IDENTIFICATION AND DIFFERENTIATION OF *BACILLUS SUBTILIS* AND A STUDY OF ITS AUTECOLOGY IN ONION RHIZOSPHERE.

by

Dominic Chung Wah Wong

B.Sc.(Hons.)Simon Fraser University 1977

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

August 1981

Approval

Name: Chung Wah (Dominic) Wong

Degree: Master of Science

Title of Thesis: Identification and differentiation of *Bacillus subtilis* and a study of its autecology in onion rhizosphere

Examining Committee:

Chairman: Dr. Robert C. Brooke

Dr. James E. Rahe, Senior Supervisor

Dr. John H. Borden

Dr. A. C. Oehlschlager

Dr. Robert S. Vernon, Research Scientist, Agriculture Canada, Vancouver, B. C., Public Examiner

Date approved: 4 September 1981

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

Identification and differentiation of *Bacillus subtilis* and a study of its autecology in onion rhizosphere

Author:

(signature)

Chung Wah (Dominic) Wong

(name)

9th September 1981

(date)

ABSTRACT

Six bacterial isolates antagonistic to Sclerotium cepivorum Berk., were confirmed to be strains of Bacillus subtilis Ehrenberg on the basis of standard morphological and biochemical criteria.

Several biochemical tests known to produce variable reactions by strains of B. subtilis did not permit differentiation of the six isolates. Differentiation of the six isolates was possible on the basis of cultural characteristics either on nutrient agar or on 10% skim milk agar, thereby suggesting that the isolates were distinct strains.

Vegetative and spore antigens were developed for the strain B2 of B. subtilis. The vegetative antiserum was found to be species-specific when tested against 10 Bacillus species, eight B. subtilis strains and 50 unidentified bacteria. The spore antiserum was less specific. It cross reacted with spores of Bacillus coagulans Hammer and Bacillus pumilis Meyer and Goltheil.

A fluorescent antibody technique was used to assess the populations of vegetative and spore cells of B. subtilis in onion root rhizosphere of bacterized and unbacterized seeds. The population survey was conducted over several weeks, after planting the seeds in the following conditions: (i) sterilized and non sterilized muck soils under greenhouse conditions, and

(ii) muck soil in the field.

Generally higher B. subtilis populations were associated with seeds treatment. Significant differences occurred during germination and early stages of plant growth under greenhouse conditions and persisted longer in sterilized than in nonsterilized soil. In the field trial only one sample period showed a significantly greater number of B. subtilis cells associated with seed treatment.

ACKNOWLEDGEMENTS

The investigations reported in this thesis were carried out under the guidance of Dr. J. E. Rahe for whose encouragement and advice I am truly grateful.

I thank Dr. R. S. Utkhede, Dr. J. H. Borden and Dr. A. C. Oehlschlager for their valuable assistance during the course of this work.

Financial assistance from the Department of Biological Sciences, S.F.U., Agriculture Canada and the National Research Council of Canada is gratefully acknowledged.

Appreciation is also extended to the faculty and staff of the Department of Biological Sciences, S.F.U.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1.

Chapter 2.

Introduction to the thesis.

Sclerotium cepivorum Berk., the causal agent of white rot of onion and other *Allium spp.*, is of long standing, world wide distribution and of considerable economic importance (41,43). The pathogen exists in soil as dormant sclerotia which can survive for many years in the absence of the host plant (43).

The disease was first recorded in the Fraser Valley of British Columbia in 1970. Since 1974 provisions of the Provincial Plant Protection Act have prohibited further onion production on known infested fields. These measures have not halted the advance of the disease, and at the present time virtually all farms producing commercial onions in the Fraser Valley are infested. "Literature suggests (3,9,29) that white rot may become a major limitation to onion production in the Fraser Valley within a few years if the present infestations cannot be checked" (40).

Chemicals can be used to control soil-borne plant pathogens by eliminating the pathogen from the soil or by reducing the inoculum level to allow economic crop production. However, no practical treatment has yet been produced that will eradicate the *S. cepivorum* fungus from the soil. Calomel (mercurous chloride) gives economic control of the disease but is hazardous

and its use is no longer permitted. The fungicide Ronilan is used in Europe, but is not registered on onions in North America. The need for an effective, cheap and safer alternative is emphasized (22). Resistant varieties would be useful but at present none are known (41).

Many cultural practices have come into use which tend to reduce the progress of diseases. Liming of soil to bring the pH up to about 7.0 reduced the incidence of white rot but did not give complete control (38). Walker (43) studied the relationship of soil temperature and moisture to the disease. He found that the pathogen was most devastating at cool temperatures (10 to 20 C) and in fairly dry soil conditions (45% of the water holding capacity). Avoidance of these conditions under field situations is not practical.

Stimulation of germination of sclerotia of S. cepivorum in the absence of host plants, thereby reducing the level of pathogenic inoculum, is an approach based on the research by Coley-Smith and his co-workers (8). Field tests conducted by Merriman et al. who injected onion oil in soil before sowing have demonstrated the potential of this method for control of white rot in dry bulb onions. However, introduction of this type of disease control into commercial practices still requires improvements in the methods, timing of application of treatment, and availability of effective germination stimulants at acceptible cost. Various attempts at biological control of white

rot have been reported and will be referred to in Chapter 1. The subject of this thesis is an evaluation of the nature and autecology of bacterial antagonists to S. cepivorum that have provided significant control of white rot when applied to onions as seed treatments.

Chapter 1.

Identification and differentiation of six bacterial isolates antagonistic to *Sclerotium cepivorum*

Introduction

In a greenhouse experiment, Ahmed and Tribe (1) used pycnidial dust prepared from *Coniothyrium minitans* as soil treatments and as seed dressing to protect onion seeds grown in *S. cepivorum* infested soil. They found that the control afforded by *C. minitans* protected the plants from white rot as well as calomel dressing. *C. minitans* was recommended for development for the practical control of white rot.

More recently, Ayers and Adams (2) using *Sporidesmium sclerotivorum* demonstrated in moist sand, steamed soil and natural soils that the mycoparasite was able to infect and destroy the sclerotia of *S. cepivorum*. They recommended the mycoparasite for biological control by direct seeding of the field to increase parasitism of the natural population of sclerotia. However, its effectiveness may be limited by its apparent inability to survive saprophytically and/or infect the hyphae of *S. cepivorum*.

The use of bacterial antagonists for the biological control of onion white rot has met with some success (40). Utkhede and Rahe (40) demonstrated that the levels of protection provided by some of the six bacterial isolates obtained from S. cepivorum sclerotia were comparable to those provided by chemical treatments. They concluded that these antagonists represented practical potential for the field control of onion white rot.

The isolates were tentatively identified in 1977 as strains of Bacillus subtilis Ehrenberg by students in Bioscience 303 at Simon Fraser University

The objective of the research reported in this chapter was to confirm the identity of the isolates and to differentiate among them, if possible.

Methods and Materials

Six test isolates were investigated (Table 1). Stock cultures were maintained on nutrient agar at 25 C. Twenty four-hour-old cultures grown at 37 C were used as inocula for morphological, cultural and biochemical studies.

Standard procedures (17) were followed for morphological studies, Gram stain, Voges-Proskaur test for production of acetylmethylcarbinol, utilization of carbohydrates, succinate and citrate, hydrolysis of starch and casein, production of

Table 1. Sources of *Bacillus subtilis* isolates and known strains.

Isolate/Strain	Source
BK	Kelowna, B.C.
BO	Olympia, Washington.
B1, B2, B4, B8.	Burnaby, B.C.
niger, *globgii*	Carolina Biological Supply Co.

urease and catalase, and the demonstration of possible anaerobic growth utilizing an overlay of sterile mineral oil over inoculated 1% glucose nutrient broth.

Growth in glucose ammonium salts medium and hydrolysis of hippurate were tested according to Cowan and Steel (11).

Growth on nutrient agar slopes at 25 C and 60 C at pH 6.0, and retardation effects due to 1% glucose on nutrient agar were tested according to Wolf and Barker (45).

To test for antagonism against S. cepivorum each bacterial strain was applied as a single streak onto potato dextrose agar (PDA) containing a freshly transferred plug from a day old culture of S. cepivorum grown on PDA (the plug was cut with a sterile 4mm dia., cork borer). The antagonism test plates were kept at 22-24 C under the normal light conditions of the laboratory and evaluated five days after inoculation. This antagonism study was conducted several times over a number of different occassions.

Photographs of the six isolates on nutrient agar were taken at the seventh day after inoculation.

Results

Morphological characteristics:

All of the six isolates formed rods whose size ranged from 0.8 x 2.4 to 0.8 x 3.2 µm. The spores were oval and centrally located. The typical spore size ranged from 0.3-0.5 x 1.0-1.6 µm.

Cultural characteristics:

The cultural characteristics for all six isolates on various media are shown in Tables 2-4 and Figures 1-6.

In nutrient broth all isolates formed a pellicle two days after inoculation and incubation at 37 C. Turbidity was slight or lacking. The sediment was slight also.

In cultivation of the bacterial isolates with *S. cepivorum* on potato dextrose agar (PDA), inhibition zones were observed between the bacterial colony and mycelial growth by the second day after inoculation and incubation under laboratory conditions (Table 4). On the third day after inoculation the fungal mycelium took on a brown colour and became black with time. By the fifth day white precipitation bands appeared in the

Table 2. Cultural characteristics of Bacillus subtilis isolates grown on nutrient agar[1].

Strains	BK	BO	B1	B2	B4	B8
Colony appearance	dense butyrous growth	feathery	dirt road image	diffuse layer on outskirts of colony	lacy	foliose
Spreading ability[2]	++	+	-	+	+++	++
Elevation	raised	raised	raised	raised	flat	raised
Colour	all were greyish white					
Surface	smooth, glistening	smooth, glistening	smooth, glistening	smooth, glistening	rough, dull	smooth, glistening

1 Cultures kept at 22 - 24 C and recorded after two weeks.

2 Spreading ability indicated by: +++ = excellent, ++ = medium, + = weak, - = none.

Table 3. Cultural characteristics of Bacillus subtilis isolates grown on 10% skim milk agar[1].

Strains	BK	BO	B1	B2	B4	B8
Growth[2]	+++	+	+++	+++	+++	+++
Topology	many ridges	flat	few ridges at edges	flat	flat	small ridges
Spreading ability[2]	+	+	-	+	+++	++
Average lytic diameter (mm) on 10 ml agar	13	6.6	15.5	13	13	7

1 Cultures kept at 22 - 24 C and recorded after four days.

2 Growth and spreading ability indicated by: +++ = excellent, ++ = medium, + = weak, - = none.

Table 4. Cultural characteristics of six Bacillus subtilis isolates grown on potato dextrose agar with Sclerotium cepivorum [1].

Strains	BK	BO	B1	B2	B4	B8
Precipitation bands[2]	+++	-	+	+++	+	-
Spreading ability[2]	+++	+++	-	-	+	+++

1 Cultures kept at 22-24 C and recorded after five days.

2 Density of precipitation bands and spreading ability indicated by: +++ = strong or excellent, ++ = medium, + = weak, - = none.

Figure 1. Bacillus subtilis isolate BK on nutrient agar on the 7th day after inoculation and incubation at 22-24 C, (7 x).

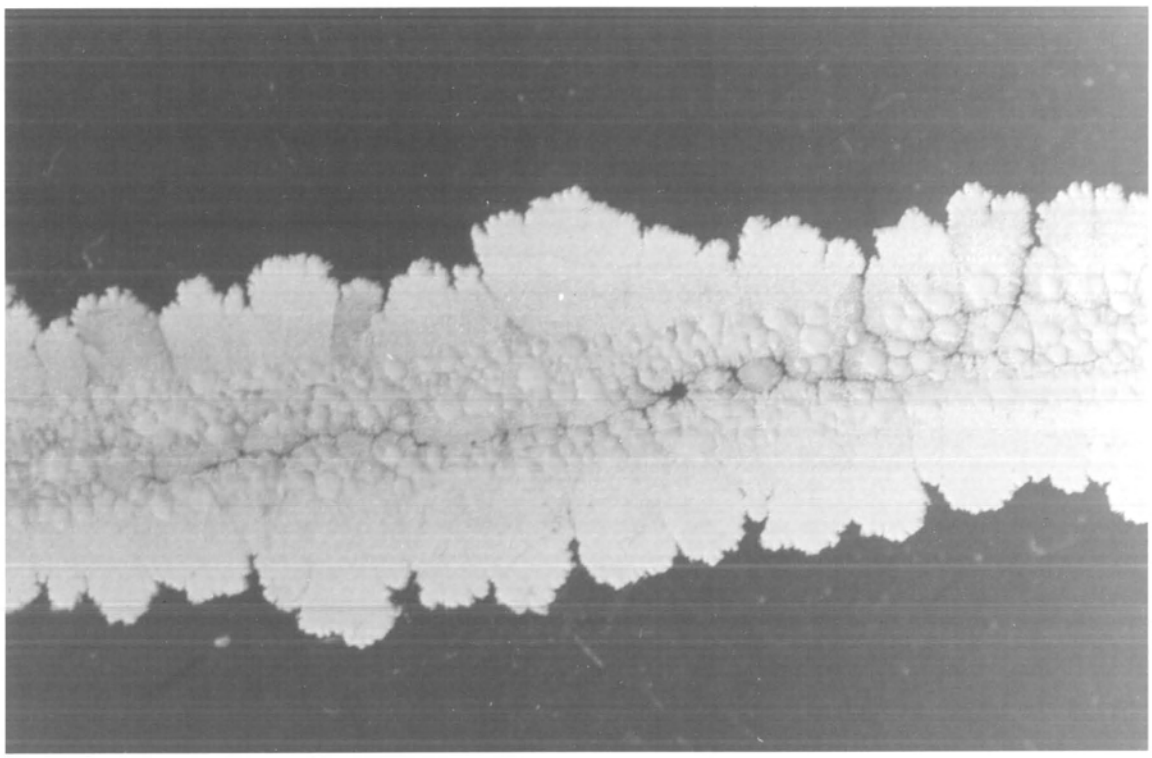


Figure 2. Bacillus subtilis isolate BO on nutrient agar on the 7th day after inoculation and incubation at 22-24 C, (5 x).

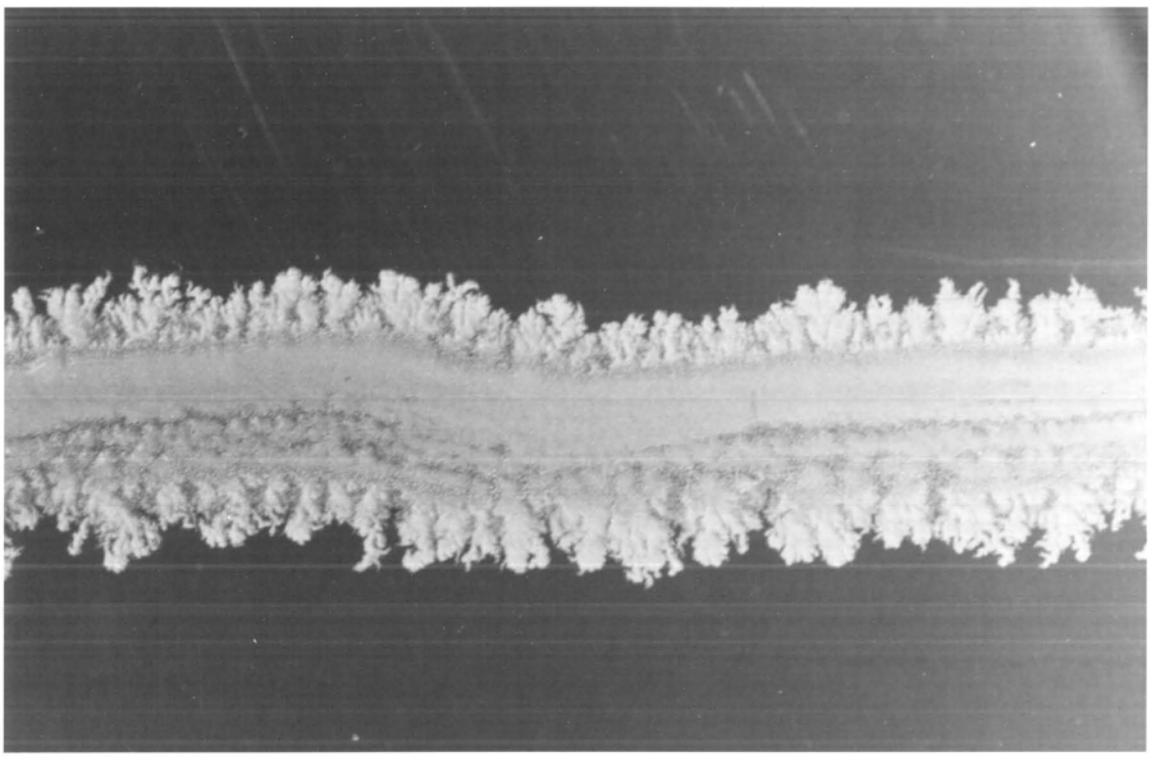


Figure 3. Bacillus subtilis isolate B1 on nutrient agar on the 7th day after inoculation and incubation at 22-24 C, (6 x).

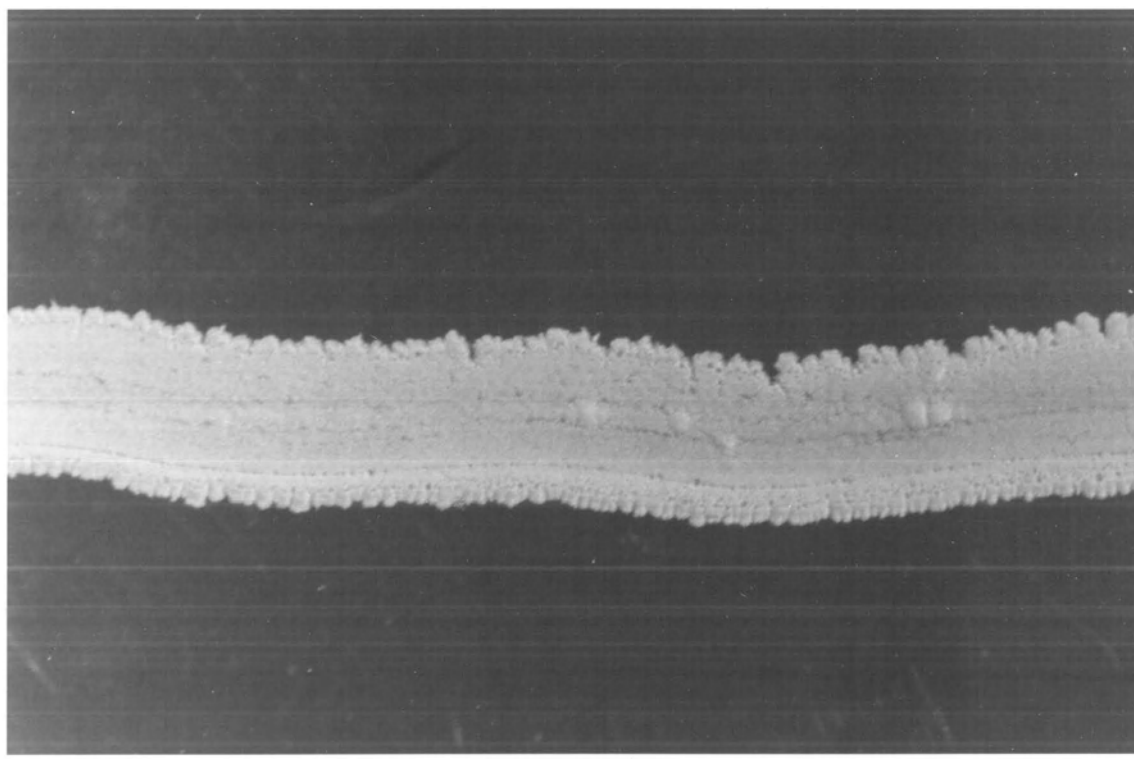


Figure 4. Bacillus subtilis isolate B2 on nutrient agar on the 7th day after inoculation and incubation at 22-24 C, (6.5 x).

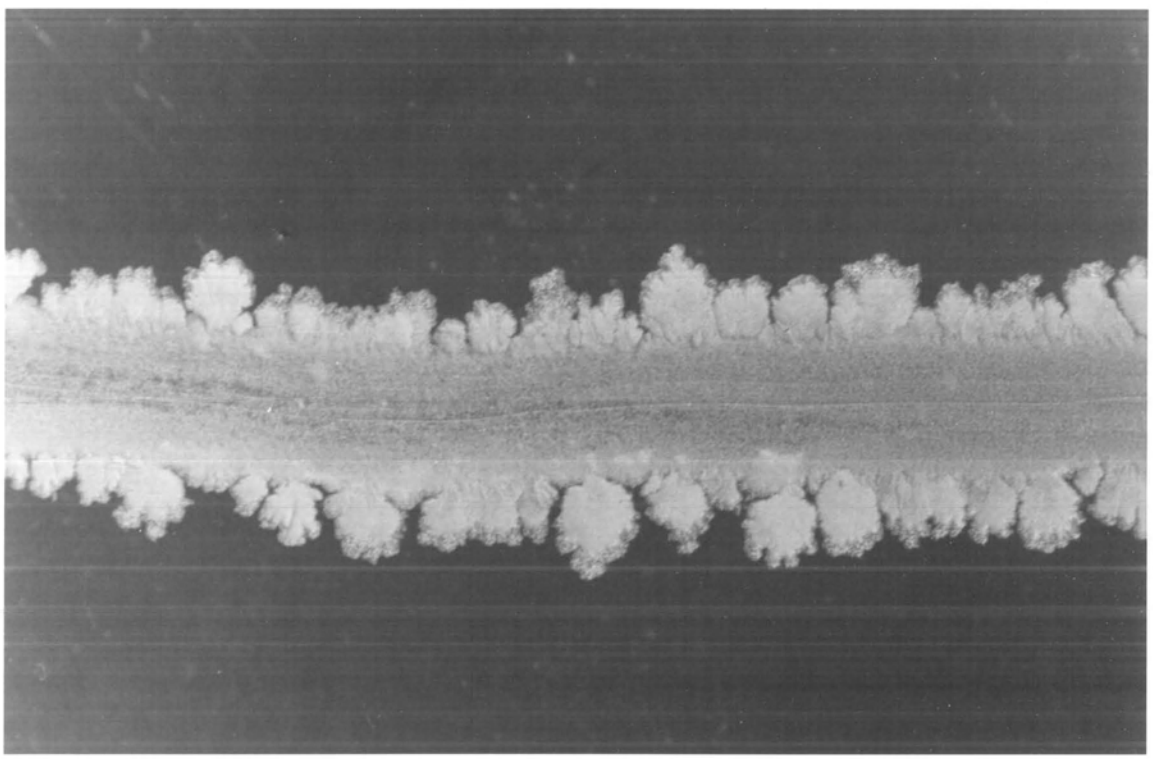


Figure 5. Bacillus subtilis isolate B4 on nutrient agar on the 7th day after inoculation and incubation at 22-24 C, (8 x).

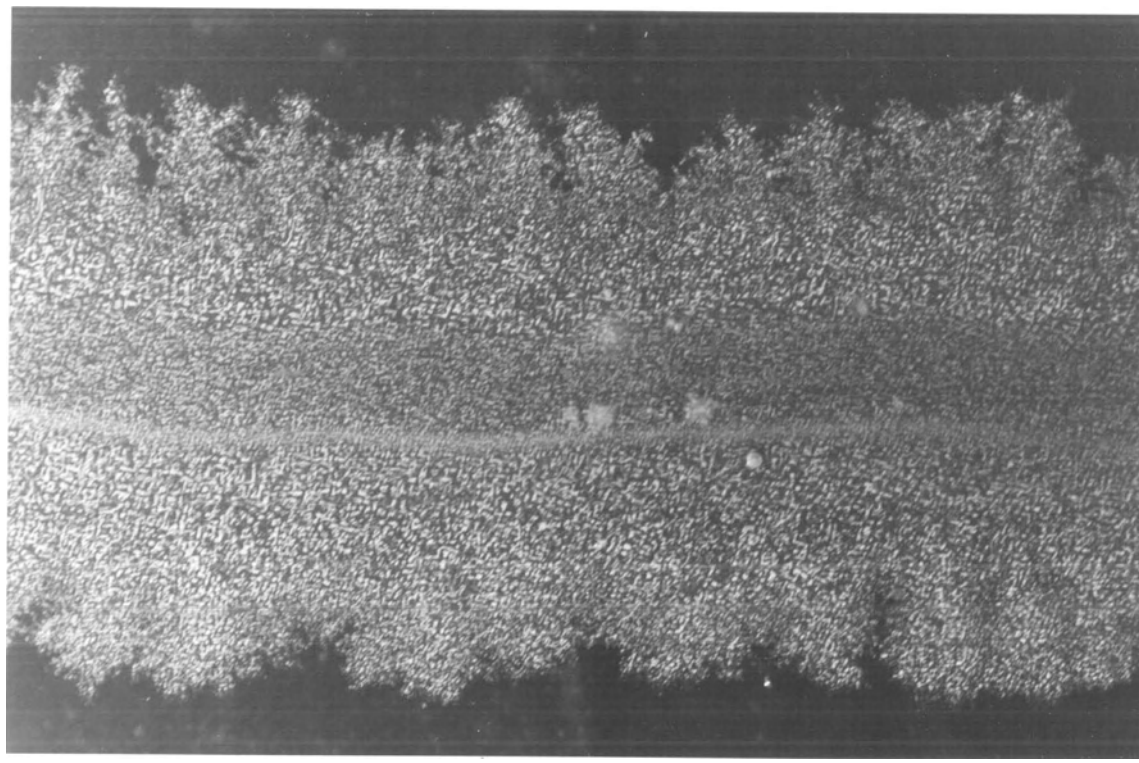
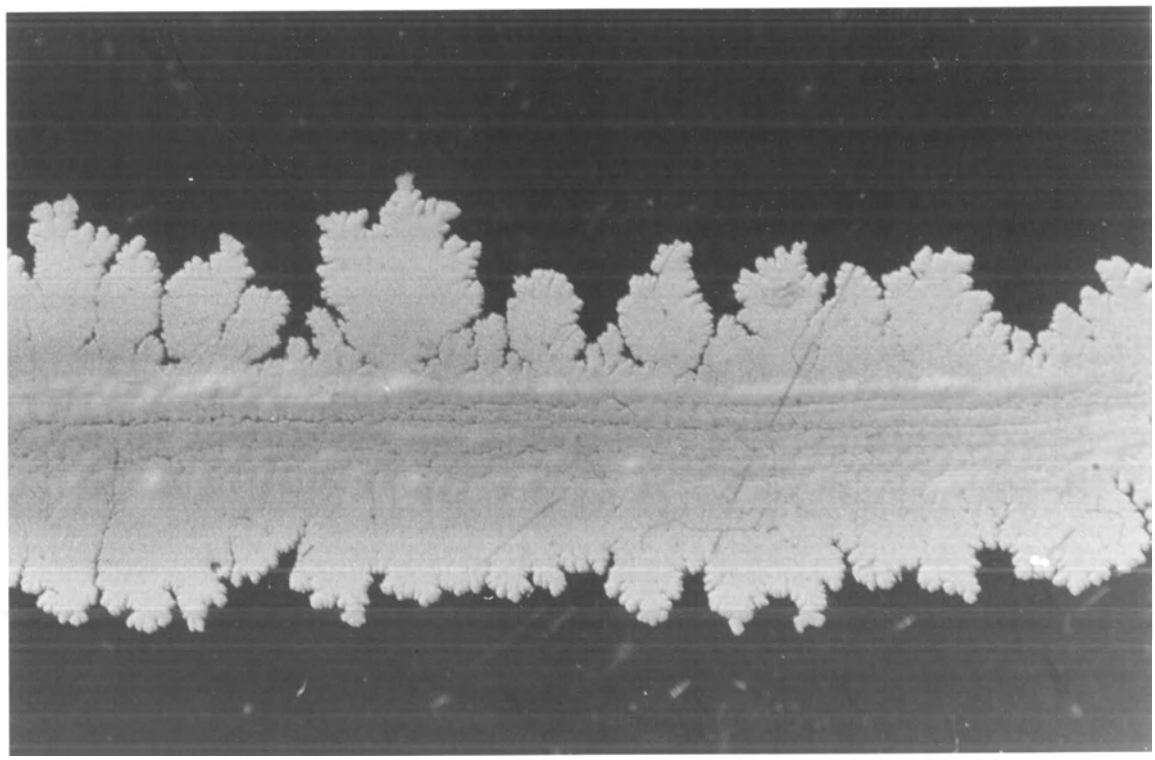


Figure 6. Bacillus subtilis isolate B8 on nutrient agar on the 7th day after inoculation and incubation at 22-24 C, (7 x).



inhibition zones for some of the bacterial strains. These precipitation bands became more conspicuous with time. Isolates B2 and BK consistently displayed the heaviest bands (Table 4).

Biochemical Characteristics.

All six isolates were Gram positive, catalase producing, aerobic spore-forming rods (the three main identifying characteristics of the genus Bacillus), and on the basis of standard biochemical criteria (19,45) all of the isolates were identified as B. subtilis. Their identities were further substantiated by the comparison of sugar fermentation patterns and other biochemical characteristics with those reported for B. subtilis by other authors(15,23,30).

Biochemical characteristics other than sugar fermentation patterns (Table 5) showed no distinguishing characteristics for any of the six isolates. Sugar fermentation patterns (Table 6) disclosed that isolate B2 showed weak acid production in D-glucose and none in D-mannitol, whereas all other isolates produced acid from these sugars. Isolate BO produced a slightly acid solution in salicin whereas the other strains did not. The remainder of the isolates exhibited no distinguishing

Table 5. Biochemical characteristics for isolates and known strains of *Bacillus subtilis*.

Test	BK,BO,B1,B2,B4,B8, *niger*, *globgii*.	Characteristics of *Bacillus subtilis*.[1]
Acid detected in glucose ammonium salt.	all positive	positive
Glucose retardory or noninhibitory.	all noninhibitory	noninhibitory
Urease production.	all grew;growth retarded	variable
Methly red reaction.	all negative	variable
Citrate utilization.	all negative	variable
Succinate utilization	all negative	variable
Aerobic growth at 60 C, pH 6 on nutrient agar.	all negative	negative
Casein hydrolysis.	all positive	positive
Starch hydrolysis.	all positive	positive
Production of acetyl-methylcarbinol.	all positive	positive
Hippurate hydrolysis.	all negative	negative
Reduction of nitrate to nitrite.	all positive	positive
Anaerobic growth in nutrient agar.	all negative	negative
Anaerobic growth in glucose broth.	all negative	negative
Growth in 7% NaCl.	all positive	positive
Pellicle in nutrient broth.	all positive	positive

1 As reported in references 15, 19, 23, and 45.

Table 6. Sugar fermentation patterns[1] for isolates and known strains of *Bacillus subtilis*.

Sugar	BK	B0	B1	B2	B4	B8	*niger*	*golgii*
D glucose	+	+	+	±	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
D mannitol	+	+	+	-	+	+	+	+
D xylose	-	-	-	-	-	-	-	-
L rhamnose	-	-	-	-	-	-	-	-
D arabinose	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-
Salicin	-	±	-	-	-	-	-	-
D sorbital	-	-	-	-	-	-	-	-

1 patterns indicated by: + = acid but no gas, ± = weak, - = negative.

characteristics.

Discussion and Conclusion.

Morphological, cultural and biochemical tests confirm the identity of the six isolates as true strains of *B. subtilis*. The behaviour of the strains in the various biochemical tests does not allow for differentiation among them. While it is possible to differentiate the strains on the basis of growth patterns on nutrient agar, 10% skim milk agar, or PDA, it is not practical. This impracticality is due to the subjective nature of growth patterns and their variability in relation to factors such as composition and age of nutrient medium, time, temperature, age of culture, possible change in cultural characteristics with repeated transfers and probably many other variables. It is obvious that more reliable, efficient and less time consuming methods are still needed for the differentiation of *B. subtilis* strains.

Chapter 2.

A quantitative autecological study of *Bacillus subtilis* in the onion root rhizosphere.

Introduction

Plants and microorganisms coexist in a state of constant interaction. Some specific interactions are of such obvious importance that they attract intense research. The benefits of symbiotic nitrogen fixation in the *Rhizobium*-legume interaction are documented extensively (36). Another example of beneficial interaction between plant and microbe is the mycorrhizal (literally, fungus root) association. It is an effective mechanism for increasing water and nutrient absorption by roots (16). Marx (25) showed that mycorrhizal fungi protected roots of shortleaf pine, *Pinus echinata* Nemnich, seedlings from infection by *Phytophthora cinnamomi* Rands.

Bacterization of seeds for biological control of plant diseases is well documented (6). Nevertheless, relatively little progress has been made towards the long range goal of increasing world food production via bacterization for biological control

(36). The goal of achieving biological control of plant pathogens by manipulation of the microbial community in which the damaging relationship is initiated remains remote.

Biological control of harmful interactions and exploitation of beneficial associations requires a "bird's eye view" of microbial ecology. Before any kind of plant-microorganism association is attained, the microbe must first interact with the microbial community of the soil. It must survive competition in the rhizosphere until the interaction with the plant host is consolidated. Data dealing with a specific microbe in a rhizosphere before establishment of an association with a plant host are virtually nonexistent (36).

The reason for this paucity of data is one of methodology. Immunofluorescence is one of the few experimental procedures to offer a method to study the autecolgy of a specific microorganism in a natural rhizosphere (14,20,24,34,37,). The technique requires facilities which are still not widely available (36). Hence application of the method for examining rhizosphere populations has been minimal.

One of the problems involved in enumerating fluorescent antibody-stained bacteria is that the field of view is extremely small and only a limited amount of soil can be tolerated in each field. Consequently, populations must be high in order to encounter a reasonable number of cells. The problem in aquatic systems is less severe since microorganisms can be concentrated

on a membrane filter (18,37).

Since the development of an acceptable system for bacterial enumeration in soil by Schmidt (34) several papers on this subject have appeared. Reyes and Schmidt (32) used a fluorescent antibody (FA) technique to enumerate the population of a particular strain of Rhizobium japonicum Kirchner, in the rhizosphere of its soybean host. More recently, Jones and Morley (20) used a fluorescent enzyme linked immunosorbent assay (ELISA) technique to study the effect of pH on host plant 'preference' for strains of Rhizobium trifolii Dangeard. Malajczuk et al. (24) used a soil sieving technique together with immunofluorescence to assess spore populations of P. cinnamomi in the rhizosphere and nonrhizosphere soils of tree grass, Xanthorahoea australis Haeck.

The long dormant interest in the autecology of plant rhizospheres is awakening in response to new experimental methods (36).

Utkhede and Rahe (40) reported that the inoculation of onion seeds with liquid cultures of several B. subtilis strains provided varying degrees of the biological control of onion white rot. I am not aware of any published autecological study of B. subtilis in the onion root rhizosphere. This study was undertaken to (i) evaluate the effect of onion seed bacterization on rhizosphere populations of B. subtilis, and (ii) to determine whether immunofluorescence would differentiate

the six newly identified bacterial strains (Chapter 1).

This chapter describes the preparation of antisera against the strain of B. subtilis (B2) that was most effective in controlling onion white rot (40). The specificities of the antisera were tested against diverse bacterial isolates from soil and laboratory cultures. Gamma immunoglobulins (Ig) from the antisera were isolated and purified for use in the FA technique. Immunofluorescence was then used to assess B. subtilis populations in onion root rhizosphere soils under various conditions.

Methods and Materials.

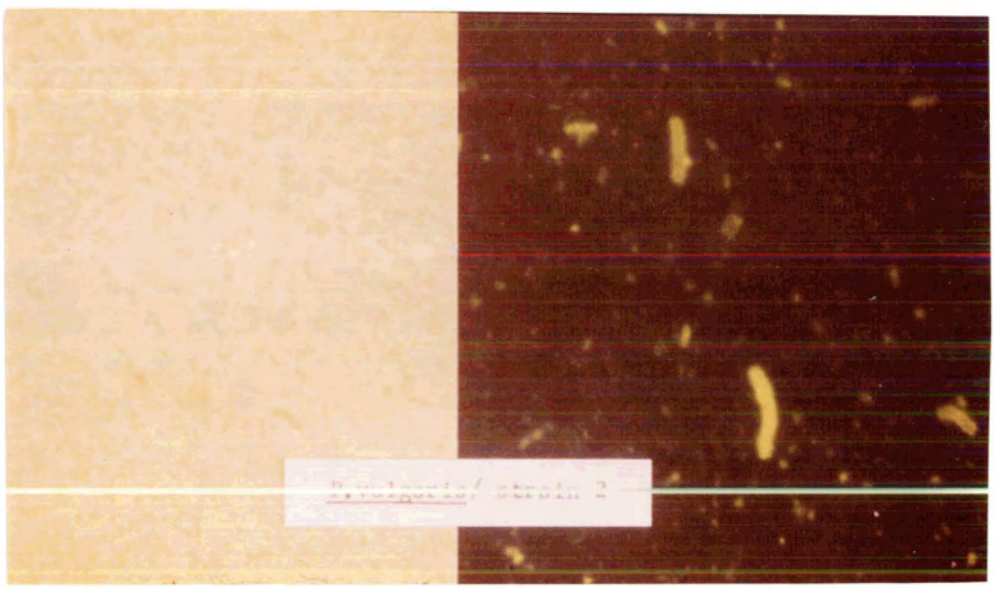
A. Fluorescent antibody preparation and evaluation of specificity.

Antisera against flagellar(H), somatic(O) and spore (S) cells of B. subtilis strain B2 were prepared from white New Zealand rabbits according to procedures described by Norris and Wolf (28). Gamma immunoglobulins (Ig) of high titer antisera were isolated and purified by precipitating three times with ammonium sulphate and desalting by dialysis in phosphate buffered saline(PBS) pH 7.2 (14). These Ig were used for all subsequent FA studies.

An indirect FA technique was used to evaluate the specificity of the antibodies. This technique requires two binding reactions. Firstly, rabbit (primary) antibacteria Ig are bound to the bacteria. Secondly, antirabbit fluorescein-conjugate goat (secondary) Ig are bound to the rabbit proteins (Figure 7). A brighter fluorescence is attained with this method than with the direct method (21)(in the direct technique the fluorescein molecule is conjugated directly to rabbit antibacteria Ig). Caprine antirabbit Ig fluorescein isothiocyanate was obtained in lyophilized form from BBL, Cockeysville MD, USA.

For the specificity studies bacteria were heat fixed onto glass slides. This was followed by seperate 30 min incubations with primary and secondary antibodies. The incubations were performed under moist conditions in the dark at room temperature. Preparations were rinsed at the end of each incubation with PBS pH 7.2. The preparations were dried with tissue and covered with a drop of Difco FA mounting fluid. A coverslip(#=1 1/2, 22mm sq.) was then gently lowered onto the slide. FA-stained preparations were viewed with a Zeiss microscope equipped with epiillumination at 1000x magnification. Illuminiation was provided by a HBO 50 mercury source , an exciter filter BP450-490, chromatic beam splitter FT510 and barrier filter LP520. Reactions of the fluorescing bacteria were rated visually: -(none), +(dim), ++(medium bright), +++(very

Figure 7 (a). Bacillus subtilis and Proteus vulgaris under tungsten illumination. (b). Same field of view showing fluorescent B. subtilis among P. vulgaris using epifluorescence, (1000 x).



(a) (b)

bright).

B.Population studies.

1.Pot trials under greenhouse conditions.

(a) Rhizosphere soil.

B. subtilis populations in rhizosphere soils of bacteria-treated and untreated onion seeds (cv. Autumn Spice) were compared at 5, 10, 14, 21 and 42 days after planting. At zero time, onion seeds were coated with bacteria by immersing them in a 10-day-old potato dextrose broth culture of B2 for 5 min. Three samples of ten seeds each were transferred into Erlenmyer flasks containing 30 ml of 0.85% saline and mixed thoroughly for 3 min. Appropriate volumes were filtered through Nucleopore filters (25 mm dia., 0.4um pore) and bacterial counts were obtained using the FA technique.

Soil for this experiment was collected from a S. cepivorum-infested commercial vegetable farm in Burnaby, British Columbia.

For sterilization, soil was placed in metal trays, covered with aluminum foil, and kept wet for 30 min at 120 C.

Seeds for three replications per treatment were placed in holes 0.5 cm dia., spaced 2 to 3 cm apart in soil in 15 x 13 x 5 cm plastic trays. Three seeds per hole were sown. There were

three trays per treatment. The trays were then placed in a growth chamber at 18 C with 12 h photoperiod (Radiant flux 96.4 uE m^{-2} sec^{-1} PAR measured at plant height with Ll-cor Quantum sensor type Ll-190S coupled to a Ll-185 Quantum meter). Soil moisture was maintained below field capacity.

Samples consisted of ten plants per replication. Whole root systems were dug carefully and subjected to gentle shaking. The soil still adhering was designated as rhizosphere soil. Dislodged soil was discarded. Roots and adhering soil from ten plants (comprising a sample) were detached and agitated for 10 sec in a minimal amount of solution containing 0.85 % saline, 10 ug/ml polymyxin B sulphate and 10 ug/ml cycloheximide. To isolate and determine the nature of any bacteria antagonistic to S. cepivorum , loopfuls of the suspension were then streaked onto PDA plates containing actively growing cultures of S. cepivorum. Bacteria antagonistic to S. cepivorum were examined morphologically and immunologically.

A modification of Schmidt's procedures (34) was used to obtain direct counts of FA-reacting bacteria in the onion root rhizosphere as well as in nonrhizosphere soil. Additional diluant (0.85 % saline, 0.05% Tween 80) was added to the root suspension which was then blended for 3 min using a Sorvall Omni-mixer at a 3.5 setting. This mixture was poured into a graduated cyclinder, brought up to 100 ml with additional diluant and stirred thoroughly for 2 min. After 30 min of

settling, appropriate volumes of supernatant were prefiltered using a 47 mm dia. Nucleopore polycarbonate filter (2.0 um pore size). For the field samples a further 1:10 dilution was necessary for 10-week and older plants because the undiluted suspension was too thick to prefilter. Bacteria were then collected onto an India ink-pretreated Nucleopore filter (25 mm dia. 0.4 um pore). The effective filtration surface was treated sequentially with 0.1 ml of gelatine-rhodamine conjugate (to eliminate nonspecific binding), primary and secondary antibodies. Each treatment was incubated for 30 min in dark moist conditions followed by washing with copious amounts of PBS pH 7.2. The filters were then placed onto glass slides with a drop of Difco FA mounting fluid and covered with a #=1 1/2, 22mm sq. cover slip. Fluorescing bacteria in 30 fields per filter were enumerated. One filter each for vegetative and spore cells per sample was counted. The number of bacteria per ten plants was estimated using the formula:

$$\#/10 \text{ plants} = N \times A \times D/a.$$

where N=average # of bacteria per field.
A=effective filtering area.
a=area of microscopic field.
D=dilution factor.

Preliminary studies had shown that there was no correlation between the number of bacteria with either air-dried soil or air dried plant root weight. Therefore, the number of bacteria per ten plants was chosen as the arbitrary basis for presentation of

data, except for the comparison of levels of B. subtilis between rhizosphere and nonrhizosphere soils where the number of bacteria is expressed per gram of air dried soil. Rhizosphere soil was collected by filtration through a Whatman # 1 filter (preweighed) which was then air dried at 22-24 C for four days.

(b) Nonrhizosphere soils.

At 5, 14 and 42 days after planting approximately 2 cc volumes of nonrhizosphere soil from each of three replications of sterilized and nonsterilized soil were scooped from between plants at a depth of 2 to 4 cm below the soil surface. These samples were then subjected to the procedures described for the quantification of B. subtilis in the onion root rhizosphere. Bacteria inhibitory to S. cepivorum were isolated by the streaking technique and examined morphologically and immunologically.

2.Field trial.

The field trial was conducted beginning May 2, 1980 in Burnaby, British Columbia on the commercial vegetable farm from which soil for the pot trials was obtained. Onions, cv. Autumn Spice, were grown on raised beds containing 5 rows 1.75 m long,

spaced 25 cm apart and double seeded at 7 cm spacing. Plants for sampling were taken at random from the three middle rows. Each bed constituted a replication of which there were three for each treatment. The beds were prepared parallel with every alternate bed being planted with treated seeds.

During the growing period samples were taken at 28, 49, 70, 91, 112 and 133 days after planting and subjected to procedures already described for enumeration of bacteria.

All results were transformed $x' = \sqrt{(x + 0.5)}$ before analysis by ANOVAR and the Newman-Keul's test at 5% level of significance.

Results

A.Specificity of *B. subtilis* antisera.

Vegetative antibodies, flagellar(H) and somatic(O), cross reacted with antigens of each other but not with spores (Table 7). Spore antibody did not cross react with either flagellar or somatic antigens (Table 7).

Flagellar and somatic antibodies of *B. subtilis* B2 did not cross react with any of the flagellar or somatic antigens of the other *Bacillus* species tested (Table 8). The spore antibody cross reacted with spores of *B. pumilis* and *B. coagulans*(Table 8).

With the exception of one bacterial isolate from the soil there was no cross reactivity shown by either flagellar or spore antibodies (Table 9). This one exception was later identified as a strain of *B. subtilis*.

B. Population studies.

1.Pot trial under greenhouse conditions.

(a).Sterilized soil.

Populations of the vegetative cells of *B. subtilis*

Table 7. Cross reactivity of Bacillus subtilis B2 antibodies with flagellar(H), somatic(O), and spore(S) antigens of B.subtilis strains .

Strains	Antigens	Antibodies[1]		
		H	O	S
BK	H	+	+	-
	O	+	+	-
	S	-	-	++
BO	H	++	+	-
	O	+	+	-
	S	-	-	++
B1	H	+++	++	-
	O	+++	++	-
	S	-	-	++
B2	H	+++	++	-
	O	+++	++	-
	S	-	-	++
B4	H	+++	++	-
	O	+++	++	-
	S	-	-	++
B8	H	+	+	-
	O	+	+	-
	S	-	-	++

1 Visual rating indicated by:

+++ = very bright, ++ = medium bright, + = dim, - = none.

Table 8. Cross reactivity of Bacillus subtilis (B2) flagellar(H), somatic(O) and spore(S) antibodies with H, O and S antigens of other Bacillus species.

Bacillus species	Antigens	Antibodies[1]		
		H	O	S
brevis	H	-	-	-
	O	-	-	-
	S	-	-	-
cereus	H	-	-	-
	O	-	-	-
	S	-	-	-
circulans	H	-	-	-
	O	-	-	-
	S	-	-	-
coagulans	H	-	-	-
	O	-	-	-
	S	-	-	++
megaterium	H	-	-	-
	O	-	-	-
	S	-	-	-
pumilis	H	-	-	-
	O	-	-	-
	S	-	-	++

1 Visual rating indicated by: ++ = medium bright, - = none.

Table 9. Cross reactivity of Bacillus subtilis B2 flagellar(H) and spore(S) antibodies with bacterial isolates from soil.

Soil isolates	Antibodies[1]	
	H	S
24 Gram + rods	only 1 +	only 1 +
16 Gram - rods	-	-
1 Gram + cocci	-	-
9 Gram ± rods	-	-

1 + = positive reaction, - = negative reaction.

increased dramatically in the rhizospheres of plants grown from bacteria treated seeds during the first few days of growth. Vegetative cells of B. subtilis also increased in the rhizospheres of control plants, but more slowly and to lower levels. The enhancement of vegetative cell populations associated with seed treatment was significant for at least ten days, but at the 14, 21 and 42 day sampling periods rhizosphere populations for both treatment and control plants had stablized and were virtually identical (Fig. 8, Table 10).

Spore populations of B. subtilis in the rhizospheres of treated and control plants showed generally similar trends. A significant enhancement associated with seed treatment was observed at 5, 10, 14 , and 21 day sampling periods (Fig. 9, Table 10). No significant differences in vegetative or spore populations of B. subtilis between control and treatment were observed in nonrhizosphere soil (Table 11).

(b).Nonsterilized soil.

Seed treatment with bacterial strain B2 enhanced the vegetative cell population of B. subtilis over control significantly only at 5 days after planting. The population trends over time of vegetative cells exhibited by control and treated seedlings were generally similar to those observed in sterilized soil (Fig. 10, Table 12). Seed treatment had no apparent effect on spore populations of B. subtilis and there were no significant changes in rhizosphere spore populations

Figure 8. Rhizosphere populations of vegetative cells of Bacillus subtilis on onions grown in sterilized muck soil under greenhouse conditions. Bars identified by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

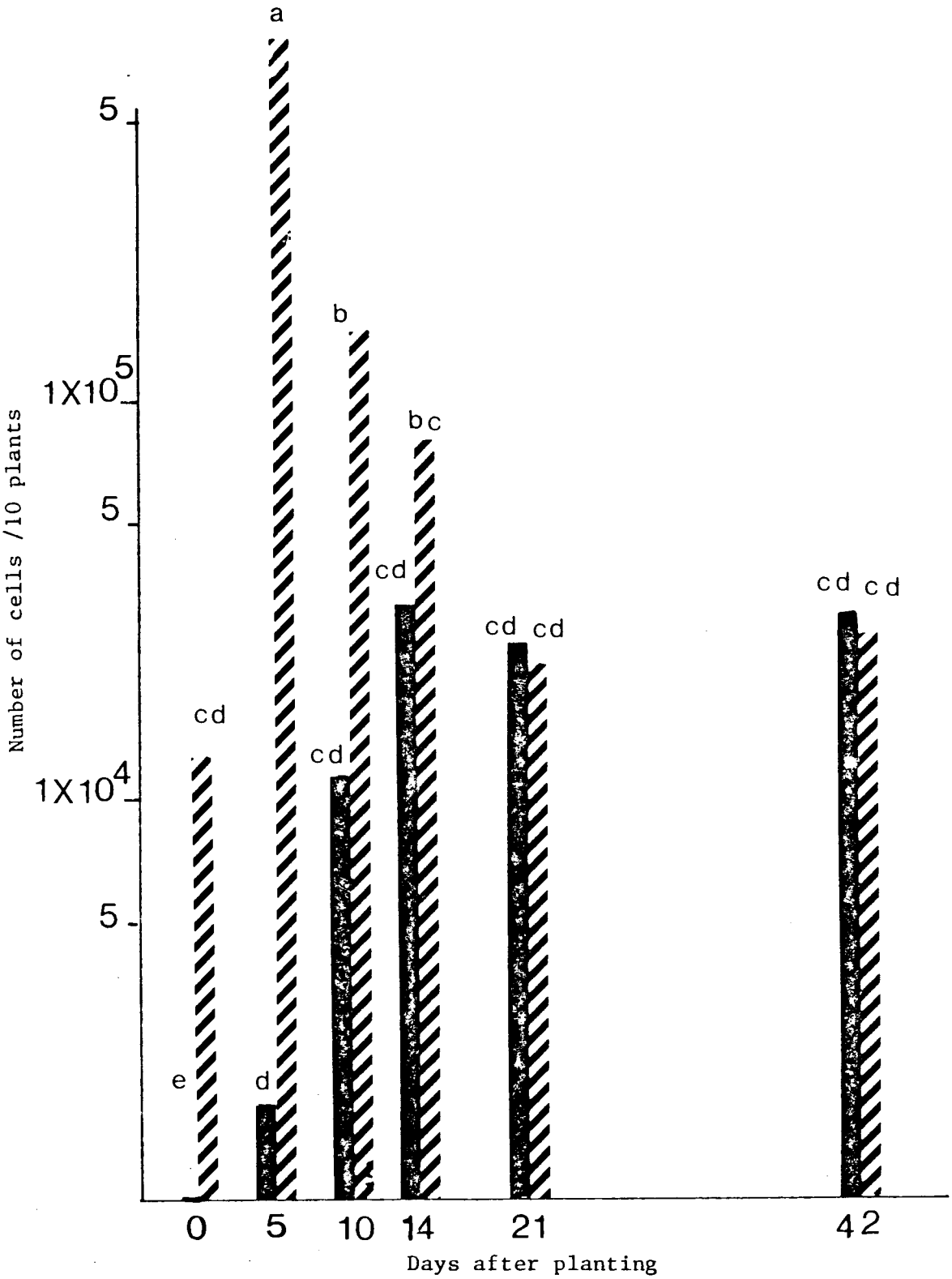

a
b
bc
cd
cd
cd
cd
cd
cd
e
d
5
1X10^5
5
1X10^4
5
Number of cells /10 plants
0
5
10
14
21
42
Days after planting

Table 10. Populations of vegetative(V) and spore(S) cells of Bacillus subtilis in rhizosphere of onions grown in sterilized muck soil,(x 10^{-4}/10plants).

Days after planting	Vegetative(V)[1]		Spore(S)[1]		V + S[1]	
	control	treatment	control	treatment	control	treatment
0	0e	1.06cd	0e	0e	0e	1.06d
5	0.17d	80.05a	2.03cd	11.29a	2.20d	91.79a
10	1.15cd	15.07b	1.47cd	6.69ab	2.61d	21.77b
14	3.20cd	8.41bc	0.82d	4.85bc	4.02cd	13.28bc
21	2.54cd	2.20cd	2.51cd	7.64ab	5.02cd	9.84cd
42	2.94cd	2.62cd	3.27bc	4.24bc	6.24cd	6.87cd

1 Values within each cell type followed by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

Figure 9. Rhizosphere populations of spore cells of Bacillus subtilis on onions grown in sterilized muck soil under greenhouse conditions. Bars identified by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

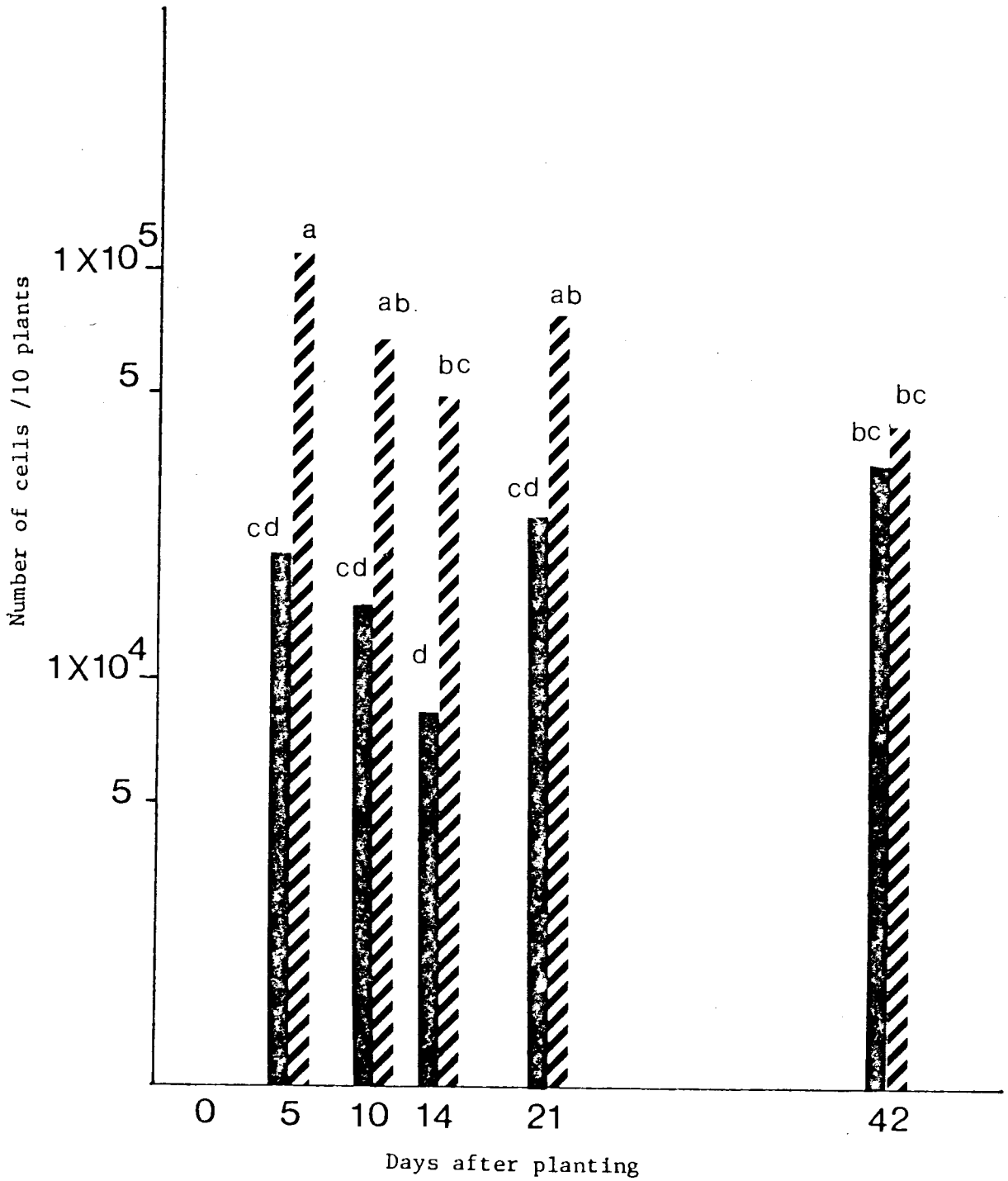

Number of cells /10 plants
1X10^5
5
1X10^4
5
a
ab
ab
bc
bc
bc
cd
cd
d
cd
0
5
10
14
21
42
Days after planting

Table 11. Comparison of populations of Bacillus subtilis in nonrhizosphere and rhizosphere soils of onions grown in sterilized muck soil, (x 10^{-5}/gm soil).

Cell type	Days after planting	Nonrhizosphere		Rhizosphere	
		control	treatment	control	treatment
Vegetative[1]	5	3.63b	4.52b	4.18b	16.20a
	14	3.83b	6.69b	22.45a	24.40a
	42	3.02b	1.53b	1.24b	2.21b
Spore[1]	5	4.33bcd	5.51bcd	9.52b	43.74a
	14	5.27bcd	5.89bcd	0.52e	3.13bcd
	42	8.02bc	4.12bcd	1.16de	2.09cde

1 Values for each cell type followed by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

Figure 10. Rhizosphere populations of vegetative cells of Bacillus subtilis on onions grown in nonsterilized muck soil under greenhouse conditions. Bars identified by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

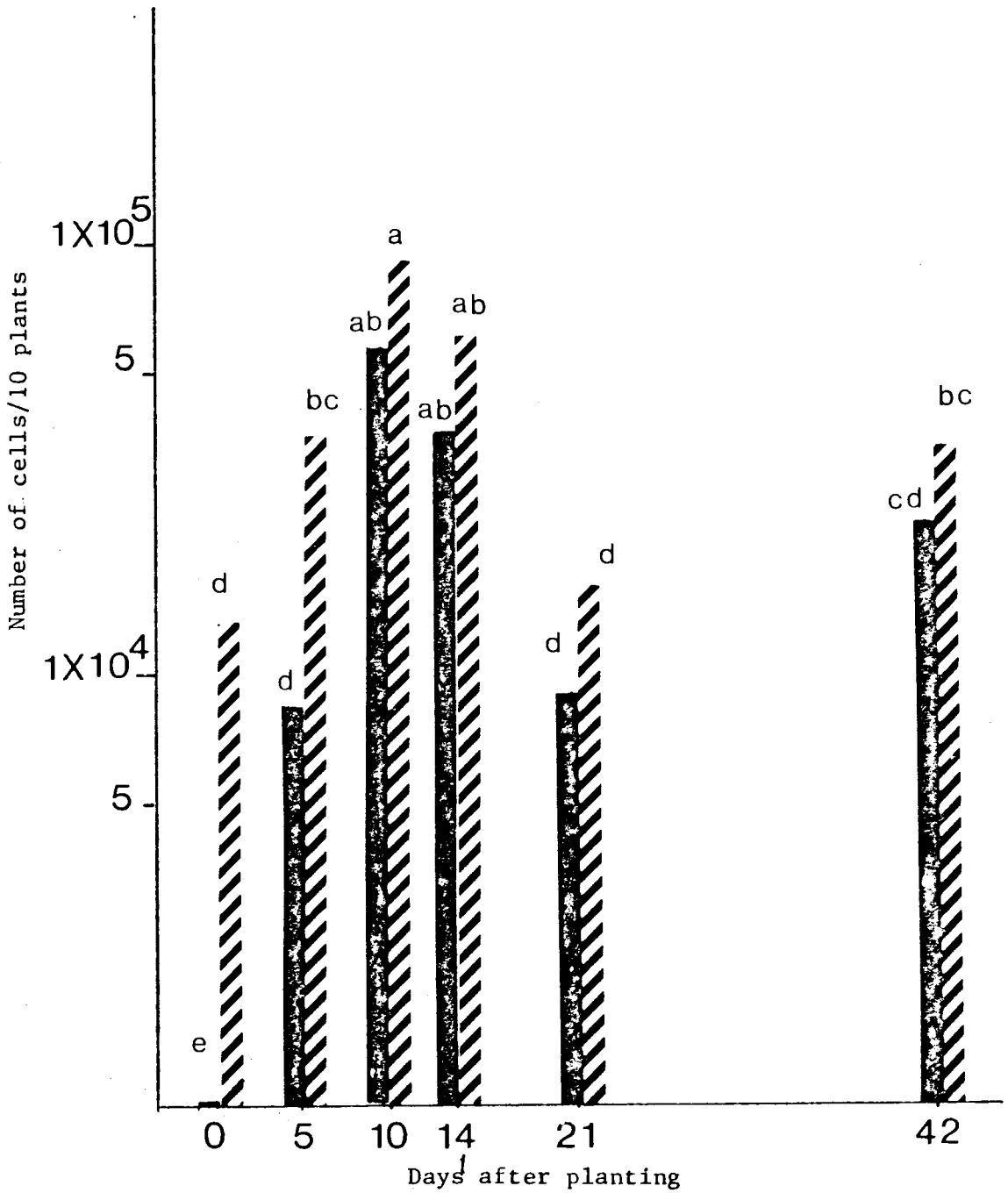

Number of cells/10 plants
1X10^5
5
1X10^4
5
e
d
d
bc
a
ab
ab
ab
d
d
cd
bc
0
5
10
14
21
42
Days after planting

Table 12. Populations of vegetative(V) and spore(S) cells of Bacillus subtilis in rhizosphere of onion plants grown in nonsterilized muck soil,(x 10^{-4}/10 plants).

Days after planting	Vegetative(V)[1]		Spore(S)[1]		V + S[1]	
	control	treatment	control	treatment	control	treatment
0	0e	1.06d	0b	0b	0e	1.06d
5	0.85d	3.64bc	1.77a	3.30a	2.63d	6.94bc
10	5.65ab	9.11a	2.30a	2.63a	7.95ab	13.08a
14	3.64ab	6.04ab	2.84a	2.38a	8.42ab	8.42ab
21	0.94d	1.26d	2.83a	3.56a	3.76cd	4.81bcd
42	2.19cd	3.30bc	3.54a	3.42a	5.74bcd	6.72bc

1 Values for each cell type followed by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

over time for either control or treated plants (Fig. 11, Table 12).

With the exception of one sample(treatment 5 days after planting) there were no significant differences in vegetative cell or spore populations between control and treatment samples of nonrhizosphere soil (Table 13).

2.Field trial.

At only one sampling period, 91 days after planting, was there a significant enhancement of vegetative cell populations for treatment over control. (Fig. 12, Table 14). The population trends for both treatment and control increased then remained steady with time. There were no significant differences in the spore populations between treatment and control (Fig. 13, Table 14).

Meteorological data for the field trial (Tables 15, 16)show adverse wet conditions that prevailed for the 1980 growing season, compared with those of previous years.

C.Antagonistic bacteria from sterilized and nonsterilized soil.

Bacteria antagonistic to S. cepivorum were isolated with a higher frequency from treated than from control plantings in both sterilized and nonsterilized soils. More antagonistic

Figure 11. Rhizosphere populations of spore cells of Bacillus subtilis on onions grown in nonsterilized muck soil under greenhouse conditions. Bars identified by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

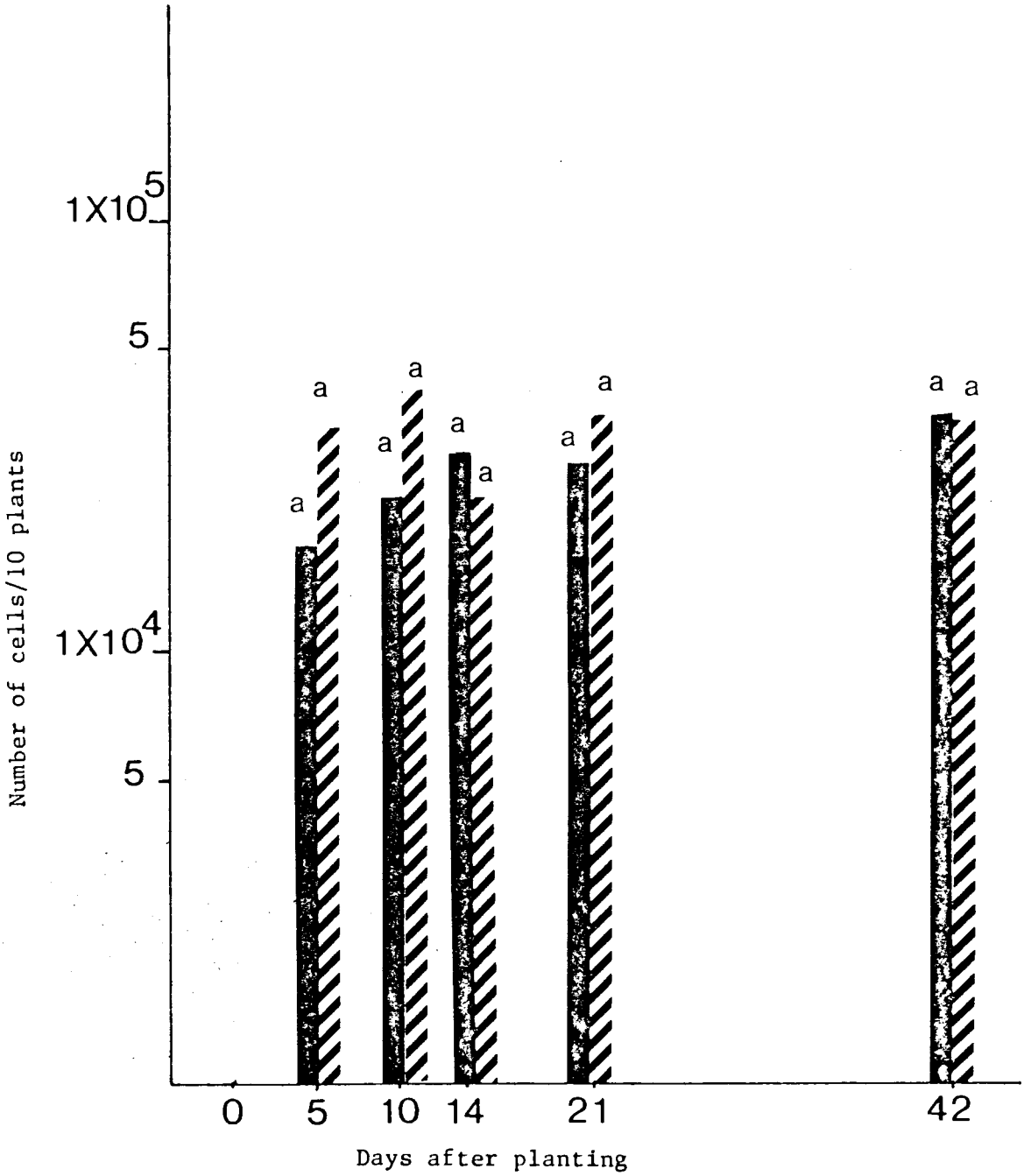

1X10^5
5
1X10^4
5
Number of cells/10 plants
a
a
a
a
a
a
a
a
a
a
0
5
10
14
21
42
Days after planting

Table 13. Comparison of populations of Bacillus subtilis in rhizosphere and nonrhizosphere soils of onions grown in nonsterilized soil, (x 10^{-5}/gm soil).

Cell type	Days after planting	Nonrhizosphere		Rhizosphere	
		control	treatment	control	treatment
Vegetative[1]	5	3.31b	15.59b	0.84b	305.49a
	14	4.49b	5.84b	2.25b	5.54b
	42	5.33b	3.78b	1.11b	1.33b
Spore[1]	5	3.71b	8.09ab	8.69ab	14.72a
	14	6.81ab	8.54ab	7.17ab	1.77b
	42	3.70b	3.46b	2.04b	2.27b

1 Values for each cell type followed by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

Figure 12. Rhizosphere populations of vegetative cells of Bacillus subtilis on onions grown in muck soil under field conditions. Bars identified by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

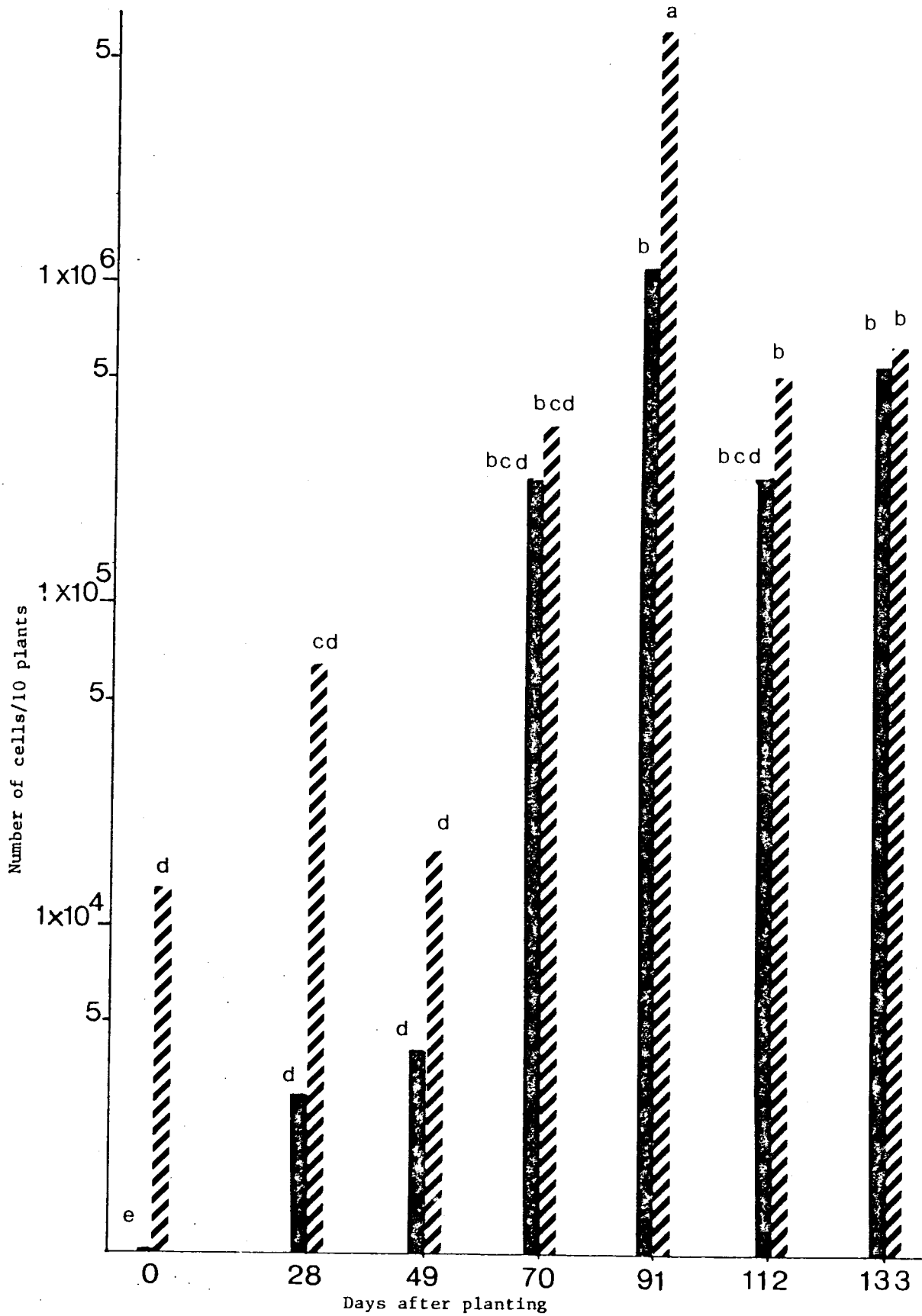

Number of cells/10 plants
5
1x10^6
5
1x10^5
5
1x10^4
5
e
d
d
cd
d
d
bcd
bcd
b
a
bcd
b
b b
0
28
49
70
91
112
133
Days after planting

Table 14. Populations of vegetative(V) and spore(S) cells of Bacillus subtilis in rhizosphere of onions grown under field conditions,(x 10^{-5}/10 plants).

Days after planting	Vegetative(V)[1]		Spore(S)[1]		V + S[1]	
	control	treatment	control	treatment	control	treatment
0	0e	0.13d	0d	0d	0e	0.13d
28	0.13d	0.67cd	1.07c	1.23c	1.58d	3.59d
49	0.04d	0.17d	0.43c	0.62c	0.82d	1.51d
70	2.27bcd	3.48bcd	17.99b	22.46ab	33.10bc	43.71bc
91	10.74b	60.22a	22.52ab	35.71a	67.06b	140.82a
112	2.52bcd	5.20b	8.01b	8.89b	19.58b	27.67c
133	5.57b	6.44b	10.66b	9.97b	31.71bc	32.11bc

1 Values for each cell type followed by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

Figure 13. Rhizosphere populations of spore cells of Bacillus subtilis on onions grown in muck soil under field conditions. Bars identified by the same letter do not differ significantly, Newman Keul's test, $P \leq 0.05$.

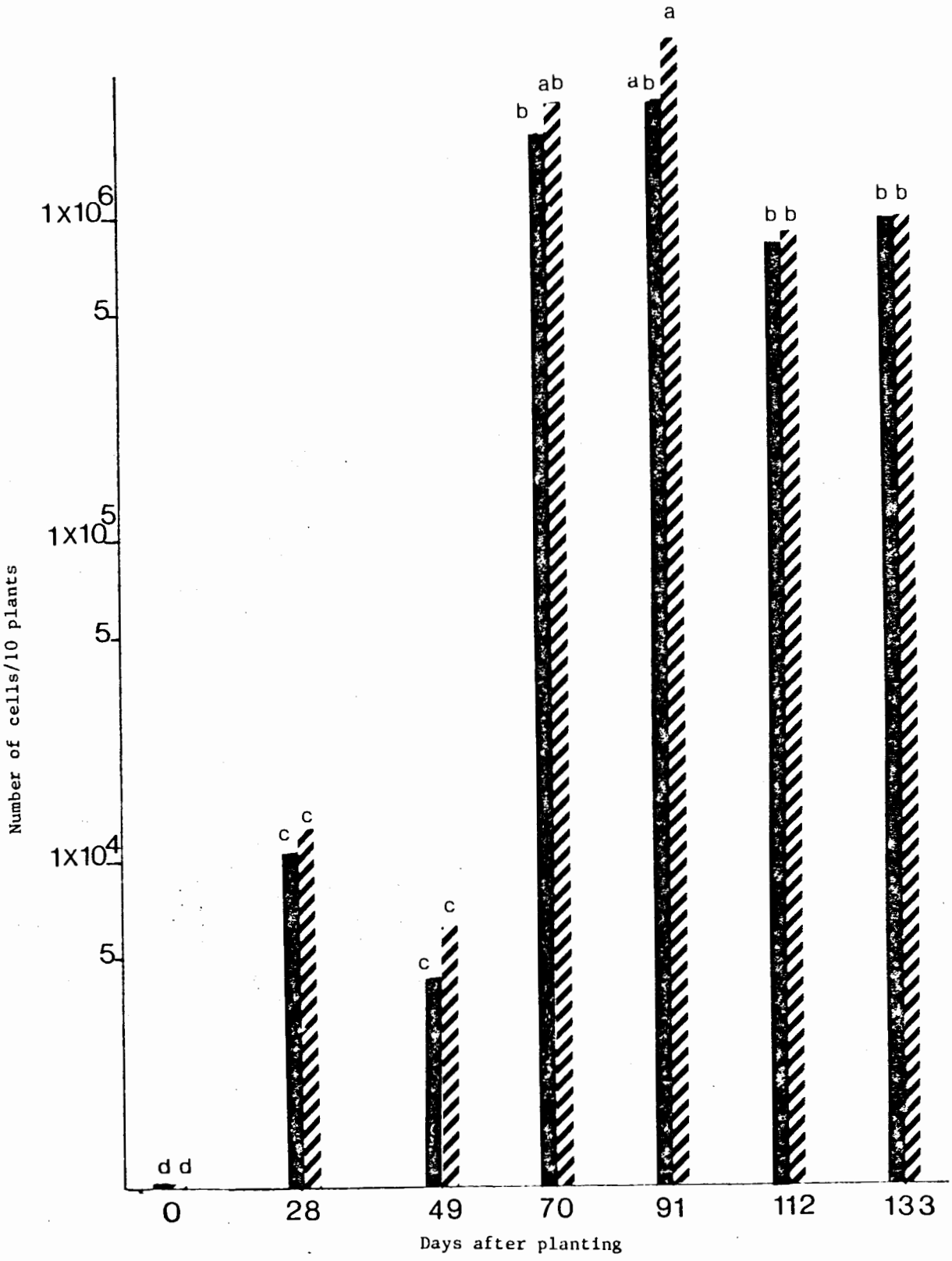

Number of cells/10 plants
1x10^6
5
1x10^5
5
1x10^4
5
d d
c c
c c
b ab
ab a
b b
b b
0
28
49
70
91
112
133
Days after planting

Table 15. Meteorological data for the 1980 growing season: mean daily temperature and rainfall for 10 day periods prior to field sampling at indicated days after planting.

Days after planting	Temperature(C)	Rainfall (mm daily).
28	12	5.4
49	14	5.4
70	11.5	5.3
91	17	0.06
112	17	2.7
133	15.5	4.1

Table 16. Summary of meteorological data for field trial area for 1976 through 1980 growing seasons[1].

	1976	1977	1978	1979	1980
Air temperature (C) 30cm above ground					
absolute range	7-27	2-29	6-32	3-30	8-21
mean daily range	14-22	11-21	10-20	15-18	8-18
Soil temperature (C) 10cm below ground					
absolute range	3-30	9-26	10-20	9-23	11-21
mean daily range	20-25	16-19	15-18	11-21	13-17
Rainfall (mm)					
total	283	218	290	196	520
daily mean for					
May	4.8	2.4	1.5	1.1	4.3
June	2.3	0.6	0.7	1.3	4.7
July	1.0	1.3	0.2	1.3	3.6
August	3.2	2.1	4.2	1.8	3.4
September	1.6	1.7	8.7	2.1	4.4

1 Data for the periods 18 May to 15 September 1976, 3 May to 15 September 1977, 16 May to 15 September 1978, 3 May to 15 September 1979 and 7 May to 12 September 1980.

bacteria were isolated from treated than from control rhizosphere samples during the early stages of plant growth (Table 17).

Discussion

A. Specificity of *Bacillus subtilis* antisera.

(1). With strains of *B. subtilis*.

Studies by Norris and Wolf (28) on *B. subtilis* and Davies (12) on *Bacillus polymxa* Prazmowski, have demonstrated that the flagellar(H) antisera can be strain specific. However, after maintaining these strains on nutrient agar for several months Davies (12) found that fresh antisera bound not only the homologous strain but other strains also. Both groups found that injection of H antigen stimulated the production of somatic(O) antibody in addition to the homologous H antibody. My results (Table 7) confirm their findings, that is that the H antisera bound the homologous O antigen as well as the H antigens of other strains. The similar degrees of fluorescence exhibited by

Table 17. Streak tests for antagonistic bacteria from sterilized and nonsterilized soil[1].

Soil treatment	Soil sampling site	Treatment[2]	Days after planting 5	10	14	21	42
Sterilized	Rhizosphere	T	+[3]	+[3]	-	-	-
		C	-	-	-	-	-
	Nonrhizosphere	T	+	N/D	+	N/D	+
		C	-	N/D	-	N/D	-
Nonsterilized	Rhizosphere	T	+[3]	+	-	-	+
		C	-	+	-	-	+
	Nonrhizosphere	T	-	N/D	+	N/D	+
		C	-	N/D	+	N/D	-

1 + = antagonistic bacteria found, - = no antagonistic bacteria found.

2 T = treatment, C = control.

3 = B2 isolated from antagonistic bacteria.

N/D = no data.

strains B1, B2, and B4 (Table 7) demonstrate that strains B1 and B4 share more of the antigenic determinants of strain B2 than do strains BK, BO and B8. Differentiation among the six strains was not possible using FA.

The spore antibody did not bind H and O antigens and vice versa (Table 7). In agglutination reactions, the spore antibody was less specific. Spore antibody cross reacted with all of the B. subtilis strains tested (Table 7). No variation in the degree of fluorescence was found. This is in agreement with the study by Norris and Wolf (28) who reported that the spores of each of the 25 B. subtilis strains they tested were agglutinated by a spore antiserum.

(2). With other Bacillus species.

Data in Table 8 confirm the finding of Norris and Wolf (28) that both flagellar and somatic antibodies are species-specific. The spore antibody cross reacted with spores of Bacillus pumilis Meyer and Goltheil, and Bacillus coagulans Hammer (Table 8). These three bacteria share at least a common antigenic determinant. The nonspecificity of the spore antibody for spores of B. pumilis confirms the similar finding of Norris and Wolf (28).

(3). With other soil bacteria.

It was essential to determine the specificity of the various antibodies prior to their utilization in a soil population survey. Of all the 50 soil bacteria tested , only one bound the H antibody (Table 9). This particular isolate was later identified as B. subtilis. Data in Tables 7 and 8 combined with this data set indicate that the H antiserum has a high degree of species specificity. The spore antibody cross reacted with only one of the soil bacteria spores that which was later identified as B. subtilis.

B.Population studies.

1. Pot trials under greenhouse conditions.

(a) Sterile soil.

Soil sterilization serves to eliminate competing organisms that would hamper the initiation of an association between plant and microbe. The data depicted in Table 10, Fig. 8 and 9

indicated that bacterization enhanced the B. subtilis population up to at least ten days. These data suggest that B. subtilis is not being established in the root rhizosphere of cv. Autumn Spice despite the initial absence of other microflora.

(b) Nonsterile soil.

Data in Table 12, Fig. 10 and 11 reinforce the finding that treatment significantly enhanced the B. subtilis population. However, these significant differences were of a shorter duration than those that occurred in sterile soil. At ten days there was no significant difference between treatment and control. This shorter duration is to be expected when one considers the competitive nature of nonsterile soil. Existing microflora can prevent the establishment of an inoculant by competition and/or antibiosis. Indeed isolations of bacteria from nonrhizosphere soil and rhizosphere soils gave evidence for the presence of bacteria antagonistic to S. cepivorum (Table 17). These antagonistic bacteria also displayed diffusible antibiotics suppressive to the growth of B2 when they were plated together on PDA.

(c) Assessment of Bacillus subtilis in nonrhizosphere soils.

Data in Tables 11 and 13 lead to several major observations. Nonrhizosphere soils from treated and untreated

pot trials contained both vegetative and spore cells of B. subtilis. Secondly, for the majority of the samples assayed the resident population in the rhizosphere of control plants was not dramatically greater than in nonrhizosphere soil. Furthermore, although treated plants demonstrated a significantly greater population at five days after planting, there was no difference at 14 days. These additional data lend support to the finding that B. subtilis is not closely associated with onion roots.

2. Field trials.

Treated seeds exhibited a significantly higher vegetative B. subtilis cell population for the 13 th week sample (Table 14, Fig. 12). This phenomenon coincided with a warm dry ten day period prior to sampling (Table 15). It suggests that climatic conditions played a role in determining the resident B. subtilis population. For the first time in three years of field trials B2 treatment of onion seeds did not give significant control of onion white rot(R.S.Utkhede and J.E.Rahe*, personal communication). This lack of control also coincided with an abnormal amount of rainfall for the 1980 growing season over similar periods for the preceeding three years (Table 16)

---

* Agriculture Canada Research Station, Summerland, B.C. and Biological Sciences, Simon Fraser University, Burnaby, B.C. , respectively.

In order for the inoculum to control the disease it must multiply around the emerging root. If it declines or dies a permanent control is unlikely. I have ascertained that the B. subtilis population in the onion root rhizosphere showed no difference between treatment and control. If Utkhede and Rahe had obtained significant control of onion white rot during the 1980 field trial it could suggest that the resident strains of B. subtilis were different in treated plants than in controls, and that this strain more effectively modified the microflora which in turn effected the control of onion white rot. Unfortunately the sensitivity of the antisera developed could not readily differentiate among the strains of B. subtilis. Attempts to reisolate and identify the inoculated B2 strain were successful only in the early stages (up to 10 days after planting) of plant growth, when its population was high (Table 17).

Conclusion

Fluorescent antibody(immunofluorescence) techniques provide a new and unique capacity to identify and enumerate specific bacteria directly in a natural environment. The method has been

used for strain detection and enumeration of several bacteria in soil(13,18,20,21,24,32,34,37). For these reasons several antisera for B. subtilis strain B2 were developed.

The cross reactivity studies demonstrated several points: (i) the vegetative antibody did not cross react with spore antigens, (ii) it could not be used to differentiate among the six B. subtilis strains, (iii) it was species specific, and (iv) the spore antibody did not cross react with the vegetative antigens(H and O). It was less specific because it bound not only B. subtilis spores but also those of B. pumilis and B. coagulans.

This is apparently the first study concerning the direct enumeration of populations of B. subtilis in rhizosphere soil of developing onion roots. In the rhizosphere of onion roots B. subtilis multiplied during the early stages of germination and seedling development, but the population then declined to levels not significantly different from those in untreated seed. Results indicated no close relationship between B. subtilis and onion roots. In order to maintain a high population of a desirable B. subtilis strain an occasional spraying of onion plants with the bacterium is recommended. It is also important that future studies evaluate conditions such as strain type, age of inoculum, soil fertility, pH, moisture, etc., that can affect the survival and proliferation of bacterial antagonists. Some of these studies are now feasible with the advent of the FA

technique.

General discussion conclusion and recommendations.

Its broad spectrum of activity, its ability to adapt to different environmental conditions and its capacity to increase yeild in some crops (27,40) should make B. subtilis an attractive biological control agent for further investigation and development. A number of different ways of applying the antagonist onto seeds such that the inoculum remains viable and in optmium numbers should be evaluated. It has been shown that the amount of inoculum applied does influence the establishment of the antagonist and its ability to control the pathogen. Penicillium oxalicum at 5 x 10/seed did not protect peas from seedling blight, but at 6 x 10/seed gave as good a control as did seed treatment with Captan (44). Brown et al. (17) found that 10 to 10 bacteria Azotobacter sp. were required for rhizosphere establishment.

Other methods for applying bacteria onto seeds to favour establishment in the rhizosphere of host plants would be to include antibiotics, nutrients, or other antagonists, directly or pelleted onto the seed. These should also be evaluated. Until recently no efficient means were available to study the effect of these different treatments. Such studies are now feasible

with the FA technique.

In the population studies of the vegetative cells of B. subtilis only the 13th week sample showed any significant population difference between bacteria-treated and/-untreated seeds. A study is recommended to determine whether this difference is real or due to chance since most of the other data seem to indicate that there was no establishment of B. subtilis in the onion rhizosphere for any long period of time. The study should try to mimic the conditions of moisture and temperature that accompanied the time period at which the significant difference occurred. Should this difference prove to be real then bacterial treatment had something to do with it, even though the FA technique did not reveal enhanced populations at earlier sampling periods.

Coley-Smith and King (10) hypothesized that bacteria "act in soil as onion rhizosphere colonists and metabolize the germination stimulants for S. cepivorum produced from onions". Sclerotial germination would thus be reduced resulting in a reduced percentage of infection. A second possible mechanism is one of antibiosis. Utkhede and Rahe (40) have demonstrated that B. subtilis strains produce antibiotics inhibitory to mycelial growth of S. cepivorum on PDA. However no evidence for such a mechanism in soil has been obtained. Both explanations are possible and could act in concert. These two hypotheses depend upon the presence of bacterial antagonists in the rhizosphere.

My study of B. subtilis in onion root rhizosphere has demonstrated that for unknown reasons B2 did not readily become established in the roots of Autumn Spice. Biotic and abiotic factors are probably involved.

Jones and Morley (20) demonstrated that soil pH affected the association of two strains of Rhizobium trifolii Dangeard, with their white clover host. Brown et al. (7) inoculated wheat grains with Azobacter cultures of different ages. They found that the populations established in the rhizosphere varied with the culture age. Viability of young inoculum decreased rapidly in one day and declined slowly over eight weeks, after which no cells were found. Old cultures, consisting entirely of encysted bacteria gave better rhizosphere establishment. Rakhno and Ryys (31) stated that it was futile to add bacteria to soils that would not support these organisms. Soils rich in minerals, organic matter, a pH near neutrality and moisture conditions favouring plant and microbial growth would give best results. Therefore, the immediate problem is to find conditions necessary for the survival and proliferation of these bacterial antagonists before any kind of mechanism is investigated. The FA technique provides an excellent vehicle for such studies.

Intensive research to control plant diseases using bacterial and fungal biocontrol agents is a recent development. The number of examples of successful biological control of plant disease is still minimal. However, the fact that some do exist

should provide stimulus for researchers to develop better control agents and methods for plant protection. The biological hazards of chemicals and their rising costs coupled with the need for a steady and reliable food supply make continued efforts for development of alternative methods such as microbial antagonists essential.

List of References

1.Ahmed, A.H.M. and Tribe, H.T. 1977. Biological control of white rot of onion (Sclerotium cepivorum) by Coniothyrium minitans . Pl. Path. 26:75-78.

2.Ayers, W.A. and Adams, P.B. 1979. Mycoparasitism of sclerotia of Sclerotinia and Sclerotium species by Sporidesesmium sclerotium. Can. J. Microbiol. 25:17-23.

3.Booer, J.R. 1946. Experiments on the control of white rot in onions. Nature(Lond.) 155:241-242.

4.Bohool, B.B. and Schmidt, E.L. 1973. Bull. Ecol Res. Commun.17:330-338.

5.Bowden, W.B. 1977. Comparison of two direct count techniques for enumerating aquatic bacteria. Appl. Environ. Microbiol. 33:1229-1232.

6.Brown, M.E. 1974. Seed and root bacterization. Ann. Rev. Phytopath. 12:181-187.

7.Brown, M.E., Burlingham, S.K. and Jackson, R.M. 1964. III Effects of artificial inoculation on crop yeilds. Plant Soil. 20:194-197.

8.Coley-Smith, J.R. and Cooke, R.C. 1971. Survival and germination of fungal sclerotia. Ann. Rev. Phytopath. 9:65-92.

9. Coley-Smith, J.R. 1959. Studies of the biology of Sclerotium cepivorum Berk. III. Host range; persistence and viability of sclerotia. Ann. Appl. Biol. 47:511-518.

10.Coley-Smith, J.R.and King, J.E. 1968. The production by species of Allium of akkyl sulphates and their effect on germination of Sclerotium cepivorumBerk. Ann. App. Biol. 64: 289-301.

11.Cowan, S.T. and Steel, K.J. 1974. Manual for the identification of medical bacteria.Cambridge University Press. 238pp.

12.Davies, S.N. 1951. The serology of Bacillus polymyxa. J. Gen. Microbiol 5:807-816.

13.Diem, H.G., Schmidt, E.L. and Dommergues, Y.R. 1978. The use

of fluorescent-antibody technique to study the behaviour of a Beijerinkkia isolate in the rhizosphere and spermosphere of rice. Ecol. Bull.(Stockholm).26:312-318.

14.Garvey, T.S., Cremer, N.E. and Sussdorf, D.H. 1977. Methods in Immunology. 3rd ed. W.A.Benjamin Inc. 545pp.

15.Hanokova-Baverova, E., Koocur, M. and Martinec. 1965. Concerning the differentiation of Bacillus subtilis and related species. J. Appl. Bact. 28:384-389.

16.Harley, J.L. 1969. The Biology of Mycorrhiza. 2nd Ed. Leonard Hill, London.334pp.

17.Harrigan, W.F. and McCance, M.E. 1966. Laboratory Mmethods in Microbiology. Academic Press. New York.362pp.

18.Hobbie ,J.E., Daley, R.J. and Jasper, S. 1977. Use of nucleopore filters for counting bacteria by fluorescent microscopy. Appl.and Environ.Microbiol. 33:1229-1232.

19.Holt, J.G. (ed.) 1977. The Shorter Bergy's Manual of Determinative Bacteriology.

20.Jones, D.G. and Morley, S.J. 1980. Effects of pH on plant preference for Rhizobium trifolii. Ann. App. Biol. 97:183-190.

21.Kawamura, A.(Ed). 1977. Fluorescent Antibody Techniques and their Applications. 2nd ed.University of Tokyo Press. Tokyo. 203pp.

22.Knight, B.C. and Wiggell, P. 1973. Collaborative ADAS trials on the control of white rot disease of salad onions. Proceedings of the 7th British Insecticide and Fungicide Conference. 2:587-596.

23.Knight, B.C.J.G. and Proom, H. 1950. A comparative survey of the nutrition and physiology of mesophilic species in the genus Bacillus. J. Gen. Microbiol. 4:508-538.

24.Malajczuk, N., Bowen, C.D. and Greenhalgh, G.C. 1978. A combined F.A.and soil sieving technique to count chlamydospores of Phytopthora cinnamomi in soil. Soil Biol.Biochem. 10:437-438.

25.Marx, D.H. 1972. Ectomycorrhizae as biological deterrents to pathogenic root infections. Ann. Rev. Phytopath. 10:429-454.

26.Merriman, P.R., Samson, I.M. and Schippers, B. 1981. Stimulation of germination of sclerotia of Sclerotium cepivorum at different depths in soil by artificial onion oil. Neth. J. Pl. Path. 87:000-000.

27.Merriman, P.R., Price, R.D., Kollmorgen, J.F., Pigott, T. and Ridge, E.H. 1974. Effect of seed inoculation with Bacillus subtilis and Streptomyces griseus on the growth of cereals and carrots. Aust. T. Agr. Res. 25:219-226.

28.Norris, J.R.and Wolf, J. 1961. A study of antigens of the aerobic spore forming bacteria. J. Appl. Bact. (24)1:42-56.

29.Ormrod, D.J. 1977. Report of the 1977 Fraser Valley onion disease survey. British Columbia Ministery of Agriculture. Cloverdale, B.C. 9p.

30.Palumbo, S.A., Rivenburg, A.E., Smith, J.L. and Kissinger, J.C. 1975. Identification of Bacillus subtilis and species from sausage products. J. Appl. Bact. 38:99-105.

31.Rakhno, P.Kh. and Ryys, O,O. 1963. The use of Azotobacter preparations. Mikrobiologiya.32:558-561.

32.Reyes, V.G. and Schmidt, E.L. 1979. Population densities of Rhizobium japonicum strain 123 estimated in soil and rhizosphere. Appl. Environ. Microbiol. 37:854-858.

33.Schank, S.C. et al. 1979. Fluorescent antibody technique to study Azospirillum brasilense associated with roots of grasses. Soil. Biol. Biochem. 11:287-295.

34.Schmidt, E.L. 1974. Quantitative autecological study of microorganisms in soil by immunofluorescence. Soil Sci. 118:141-149.

35. Schmidt, E.L. 1973. Fluorescent antibody techniques for the study of microbial ecology. Bull. Ecol. Res. Commun.(Sockholm). 17:67-76.

36.Schmidt, E.L. 1979. Initiation of plant root-microbe interactions. Ann. Rev. Microbiol. 33:355-357.

37.Strayer, R.F. and Tiedje, J.M. 1978. Application of the fluorescent antibody technique to the study of a methanogenic bacterium in lake sediments. Appl. Environ. Microbiol. 35:192-198.

38. Tims, E.C. 1948. White rot of shallot. Phytopathology. 38:378-394.

40. Utkhede, R.S. and Rahe, R.E. 1979. Biological control of onion white rot .Soil. Biol. Biochem. 12:101-104.

41. Utkhede, R.S. and Rahe, J,E. 1979. Screening commercial onion cultivars for resistance to whit rot. Phytopath. 68:1080-1083.

42. Utkhede, R.S. and Rahe, J.E. 1979 Evaluation of chemical fungicides for the control of onion white rot. Pesticide Science. 10: 414-418.

43. Walker. J.C. 1969. Plant Pathology. McGraw-Hill, New York 819pp.

44. Windels, C.E. and Kommendahl, T . 1978. Factors affecting Penicillium oxalicum as a seed treatment against seedling blight of pea. Phytopath. 68:1656-1661.

45. Wolf, J. and Barker, A.N. 1968. The genus Bacillus: Aids to the Identification of its Species. In Identification Methods for Microbiologists. Part B. The Society for Applied Bacteriology Technical Series #2. Gibbs, B.M. and Shapton, D.A.(Eds.) .Academic Press, London. 203pp.