluorescence (Monier, 1999).

Immuno-scanning electron microscopy was moderately successful with growth chamber applied vegetative cells. It appears that the affinity of the antibody is not as great as is necessary for robust scanning electron microscopy (SEM) work. The coverage of the bacterial cells with gold particles was good with pure cultures on a silicon stub (Figure 2-35). With bacteria applied to leaf samples, however, coverage was not as complete as that reported by other researchers doing similar work (Davis and Brlansky, 1991). The affinity of the antibody could be improved by concentrating the IgG fraction of the antibody by precipitation or with protein A sepharose gel chromatography. Finding bacterial cells on a leaf with SEM is much more difficult that with fluorescent microscopy. With SEM it is first necessary to find cells believed to be the BCA, then move to back-scatter mode to see the gold particle. With fluorescent microscopy, if cells are reacting they quickly draw the attention of the researcher.

BacB, a *Bacillus subtilis* biological control agent, was monitored at many different scales in this autecological study. At the leaf and microscopic scale differences in the ability of a supportive nutrient substrate to effect the colonization of the BCA were seen, while at the plant or field scale, essentially no difference in total population numbers was seen due to the nutrient. A comparison of two different interpolation methods indicated the potential for using spatial statistics to model a bacterial population on the leaf scale. This analysis also indicated some tendency for the BCA to increase in numbers around leaf veins. Another population showed increased numbers near the leaf base. The geographical scale of the observations is immensely important in any spatial analysis, and it would be interesting to extend the microscopic methods used here to a more vigorous analysis of the BCA at this scale. Maps of the BCA in the presence of the pathogen *C. beticola*, indicate that competition and antibiosis are important in the mechanism of disease control, as areas with high populations of the BCA tended to have less disease.

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OPTIMIZING A *BACILLUS* SP. FOR BIOLOGICAL CONTROL OF SUGAR BEET CERCOSPORA LEAF SPOT

Introduction

Understanding the behavior of a biological control agent (BCA) in the environment in which it is expected to perform may lead to improved performance of the BCA. If certain behaviors can be correlated with better disease control, then it may be possible to encourage these behaviors and improve disease control. Understanding mechanisms and behaviors associated with disease control will allow researchers to move from the "spray and pray" approach of biological control to a more reliable and predictable product.

One "behavior" that *Bacillus* spp. are capable of is forming endospores. Endospores are resilient structures capable of surviving desiccation, heat, oxidizing agents, and UV and γ radiation (Setlow, 1995). These qualities confer exceptional ecological advantage to Bacilli, and allow for long term storage and relatively easy commercialization. Bacilli also produce a range of antibiotic compounds, inhibitory to fungi and Stramenopila (Utkhede and Sholberg, 1986), bacteria (Zuber *et al.*, 1993) and insects (Aranda *et al.*, 1996). Antibiotic production and storability make certain Bacilli desirable organisms for commercial BCAs. *Bacillus* sp. isolates have been demonstrated

to control early leafspot of peanut (Kokalis-Burelle *et al.*, 1992), yam leaf spot (Michereff *et al.*, 1994), postharvest apple diseases (Sholberg *et al.*, 1995), grey mould of strawberries (Swadling and Jeffries, 1996), and postbloom fruit drop of citrus (Sonoda *et al.*, 1996). "Kodiak", a *B. subtilitis* isolate has been registered for control of foliar diseases by Gustafson, Inc., Plano, TX (Sonoda *et al.*, 1996).

All of these experiments were done with bacteria from a 2-5 day culture, and therefore consisted of primarily vegetative Bacilli cells, with the exception of Sonoda *et al.* (1996). The authors specifically addressed the question of what provided better control of fruit drop of citrus *(Colletotrichum acutatum)*, vegetative cells or a spore formulation. Vegetative cells were applied as a 48h culture of *Bacillus subtilis* "Kodiak" grown in Luria-Bertani broth diluted 1:1 with water, while the spore formulation was applied at a rate of 1 g/l. Disease control was superior with the fresh cells in the 3 orange groves in the trial, and significantly ($p>0.05$) better in 2 out of 3 of the groves. The authors do not speculate on mechanisms involved with this enhanced disease control, nor do they address logistical problems associated with a grower applying vegetative cells that would not be encountered by simply mixing a spore powder with water immediately before spraying.

Of these studies, only Kokalis-Burelle *et al.* (1992) experimented with selective nutrient substrates in their applications. The authors used scanning electron microscopy to demonstrate that a chitin formulation extended the habitable area of the leaf surface for a *B. cereus* BCA. This was a notable achievement given that epiphytic microorganisms are generally restricted to cell wall junctions and areas around trichomes, stomates, or

veins (Blakeman and Fokkema, 1982). Chitin applied with the BCA decreased the number of leafspots per leaf by about 44% compared to the BCA applied alone.

BacB is a *Bacillus subtilis* isolate that has been demonstrated to provide control of Cercospora leaf spot (CLS) of sugar beets (*C. beticola*) in three previous field trials (Kiewnick and Jacobsen, 1998). Critical in choosing this *Bacillus* sp. isolate as a BCA were its ability to form an endospore, its ability to use chitin and β-glucan as substrates, its ability to inhibit the growth of *C. beticola* in vitro, and its ability to effect disease control better than 40% in field and glasshouse studies. Chitinolytic, β-glucanalytic and antibiotic activity increase the chances for complex ecological mechanisms in disease control. Fungal cell walls are composed of chitin and β-glucan, so an organism capable of cleaving these polymers could potentially use a fungal pathogen as a food source.

Mechanisms of control are thought to include antibiosis, induced systemic resistance (ISR), competition, and hyperparasitism. Antibiosis and hyperparasitism have been demonstrated in vitro (Jacobsen, 1999), while ISR has been demonstrated in growth chamber studies (Braun-Kiewnick *et al.*, 1998).

The goal of this research was to determine the effect of applied BacB population density and the concentration of β-glucan on disease control. Also, given the probability of ISR as one of the mechanisms of disease control, the concept of applying the BCA several days prior to inoculation with the pathogen was examined. These hypotheses were tested with growth chamber and field studies. In addressing the hypothesis that application of the BCA as vegetative cells will result in a more active population and provide significantly better control than a spore application, these treatments were

applied to plants immediately prior to inoculation in growth chamber studies. Finally, in an attempt to optimize the BCA for use in the phyllosphere, a pre-application treatment for the spore formulation, that could be reliably used by a grower, is designed and tested against the regular spore application in field trials.

Materials and Methods

Location of field trials and variety used

The 1998 and 1999 field trials were conducted with the sugar beet cultivars HH88 and KW2262 respectively, at the Eastern Agricultural Research Center in Sidney, MT, in a Savage silt loam soil. HH88, a more susceptible cultivar, could was not available for the 1999 season.

Disease control at different levels of β-glucan and BCA

In 1998, twelve treatments (Table 3-1) were applied with a back pack CO_2 sprayer (R&D Designs Inc.) at a rate of 1400L/hectare in a randomized complete block design with six replications. Treatments were applied 4 times, at 14 day intervals, during the season, beginning at disease onset on 9 July, 1998. Freeze dried spore preparations were suspended in tap water or β-glucan (Nurture 1080, Missoula, MT) solution for the appropriate final bacterial and nutrient concentrations. Disease ratings were done 6 August and 26 August, 1998 by rating 100 leaves per plot using the KWS-scale (Shane and Teng, 1992; Smith and Martin, 1978). Data were collected with a data logger and the % disease severity was calculated. Data was analyzed with SAS proc GLM and means separated by LSD (SAS/STAT ™, 1988).

Table 3-1. Combinations of Levels of BacB and β-glucan Tested in 1998 field Experiment

Wild Type BacB (CFU/ml)	Concentration (%) β-glucan		
$1 X10^9$	0.0	0.5	1.0
$1 X 10^8$	0.0	0.5	1.0
$1 X10^6$	0.0	0.5	1.0
$1 X 10^4$		0.5	1.0
none	0.0		

In 1999, fifteen treatments (Table 3-2) were applied in a randomized complete block design with six replications 5 times, every 14 days, during the season, beginning at disease onset on 8 July, 1999, as described above. Disease ratings were done 18-Aug, 1 September, and, 16 September, 1999. Disease ratings and analysis were identical to 1998.

Table 3-2. Combinations of Levels of BacB and β-glucan Tested in 1999 Field Experiment

Wild Type BacB (CFU/ml)	Concentration (%) β-glucan		
$1 X 10^8$	0.0	0.5	1.0
$1 X10^6$	0.0	0.5	1.0
$1 X 10^4$	0.0	0.5	1.0
$1 X 10^2$	0.0	0.5	1.0
none	0.0	0.5	1.0

Effect of applying BCA early

Induced systemic resistance appears to be a mechanism in disease control affected by BacB. Braun-Kiewnick *et al.* (1998) demonstrated a peak in peroxidase and glucanase activity between 3 and 7 days after application with BacB. An experiment was designed to determine the effect of applying BacB before inoculation with *C. beticola*. The growth chamber experiments were performed with 6 plants (cultivar HH88) per

treatment. There was an untreated control, and the BCA was applied 5, 3, 1, and 0 days before inoculation for a total of five treatments.

BacB treatments were prepared by adding the appropriate amount of spore formulation to PBS with 0.1% methyl cellulose for a final concentration of 10^8 CFU/ml. *C. beticola* inoculum was prepared by growing a pure cuture on V-8 agar for approximately 1 month at 24°C with 12 hours of light per day[1]. Spores were harvested by depositing 10 ml of sterile 0.1% methyl cellulose on a plate, then scraping well with a rubber policeman. The spore and mycelium mix was moved to a sterile container and macerated with a hand held grinder (Omni International *TH*). Spore concentration was checked with a hemacytometer, and diluted to $1X10^4$ spores/ml. BacB treatments *and C. beticola* inoculum were applied with a handheld sprayer at a rate of 10 ml/plant allowing for runoff

All 30 plants were moved into a growth chamber after the 5-day treatment was applied. The growth chamber was held at a temperature of 28°C for 12 hours and 26°C for 12 hours. Following the day 0 treatment, all but the control plants were inoculated with *C. beticola* spores at $1 X 10^4$ spores/ml. Plants were exposed to 100% humidity with two ultrasonic humidifiers for 48 hours continuously, then intermittently for 1 hour

[1] A single isolate of *C. beticola* was obtained from infected sugar beet leaf tissue periodically as follows:. A *C. beticola* lesion is placed in a humidity chamber for 24-48h; a 50 µl drop of sterile water is placed on the sporulating lesion and the spores are gently agitated from the lesion with a sterile needle; the water drop is sucked back up, and deposited on water agar optionally amended with 50 ppm streptomycin; another 50-100µl of water are added to the plate and the spores are spread around the center of the plate and the plate is incubated at 24°C for 18-24 h; the plate is then searched with a light microscope with a 4X lens for germinating *C. beticola* spores; a single germinating spore is transferred to PDA optionally amended with 50 ppm streptomycin, and incubated for 4-7 days; when a pure colony has grown to about 2.5 cm diameter, then an approximately 1 cm diameter agar plug is suspended in 10 ml sterile water and blended or macerated until a dark suspension results; this suspension is drop plated onto V-8 agar by depositing about 5, 100µl drops per plate. The V-8 agar plates are then incubated for about 1 month at 24°C with 12 hours

on and 1 hour off during the next two 12 hour night periods. Disease ratings were done with the KWS scale (Shane and Teng, 1992; Smith and Martin, 1978). Data were entered into a data logger and the percent disease severity and area under disease progress curve were calculated. Data was analyzed with SAS proc GLM and means separated by LSD (SAS/STAT ™, 1988). This experiment was repeated twice.

Log phase cells versus spores

Log phase cells were compared to a spore application for their abilities to effect disease control of *C. beticola* in growth chamber experiments done in May 1998 and in the winter and spring of 1999. In the May 1998 experiment, log phase cells were prepared by inoculating 50 ml of TSA with a loop of BacB from an agar plate, and growing on a shaker (Lab-Line® Orbit Environ-Shaker) with 50 rpms for 24h at 28 °C. The culture was centrifuged for 12 minutes at 10,000 rpm, the broth was decanted, and the pellet was resuspended in 30 ml potassium buffer solution (PBS –6.8g KH_2PO_4, 1.16g NaOH, 8.5g NaCl in 1 L distilled water). The concentration was adjusted to 2 X 10^8 CFU/ml using a spectrophotometer and established cell concentration curve at 600 nm. This was mixed with an equal amount of 0.2% methyl cellulose with 2% β-glucan for a final bacterial concentration of 10^8 CFU/ml in 0.1% methyl cellulose and 1% β-glucan. The spore treatment was prepared by adding the appropriate amount of spore formulation to PBS with 0.1% methyl cellulose with 1% β-glucan for a final

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of light per day. It will take the scrapings from about 7 plates to obtain a 500 ml solution of $1X10^4$ spores/ml.

concentration of 10^8 CFU/ml. Bacterial concentrations were confirmed by spiral plating onto 5% TSA, incubating for 24 – 48 hours and counting.

Cercospora beticola inoculum was prepared and the infection period was done as described above. Treatments and inoculum were applied as above, except inoculum was applied immediately after the BCA treatment. (see Effect of applying BCA early).

The experiment was repeated several times between January and May 1999 as described above, but the β-glucan was eliminated from the treatments.

Pre-application treatment

A set of experiments were performed to determine to what extent vegetative growth of BacB could be induced from the spore formulation in a short period of time and without relying on fermentation equipment. We were especially interested in developing a method that could conceivably be commercialized. Though more involved than mixing a powder and spraying immediately it would be relatively easily for a grower to use.

Germination in milk with shaking and no shaking. Fifty ml of 2% milk were pipetted into sterile 125 ml erlenmeyer flasks covered with aluminum foil. Treatments included shaking and no shaking. Shaking was done on a shaker (Lab-Line® Orbit Environ-Shaker; 50 rpms; 24°C), while the no shaking, or anaerobic treatment was done by placing flasks on a lab bench (24°C). Three flasks were used for each treatment with milk. In addition, two flasks were prepared with 50ml tryptic soy broth (TSB) and autoclaved. One of these was given the shaker treatment and one was set on the lab

bench. BacB was inoculated into each of the flasks as follows: 0.12g of spore formulation were mixed with 8.2 ml of water and pasteurized at 80°C for 20min; 1 ml of this solution was pipetted into each flask for a total concentration of 10^8 CFU/ml. The pasteurization step serves two purposes. It ensures that the experiment starts with 100% spores, and also breaks up the clumps that are common in the dehydrated spore formulation. This is necessary to accurately determine percent spores. Total CFU/ml and percent spores were determined for each flask at 0, 9, 14, 21, and 38 hours by making the appropriate dilutions and spiral plating (Spiral Systems Model C) onto 5%TSA, incubating for 24-48h at 28°C, then counting. Percent spores were determined by plating an aliquot of the solution before and after pasteurization. Samples were pasteurized for 20 min. at 80°C. Percent spores equals:

$$(CFU_{\text{after pasteurization}} / CFU_{\text{before pasteurization}}) * 100$$

Effect of concentration of BacB spores at time 0 on vegetative growth. This experiment was done to determine what effect the quantity of TSB (or concentration of BacB spores) would have on vegetative cell growth, starting with a constant number of BacB spores. There were 3 replications per treatment and the 3 treatments were 1 ml TSB, 10ml TSB, and 100ml TSB. All solutions were autoclaved and allowed to cool, before the pasteurized spore application was added. The quantity of spore application added yielded a concentration of 5 X 10^{10}, 5X 10^9, and 5 X 10^8 for the 1 ml, 10 ml and 100ml treatments respectively. The spore application was prepared in a manner similar to that described above (see Germination in milk…). The treatments were shaken well

and set on the lab bench. The solutions were plated as described above 20 h after inoculating the TSB and percent spores was determined.

Effect of form of BacB, β-glucan, and application timing on disease

To determine the effect of the form of BacB, β-glucan, and timing of application of disease severity, nine treatments (Table 3-3) were applied in a randomized complete block design with six replications. The early application treatments were begun 1 July, 1999, and the regular application treatments were begun 8 July, 1999. Treatments for the early application and regular application were applied 6 and 5 times respectively, every 14 days. Disease ratings and analysis were done identically to the 1999 field experiment described above. The log phase treatment was prepared by mixing 0.3g of the spore formulation with 40 ml of sterile TSA approximately 24 h prior to spraying. The solution was shaken well at time 0 and immediately before diluting to the spray volume of 2000 ml. Spore treatments were mixed immediately before spraying by diluting 0.3 g of the spore formulation and 40 ml of TSA to 2000ml, giving a final TSA concentration of 0.06% (1.2g TSA/2000ml).

Table 3-3. Nine Treatments in Field Experiment Assessing the Effect of Applying BacB Early, and Comparing Log Phase Cells to Spores, With and Without β-glucan. All Biocontrol Treatments Contained Approximately 10^8 CFU/ml and 0.06% TSB.

Early application	Regular application
BacB vegetative	BacB vegetative
BacB vegetative + 1% β-glucan	BacB vegetative + 1% β-glucan
BacB spores	BacB spores
BacB spores + 1% β-glucan	BacB spores + 1% β-glucan
Control (no application)	

Results

Disease control at different levels of β-glucan and BCA

The extreme disease pressure in 1998 resulted in an average percent disease severity (APDS) value of 9.4 in the untreated check (Table 3-4). The three treatments with the lowest APDS were BacB at 10^8 CFU/ml + 0% β-glucan, BacB at 10^9 CFU/ml + 1% β-glucan, and BacB at 10^6 CFU/ml + 1% β-glucan. A defoliating hailstorm on 1 September, 1998 prevented the three disease ratings necessary for an area under disease progress curve calculation.

Table 3-4. Average Percent Disease Severity for Different Levels of BacB and β-glucan in 1998 Field Experiment.

Treatment		Average Percent Disease Severity		
Level of BacB (CFU/ml)	Level of β-glucan (%)	6 August	26 August	
0	0.0	0.66[1]	9.40	A[2]
1 X 10^8	1.0	0.80	8.37	AB
1 X 10^4	1.0	0.77	7.80	ABC
1 X 10^9	0.5	0.84	7.58	ABC
1 X 10^6	0.5	0.76	7.52	ABC
1 X 10^4	0.5	0.58	7.00	BCD
1 X 10^8	0.5	0.69	6.79	BCD
1 X 10^6	0.0	0.77	6.71	BCDE
1 X 10^9	0.0	0.98	6.11	CDE
1 X 10^6	1.0	0.51	5.11	DE
1 X 10^9	1.0	0.77	4.85	DE
1 X 10^8	0.0	0.76	4.56	E

[1]P>0.54 for treatment effect.
[2]Means sharing the same letter are not significantly different at p>0.05. Means were separated by LSD.

The treatments across the bacterial levels of 1X10^6, 1X10^8, and 1X10^9 CFU/ml were analyzed in an orthogonal factorial analysis for main effects and interaction. The

results of this statistical analysis indicated that the most significant element of the experiment was the interaction between level of nutrient and level of bacteria (Table 3-5). Given this interaction, averages across bacterial levels or nutrient levels were not made. Instead, this interaction is portrayed in a scatter plot (Figure 3-1).

Table 3-5. Analysis of Variance for Factors and Interactions Effecting Disease Severity of Cercospora Leaf Spot in 1998 Field Experiment.

Source	DF	F	P>F
Model	13	2.9	0.005
Block	5	3.34	0.013
Nutrient	2	2.76	0.075
Bacteria	2	0.18	0.839
Nutrient X Bacteria	4	3.78	0.011

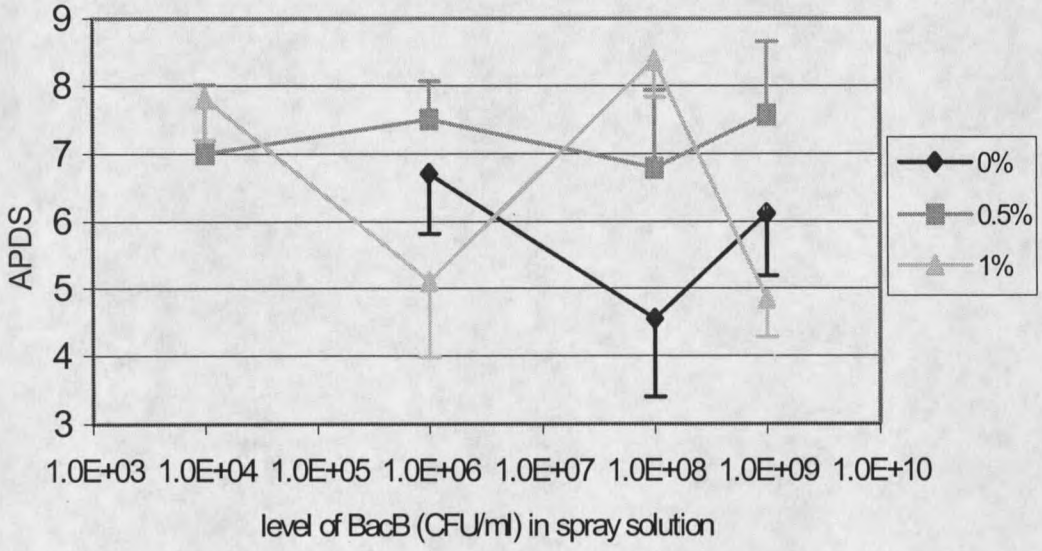


Figure 3-1. Interactions Between Level of BacB and Percent β-glucan. Bars represent 1 standard error.

As seen in the scatter plot (Figure 3-1), disease severity fluctuates widely at the 1% level of β-glucan. Disease decreases as BacB increases from $1X10^4$ CFU/ml to $1X10^6$, than increases as bacteria increases to $1X10^8$, only to decrease again at $1X10^9$. Disease severity remains fairly stable at the the 0.5% level of β-glucan, and these treatments provided the least disease control on average. Conversely, the 0% β-glucan level provided the best disease control. Disease severity was lowest at the $1X10^8$ CFU/ml level of bacteria, though not significantly different than $1X10^6$ or $1X10^9$ levels at α=0.05.

There was less disease pressure in 1999, with the 0 BacB + 0.5% β-glucan treatment reaching an average percent disease severity of 4.84 (Table 3-6). Treatment effects were not significant at any of the times that disease ratings were done nor was there a significant treatment effect for the area under disease progress curve.

Similarly, when this experiment was analyzed as a factorial, neither the model nor any of the main effects or interactions was significant (Table 3-7). Therefore, no averages across levels of bacteria or nutrient are considered.

Table 3-6. Average Percent Disease Severity (APDS) and Area Under Disease Progress Curve (AUDPC) of Cercospora Leaf Spot for Different Levels of BacB and β-glucan in 1999.

Treatment		APDS [1]			AUDPC [2]
BacB (CFU/ml)	(%)β-glucan	18Aug	1Sept	16Sept	
0	0.5	0.06	1.61	4.84	60.22
$1X10^2$	1.0	0.06	1.49	4.81	58.14
$1X10^6$	1.0	0.05	1.33	4.78	55.43
$1X10^4$	0.0	0.08	1.12	4.69	52.04
0	1.0	0.05	1.47	3.93	51.19
0	0.0	0.06	1.61	3.53	50.16
$1X10^4$	0.5	0.06	1.34	3.96	49.59
$1X10^8$	1.0	0.06	1.15	4.12	47.93
$1X10^6$	0.5	0.05	1.22	3.85	46.88
$1X10^6$	0.0	0.05	1.15	3.7	44.80
$1X10^4$	1.0	0.05	0.93	4.13	44.76
$1X10^8$	0.5	0.06	1.06	3.82	44.44
$1X10^2$	0.0	0.06	1.3	3.18	43.16
$1X10^8$	0.0	0.05	0.9	3.77	41.70
$1X10^2$	0.5	0.06	1.11	3.15	40.19

[1] Treatment effect 18 Aug $p>0.65$, 1 Sept $p>0.34$, 16 Sept>0.65.
[2] Area under disease progress curve $p>0.67$.

Table 3-7. Analysis of Variance for Factors and Interactions Effecting Disease Severity of Cercospora Leaf Spot in 1999 Field Experiment.

Source	DF	F	P>F
Model	19	0.91	0.57
Block	5	1.23	0.30
Nutrient	2	0.76	0.47
Bacteria	4	0.77	0.55
Nutrient X Bacteria	8	0.82	0.58

Effect of applying BCA early

The hypothesis that applying BacB prior to inoculation with *C. beticola* would induce systemic resistance in sugar beet, and result in superior disease control compared to applying the BCA immediately prior to the pathogen was tested in a growth chamber experiment. The experiment was done once in April, 1999, and once in May, 1999, and

spore formulation with no nutrient was used for all treatments. In the first trial, all treatments had significantly lower AUDPC values 18 days after application than the untreated control (Table 3-8). There was no significant difference in disease control between applications applied 0, 1, or 5 days before inoculation, while the application applied 3 days before inoculation did provide significanly better control than the other treatments.

Table 3-8. Effect of Applying BacB Spore Formulation at 10^8 CFU/ml Before Inoculation with *C. beticola* on Severity of Cercospora Leaf Spot, in Growth Chamber April, 1999.

Treatment (days before inoculation with C. beticola)	APDS[1]			AUDPC[1]
	Day9	Day14	Day18	
Inoculated control	0.7 A	3.5 A	11.9 A	41 A
0	0.3 BC	2.1 B	10.8 A	32 B
1	0.4 AB	2.2 B	9.4 A	30 B
5	0.4 BC	1.9 B	9.7 A	29 B
3	0.3 BC	1.3 BC	6.6 B	19 C
Uninoculated control	0.1 C	0.1 C	0.7 C	2 D

[1]Means followed by the same letter are not significantly different at $p>0.05$.

When the above experiment was repeated in May, 1999, a severe spider mite infestation prevented the recording of three days of APDS values, which are necessary for an AUDPC calculation. However, by day 17 disease severity was severe enough, though not as severe as that recorded in the April, 1999 experiment (Table 3-9), to discern some differences between treatments. A similar trend had begun to emerge, with treatments applied earlier providing better disease control. Treatments applied 0, 1, and 3 days before inoculation with *C. beticola* did not provide significantly better control than the inoculated control. The application made 5 days before inoculation did provide

significantly better control than the inoculated control, but was not significantly better than any of the other treatments.

Table 3-9. Effect of Applying BacB Spore Formulation at 10^8 CFU/ml Before Inoculation with *C. Beticola* on Disease Severity of Cercospora Leaf Spot, in Growth Chamber May, 1999.

Treatment (days before inoculation with *C. beticola*)	APDS[1] Day 17
Control	3.18 A
1	2.3 AB
0	1.8 ABC
3	1.35 ABC
5	1.1 BC
Uninoculated control	0.005 C

[1]Means followed by the same letter are not significantly different at $p>0.05$.

Disease control with vegetative cells vs. spores

The ability of fresh cells of BacB to control disease was compared to a spore formulation in growth chamber experiments. Disease control was significantly better with vegetative cells or with a combination of vegetative cells and spores, than with spores alone in a May, 1998 experiment (Table 3-10).

Table 3-10. Effect of Applying BacB at 10^8 CFU/ml as a Spore Formulation or Vegetative Cells on Severity of Cercospora Leaf Spot, in Growth Chamber May, 1998.

Treatment	AUDPC[1]
Control	73 A
BacB spores +1% β-glucan	56 B
BacB vegetative +1% β-glucan	38 C
Spore & vegetative +1% β-glucan	37 C

[1]Means followed by the same letter are not significantly different at $p>0.05$.

The above experiment was repeated three times in the winter and spring of 1999. In only one of these experiments was sufficient disease severity achieved to decipher

differences between the control and treatments. In this experiment (Table 3-11), no β-glucan was included in any of the treatments, and no significant differences were seen between spore applications and vegetative cell applications. BacJ, another *Bacillus* sp. BCA used in our lab, was also included in the experiment, and similar results were recorded for this BCA.

Table 3-11. Effect of Applying BacB at 10^8 CFU/ml as a Spore Formulation or Vegetative Cells on Severity of Cercospora Leaf Spot, in Growth Chamber April 1999.

Treatment	AUDPC[1]
Control	45.6 A
BacJ spores	18.7 B
BacJ vegetative	18.2 B
BacB spores	17.4 B
BacB vegetative	15.4 B

[1]Means followed by the same letter are not significantly different at p>0.05.

Formulation of pre-application treatment

Germination in milk with shaking and no shaking. Three flasks containing 2% milk were inoculated with spores and placed on a shaker, while three flasks containing milk were inoculated and left on a lab bench at 24°C. One flask containing TSB was shaken, while one flask containing TSB was placed on a lab bench. After inoculation with 100% BacB spores, total cells increased for all treatments, but most dramatically for the TSB shaker treatment (Figure 3-2). After 21 hours logarithmic growth had ceased for the shaker treatments, and the stationary phase was achieved. The anaerobic treatments had not leveled off by 38 hours.

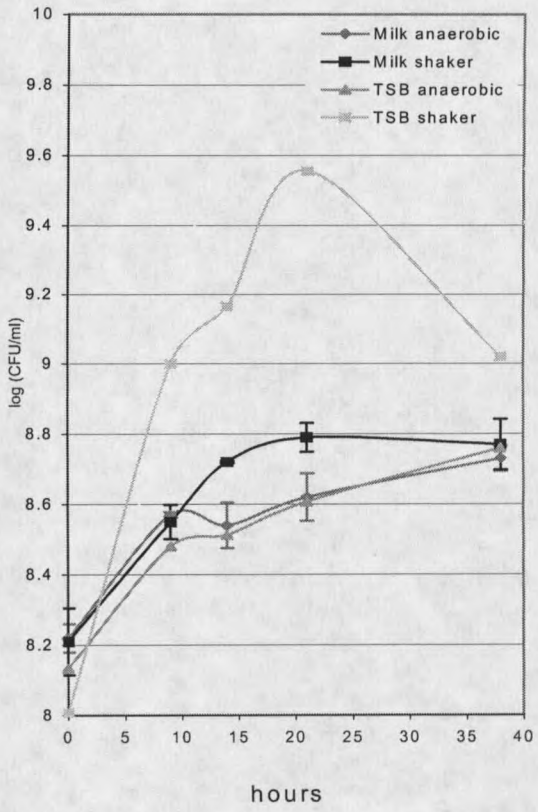


Figure 3-2. Total BacB CFU/ml with Shaker and Anaerobic Cultures. Treatments were started with 100% BacB spores at a concentration of approximately $1X10^8$CFU/ml. There were 3 replications for the two milk treatments and only one replication for each TSB treatment. The two shaker treatments appear to have reached a stationary phase after 21 hours, while the anaerobic treatments had not leveled off by 38 hours after inoculation of the media.

Percent spores decreased through time for all treatments (Figure 3-3). The TSB shaker treatment showed the most dramatic decrease, with a mere 0.2% spores at 9hrs. Percent spores remained near 0 at 14 and 21 hrs, then increased slightly to 3.6 at 38 hrs. The TSB anaerobic treatment decreased to 13% at 9hrs, and remained below 20% for the rest of the experiment, with only 3% at 38hrs. The two milk treatments declined more slowly than either TSB treatment, and only the aerobic treatment went below 20% spores. The aerobic treatment had significantly fewer spores than the anaerobic treatment at 14

hrs ($p>0.09$), and a less significant difference at 24 hours ($p>0.19$) and were essentially the same at 38 hrs.

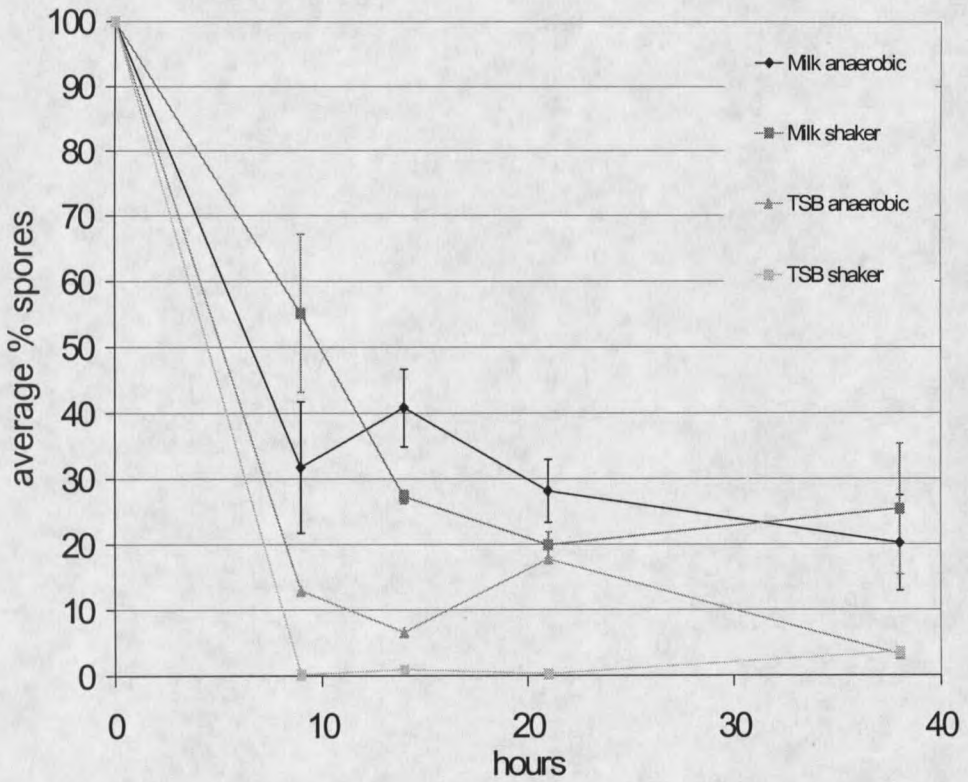


Figure 3-3. Average Percent Spores of BacB through time with Shaker and Anaerobic Cultures. Treatments were started with 100% BacB spores. There were 3 replications for the two milk treatments and only one replication for each TSB treatment. TSB induced germination and supported vegetative growth more effectively than milk.

Germination in different quantities of TSB. Given the dramatic decrease in spores over a 20 hr period in TSB (Figure 3-3) this media was chosen for a pre-application treatment. An experiment was conducted to determine the effect of different volumes of TSB on germination of BacB endospores. Percent spores decreased with increasing TSB (Table 3-12). The 100 ml volume of TSB was significantly different than the 1 ml volume ($p>0.05$) and the 10ml volume ($p>0.1$). The 10 ml volume was not

significantly different in level of vegetative growth than the 1ml volume. The pre-appliation treatment used for the field trial in 1999 (vegetative treatment) is a scale up of the 10 ml volume of TSB. Four times the amount of spore formulation was added to 40 ml of TSB, though the dried spore formulation was added directly to the TSB and not pasteurized first.

Table 3-12. Effect of Concentration of BacB Spores atTime Zero on Spore Germination in TSB after 20 Hours.

Volume of TSB (ml)	CFU/ml at time 0	% spores at 20 hrs[(1)]
1	$5X10^{10}$	50.5 A [A]
10	$5X10^{9}$	39.0 AB [A]
100	$5X10^{8}$	17.6 B [B]

[(1)]Means followed by the same letter are not significantly different at $p>0.05$. Bracketed letter indicates $p>0.1$.

Effect of form of BacB, β-glucan, and application timing on disease

This experiment was planted near the experiment testing disease control at different levels of BacB and β-glucan. However, the coefficient of variation (C.V.) for the AUDPC for this experiment was 17.43, while the other experiment had a C.V of 33.43. This reduced variation within treatments resulted in discernable differences and a highly significant treatment effect (Table 3-13). Disease pressure was similar, with an APDS of 5.28 for the untreated control on 16 September 1999. The four best treatments were the four treatments with no β-glucan, and all treatments provided significantly better control than the untreated control.

Table 3-13. Average Percent Disease Severity (APDS) and Area Under Disease Progress Curve (AUDPC) of Cercospora Leaf Spot for Early Applications of BacB at 10^8 CFU/ml as a Spore Formulation or as Vegetative Cells at Different Levels of β-glucan (0% or 1%) in 1999 Field Trial.

Treatment	APDS[1]			AUDPC[1]
	18Aug	1 Sept	16 Sept	
Untreated control	0.10	1.94 A	5.28 A	68.42 A
Spore + 1%β-gluc	0.07	1.35 B	4.45 AB	53.42 B
Vegetative +1%β-gluc	0.09	1.25 BC	3.86 BC	47.77 BC
Spore + 1%β-gluc (early)	0.07	1.22 BC	3.79 BC	46.64 BC
Vegetative + 1%β-gluc (early)	0.07	1.36 B	3.46 C	46.26 BC
Vegetative (early)	0.08	1.19 BC	3.67 BC	45.48 BC
Spore	0.07	1.06 BC	3.61 BC	42.91 C
Vegetative	0.07	0.97 C	3.43 C	39.56 C
Spore (early)	0.06	1.08 BC	3.13 C	39.45 C

(1)Means followed by the same letter are not significantly different at p>0.05. Treatment effect for 18 August p>0.59.

This test was also analyzed for nutrient, timing of application, and form of application (vegetative or spore) effects, as well as interactions between these factors (Table3-14). There was a highly significant nutrient effect (p>0.009), no significant form effect (p>0.729), and no significant time effect (0.55). There was a trend toward a significant form by time interaction (p>0.136), and less of a nutrient by time (p>0.271) or form by nutrient interaction (p>0.371).

Table 3-14. Analysis of Timing of Application, Form of Application, and Nutrient Factors and Interactions Effecting Disease Severity of Cercospora Leaf Spot in 1999 Field Trial.

Source	DF	F	P>F
Model	11	2.14	0.040
Block	5	2.19	0.077
Form	1	0.12	0.729
Nutrient	1	7.71	0.009
Time	1	0.37	0.545
Form X Time	1	2.33	0.136
Form X Nutrient	1	0.82	0.371
Nutrient X Time	1	1.25	0.271

There was significantly less disease without β-glucan, than with β-glucan, the vegetative treatments were better than spore treatments, and early applications were better than the regularly timed applications (Table 3-15). Vegetative cells were less influenced by the early application than was the spore application, accounting for the trend toward a significant form by time interaction (Table 3-16).

Table 3-15. Area Under Disease Progress Curve (AUDPC) of Cercospora Leaf Spot at Different Levels of b-glucan (0% and 1%), Different Forms of Application (Spore and Vegetative) and Different Application Times (Earl and Regular) in Field Trial 1999.

Main Effects	AUDPC[1]
Level of nutrient	
1%	48.52
0%	41.85
Form of application	
Spore	45.60
Vegetative	44.77
Timing of application	
Regular	45.91
Early	44.46

[1]Nutrient effect p>0.009, Form effect p>0.729, Time effect p>0.548.

Table 3-16. Area Under Disease Progress Curve (AUDPC) of Cercospora Leaf Spot for for Form by Timing Interaction in Field Trial 1999.

Form and timing of application	AUDPC[1]
Vegetative early	45.87
Vegetative regular	43.66
Spore early	43.04
Spore regular	48.17

[1]Form X Time interaction p>0.14

Discussion

A major goal of this research was to determine a minimum density of BacB and β-glucan needed to provide disease control. Data indicating that induced systemic

resistance is important in the mechanism of control encouraged us to investigate the effect of application timing on disease control. Ecological data indicated that a high percentage of the BCA, sprayed as a spore formulation, remained in the spore form 4 days after application (Table 2-6). This encouraged us to look more closely at the effect on disease control of the form of the BCA when it is applied. Growth chamber experiments showed that vegetative cells could improve disease control compared to a spore application. Spore formulations are good for their ease of commercialization and storability, but may not be the best form of the BCA for application. A good commercial BCA product must be able to be used dependably without contamination, or relying on fermentation equipment. Therefore, attempts were made to develop a pre-application treatment for the spore formulation that would encourage germination of spores and vegetative growth without fermentation equipment. This treatment was compared to the straight spore formulation in the field. Some of the treatments were applied earlier, in a further effort to optimize the BCA for control of Cercospora leaf spot.

Field disease control data from 1998 indicated that β-glucan has the potential to increase disease. Treatments with no β-glucan provided consistently better control than those with β-glucan (Table 3-4). β-glucan may favor disease by acting as a sticky substance for *C. beticola* conidia to adhere to, or by increasing the relative humidity on the leaf surface. However, even more important than the nutrient effect was the interaction between level of nutrient and level of BCA (Table 3-5, Figure 3-1). Disease severity at the 1% level of β-glucan varied widely between the 4 levels of BacB tested. Ecological data collected during the same season, with a rifampicin resistant strain of

BacB (BacB Rif+), indicated that the nutrient substrate has the potential to decrease the percent spores of the BCA population (Table 2-6). These data suggest that β-glucan may induce a more active population, at least at the 1% level. The 0.5% level of β-glucan consistently resulted in the highest disease, across all levels of the BCA tested. Percent spore data (Table 2-6) indicated that this level of nutrient did not significantly decrease percent spores in a population of BacB Rif+, further supporting the hypothesis that activity of the BCA population may be responsible for the interaction between nutrient and level of bacteria.

While the nutrient effect alone was significant ($p>0.075$) in 1998, the level of bacteria effect was not significant ($p>0.839$). Given the highly significant nutrient by bacteria interaction (0.011), it is necessary to consider increases in BCA in the spray solution at each level of β-glucan independently (Figure 3-1). There is clearly no increase in disease control at the 0.5% level, across the concentrations of BCA examined. As discussed previously, disease control varied widely at the 1% level, with the $1X10^6$ and $1X10^9$ CFU/ml levels of BacB providing significantly better control than the $1X10^4$ or $1X10^8$ CFU/ml levels. The $1X10^8$ CFU/ml level of BacB appears to have resulted in unusually high disease that could only be explained by the negative effects of β-glucan superceding any positive effects of the more active BCA population. Indeed, the $1X10^8$ CFU/ml level of BacB with 0% β-glucan provided the best disease control, though the level of control was not significantly different than the $1X10^9$ CFU/ml level. The $1X10^9$ CFU/ml level of BacB did not provide significantly better control than the $1X10^6$ level, indicating that a relatively low concentration of BCA can effect disease control without

any nutrient source. All levels of BCA tested at 0% β-glucan provided significantly better control than the untreated control.

The answer to the question of an optimum concentration of BacB and level of β-glucan to effect superior disease control with the spore formulation is a concentration of $1X10^8$ CFU/ml BacB with 0% β-glucan. However, there are complex interactions between the host plant, nutrient film, BacB, and *C. beticola*. In the absence of any nutrient, the BCA was active enough to effect disease control at a relatively high concentration of $1X10^8$ CFU/ml. On the other hand, at the lower application level of $1X10^6$ CFU/ml, disease control was equally good. The intricacies of these interactions may lie in the different methods of colonization employed by the BCA with and without the nutrient. Without the nutrient substrate, perhaps an intimate interaction emerges between the host plant and BCA that is not fostered in the presence of the nutrient.

The 1998 experiment examining disease control at different levels of BCA and β-glucan was not a balanced orthogonal experiment. This experiment was repeated in 1999 as a fully balanced orthogonal experiment (Table 3-2). The disease pressure was much lower in the cooler 1999 season and no significant differences were seen between any of the treatments (Table 3-6). When analyzed as a factorial there were no significant nutrient, bacteria, or nutrient X bacteria effects (Table 3-7). There is always high variation in Cercospora leaf spot ratings, and the rating system used was apparently not sensitive enough to detect differences with this high variation.

Induced systemic resistance refers to the cascade of genetic events resulting in the production of pathogenesis related (PR) proteins, such as chitinases, glucanases, or

peroxidases. The gene cascade is most commonly induced by attack by some incompatible pathogen. For example, a fungus or bacterium pathogenic to wheat, that landed on a sugar beet leaf, might elicit a compound to help it gain entry into the plant. Receptors on sugar beet leaf cells recognize this compound, and the gene cascade is induced. The production of PR proteins prevents infection of the incompatible pathogen (Van Loon, 1999).

Braun-Kiewnick et al. (1998) found that BacB, applied as fresh cells, has the ability to induce pathogenesis related proteins in sugar beet. The authors demonstrated a peak in peroxidase and glucanase activity 3 to 7 days after application. In this study, a growth chamber experiment was done to determine if applying BacB prior to inoculation with *C. beticola* would provide better disease control than inoculation immediately following BCA application. BacB spores, which have demonstrated good control in growth chamber experiments, were applied 0, 1, 3, and 5 days before inoculation with *C. beticola.* In the first growth chamber trial in April, 1999, all treatments provided significantly better disease control than the uninoculated control (Table 3-9). The treatment applied 3 days before inoculation with the pathogen provided significantly better control than the other applications. The application done 5 days before inoculation was better than 1 day before, and 1 day before was better than the application done immediately before inoculation, though none of these treatments were significantly different from each other. It is likely that BacB, not known to be a pathogen to any plant, including sugar beets, is producing a compound that elicits an ISR response in sugar beet. When given several days for this response to build, the sugar beet is prepared for

infection by pathogens. These plants with elevated defenses than have an advantage when challenged with *C. beticola.*

The endospore is perhaps the most important component of the ecology of *Bacillus* spp. Although the endospore provides protection from environmental flux, it is not likely this dormant stage of BacB is contributing to disease control. Fresh cells of BacB were compared against a spore formulation for their ability to effect disease control in a growth chamber test. The first time this test was done, the fresh cells provided significantly better disease control than the spore formulation (Table 3-10). This is consistent with what Sonoda *et al.* (1996) found in their study of a *Bacillus subtilis* BCA isolate marketed as "Kodiak". When repeated, this test again showed better control by vegetative cells, though not significantly better than the spore application.

Considering the mechanisms hypothesized to be responsible for disease control by BacB – competition, antibiosis, ISR, or hyperparasitism – certainly vegetative cells would be more responsible for these activities than endospores. But, the fact that spores do provide disease control, as seen in this May 1998 growth chamber experiment (Table 3-10) and in the field in 1998, indicates that a portion of the spores do germinate and are active in disease controlling activities. Furthermore, when this growth chamber experiment was repeated in April, 1999, no differences could be discerned between vegetative cell and spore applications (Table 3-11). Two of the proposed mechanisms, competition and antibiosis, could just as well be provided by endospores as by vegetative cells. The spore certainly occupies space, and it is not known whether the compound(s) responsible for antibiosis *in vitro* are present on the exterior of the cell. Some antibiotic

compounds on the exterior of the endospore would certainly be of ecological advantage to the microorganism.

Though it may be easy for a microbiology lab to ferment a BCA for a 48-72 hour period, centrifuge the cells into a pellet, and spray the BCA plus nutrient broth onto plants, this is not practical for a grower. We have hypothesized that the BCA will be more active and provide better disease control in the vegetative state than in the spore form. Growth chamber experiments have provided mixed evidence as to whether or not this is indeed the case. Nevertheless, we undertook a series of experiments to develop a reliable method that a grower could use to encourage vegetative cell growth from a spore application. This would allow for the ease of storability provided by the spore formulation, while contributing the activity of vegetative cells at the time of application.

One set of pre-application experiments considered 2% milk as a substrate for promoting vegetative growth from a spore formulation. Milk was chosen because it is nutritious, easily available and relatively free of microbes. Milk did indeed promote cell growth (Figure 3-2) and reduce percent spores (Figure 3-3) over a 38 hour period but not to the extent of tryptic soy broth (TSB). There was also contamination in most of the milk containers after this short period. Therefore, TSB was chosen as a medium for the pre-application treatment.

It was decided to use a small amount of TSB, 40ml, for the pre-application treatment. This amount was also added to the spore treatments at the time of application, to control for the TSB added with the vegetative cell application. The small amount was chosen, because we wanted to know the effect on disease control of allowing cells to

germinate before application, not the effect of TSB. Enough of the spore formulation was added to the 40 ml of TSB to provide a final concentration of $1X10^8$ CFU/ml in the 2000 ml volume of the spray solution. This resulted in approximately $5X10^9$ CFU/ml in the 40ml TSB solution at the beginning of the 24 hour period. Table 3-12 shows that this concentration of spores, in a scaled down version of the pre-application experiment, resulted in an average of 39% spores after 20 hours. Concentration of BacB spores at time 0 is definitely important in inducing germination and vegetative growth, and starting with a less concentrated solution (using more TSB) in a future pre-application experiment should be considered.

When the pre-application, or vegetative cell, treatment was compared to a spore application, there was no indication that the pre-application treatment resulted in improved disease control. What was made clear by this experiment is that 1% β-glucan increased disease severity in all treatments. β-glucan was originally included in spray solutions because it was thought that the selective nutrient substrate would increase the habitable area of the leaf for the BCA, similar to how a chitin formulation increased the habitable area of a *Bacillus cereus* BCA on peanut leaves (Kokalis-Burelle et al., 1992). Chitin application also increased the percentage of chitinolytic microorganisms present on the leaf surface, and was itself able to decrease disease as compared to untreated plants. Interestingly, the peanut disease being controlled by Kokalis-Burelle et al. (1992), early leafspot of peanuts, is also caused by a *Cercospora* sp., *C. arachidicola*. A major difference between these two studies, besides the crops being studied, is the amount of spray used per acre. Kokalis-Burelle et al. (1992) applied 650 L/ha, while we sprayed

140L/ha, similar to fungicide spray volumes commonly used by farmers for Cercospora leaf spot control in sugar beets.

There was a significant nutrient effect, but no significant effect due to applying treatments early, or, surprisingly due to the form of the BCA. There was a significant form X time interaction ($p>0.14$). In the spore form, timing of application was more important than with the vegetative cell form (Table 3-16). This may be explained by the ISR mechanism. If it takes the spore a longer time to germinate and "alert" the plant to its presence, then any gene cascades and PR protein production would be delayed as compared to vegetative cells.

In any case, it is clear that a spore application with no β-glucan can provide disease control. The bulk of the ecological evidence suggest that β-glucan enhances vegetative growth (Table 2-6), including the development of areas of dense vegetative growth (Figures 2-8, 2-9). However, there is some degree of germination in the absence of β-glucan on the leaf surface. This trade-off – less activity for escaping the detrimental effects of the nutrient film on the leaf – apparently is justified. The formulation of vegetative cells tested here did not provide better control than a spore formulation, but another formulation of vegetative cells might. Germinating cells in more TSB, though not as economical or practical as a small amount of TSB may provide a more active population and better disease control. Also, mixing of a large amount of fermented broth with the spray solution was not detrimental to disease control of fruit drop of citrus as reported by Sonoda et al. (1996).

Biological control of Cercospora leaf spot is not an easy proposition. Fields not sprayed with fungicides literally wilt and turn brown in severe disease years, such as 1998. Even in less severe disease years, such as 1999, the cost of a fungicide program is rewarded in increased root tonnage, increased sugar content, and decreased impurities. Fungicide use is intense in all areas where sugar beets are grown, and resistance in *C. beticola* to commonly used fungicides is increasing. The idea of using a BCA as part of a disease control strategy is appealing. The multiple mechanisms involved in the control of *C. beticola* with BacB indicates the potential for using this organism to manage resistance. However, acceptable disease control has not been achieved by BacB alone. This study has taken an ecological approach in an attempt to optimize this *Bacillus* sp. for biological control. More investigations into increasing the activity of the population before application, and then maintaining activity *in situ* are necessary. The pre-application treatments presented here may be a step in the right direction, that could benefit other *Bacillus* sp. BCA programs.

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