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The Investigation of Several Species of Bacillus to Determine their Potential as Biocontrol Organisms on Wheat

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THE INVESTIGATION OF SEVERAL SPECIES OF *BACILLUS* TO DETERMINE THEIR POTENTIAL AS BIOCONTROL ORGANISMS ON WHEAT

BY

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BIOCONTROL ORGANISMS ON WHEAT

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Doctor of Philosophy, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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THE INVESTIGATION OF SEVERAL SPECIES OF *BACILLUS* TO DETERMINE THEIR POTENTIAL AS BIOCONTROL ORGANISMS ON WHEAT

Abstract

VENANCE H. LENGKEEK

Under the supervision of Dr. Jack D. Otta

Of twenty *Bacillus* species tested, only five were capable of *in vitro* inhibition of several wheat pathogens. The mode of inhibition of these five *Bacillus* species was due to the production of an extracellular peptide antibiotic which caused disruption of the plasma membrane. Various other physical and chemical characteristics of the antibiotics were also investigated. When applied as seed treatments on spring wheat, the five *Bacillus* species did not produce significant yield differences. Low soil temperatures at spring wheat planting may have been responsible for the failure of response. The five *Bacillus* species, *B. subtilis* var. *niger* (originally received as *B. globigii*), *B. subtilis*, *B. polymyxa*, *B. thuringiensis* subsp. *sotto* and *B. subtilis* (originally received as *B. uniflagellatus*) required a temperature of 14 C or higher to grow and a temperature of 16 C or higher to produce their antibiotic(s). Our results also indicated that six phytopathogenic organisms, used as test organisms, are capable of growth at temperatures of 10 and 12 C. Soil temperatures at spring wheat planting time are generally 7-10 C and may be too low to allow for *Bacillus* success as a biocontrol agent.

INTRODUCTION

Control of one organism by another, biological control, is a phenomenon that is currently receiving much attention. Baker and Cook (7) define biological control as "the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host or antagonist, or by mass introduction of one or more antagonists."

Microorganisms such as bacteria, actinomycetes and fungi may have potential as biological control agents (5, 7, 13, 14, 20, 38, 40, 47, 48, 49, 50, 54, 58, 64, 70, 71, 72). In many instances the presence of these microorganisms in a soil is responsible for low plant disease incidence though both host and pathogen are present (7, 8, 23, 36, 60, 71).

Biological control by microorganisms is largely through passive antagonism and is accomplished through accidental contact with the pathogen. Antagonistic effect is dependant upon the group rather than the individual (7), and works through antibiosis and lysis, competition, and/or parasitism and predation (1, 2, 3, 18, 61).

An organism should satisfy several requirements to be useful as a biological control agent (7). It should produce large quantities of inoculum; resist, escape or tolerate other organisms; germinate and grow rapidly; invade and occupy organic substrates; grow near or in the pathogens environment; produce, even on simple substrates, a broad spectrum, highly toxic antibiotic that retains its potency and is not inhibitory to other associated antagonists or harmful to the

host plant; be adapted to large scale commercial production; and be more adaptable to environmental changes than the pathogen. No organism meets all these qualifications, but some spore forming bacteria of the genus Bacillus (Fischer) satisfy several of them (2, 7, 9, 13). They are easily grown on most media, germinate and grow rapidly, efficiently produce antibiotics, readily form resistant spores and are widely distributed in soils.

Several Bacillus species have been studied as biological control agents for reducing plant diseases (7, 13, 16, 20, 37, 38, 44, 46, 48, 53, 58, 66, 71). Merriman et al. (47) applied 10^6 to $3x10^7$ Bacillus subtilis A 13 cells per oat (Avena sativa L. 'Avon') seed and found significant yield increases of 40-45 per cent. Price (57) inoculated barley (Hordeum vulgare L.), wheat (Triticum aestivum L.) and oats (Avena sativa L.) with 10^6-10^7 B. subtilis A 13 cells per seed and found increased tillering and dry weight of wheat and oats and increased yields of oats and barley. Mann (44) found that addition of Bacillus uniflagellatus to soil of tobacco plants (Nicotiana tabacum L. 'Samsun' and 'Xanthi-nc') reduced local lesions in 'Xanthi-nc' and systemic infection in 'Samsun' caused by tobacco mosaic virus (TMV). Kommedahl (38) inoculated corn (Zea mays L. 'Minhybrid 508, 5302 and 6302') with Captan, Chaetomium globosum and B. subtilis in 1968, 1969 and 1970. B. subtilis had no effect in 1968 and 1970 on the average yield of three corn hybrids, but improved yields significantly in 1969. Differences in soil temperature and moisture during the three years of testing led Kommedahl to postulate that "there may be a relationship between the effectiveness of some organisms as seed

treatments and soil moisture and temperature." The inconsistancy in the effect of the antagonists may be due to "factors of soil temperature and soil moisture, with B. subtilis needing a moist soil to be effective" (38).

The success of a Bacillus species in biological control depends primarily upon production of an antibiotic inhibitory to certain plant pathogens (7, 16, 17, 19, 20, 28, 38, 44, 52, 53, 54, 56, 58, 62, 65, 66, 69, 71). However, growth-stimulatory factors produced by Bacillus subtilis may also be responsible for increased yields in certain plants (46, 47).

Over 62 antibiotics produced by various Bacillus species have been reported (6, 22, 25, 33, 39, 56, 60, 67). Thirty of these are produced by Bacillus subtilis. Of these 30 only fungistatin, bacillomycin, bulbiformin, eumycin, fungocin, mycosubtilin, mycobacillin, toximycin and two other unnamed compounds are chiefly antifungal in nature. Korzybski et al. (39) lists the spectrum of activity of several of these antibiotics.

Frobisher (22) states that the mode of action of bacillary antibiotics is that of a surfactant. Cell membranes are quickly destroyed due to destruction of the lipid fraction, but the cell wall remains intact. Gottlieb and Shaw (25) conclude that the main effect of bacillary antibiotics (eg. bacitracin) is the "disruption of the integrity of the cytoplasmic membrane." Newton (51) found that certain Bacillus-produced antibiotics affect cell membrane permeability and thus allow for rapid leakage of low molecular weight materials (eg. inorganic ions, amino acids, purines and pyrimidines) from the

cell. Vasudeva (67) found that bulbiformin, produced by B. subtilis, induced characteristic "bulb-like" formations on germinating fungal spores and hyphal ends.

Bacillary antibiotics all have similar chemical constituents. All are polypeptides and contain rare D-series amino acids as well as non-protein amino acids (11, 25, 32, 33, 39). All normally contain ornithine or di-aminobutyric acid (25, 32) and may be long-chained, branched or cyclic in structure. In general they have a low molecular weight of around 1400 and contain approximately 10 amino acids per molecule (32).

The fact that antibiotics arise late in the growth cycle, after the exponential growth phase but preceeding sporulation (25, 32), has led to theories that antibiotic synthesis may be related to spore formation (10). However, these theories are still controversial (32).

Several authors (31, 32) suggest that bacillary antibiotics are either by-products of metabolism or products of incomplete metabolism which do not accumulate under conditions most favorable for growth of the producing culture. When conditions become less favorable for growth these by-products are bound by specific enzymes to form a particular antibiotic (32). The exact process of antibiotic formation is unknown.

Simple assay procedures have been developed to test for antibiotic production in vitro (7, 15, 17, 29, 53, 54, 71). The majority of these procedures involve inoculation of the pathogen and test organism on the same plate, followed by observation and measurement of the mutual effects of their growth. If antibiotic production

occurs, inhibition zones are formed in which the pathogen will not grow. Various media have been used to conduct the assays, but Wood and Tveit (71) suggest using media "as close as possible to the natural substrate" to test for antibiotic production. Antibiotic assays on agar are a primary method of determining if antibiotics are produced, but do not necessarily indicate antibiotic production in the soil (4, 7, 71).

In the past the media used and the conditions involved for assaying for antibiotic production in vitro have generally favored the antagonist (7). Wood and Tveit (71) have suggested that "a range of temperatures be used in screening tests and that organisms selected for further tests in disease control should have growth-temperature relations similar to those of the pathogen. Tests of antagonism in pure culture are of greatest value when conditions approach, as closely as possible, those at the site of infection. The temperature for screening tests should fall within the range in which the disease would be expected to develop, and nutrient levels should be those of natural substrate wherever possible."

The success of a Bacillus organism as a seed-treatment biological control agent depends upon its ability to grow in the soil, produce its antibiotic in the soil and produce an antibiotic which maintains its biological activity in the soil (41). Growth in the soil may be the most important factor since growth preceeds antibiotic production (25, 32).

The ability of a Bacillus to grow in the soil is influenced by three main environmental conditions; soil moisture, soil temperature

and soil nutrients and organic matter (2). Other factors such as pH, O_2 and CO_2 content and mineral content may also, to a more limited extent, affect the growth of a Bacillus organism in the soil.

Kommedahl (38) found that Bacillus subtilis required more soil moisture to be successful in increasing corn yield than did the bio-control Chaetomium globosum. Alexander (2) states that "maximum bacterial density is found in regions of fairly high moisture content often at levels of 50 to 75 per cent of the soils' moisture-holding capacity."

Temperature governs all biological processes, and is a prime factor affecting bacteria. Gordon (24) states that the minimum temperature for Bacillus subtilis growth is between 5 and 20 C, while the optimum growth temperature is between 28 and 40 C (9, 43). Chang and Kommedahl (16) found that B. subtilis, grown in vitro, required a temperature of 16-30 C for good growth. At a temperature of 10 C the organism did not grow at all. Temperature not only affects the ability of the bacteria to grow, but also affects the organisms' ability to produce its inhibitory substance. Vasudeva et al. (65) found that temperatures between 24 and 34 C resulted in production of the greatest amounts of the inhibitory substances formed by B. subtilis.

Population size of microorganisms is directly related to organic matter and nutrient content of the soil (2, 22). It has also been found that the quality as well as quantity of antibiotics produced by various Bacillus species depends upon the nutrients available (12, 20, 66, 67, 68). Addition of organic as well as inorganic compounds directly influences Bacillus growth as well as antibiotic activity.

Thus, the biological control method of "green manuring" not only influences Bacillus growth but also antibiotic production.

When moisture, temperature and organic matter content are optimum for Bacillus in the soil, their numbers may vary from 10^6 to 10^7 or more organisms per gram (2). Commonly, some 5 to 20 per cent of the organisms of the A horizon are strains of Bacillus, with B. megaterium, B. cereus and B. subtilis most frequently encountered. In areas where conditions are not optimum, Bacillus species are probably found in the spore state, persisting in this dormant condition for many years.

Antibiotics produced in the soil by organisms of the genus Bacillus must remain active for a period of time for the bacteria to be successful as a biocontrol organism. The only sure method of determining antibiotic production or maintanence in soil is assaying for antibiotic presence. Several methods have been used to extract and assay antibiotics from the soil (26, 30, 34, 35, 45, 59, 63, 66).

Vasudeva et al. (66), using an n-butanol extraction method, found that Bacillus subtilis antibiotic production peaked in the soil at 7 days. They also found recovery rates of bulbiformin to be 50% using this method, and found that bulbiformin activity decreased 50% in ordinary, unsterilized soil in 7 days and was completely destroyed in 28 days.

Other antibiotics are known to be broken down in the soil (26, 30, 34, 45, 59). Christensen and Davies (17) reported that an antifungal substance produced by Bacillus mesentericus was destroyed or removed in sand, clay and loam, with clay being the most efficient.

Vasudeva (65) found that an antifungal substance produced by Bacillus subtilis lost its active principle rapidly when filtered through soil.

Antibiotic inactivation in soil may be the result of one or more of three distinct processes (55): intrinsic chemical instability of the antibiotic molecule, adsorption on clay minerals and organic matter, or microbial degredation.

Investigators (17, 65) have found that bacillary antibiotics tend to be adsorbed on clay particles very readily. The adsorption by clay minerals results in expansion of the crystal lattice and flocculation of the clay (55) resulting in a "trapped" antibiotic which loses its biological activity.

Since antibiotics are natural products, they are probably all susceptible to microbial attack and degredation in the soil. The rate of decomposition is dependant upon the nature of the antibiotic and is influenced by the physical, chemical and biological characteristics of the soil (55). Vasudeva et al. (66) found that bulbiformin was degraded more rapidly in non-sterile soils than in sterile soil. This difference can probably be explained on the basis of microbial activity.

The objective of this research was to determine the effect of coating wheat seed with several species of the genus Bacillus known to be antagonistic towards various wheat pathogens and, to determine what environmental factors in the soil may determine the success or failure of these species as biological control agents.

MATERIALS AND METHODS

Organisms studied. - Nineteen Bacillus cultures were obtained from P. R. Middaugh, Microbiology Dept., South Dakota State University, Brookings, South Dakota. The cultures obtained were Bacillus brevis Migula, B. cereus Frankland and Frankland, B. cereus var. mycoides (Flügge) Smith, Gordon and Clark, B. circulans Jordan, B. coagulans Hammer, B. cylindricus Meyer and Blau, B. licheniformis (Weigmann) Chester, B. ligneus Macé, B. macerans Schardinger, B. megaterium de Bary, B. polymyxa (Prazmowski) Macé, B. pumilis Meyer and Gottheil, B. sphaericus Meyer and Neide, B. subtilis (Ehrenberg) Cohn, B. subtilis var. niger Smith, Gordon and Clark (originally received as B. globigii Migula), B. subtilis var. niger Smith, Gordon and Clark, B. thuringiensis Berliner, B. thuringiensis subsp. sotto Heimpel and Angus, B. thuringiensis subsp. subtoxicus Heimpel and B. subtilis (Ehrenberg) Cohn (originally received from Dr. E. W. Mann, Hershey Chocolate Company, as B. uniflagellatus Mann). For simplicity sake, cultures received under a different name than listed in Bergey's Manual of Determinative Bacteriology (8th ed., 1974) will be referred to by the original name. These particular Bacillus species were chosen for several reasons. B. cereus, B. megaterium and B. subtilis represent the most important Bacillus species found in the soil, while B. brevis, B. circulans, B. licheniformis, B. polymyxa and B. pumilis are important producers of antibiotics. B. thuringiensis and B. uniflagellatus have been used as biological control organisms and B. coagulans, B. cylindricus, B. ligneus, B. macerans and B. sphaericus were originally isolated from a soil habitat and are naturally

occurring organisms in the soil.

All Bacillus cultures were tested for ability to inhibit, in vitro, seven different wheat pathogens, Fusarium roseum f. cerealis (Cke.) Snyder and Hanson, Gleosporium bolleyi Sprague (cultures G-2 3uc, G-2 1tc2, G-2 1tal and G-2 4ual), Helminthosporium sativum Pamm, Pythium graminicola Vanterpool (culture 169), Rhizoctonia solani Kuehn (cultures R-4 and R-267), Pseudomonas syringae Van Hall and P. atrofaciens (McCullock) Stevens (cultures 529 and 530). The Fusarium and Helminthosporium cultures were obtained from G. W. Buchenau, Plant Science Dept., South Dakota State University, Brookings, South Dakota, the Gleosporium and Pseudomonas cultures from J. D. Otta, Plant Science Dept., South Dakota State University, Brookings, South Dakota, and the Pythium and Rhizoctonia cultures from G. Semenuik, Professor Emeritus, Plant Science Dept., South Dakota State University, Brookings, South Dakota. The Fusarium, Helminthosporium, Gleosporium, Pythium and Rhizoctonia cultures were isolated from root or crown lesions on wheat. The Pseudomonas cultures were isolated from necrotic wheat leaves.

Antibiotic assay. - The basal medium consisted of a glucose yeast-extract agar (44), containing agar, 20 g; glucose, 10 g; yeast extract, 2 g; but modified to contain 2 ml soil extract. These nutrients were added to 1 liter of distilled water. The soil extract was prepared by suspending 77 g of soil and 0.2 g of Na_2CO_3 in 200 ml of distilled water. This solution was allowed to settle for 24 hr at 22 C before being filtered through cheesecloth. The extract was sterilized before use by autoclaving one hr at 121 C at 1.1 kg/cm^2.

Petri dishes, each containing 10 ml of agar, were prepared. Two

circles, 9 and 40 mm in diameter were centered and drawn on the bottom of the plates. The fungal organisms were grown on glucose yeast-extract agar and a 9 mm diameter section of hyphae and medium was removed and placed on the basal medium in the center of the plates. Each Bacillus culture was streaked at four places on the periphery of the larger circle. Testing the Pseudomonas species against the Bacillus species involved a different procedure. A line was drawn on the bottom through the center of the plate and the Bacillus species were streaked on the medium above this line. The Pseudomonas species were then streaked at right angles to the Bacillus species.

The inoculated plates were incubated at 22 C for seven days to allow for pathogen and bacterial growth and antibiotic production. Zones of inhibition were recorded and rated on a scale of 0-4; 0 indicating no effect of the Bacillus species on pathogen growth, 1 indicating slight inhibition, 2 indicating moderate inhibition, 3 indicating extreme inhibition and 4 indicating total inhibition. Only those Bacillus species which inhibited the pathogens in vitro were used for further studies since those which produced no inhibition zones on agar probably would not produce inhibitory substances in the soil (7).

Electron microscopy. - Helminthosporium sativum mycelium from the zone of inhibition was studied with the electron microscope as follows: Agar sections containing mycelium were taken from the edge of the inhibition zones and transferred to 5% glutaraldehyde in 0.05m potassium phosphate buffer (pH 7.0) for 15 minutes followed by post fixation for 15 min in 1% OsO_4. The mycelium was dehydrated in a graded series of acetone. Epoxy resins were used for the embedding

medium. The mycelial sections were placed vertically in micromold (Micron Product) capsules and plastic was added. The plastic was allowed to soak into the material for 3-4 hours and the capsules were then placed into a 60 C oven for 24 hours to complete polymerization of the plastic. Thin sectioning of the plastic-embedded material was accomplished with a Porter - Blum - MT-2 ultramicrotome using a glass knife. Thin sections were placed on a 200 mesh grid and stained in a 2% uranyl acetate solution for 20-30 minutes followed by staining in lead citrate for 2 minutes. The thin sections were observed with a Hitachi HU-12 electron microscope.

Characterization of antibiotic properties. - Although attempts were made to isolate and purify the antibiotic(s) from the five Bacillus species, yields were too low to allow further work. Thus a semi-concentrated antibiotic solution, which was active at fairly high dilutions (1:1000), was used for study of the properties of the antibiotic(s).

The semi-concentrated solution of the antibiotic(s) produced by each Bacillus culture was prepared as follows: The cultures were grown in 100 ml glucose yeast-extract broth for four days at 32 C. This solution was centrifuged at 10,000 rpm for 30 minutes at 4 C in a Sorval RC-2 centrifuge. The supernatant liquid was saved and evaporated to 1/10 volume on a Buchler Flash-Evaporator. The remaining solution was adjusted to pH 2.5 and sterilized at 121 C at 1.1 kg/cm^2 for 15 minutes. Preliminary studies had shown the antibiotic solutions to be heat stable, even upon autoclaving, if the pH was adjusted to 2.5 prior to heating.

Paper chromatography of the semi-concentrated antibiotic solutions utilizing Silica Gel Impregnated Glass Fiber Sheets (ITLC^{tm} SG, Gelman Instrument Co.) in n-butanol - acetic acid - water (6:2:2) was used to separate the solutions into various fractions. The fractions were tested for presence of peptides by ninhydrin reaction and biuret reaction. The fractions were also tested for antibiotic activity. Ninhydrin in a 0.15% ethanol solution was sprayed on the chromatographic strips and the color developed at 60 C for 30 minutes. The semi-concentrated antibiotic solution as well as the chromatographic strips were tested for the presence of amino groups by use of the biuret reaction. Four ml of biuret reagent was added to 1 ml of the semi-concentrated antibiotic solution, allowed to set for 15 min and read at 540 nm. The antibiotics were extracted from the chromatographic strips with 40 ml of 95% ethanol and mixed 1:4 with biuret reagent. The chromatographic strips spotted with the various semi-concentrated antibiotic solutions and developed in butanol, acetic acid and water were assayed for antibiotic activity by placing the strips in a flat-bottomed cake pan, covering with a thin film of glucose yeast-extract agar acidified to pH 4.0 and spraying with an aqueous suspension of Helminthosporium sativum spores.

Recovery of the antibiotics from the soil was based on the methods of Vasudeva et al. (66) using n-butanol for the extraction solvent.

Phytotoxic effects of Bacillus organisms and bacillary antibiotics. - Since the bacteria were to be applied as seed treatments to wheat, several experiments were performed to determine if the bacteria, or their antibiotics, would have any phytotoxic effects on

germination, root length or yield.

Spring wheat seeds (Triticum aestivum L. 'Protor') were immersed in the semi-concentrated antibiotic solution for 1 hr, placed on filter paper and air dried at 22 C. Three seeds were planted per pot with four replications. Emergence was used as an indicator of germination. Seeds treated similarly with the antibiotic solution were placed on filter paper in a petri plate and replicated five times. The filter paper was saturated with 5 ml of the antibiotic solution and root measurements were recorded after 3 days at 22 C.

The effect of the antibiotic solution as a direct injection or foliar spray on yield was determined. Wheat was planted to allow five plants per pot after thinning. Each treatment was replicated three times. When the plants reached a height of 10-13 cm they were inoculated intervascularly with 0.33 ml antibiotic solution at three sites; one in the stem below the first leaf, one in the first leaf and one in the stem above the first leaf; or atomized with 0.5 ml of the solution. Injections were made once, while atomizing was repeated at the "boot" stage.

Seed-treatment with the various Bacillus species was accomplished in the following manner. Each culture was streaked for isolation on glucose yeast-extract agar. An isolated colony was transferred to 10 ml glucose yeast-extract broth and incubated on a rotory shaker for 48 hr. One ml of this broth was inoculated onto glucose yeast-extract agar plates, spread evenly with an L-rod and incubated for 4 days at 32 C. The cells were washed off the agar with sterile distilled water and the cell concentration was adjusted to

0.03-0.05 OD_{650}. This would allow each seed to be inoculated with 10^6-10^7 bacterial cells. Gelatin was added to achieve a 3% final concentration to aid in adherance of the bacteria to the seed coat. Seeds were immersed in this solution for 1 hr, placed on filter paper and air dried at 22 C. These treated seeds were used to determine the effect of the bacterial treatments on germination, root length and yield. Germination and root length studies utilized methods similar to those in the antibiotic seed-coating studies, except that in the root-length studies the seeds were germinated and grown on glucose yeast-extract agar. In determining the effects of the bacteria on phytotoxicity, two procedures were again used, direct injection and foliar spray. Yield was taken as an index to determine phytotoxicity. Suspensions of the various Bacillus cells (0.33 ml) were injected into a series of plants as in the antibiotic injection procedure, or 0.5 ml suspensions of the bacterial cells were atomized onto a second series of plants. The Bacillus suspensions used for the injections and atomizings contained 10^6-10^7 cells per ml. Injections were made once, while atomizing was repeated at the "boot" stage.

Root-lesion counts and field response of wheat to several Bacillus species seed-treatments. - Wheat treated with the Bacillus cultures under study and a water control was planted in the greenhouse and in the field in split-plot designs. Each of 6 different Bacillus species seed-treatments was subjected to each of the 6 pathogens. Root-lesion counts or yield results were taken. All seeds were inoculated with a particular Bacillus species as described earlier. Five seeds were planted per pot in the greenhouse in

non-sterile soil and thinned to three plants. Hill plots were used in the field with each hill planted with 10 seeds. Four replications of pots were used in the greenhouse and six hills in the field.

Before planting, each pot or hill plot was inoculated with one of the pathogens. Each fungal pathogen was grown on five acidified (pH 3.5-4.0) glucose yeast-extract agar plates until they covered the entire agar surface. The pseudomonad was evenly spread on glucose yeast-extract agar and allowed to grow until a "lawn" was present. The fungal or bacterial growth, including the agar, was combined for each culture and mixed with 800 ml sterile distilled water in a Waring blender. This mixture was "whipped" for 10-15 seconds. Fifteen ml of this solution was added to each pot or hill plot at a depth of 4-8 cm. Seeds treated with the various Bacillus species were immediately planted in the inoculated area and soil pressed firmly over them.

Plants in the greenhouse were allowed to grow to a height of 10-12 cm and were then separated from the soil by washing. The number of root-lesions per plant were counted and one in every four lesions was plated on glucose yeast-extract agar acidified to pH 3.5-4.0 to determine the causal agent. Lesions from the pseudomonad inoculated pots were placed on glucose yeast-extract agar. Wheat in the field was allowed to grow until mature when yield data were taken.

Low temperature effects on pathogen and Bacillus growth and pathogen inhibition. - Since previous studies by the author had shown that, in eastern South Dakota, soil temperature is quite low (7-10 C) at the normal spring wheat planting time, several experiments were performed to determine what effect low temperatures would have on

seed-treatments which utilized Bacillus species. Other preliminary studies by the author had shown that antibiotic activity and growth of the five Bacillus species under study ceased somewhere at a temperature less than 17 C. Because of this, the low temperatures used were 10, 12, 14 and 16 C.

The first experiments involved use of the antibiotic assay method previously described with incubation temperatures of 10, 12, 14 and 16 C. Each pathogen was inoculated onto a plate containing glucose yeast-extract agar or soil extract agar. The soil extract agar contained agar, 15 g; glucose, 1 g; K_2HPO_4, 0.5 g; soil extract, 100 ml. These nutrients were added to 900 ml tap water and the pH adjusted to 6.8-7.0. Plates inoculated with Pythium, Rhizoctonia and Pseudomonas were incubated for 6 days, those inoculated with Fusarium, Gleosporium and Helminthosporium for 12 days. This allowed time for growth of the pathogen and the five species of Bacillus and for antibiotic production. Pathogen and bacterial growth and pathogen inhibition were rated on a scale of 0-4; 0 indicating no growth or inhibition, 1 indicating slight growth or inhibition, 2 indicating moderate growth or inhibition, 3 indicating good growth or extreme inhibition and 4 indicating excellent growth or total inhibition.

This experiment was further modified to include testing of bacterial and antibiotic-treated wheat seed. Wheat was inoculated with B. uniflagellatus, B. subtilis and the antibiotic(s) from each as described earlier. Two treated seeds were placed on the periphery of glucose yeast-extract agar plates for each treatment and the plates were replicated three times. H. sativum from 7 day-old cultures was

placed in the center of the plate. This culture was used since it grew quite rapidly and formed clear, sharp zones of inhibition. The plates were incubated at 10, 12, 14 and 16 C for 12 days to allow for wheat germination and growth of the pathogen and Bacillus species. H. sativum growth and inhibition and B. subtilis and B. uniflagellatus growth were rated on a scale of 0-4.

A second series of experiments involving low temperatures involved determination of Bacillus species colony forming units (CFU) in the soil. Twenty-five g of soil were placed in petri plates and autoclaved for one hour at 1.1 kg/cm^2. The five Bacillus species were grown in glucose yeast-extract broth for 24-36 hours. The cells were washed off the agar with sterile distilled water and the cell concentration was adjusted to 0.03-0.05 OD_{650} with a Spectronic 20. Four ml of each diluted bacterial suspension was added to each of three plates and mixed into the soil. Plate counts of this initial inoculum were made to determine the number of cells added to the soil. Soil-plates were incubated at temperatures of 10, 12, 14 and 16 C for 1, 2, 4 and 8 days. At each sample date 10-fold dilution series were made of the samples. One-tenth ml of each dilution was plated on glucose yeast-extract agar. The CFU found on the sample dates were compared to the initial CFU determination.

The effects of seed inoculation at various locations and dates in the field. - Since it appeared that low temperature may affect the Bacillus species under study, an experiment was initiated to correlate soil temperature differences with the Bacillus species success as shown by yield response. Field plots were utilized in

three locations, the Southeast Experiment Farm at Centerville, South Dakota, the Plant Science Farm at Brookings, South Dakota and the North Sioux Valley Research Station at Watertown, South Dakota. Stations were approximately 60 miles apart, in a line, north to south. Watertown is in northeastern South Dakota, Brookings in east-central and Centerville in southeastern South Dakota. Two planting dates, April 15 and May 5, were used per location. Hill plots were used and each plot was inoculated with one of the pathogens and planted with Protor spring wheat which had been treated as described earlier with one of the five Bacillus species being studied. The Bacillus species used were the five that gave consistant inhibition zones against the wheat pathogens. Each treatment was replicated 10 times in a randomized complete block design. Fifteen seeds were sown at 3-4 cm depth in the inoculated plots and firmly covered with soil. Hill plots were inoculated as follows: Each fungal pathogen was grown on glucose yeast-extract agar for seven days. Pseudomonas syringae was streaked on glucose yeast-extract agar. A 3 mm x 3 mm section (or one loop-ful of the pseudomonad) of fungal-embedded agar was inoculated into 600 ml glucose yeast-extract broth and allowed to incubate 7 days. Each solution was then poured into a Waring blender and "whipped" for 15 seconds. Each hill plot received 10 ml of this solution. Seeds were planted directly into the inoculum. Soil temperatures were recorded daily at 8:00 a.m. at each location at 8 cm depth. Yield data were taken from each hill plot at each location.

RESULTS

Antibiotic assays. - Of the 20 Bacillus cultures tested for inhibition of the seven wheat pathogens, only five, B. globigii, B. polymyxa, B. subtilis, B. thuringiensis subsp. sotto and B. uniflagellatus, gave consistant zones of inhibition against most of the fungal pathogens (Table 1). Fusarium roseum f. cerealis, however, seemed to be relatively non-sensitive to each of the 20 Bacillus species with B. globigii, B. polymyxa, B. subtilis, B. thuringiensis subsp. sotto and B. uniflagellatus producing an inhibition rating of less than 1 on a scale of 0-4. Gleosporium bolleyi, Helminthosporium sativum, Pythium graminicola and Rhizoctonia solani were inhibited severely by the same five Bacillus species and were also slightly inhibited by several of the other Bacillus speices. Both Pseudomonas syringae and P. atrofaciens inhibited growth of the Bacillus species. This inhibition was quite pronounced against Bacillus cereus var. mycoides, B. polymyxa, B. subtilis and B. thuringiensis subsp. sotto, and was probably due to the production of a toxin by the Pseudomonas species (27).

Since the reactions of different isolates of a given pathogenic organism were similar, it was decided to use only one culture of each pathogen for further study. The organisms chosen from those originally having more than one isolate were Gleosporium bolleyi (G-2 3uc), Rhizoctonia solani (R-276) and Pseudomonas syringae.

Sections of media containing hyphal tips were taken at the periphery of the inhibition zone to help determine the mechanism(s) of the inhibition that was occurring. These sections were placed in

TABLE 1. Antibiotic activity of 20 Bacillus species against 6 wheat pathogens. The results shown are based on a rating scale: 0 = no effect of Bacillus vs. pathogen; 1 = slight inhibition; 2 = moderate inhibition; 3 = extreme inhibition; 4 = total inhibition. Results are average of 4 replications.

	Organisms pathogenic on wheat					
Bacillus species	Fusarium roseum f. cerealis	Gleosporium bolleyi (G-2 3uc)	Helminthosporium sativum	Pythium graminicola (169)	Rhizoctonia solani (R-276)	Pseudomonas syringae
B. brevis	0	0	0	1	0	0
B. cereus	0	0	0	0	0	0
B. cereus var. mycoides	0	0	0	2	1	4[a]
B. circulans	0	0	0	0	0	0
B. coagulans	0	0	0	0	0	0
B. cylindricus	0	1	0	0	0	0
B. licheniformis	0	0	0	0	0	1[a]
B. ligneus	0	2	0	1	3	0
B. macerans	0	1	0	1	2	0
B. megaterium	0	0	0	1	2	0
B. polymyxa	0	3	3	2	4	2[a]
B. pumilis	0	1	0	0	0	0
B. sphaericus	0	1	0	0	0	0
B. subtilis	1	3	3	2	4	2[a]
B. globigii	1	3	3	3	3	0
B. subtilis var. niger	0	1	0	3	1	0
B. thuringiensis	0	0	0	0	0	0
B. thuringiensis subsp. sotto	0	3	1	2	2	2[a]
B. thuringiensis subsp. subtoxicus	0	0	0	0	0	0
B. uniflagellatus	0	3	4	2	4	0

[a]Reverse inhibition, i.e. Pseudomonas inhibited Bacillus.

water on glass slides and observed under high power on a Nikon light microscope and photomicrographs were taken. Fungal mycelium at the periphery of the zone of inhibition is very swollen and disrupted (Fig. 1) with "bulb-like" formations as described by Vasudeva (67). The germ tubes of germinating spores also had bulb formations present. All of the fungal pathogens tested had similar signs of hyphal-tip swelling with Helminthosporium sativum, Gleosporium bolleyi and Fusarium roseum f. cerealis producing the more distinctive formations. All five Bacillus species elicited the response with B. globigii, B. subtilis and B. uniflagellatus giving the most pronounced reaction.

Fungal ultrastructure in response to bacillary antibiotics. - The ultrastructural differences between hyphae affected by bacillary antibiotics (abnormal) and normal hyphae can be seen in Fig. 2. The normal mycelium is filled with a dense cytoplasmic matrix and contains a well defined nucleus and other globular bodies. Ground cytoplasm in the abnormal mycelium is less dense, and both the nucleus and globular bodies also show signs of degradation. The collapse and degeneration of the nucleus, nuclear envelope and globular bodies is even more evident in Fig. 3. The globular bodies may be either lysozomes or mitochondria, but accurate identification is not possible because of the degeneration that has occurred. The cell walls of both the normal and abnormal mycelium are quite similar and show very little disruption, while the plasma membrane is very disrupted in the abnormal mycelium (Fig. 3). All plasma membranes are very disrupted in the mycelium contacting the bacillary antibiotic.

Antibiotic properties. - The antifungal assay of thin layer

Fig. 1. Mycelium of Helminthosporium sativum showing "bulb-like" formation and hyphal-tip swelling. The mycelium has been disrupted by the action of an antibiotic formed by Bacillus uniflagellatus.

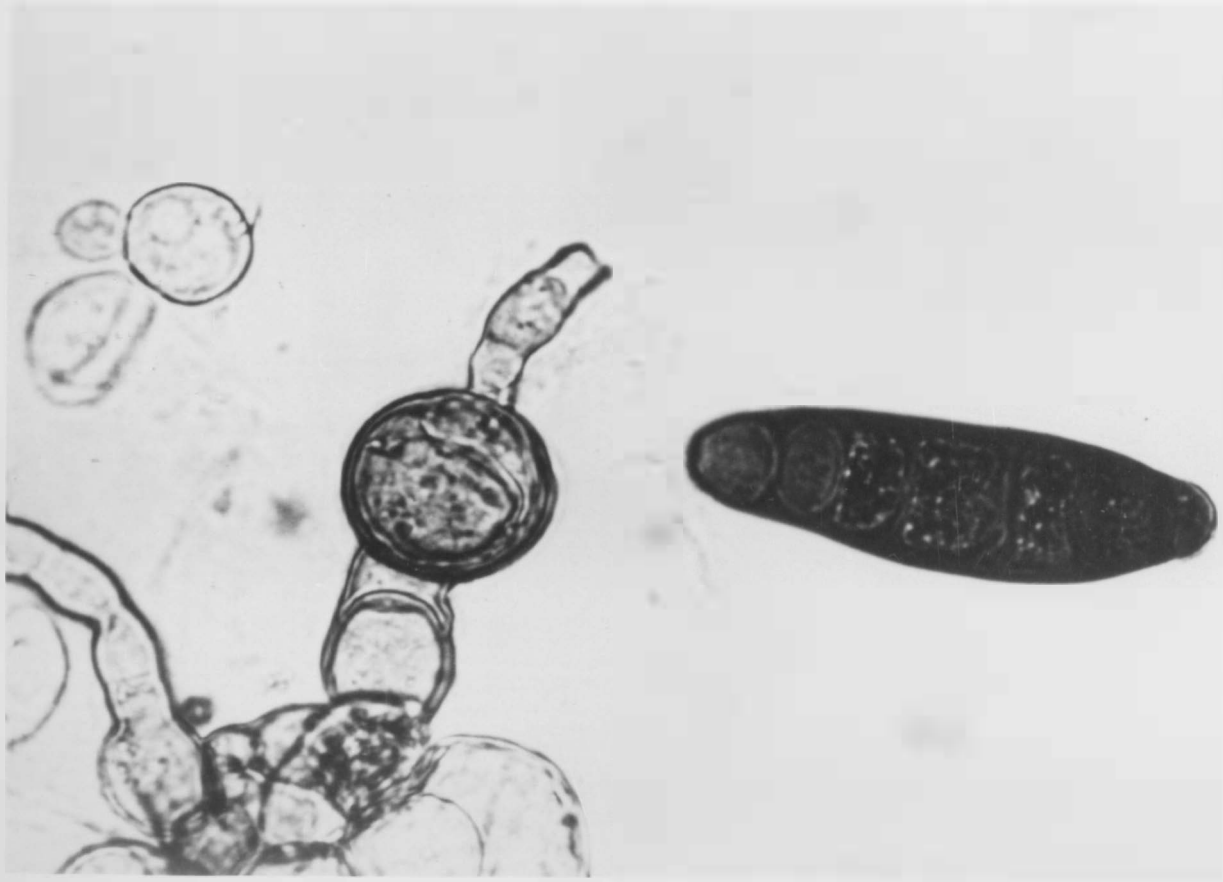


Fig. 2. Electron micrograph of Helminthosporium sativum showing normal mycelium (A) and mycelium affected by an antibiotic formed by Bacillus uniflagellatus (B). Cym: cytoplasmic matrix; gb: globular bodies; n: nucleus. Magnification is 4000X.

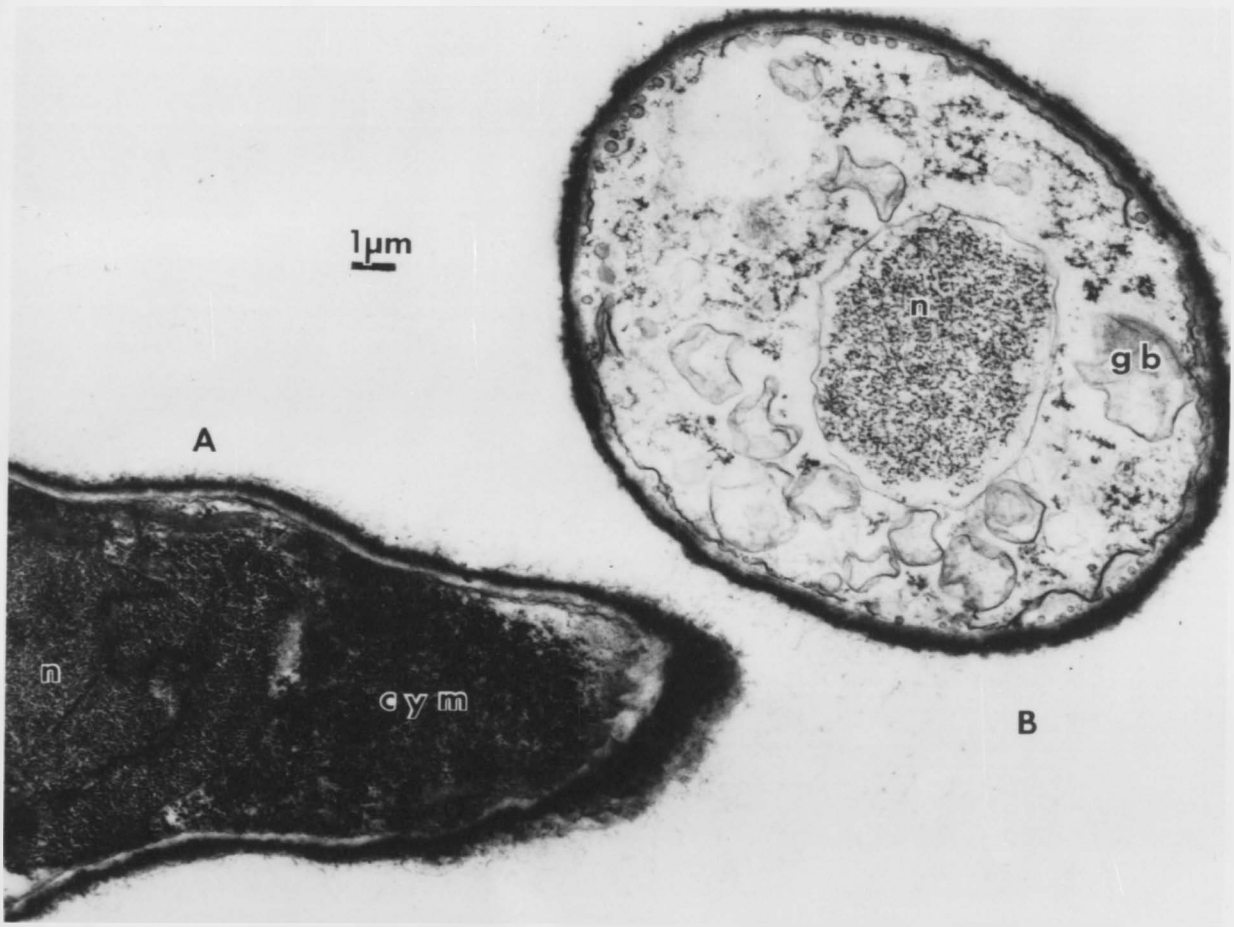
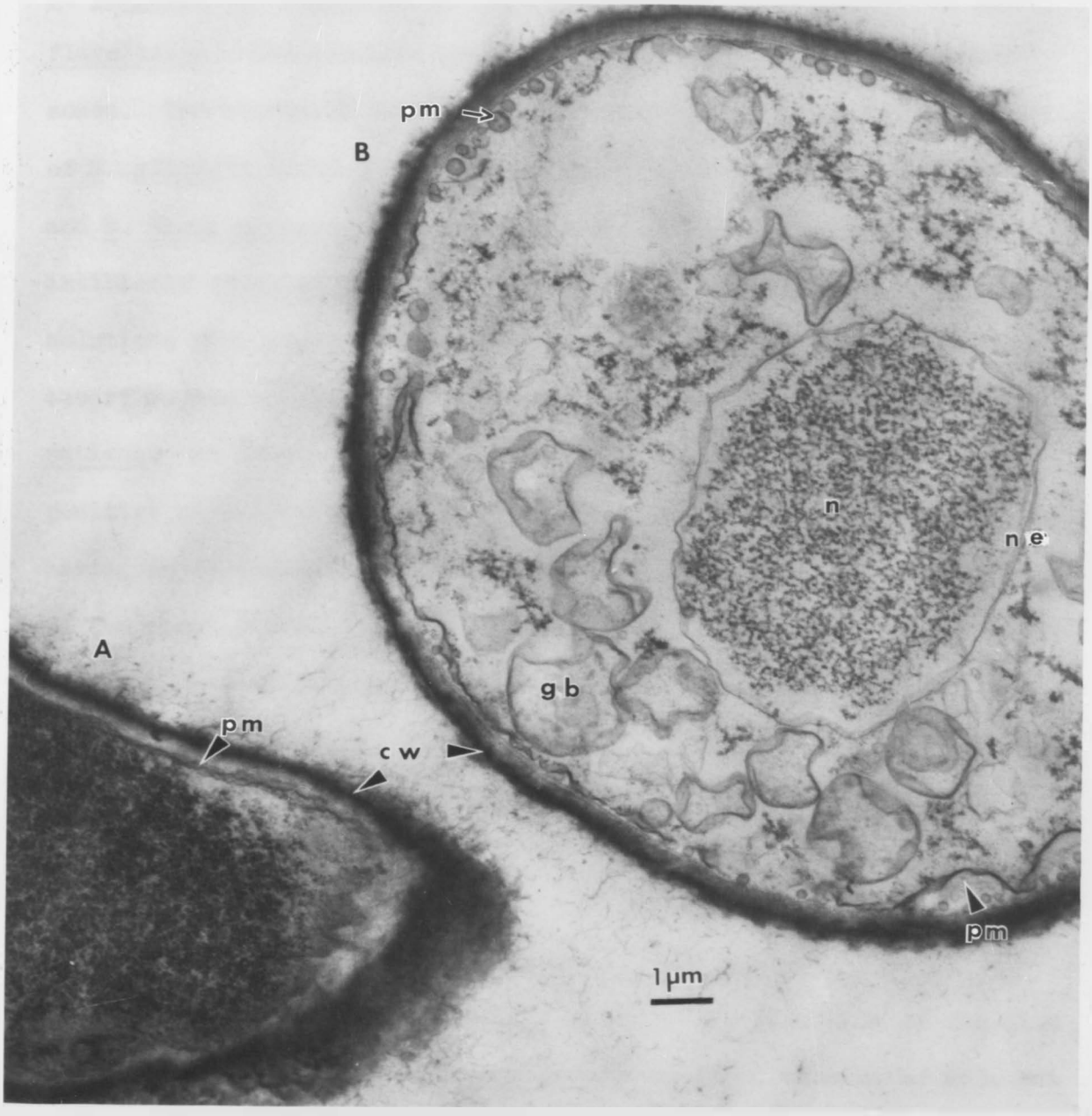

1µm
n
gb
A
n
cym
B

Fig. 3. Electron micrograph of Helminthosporium sativum showing collapse and degeneration of the nucleus (n), nuclear envelope (ne), plasma membrane (pm) and globular bodies (gb) of mycelium affected by an antibiotic formed by Bacillus uniflagellatus. Plasma membrane disruption is characterized by a "curling" of the membrane as shown by the arrow. Cw: cell wall; B: abnormal mycelium; A: normal mycelium. Magnification is 7000X.


pm
B
n
n e
A
g b
pm
c w
pm
1 µm

chromatograms of antibiotic preparations gave inhibitory zones having rf values of 8.0-8.5 for solutions of Bacillus globigii, B. polymyxa, B. subtilis, B. thuringiensis subsp. sotto and B. uniflagellatus. The glucose yeast-extract control gave no inhibition zones. The ninhydrin reaction was positive for antibiotic solutions of B. globigii (rf:8.3), B. polymyxa (rf:7.7), B. subtilis (rf:8.0) and B. thuringiensis subsp. sotto (rf:8.5) but negative for the antibiotic solution of B. uniflagellatus. The rf values of the solutions giving a positive ninhydrin test corresponded closely to the rf values of the spot showing inhibition of Helminthosporium sativum. At first the glucose yeast-extract control also gave a positive ninhydrin test, however, there was no fraction in the control having an rf value of 8.0-8.5 which gave a positive test. Removal of the yeast-extract from the growth medium also prevented the control from giving a positive ninhydrin test. The Bacillus cultures grown in glucose medium all gave positive ninhydrin reactions at rf values 7.7-8.5, except for B. uniflagellatus which gave a negative ninhydrin test.

The biuret test was positive only for the antibiotic solutions of B. thuringiensis subsp. sotto and B. uniflagellatus.

The semi-concentrated antibiotic solutions from each of the five Bacillus species tested were heat stable at 100 C at a pH of 2.5, but lost their activity when heated at 100 C at pH 8.0. The antibiotic activity was retained for 365 days when left standing at 22 C in both light and dark conditions.

Phytotoxic effects of Bacillus organisms and bacillary

antibiotics. - Wheat is affected by treatments with bacillary antibiotics applied either as seed inoculants or foliar applications (Table 2). There also is a response of wheat to seed treatments and foliar applications of the five Bacillus species (Table 2). Germination was not significantly ($P \leq 0.05$) affected by seed treatment with either the bacteria or the antibiotics. However, root length was significantly increased in seeds treated with the B. globigii antibiotic solution, but showed no differences due to treatment with any of the other Bacillus species.

Plants receiving the direct antibiotic treatment showed no significant differences ($P \leq 0.05$) in yield among the various treatments. Plants injected with Bacillus uniflagellatus, B. subtilis and B. polymyxa were not significantly different in yield from the water control, but were significantly higher in yield than plants injected with the glucose yeast-extract agar control, B. thuringiensis subsp. sotto and B. globigii (Table 2). Yield responses to the foliar spray showed significant differences in treatments with both the antibiotics and the Bacillus and was the only measurement showing significant differences under both treatments (Table 2).

Although root length, in response to the bacillary seed treatments, was not significantly different from the water control, roots from seeds treated with the water control were consistently longer than those treated with the Bacillus cultures. At recording time, the bacterial population surrounding the roots was quite low (Fig. 4A). It was thought that this experiment should be modified so that the wheat would be germinating and growing in a high concentration of

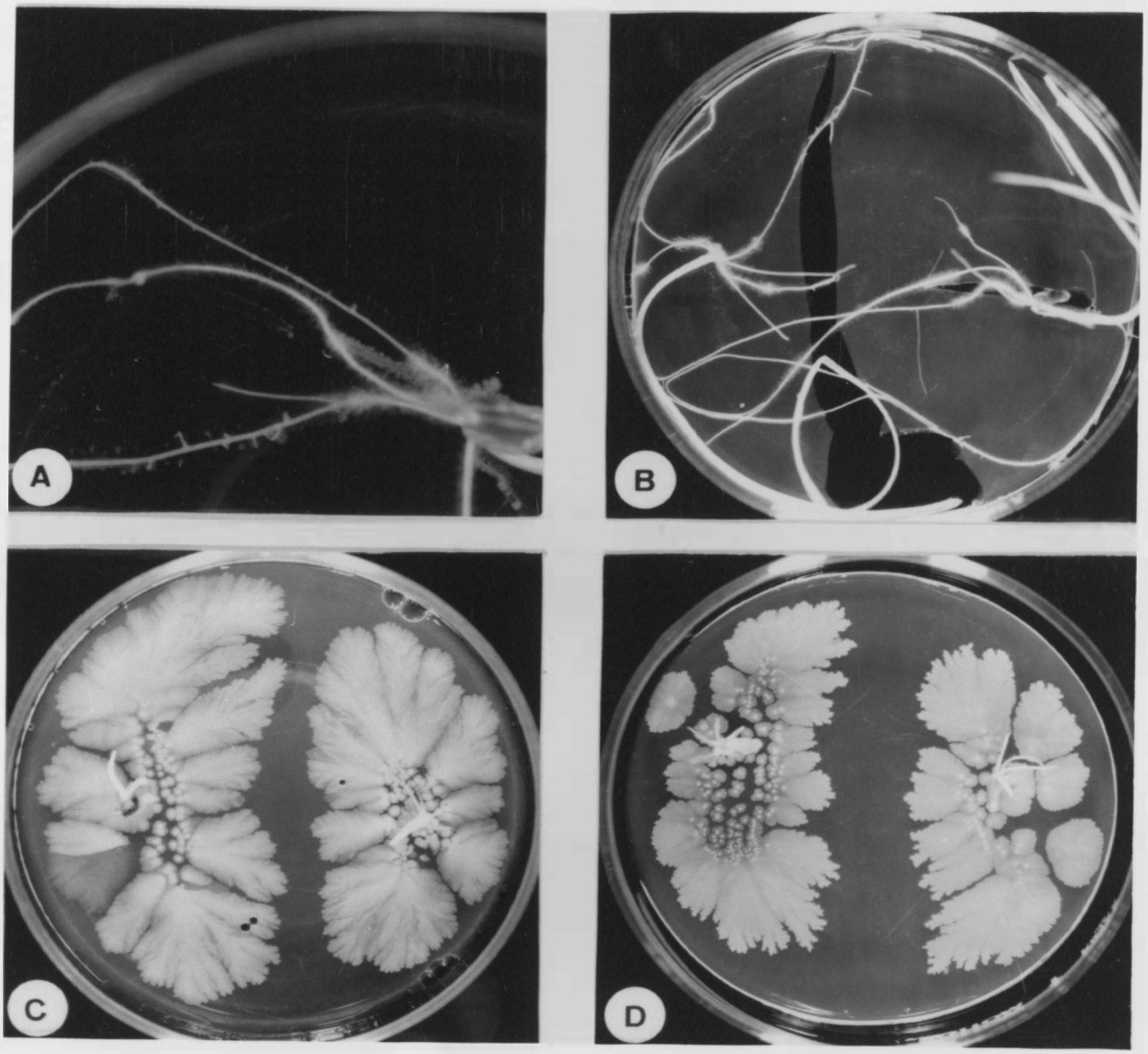
TABLE 2. Phytotoxic effects of five Bacillus species and their antibiotics on germination, root length and yield of wheat. All observations are averages based on three replications. Plants were used in triplicate to determine root length and yield due to direct injection and foliar spray.[a]

	Antibiotic solution treatment				Bacillus treatment			
			Yield (g)				Yield (g)	
Bacillus	Germination[b]	Root length (mm)	Direct injection	Foliar spray	Germination[b]	Root length (mm)	Direct injection	Foliar spray
B. globigii	1.67a	17.60 b	1.48a	1.83a	2.90a	7.26a	0.67 bc	1.49a
B. polymyxa	0.67a	8.60a	1.65a	1.42ab	3.00a	10.00a	1.53a	1.13ab
B. subtilis	2.00a	10.47a	1.23a	1.39ab	2.90a	10.53a	1.54a	1.43a
B. thuringiensis subsp. sotto	1.00a	10.47a	1.23a	1.78a	3.00a	10.73a	0.95 bc	1.28ab
B. uniflagellatus	2.00a	6.00a	1.12a	1.08 b	3.00a	5.66a	1.66a	0.88 b
Glucose yeast-extract broth control	0.67a	———	0.86a	1.47ab	3.00a	———	1.04 b	———
H_2O control	2.34a	10.80a	0.77a	1.12 b	3.00a	12.27a	1.38a	1.16ab

[a]Numbers in columns with similar letters are not significantly different at $P \leq 0.05$ using Duncan's multiple-range test.

[b]The number of plants emerged from three seeds planted.

Fig. 4 - (A to D). At an incubation temperature of 22 C for 3 days, (A) shows low populations of Bacillus subtilis surrounding the seed and roots causing no phytotoxic response. (B) is the control showing no bacterial growth, (C) represents populations of 10^{12}-10^{15} cells/root of B. subtilis and (D) represents populations of 10^{12}-10^{15} cells/root of B. uniflagellatus. Both (C) and (D) show a phytotoxic response of the Bacillus species on seedling growth. Seeds inoculated in (B), (C) and (D) were incubated at 32 C for 12 days with bacteria.


A
B
C
D

bacteria. Therefore, seeds were treated with B. subtilis and B. uniflagellatus and their antibiotics and placed on glucose yeast-extract agar at 32 C - the optimum growth temperature for the Bacillus species - for 12 days and root length recorded. The results (Fig. 4B-D) show a high population of the bacteria surrounding the roots and seed. Both the roots and coleoptile of the control were well developed while the roots of the bacterial-treated seeds were very necrotic and much reduced. There is a significant ($P \leq 0.05$) reduction in root length of the B. uniflagellatus and the B. subtilis treated seeds over the control (Table 3).

It was thought that, since the presence of a Bacillus species on a plant may have an influence on yield (21), perhaps Bacillus species inoculated onto seed may lead to an epiphytic population of the bacterium on the plant. An experiment was designed to determine if a Bacillus species, applied as a seed treatment, could become established as an epiphyte on wheat. B. globigii was used as the test organism because it produces brick-red colonies on glucose yeast-extract agar. Wheat seeds were inoculated with this Bacillus species as described earlier and ten seeds planted per pot. Both the pot and the soil were sterilized and planted to prevent contamination. The plants were allowed to grow to a height of 10 cm and then harvested 1 cm above the soil level. Two plants per pot were harvested and two leaves from each were pressed onto glucose yeast-extract agar for one minute. The leaves were removed and the plates incubated at 32 C for 48 hours. Two other plants were harvested and surface sterilized by sequential immersion for 1 minute in 70% alcohol, 10% sodium hypochlorite and

TABLE 3. Effects of seed treatments with B. uniflagellatus and B. subtilis, and their antibiotics, on root length of wheat seedlings. Data represents mean of four replications.[a]

Treatment	Root length (mm)
Glucose yeast-extract broth	112.50a
B. uniflagellatus antibiotic solution	107.25a
B. subtilis antibiotic solution	108.25a
B. uniflagellatus	10.25 b
B. subtilis	9.00 b

[a]Numbers in columns with similar letters are not significantly different at $P \leq 0.05$.

three changes of sterile distilled water. The leaves were placed in a mortar with 1 ml of sterile distilled water per leaf and ground with a pestle for 30-40 seconds. Serial dilutions of the titurate were heat shocked for 30 minutes at 65 C to induce sporulation and reduce numbers of non-sporulating organisms, and were then plated on glucose yeast-extract agar for 48 hours at 32 C. Use of the remaining plants involved the same procedure as above, but the plants were not surface sterilized. The results (Table 4) indicate that Bacillus globigii applied as a seed inoculant does not become either systemic or epiphytic. Evidentally the organism remains in the soil, or on the seed coat, and any contact with the leaves seems to be purely accidental.

Root-lesion counts and yield data. - Neither the Bacillus or pathogen treatments nor the Bacillus-pathogen interactions produced any significant difference ($P \leq 0.05$) in root-lesion counts in the greenhouse (Table 5). In addition, wheat yield from hill plots in the field were also not significantly different between the Bacillus treatments, the pathogen treatments or the Bacillus-pathogen interaction (Table 6). None of the bacterial seed treatments produced a significant difference in yield when compared to each other or to the control, and no pathogen was more important than another in reducing yield. Soil moisture content and soil temperatures were taken from planting (May 8) to harvest (July 22). Soil moisture remained quite high from planting to mid-June, while soil temperature from planting to emergence (May 14) was quite low; averaging 12 C at 10:00 a.m. (Fig. 5).

TABLE 4. Recovery of B. globigii from wheat leaves grown from seed inoculated with the bacterium.

Seed treatment	Leaf surface	Sterile macerated leaf[a]		Non-sterile macerated leaf[a]	
		Dilution			
		10^{-1}	10^{-2}	10^{-1}	10^{-2}
Water control	0	6	0	0	0
B. globigii	0	0	0	0	0

[a]Values represent the mean number of B. globigii colonies found per six replications.

TABLE 5. Root-lesion counts of wheat grown in the greenhouse from bacterial treated seed and tested against infection by six wheat pathogens.[a]

Pathogen	Number of root lesions on wheat grown from seed treated with[bc]					
	H_2O	*B. polymyxa*	*B. subtilis*	*B. globigii*	*B. thuringiensis* subsp. *sotto*	*B. uniflagellatus*
Fusarium roseum f. cerealis	8.00	5.75	4.00	6.00	7.25	6.25
Gleosporium bolleyi	7.50	5.25	4.74	4.25	4.25	5.25
Helminthosporium sativum	5.75	4.25	7.00	7.75	7.25	5.75
Pythium graminicola	3.25	1.75	5.00	5.75	6.50	8.00
Rhizoctonia solani	5.75	4.75	3.75	8.00	4.00	4.25
Pseudomonas syringae	6.50	7.75	6.50	5.75	8.00	6.00

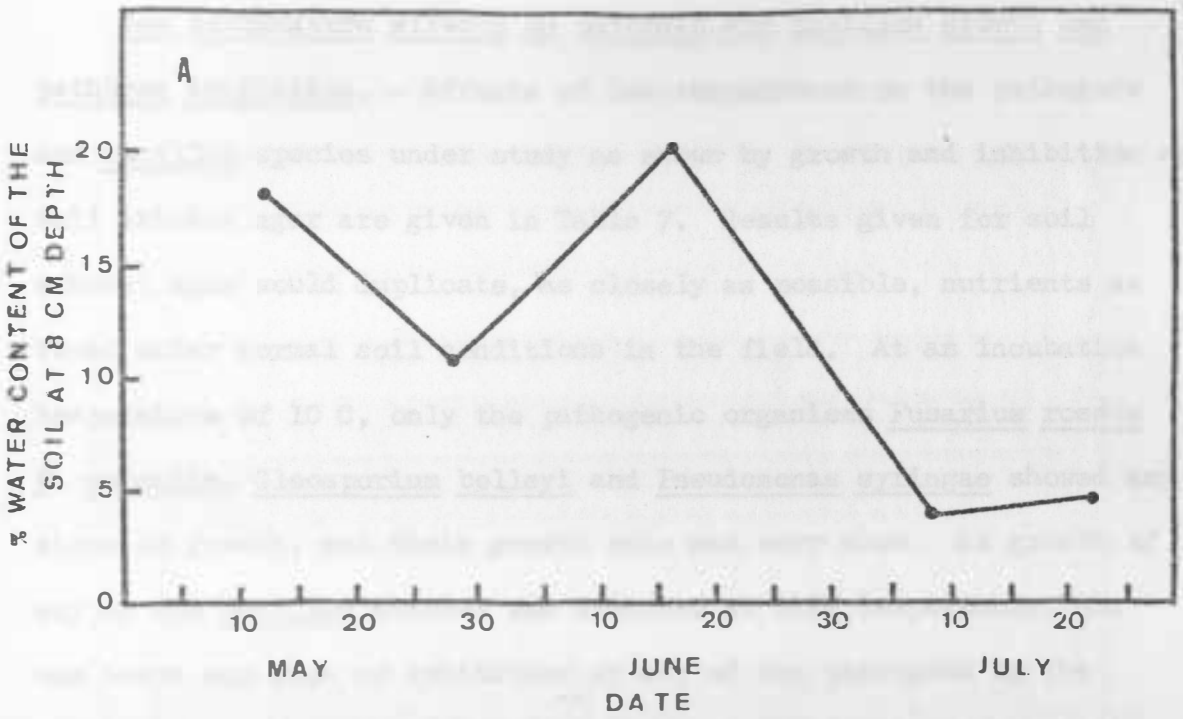
[a] Lesions found are a result of the particular pathogen inoculated in the pot.
[b] All root lesion count differences are non-significant at $P \leq 0.05$.
[c] Values represent mean of 4 replications.

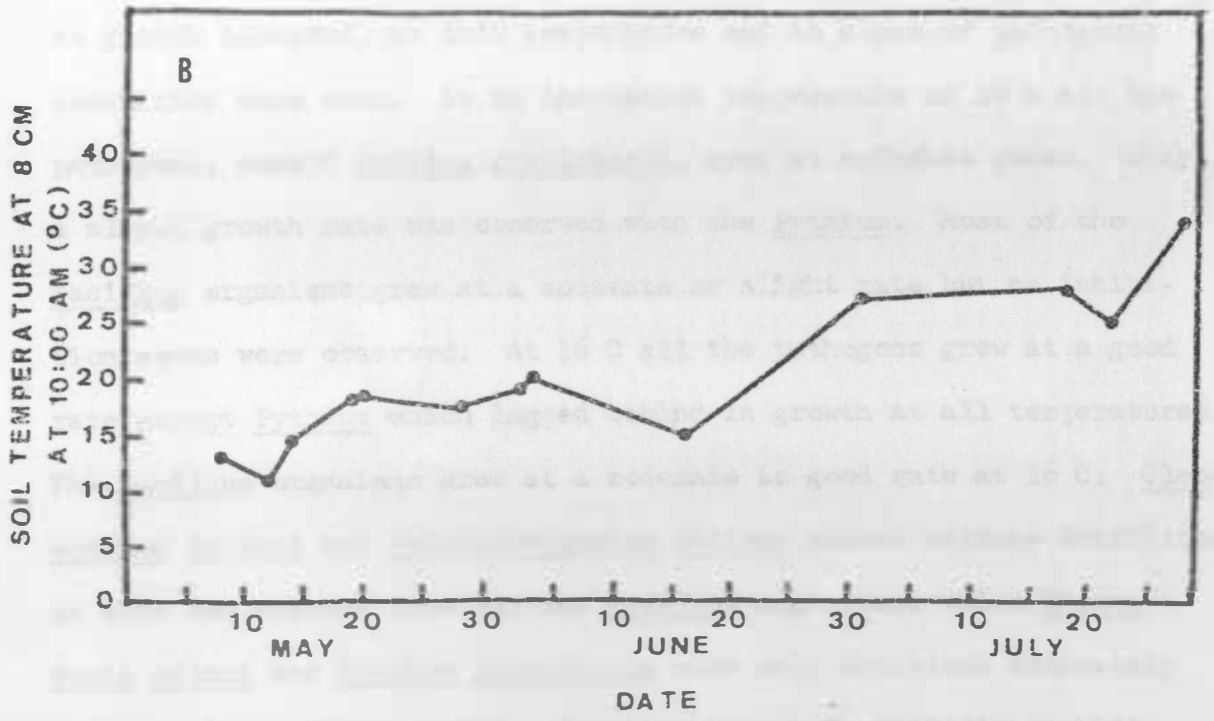
TABLE 6. Yield effect of seed inoculation with Bacillus species when wheat is challenged with various pathogens at the Plant Science Farm at Brookings.

Pathogen	Yield of wheat grown from seed treated with[abc]					
	H_2O	B. polymyxa	B. subtilis	B. globigii	B. thuringiensis subsp. sotto	B. uniflagellatus
Fusarium roseum f. cerealis	13.18	12.39	10.27	9.21	11.94	11.04
Gleosporium bolleyi	14.72	11.31	14.51	12.90	15.06	14.48
Helminthosporium sativum	10.12	14.66	13.96	8.32	13.77	9.81
Pythium graminicola	12.32	14.23	12.51	14.75	9.46	10.55
Rhizoctonia solani	16.97	11.47	16.37	11.23	13.70	9.80
Pseudomonas syringae	12.48	12.09	9.92	13.44	15.34	9.85

[a]All yield differences are non-significant at $P \leq 0.05$.
[b]Values represent mean of 6 replications.
[c]All data is expressed in grams per hill plot.

Fig. 5 - (A to B). The water content of the soil at a depth of 8 cm (A), and soil temperature taken at 10:00 a.m. at 8 cm (B) from field plots at Brookings, S. D.


A
% WATER CONTENT OF THE SOIL AT 8 CM DEPTH
0
5
10
15
20
10
20
30
10
20
30
10
20
MAY
JUNE
JULY
DATE


B
SOIL TEMPERATURE AT 8 CM AT 10:00 AM (°C)
0
5
10
15
20
25
30
35
40
10
20
30
10
20
30
10
20
MAY
JUNE
JULY
DATE

Low temperature effects on pathogen and Bacillus growth and pathogen inhibition. - Effects of low temperature on the pathogens and Bacillus species under study as shown by growth and inhibition on soil extract agar are given in Table 7. Results given for soil extract agar would duplicate, as closely as possible, nutrients as found under normal soil conditions in the field. At an incubation temperature of 10 C, only the pathogenic organisms Fusarium roseum f. cerealis, Gleosporium bolleyi and Pseudomonas syringae showed any signs of growth, and their growth rate was very slow. No growth of any of the Bacillus species was observed at this temperature, nor was there any sign of inhibition of any of the pathogens by the Bacillus species. At 12 C all the pathogenic organisms showed signs of growth with F. roseum f. cerealis and G. bolleyi showing a good growth rate. The Bacillus organisms' growth rates were slight, or no growth occurred, at this temperature and no signs of pathogenic inhibition were seen. At an incubation temperature of 14 C all the pathogens, except Pythium graminicola, grew at moderate rates. Only a slight growth rate was observed with the Pythium. Most of the Bacillus organisms grew at a moderate or slight rate but no inhibition zones were observed. At 16 C all the pathogens grew at a good rate except Pythium which lagged behind in growth at all temperatures. The Bacillus organisms grew at a moderate to good rate at 16 C. Gleosporium bolleyi and Helminthosporium sativum showed extreme inhibition at this temperature from all the Bacillus organisms, while Rhizoctonia solani and Pythium graminicola were only inhibited moderately by several Bacillus species. Fusarium roseum f. cerealis was not

TABLE 7. The growth of six wheat pathogens and five Bacillus species and pathogen inhibition on soil extract-agar at 10, 12, 14 and 16 C.

Pathogen[b]	Bacillus	Pathogen growth (C)[a]				Bacillus growth (C)[a]				Inhibition (C)[a]			
		10	12	14	16	10	12	14	16	10	12	14	16
F. roseum f. cerealis	B. globigii	2	3	3	3	0	0	0	1	0	0	0	0
F. roseum f. cerealis	B. polymyxa	2	3	3	3	0	0	0	1	0	0	0	0
F. roseum f. cerealis	B. subtilis	2	3	3	3	0	0	0	1	0	0	0	0
F. roseum f. cerealis	B. thuringiensis subsp. sotto	2	3	3	3	0	0	0	1	0	0	0	0
F. roseum f. cerealis	B. uniflagellatus	2	3	3	3	0	0	0	1	0	0	0	0
G. bolleyi	B. globigii	1	3	3	3	0	2	2	3	0	0	0	3
G. bolleyi	B. polymyxa	1	3	3	3	0	1	2	3	0	0	0	3
G. bolleyi	B. subtilis	1	3	3	3	0	1	2	3	0	0	0	3
G. bolleyi	B. thuringiensis subsp. sotto	1	3	3	3	0	1	2	3	0	0	0	3
G. bolleyi	B. uniflagellatus	1	3	3	3	0	1	2	3	0	0	0	3
H. sativum	B. globigii	0	2	2	3	0	0	1	3	0	0	0	4
H. sativum	B. polymyxa	0	2	2	3	0	0	1	3	0	0	0	4
H. sativum	B. subtilis	0	2	2	3	0	0	1	3	0	0	0	4
H. sativum	B. thuringiensis subsp. sotto	0	2	2	3	0	0	1	3	0	0	0	4
H. sativum	B. uniflagellatus	0	2	2	3	0	0	1	3	0	0	0	4
P. graminicola	B. globigii	0	1	1	2	0	1	2	2	0	0	0	2
P. graminicola	B. polymyxa	0	1	1	2	0	0	2	2	0	0	0	2
P. graminicola	B. subtilis	0	1	1	2	0	0	2	2	0	0	0	0
P. graminicola	B. thuringiensis subsp. sotto	0	1	1	2	0	0	2	2	0	0	0	0
P. graminicola	B. uniflagellatus	0	1	1	2	0	0	2	2	0	0	0	0
P. syringae	B. globigii	1	2	2	3	0	0	2	3	0	0	0	0
P. syringae	B. polymyxa	1	2	2	3	0	0	2	3	0	0	0	0
P. syringae	B. subtilis	1	2	2	3	0	0	2	3	0	0	0	0
P. syringae	B. thuringiensis subsp. sotto	1	2	2	3	0	0	2	3	0	0	0	0
P. syringae	B. uniflagellatus	1	2	2	3	0	0	2	3	0	0	0	0
R. solani	B. globigii	0	1	2	3	0	0	0	2	0	0	0	0
R. solani	B. polymyxa	0	1	2	3	0	0	0	2	0	0	0	2
R. solani	B. subtilis	0	1	2	3	0	0	1	2	0	0	0	1
R. solani	B. thuringiensis subsp. sotto	0	1	2	3	0	0	1	2	0	0	0	1
R. solani	B. uniflagellatus	0	1	2	3	0	0	1	2	0	0	0	0

[a]Based on a rating scale of 0-4. 0 = no growth or inhibition, 1 = slight growth or inhibition, 2 = moderate growth or inhibition, 3 = good growth or extreme inhibition and 4 = excellent growth or total inhibition.

[b]Plates inoculated with Pseudomonas, Pythium or Rhizoctonia were incubated for six days. Plates inoculated with Fusarium, Gleosporium or Helminthosporium were incubated for 12 days.

inhibited at 16 C by any of the Bacillus species.

When glucose yeast-extract agar was used for the assay medium the results were quite different than with soil extract agar (Table 8). At 10 C G. bolleyi, H. sativum, P. syringae and R. solani grew at a slight growth rate. F. roseum f. cerealis and P. graminicola showed no growth; the same was true of all the Bacillus species. No inhibition was noted at 10 C. At an incubation temperature of 12 C all pathogens and all Bacillus species grew at a growth rating of good to excellent and inhibition of the pathogens was extreme to total, indicating active production of an antibiotic by the Bacillus species. Results at incubation temperatures of 14 C and 16 C are similar to those obtained at 12 C.

The results for wheat treated with B. subtilis and B. uniflagellatus, and their respective antibiotics and assayed against Helminthosporium sativum at temperatures of 10, 12, 14 and 16 C are given in Table 9.

When grown on soil extract agar the fungus showed a growth rating of moderate to excellent over all temperature ranges, while the only bacterial growth observed was a limited amount at 16 C. There was no sign of pathogen inhibition at any incubation temperature. On the richer glucose yeast-extract agar the fungus showed slight to excellent growth over all temperature ranges, while moderate to good bacterial growth was recorded at 12 C. Inhibition zones are noted at 12 C indicating antibiotic production by the bacteria at this temperature. Coating the seeds with the antibiotic gave no fungal inhibition indicating that the antibiotic was not

TABLE 8. The growth of six wheat pathogens and five Bacillus species and pathogen inhibition on glucose yeast-extract agar at 10, 12, 14 and 16 C.

Pathogen[b]	Bacillus	Pathogen growth (C)[a]				Bacillus growth (C)[a]				Inhibition (C)[a]			
		10	12	14	16	10	12	14	16	10	12	14	16
F. roseum f. cerealis	B. globigii	0	3	3	4	0	3	3	4	0	2	3	4
F. roseum f. cerealis	B. polymyxa	0	3	3	4	0	3	3	4	0	3	3	4
F. roseum f. cerealis	B. subtilis	0	3	3	4	0	3	3	4	0	3	3	4
F. roseum f. cerealis	B. thuringiensis subsp. sotto	0	3	3	4	0	3	3	4	0	3	3	4
F. roseum f. cerealis	B. uniflagellatus	0	3	3	4	0	3	3	4	0	3	3	4
G. bolleyi	B. globigii	1	2	2	4	0	3	3	4	0	2	3	4
G. bolleyi	B. polymyxa	1	2	2	4	0	3	3	4	0	2	3	4
G. bolleyi	B. subtilis	1	2	3	4	0	3	4	4	0	2	3	4
G. bolleyi	B. thuringiensis subsp. sotto	1	2	2	4	0	3	3	4	0	2	3	4
G. bolleyi	B. uniflagellatus	1	2	2	4	0	3	3	4	0	2	3	4
H. sativum	B. globigii	1	3	3	4	0	3	3	4	0	3	3	4
H. sativum	B. polymyxa	1	3	3	4	0	3	3	4	0	3	3	4
H. sativum	B. subtilis	1	3	3	4	0	3	3	4	0	3	3	4
H. sativum	B. thuringiensis subsp. sotto	1	3	3	4	0	3	3	4	0	3	3	4
H. sativum	B. uniflagellatus	1	3	3	4	0	3	3	4	0	3	3	4
P. graminicola	B. globigii	0	2	3	4	0	2	3	4	0	2	2	4
P. graminicola	B. polymyxa	0	2	3	4	0	2	3	4	0	2	2	4
P. graminicola	B. subtilis	0	2	3	4	0	2	3	4	0	2	2	4
P. graminicola	B. thuringiensis subsp. sotto	0	2	3	4	0	2	3	4	0	2	2	4
P. graminicola	B. uniflagellatus	0	2	3	4	0	2	3	4	0	2	2	4
P. syringae	B. globigii	1	4	4	4	0	4	4	4	0	0	0	0
P. syringae	B. polymyxa	1	4	4	4	0	4	4	4	0	0	0	0
P. syringae	B. subtilis	1	4	4	4	0	4	4	4	0	0	0	0
P. syringae	B. thuringiensis subsp. sotto	1	4	4	4	0	4	4	4	0	0	0	0
P. syringae	B. uniflagellatus	1	4	4	4	0	4	4	4	0	0	0	0
R. solani	B. globigii	1	4	4	4	0	3	4	4	0	4	4	4
R. solani	B. polymyxa	1	4	4	4	0	3	4	4	0	3	1	1
R. solani	B. subtilis	1	4	4	4	0	3	4	4	0	3	4	4
R. solani	B. thuringiensis subsp. sotto	1	4	4	4	0	3	4	4	0	3	4	4
R. solani	B. uniflagellatus	1	4	4	4	0	3	4	4	0	3	4	4

[a] Based on a rating scale of 0-4. 0 = no growth or inhibition, 1 = slight growth or inhibition, 2 = moderate growth or inhibition, 3 = good growth or extreme inhibition and 4 = excellent growth or total inhibition.

[b] Plates inoculated with Pseudomonas, Pythium or Rhizoctonia were incubated for six days. Plates inoculated with Fusarium, Gleosporium or Helminthosporium were incubated for 12 days.

TABLE 9. Temperature effects on inhibition of Helminthosporium sativum on soil extract agar and glucose yeast-extract agar by seed treated with Bacillus subtilis and B. uniflagellatus, and their antibiotics.[b]

Seed treatment	Soil extract agar[a]												Glucose yeast-extract agar[a]											
	Bacillus growth (C)				Fungal growth (C)				Fungal inhibition (C)				Bacillus growth (C)				Fungal growth (C)				Fungal inhibition (C)			
	10	12	14	16	10	12	14	16	10	12	14	16	10	12	14	16	10	12	14	16	10	12	14	16
Control	0	0	0	0	2	3	4	3	0	0	0	0	0	0	0	0	2	3	4	2	0	0	0	0
B. subtilis	0	0	0	1	2	2	2	2	0	0	0	0	0	3	3	3	2	1	4	4	0	4	4	3
B. subtilis antibiotic	0	0	0	0	2	3	4	3	0	0	0	0	0	0	0	0	3	4	4	4	0	0	0	0
B. uniflagellatus	0	0	0	0	2	3	3	3	0	0	0	0	0	2	1	0	2	1	2	2	0	2	1	1
B. uniflagellatus antibiotic	0	0	0	0	2	3	3	2	0	0	0	0	0	0	0	0	2	2	2	3	0	0	0	0

[a]Based on a rating scale of 0-4. 0 = no growth or inhibition, 1 = slight growth or inhibition, 2 = moderate growth or inhibition, 3 = good growth or extreme inhibition and 4 = excellent growth or total inhibition.

[b]Plates were incubated for 12 days.

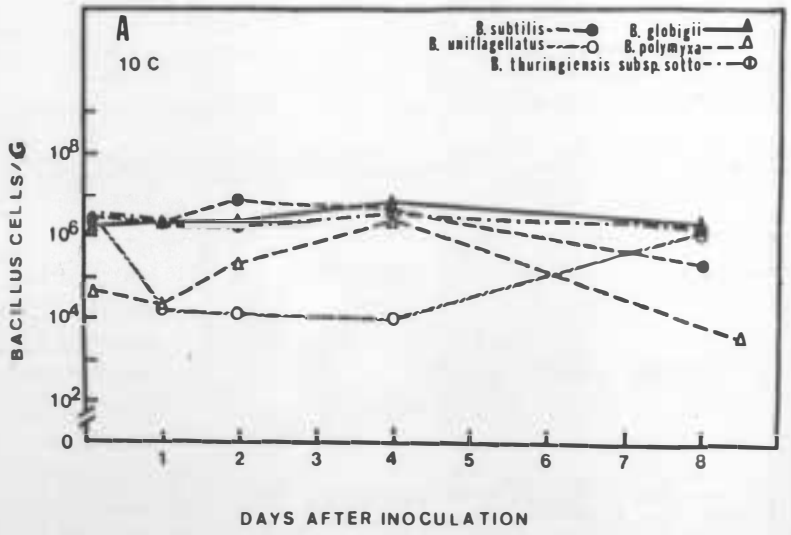
effective when applied as a seed treatment.

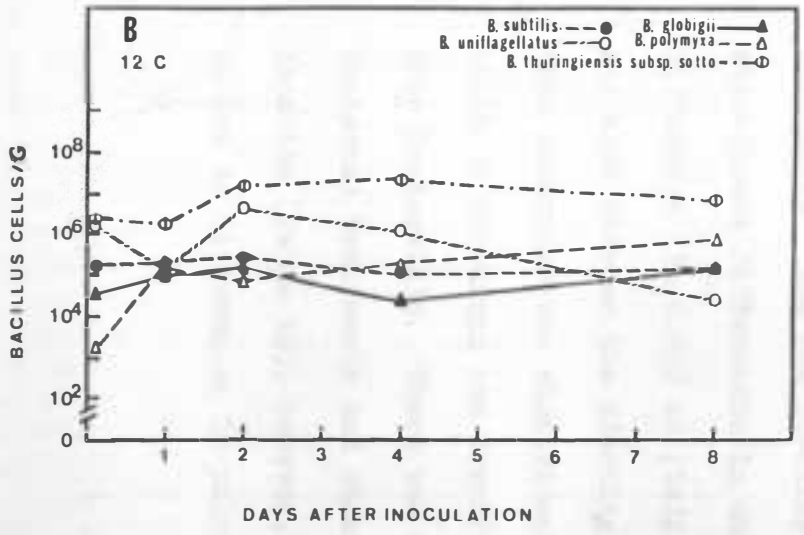
Low soil temperatures also influenced Bacillus species growth in the soil (Fig. 6). The colony forming units (CFU) of all five Bacillus species in the soil at a temperature of 10 C declined with time (Fig. 6). The trend at 12 C for B. globigii, B. subtilis and B. thuringiensis subsp. sotto was towards stability, while B. polymyxa increased initially then remained stable and B. uniflagellatus tended to decrease (Fig. 6). At a soil temperature of 14 C the trend for growth was towards increasing population (Fig. 6). The trend for growth at 16 C was towards stability, except for B. globigii which showed a consistant decrease in population size (Fig. 6). Since the period at which a Bacillus species may have the greatest potential for reducing plant disease is that time from germination to emergence (7), the most important data for Bacillus species growth in the soil was obtained during the 8 days incubation at the various low temperatures (Fig. 6).

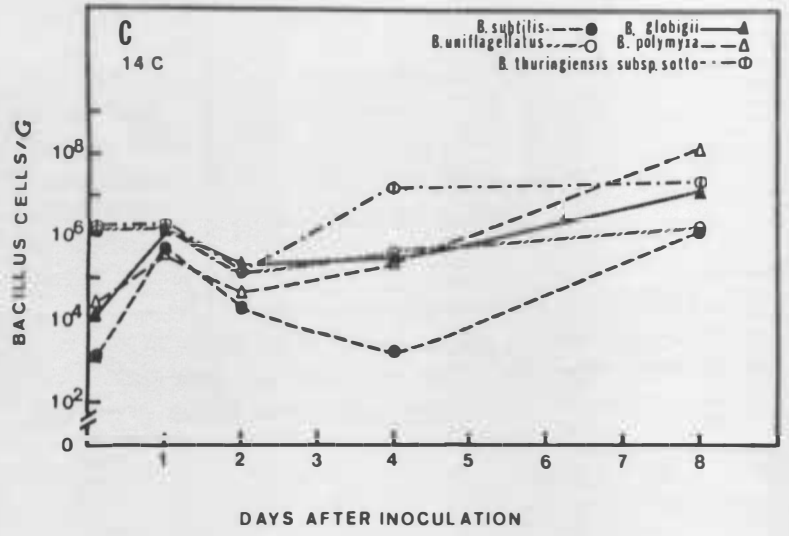
The effects of seed inoculation at various locations and dates in the field. - This experiment was designed to try and correlate soil temperature at the three locations and the two planting dates within each location with Bacillus response as measured by yield. However, the average soil temperatures between the three locations and between the two plantings in each location from the time of planting to emergence was not significantly different.

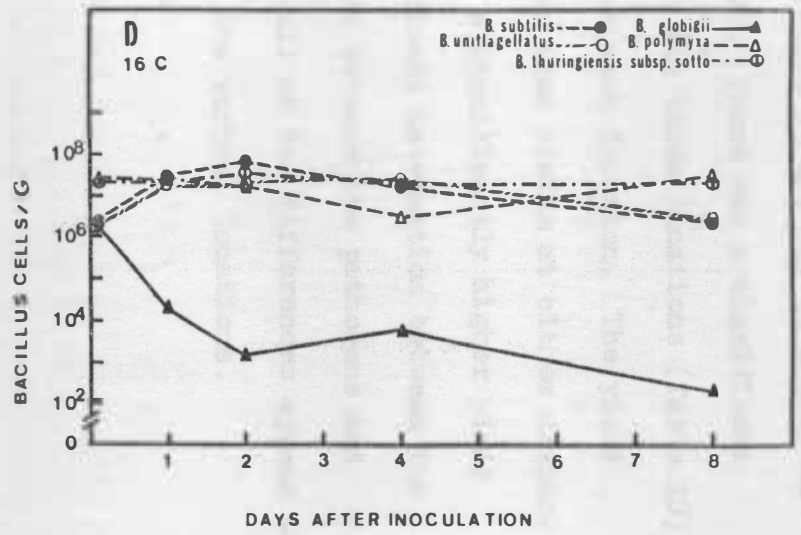
Yield of hill plots planted with the various bacteria-treated wheat and inoculated with the various pathogens showed no significant differences between the Bacillus species treatments or pathogen

Fig. 6 - (A to D). The population trends of five Bacillus species in sterile soil incubated at temperatures of 10, 12, 14 and 16 C. Incubation was continued from inoculation to final sampling 8 days later. Population is measured as colony forming units (CFU)/ g of soil.


A
10 C
B. subtilis
B. globigii
B. uniflagellatus
B. polymyxa
B. thuringiensis subsp. sotto
BACILLUS CELLS/G
DAYS AFTER INOCULATION


B
12 C
B. subtilis
B. globigii
B. uniflagellatus
B. polymyxa
B. thuringiensis subsp. sotto
BACILLUS CELLS/G
DAYS AFTER INOCULATION


C
14 C
B. subtilis
B. globigii
B. uniflagellatus
B. polymyxa
B. thuringiensis subsp. sotto
BACILLUS CELLS/G
DAYS AFTER INOCULATION


D
16 C
B. subtilis
B. globigii
B. uniflagellatus
B. polymyxa
B. thuringiensis subsp. sotto
BACILLUS CELLS/G
DAYS AFTER INOCULATION

treatments. The two planting dates (April 15 and May 5) showed no significant differences in wheat yield. There was a significant difference ($P \leq 0.05$) in yield between the three locations (Table 10) and also between the planting dates at each location. The yield from Watertown was significantly lower than yields at either Centerville or Brookings and Brookings had a significantly higher yield than Centerville. There was a significant interaction between the bacterial treatments and location, and between the pathogens and location (Table 10); however, almost all of the differences appear to be due to differences in rainfall at the various locations.

TABLE 10. The wheat yield from various Bacillus treated wheat and from wheat exposed to various pathogens at three locations; Watertown, Centerville and Brookings.[a]

Location	Bacillus seed treatment	Yield[b]	Pathogen inoculation	Yield[b]
Watertown	B. globigii	2.3800a	F. roseum f. cerealis	3.0717a
Watertown	B. polymyxa	2.5715a	G. bolleyi	2.6317a
Watertown	B. subtilis	2.4267a	H. sativum	2.5433a
Watertown	B. thuringiensis subsp. sotto	2.6950a	P. graminicola	2.4452a
Watertown	B. uniflagellatus	2.9142a	R. solani	2.4717a
Watertown	Control	2.6817a	P. syringae	2.5658a
Total yield		2.6222a		2.6222a
Centerville	B. globigii	16.8317a c	F. roseum f. cerealis	17.9617a c
Centerville	B. polymyxa	17.0458a c	G. bolleyi	16.8292a c
Centerville	B. subtilis	18.4125a c	H. sativum	17.5083a c
Centerville	B. thuringiensis subsp. sotto	18.8833a c	P. graminicola	18.7642a c
Centerville	B. uniflagellatus	17.6108a c	R. solani	17.5883a c
Centerville	Control	16.7317a c	P. syringae	16.8392a c
Total yield		17.5860a c		17.5860a c
Brookings	B. globigii	27.1675 bc	F. roseum f. cerealis	26.6517 bc
Brookings	B. polymyxa	25.1050 bc	G. bolleyi	29.6133 bc
Brookings	B. subtilis	33.5692 bc	H. sativum	30.2492 bc
Brookings	B. thuringiensis subsp. sotto	33.8867 bc	P. graminicola	30.0082 bc
Brookings	B. uniflagellatus	34.8692 bc	R. solani	29.7983 bc
Brookings	Control	22.3942a c	P. syringae	29.8633 bc
Total yield		29.3628 bc		29.3628 bc

[a]All data is expressed in grams per hill plot.

[b]Numbers in columns with similar letters are not significantly different at $P \leq 0.05$ using Duncan's multiple range test.

DISCUSSION

Not all members of the genus Bacillus tested were capable of producing antibiotics that were antifungal in nature. Bacillus globigii, B. polymyxa, B. subtilis, B. thuringiensis subsp. sotto and B. uniflagellatus were responsible for 68% of the total inhibition exhibited by the 20 Bacillus species against the seven pathogens. Since Wood and Tveit (71) and Baker and Cook (7) have suggested that only those species which are able to promote inhibition in vitro have potential of producing that inhibition in the soil, only these five Bacillus species showed promise as possible biological control organisms.

The difference in reaction of certain Bacillus species to the inhibitory substance produced by the Pseudomonas species, or the difference in reaction of certain fungal pathogens to the various Bacillus species is not known but must be related to the ability of the organism to escape toxins or be insensitive to them.

The mode of action of the antibiotics produced by the five Bacillus species seems to be the destruction of the unit membrane. This destruction is indicative of bacillary antibiotics and has been found and described by Frobisher (22), Gottlieb and Shaw (25) and Newton (51).

The antibiotics from the five Bacillus species were heat stable at a pH of 2.5, but rapidly lost activity when heated at pH 8.0. Eumycin, mycosubtilin and mycobacillin show similar lability (39). The antibiotics appear to be chiefly antifungal and do not inhibit Escherichia coli or Staphlococcus aureus as does eumycin. Although no attempt was made to chemically define or physically name the

antibiotics, preliminary studies seem to indicate that the inhibitory substance(s) produced by B. globigii and B. subtilis are very similar to fungocin (56). The antibiotics produced by B. uniflagellatus and B. thuringiensis subsp. sotto appear to have slightly different properties and may be similar to mycobacillin (42). The antibiotic produced by B. polymyxa seems to be slightly different in activity than the other antibiotics. It is surprising that B. polymyxa produces an antibiotic that is antifungal in nature since Pratt and Dufrenoy (56) list the spectrum for antibiotic activity for B. polymyxa as mostly gram negative bacteria. However, Korzybski et al. (39) state that sensitivity of test organisms as well as specific antibiotic composition is very dependant upon the composition of the medium. This is probably true for all the bacillary antibiotics. Composition of the medium may directly influence what specific antibiotic is formed; and, since all bacillary antibiotics are very similar in chemical composition and mode of action, it may be possible for one Bacillus species to produce more than one antibiotic. Also, the same antibiotic, or one very similar, may be produced by more than one Bacillus species (39). Since all antibiotic production from the five Bacillus species was on a glucose yeast-extract medium, the antibiotic(s) produced may be very similar in chemical structure, physical properties and mode of action.

The antibiotic(s) appear to be rapidly destroyed or adsorbed in the soil as all methods used in trying to recover them were negative. The n-butanol used in the extraction method of Vasudeva et al. (66) proved to be more toxic to the fungal test organisms than did

concentrated solutions of the antibiotic(s). Several other extraction methods and assay procedures were used (17, 25, 39, 56, 65) but no antibiotic activity was observed. It appears that the antibiotic(s) produced by the five Bacillus species rapidly lose their activity when placed in, or produced in the soil. They would have to be produced continually and in high quantities to be active enough to inhibit pathogen growth.

Why certain Bacillus species, or their antibiotic solutions, have an effect on plant yield or root length is not known. There seems to be no relationship between the Bacillus treatments, or among their antibiotic treatments, in terms of effects on yield or root length. Some bacillary or antibiotic treatments showed increased yields over the control, and some showed decreased yields. Those treatments which showed an increase in one area, showed a decrease or no response in another and there was no set pattern that developed among any of the treatments. The results are hard to interpret but perhaps the differences in response are due to a phytotoxic effect of the bacteria or bacillary antibiotic solutions, or to growth stimulatory substances produced by the Bacillus species and present in the antibiotic solutions. However, the inconsistency of the data and the degree of variation between the replications tend to question the validity of these results. More study should be done in this area before any solid conclusions can be drawn.

A high concentration of cells of some Bacillus species surrounding the germinating seed and growing seedling does have a phytotoxic, or phytopathogenic effect on root length. Roots having a

high concentration of bacterial cells surrounding them were very much reduced and necrotic. The Bacillus species could be isolated from the lesions indicating that the organism used in seed treatment was the causal agent. Studies showed the concentration of bacterial cells surrounding the necrotic roots to be between 10^{12}-10^{15} cells/root. Whether Bacillus species numbers ever get this high surrounding or on the germinating seed and growing seedling in a field situation is not known; neither is the occurrence of the phytotoxic effect if numbers do get this high. In vitro, however, this phytotoxic effect can readily be observed. Several Bacillus species seem to have the potential of becoming pathogenic at high concentrations, yet inoculated seeds are coated with 10^{6}-10^{7} cells, a fairly high concentration in itself. If environmental conditions - temperature, moisture, nutrients - would ever become optimum for a rapid flush of bacterial growth, the possibility exists that the bacteria could become pathogenic instead of being beneficial or neutral in effect.

No significant effects of wheat yield or plant response, due to Bacillus species seed inoculation, were noted in any of the field trials. Environmental factors - in particular low soil temperatures - at planting and 1-2 weeks after may have been responsible for the failure of response. The Bacillus organisms must grow in the soil before they can produce their antibiotic(s) (32), and the production of the antibiotic(s) determines the success of a Bacillus species in reducing disease incidence and thereby increasing yield (7). If the Bacillus species does not grow or increase in logarithmic or exponential rates, the antibiotic fails to be formed since production of the

antibiotic is late in the growth cycle after the exponential phase and is dependant upon this phase for metabolite synthesis (32).

Spring wheat is planted as early in the spring as possible, in eastern South Dakota, from April 1 to mid-May. Soil temperatures at planting depth at this time were low during this study, averaging from 7-10 C and did not increase rapidly over a 1-2 week period. The low temperatures during this period reached near freezing while high temperatures were seldom much above 15-18 C. The average soil temperature from Watertown, Brookings, and Centerville, S. D. at a depth of 8 cm taken at 8:00 a.m. from April 15 to May 15, 1976 was 8.27 C. Our studies indicate that from the period of planting to emergence, at which time a Bacillus species has the greatest potential of reducing plant disease, the soil temperatures were too low to allow rapid enough growth for the bacteria to produce their antibiotic(s). Temperature in the field must average or be higher than 14 C before Bacillus species growth will occur, and 16 C or higher is needed before antibiotic production is noticed in vitro. Even if nutrients in the soil should increase from exudates of the germinating seed or from the growing seedling, or if organic matter content is high, a soil temperature of 12 C or higher is needed for growth and antibiotic production by Bacillus species. From our studies in vitro, it seems that whenever inhibition of the pathogen by a Bacillus species is noticed, the bacterial growth - rated on a scale of 0-4 - is listed as a 3 or 4. Ten fold serial dilutions gave most probable number (MPN) estimates of a bacterial population listed at 3-4 on our scale of 0-4 tc be between 10^{10}-10^{12} cells per ml. This means that the Bacillus

species must increase from the initial inoculum numbers (MPN = 10^2-10^4 cells/ml) to 10^{10}-10^{12} cells/ml in 12 days before antibiotic production occurs in high enough quantity and quality to inhibit the phytopathogens.

It is very unlikely that Bacillus species numbers would ever increase this rapidly or to this high a population level in the soil, especially in spring when soil temperatures are low.

Our data indicates that the greatest increase in Bacillus species numbers in the soil occurred over a period of 8 days at a temperature of 14 C and was an increase of 10^4 cells from 3.80×10^4 cells/ml to 1.16×10^8 cells/ml. This increase is probably not a rapid enough increase to cause production of the antibiotic. Therefore, even though bacterial growth is noted in the soil at 14 C, it is doubtful that this increase would be rapid enough to insure antibiotic production.

If Bacillus species numbers would ever reach levels of 10^{10} to 10^{12} cells/ml in the soil, there is a possibility of not only antibiotic production, but, more importantly, phytotoxicity, since, at population levels of 10^{10} to 10^{12} and slightly higher, the Bacillus species show phytopathogenic capabilities.

Our results also indicate that the phytopathogenic organisms Fusarium roseum f. cerealis, Gleosporium bolleyi, Helminthosporium sativum, Pythium graminicola, Pseudomonas syringae and Rhizoctonia solani are capable of growth at temperatures of 10 and 12 C. At these temperatures they would have the ability to invade a host plant before the Bacillus species biocontrol organism could grow, or produce

its antibiotic. Why we had no response of our pathogens on yield when we inoculated them into our field plots is not known. Perhaps soil temperature and/or soil moisture was not conducive to growth and invasion or infection, or our method of inoculation was not suitable. It has been suggested that perhaps the pathogens should have been inoculated directly on the seed as a spore or water suspension, followed by inoculation with the Bacillus organism (Dr. Semenuik, personal communication). Studies in vitro have shown this method to be superior over the liquid inoculation in causing pathogenicity and it is thought this method should be used in any future field trials.

Data from the three research farms in eastern South Dakota, and from the two planting dates within each location, suggests that moisture became the critical factor affecting yields of wheat planted late in the spring. The year 1976 was very dry and the significant yield differences between the three locations and between the planting dates in each location can be explained in terms of rainfall. The location that had the least rain had the lowest yields, and wheat planted later in the spring, on May 5, missed the early rains and had yields correspondingly lower than wheat planted on April 15.

Spring wheat must be planted as early in the spring as possible to take advantage of the normal early spring rains. Yet the earlier wheat is planted, the lower the soil temperature, and the lower the soil temperature the less chance the Bacillus organism will have of growing, producing its antibiotic and becoming a successful biological control organism. If we wait with wheat planting until the soil is warm enough for bacterial growth and antibiotic production, the early

spring rains may be missed and the wheat yield may be severely limited due to lack of rainfall.

Several Bacillus species have potential of reducing wheat disease incidence due to antibiotic inhibition of phytopathogens. However, low soil temperatures in the spring would probably prevent bacterial growth and antibiotic production in the soil environment and would limit the success of a Bacillus species as a biological control agent in reducing wheat disease. Perhaps the organism would be more successful on a warm season crop, or in an environment where soil temperatures are from 16-20 C at planting time.

NOTE ADDED IN PROOF

Since this manuscript was submitted to the committee a very recent study has come to our attention. In a paper on the effect of Bacillus species on increased growth of several crops, Broadbent et. al. state that in general "results of glasshouse or growth-cabinet experiments are reproducible, but field trials often are not." Field conditions often vary owing to uncontrolled physical or biological factors. Factors of soil water potential, nutrients and temperature may influence inoculum growth and information is needed concerning the relationship of these factors to successful seed and root bacterization. (BROADBENT, P. K., K. F. BAKER, N. FRANKS, and J. HOLLAND. 1977. Effect of Bacillus spp. on increased growth of seedlings in steamed and in nonsteamed soil. Phytopathology 67:1027-1034.).

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