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.

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

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APPROVAL

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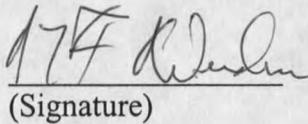
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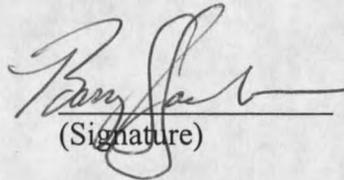
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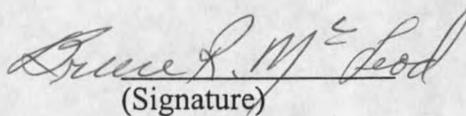
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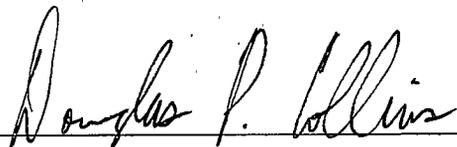(Signature)   (Date)

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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 1×10^6 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°C 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 (10^7 CFU/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 $1 \times 10^8 CFU/ml$ 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^oC, 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 1×10^8 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 1×10^4 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$^+$™ ; 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$^+$™ (Gamma Design Software, MI). GS$^+$™ 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$^+$™ 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