STUDIES ON THE ROLE OF SOIL BACTERIA IN THE BIOLOGY AND CONTROL OF ONION WHITE ROT

by

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Studies on the Role of Soil Bacteria in the Biology and Control of Onion White Rot

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ABSTRACT

White rot, an onion disease caused by the fungus *Sclerotium cepivorum* Berk., is a serious problem in the Fraser Valley of British Columbia and also in many other parts of the world. This thesis examines the effects of selected soil bacteria on the growth of onion seedlings and on the germination of sclerotia of *S. cepivorum*.

Antibiotic-tolerant strains of the various soil bacteria were developed and used to assess and compare the abilities of these bacteria to colonize and persist in onion seedling rhizosphere and root-zone soils. *Bacillus subtilis* B-2, a bacterium previously shown to have potential for biological control of white rot, was found to be a poor rhizosphere colonizer compared with the other bacterial isolates tested. Seed bacterization with three of five different soil bacteria caused significant increases in shoot height, shoot and root dry weights of onion seedlings over non-bacterized controls. These same three bacteria caused significant reductions in the estimated indigenous rhizosphere and nonrhizosphere root-zone soil bacterial and fungal populations compared with controls.

Germination of sclerotia of *S. cepivorum* was significantly enhanced by soil treatments with diallyl disulphide and also by onion seedlings bacterized with the same three bacterial strains shown to enhance seedling growth. These three bacterial treatments which enhanced sclerotial germination significantly reduced bacterial and fungal populations associated with sclerotia.

For effective disease control it is important to have knowledge of the population dynamics of introduced bacteria in seedling rhizosphere and root-zone soils over time, and to understand the ecological relationship between onion seed bacterization and germination of sclerotia of *S. cepivorum*. The techniques and bioassays developed during this research have proven to be useful in obtaining such information. The results obtained in this research lead to the conclusion that the effects of bacterization on onion seedling growth and germination of sclerotia of *S. cepivorum* are indirect. These effects were not related to the relative rhizosphere and/or root-zone soil populations of the bacterial strains evaluated, but to the relative abilities of these strains to suppress populations of indigenous rhizosphere, root-zone soil or sclerotial mycosphere microorganisms.

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DEDICATION

TO MY MOTHER MUNAGALA ESWARAMMA

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INTRODUCTION TO THE THESIS

White rot, an important disease of onion and other *Allium* spp. caused by the fungus *Sclerotium cepivorum* Berk., (74) was first discovered in England by M. J. Berkeley in 1841. Since then it has been described in many other countries (147). The disease was first recorded in the commercial onion growing areas of the Fraser Valley of British Columbia, Canada in 1970 (105). The disease causes severe yield losses (1, 41, 42, 96) and is at present a major problem to the onion growers in the Fraser Valley (145).

The pathogen, *S. cepivorum* is a facultative saprophyte whose pathogenic activities under natural conditions are restricted to *Allium* spp. (25, 27). It survives in soil as sclerotia. The sclerotia can withstand the extremes of wetness and dryness in the soil (31, 109). These sclerotia remain dormant in non-sterile soil until stimulated to germinate by root exudates from *Allium* spp. (30, 32, 34, 51, 76, 77). The sclerotia remain viable in soil for many years even in the absence of host *Allium* spp. (25, 122). Coley-Smith (27) reported that sclerotia buried in soil near Hull, UK persisted for 10 years with very little loss in viability. Crowe and Hall (40) recovered viable sclerotia from fields in California where no onions had been grown for 15 years. In contrast, Leggett *et al*. (86) reported substantial decay of sclerotia buried for less than one year in muck soil in the Fraser Valley of B. C.

The symptoms of white rot are yellowing and dying of the outer leaves of the onion plant beginning at the tips and progressing downwards (147). Roots and scales of onion bulbs are attacked by the fungus, which forms a superficial white, fluffy mycelium thereon. It later produces black, spherical

sclerotia that range from 0.2 to 0.6 mm in diameter. The sclerotium has an outer rind of one to two layers of rounded, thick pigmented cells enclosing a large medullary tissue of hyphae. They are released into the soil from diseased plant tissue either at harvest time or when the infected plants decay.

Although sclerotia germinate on a variety of culture media in sterile conditions, they will germinate in soil only in the presence of *Allium* spp. or of *Allium* extracts (35). Coley-Smith *et al*. (27) have shown that the dormancy of sclerotia of *S. cepivorum* in the field is a condition imposed by the soil microflora, since they germinate readily in sterile soil. They are held in dormant condition by soil fungistasis (2, 35, 77), and are specifically stimulated by active compounds associated with aqueous extracts and exudates of *Allium* spp. (26, 32, 33). The active volatile compounds in extracts appear to be alkyl sulphides and in particular n-propyl and allyl suphides (28). Sclerotia show low response to methyl sulphides and high response to n-propyl and allyl derivatives (28). Methyl sulphides are produced by a wide range of plants whereas n-propyl and allyl sulphides are produced only by *Allium* spp. (28). These volatile compounds are not produced by intact onion plants. Intact onion plants exude small quantities of alkyl cysteine sulphoxides. These latter compounds are thermostable, water soluble and diffusible, and are metabolized by bacteria to generate stimulatory volatile alkyl sulphides which are reported to act as stimulants for the initiation of sclerotial germination (77). Coley-Smith and Dickinson (29) and Dickinson and Coley-Smith (47) have also reported that sclerotia of *S. cepivorum* enhance microbial activity. Thus, soil bacteria and/or bacteria associated with sclerotia may mediate the

stimulatory effect of *Allium* root exudates.

The primary functions of the sclerotium are to perennate the fungus, and to bring about successful contact between the pathogen and host tissue. Therefore, sclerotial germination is an important phase at which the control of white rot can be implemented. Complete control of white rot of onions has not been achieved. Control of white rot by chemicals, including calomel (8, 42), PCNB (117), dichloran (55), iprodione (53, 54, 140) and vinclozolin (140) has been reported. Among these chemicals only dichloran is registered in Canada for use on onion white rot. Vinclozolin and iprodione have significantly reduced white rot in field trials (140) and may provide a short term solution to the white rot problem. They likely cannot be relied upon in the long term because there is an evidence that the pathogen can develop resistance to them (89). Cultural practices such as the use of organic amendments, altering sowing times (117), and the use of onions as a trap crop for reducing the *S. cepivorum* inoculum in soil (95) have been tried, but have been of little success in controlling white rot. Use of onion oil in field trials to stimulate germination of sclerotia in the absence of the host has been tried (96, 99, 142). This method was partially effective because a substantial proportion of sclerotia germinated in the absence of the host, decayed quickly and did not form secondary sclerotia (25, 40, 142). However control was incomplete, and implementation of this method would be very expensive.

Very little work is reported in the literature on the biological control of onion white rot. Utkhede and Rahe studied control of onion white rot by seed bacterization with six different isolates of *Bacillus subtilis* recovered from

sclerotia of *S. cepivorum* (141, 144). Among the six isolates *B. subtilis* B-2 provided significant levels of season long control of onion white rot in 1978 (141). Significant control was also obtained in similar field trials in 1979 (143), but not in 1980 (143) and 1981 (Rahe and Utkhede unpublished data). No information is available in their study on either the initial colonization of onion seeds by *B. subtilis* or on subsequent colonization and persistence of the bacteria on growing roots during the season.

A fluorescent antibody technique was used to study vegetative and spore cell populations of *B. subtilis* in the rhizospheres of onions grown from bacterized and non-bacterized seeds (153). The success of this study was limited by lack of strain specificity of the antibodies used.

Bacterization is the process of improving plant growth or controlling soil borne plant diseases by introducing specific bacterial strains to seeds of agricultural crops (16). Little information is available concerning the effects of soil pH, moisture and temperature on the ability of specific bacteria to colonize and persist on roots. Failures of a beneficial bacterium to improve plant growth or control plant disease could be the result of failure of the bacterium to establish and/or function in plant root ecosystem. One possible indirect mechanism has been suggested to explain the beneficial influence of specific rhizobacteria on plant growth. This mechanism is the reduction of deleterious indigenous microflora including both bacterial and fungal pathogens in the soil root ecosystem (83, 124). A strategy to identify specific bacteria as plant growth promoters or biological control agents must select not only for bacteria that are capable of antagonism against soil borne root pathogens, but also for their ability to effectively colonize roots. Thus, studies on the

population dynamics of a specific bacterial isolate in the rhizosphere and nonrhizosphere root-zone soils is basic to the logical development of its use for biological control of soil borne diseases and/or for promotion of plant growth.

This thesis represents a comparative study of the population dynamics of *B. subtilis* B-2 and four other selected onion rhizobacteria in onion rhizosphere and nonrhizosphere root-zone soils, and associated biological effects of seed bacterization on onion seedlings under laboratory and field conditions. More importantly a part of this research was designed to gain a better understanding of the possible ecological relationships among coexisting *S. cepivorum* and the introduced rhizosphere/root-zone bacterial microflora.

CHAPTER I

GENETIC MARKING OF *BACILLUS SUBTILIS* B-2 FOR ANTIBIOTIC-TOLERANCE

INTRODUCTION

Incorporation of antibiotic-tolerance markers into bacteria is useful for studying the population dynamics of specific bacteria under laboratory or field conditions (80). It allows isolation of the marked bacteria from inoculated plant tissue by plating onto antibiotic amended media. The feasibility of long term ecological studies of bacteria in laboratory and field environments depends on the stability of the markers used to distinguish one strain from another (72), on the stability of the marked strains, and on the degree to which the marked strains are representative of the parental wild type.

Streptomycin resistance is a marker frequently used in genetic and population studies of *Rhizobium* spp. in soil (14, 78, 88, 126, 127). Strains of *Erwinia amylovora* resistant to streptomycin have been used to study the movement and persistence of this bacterium in stem and root of apple (60), and also in studies concerned with the mode of penetration and movement of the bacterium in apple leaf and stem tissue under greenhouse conditions (87). Hsieh *et al.* (65) used a streptomycin resistant strain of *Xanthomonas oryzae* to follow infection of rice seed. Stall and Cook (132) studied the multiplication of *Xanthomonas vesicatoria* in susceptible and resistant peppers (*Capsicum annum* L.) using a streptomycin resistant strain.

To the author's knowledge there has been no study using strains of *B. subtilis* marked with antibiotic-tolerance for the study of its survival in

biologically active soil.

The present chapter deals with: (i) the isolation of a genetically marked strain of *B. subtilis* B-2 tolerant to combinations of streptomycin, cycloheximide and benomyl from a parental strain, (ii) comparisons of several characteristics of the marked strain with the parental strain, and evaluation of its stability in pure culture and in soil under controlled conditions.

MATERIALS AND METHODS

Source of parental wild type bacterial isolate

B. subtilis B-2, isolated by Utkhede and Rahe (141) from sclerotia of *S. cepivorum* recovered from naturally infested onions grown on muck soil near Cloverdale, British Columbia, Canada in 1979, was used for this study. This isolate was of particular interest because of its potential for biological control of onion white rot (141, 144).

Genetic marking of B. subtilis B-2 for antibiotic-tolerence

B. subtilis B-2, previously maintained on potato dextrose agar (PDA), was grown in a sterile 250 ml flask containing 10 ml of sterilized tryptic soy broth (TSB) on a reciprocating mechanical shaker for 8 hr at 24-25 C. A bacterial pellet was prepared by centrifugaton for 20 min at 2000 rpm. The pellet was washed with sterile water and suspended in 10 ml of sterilized 0.1 M $MgSO_4$. Serial dilutions of the suspension were made and 0.1 ml aliquots from appropriate dilutions were spread onto triplicate plates containing PDA, and PDA amended with 120 ug/ml streptomycin (S) (Sigma Chemical Company,

St. Louis, MO. 63178 USA), 100 ug/ml cycloheximide (C) (Sigma chemical company) and 30 ug/ml benomyl (B) (Plant Products Co Ltd., Ontario, Canada) (S_{120}CB-PDA). The inoculated plates were incubated for 3-4 days at 24-25 C. Colony counts were made and the frequency of S_{120}CB-tolerant genotypes occurring in the parental wild type populations was estimated. Three colonies developing on S_{120}CB-PDA were streaked onto PDA, allowed to grow for 48 hr, and then streaked back onto S_{120}CB-PDA as a gross check for stability of tolerance. This procedure was repeated while increasing the streptomycin concentration in increments of 20 ug/ml until the level of tolerance of the S_{120}CB-tolerant isolates of *B. subtilis* B-2 was increased to 300 ug/ml (S_{300}CB-PDA).

The proportions of antibiotic-tolerant cells within colonies developing on S_{120}CB-PDA and S_{300}CB-PDA were estimated at the times such colonies were first obtained. Cells from representative colonies were transferred from these media and grown in TSB for 8 hr. Bacterial pellets were made, washed and suspended in 0.1 M $MgSO_4$ as described above. Serial dilutions were made and 0.1 ml aliquots from the appropriate dilutions were spread onto triplicate plate of S_{120}CB-PDA and PDA in the case of isolates taken from S_{120}CB-PDA, and onto S_{300}CB-PDA and PDA in the case of isolates from S_{300}CB-PDA. Colonies on each plate were counted after incubation of the plates for 3-4 days at 24-25 C.

One S_{300}CB-tolerant strain was arbitrarily chosen as the test isolate for subsequent studies. This marked strain was maintained on S_{300}CB-PDA except where otherwise indicated.

Comparison of cultural and biochemical characteristics of the parental and the marked strains of B. subtilis B-2

Twentyfour-hour cultures of *B. subtilis* B-2 representing the parental strain from PDA and the marked strain from S_{300}CB-PDA were used for cultural and biochemical comparisons. Standard procedures (62) were followed for measuring cultural characteristics such as colony appearance, spreading ability, elevation, colour and shape of the colonies. Biochemical tests included the Voges-Proskaur test for production of acetylmethyl carbinol, utilization of succinate and citrate, hydrolysis of starch and casein, and the production of ammonia (62). Growth in glucose ammonium salts medium and hydrolysis of hippurate were tested according to Cowan and Steel (39). Sugar fermentation abilities of the isolates were compared utilizing D-glucose, sucrose, D-mannitol, D-xylose, L-rhamnose, D-arabinose, lactose, salicin and D-sorbitol according to Harrigan and Maccance (62). There were four replications of each test for each strain.

Antagonism against *S. cepivorum* on PDA plates was compared at each step of the tolerance selection procedure between the parental strain and the S_{300}CB-tolerant isolate. The test strain was streaked approximately 2.5 cm from the edge of a 9 cm diametre petri plate containing PDA and allowed to grow for 24 hr before a 5 mm diametre core of *S. cepivorum* was placed 2.5 cm from the edge on the opposite side. The core was taken from the edge of an actively growing colony of *S. cepivorum* on PDA with a No. 2 cork borer. The plates were incubated at 24-25 C and evaluated subjectively for possible differences in the nature and magnitude of the inhibition zone between the test bacterial strain and *S. cepivorum* after addition of the *S.*

cepivorum cores.

Survival of the parental and the marked strains of B. subtilis B-2 in muck soil

Freshly collected muck soil from a commercial onion field in Cloverdale, British Columbia was used as an incubation medium for evaluating survival of the parental and marked strains of *B. subtilis* B-2. Soil pH was approximately 4.5 (determined using a thick suspension of soil in 0.01 M $CaCl_2$). The moisture content of the soil was estimated by drying a 40 g sample overnight in an oven at 110 C. The oven-dried soil was weighed and percentage of moisture was calculated by the following formula:

$$\text{Moisture percentage} = \frac{\text{Wet soil weight} - \text{Oven dry soil weight}}{\text{Oven dry soil weight}} \times 100$$

The estimated moisture percentage was used to adjust the remaining soil to 80% moisture. In experiments requiring sterile soil, moisture adjusted soil was placed in metal trays covered with aluminum foil, and autoclaved for 1 hr on each of 3 consecutive days. No microorganisms were detected when autoclaved soil was plated onto PDA. Fresh cultures of the parental and the marked strains of B-2 were grown separately in sterile 1000 ml flasks containing 500 ml of sterilized TSB incubated on a reciprocating shaker at 24-25 C for 8 hr. Bacterial pellets were prepared, washed and suspended in sterilized 0.1 M $MgSO_4$ as described previously. Suspensions (10 ml) containing 1.90×10^9 cells/ml of the parental strain were added to 1000 g quantities of sterilized soil, and of the marked strain to 1000 g quantities of

both sterilized and non-sterilized soil. Bacteria and soil, contained in sterile polyethylene bags, were mixed thoroughly and the bags were incubated in a growth chamber at 22 C. There were five replications for each treatment. Recoveries of the marked strain from initially sterilized and non-sterilized soils and of the parental strain from initially sterilized soil were estimated at 0, 4, 8, 21, 42, 60 and 90 days following inoculation. At each sampling period 10 g of soil was removed aseptically from the bags and placed into a sterile 500 ml flask containing 100 ml of sterilized 0.1 M $MgSO_4$. The suspension was agitated for 20 min by gentle magnetic stirring. Serial dilutions of the agitated suspensions were then prepared and 0.5 ml aliquots from appropriate dilutions were plated onto PDA or S_{300}CB-PDA in triplicate. The plates were incubated at 24-25 C. Colony counts were made after 72 hr and populations of the parental and the marked strains recovered from soil were estimated and recorded as colony forming units (cfu) per g dry wt. of soil.

The data were analyzed by analysis of variance and Student Newman-Keul's test at 5% level of significance.

RESULTS

Genetic marking of B. subtilis B-2 for antibiotic-tolerance

The estimated frequency of S_{120}CB-tolerant genotypes occurring in the parental wild type populations was approximately 1 in 3.64×10^6 cfu plated. The frequency of S_{120}CB-tolerant genotypes occurring in the S_{120}CB-tolerant populations was approximately 1 in 1.20 cfu plated. The frequency of S_{300}CB-tolerant genotypes occurring in S_{300}CB-tolerant populations was

approximately 1 in 1.03 cfu plated.

Growth of the marked strain on PDA and on S_{300}CB-PDA was similar in appearance to that of the parental strain on PDA (Fig. 1). The marked strain retained the cultural characteristics of the parental strain, except its spreading ability on S_{300}CB-PDA, this being weak on S_{300}CB-PDA and moderate on PDA (Table 1). None of the biochemical tests distinguished among the parental and the marked strains with the possible exception of the methyl red test (Table 2). Among the four replicates of the methyl red test, the parental strain gave all positives and the marked strain gave two positives and two negatives. Similarity of the parental and the marked strain was further indicated by comparison of sugar fermentation patterns (Table 3). The marked strain behaved somewhat differently from the parental strain only in presence of D-sorbitol. Both strains showed similar types and levels of inhibition against *S. cepivorum* in dual culture test (Fig. 2).

Survival of the parental and the marked strains of B. subtilis B-2 in muck soil

Recovery data for the populations of the parental strain from initially sterilized soil, and of the marked strain from initially sterilized and non-sterilized soils following inoculations are shown in Table 4. There were no significant differences in survival between the parental and the marked strains in initially sterilized soil for at least 90 days following inoculation. The parental strain was not recovered on S_{300}CB-PDA. Slight decreases in recovery of both of the strains, compared with the populations introduced into the soil samples, were observed at zero time (within 24 hr) after inoculation of soils. Populations of the parental strain in initially sterilized soil and of

Figure 1. Appearance of the parental and the marked strains of *Bacillus subtilis* B-2 on 7th day after inoculation and incubation at 24-25 C.

a. Parental strain on PDA
b. Marked strain on PDA
c. Marked strain on S_{300}CB-PDA

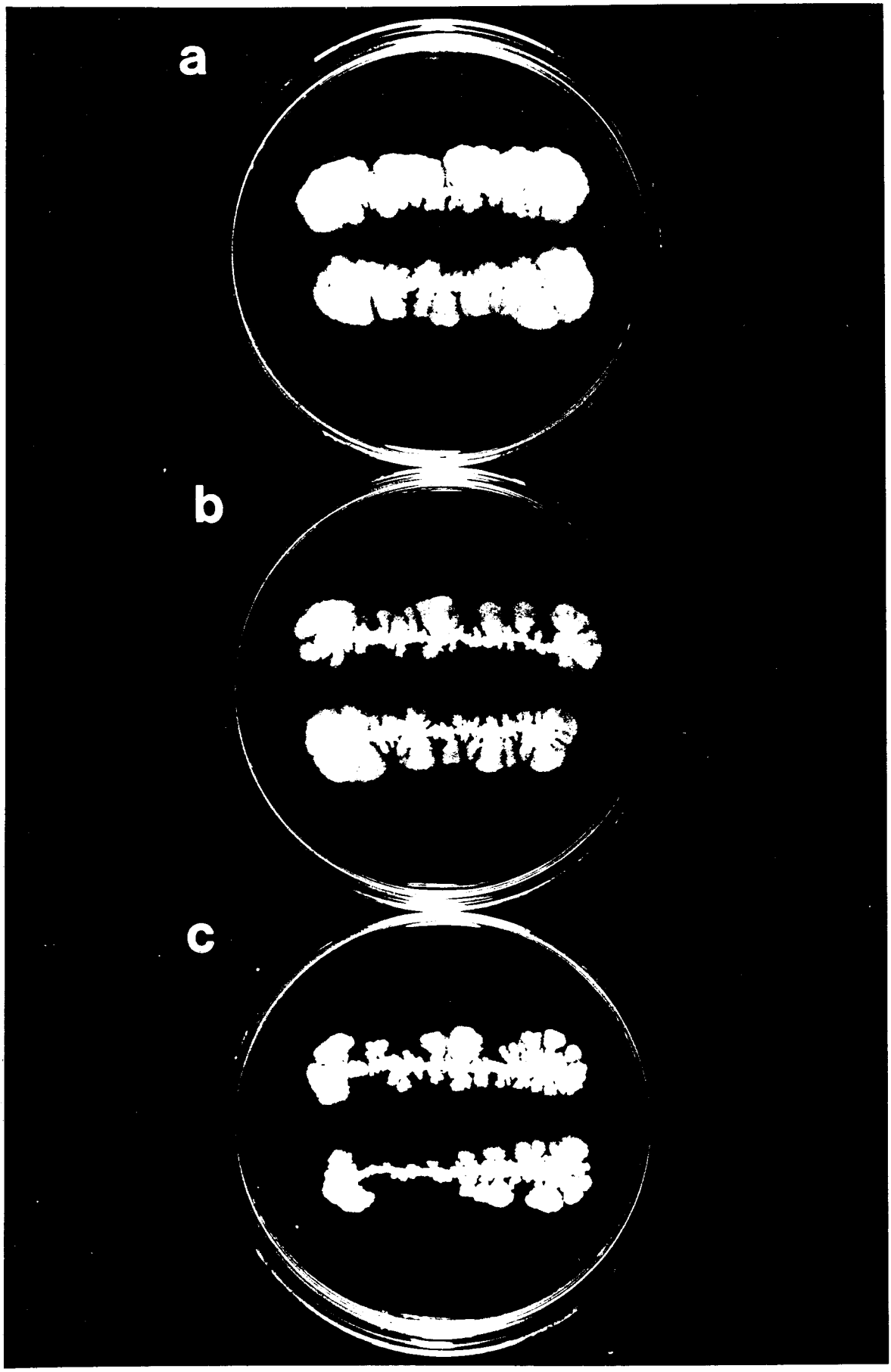
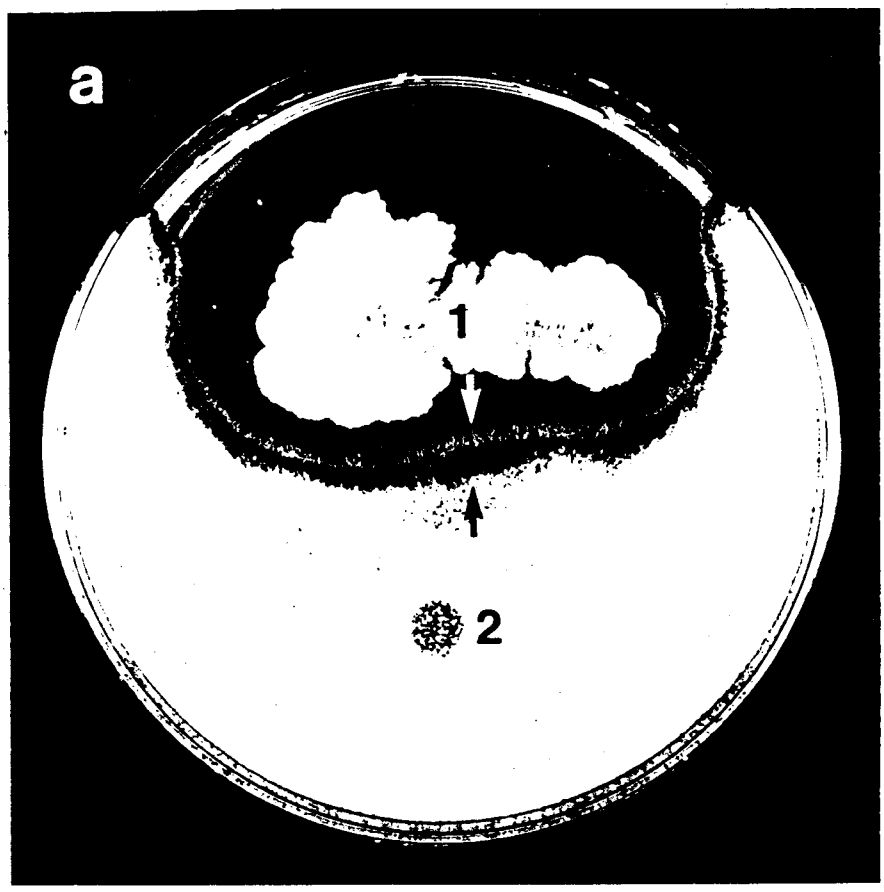

a
b
c

Figure 2. Antagonistic interaction of the parental and the marked strains of *Bacillus subtilis* B-2 (1) and Sclerotium cepivorum (2) on potato dextrose agar (arrows represent the site of antagonistic interaction).

a. Between the parental strain and *S. cepivorum*.
b. Between the marked strain and *S. cepivorum*.


a
1
2

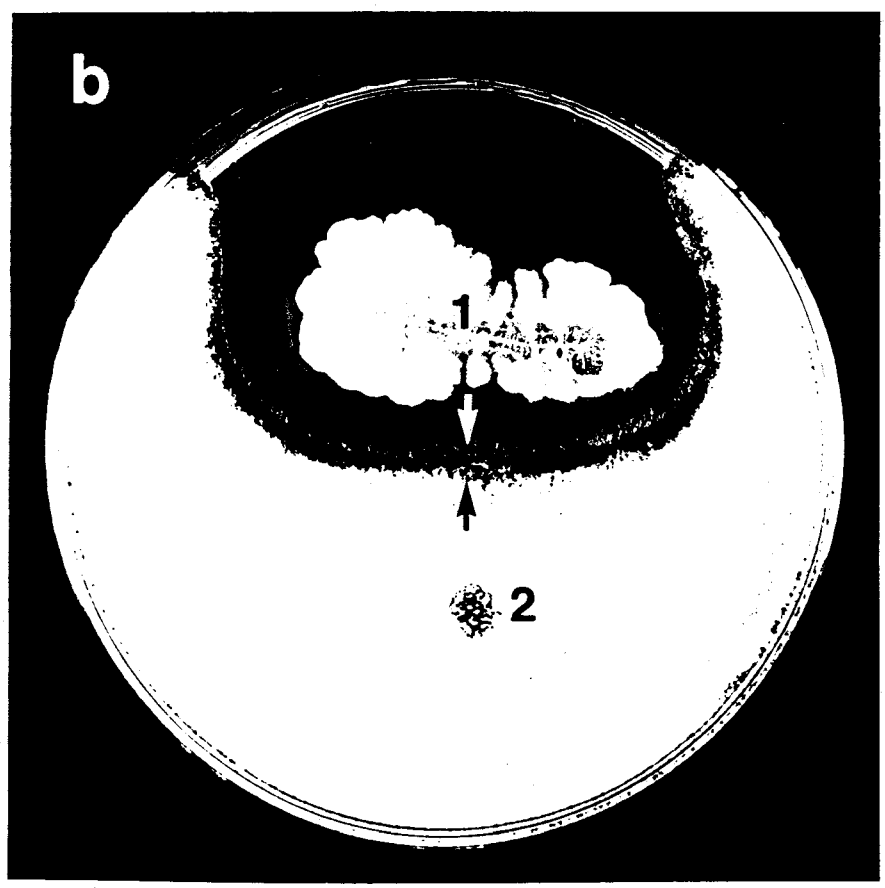

b
1
2

Table 1. Comparison of cultural characteristics[1] of the parental and the marked strains of *Bacillus subtilis* B-2.

Characters measured	Parental strain	Marked strain	
	On PDA[2]	On PDA	On S_{300}CB-PDA[2]
Colony appearance	Diffuse, rough layer on outer surface of colony	Diffuse, rough layer on outer surface of colony	Slightly diffuse, rough layer on outer surface of colony
Spreading ability	Moderate	Moderate	Weak
Elevation	Raised	Raised	Raised
Colour	Greyish-white	Greyish-white	Greyish-white
Surface	Smooth, glistening	Smooth, glistening	Smooth, glistening

[1] Cultures kept at 24-25 C and recorded after 7 days.

[2] PDA=potato dextrose agar; S_{300}CB-PDA=PDA amended with 300 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl.

Table 2. Comparison of biochemical test responses[1] between the parental and the marked strains of *Bacillus subtilis* B-2.

Biochemical tests	Parental strain	Marked strain
Production of ammonia	+	+
Utilization of citrate	-	-
Utilization of succinate	-	-
Reduction of nitrate	+	+
Hydrolysis of starch	+	+
Hydrolysis of casein	+	+
Hydrolysis of hippurate	-	-
Voges-Proskaur test	+	+
Methyl red test	+	±
Anaerobic growth in glucose broth	-	-
Growth in 7% $NaCl_2$	+	+
Detection of acid in glucose ammonium salt	+	+

[1] Test responses: +=positive reaction
-=negative reaction
±=variable

Table 3. Comparison of sugar fermentation patterns[1] between the parental and the marked strains of *Bacillus subtilis* B-2.

Sugar	Parental strain	Marked strain
D-glucose	±	±
Sucrose	+	+
D-mannitol	-	-
D-xylose	-	-
L-rhamnose	-	-
D-arabinose	-	-
Lactose	-	-
Salicin	-	-
D-sorbitol	-	*

[1] Patterns indicated by: + = acid but no gas
± = weak
- = negative
* = variable

Table 4. Recovery of the parental and the marked strains of *Bacillus subtilis* B-2 added to muck soil at 1.90×10^7 cfu/g dry wt. of soil.

Strain type	Recovery medium[1]	Recovery time (days)[2] 0	4	8	21	42	60	90
		Recovery from sterilized soil (cfu/g dry wt. of soil)[3]						
Parental	PDA	$1.44 \pm 0.11 \times 10^7$b	$4.18 \pm 0.29 \times 10^6$b	$3.29 \pm 0.09 \times 10^6$b	$2.59 \pm 0.14 \times 10^6$b	$1.18 \pm 0.17 \times 10^6$b	$3.09 \pm 2.63 \times 10^5$b	$8.15 \pm 0.11 \times 10^4$b
	S_{300}CB-PDA	0	0	0	0	0	0	0
Marked	PDA	$1.54 \pm 0.13 \times 10^7$b	$4.09 \pm 0.08 \times 10^6$b	$3.24 \pm 0.16 \times 10^6$b	$2.41 \pm 0.09 \times 10^6$b	$1.11 \pm 0.07 \times 10^6$b	$3.14 \pm 0.07 \times 10^5$b	$8.02 \pm 0.08 \times 10^4$b
	S_{300}CB-PDA	$1.49 \pm 0.10 \times 10^7$b	$4.12 \pm 0.05 \times 10^6$b	$3.32 \pm 0.19 \times 10^6$b	$2.39 \pm 0.08 \times 10^6$b	$1.04 \pm 0.08 \times 10^6$b	$3.00 \pm 0.13 \times 10^5$b	$8.19 \pm 0.13 \times 10^4$b
		Recovery from non-sterilized soil (cfu/g dry wt. of soil)[3]						
Marked	S_{300}CB-PDA	$1.38 \pm 0.09 \times 10^7$b	$3.01 \pm 0.07 \times 10^6$a	$2.20 \pm 0.07 \times 10^6$a	$1.29 \pm 0.04 \times 10^6$a	$8.63 \pm 0.31 \times 10^5$a	$1.20 \pm 0.03 \times 10^5$a	$5.60 \pm 0.10 \times 10^4$a

[1] PDA=potato dextrose agar; S_{300}CB-PDA=PDA amended with 300 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl.

[2] Mean ($\pm$=S. E) of 5 replications, 10 g soil each.

[3] Non-zero means within a column followed by the same letter do not differ significantly according to Student Newman-Keul's test ($p=0.05$).

the marked strain in initially sterilized and non-sterilized soils declined at each subsequent sampling period at similar rates. Populations of the marked strain recovered from the initially sterilized soil on PDA and on S_{300}CB-PDA were not significantly different. A significant decrease in the apparent survival (recoveries) of the marked strain in non-sterilized soil compared to that in initially sterilized soil was observed with time.

DISCUSSION

It is essential that genetically marked isolates of bacteria intended for use in ecological studies under field conditions be tested in the laboratory with regard to similarity to wild type and stability of the marker character. Strains of *B. subtilis* B-2 tolerant to the combinations of 300 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl in PDA were isolated from the parental wild type isolate by a non-mutagenizing reselection procedure. The marked strain of B-2 selected for subsequent study was similar to the parental strain as regards its ability to inhibit *S. cepivorum*, and its morphological and biochemical characteristics. Stability of the marked character in *B. subtilis* B-2 in short term (8hr) culture and, more importantly in sterilized and non-sterilized soil during long term incubation appears to present no problem, since loss of combined tolerance to S_{300}CB-PDA was not encountered in this study.

Streptomycin was selected for genetic marking of *B. subtilis* B-2 because of its known broad spectrum of antibacterial activity (126). It is an aminoglycoside that interferes with the function of a protein in the 30-S

subunit of the ribosome (126). Cycloheximide and benomyl were selected for additional markers, because of their fungicidal activity (84). Combined tolerance to 300 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl in PDA provides a potentially powerful tool for recovery of a marked bacterium representative of *B. subtilis* B-2 from biologically complex environments. The S_{300}CB-PDA medium permits selective recovery of the B-2 marker by excluding undesirable microorganisms that would otherwise interfere with the growth of the test bacterium on the isolation plates. The use of a marked strain can thus provide a rapid and reliable means of monitoring the persistence and movement of *B. subtilis* B-2 in soil and plant root environments, and its response to various environmental variables.

Addition of either the parental or the marked strain of *B. subtilis* B-2 to muck soil did not result in as rapid a decline as often occurs when bacteria are added to soil. For example, the population of *Agrobacterium tumefaciens* added to non-sterilized soil decreased almost four log units in 8 weeks (46); *Micrococcus luteus* rapidly lost viability, with less than 1% of its initial population surviving 2 weeks after addition to soil (23); *Escherichia coli* decreased six log units in 24 days when added to soil (79). In contrast, rhizobia added to soil survived well, with numbers decreasing only one log unit in 8 weeks (43). Population densities of *Pseudomonas putida* increased after addition to soil; after 5 weeks numbers were often higher than the initial population densities (49). In the present study population densities of parental and marked strains of B-2 decreased slightly within 24 hr after addition to soil and then declined slowly with time. Although *B. subtilis* is considered to be a soil bacterium, it apparently failed to multiply in both the

sterilized and non-sterilized soils tested. Instead, both the parental and the marked strains declined at a comparable rate in sterilized soil. The decline of the marked B-2 was higher in non-sterilized soil than in sterilized soil. This may be due to the presence of competitors, predators, and other organisms in non-sterilized soil that are detrimental to B-2. Predation by indigenous protozoa was responsible for the observed decline of *Rhizobium* spp. and *Xanthomonas* spp. in non-sterilized soil (43-45, 61, 106, 112). In sterile soil, however, these adverse effects would not occur.

The persistence of B-2 marked strain in sterilized and non-sterilized soil for at least for 90 days following artificial inoculation, and the recovery of comparable populations of the parental and the marked strains from sterilized soil is evidence of the long term stability of the S_{300}CB marker in soil. The S_{300}CB marked strain is thus considered to be a useful tool for further studies on the population dynamics of *B. subtilis* B-2 in the onion root-soil ecosystem under laboratory and field conditions.

CHAPTER II

RHIZOSPHERE COLONIZATION AND BIOLOGICAL EFFECTS RELATED TO BACTERIZATION OF ONION SEEDS WITH *BACILLUS SUBTILIS* B-2

INTRODUCTION

Studies on the population dynamics of bacterial isolates in the rhizosphere are basic to the logical development of their use for biological control of soil-borne pathogens or for promotion of plant growth (80). Until recently most studies of plant-bacterial associations reported in the literature have dealt with populations of general bacterial groups such as amino acid-requiring, Gram-positive vs Gram-negative, or aerobic vs anaerobic bacteria, rather than with the quantitative estimation of specific bacterial strains in the rhizosphere during different stages of plant growth (6, 15, 16, 50, 69, 103, 114). Thus, little information is available on the initial colonization of seeds by specific bacterial groups and their subsequent growth and persistence in the rhizosphere.

Considerable data are available on the use of selected soil bacteria such as *Azotobacter, Bacillus, Clostridium, Streptomyces* and *Pseudomonas* as seed inoculants for the stimulation of growth of agricultural crops (11-13, 19, 20, 37, 97, 98, 100). Renewed interest in the use of phytostimulatory *Bacillus* in Australia has followed the work of Broadbent *et al.* (12), and a number of field trials have shown promising enhancement of growth and yield of a range of nursery, vegetable, cereal and field crops by *Bacillus* spp. (13, 94, 98, 100). Although highly significant increases in crop yield have sometimes

resulted from seed bacterization with specific bacteria (13, 22, 97), the results have often been erratic thus raising questions about the causes of such variation and the significance of the effects (38, 101, 116). These studies did not relate effects on plant growth to rhizosphere colonization by the introduced test bacteria. Various edaphic factors such as soil pH, moisture and temperature greatly affect bacterial colonization and persistence in the rhizosphere (64).

Utkhede and Rahe studied control of onion white rot, caused by the fungus *S. cepivorum*, by seed bacterization with six different isolates of *B. subtilis* recovered from sclerotia of *S. cepivorum* (141, 144). Among the six isolates *B. subtilis* B-2 provided significant levels of season long control of onion white rot in 1978 (141). Significant control was also obtained in a similar field trial in 1979, but not in trials conducted in 1980 and 1981 (Rahe and Utkhede unpublished data). No information was available in their study on either the initial colonization of onion seeds by *B. subtilis* or on subsequent colonization and persistence of the bacteria on growing roots during the season. It seems logical that, for successful biological control of plant diseases by seed bacterization, the inoculants must multiply in the rhizosphere and inhibit pathogens either by competition or antibiosis (13, 16). Knowledge of factors affecting the persistence of *B. subtilis* strains in the rhizosphere when introduced onto seed is thus important for successful control of onion white rot.

A fluorescent antibody technique was used to assess vegetative and spore cell populations of *B. subtilis* in the rhizospheres of onions grown from bacterized and non-bacterized seeds (153). The success of this technique was

limited by lack of strain specificity of the antibodies used. Strain identification in studies on the ecology of *B. subtilis* in the onion rhizosphere presents a major difficulty, particularly when several strains are to be evaluated. In situations in which *B. subtilis* is present in the soil prior to sowing, it is necessary to use techniques that distinguish between strains already in the soil and those introduced onto the seed. The use of marked strains allows study of the population dynamics of specific strains in the presence of indigenous populations of soil inhabiting bacteria (80).

A strain of *B. subtilis* B-2 marked with antibiotic tolerance that permitted its selective recovery from microbially active muck soil was developed (Chapter I), and experiments were carried out to evaluate the effects of soil pH, moisture and temperature on its colonization of the onion seedling rhizosphere from bacterized seed, and associated effects on the growth of onion seedlings.

MATERIALS AND METHODS

Source of bacterial isolate and bacterization of onion seeds

A genetically marked strain of *B. subtilis* B-2 tolerant to the combinations of 300 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl in PDA (S_{300}CB-PDA) (Chapter I) was used throughout this investigation.

Onion seeds cv. Autumn Spice (Stokes Seeds Ltd., St. Catharines, Ontario, Canada) were surface sterilized in 0.1% $HgCl_2$ solution for 5 min, rinsed briefly in 70% ethanol and rinsed four times with sterile distilled water. Fresh

bacterial culture of S_{300}CB-tolerant *B. subtilis* B-2, previously maintained on S_{300}CB-PDA was produced in a sterile 500 ml flask containing 250 ml of sterilized TSB incubated on a continuously reciprocating shaker at 24-25 C for 8-10 hr. A bacterial pellet was prepared, washed and suspended in sterile 0.1 M $MgSO_4$ as described in Chapter I. Surface sterilized onion seeds were bacterized by suspending the seeds in the bacterial suspension for 10 min, and then dried in flowing sterile air for 1-2 hr. At zero time five samples, each containing five bacterized onion seeds, were transferred into sterile 250 ml flasks containing 10 ml of sterile 0.1 M $MgSO_4$ and stirred magnetically for 10 min. Serial dilutions of the $MgSO_4$ washing suspension were made, and 0.1 ml aliquots of the appropriate dilutions were plated onto S_{300}CB-PDA in triplicate. Plates were incubated for 72 hr at 24-25 C. Colony counts were made and recorded as cfu/seed. Surface sterilized onion seeds treated with an identical amount of sterile 0.1 M $MgSO_4$ solution as above served as non-bacterized controls.

Soil treatments and experimental design

Muck soil collected from an onion field in Cloverdale, B. C., was used for all experiments. Three pH regimes (4.5, 5.5 and 6.5), 2 moisture levels (80% and 55%) and 2 temperatures (22-25 C and 17-19 C) were selected to evaluate the effects of these physical environmental variables on onion rhizosphere colonization by *B. subtilis* B-2, and on the growth of onion seedlings. The three pH levels were obtained by adding appropriate amounts of calcium hydroxide to the soil, and the soil was mixed thoroughly and stored in clay pots for 30-40 days outdoors before use. The moisture content of the soil was estimated as described in Chapter I and adjusted to 80% and

55%. The various soil treatments (2 moisture levels x 3 pH's) were allocated to disposable rectangular, 5.5 cm deep plastic pots of approximately 110 cc capacity (Westcan Horticultural Supply, Calgary, Alberta). Each pot received approximately 75 cc of the appropriate soil treatment. Individual treatment pots were placed in 28 cm x 50 cm x 5 cm plastic propagating flats without drain holes, 36 per flat. Each flat contained six replicate pots (one for each sampling period) of each of the six soil treatments, arranged in a randomized complete block design. There were four replicate flats for each temperature. Four bacteized or non-bacterized onion seeds were sown in each treatment pot. To maintain the low soil temperature (17-19 C) the flats containing seedlings were floated on a water bath maintained at about 12 C. The high soil temperature (22-25 C) occurred naturally in the flats arranged on a bench at ambient laboratory temperature. The experiments ran for 14 weeks with a 14 hr photoperiod. Sterile water was added periodically on a weight basis to the pots to replace moisture lost during incubation.

Effect of different soil pH, moisture and temperature regimes on populations of B. subtilis B-2 marker in onion seedling rhizospheres

Populations of the marked *B. subtilis* B-2 in the rhizospheres of seedlings growing from bacterized and non-bacterized control seeds were estimated at 2, 4, 6, 8, 11, and 14 weeks after seeding by dilution plating onto S_{300}CB-PDA. One plant per treatment per replication was removed and shaken to remove excess soil. The entire root system and soil adhering after gentle shaking was detached from the shoot and designated as rhizosphere. The root comprising a sample was cut into approximately 5 mm segments, using a surface sterilized surgical blade. The segments were placed in a 250

ml flask containing 10 ml of sterile 0.1 M $MgSO_4$ and washed for 20 min by gentle magnetic stirring. Serial dilutions of the washing suspension were prepared and 0.5 ml aliquots from the appropriate dilutions were plated onto S_{300}CB-PDA in triplicate. Plates were incubated for 72 hr at 24-25 C and colony counts were made and recorded as cfu per plant. All root samples were processed within 24 hr of harvesting. A backward elimination procedure ($P \geq 0.05$) (48, 59) using GLIM (4) was used to obtain a best fit regression of the colony count data on time to a linear model.

Measurement of growth of onion seedlings

Growth of seedlings was measured at 2, 4, 6, 8, 11 and 14 weeks following seeding. Individual seedlings were removed from each treatment pot and shaken to remove excess soil. Height of the shoot was recorded. Roots and shoots were seperated, washed, air dried, and weighed. The data were analyzed by analysis of variance and Student Newman-Keul's test at 5% level of significance.

RESULTS

Effect of different soil pH, moisture and temperature levels on populations of B. subtilis B-2 marker in onion rhizospheres following seeding

Populations of the B-2 marker in onion rhizospheres declined rapidly within the first 14 days after seeding, from 4.80×10^6 cfu/seed on day zero to an average of 1.06×10^3 cfu/plant at day 14, and less rapidly during the next 12 weeks following seeding irrespective of the soil treatments. An average population of 9.45×10^1 cfu/plant was recovered at 14 weeks following seeding.

Though the recovered poulations were low, the B-2 marker persisted in onion rhizospheres throughout the 14 week duration of the experiment in all treatments.

No B-2 marker populations were detected from the rhizospheres of onion seedlings grown from non-bacterized control seeds throughout the experiment, irrespective of the soil treatments.

Regression curves showing the effects of the moisture, temperature and pH treatments on rhizosphere populations of *B. subtilis* B-2 marker are shown in Fig. 3. Every regression model attempted substantially underestimated the observed populations of zero time (4.80×10^6 or 15.38 ln cfu/seed). The regression of ln cfu/plant on ln time gave an overall $r^2=0.92$ for the data from 2 through 14 weeks following seeding. The significance levels of the various factors and interactions evaluated in the backward elimination procedure, and the components of the final model used to present the data shown in Fig. 3 are listed in Table 5. Soil pH, temperature and moisture, and interactions of temperature x moisture and temperature x pH were significant ($P \leq 0.05$); pH x moisture and pH x moisture x temperature interactions were not significant. Fig. 3 shows that higher populations of *B. subtilis* B-2 marker were maintained at 22-25 C than at 17-19 C. The differential effect of temperature on rhizosphere populations of the B-2 marker was expressed mainly between 0 and 2 weeks in the high soil moisture regime, whereas they occurred over the 14 week duration of the experiment in the low soil moisture regime. Overall, the most favourable treatment for persistence of B-2 in onion seedling rhizospheres was the high temperature, high moisture, high pH regime; temperature appreared to be the most important variable.

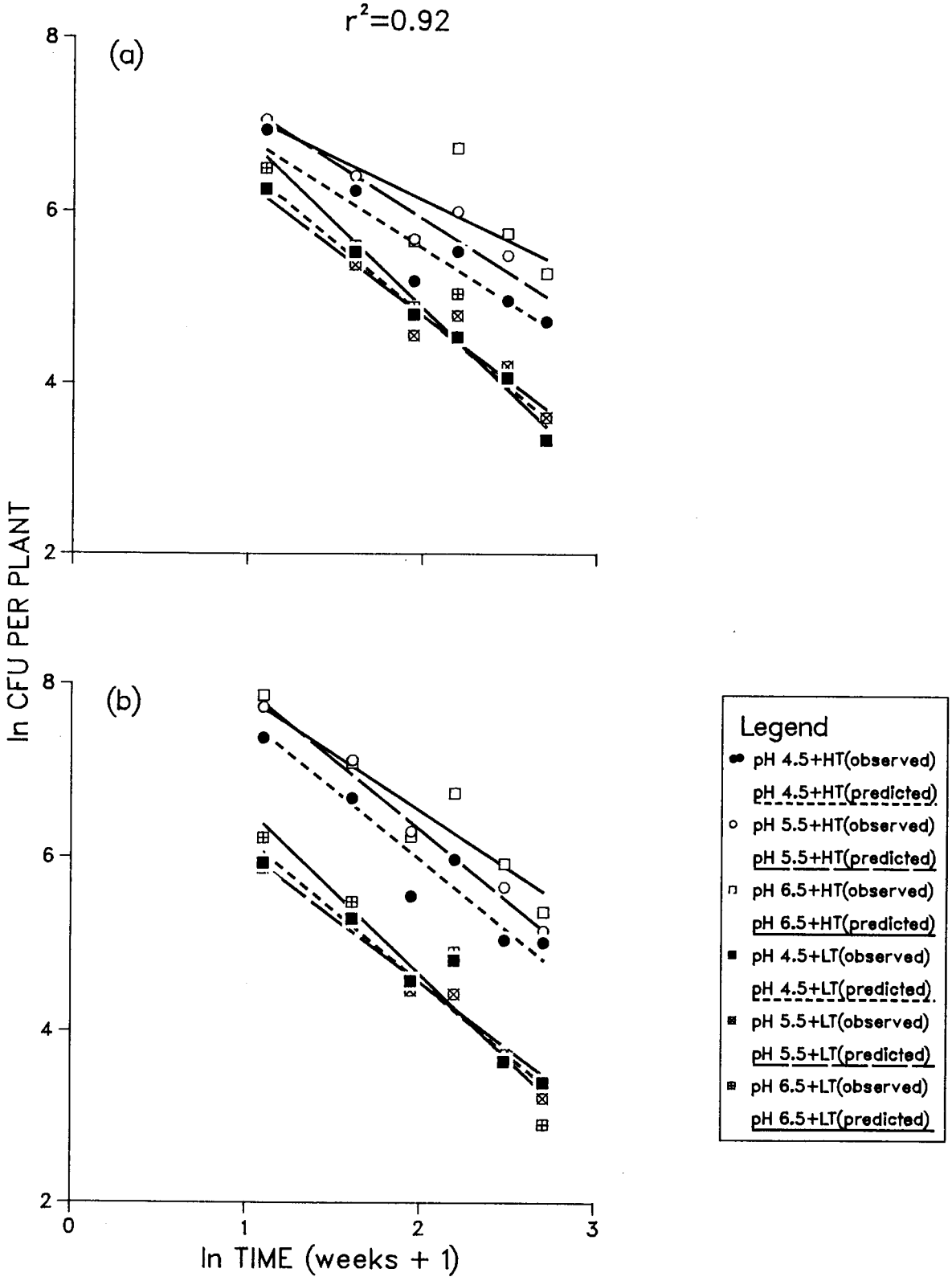
Table 5. Effects of various factors and interactions evaluated by backward elimination in the derivation of the regression model for effects of pH, temperature and moisture on the survival of *Bacillus subtilis* B-2 marker in onion seedling rhizospheres.

#	Tested against	Regression model	Variable removed	D.F	SS(error)	F-Value	P-Value
1	-	P+T+M+PxT+PxM+TxM+PxTxM+TI+PxTI+TxTI+MxTIxPxTxTI+PxMxTI+TxMxTI+PxTxMxTI+R	-	261	26.07	-	-
2	1	P+T+M+PxT+PxM+TxM+PxTxM+TI+PxTI+TxTI+MxTI+PxTxTI+PxMxTI+TxMxTI+R	PxTxMxTI	263	26.09	0.101	0.904
3	2	P+T+M+PxT+PxM+TxM+TI+PxTI+TxTI+MxTI+PxTxTI+PxMxTI+TxMxTI+R	PxTxM	265	26.22	0.655	0.520
4	3	P+T+M+PxT+TxM+TI+PxTI+TxTI+MxTI+PxTxTI+PxMxTI+TxMxTI+R	PxM	267	26.60	1.920	0.149
5	4	P+T+M+PxT+TxM+TI+PxTI+TxTI+MxTI+PxTxTI+TxMxTI+R	PxMxTI	269	26.67	0.351	0.704
6	5	P+T+M+PxT+TxM+TI+PxTI+TxTI+MxTI+PxTxTI+TxMxTI[1]	R	272	26.94	0.908	0.438
7	6	P+T+M+TxM+TI+PxTI+TxTI+MxTI+PxTxTI+TxMxTI	PxT	274	28.37	7.219	<0.0001
8	6	P+T+M+TI+PxTI+TxTI+MxTI+P.T.TI+TxMxTI	TxM	273	29.27	23.525	<0.0001
9	6	P+T+M+TI+PxTI+TxTI+MxTI+TxMxTI	PxTxTI	274	29.00	10.399	<0.0001
10	6	P+T+M+TI+PxTI+TxTI+MxTI	TxMxTI	273	27.68	7.471	0.007

[1]Final model.
(P=pH, T=temperature, M=moisture, TI=time, R=reps).

Figure 3. Regression curves showing the effects of different soil pH and temperature regimes at two soil moisture levels on populations of *Bacillus subtilis* B-2 marker in onion seedling rhizospheres following bacterized seeding (HT=higher temperature (22-25 C); LT=lower temperature (17-19 C)). Overall $r^2=0.92$.

(a) Populations at 55% soil moisture
(b) Populations at 80% soil moisture


r^2=0.92
(a)
(b)
8
6
4
2
0
1
2
3
ln CFU PER PLANT
ln TIME (weeks + 1)
Legend
pH 4.5+HT(observed)
pH 4.5+HT(predicted)
pH 5.5+HT(observed)
pH 5.5+HT(predicted)
pH 6.5+HT(observed)
pH 6.5+HT(predicted)
pH 4.5+LT(observed)
pH 4.5+LT(predicted)
pH 5.5+LT(observed)
pH 5.5+LT(predicted)
pH 6.5+LT(observed)
pH 6.5+LT(predicted)

affecting persistence.

Effect of soil and bacterized treatments on the growth of onion seedlings

Data on the overall effects of soil pH, moisture and temperature, and seed bacterization with *B. subtilis* B-2 marker on the growth of onion seedlings at 14 weeks are presented in Table 6. Seed bacterization with *B. subtilis* B-2 marker caused significant increases in shoot height (Fig. 4), dry weight (Fig. 5) and root dry weight (Figs. 6, 7) of onion seedlings over non-bacterized controls, irrespective of the soil treatments. Increases in shoot dry weight ranged from 12-94%, root dry weight from 13-100% and shoot height from 12-40% over control. Mean increases associated with bacterization over all environmental conditions were 18.4% for shoot height, 20.2% for shoot dry weight and 35.5% for root dry weight. Increases in shoot height and weight of onion seedlings were greatest at low temperature and high moisture at all pH regimes (Figs. 4, 5). Root weight was similarly affected by temperature (Fig. 7), but was significantly increased at pH 6.5 compared to 5.5 and 4.5 (Fig. 6).

Table 6. Overall effects of soil pH, moisture, temperature and seed bacterization with *Bacillus subtilis* B-2 marker on the growth of onion seedlings at 14 weeks after seeding.

Treatments	Range	Shoot height (cm)[1]	Shoot dry weight (mg)[1]	Root dry weight (mg)[1]
pH	4.5	23.56 ± 1.79 a[2]	33.53 ± 5.41 a[2]	2.95 ± 0.32 a[2]
	5.5	22.94 ± 1.71 a	34.41 ± 4.43 a	3.18 ± 0.41 b
	6.5	23.00 ± 1.97 a	34.40 ± 5.39 a	3.79 ± 0.52 c
Moisture	55%	22.61 ± 1.78 a	33.13 ± 5.21 a	2.93 ± 0.41 a
	80%	23.72 ± 1.82 b	35.09 ± 4.86 b	3.68 ± 0.43 b
Temperature	22-25 C	20.57 ± 1.30 a	25.02 ± 1.96 a	2.93 ± 0.47 a
	17-19 C	25.76 ± 1.22 b	43.20 ± 2.31 b	3.69 ± 0.36 b
Bacterization		25.16 ± 1.54 b	37.31 ± 4.87 b	3.82 ± 0.41 b
Non-bacterization		21.25 ± 1.41 a	31.04 ± 4.66 a	2.82 ± 0.26 a

[1] Mean (± =S. E) of 4 replications, one plant each.

[2] Means within a column followed by the same letter do not differ significantly according to Student Newman-Keul's test (p = 0.05).

Figure 4. Influence of seed bacterization with *Bacillus subtilis* B-2 marker on shoot height of onion seedlings grown at various moisture and temperature regimes.

C, C1, C2, C3 = controls
T, T1, T2, T3 = bacterized treatments
C, T = height at 55% moisture and high temperature
C1, T1 = height at 80% moisture and high temperature
C2, T2 = height at 55% moisture and low temperature
C3, T3 = height at 80% moisture and low temperature

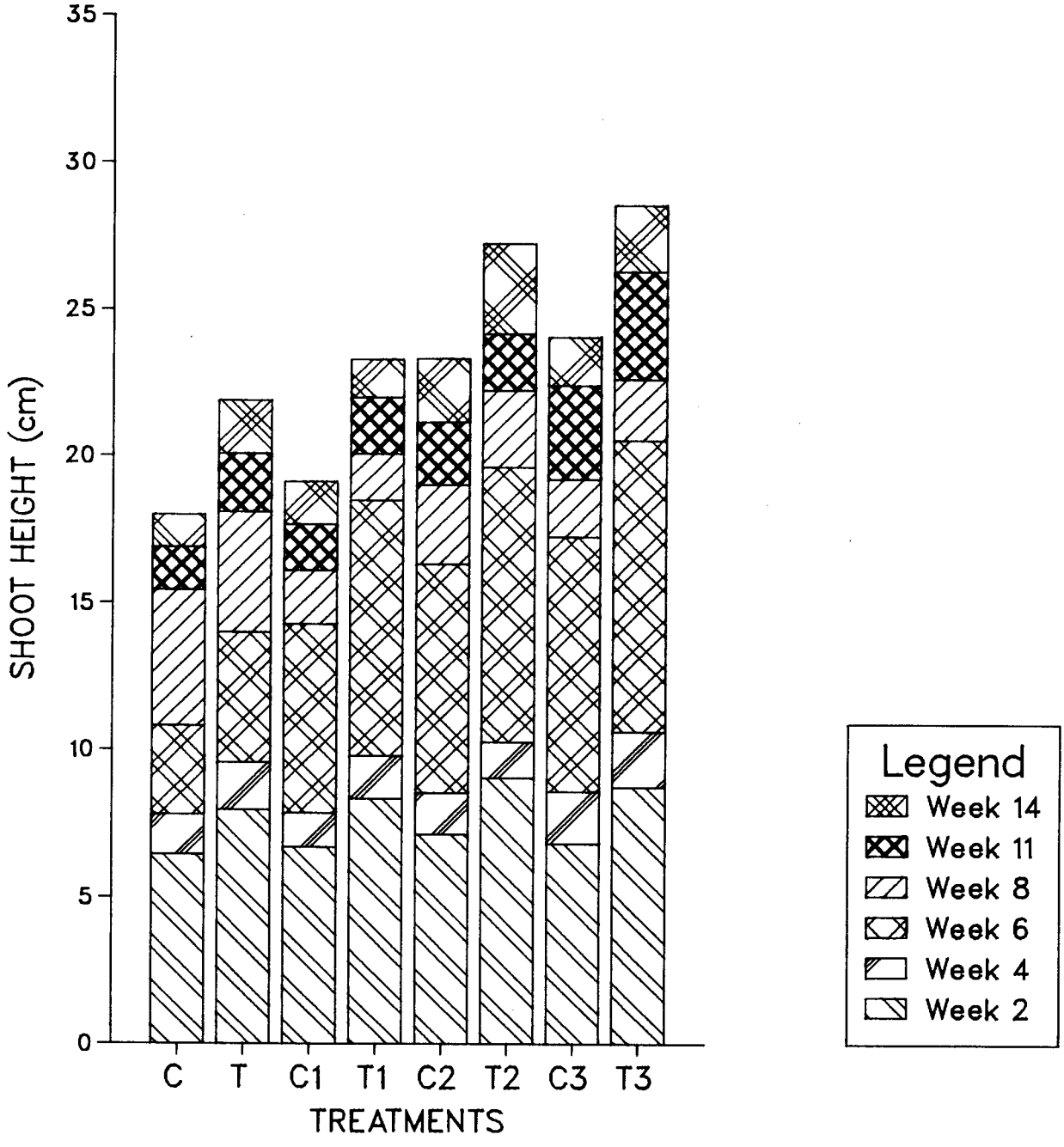

35
30
25
20
15
10
5
0
SHOOT HEIGHT (cm)
C
T
C1
T1
C2
T2
C3
T3
TREATMENTS
Legend
Week 14
Week 11
Week 8
Week 6
Week 4
Week 2

Figure 5. Influence of seed bacterization with *Bacillus subtilis* B-2 marker on shoot dry weight of onion seedlings grown at various moisture and temperature regimes.

C, C1, C2, C3 = controls
T, T1, T2, T3 = bacterized treatments
C, T = shoot dry weight at 55% moisture and high temperature
C1, T1 = shoot dry weight at 80% moisture and high temperature
C2, T2 = shoot dry weight at 55% moisture and low temperature
C3, T3 = shoot dry weight at 80% moisture and low temperature

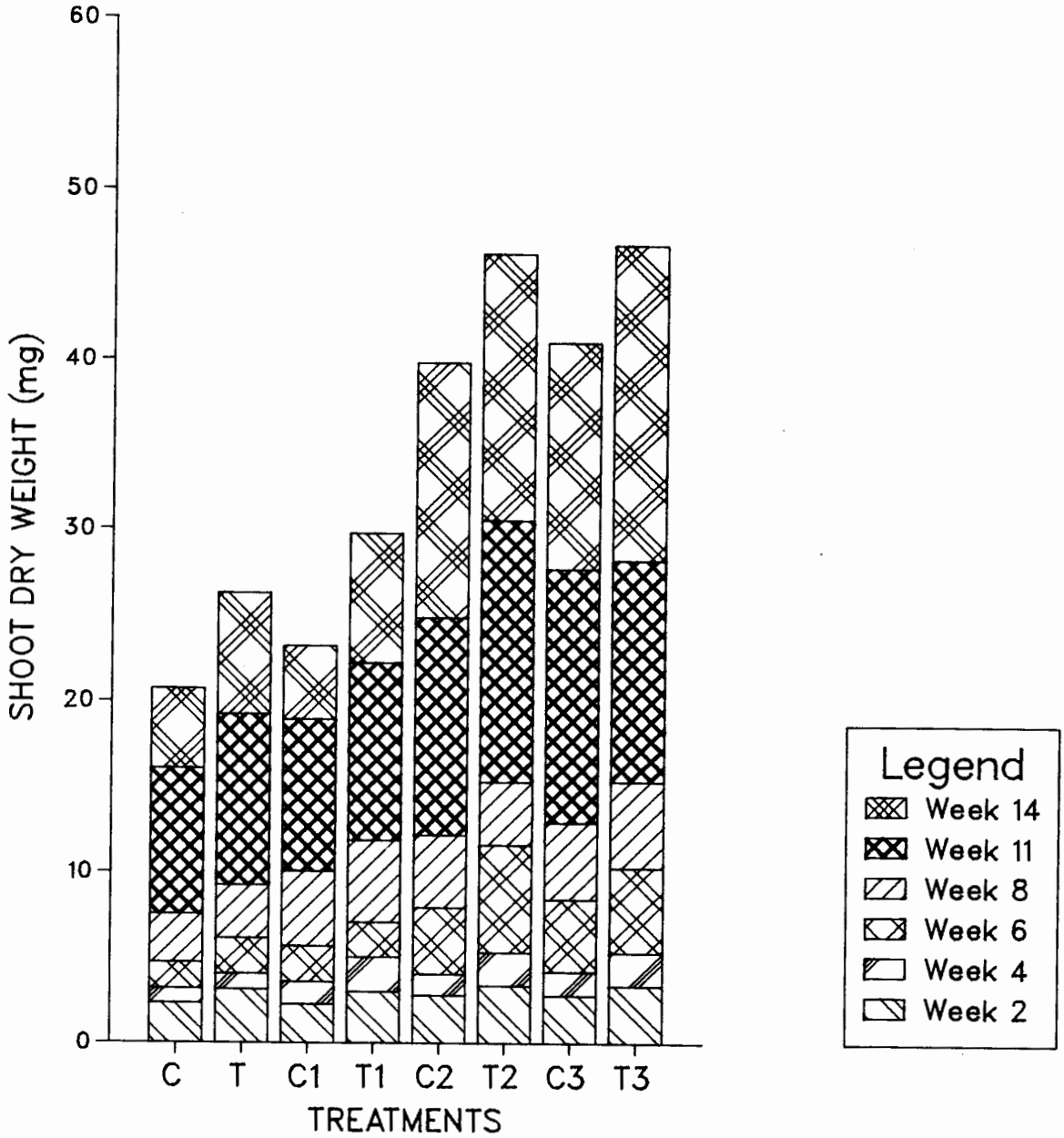

60
50
40
30
20
10
0
SHOOT DRY WEIGHT (mg)
C
T
C1
T1
C2
T2
C3
T3
TREATMENTS
Legend
Week 14
Week 11
Week 8
Week 6
Week 4
Week 2

Figure 6. Influence of *Bacillus subtilis* B-2 marker on root dry weight of onion seedlings grown at various pH ranges of muck soil.

C, C1, C2 = controls
T, T1, T2 = bacterized treatments
C, T = root dry weights at pH 4.5
C1, T1 = root dry weights at pH 5.5
C2, T3 = root dry weights at pH 6.5

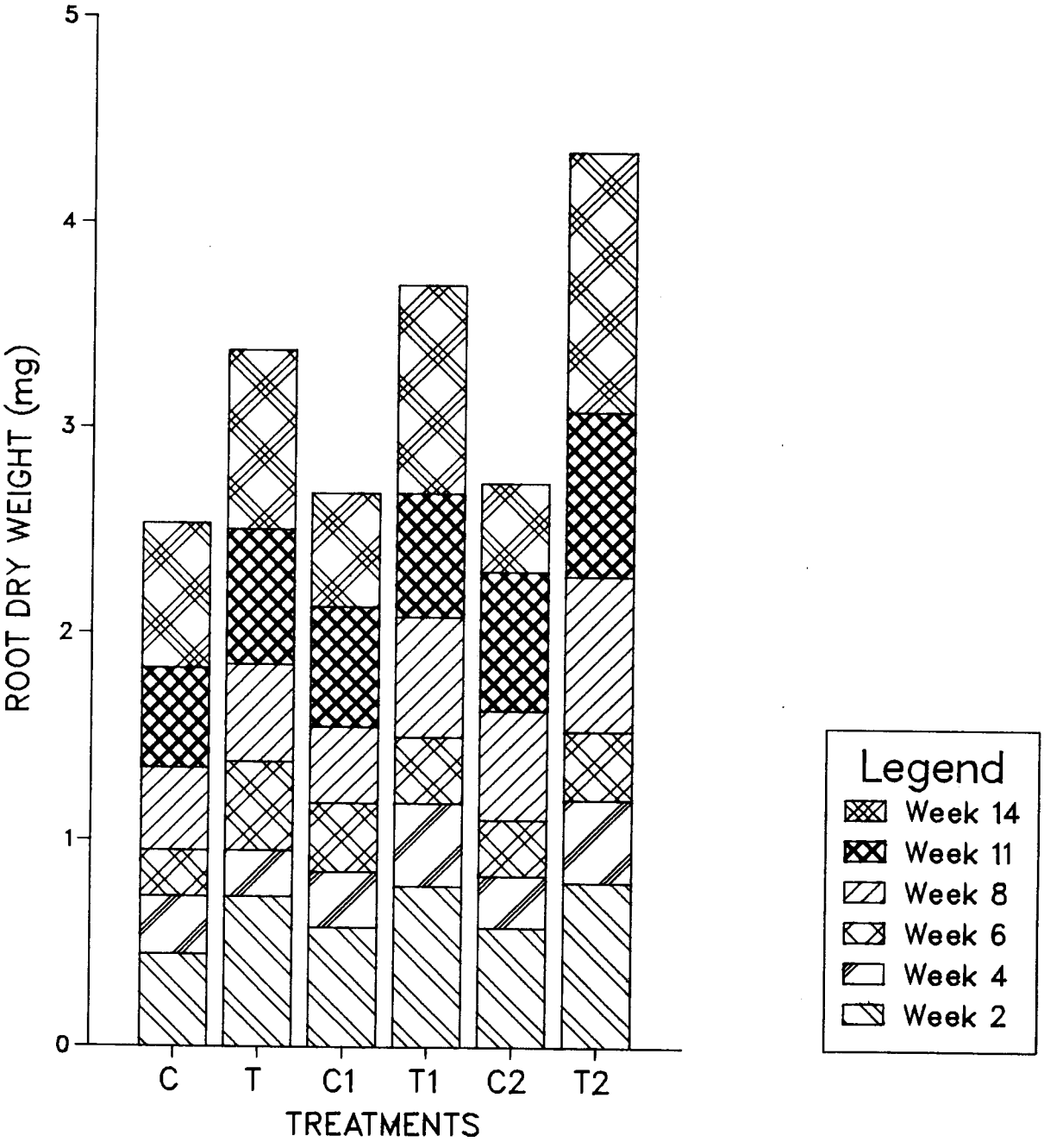
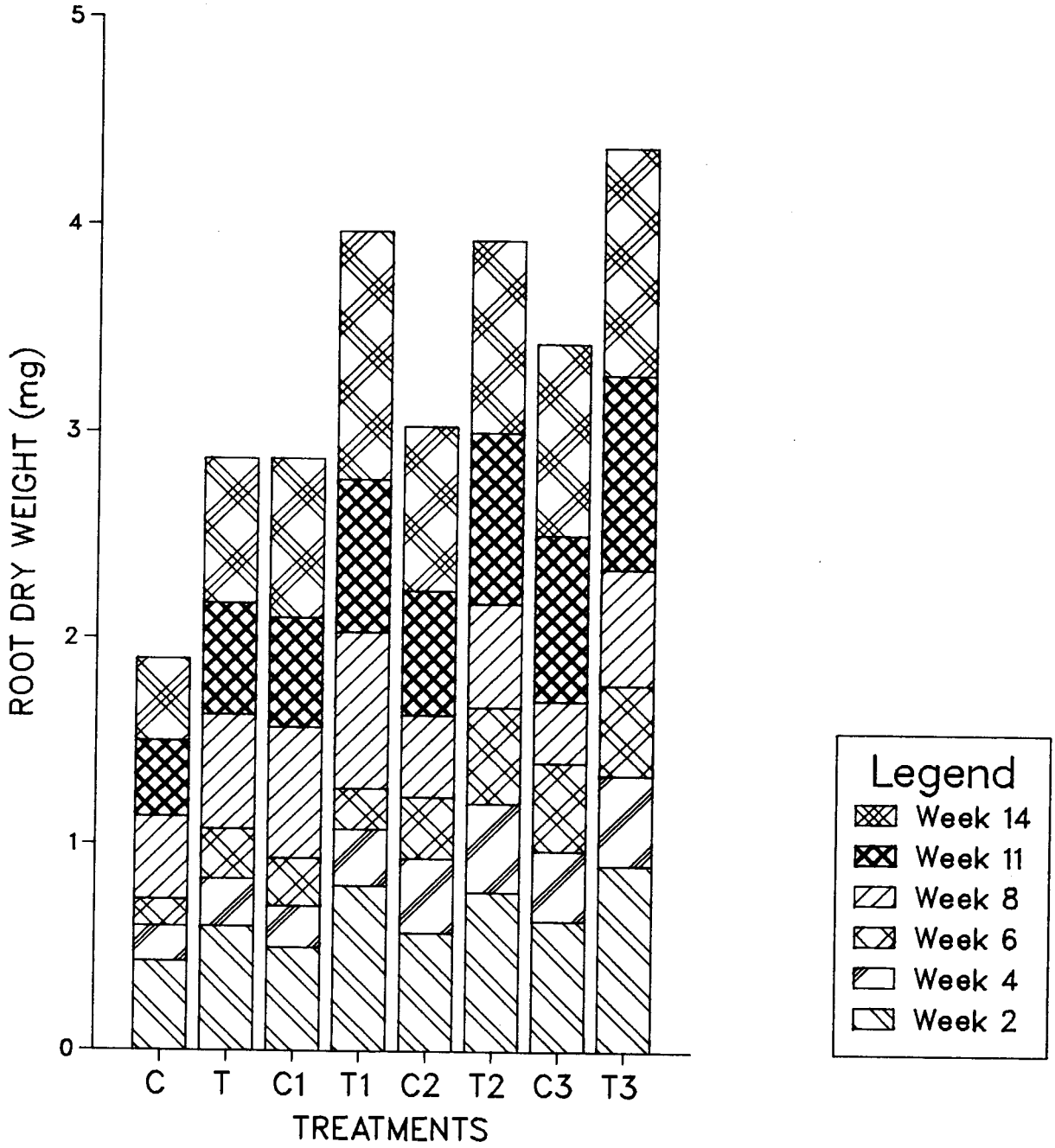

ROOT DRY WEIGHT (mg)
5
4
3
2
1
0
C
T
C1
T1
C2
T2
TREATMENTS
Legend
Week 14
Week 11
Week 8
Week 6
Week 4
Week 2

Figure 7. Influence of *Bacillus subtilis* B-2 marker on root dry weight of onion seedlings grown at various moisture and temperature regimes.

C, C1, C2, C3 = controls
T, T1, T2, T3 = bacterized treatments
C, T = root dry weight at 55% moisture and high temperature
C1, T1 = root dry weight at 80% moisture and high temperature
C2, T2 = root dry weight at 55% moisture and low temperature
C3, T3 = root dry weight at 80% moisture and low temperature


5
4
3
2
1
0
ROOT DRY WEIGHT (mg)
C
T
C1
T1
C2
T2
C3
T3
TREATMENTS
Legend
Week 14
Week 11
Week 8
Week 6
Week 4
Week 2

DISCUSSION

Populations of a genetically marked strain of *B. subtilis* B-2 were successfully monitored in onion seedling rhizospheres for 14 weeks. Populations of B-2 marker in onion seedling rhizospheres as low as 10 cfu/plant were recovered on S_{300}CB-PDA without interference from growth of soil fungi or bacteria, thus confirming the applicability of the method for the study of the population dynamics of a specific bacterial strain occurring at low levels in a complex, biologically active environment.

The procedure is useful because (i) it allows quantitative estimates of changes in population levels of the marked strain within the rhizosphere associated with various soil treatments, and (ii) it aids in the interpretation of the influence of seed bacterization on the growth of onion seedlings.

Recovery of the marked strain of *B. subtilis* B-2 from onion rhizospheres showed that its populations declined rapidly during the first 2 weeks following seeding in all treatments, but also that it persisted under all test conditions on roots for the next 12 weeks. High soil moisture at high temperature appears to be most favourable to survival of *B. subtilis* B-2 in onion rhizospheres. The results suggest that the effect of soil moisture is temperature dependent.

The rapid decline of populations irrespective of soil treatments, suggests that *B. subtilis* B-2 did not preferentially establish in the rhizospheres of onion seedlings grown from bacterized seeds. These findings are similar to those of Wong (153). Using a fluorescent antibody technique Wong (153) was

unable to show a significant enhancement of rhizosphere *B. subtilis* over controls, at 14 and 42 days post seeding. This was likely due to the fact that the fluorescent antibody technique did not provide selective differentiation between B-2 and indigenous strains, as Wong (153) found substantial populations of *B. subtilis* in control rhizospheres soon after seeding. The specificity of the marker strain technique permitted the detection of significant long term differences in the rhizosphere populations of the marked strain resulting from seed bacterization.

My results also suggest that the failure of onion white rot control in 1980 and 1981 field trials (Rahe and Utkhede unpublished data) may have been due to the failure of *B. subtilis* B-2 to establish in onion rhizospheres due to continuous cool and wet conditions which prevailed in those years. My results suggest that 17-19 C strongly supressed B-2 populations in the rhizosphere relative to temperatures of 22-25 C, whereas high soil moisture enhanced populations only at the higher temperature. Soil temperatures in the field following seeding are typically well below 17-19 C. Thus, it is possible that the potential of *B. subtilis* B-2 as a biocontrol agent of onion white rot may be restricted to relatively wet and warm conditions.

Although *B. subtilis* B-2 did not appear to be preferentially associated with the seedling rhizospheres, it nevertheless had a significant positive influence on the growth of onion seedlings. I hypothesize that although B-2 may not be an active onion rhizosphere colonist, it could persist in nonrhizosphere root-zone soil and in some way influence the growth of onion seedlings.

The mechanism by which *B. subtilis* B-2 promoted increased onion seedling growth is unclear. It may include elaboration of products which directly stimulate plant growth and/or affect the composition of the root microflora by antagonism. The data obtained suggest that plant growth response is associated with seed bacterization and may result from rhizosphere colonization by *B. subtilis* B-2 at low levels, or from possible activity in the nonrhizosphere root-zone soil. The observed growth responses were not proportional to rhizosphere populations of *B. subtilis* B-2; seedling growth was greatest under the low temperature (17-19 C) regime, whereas B-2 rhizosphere populations were most favoured by the high temperature (22-25 C) regime.

The observed enhancement of onion seedling growth associated with seed bacterization with *B. subtilis* B-2 is consistent with published reports on stimulation of plant growth by *Bacillus* spp. in various agricultural crops and vegetables (12, 13, 97, 113, 115, 116). Cooper (38) reported that more than 35 million hectares of crop lands were treated with strains of *Azotobacter* spp. and *Bacillus* spp., giving yield increases ranging from 10 to 20%. Greatest benefits were reported on vegetable crops (100).

Some of the variability in growth and yield increase responses associated with bacterization is probably due to the failure of the introduced bacteria to colonize the rhizosphere, although there is a lack of data dealing with this aspect. Absence of rhizosphere colonization could be due to ineffective strains, low viability of the introduced bacterial isolates, and/or unfavourable effects of soil moisture, soil pH and temperature. Broadbent *et al.* (13) reported field trial variability of yield from season to season using

Bacillus spp. and attributed this to uncontrolled physical or biological factors, but did not provide data on rhizosphere colonization by the *Bacillus* spp. These and other studies provide very little data on the ability of specific bacteria to colonize the rhizosphere, presumably due to lack of methods to identify the introduced bacterial strains. Where data on the population dynamics of specific bacteria are available, identification has generally been based on colony morphology (22, 115) *in vitro* requirements for particular amino acids (50), antigenic reactions (22), or biochemical reactions *in vitro* (6). The use of the genetically marked strain of *B. subtilis* B-2 tolerant to antibiotics permitted a precise determination of onion rhizosphere colonization by the marker strain.

CHAPTER III

A COMPARATIVE POPULATION STUDY OF *BACILLUS SUBTILIS* B-2 AND OTHER RHIZOBACTERIA IN ONION SEEDLING RHIZOSPHERES AND NONRHIZOSPHERE ROOT-ZONE SOILS, AND THEIR EFFECTS ON INDIGENOUS MICROFLORA

INTRODUCTION

Both direct and indirect mechanisms have been suggested to explain the positive influence of certain bacteria on plant growth (124). Hypothesized direct mechanisms are that bacteria elaborate substances that directly stimulate plant growth such as nitrogen, phytohormones (18-21, 66, 70, 75), and/or compounds which promote the mineralization of phosphates (5, 17, 75) in the root-zone. A popular hypothesis for an indirect mechanism is that the populations of various fungal and bacterial pathogens in the root system are reduced by the bacteria introduced via seed or root bacterization (75). The hypothesis that elaboration of bacterial products is involved in enhancing plant growth and yield increase is controversial due to lack of supportive data. A conclusion of studies with *Azotobacter* spp., *Bacillus* spp., and *Clostridium* spp. was that increased plant growth resulted from bacterial nitrogen fixation or solubilization of soil phosphates (16, 18, 75). But Mishustin and Naumova (101) calculated that the amount of nitrogen or phosphates which would be made available to plants as a result of bacterial metabolism could not possibly account for the observed growth enhancement. Several authors have suggested that plant hormones which are produced *in vitro* by *Bacillus* spp. and fluorescent Pseudomonads may increase plant growth (16, 18, 50, 63, 66, 75, 131). However direct evidence of hormonal activity in the rhizosphere is

lacking. Similarly, although microbial production of antibiotics has been demonstrated in soil organic matter (154), their detection in the rhizosphere remains difficult. Increase in plant growth by bacteria interacting with the indigenous microflora is considered to be a favourable hypothesis (82), but also suffers from insufficient supporting data.

My earlier studies on the population dynamics of the genetically marked strain of *B. subtilis* B-2 in onion seedling rhizospheres showed that although B-2 was not preferentially associated with onion rhizospheres, it nevertheless had a significant influence on the growth of onion seedlings under controlled conditions (Chapter II). These results prompted this comparative population study of *B. subtilis* B-2 and other selected rhizobacteria in both onion rhizospheres and nonrhizosphere root-zone soils.

The following experimental objectives were accomplished: (i) a comparative study of the population dynamics of *B. subtilis* B-2 and four other selected rhizobacteria in onion rhizosphere and nonrhizosphere root-zone soil following seed bacterization, (ii) an evaluation of the effects of the test isolates on the growth of onion seedlings, and (iii) an evaluation of the effects of the test isolates on indigenous soil and root microflora.

MATERIALS AND METHODS

Source of bacterial isolates and their characterization

Genetically marked strains of *B. subtilis* B-2 (Chapter I), and four other rhizobacteria were used in this study. The latter four rhizobacteria were isolated from the rhizospheres of commercial onions (cv. Autumn Spice)

growing in muck soil in Cloverdale, B. C., during the summer of 1983. Serial dilutions were made from the washings of onion roots and plated onto Thornton's agar (TA) (73). Isolates representative of colony types present in greatest numbers were selected. The parental isolates of the marked strains used in this study were designated UI:2, UI:1, B and W. The method described in Chapter I was followed for the isolation of genetically marked strains tolerant to the combination of 300 ug/ml streptomycin (S), 100 ug/ml cycloheximide (C) and 30 ug/ml benomyl (B) in PDA (S_{300}CB-PDA). The frequencies of S_{120}CB- and S_{300}CB-tolerant genotypes occurring in the parental populations and in colonies derived from S_{300}CB-PDA plates were estimated. Standard procedures (62) followed for cultural studies and for biochemical tests included hydrolysis of starch, reduction of nitrates, and production of indole and H_2S that were used to compare the parental strains with their respective marked strains. Comparision between the parental and the marked strains for inhibition of *S. cepivorum* in dual culture was done as described in Chapter I.

Bacterized and non-bacterized onion seed treatments

S_{300}CB-tolerant strains of *B. subtilis* B-2, and of rhizobacteria UI:2, B, UI:1, and W were used for seed bacterization on onion cv. Autumn Spice. The seeds were surface sterilized as described in Chapter II. For each bacterium, fresh bacterial culture previously maintained on S_{300}CB-PDA was grown in 500 ml flasks containing sterilized 250 ml of TSB, and the method described in Chapter II was followed for the preparation of bacterial pellets, seed bacterization and non-bacterized control treatments. At zero time, the numbers of cfu/seed for each bacterium from the bacterized seeds were estimated as

described in Chapter II.

Estimation of genetically marked bacterial populations in onion rhizosphere and nonrhizosphere root-zone soils of field grown onions

A field trial was conducted in the summer of 1985 in Cloverdale, B. C., on muck soil in which onions had been grown commercially for the past four years. Soil pH in the trial field was approximately 4.5 (determined using a thick suspension of soil in 0.01 M $CaCl_2$). Meteorological data were recorded in the centre of the experimental area. Air temperature (30 cm above ground) during the trial ranged from 15 to 30 C. The soil temperatures (10 cm below the surface) ranged from 10 to 26 C. This field trial included five bacterized treatments (B-2, UI:2, UI:1, B, and W) and a non-bacterized control treatment with five replicates per treatment. The individual treatment plots contained five rows 2 m long, spaced 25 cm apart. These were arranged in a randomized complete block design. The centre three rows were double seeded by hand with bacterized or non-bacterized onion seeds at 7 cm spacing. Guard rows were seeded with cv. Taurus with a tractor mounted Stanhay Precision Seeder. The trial was seeded on 19 May 1985. Due to poor germination only one sampling was possible; this was done 30 days following seeding. Samples consisted of five plants per replication per treatment. Plants were dug at random from the three middle rows of plots, shaken gently to dislodge adhering soil, placed in polyethylene bags and kept cool at about 4 C until processing. All samples were processed within 24 hr of collection. Roots and adhering soil (designated rhizosphere soil) from five seedlings were detached, cut into small pieces (5 mm) using surface sterilized scissors, and placed immediately into 500 ml flasks containing 10 ml of sterile 0.1 M

$MgSO_4$. The method described in Chapter II was followed for the quantification of populations of the marked bacterial strains.

Soil samples were collected from the region 3-7 cm around where the onion seedlings had been removed and designated nonrhizosphere root-zone soil. At least five cores of soil were collected at random within this region to a depth of 10 cm with a soil sampler (2.5 x 10 cm) and consolidated to give one sample from each replication of each treatment. Samples were placed in sterile polyethylene bags, and kept cool at about 4 C until processed. All samples were processed within 48 hr of field sampling. Each composite sample was mixed thoroughly and then sifted through a 2 mm sieve. A 10 g subsample from each composite sample was weighed in previously tared glass petri dish, dried overnight in an oven at 105 C, and then reweighed to determine the moisture content. A 10 g subsample from each composite sample was placed into a 500 ml flask containing 100 ml of sterile 0.1 M $MgSO_4$ and then subjected to the procedure described for the quantification of the marked bacterial populations in the rhizosphere. Colony counts were made and the populations of the five marked strains in the nonrhizosphere root-zone soil were determined and reported as cfu/g dry wt. of soil.

Evaluation of the effects of seed bacterization treatments on the growth of onion seedlings

Individual onion seedlings were dug at random from the three middle rows of the plot, at 30 days following bacterized seeding. Samples consisted of five plants for each of the five replications per treatment. Height of the

shoot system was recorded. Roots and shoots were seperated, washed, air dried, and weighed.

Effect of bacterized seed treatments on indigenous rhizosphere and nonrhizosphere root-zone soils microflora

The effect of seed bacterization treatments on populations of naturally occurring bacteria and fungi in rhizosphere and nonrhizosphere root-zone soils was determined 30 days following seeding. The washed suspensions of onion rhizosphere and nonrhizosphere root-zone soils in 0.1 M $MgSO_4$ which were used earlier for the quantification of the marker bacterial populations were used for the estimation of total indigenous soil-root bacterial and fungal populations. Bacterial populations were estimated on TA (73) and fungal populations on rose bengal agar (RBA) (73). One tenth ml aliquots from appropriate dilutions of the washed suspensions representing each field replication were plated onto TA and RBA plates in triplicate. The plates were incubated at 24-25 C. Colony counts of bacteria and fungi were recorded after 3 and 7 days incubation, respectively. The populations of bacteria and fungi were estimated as cfu/plant in the rhizosphere and as cfu/g dry wt. of soil in the root-zone soil. No attempt was made to further identify the bacteria and fungi.

The data were analyzed by analysis of variance and Student Newman-Keul's test at 5% level of significance.

Table 7. Frequency of S_{120}CB-tolerant phenotypes[1] occurring in the parental wild type populations of four selected rhizobacteria.

Bacterial strains[2]	Colonies recorded		Frequency of tolerance
	On PDA[3]	On S_{120}CB-PDA[3]	
UI:2	$4.43 \pm 0.71 \times 10^8$	$9.88 \pm 2.34 \times 10^1$	$1:4.52 \times 10^6$
UI:1	$5.25 \pm 0.45 \times 10^8$	$1.14 \pm 0.02 \times 10^2$	$1:4.61 \times 10^6$
B	$6.74 \pm 0.42 \times 10^8$	$1.74 \pm 0.12 \times 10^2$	$1:3.87 \times 10^6$
W	$7.32 \pm 0.43 \times 10^8$	$1.14 \pm 0.25 \times 10^2$	$1:6.42 \times 10^6$

[1] S_{120}CB-tolerant phenotypes are colonies developing from parental isolates on potato dextrose agar amended with 120 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl (S_{120}CB-PDA).

[2] UI:2, UI:1, B and W were obtained from the rhizospheres of commercial onions (cv. Autumn Spice) grown in muck soil at Cloverdale, B. C.

[3] Mean ($\pm$=S. E) of 5 replications.

Table 8. Frequency of S_{120}CB-tolerant phenotypes[1] occurring in S_{120}CB-tolerant populations[2] of four selected rhizobacteria.

Bacterial strains[3]	Colonies recorded		Frequency of tolerance
	On PDA[4]	On S_{120}CB-PDA[4]	
UI:2	$5.70 \pm 0.51 \times 10^8$	$5.45 \pm 0.56 \times 10^8$	1:1.05
UI:1	$4.43 \pm 0.67 \times 10^8$	$3.79 \pm 0.81 \times 10^8$	1:1.17
B	$6.45 \pm 0.12 \times 10^8$	$5.78 \pm 0.15 \times 10^8$	1:1.12
W	$5.34 \pm 0.55 \times 10^8$	$4.48 \pm 2.00 \times 10^8$	1:1.19

[1] S_{120}CB-tolerant phenotypes are colonies developing from S_{120}CB-tolerant populations on potato dextrose agar amended with 120 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl (S_{120}CB-PDA).

[2] S_{120}CB-tolerant populations are colonies developing from parental isolates on potato dextrose agar amended with 120 ug/ml streptomycin, 100 ug/ml cycloheximide, 30 ug/ml benomyl (S_{120}CB-PDA).

[3] UI:2, UI:1, B and W were obtained from the rhizospheres of commercial onions (cv. Autumn Spice) grown in muck soil at Cloverdale, B. C.

[4] Mean ($\pm$=S. E) of 5 replications.

Table 9. Frequency of S_{300}CB-tolerant phenotypes[1] occurring in S_{300}CB-tolerant populations[2] of four selected rhizobacteria.

Bacterial strains[3]	Colonies recorded		Frequency of tolerance
	On PDA[4]	On S_{300}CB-PDA[4]	
UI:2	$3.78 \pm 0.52 \times 10^8$	$3.69 \pm 0.48 \times 10^8$	1:1.02
UI:1	$4.53 \pm 0.37 \times 10^8$	$3.62 \pm 0.34 \times 10^8$	1:1.25
B	$7.38 \pm 0.34 \times 10^8$	$7.08 \pm 0.31 \times 10^8$	1:1.04
W	$6.18 \pm 0.64 \times 10^8$	$5.78 \pm 0.48 \times 10^8$	1:1.07

[1] S_{300}CB-tolerant phenotypes are colonies developing from S_{300}CB-tolerant populations on potato dextrose agar amended with 300 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl (S_{300}CB-PDA).

[2] S_{300}CB-tolerant populations are colonies developing from potato dextrose agar amended with 300 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl.

[3] UI:2, UI:1, B and W were obtained from the rhizospheres of commercial onions (cv. Autumn Spice) grown in muck soil at Cloverdale, B. C.

[4] Mean ($\pm$=S. E) of 5 replications.

Table 10. Cultural characteristics[1] of parental isolates and S_{300}CB-tolerant derivatives of four selected rhizobacteria used for seed bacterization treatments[2].

Characters measured	Marked strains[3]			
	UI:2	UI:1	B	W
Colony appearance	Rough layer on outer surface	Slightly rough on outer surface	Smooth layer on outer surface	Smooth layer on outer surface
Spreading	Moderate	Moderate	Moderate	Weak
Elevation	Raised	Raised	Raised	Flat
Colour	Greyish-white	Greyish-white	Greyish-white	White
Surface	Smooth, glistening	Smooth, glistening	Smooth, watery-type	Smooth, glistening

[1] Cultures grown on potato dextrose agar at 22-25 C and evaluated after 5-7 days.

[2] Features of parental strains and S_{300}CB-tolerant derivatives were similar.

[3] UI:2, UI:1, B and W were obtained from the rhizospheres of commercial onions (cv. Autumn Spice) grown in muck soil at Cloverdale, B. C.

Table 11. Comparison of biochemical tests[1] and antagonistic reaction between parental isolates (P)[2] and S_{300}CB-tolerant strains (M) of four selected rhizobacteria used for seed bacterization treatments.

	Strains							
	UI:2		UI:1		B		W	
Tests	P	M	P	M	P	M	P	M
Gram-reaction	+	+	-	-	-	-	+	+
Reduction of nitrates	+	+	+	+	+	+	-	-
Indole production	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	-	-	-	-
Sugar fermentation:								
Glucose	•	•	•	•	•	•	•	•
Sucrose	•	•	•	•	•	•	•	•
Lactose	-	-	-	-	+	+	±	±
Antagonistic reaction:								
With *Sclerotium cepivorum*	+	+	-	-	+	+	-	-

[1] Biochemical test responses: + =positive; - =negative; ± =variable; • =acid production.

[2] UI:2, UI:1, B and W were obtained from the rhizospheres of commercial onions (cv. Autumn Spice) grown in muck soil at Cloverdale, B. C.

RESULTS

Strains of UI:2, UI:1, B, and W tolerant to the combinations of 300 ug/ml streptomycin, 100 ug/ml cycloheximide, and 30 ug/ml benomyl in PDA (S_{300}CB-PDA) and capable of growing on S_{300}CB-PDA were obtained. The frequencies of occurrence of SCB-tolerant phenotypes in the parental wild type populations are shown in Tables 7, 8 and 9. The marked strains expressed colony morphology, cultural characteristics (Table 10), biochemical tests and inhibition of *S. cepivorum* in dual culture comparable to these features in the respective parental wild types (Table 11).

Comparison of marked populations of B. subtilis B-2 and four other selected marked rhizobacteria applied to seeds and recovered from onion rhizosphere and nonrhizosphere root-zone soils 30 days after seeding

Estimated populations of the five genetically marked strains on bacterized seeds, and in the rhizosphere and nonrhizosphere root-zone soil of field grown onion seedlings 30 days after seeding are shown in Table 12. Populations per plant at 30 days were lower than on seeds at the time of planting in all cases, with the greatest declines occurring for B-2 and W. All marked strains were recovered from root-zone soil in substantial numbers. Among the five bacteria UI:2 and B maintained the highest populations in both rhizosphere and root-zone soil. Populations of all the bacterial strains were greater in nonrhizosphere root-zone soil than in rhizosphere soil. No marked bacterial populations were detected from rhizosphere and nonrhizosphere root-zone soils of non-bacterized control seedlings. Overall, UI:2 and B were comparatively good colonizers in both the rhizosphere and

nonrhizosphere root-zone soils, UI:1 and W were intermediate and B-2 was poor.

Effects of marked strains of B. subtilis B-2 and four other rhizobacteria on growth of onion seedlings

Seed bacterization treatments with the marked strains of B-2, UI:2 and B caused significant increases in shoot height , and shoot and root dry weights of onion seedlings over control and seed bacterization treatments UI:1 and W, 30 days following seeding (Table 13). The increases in shoot height over control ranged from 22% to 39%, in shoot dry weight from 23% to 32%, and in root dry weight from 32% to 46% over control. Seed treatments with UI:1 and W did not show any effect on shoot height or shoot and root dry weight of onion seedlings compared to control.

Effects of seed bacterization on populations of total indigenous bacteria and fungi in onion seedling rhizosphere and root-zone soils

Bacterization of seeds with the marked strains resulted in significant reductions over controls in the estimated populations of total indigenous bacteria and fungi in the rhizosphere and nonrhizosphere root-zone soils 30 days following seeding (Tables 14, 15). Reductions of indigenous bacteria by the five strains used for seed bacterization ranged from 21% to 71% in rhizosphere and 10% to 44% in nonrhizosphere root-zone soil. Strains B-2 and UI:2 were the most effective and strains W and UI:1 least effective in suppressing indigenous bacterial populations both in rhizosphere and root-zone soils.

Table 12. Comparison of populations of *Bacillus subtilis* B-2 marked with four selected marked rhizobacterial populations applied to seeds and recovered from onion rhizosphere and nonrhizosphere root-zone soils at 30 days after seeding.

Bacterial strains[1]	Populations in rhizosphere soil (cfu/seed or plant)[2]		Populations in nonrhizosphere root-zone soil (cfu/g dry wt. of soil)[3]
	Before seeding	30 days after seeding	30 days after seeding
B-2	$7.21 \pm 0.51 \times 10^5$	$7.60 \pm 0.24 \times 10^3$ a[4]	$8.70 \pm 1.02 \times 10^4$ a[4]
B	$1.65 \pm 0.93 \times 10^6$	$1.85 \pm 0.18 \times 10^5$ e	$9.84 \pm 0.83 \times 10^5$ e
W	$8.48 \pm 0.25 \times 10^5$	$2.20 \pm 1.53 \times 10^4$ b	$2.15 \pm 0.08 \times 10^5$ c
UI:1	$5.43 \pm 0.23 \times 10^5$	$7.83 \pm 0.61 \times 10^4$ c	$1.50 \pm 0.16 \times 10^5$ b
UI:2	$4.80 \pm 0.31 \times 10^5$	$1.29 \pm 0.10 \times 10^5$ d	$5.15 \pm 0.36 \times 10^5$ d
Control	0	0	0

[1] B-2 is an isolate of *Bacillus subtilis* obtained from sclerotia of *Sclerotium cepivorum* and B, W, UI:1 and UI:2 were obtained from the rhizospheres of commercial onions (cv. Autumn Spice). Both sclerotia and onions were from naturally infested muck soil from Cloverdale, B. C.

[2] Mean ($\pm$=S. E) of 5 replications, 5 seeds or plants each.

[3] Mean ($\pm$=S. E) of 5 replications, 10 g of soil each.

[4] Non-zero means within a column followed by the same letter do not differ significantly according to Student Newman-Keul's test ($p=0.05$).

Table 13. Comparison of *Bacillus subtilis* B-2 marker with the marked strains of four selected rhizobacteria in increasing shoot height, shoot and root dry weights of onion seedlings 30 days after seeding in a field trial.

Bacterial strains[1]	Shoot height (cm)[2]	% Increase compared to control	Shoot dry weight (mg)[2]	% Increase compared to control	Root dry weight (mg)[2]	% Increase compared to control
B-2	8.01±0.11 c[3]	38.60	8.60±0.64 c[3]	32.30	0.83±0.02 b[3]	40.70
B	7.05±0.30 b	22.00	8.00±0.55 bc	23.10	0.78±0.01 ab	32.20
W	5.38±0.24 a	-6.90	6.32±0.32 ab	-3.10	0.59±0.01 a	0
UI:1	5.81±0.18 a	0.50	6.10±0.36 a	-6.20	0.61±0.01 a	3.40
UI:2	7.97±0.39 c	37.90	8.40±0.51 c	29.20	0.86±0.02 bc	45.80
Control	5.78±0.17 a	0	6.50±0.24 ab	0	0.59±0.01 a	0

[1] B-2 is an isolate of *Bacillus subtilis* obtained from sclerotia of *Sclerotium cepivorum* and B, W, UI:1, and UI:2 were obtained from the rhizospheres of commercial onions (cv. Autumn Spice). Both sclerotia and onions were from naturally infested muck soil from Cloverdale, B. C.

[2] Mean (± =S. E) of 5 replications, 5 plants each.

[3] Means within a column followed by the same letter do not differ significantly according to Student Newman-Keul's test (p = 0.05).

Table 14. Effect of seed bacterization treatments on populations of total bacteria in onion seedling rhizosphere and nonrhizosphere root-zone soils at 30 days after seeding.

Bacterial strains[1]	Populations in rhizopshere soil (cfu/plant)[2]	% Change compared to control	Populations in nonrhizosphere root-zone soil (cfu/g dry wt.of soil)[3]	% Change compared to control
B-2	$1.74 \pm 0.16 \times 10^5$ b[4]	-59.00	$2.12 \pm 0.11 \times 10^6$ a[4]	-43.80
B	$2.21 \pm 0.11 \times 10^5$ c	-47.90	$2.54 \pm 0.04 \times 10^6$ b	-32.60
W	$3.37 \pm 0.32 \times 10^5$ d	-20.50	$2.92 \pm 0.04 \times 10^6$ c	-22.60
UI:1	$3.35 \pm 0.29 \times 10^5$ d	-21.00	$3.39 \pm 0.18 \times 10^6$ d	-10.10
UI:2	$1.25 \pm 0.07 \times 10^5$ a	-70.50	$2.12 \pm 0.17 \times 10^6$ a	-43.80
Control	$4.24 \pm 0.25 \times 10^5$ e	0	$3.77 \pm 0.16 \times 10^6$ d	0

[1] B-2 is an isolate of *Bacillus subtilis* obtained from sclerotia of *Sclerotium cepivorum* and B, W, UI:1 and UI:2 were obtained from the rhizospheres of comercial onions (cv. Autumn Spice). Both sclerotia and onions were from naturally infested muck soil from Cloverdale, B. C.

[2] Mean ($\pm$=S. E) of 5 replications, 5 plants each.

[3] Mean ($\pm$=S. E) of 5 replications, 10 g of soil each.

[4] Means within a column followed by the same letter do not differ significantly according to Student Newman-Keul's test ($p=0.05$).

Table 15. Effect of seed bacterization treatments on populations of total fungi in onion seedling rhizosphere and nonrhizosphere root-zone soils at 30 days after seeding.

Bacterial strains[1]	Populations in rhizopshere soil (cfu/plant)[2]	% Change compared to control	Populations in nonrhizosphere root-zone soil (cfu/g dry wt.of soil)[3]	% Change compared to control
B-2	$1.24 \pm 0.25 \times 10^3$ a[4]	-91.20	$2.34 \pm 0.17 \times 10^4$ a[4]	-86.20
B	$2.04 \pm 0.43 \times 10^3$ a	-85.50	$2.72 \pm 0.27 \times 10^4$ a	-83.90
W	$5.25 \pm 0.48 \times 10^3$ b	-62.80	$6.94 \pm 0.77 \times 10^4$ b	-58.90
UI:1	$4.73 \pm 0.48 \times 10^3$ b	-66.50	$4.71 \pm 0.47 \times 10^4$ b	-72.10
UI:2	$1.95 \pm 0.18 \times 10^3$ a	-86.20	$2.29 \pm 0.05 \times 10^4$ a	-86.00
Control	$1.41 \pm 0.07 \times 10^4$ c	0	$1.69 \pm 0.11 \times 10^5$ c	0

[1] B-2 is an isolate of *Bacillus subtilis* obtained from sclerotia of *Sclerotium cepivorum* and B, W, UI:1 and UI:2 were obtained from the rhizospheres of commercial onions (cv. Autumn Spice). Both sclerotia and onions were from naturally infested muck soil from Cloverdale, B. C.

[2] Mean ($\pm$=S. E) of 5 replications, 5 plants each.

[3] Mean ($\pm$=S. E) of 5 replications, 10 g of soil each.

[4] Means within a column followed by the same letter do not differ significantly according to Student Newman-Keul's test ($p=0.05$).

Reductions in estimated populations of indigenous fungi in the rhizosphere and nonrhizosphere root-zone soil associated with seed bacterization ranged from 63% to 91% in rhizosphere and 59% to 86% in root-zone soil compared to controls. Strains B-2, UI:2 and B were the most effective and strains W and UI:1 the least effective suppressors of indigenous fungal populations in both rhizosphere and root-zone soils.

DISCUSSION

This comparative population study of *B. subtilis* B-2 and four other selected marked rhizobacteria shows that UI:2, UI:1, B, and W each persisted in greater numbers both in onion rhizosphere and nonrhizosphere root-zone soils than did B-2. It also suggests that *B. subtilis* B-2 was preferentially associated with the nonrhizosphere root-zone soil compared with the rhizosphere soil to a greater extent than were the other four bacteria. Results obtained here are consistent with earlier laboratory (Chapter II) and laboratory and field (153) studies indicates that *B. subtilis* B-2 establishes poorly in onion rhizosphere. Among the four other rhizobacteria UI:2 and B were the most persistent in both rhizosphere and nonrhizosphere root-zone soils. Populations of all the bacterial strains evaluated were greater in nonrhizosphere root-zone soil than in rhizosphere soil. At least in the case of the bacteria studied here, those obtained from the test host plant roots under natural conditions were the best rhizosphere and root-zone soil colonizers; UI:2, UI:1, B, and W were obtained from onion roots, whereas B-2 was obtained from sclerotia of *S. cepivorum* (Chapter I).

Onion seedling growth promotion by seed bacterization was not correlated with populations of the various bacterial isolates. B-2, UI:2 and B enhanced seedling growth the most; this effect appears related to their ability to reduce populations of indigenous rhizosphere and nonrhizosphere root-zone soil microflora, rather than to their own relative populations in these environments. These results are consistent with the hypothesis of growth enhancement resulting from indirect effects of the introduced bacteria B-2, UI:2 and B (80, 136). This hypothesis is supported by the observation that B-2, UI:2 and B were all antagonistic to growth of *S. cepivorum* whereas UI:1 and W were not (Fig. 2, Table 11). All bacterial strains suppressed indigenous rhizosphere and root-zone soil microflora, but these effects were clearly greatest for B-2, UI:2 and B against both fungi and bacteria (Tables 14, 15).

Kloepper and Schroth (83) reported that inoculation of potato seed pieces with plant growth promoting rhizobacteria (PGPR) resulted in generally decreased population densities of indigenous Gram-positive bacteria and fungi in the root-zone soil. Suslow (133) observed both a reduction in the total population density of root colonizing fungi and a shift in the density of particular components of the fungal microflora following inoculation of sugar beet seed with PGPR.

Investigations with rhizobacteria suggest that there are a large number of relatively weak plant pathogens that damage roots and reduce plant growth (80, 135, 136). Parasitic and non-parasitic bacteria and fungi colonizing plant roots can cause disease or reduced plant vigor (118, 152). Other workers have speculated that reduction of parasitic and non-parasitic rhizosphere microorganisms by fumigation or chemical seed treatments contributes in part

to the achievement of enhanced plant growth (9, 10, 97, 118). Our studies establish that reductions of indigenous microflora by seed bacterization treatments with B-2, UI:2 and B were associated with increased plant growth.

Seed bacterization treatments with UI:1 and W had no effect on the growth of onion seedlings, though these treatments had significant effect on the reduction of fungal and bacterial population levels in onion rhizosphere and root-zone soils. This is not inconsistent with the interpretation that B-2, UI:2 and B may have enhanced seedling growth via suppression of indigenous microflora since how the phenomenon of growth enhancement is affected by various interacting factors, such as the composition of the qualitative soil microflora, nutrient status of the soil available to the bacteria, and/or environmental stress, is currently unknown. Different rhizobacteria presumably are antagonistic to a different spectrum of rhizosphere microflora. The indirect evidence for antibiotic production in the root-zone soil as measured by plant response and reduction of indigenous microflora supports the claims of other workers that antibiotics may be produced and functionally active in a natural environment. It also supports suggestions that the mode of action of growth promoting bacteria may involve root-zone antagonism (80).

The use of the genetically marked bacterial strains tolerant to antibiotics reported here allowed a precise estimation of the population dynamics of these bacteria in onion rhizosphere and root-zone soils under field conditions. The capacity of *B. substilis* B-2, UI:2, and B to markedly change the indigenous microflora suggests that they have potential to alter the composition of the rhizosphere resulting in increased plant growth. This mechanism could provide a valuable basis for improving crop yield and for

control of various soil-borne root pathogens. I conclude that effort should be focussed on manipulation of the rhizosphere and root-zone soil environment by selective treatments such as seed bacterization to shift the composition of the root-zone microflora to favour onion growth. The possibilities of treating seeds with specific rhizobacteria which are antagonistic to specific soil-borne root pathogens should be explored.

CHAPTER IV

COLONIZATION OF ONION RHIZOSPHERE BY BACTERIA ADDED TO SOIL

INTRODUCTION

It is widely recognized that biological control of soil borne diseases is a distinct possibility for the future and can be successfully exploited in modern agriculture (3, 36, 37, 56-58, 107, 108) especially within the framework of integrated pest management systems (93, 108, 110). Several workers have succeeded in displacing the deleterious components of the indigenous root microflora to obtain biological control of soil-borne diseases (84, 119, 120, 128-130, 149) and increased plant growth (81-83, 138 and Chapter III). The currently predominant view is that colonization of plant roots by bacteria must occur before subsequent effects of the bacteria on promotion or limitation of plant growth can take place (123). A strategy to identify specific bacteria as plant growth promoters or biological control agents must select not only for bacteria that are capable of antagonism against plant pathogens, but also for their ability to effectively colonize roots (134).

While root colonization alone is not predictive of a beneficial plant response, it is a logical starting point from which to select either biological control agents or plant growth promoting bacteria. Past studies on screening of various soil and rhizobacteria for the ability to colonize plant roots have involved introducing bacteria onto seeds, tubers or bulbs, subsequent sampling of the root system for qualitative or quantitative estimation of these bacteria at intervals in plant development (83, 91, 126, 148, 153, Chapter II, Chapter

111). These screenings are both time consuming because the plant development requires considerable time and difficult because of the problem posed by monitoring for a specific organism in a biologically complex enivironment. Therefore, a rapid assay which gives evidence of colonization by introduced bacteria in the rhizosphere would be a valuable tool.

Bennett and Lynch (7) developed a closed test tube technique for assessing bacterial root colonizing ability under gnotobiotic conditions which proved useful for estimating specific microbial interactions in the rhizosphere. However, in sterilized soils, populations of Pseudomonads are enhanced but plant growth promotion may not be realized (83). Therefore it is not possible to extrapolate from results obtained in sterile environments to those expected under field conditions. Scher *et al.* (121) also developed a closed tube assay for measuring the root colonization capacity of bacteria on maize in non-sterilized soil and sand by introduction of bacteria onto seeds. Though seed treatment is an attractive method for introducing bacteria into the soil-plant environment, seed inoculants are frequently ineffective in preventing root disease (85, 90, 97), perhaps because these organisms are either poor root surface colonizers (111, 139, 151) or because they are incapable of mobility within the soil profile (92). Little or no information has been reported regarding movement of specific bacterial strains from the soil and their ability to colonize the growing plant root rhizosphere. Hence, studies were conducted to assess the colonization potential of several selected bacterial strains in onion rhizospheres. The effect of these bacteria in increasing onion seedling growth is discussed in the context of possible mechanisms of growth enhancement.

MATERIALS AND METHODS

Source of bacterial strains

Genetically marked strains of *B. subtilis* B-2 (Chapter I), rhizobacteria: UI:2, UI:1, B and, W (Chapter III), and an additional five isolates of *B. subtilis*: Bact-8, Bact-K, Bact-O, EBW-1 and AB-9 were used in this study. The later five isolates of *B. subtilis* were isolated by Utkhede and Rahe (141) from sclerotia of *S. cepivorum* obtained from various sources: Bact-8, sclerotia from naturally infested muck soils of the Fraser Valley of British Columbia, Canada; Bact-K, sclerotia from mineral soil near Kelowna, B. C., Bact-O, sclerotia from mineral soil near Olympia, WA, USA; EBW-1, sclerotia from J. R. Coley-Smith, University of Hull, UK; AB-9, sclerotia from I. D. Geard, Department of Agriculture, New Town Research Centre, DSIR, Private Bag, Christchurch, New Zealand. These additional isolates were also marked for tolerance to the combination of 300 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl in PDA (S_{300}CB-PDA) in the same manner as described in Chapter I.

Bacterial soil treatments

Fresh muck soil collected from Cloverdale, B. C., from a field in which onions had been grown for the past four years was used. The pH was approximately 4.5. Soil was sifted through a 2 mm sieve and the moisture content was adjusted to 80%. Suspensions of the ten marked bacterial strains in 0.1 M $MgSO_4$ were prepared as described in Chapter I and bacterial counts were estimated with a haemocytometer. Suspensions were adjusted to 2.4×10^{10} cells/ml, and 20 ml of each suspension was added to a seperate 500 g (dry

wt.) quantities of soil and mixed promptly by hand in sterile polyethylene bags, to give an initial population in each case of 9.60×10^8 cfu/g dry wt. of soil. Soil amended with 20 ml of sterile 0.1 M $MgSO_4$ served as control.

Disposable 5.5 cm deep rectangular plastic packs (pots) of approximately 110 cc were filled with 80 g of bacterized or control soil. Each treatment was replicated five times. At zero time (within 24 hr after bacterization) population levels of the marked bacterial strains in soils were estimated by dilution plating as described in Chapter III and reported as cfu/g dry wt. of soil. Five surface sterilized onion seeds (cv. Autumn Spice) were sown in each treatment pot. Seeded treatment pots were arranged on a plant growth bench in a randomized complete block design and incubated at 24-25 C with 14 hr photoperiod. Sterile water was added periodically to the pots on weight basis to replace moisture lost during incubation.

Rhizosphere colonization assay

Onion rhizosphere colonization by the ten marked bacterial strains was assessed by estimating their populations in the rhizosphere at 30 days after seeding. Individual seedlings were removed carefully from the treatment pots and shaken gently to dislodge adhering soil. The tops were removed and root samples were cut into 5 mm segments with a sterile scalpel. The root segments were placed immediately into sterile 250 ml flasks containing 10 ml of sterilized 0.1 M $MgSO_4$ and subjected to the rhizosphere procedure described in Chapter II. Populations of the marked bacterial strains in nonrhizosphere root-zone soil were also estimated. The root-zone soil (without roots) was removed from each treatment pot and mixed in a polyethylene

bag. Subsamples (10 g) were placed in 500 ml flasks containing 100 ml of sterilized 0.1 M $MgSO_4$ and subjected to the nonrhizosphere root-zone soil procedure described in Chapter III. Colony counts were made and populations of the ten marked bacterial strains in the rhizosphere and root-zone soils were recorded as cfu/plant and cfu/g dry wt. of soil respectively.

Effect of soil bacterization on the growth of onion seedlings

Individual onion seedlings were removed from each treatment pot at 30 days after seeding and were shaken to remove excess soil. Height of the shoot was recorded. Roots and shoots were seperated, washed, air dried, and weighed. There were five replications per each bacterial soil treatment and control, one plant per replication.

The data were analyzed by analysis of variance and Student Newman-Keul's test at 5% level of significance.

RESULTS

Rhizosphere colonization assay

Data summarizing the abilities of the ten marked bacterial strains to colonize the rhizospheres of onion seedlings from bacterized soil are shown in Table 16. All of the marked bacterial strains tested were able to colonize the onion seedling rhizospheres, and their populations in the rhizospheres at 30 days after seeding ranged from 9.07×10^2 to 1.30×10^5 cfu/plant. Initial populations of these strains in the soil estimated by dilution plating within 24 hr after bacterization, ranged from 1.18×10^7 to 1.79×10^7 cfu/g dry wt. of soil (average 1.63×10^7 cfu/g dry wt. of soil). Populations in the nonrhizosphere root-zone

soil at 30 days following inoculation averaged 1.65×10^6 cfu/g dry wt. of soil. Marked bacteria were not detected in control treatments from either rhizosphere or nonrhizosphere root-zone soil at any of the times of analysis. Among the ten marked bacterial strains, B and AB-9 in the rhizosphere, and B, UI:2 and W in the nonrhizosphere root-zone soil maintained the highest populations. In contrast B-2, Bact-K, Bact-8 and Bact-0 in the rhizosphere and B-2, EBW-1, Bact-0 in the nonrhizosphere root-zone soil were recovered in lower numbers.

Effect of bacterial soil treatments on the growth of onion seedlings

Soil treatments with the marked bacterial strains had no significant influence on height and dry weight of shoots of onion seedlings compared to controls at 30 days following inoculation (Table 17). However, a significant increase in root dry weight occurred in the AB-9, Bact-K, and B bacterization treatments (Table 17). These increases ranged from 36% to 42% over control.

DISCUSSION

The technique reported here permits simple and rapid comparison of the rhizosphere colonizing abilities of bacterial strains on onions. Under the present experimental conditions all of the marked bacterial strains added to the non-sterilized muck soil were recovered from onion seedling rhizospheres, but at markedly different levels. It is difficult to define what constitutes a rhizosphere 'colonizer' because the experimental definition of 'rhizosphere' does not totally exclude nonrhizosphere root-zone soil. Nevertheless, marked strains of B and AB-9, UI:2, W and UI;1 occurred in comparatively high populations

Table 16. Populations of marked bacterial strains in onion rhizosphere and nonrhizosphere root-zone soils 30 days after addition of bacteria to soil at an initial concentration of 9.60×10^8 cfu/g dry weight of soil.

Bacterial strains[1]	Populations in rhizosphere soil (cfu/plant)[2]	Populations in nonrhizosphere root-zone soil (cfu/g dry wt. of soil)[3]	
	30 days	0 day	30 days
B-2	$9.07 \pm 1.12 \times 10^2$ a[4]	$1.18 \pm 0.04 \times 10^7$ a[4]	$4.31 \pm 0.35 \times 10^5$ c[4]
AB-9	$5.77 \pm 0.23 \times 10^4$ f	$1.67 \pm 0.08 \times 10^7$ a	$1.21 \pm 0.07 \times 10^6$ e
EBW-1	$6.76 \pm 0.27 \times 10^3$ d	$1.72 \pm 0.01 \times 10^7$ a	$2.63 \pm 0.26 \times 10^5$ a
Bact-K	$9.60 \pm 1.42 \times 10^2$ a	$1.78 \pm 0.04 \times 10^7$ a	$8.98 \pm 0.74 \times 10^5$ d
Bact-8	$1.97 \pm 0.22 \times 10^3$ b	$1.77 \pm 0.02 \times 10^7$ a	$5.12 \pm 0.41 \times 10^5$ c
Bact-0	$2.65 \pm 0.19 \times 10^3$ c	$1.54 \pm 0.05 \times 10^7$ a	$3.40 \pm 0.25 \times 10^5$ b
B	$1.29 \pm 0.01 \times 10^5$ g	$1.77 \pm 0.11 \times 10^7$ a	$6.14 \pm 0.34 \times 10^6$ h
UI:1	$1.84 \pm 0.17 \times 10^4$ e	$1.32 \pm 0.09 \times 10^7$ a	$1.36 \pm 0.03 \times 10^6$ e
UI:2	$2.54 \pm 0.26 \times 10^4$ e	$1.72 \pm 0.06 \times 10^7$ a	$2.91 \pm 0.05 \times 10^6$ g
W	$2.43 \pm 0.18 \times 10^4$ e	$1.79 \pm 0.02 \times 10^7$ a	$2.39 \pm 0.20 \times 10^6$ f
Control	0	0	0

[1] B-2, AB-9, EBW-1, Bact-K, Bact-8, Bact-0 are strains of *Bacillus subtilis*. B-2 and Bact-8 were isolated from sclerotia of *Sclerotium cepivorum* recovered from naturally infested muck soils from near Cloverdale, B. C; AB-9 is from sclerotia obtained from New Zealand (I. D. Geard); Bact-K is from sclerotia recovered from a mineral soil near Kelowna, B.C., Bact-0 from sclerotia recovered from mineral soil near Olympia, WA., EBW-1 from UK (Coley-Smith); strains B, UI:1, UI:2, and W were from onion roots grown in muck soil near Cloverdale, B. C.

[2] Mean ($\pm$=S. E) of 5 replications, one plant each.

[3] Mean ($\pm$=S. E) of 5 replications, 10 g soil each.

[4] Non-zero means within a column followed by the same letter do not differ significantly according to Student Newman-Keul's test ($p=0.05$).

Table 17. Effect of bacterial soil treatments on shoot height, shoot and root dry weight of onion seedlings.

Bacterial strains[1]	Shoot height (cm)[2]	% Increase compared to control	Shoot dry weight (mg)[2]	% Increase compared to control	Root dry weight (mg)[2]	% Increase control control
B-2	8.86 ± 0.37 bc[3]	25.50	6.68 ± 0.67 a[3]	24.20	0.76 ± 0.04 bc[3]	22.60
AB-9	9.08 ± 0.35 c	28.60	6.92 ± 0.49 a	28.60	0.88 ± 0.07 c	42.00
EBW-1	7.54 ± 0.36 abc	6.80	5.66 ± 0.44 a	5.20	0.56 ± 0.05 a	-9.70
Bact-K	8.08 ± 0.69 abc	18.10	7.10 ± 0.23 a	32.00	0.86 ± 0.03 c	38.70
Bact-8	8.70 ± 0.72 bc	27.20	5.86 ± 0.43 a	8.90	0.76 ± 0.05 bc	22.60
Bact-0	7.34 ± 0.36 abc	4.00	5.58 ± 0.51 a	3.80	0.62 ± 0.04 ab	0
B	8.56 ± 0.65 bc	25.20	6.74 ± 0.18 a	25.20	0.84 ± 0.03 c	35.50
UI:1	6.70 ± 0.36 ab	-2.10	5.28 ± 0.29 a	-1.90	0.64 ± 0.02 ab	3.20
UI:2	8.90 ± 0.32 bc	30.10	6.62 ± 0.26 a	23.10	0.74 ± 0.05 abc	19.40
W	6.10 ± 0.42 a	-10.80	5.38 ± 0.38 a	0	0.62 ± 0.01 ab	0
Control	7.06 ± 0.42 abc	0	5.38 ± 0.16 a	0	0.62 ± 0.02 ab	0

[1] B-2, AB-9, EBW-1, Bact-K, Bact-8, Bact-0 are strains of *Bacillus subtilis*; B-2 and Bact-8 were isolated from sclerotia of *Sclerotium cepivorum* recovered from naturally infested muck soils from Cloverdale, B. C., AB-9 is from sclerotia obtained from New Zealand (I. D. Geard); Bact-K is from sclerotia recovered from a mineral soil near Kelowna, B.C., Bact-0 from sclerotia recovered from mineral soil near Olympia, WA., EBW-1 from UK (Coley-Smith) strains B, UI:1, UI:2 and W were from onion roots grown in muck soil near Cloverdale, B. C.

[2] Mean (± =S. E) of 5 replications, one plant each.

[3] Means within a column followed by the same letter do not differ significantly according to Student Newman-Keul's test (p=0.05).

in the rhizospheres of onion seedlings 30 days after bacterization of the muck soil growth medium. More importantly, strains AB-9 and EBW-1 showed disproportionately higher populations in seedling rhizospheres as compared to nonrhizosphere root-zone soil. The relative proportional distribution of bacteria between soil and seedling rhizospheres following bacterization may be a good indicator of relative rhizosphere colonizing ability for different bacteria. Although experimental estimation of these ratios requires analyses of bacterial populations in both rhizosphere and root-zone soils, the resulting data provide a more comprehensive picture of the comparative behavior of different bacteria in the plant rhizosphere/root-zone soil environment.

Although the bacterial strains showed varying abilities to colonize seedling rhizospheres, none of them influenced shoot height or shoot dry weight of onion seedlings. In contrast, strains Bact-K, AB-9 and B significantly enhanced root dry weight. These results are not in conflict with those reported in Chapter III, where B-2, UI:2 and B enhanced shoot height and dry weight. Although enhancement of these parameters was not significant at the 95% confidence level, the relative effects of these three strains was consistent with their performance in the earlier experiments.

The biotic status of the soil markedly affects the colonization of microorganisms on roots. The advantage of this system over sterile conditions is that the ability of a bacterial strain to compete in soil with resident microflora, as well as its ability to persist in root-zone soil is required for successful rhizosphere colonization. Bowen (9, 10) and Scher *et al.* (121) stated that the behaviour of bacteria in the soil root-ecosystem can be fully understood only when non-sterilized field soils are used for rhizosphere

studies. *Enterobacter cloacae* moved from treated seeds onto the entire surface of roots growing in sterile soil or in soil free moist chambers (24). However, if seeds treated with *E. cloacae* were planted in non-sterilized soil, only the upper one-fourth of the root was colonized by *E. cloacae*. That conclusions from a sterile soil colonization test may not be extended to greenhouse or field tests is also apparent from the observation of James *et al.* (71) that two bacteria, strain WD-13 and a *Serratia* strain, were good colonizers under sterile conditions but were not effective colonizers in non-sterilized field soil.

Rovira (114) reported that many bacterial strains colonizing roots of oats and tomatoes were Gram-negative. Scher *et al.* (121) also found a lack of maize root colonization by Gram-positive strains both in non-sterilized and sterilized soil. In my studies only two rhizobacteria, B and UI:1, are Gram-negative; all other strains were Gram-positive. Several of the Gram-positive isolates proved to be rhizosphere colonizers, which contradicts the generalizations of Rovira (114) and Scher *et al.* (121). The colonization measured in my assay is primarily at 30 days following inoculation, which may not predict the long term colonization and survival potential under field conditions. Nevertheless, for successfull colonization and survival during the growth of onion seedlings, a bacterial strain must colonize the root system soon after seed germination. Population studies with rhizobacteria on sugar beets (137), *Pseudomonas* on potato (84), and fluorescent Pseudomonadas on wheat (148, 149) showed that bacterial strains which colonized roots immediately after plant emergence continued to colonize the developing root system throughout the season. Therefore I anticipate that many of the strains which are shown to be rhizosphere colonizers under my test conditions would

likely be successful onion rhizosphere colonizers under field conditions. The experimental rhizosphere colonization assay presented here appears to be a promising tool for studying population dynamics of the microflora in the root-soil interface and for the development of plant growth promoting and/or biocontrol agents.

CHAPTER V

EFFECT OF ONION SEED BACTERIZATION ON THE GERMINATION OF SCLEROTIA OF *SCLEROTIUM CEPIVORUM* IN MUCK SOIL

INTRODUCTION

Sclerotia of *S. cepivorum* Berk., the causal agent of white rot of *Allium* spp., are held in a dormant condition by fungistasis in non-sterile soil (2, 35, 77) and are specifically stimulated to germinate by active compounds associated with aqueous extracts and exudates of plants of the genus *Allium* (26, 33). The active components include various volatile sulfur compounds possessing a divalent sulfur covalently bonded to an alkyl group containing 3-5 carbons (68). Schwimmer and Friedman (125) suggested that these compounds are primarily produced by cellular disruption; the active volatile sulfur compounds are released when substrates such as propenyl cysteine sulfoxide interact with onion enzymes. Coley-Smith and King (34) hypothesize that sulfoxide compounds are exuded by intact roots into the surrounding soil (root ecosystem), where they are metabolized to sulfides and other volatile compounds by soil bacteria. Experimental evidence reported by Ikeshoji (67) showed that rhizosphere bacteria were actively involved in the release of precursors from root cells and that these were metabolized into volatile compounds mostly by bacteria. He suggested that rhizosphere bacteria cause mechanical injury to root epithelium, which releases alliinase to generate propyl thio-compounds.

Soil microflora, including *Bacillus* spp., have been shown to metabolize non-volatile alkyl and alkenyl cysteine sulfoxides to volatile sulfur containing compounds which activate dormant sclerotia (28, 34, 77). Murakami (102) found that *B. subtilis* possesses an enzyme which catalyses S-methyl cysteine sulphoxide. Bacteria such as intestinal coli-types and *Psuedomonas cruceviae* were involved in conversion of cysteine sulphoxides into disulphides and methyl thiosulphinate, respectively (104, 146).

In view of these considerations, experiments were carried out to investigate the effect of diallyl disulphide, bacterized onion seedlings with the marked strains of *Bacillus subtilis* B-2, four selected onion rhizobacteria and non-bacterized onion seedlings on the germination of sclerotia of *Sclerotium cepivorum* and on the total bacterial and fungal populations associated with sclerotia under controlled conditions.

MATERIALS AND METHODS

Source of sclerotia and bacterial isolates

Sclerota of *S. cepivorum* was collected in October 1983 from naturally infected onions grown in muck soil at Cloverdale, British Columbia. Sclerotia were kept in muck soil at 17 C in a polyethylene bag until needed. Antibiotic-tolerant strains of *B. subtilis* B-2 (Chapter I) and four rhizobacteria: UI:2, UI:1, B and W (Chapter III) were used.

Bacterized and non-bacterized onion seed treatments

Seeds of onion cv. Autumn Spice were surface sterilized in 0.1% $HgCl_2$ solution for 5 min, rinsed briefly in 70% ethanol and washed with sterile distilled water four times. For each bacterium, fresh bacterial culture previously maintained on S_{300}CB-PDA was grown in 500 ml flasks containing 250 ml of sterilized TSB incubated on a continuously reciprocating shaker at 24-25 C for 8-10 hr. The bacterial pellet was prepared as described in Chapter I and suspended in sterile 0.1 M $MgSO_4$. Surface sterilized onion seeds were placed in the bacterial suspension for 10 min, then air dried for 1-2 hr. At zero time five samples of 5 bacterized onion seeds each were transferred into a 250 ml flask containing 10 ml of sterile 0.1 M $MgSO_4$ and washed for 10 min by magnetic stirring. Colony counts were made as described in Chapter II. Onion seeds surface sterilized as above and treated with sterile 0.1 M $MgSO_4$ solution served as non-bacterized treatments.

Soil treatments

Freshly collected muck soil from an onion field in Cloverdale, B. C., with pH of approximately 4.5 was used for all experiments. The moisture content was adjusted to 70% and maintained at this level throughout the experiment. Sterilization of the moist soil was done as described in Chapter I.

Diallyl disulphide (DADS) (ICN Pharmaceuticals, Inc., Life Sciences Group, Plain View, NY) was added to sterilized or non-sterilized muck soil at the rate of 10 ul/40 cc soil and mixed thoroughly. Sterilized or non-sterilized muck soil, without further treatment was used as control.

Sclerotial germination bioassay

Bioassays evaluating the effects of bacterized and non-bacterized onion seedlings and DADS on germination of sclerotia of *S. cepivorum* were carried out in a well-ventilated controlled environment chamber at 17 C under fluorescent light with 14 hr photoperiod. The various treatments were contained in disposable rectangular plastic paks (pots) 5.5 cm deep of approximately 110 cc capacity; these in turn were housed in wide mouth rectangular glass 'cookie' jars (approximately 13 x 13 x 20 cm; 3.6 l capacity) covered with four layers of cheeshcloth.

Each pot contained approximately 75 cc of the appropriate soil treatment and twenty sclerotia of *S. cepivorum*. Sclerotia were placed either 3 cm below the soil surface in a small nylon mesh bag (buried treatment) or arranged in a 4 x 5 grid pattern on a 1 cm dia nylon mesh disc on the soil (surface treatment). Twenty onion seeds were sown in each pot receiving the bacterized or non-bacterized seedling treatments; additional pots with the various soil treatments and sclerotia served as unseeded controls. Three pots for each treatment were placed in individual cookie jars, and there were four replicate cookie jars for each treatment in all experiments. Sterile water to replace moisture lost during incubation was added periodically on a weight basis to the pots in each jar.

Three 21 day bioassays were carried out. A preliminary experiment revealed that there was no difference between 19 and 29 days in the % germination of sclerotia of *S. cepivorum*. Therefore germination bioassay was terminated at 21 days. Bioassay 1 was started on 20 January 1985 and

compared DADS, bacterized (*B. subtilis* B-2) (BT) and non-bacterized onion seedlings (NBT) and control (C) treatments on the germination of sclerotia of *S. cepivorum* placed on the surface of sterilized and non-sterilized soil. Bioassay 2 was started on 8 July, and evaluated the same treatments as in bioassay 1 plus a fifth treatment consisting of two each of BT and C pots together in the same cookie jar (BT/C; C/BT), and all treatments were evaluated against sclerotia both on the surface and buried. Bioassay 3 was begun on 8 October and compared C, DADS, NBT seedlings, and five different marked bacterial seed treatments (*B. subtilis* B-2, B, UI:1, UI:2, and W).

Each treatment was replicated four times in each bioassy, and the treatment cookie jars were arranged in a randomized complete block design within the growth chamber. One pot was removed from each replicate jar at each sampling period. There were three sampling times in bioassays 1 and 2 (7, 14, and 21 days), and two in bioassay 3 (14 and 21 days).

Effect of DADS, and bacterized and non-bacterized onion seedlings on sclerotial germination

At each sampling period one nylon mesh disc and one nylon mesh bag (in the case of bioassays involving buried sclerotia) were removed aseptically from one pot from each replicate jar. Sclerotial germination was scored using a dissecting microscope. The data were analyzed by stepwise regression (48) using GLIM (4) after transforming the data for sclerotial germination to log odds {log(P/1-P) where P=proportion of sclerotial germination)} (59). The best fit linear regressions for the various treatments in the log odds scale (predicted) were transformed back to the proportion scale and plotted against

time.

At each sampling period sclerotia recovered as germinated were plated onto PDA (4 sclerotia per plate) amended with 1000 ug/ml chloromphenicol (CP) (Sigma Chemical Company), and 1000 ug/ml streptomycin (S) (CPS-PDA) for the confirmation of germination. Plates were incubated in plastic bags at 17 C for 4-15 days and colonies typical of *S. cepivorum* were recorded. Non-germinated sclerotia from the circular nylon mesh discs and bags were surface sterilized in 0.1% NaOCl for 5 min, washed with six changes of sterile distilled water, and plated onto PDA (4-5 sclerotia/plate) and incubated 6-15 days to determine viability. The data were subjected to a stepwise regression analysis (59).

Effect of DADS, and bacterized and non-bacterized onion seedlings on total bacterial and fungal populations associated with surface and buried sclerotia

Bacterial and fungal populations associated with surface and buried sclerotia in bioassay 3 were estimated using a dilution plate technique and two selective media. Bacteria were isolated on Thornton's agar (TA) (73) and fungi on rose bengal agar (RBA) (73). Three to five non-germinated sclerotia from each treatment at each sampling period including zero time were rinsed in distilled water and placed into 1 ml test tubes each containing 0.5 ml of sterile distilled water. The sclerotia in a tube were crushed with a sterile glass rod and the resulting suspension was added to a test tube containing 9.5 ml of sterile distilled water. Serial dilutions were made and 0.5 ml aliquots from appropriate dilutions were spread onto triplicate RBA plates, and 0.1 ml aliquots onto triplicate TA plates. The plates were incubated at 24-25

C. Bacterial and fungal colonies were counted after 4 and 7 days incubation, respectively. The populations of bacterial and fungal colonies obtained from both surface and buried sclerotia were estimated and recorded as ln cfu/sclerotium at 0, 14 and 21 days. The data were analyzed by stepwise regression (59).

Estimation of genetically marked bacterial populations in onion rhizosphere and nonrhizosphere root-zone soil

Populations of the marked bacterial strains in the rhizosphere and nonrhizosphere root-zone soil following bacterized and non-bacterized seeding were estimated as described in Chapter III at 0, 7, 14 and 21 days in bioassay 1 and 2, and at 0, 14, and 21 days in bioassay 3. Samples consisted of five plants per replication, and there were four replications per treatment. Colony counts were made and populations of the marked bacterial strains in the rhizosphere and nonrhizosphere root-zone soil were recorded as cfu/plant and cfu/g dry wt. of soil respectively. The data were analysed by analysis of variance and Newman-Keul's test at 5% level of significance.

RESULTS

Effect of DADS, and bacterized and non-bacterized onion seedlings on sclerotial germination

Curves derived from logistic linear regressions showing the effects of DADS, and bacterized and non-bacterized onion seedlings on the proportions of sclerotia germinating in bioassays 1, 2 and 3 are shown in Figs. 8, 9 and 10 respectively. The significance levels of the various factors and interactions evaluated in the stepwise regression analyses, and the components of the final models used to present the data shown in Figs. 8, 9 and 10 are listed in Tables 18, 19 and 20 respectively. There were no significant differences for a given treatment between sterilized and non-sterilized soil. Therefore, pooled data for sterilized and non-sterilized soils were are represented in Figs. 8, 9 and 10.

Both DADS and seedlings grown from bacterized onion seeds enhanced the germination of surface and buried sclerotia significantly (P=0.05) over controls in all bioassays. Onion seedlings grown from non-bacterized surface sterilized seeds did not enhance the germination of either surface or buried sclerotia of *S. cepivorum* over that observed in control pots. The level of enhancement was greater for sclerotia on the soil surface than for buried sclerotia. Practically all sclerotial germination observed in the three bioassays was of the mycelial type; "plug" or "eruptive" germination was rarely seen. Of 477, 906, and 1420 sclerotia scored as germinated under the dissecting microscope in bioassays 1, 2, and 3 respectively and plated onto CPS-PDA, 99% yielded colonies typical of *S. cepivorum*.

No significant differences were found in the proportions of viable sclerotia in the non-germinated populations of *S. cepivorum* among the various treatments and controls. Also no differences in the viability of non-germinated sclerotia were observed among sclerotia placed on the surface of the soil or buried, whether in sterilized or in non-sterilized soil. The mean proportions of viable sclerotia in non-germinated populations of sclerotia of *S. cepivorum* for all treatments in bioassays 1, 2 and 3 were 0.94, 0.88 and 0.87, respectively.

In bioassay 2, germination of sclerotia in control pots was enhanced significantly when control pots were incubated in the same cookie jar with other pots containing onion seedlings bacterized with *B. subtilis* B-2 (Fig. 9). In bioassay 3, the various bacteria differed in their ability to induce germination of sclerotia of *S. cepivorum*; bacterial strains B-2, UI:2 and B caused significantly (P=0.05) higher levels of germination than did strains UI:1 and W when these strains were introduced via seed bacterization.

Effect of DADS, and bacterized and non-bacterized onion seedlings on populations of total bacteria and fungi associated with surface and buried sclerotia

Regression curves showing the effect of DADS, bacterized and non-bacterized onion seedlings on populations of total bacteria and fungi associated with surface and buried sclerotia in bioassay 3 are shown in Figs. 11 and 12. The significance levels of the various factors and interactions evaluated in the backward elimination procedure used for analyses of the data, and the components of the final models used to present the data shown in

Figs. 11 and 12 are listed in Tables 21 and 22, respectively. Larger numbers of bacteria and fungi were associated with sclerotia on the soil surface than with buried sclerotia in both sterilized and non-sterilized soil irrespective of the treatments. Higher numbers of bacteria and fungi were associated with sclerotia in sterilzed soil than in non-sterilized soil, irrespective of treatments on placement of sclerotia. There were significant treatment differences in the numbers of bacteria and fungi isolated from sclerotia. The effects of treatments on bacteria and fungi associated with sclerotia were similar in sterilized and non-sterilized soil.

Overall, the level of reduction of bacterial populations associated with surface or buried sclerotia was significantly greatest by DADS, B-2, UI:2 and B, intermediate by UI:1 and W, and least by NBT and C, both in sterilized and non-sterilized soils. There were no significant differences within DADS, B-2, UI:2 and B; between UI:1 and W; nor between NBT and C. Irrespective of the placement of sclerotia and soil, the overall level of reduction of fungal populations associated with sclerotia was significantly highest by B-2 and UI:2, intermediate by B, and least by UI:1, W, NBT and C. Fungal populations were significantly increased by DADS. There were no significant differences between UI:2 and B-2, and among UI:1, W, NBT and C.

Correlation between the proportion of sclerotial germination and populations of total bacteria or fungi associated with sclerotia

Plots of the estimated sclerotial mycosphere populations of fungi and bacteria against the proportions of sclerotia germinating in the various seedling treatments evaluated in bioassay 3 are shown in Fig. 13.

Table 18. Effects of various factors and interactions evaluated by forward elimination in the derivation of the regression model for effects of diallyl disulphide, bacterized and non-bacterized onion seedlings on the germination of sclerotia of *Sclerotium cepivorum* in muck soil (bioassay 1).

#	Tested against	Regression model	Variable added	Log likelyhood	Improvement Chi-square	D.F	P-value
0	-	EMPTY	-	789.50	-	-	-
1	0	TI	TI	181.20	608.30	1	<0.0001
2	1	TI+TIxTRT[1]	TIxTRT	65.63	115.57	3	<0.0001
3	2	TI+TIxTRT+TIxS	TIxS	64.99	0.64	1	0.424
4	2	TI+TIxTRT+TIxSxR	TIxSxTRT	64.68	0.31	3	0.958

[1]Final model.
(TI=time, TRT=treatment, S=soil, R=reps).

Figure 8. Effect of diallyl disulphide, and bacterized and non-bacterized onion seedlings on the germination of sclerotia of *Sclerotium cepivorum* (bioassay 1).

BT=onion seed bacterization with *Bacillus subtilis* B-2 marker
NBT=non-bacterized onion seedlings
DADS=soil treated with 10 ug/40 cc of soil with diallyl disulphide
C=control (soil only)

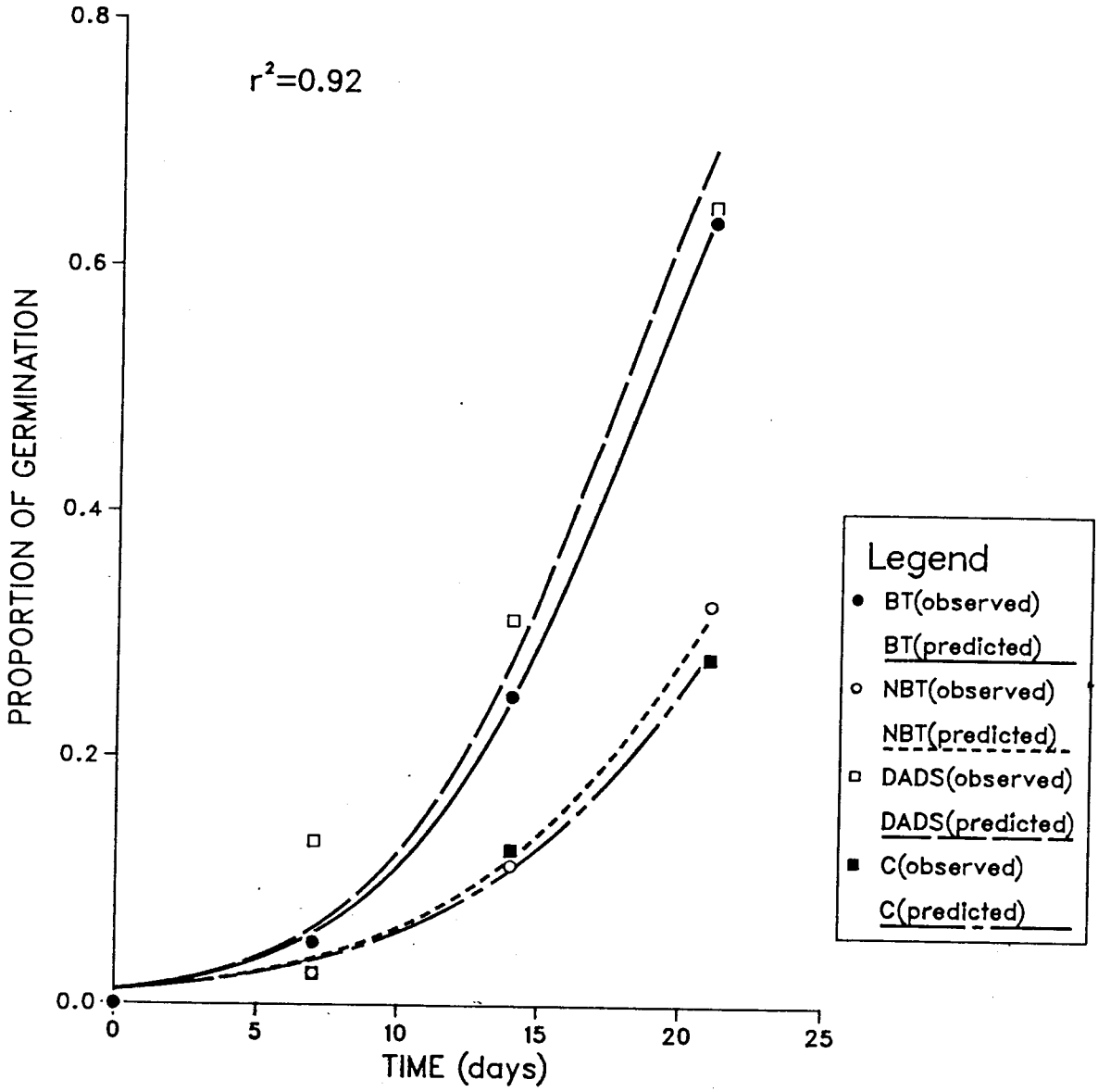

r^2=0.92
PROPORTION OF GERMINATION
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
TIME (days)
Legend
BT(observed)
BT(predicted)
NBT(observed)
NBT(predicted)
DADS(observed)
DADS(predicted)
C(observed)
C(predicted)

Table 19. Effects of various factors and interactions evaluated by forward elimination in the derivation of the regression model for effects of diallyl disulphide, bacterized and non-bacterized onion seedlings on the germination of sclerotia of *Sclerotium cepivorum* in muck soil (bioassay 2).

#	Tested against	Regression model	Variable added	Log likelyhood	Improvement Chi-square	D.F	P-Value
0	-	EMPTY	-	1671.0	-	-	-
1	0	TI	TI	677.4	993.6	1	<0.0001
2	1	TI+TIxTRT	TIxTRT	309.3	368.1	5	<0.0001
3	2	TI+TIxTRT+TIxP[1]	TIxP	239.0	70.3	1	<0.0001
4	3	TI+TIxTRT+TIxP+TIxTRTxP	TIxTRTxP	229.9	9.1	5	0.105
5	3	TI+TIxTRT+TIxP+TIxS	TIxS	238.7	0.3	1	0.584
6	3	TI+TIxTRT+TIxP+R	R	229.4	0.5	2	0.779

[1]Final model.
(TI=time, TRT=treatment, P=position, S=soil, R=reps).

Figure 9. Effect of diallyl disulphide, and bacterized and non-bacterized onion seedlings on the germination of (a) suface and (b) buried sclerotia of *Sclerotium cepivorum* (bioassay 2).

BT=onion seed bacterization with *Bacillus subtilis* B-2 marker
NBT=non-bacterized onion seedlings
DADS=soil treated with 10 ug/40 cc of soil with diallyl disulphide
C=control (soil only)
BT/C (data from BT), C/BT(data from C)=two each of BT and C pots placed together in the same cookie jar

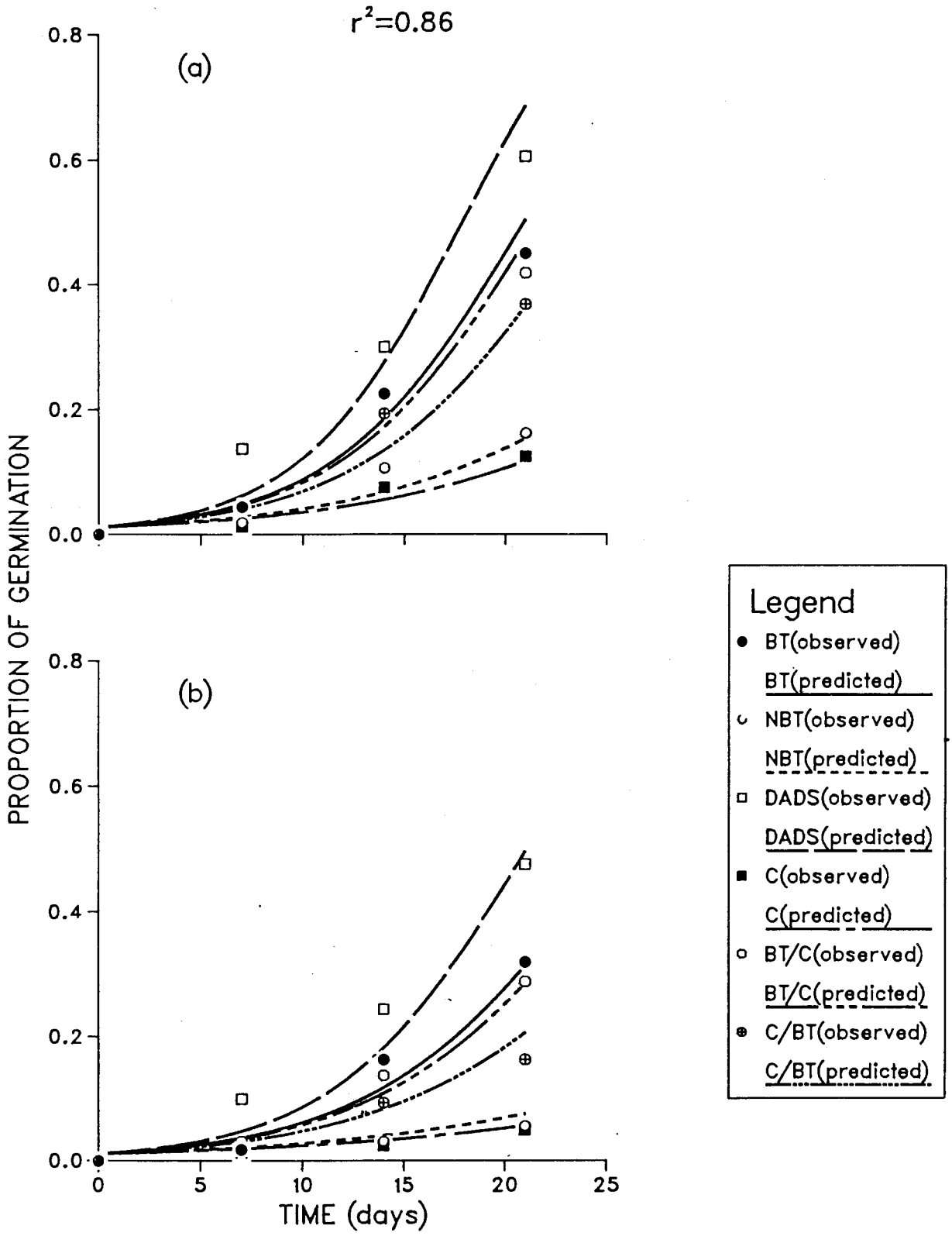

r²=0.86
(a)
(b)
PROPORTION OF GERMINATION
TIME (days)
0.0
0.2
0.4
0.6
0.8
0
5
10
15
20
25
Legend
BT(observed)
BT(predicted)
NBT(observed)
NBT(predicted)
DADS(observed)
DADS(predicted)
C(observed)
C(predicted)
BT/C(observed)
BT/C(predicted)
C/BT(observed)
C/BT(predicted)

Table 20. Effects of various factors and interactions evaluated by forward elimination in the derivation of the regression model for effects of diallyl disulphide, bacterized and non-bacterized onion seedlings on the germination of sclerotia of *Sclerotium cepivorum* in muck soil (bioassay 3).

#	Tested against	Regression model	Variable added	Log likelyhood	Improvement Chi-square	D.F	P-value
0	-	EMPTY	-	1940.0	-	-	-
1	0	TI	TI	521.8	1418.2	1	<0.0001
2	1	TI+TIxTRT	TIxTRT	266.9	254.9	7	<0.0001
3	2	TI+TIxTRT+TIxP[1]	TIxP	209.4	57.5	1	<0.0001
4	3	TI+TIxTRT+TIxP+TIxTRTxP	TIxTRTxP	208.8	0.6	7	0.999
5	3	TI+TIxTRT+TIxP+TIxS	TIxS	208.8	0.6	1	0.439
6	3	TI+TIxTRT+TIxP+R	R	208.7	0.7	3	0.873

[1]Final model.
(TI=time, TRT=treatment, P=position, S=soil, R=reps).

Figure 10. Effect of diallyl disulphide, and bacterized and non-bacterized onion seedlings on the germination of (a) surface and (b) buried sclerotia of *Sclerotium cepivorum* (bioassay 3).

B-2, UI:2, B, UI:1 and W are marked bacterial strains used for onion seed bacterization
NBT=non-bacterized onion seedlings
DADS=soil treated with 10 ug/40 cc of soil with diallyl disulphide
C=control (soil only)

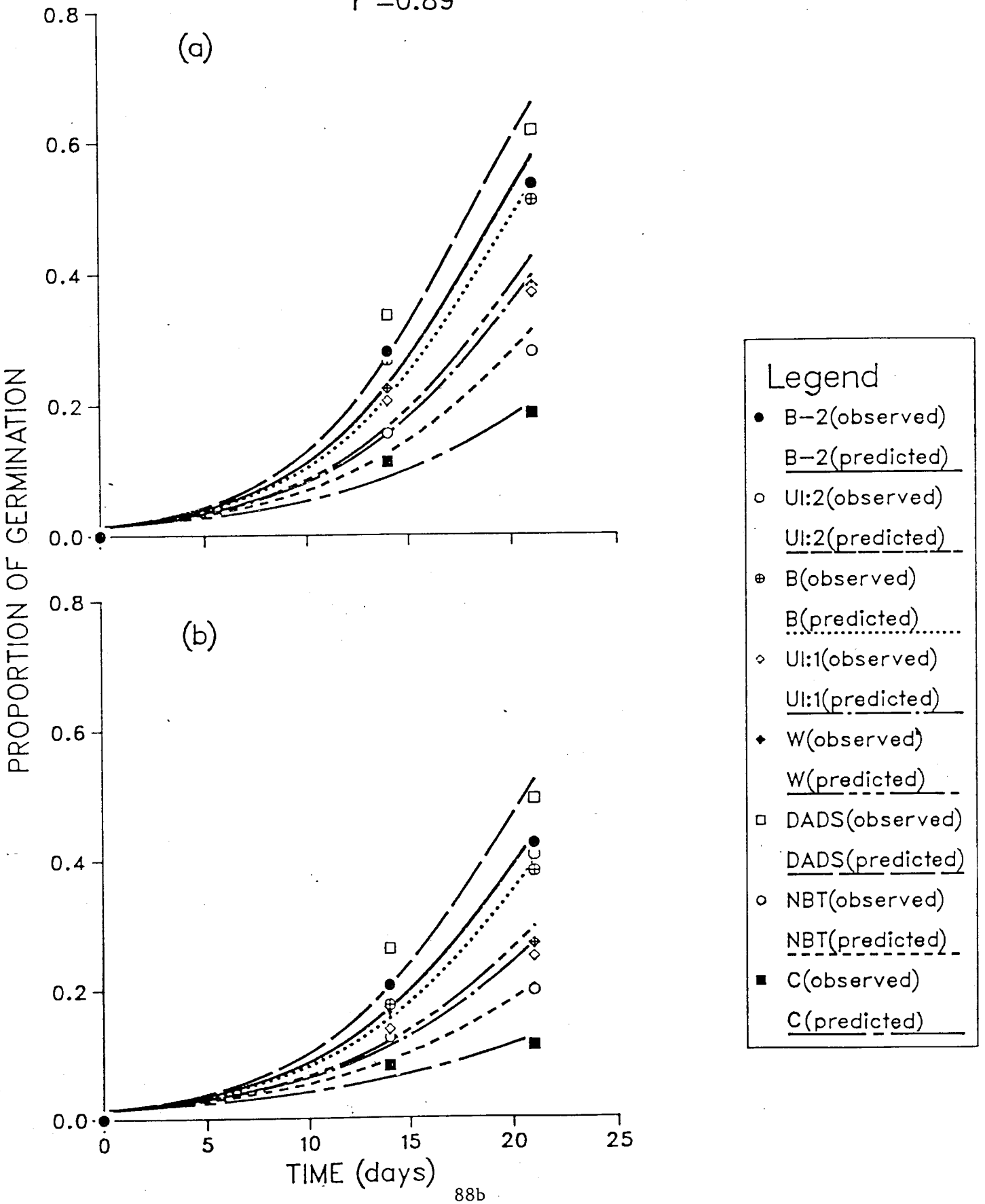

r^2=0.89
(a)
(b)
PROPORTION OF GERMINATION
TIME (days)
0.0
0.2
0.4
0.6
0.8
0
5
10
15
20
25
Legend
B−2(observed)
B−2(predicted)
UI:2(observed)
UI:2(predicted)
B(observed)
B(predicted)
UI:1(observed)
UI:1(predicted)
W(observed)
W(predicted)
DADS(observed)
DADS(predicted)
NBT(observed)
NBT(predicted)
C(observed)
C(predicted)

Table 21. Effects of various factors and interactions evaluated by backward elimination in the derivation of the regression model for effects of diallyl disulphide, bacterized and non-bacterized seedlings on total bacterial populations associated with sclerotia of *Sclerotium cepivorum* in bioassay 3.

#	Tested against	Regression model	Variable removed	D.F	SS(error)	F-Value	P-Value
1	-	S+P+T+R+SxP+SxT+PxT+SxPxT+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI+SxPxTxTI	-	317	4.926	-	-
2	1	S+P+T+R+SxP+SxT+PxT+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI+SxPxTxTI	SxPxT	324	4.927	0.009	1.000
3	2	S+P+T+R+SxP+SxT+PxT+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	SxPxTxTI	331	5.069	1.334	0.233
4	3	S+P+T+R+SxT+PxT+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	SxP	332	5.070	0.065	0.799
5	4	S+P+T+R+SxT+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	PxT	339	5.074	0.037	0.999
6	5	S+P+T+R+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	SxT	346	5.082	0.076	0.999
7	6	S+T+R+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	P	347	5.085	0.204	0.652
8	7	T+R+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	S	348	5.090	0.341	0.559
9	8	T+R+SxTI+TI+PxTI+TxTI+SxTxTI+PxTxTI	SxPxTI	349	5.122	2.188	0.140
10	9	R+SxTI+TI+PxTI+TxTI+SxTxTI+PxTxTI	T	356	5.297	1.703	0.107
11	10	SxTI+TI+PxTI+TxTI+SxTxTI+PxTxTI[1]	R	359	5.317	0.448	0.719
12	11	SxTI+TI+PxTI+TxTI+SxTxTI	PxTxTI	366	5.892	5.546	<0.0001
13	11	SxTI+TI+PxTI+TxTI	SxTxTI	366	6.141	7.948	<0.0001

[1]Final model.
(S=soil, P=position, T=treatments, R=reps, TI=time).

Figure 11. Effect of diallyl disulphide, and bacterized and non-bacterized onion seedlings on total bacterial populations associated with sclerotia of *Sclerotium cepivorum* in bioassay 3. (a) surface sclerotia in sterilized soil, (b) buried scleotia in sterilized soil, (c) surface sclerotia in non-sterilized soil, and (d) buried sclerotia in non-sterilized soil.

B-2, UI:2, B, UI:1 amd W are the marked bacterial strains used for onion seed bacterization
NBT=non-bacterized onion seedlings
DADS=soil treated with 10 ug/40 cc of soil with diallyl disulphide
C=control (soil only)

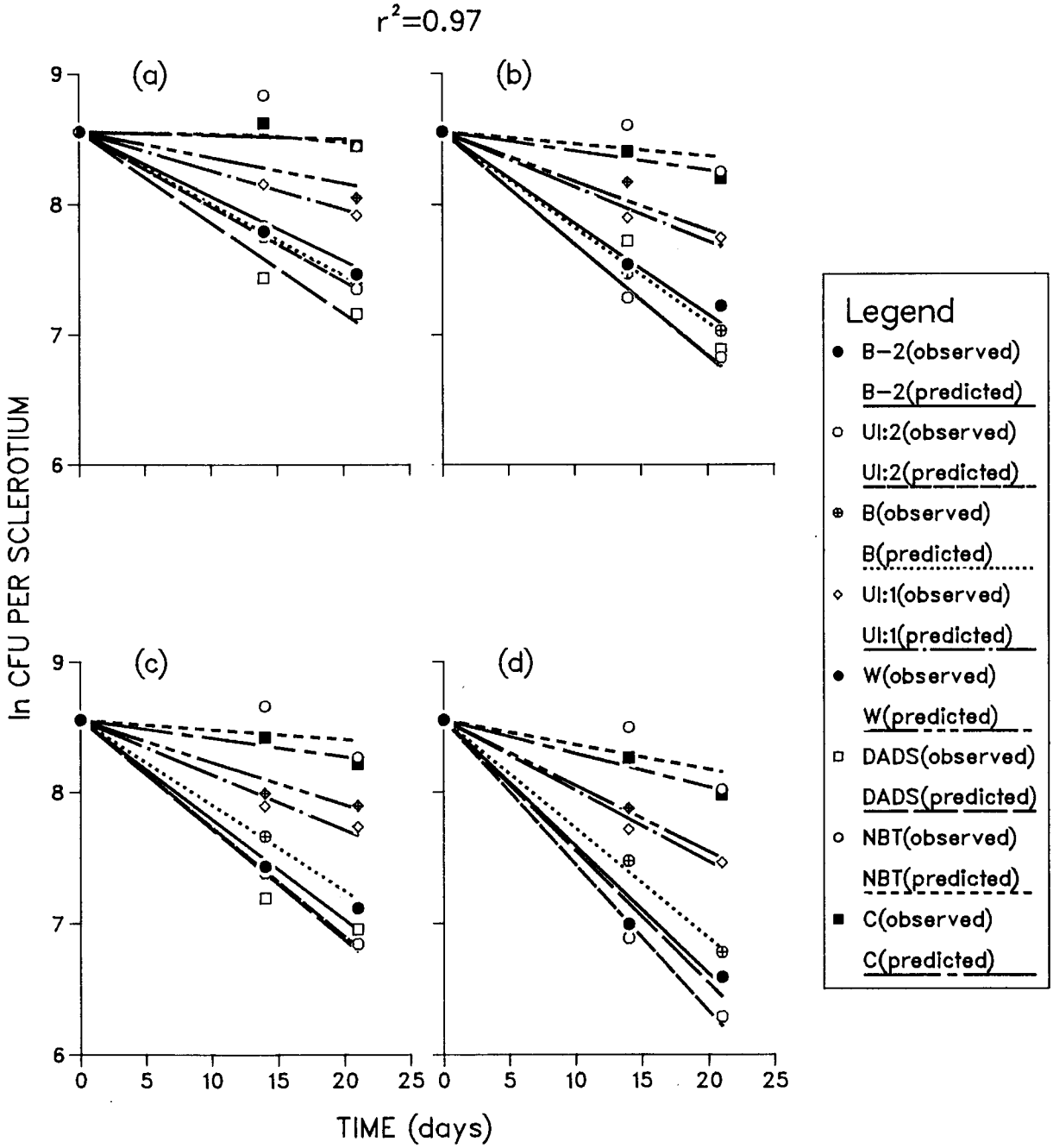

r^2=0.97
(a)
(b)
(c)
(d)
ln CFU PER SCLEROTIUM
TIME (days)
9
8
7
6
0
5
10
15
20
25
Legend
B−2(observed)
B−2(predicted)
Ul:2(observed)
Ul:2(predicted)
B(observed)
B(predicted)
Ul:1(observed)
Ul:1(predicted)
W(observed)
W(predicted)
DADS(observed)
DADS(predicted)
NBT(observed)
NBT(predicted)
C(observed)
C(predicted)

Table 22. Effects of various factors and interactions evaluated by backward elimination in the derivation of the regression model for effects of diallyl disulphide, bacterized and non-bacterized seedlings on total fungal populations associated with sclerotia of *Sclerotium cepivorum* in bioassay 3.

#	Tested against	Regression model	Variable removed	D.F	SS(error)	F-Value	P-Value
1	-	S+P+T+R+SxP+SxT+PxT+SxPxT+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI+SxPxTxTI	-	317	4.308	-	-
2	1	S+P+T+R+SxP+SxT+PxT+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI+SxPxTxTI	SxPxT	324	4.344	0.378	0.915
3	2	S+P+T+R+SxP+SxT+PxT+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	SxPxTxTI	331	4.463	1.268	0.266
4	3	S+P+T+R+SxT+PxT+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	SxP	332	4.468	0.371	0.543
5	4	S+P+T+R+SxT+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	PxT	339	4.503	0.372	0.918
6	5	S+P+T+R+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	SxT	346	4.538	0.376	0.916
7	6	S+T+R+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	P	347	4.541	0.229	0.633
8	7	T+R+SxTI+TI+PxTI+TxTI+SxPxTI+SxTxTI+PxTxTI	S	348	4.582	3.133	0.077
9	8	T+R+SxTI+TI+PxTI+TxTI+SxTxTI+PxTxTI	SxPxTI	349	4.703	9.189	0.003
10	9	R+SxTI+TI+PxTI+TxTI+SxTxTI+PxTxTI	T	356	4.720	0.180	0.989
11	10	SxTI+TI+PxTI+TxTI+SxTxTI+PxTxTI[1]	R	359	4.830	2.766	0.042
12	11	SxTI+TI+PxTI+TxTI+SxTxTI	PxTxTI	366	5.418	6.245	<0.0001
13	11	SxTI+TI+PxTI+TxTI	SxTxTI	366	5.050	2.336	<0.0001

[1]Final model.
(S=soil, P=position, T=treatments, R=reps, TI=time).

Figure 12. Effect of diallyl disulphide, and bacterized and non-bacterized onion seedlings on total fungal populations associated with sclerotia of *Sclerotium cepivorum* in bioassay 3. (a) surface sclerotia in sterilized soil, (b) buried scleotia in sterilized soil, (c) surface sclerotia in non-sterilized soil, and (d) buried sclerotia in non-sterilized soil.

B-2, UI:2, B, UI:1 amd W are the marked bacterial strains used for onion seed bacterization
NBT=non-bacterized onion seedlings
DADS=soil treated with 10 ug/40 cc of soil with diallyl disulphide
C=control (soil only)

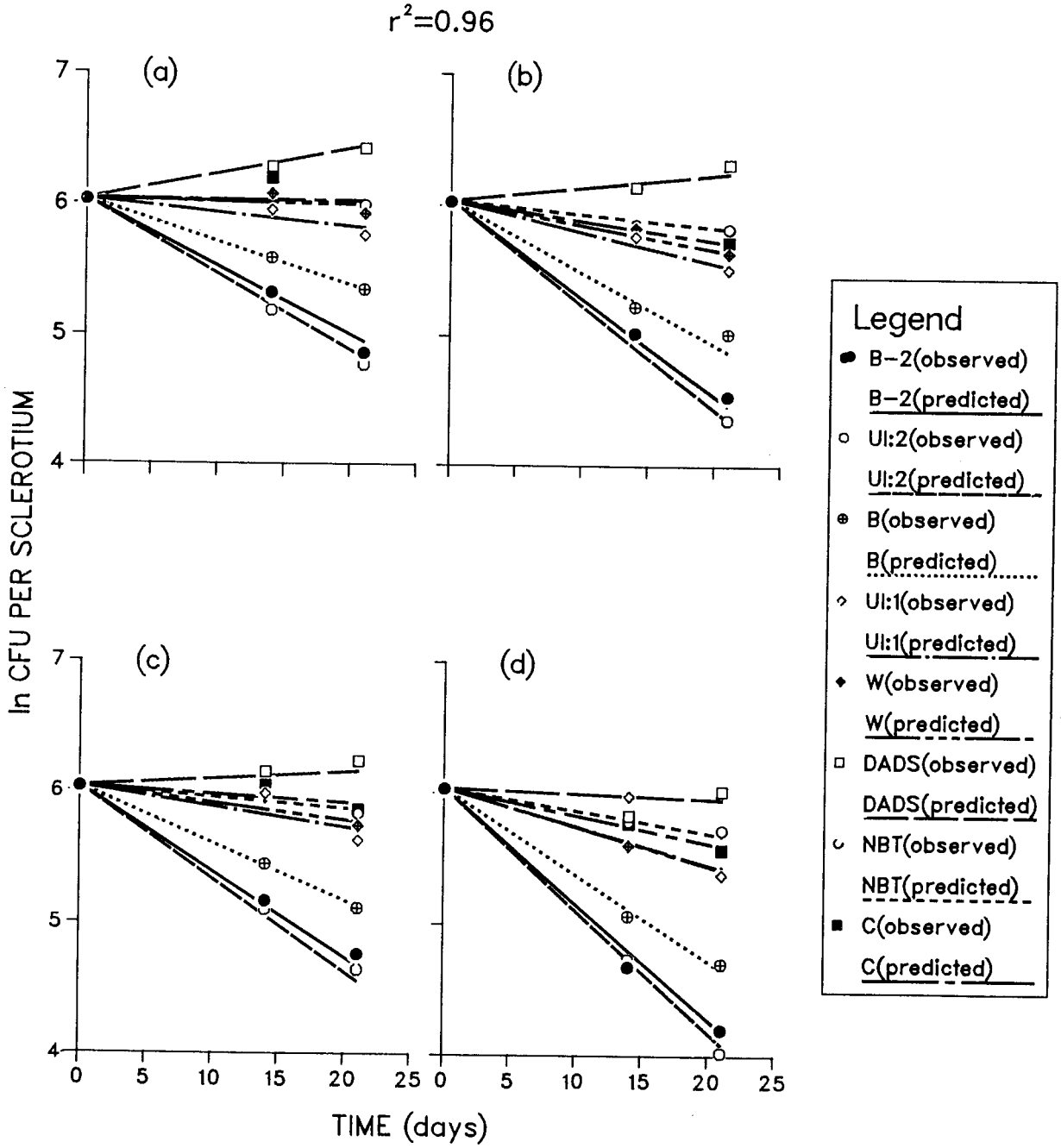
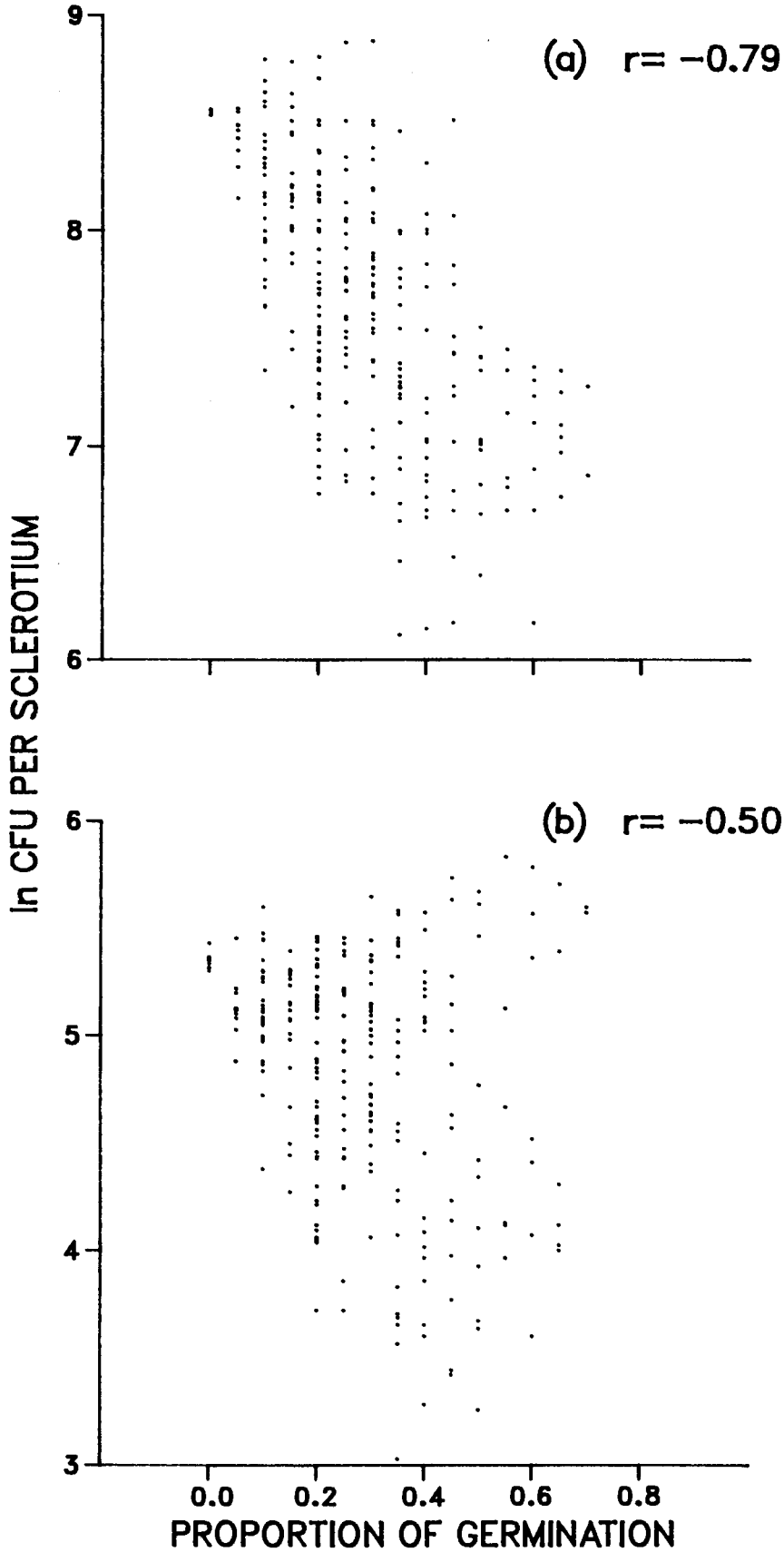

r^2=0.96
(a)
(b)
(c)
(d)
ln CFU PER SCLEROTIUM
TIME (days)
Legend
B-2(observed)
B-2(predicted)
UI:2(observed)
UI:2(predicted)
B(observed)
B(predicted)
UI:1(observed)
UI:1(predicted)
W(observed)
W(predicted)
DADS(observed)
DADS(predicted)
NBT(observed)
NBT(predicted)
C(observed)
C(predicted)

Figure 13. Correlation between the proportions of sclerotial germination and populations of total bacteria (a), and fungi (b) associated with sclerotia of *Sclerotium cepivorum*.


(a) r= −0.79
(b) r= −0.50
ln CFU PER SCLEROTIUM
9
8
7
6
6
5
4
3
0.0
0.2
0.4
0.6
0.8
PROPORTION OF GERMINATION

A significant inverse correlations between the proportion of sclerotial germination and populations of bacteria (P=0.01) and fungi (P=0.05) associated with sclerotia of *S. cepivorum* was obtained.

Estimation of Populations of genetically marked bacterial strains in rhizosphere and nonrhizosphere root-zone soil

Data showing the rhizosphere and root-zone soil populations of genetically marked bacterial strains for bioassays 1 and 2 are shown in Table 23, and for bioassay 3 shown in Table 24. All the marked bacterial strains persisted in both the rhizosphere and nonrhizosphere root-zone soil during the entire period of the experiments. There were differences in the magnitude of rhizosphere and nonrhizosphere root-zone soil populations of the different marked strains. Populations of the marked strains in all bioassays were higher in nonrhizosphere root-zone soil than in the rhizosphere. However a significant enhancement of the rhizosphere and the nonrhizosphere root-zone soil populations in sterilized soil over non-sterilized soil was observed following seed bacterization in all three bioassays. Marked bacterial populations were not detected from the rhizosphere or nonrhizosphere root-zone soils of non-bacterized onion seedlings either in sterilized or non-sterized soil in any of the bioassays.

Table 23. Populations of *Bacillus subtilis* B-2 marker in onion rhizosphere and nonrhizosphere root-zone soils in bioassay 1 and 2.

Soil type[1]	Populations in rhizosphere soil[2] (cfu/seed or plant)				Populations in nonrhizosphere root-zone soil[3] (cfu/g dry wt. of soil)		
	Time in days						
	0	7	14	21	7	14	21
				Bioassay 1[4]			
SS	$8.62 \pm 0.16 \times 10^5$ a	$2.73 \pm 0.16 \times 10^4$ b	$2.05 \pm 0.27 \times 10^4$ b	$6.33 \pm 0.97 \times 10^3$ b	$3.60 \pm 0.08 \times 10^6$ b	$2.10 \pm 0.12 \times 10^6$ b	$7.08 \pm 0.94 \times 10^5$ b
NSS	$8.62 \pm 0.16 \times 10^5$ a	$2.21 \pm 0.12 \times 10^4$ b	$1.30 \pm 0.06 \times 10^4$ a	$2.93 \pm 0.25 \times 10^3$ a	$2.53 \pm 0.07 \times 10^6$ a	$1.06 \pm 0.08 \times 10^6$ a	$2.86 \pm 0.43 \times 10^5$ a
				Bioassay 2[4]			
SS	$1.19 \pm 0.05 \times 10^6$ a	$3.24 \pm 0.04 \times 10^4$ b	$6.03 \pm 0.41 \times 10^3$ b	$3.91 \pm 0.24 \times 10^3$ b	$2.56 \pm 0.24 \times 10^6$ b	$1.87 \pm 0.07 \times 10^6$ b	$1.20 \pm 0.86 \times 10^5$ b
NSS	$1.19 \pm 0.05 \times 10^6$ a	$2.07 \pm 0.07 \times 10^4$ a	$4.38 \pm 0.38 \times 10^3$ a	$2.53 \pm 0.06 \times 10^3$ a	$2.09 \pm 0.36 \times 10^6$ a	$1.14 \pm 0.09 \times 10^6$ a	$5.51 \pm 0.28 \times 10^5$ a

[1] SS=sterilized muck soil; NSS=non-sterilized muck soil.
[2] Mean (±=S. E) of 4 replications, one plant each.
[3] Mean (±=S. E) of 4 replications, 10 g soil each.
[4] Means within a column followed by the same letter do not differ significantly according to student Newman-Keul's test (p=0.05).

Table 24. Populations of the marked bacterial strains in onion rhizosphere and nonrhizosphere root-zone soils in bioassay 3.

Bacterial strains[1]	Soil type[2]	Populations in rhizosphere[3] (cfu/seed or plant)			Populations in nonrhizosphere root-zone soil (cfu/g dry wt. of soil)[4]	
		Time in days				
		0	14	21	14	21
B-2	SS	$4.50 \pm 0.25 \times 10^5$	$1.64 \pm 0.11 \times 10^4$	$1.65 \pm 0.16 \times 10^3$	$1.86 \pm 0.06 \times 10^6$	$1.25 \pm 0.05 \times 10^6$
	NSS	$4.50 \pm 0.25 \times 10^5$	$1.30 \pm 0.07 \times 10^4$	$1.15 \pm 0.08 \times 10^3$	$1.51 \pm 0.06 \times 10^6$	$1.08 \pm 0.03 \times 10^6$
UI:2	SS	$3.23 \pm 0.39 \times 10^5$	$5.29 \pm 0.17 \times 10^5$	$4.36 \pm 0.15 \times 10^5$	$2.83 \pm 0.11 \times 10^6$	$2.07 \pm 0.07 \times 10^6$
	NSS	$3.23 \pm 0.39 \times 10^5$	$4.60 \pm 0.18 \times 10^5$	$3.72 \pm 0.16 \times 10^5$	$2.53 \pm 0.10 \times 10^6$	$1.63 \pm 0.06 \times 10^6$
B	SS	$5.83 \pm 0.93 \times 10^6$	$9.73 \pm 0.58 \times 10^6$	$1.03 \pm 0.04 \times 10^6$	$3.80 \pm 0.15 \times 10^6$	$3.53 \pm 0.08 \times 10^6$
	NSS	$5.83 \pm 0.93 \times 10^6$	$7.89 \pm 0.21 \times 10^6$	$9.27 \pm 0.29 \times 10^5$	$3.37 \pm 0.11 \times 10^6$	$3.09 \pm 0.09 \times 10^6$
UI:1	SS	$5.33 \pm 0.04 \times 10^5$	$2.99 \pm 0.25 \times 10^5$	$2.17 \pm 0.25 \times 10^5$	$2.48 \pm 0.10 \times 10^6$	$1.96 \pm 0.04 \times 10^6$
	NSS	$5.33 \pm 0.04 \times 10^5$	$2.44 \pm 0.24 \times 10^5$	$1.55 \pm 0.04 \times 10^5$	$2.16 \pm 0.07 \times 10^6$	$1.67 \pm 0.06 \times 10^6$
W	SS	$1.24 \pm 0.12 \times 10^6$	$5.83 \pm 0.51 \times 10^6$	$4.83 \pm 0.26 \times 10^5$	$3.04 \pm 0.10 \times 10^6$	$2.79 \pm 0.04 \times 10^6$
	NSS	$1.24 \pm 0.12 \times 10^6$	$4.87 \pm 0.37 \times 10^6$	$3.75 \pm 0.11 \times 10^5$	$2.73 \pm 0.09 \times 10^6$	$2.49 \pm 0.06 \times 10^6$
NBT	SS	0	0	0	0	0
	NSS	0	0	0	0	0

[1] B-2 is an isolate of *Bacillus subtilis*, obtained from *sclerotium cepivorum* and UI:2, B, UI:1 and W strains were obtained from the rhizospheres of commercial onions (cv. Autumn spice). Both sclerotia and onions were from naturally infested muck soil Cloverdale, B.C., NBT=non-bacterized onion seeds.

[2] SS=sterilized muck soil; NSS=non-sterilized muck soil.

[3] Mean (±=S. E) of 4 replications, one plant each.

[4] Mean (±=S. E) of 4 replications, 10 g soil each.

DISCUSSION

The initial reason for carrying out the work described in this chapter was to develop a suitable bioassay technique to help in future studies to identify the principle in *Allium* spp. which stimulates germination of sclerotia of *S. cepivorum* in sterilized and non-sterilized muck soil. Data presented here support the report of Coley-Smith and King (34) that bacteria could function to enhance the germination of sclerotia of *S. cepivorum*. However the nature of the stimulation for germination of sclerotia of *S. cepivorum* in the field and its relationship to disease incidence has been the subject of many studies (26, 33, 34).

Enhanced germination of sclerotia of *S. cepivorum* was associated with seed bacterization in bioassays 1, 2 and 3. Similar results were observed with DADS, a known synthetic germination stimulant (52). The marked bacterial strains persisted throught the period of the bioassays both in onion rhizosphere and nonrhizosphere root-zone soils.

I suggest two possible mechanisms to explain the findings of this study on the effect of onion seed bacterization on the germination of sclerotia of *S. cepivorum*. One possible mechanism is that non-volatile sulfur compounds released from onion root exudates into the surrounding soil may be metabolized by the marked bacterial isolates used for seed bacterization into volatile stimulatory sulfur compounds. These compounds may then diffuse into or onto the soil and stimulate sclerotia of *S. cepivorum* which were placed on the surface or buried in the soil to germinate.

In the case of non-bacterized, surface sterilized onion seedlings, non-volatile sulfur compounds might not be converted into volatile forms, hence the lower percent germination of sclerotia of *S. cepivorum* that was observed. Similarly low levels of germination were observed in the control treatment (soil only).

Coley-Smith and King (34) have shown that volatile compounds produced from the non-volatile sulphide precursors allyl cysteine and n-propylcysteine by bacterial degradation play a major role in the induction of sclerotial germination. Intestinal coli-types are known to be capable of reducing S-allyl-L-cysteine sulphoxides to disulphides (146). Murakami (102) found that *B. subtilis* possesses an enzyme which catalyses the production of methyl thiosulphinate, pyruvate and ammonia from S-methyl cysteine sulphoxide. Nomura *et al.* (104) showed that S-allylcysteinase resembles an alliinase which has been isolated from *Pseudomonas cruceviae* and catalyzes the conversion of S-methyl-L-cysteine sulphoxide to methyl thiosulphinate pyruvic acid and ammonia. Whitaker (150) showed that S-propenyl-1-cysteine sulphoxide was a major non-volatile sulfide precursor in intact onions. Without enzymatic activity (150), or soil bacteria (67) intact onions did not release any volatile compounds into the soil. My results substantiate the above reports, and offer a clear indication that onion seed bacterization plays a role in the onion root ecosystem as a promoter of sclerotial germination.

A second possible mechanism is that the germination of sclerotia of *S. cepivorum* was an indirect response to the effects of onion seed bacterization or DADS on mycoflora associated with sclerotia. Bacterization treatments and DADS may have allowed the sclerotia to germinate by a specific reversal of

the inhibitory effect of this resident sclerotial mycoflora. The reduction of bacteria and fungal populations associated with surface and buried sclerotia by strains B-2, UI:2 and B and bacterial populations by DADS was inversely correlated with the proportion of sclerotial germination observed in these treatments (Fig. 13). These findings substantiate the conclusion of Coley-Smith *et al.* (30) that *Allium* spp., and their extracts either override or reduce the mycostatic effect which maintains sclerotial dormancy in the case of *S. cepivorum.*

Overall, the persistence of the marked bacterial strains in the rhizosphere and nonrhizosphere root-zone soils throughout the bioassays demonstrates that the onion seed bacterization could influence the germination of sclerotia of *S. cepivorum.*

CONCLUSION

Biological control of soil borne diseases is a distinct possibility for the future, especially within the framework of integrated pest management systems. Studies on the population dynamics and activities of bacterial isolates in plant rhizosphere and root-zone soils are basic to the logical development of their use for biological control of soil borne pathogens or for promotion of plant growth.

Incorporation of antibiotic markers into bacteria is useful for studying the population dynamics of specific soil bacteria under laboratory or field conditions. Six strains of *B. subtilis* and four other selected onion rhizobacteria tolerant to the combinations of 300 ug/ml streptomycin, 100 ug/ml cycloheximide and 30 ug/ml benomyl in PDA were isolated from the parental wild type isolates by a non-mutagenizing reselection procedure. The S_{300}CB-PDA medium permitted selective recovery of the marked bacterial strains from onion seedling rhizosphere and nonrhizosphere root-zone soils by excluding practically all other microorganisms.

A comparative population study of *B. subtilis* B-2 and UI:2, B, UI:1 and W showed that UI:2, B, UI:1 and W persisted in greater numbers both in onion rhizosphere and nonrhizosphere root-zone soils than did B-2. Overall, UI:2 and B were comparatively good colonizers in both the rhizosphere and nonrhizosphere root-zone soils, UI:1 and W were intermediate and B-2 was poor. This study revealed that *B. subtilis* B-2 was preferentially associated with the nonrhizosphere root-zone soil compared with the rhizosphere soil to a greater extent than were the other four bacteria. The onion rhizosphere

colonization assay reported in the thesis is a simple and rapid technique to compare rhizosphere colonizing abilities of bacterial strains on onions. All of the marked bacterial strains were able to colonize the onion seedling rhizospheres. Among the ten marked bacterial strains B and AB-9 in the rhizosphere and B, UI:2 and W in the nonrhizosphere root-zone soil maintained the highest populations. In contrast B-2, Bact-K, Bact-8 and Bact-O in the rhizosphere and B-2, EBW-1, Bact-O in the non-rhizosphere were recoverd in lower numbers. Although, root colonization alone is not predictive of a beneficial plant response, it is a logical starting point from which to select either biological control agents or plant growth promoting bacteria. The use of marked strains is useful because (i) it allows quantitative estimates of changes and the distribution of populations of the marked strains within the rhizosphere and nonrhizosphere root-zone soils, and (ii) it aids in the interpretation of the influence of seed bacterization on the growth of onion seedlings.

Seed bacterization with *B. subtilis* B-2, UI:2 and B marker strains caused significant increases in shoot height, dry weight and root dry weight of onion seedlings over non-bacterized controls and seed bacterization treatments UI:1 and W. Seed treatments with UI:1 and W did not show any effect on shoot height or shoot and root dry weight of onion seedlings over control. Bacterization of onion seeds with the marked strains resulted in significant reductions over controls in the estimated populations of total indigenous bacteria and fungi in the rhizosphere and nonrhizosphere root-zone soils 30 days following seeding in a field trial. Strains B-2, B and UI:2 were the most effective and strains W and UI:1 the least effective suppressors of

indigenous bacterial and fungal populations both in rhizosphere and root-zone soils. Onion seedling growth promotion by seed bacterization was not correlated with populations of the various bacterial isolates. B-2, UI:2 and B enhanced seedling growth the most; this effect appears related to their relative abilities to suppress indigenous bacterial and fungal populations in onion seedling rhizosphere and root-zone soil environments. These results are consistent with the hypothesis that growth enhancement results from indirect effects of the introduced bacteria B-2, UI:2 and B (80, 136). This hypothesis is supported by the observation that B-2, UI:2 and B were all antagonistic to growth of *S. cepivorum* whereas UI:1 and W were not (Fig. 2, Table 11).

Germination of sclerotia of *S. cepivorum* was enhanced by soil treatments with DADS a known synthetic germination stimulant and also by seed bacterization with some but not all of the strains evaluated. I suggest two possible mechanisms to explain the findings of this study on the effect of onion seed bacterization on the germination of sclerotia of *S. cepivorum*. One possible mechanism is that non-volatile sulfur compounds in onion root exudates released into the soil may have been metabolized into volatile stimulatory sulfur compounds by the marked bacterial isolates used for seed bacterization (34). These compounds may have then diffused into or onto the soil and stimulated sclerotia of *S. cepivorum* which were placed on the surface or buried in the soil to germinate. A second possible mechanism is that the germination of sclerotia of *S. cepivorum* was an indirect response to the effects of onion seed bacterization or DADS on mycoflora associated with sclerotia. Bacterization treatments and DADS may have allowed the sclerotia to germinate by a specific reversal of the inhibiting effect of this

resident sclerotial mycoflora. The reduction of bacteria and fungal populations associated with surface and buried sclerotia by strains B-2, UI:2 and B and by DADS was inversely correlated with the proportion of sclerotial germination observed in these treatments (Fig. 13).

For effective disease control it is important to have knowledge of the population dynamics of introduced bacteria in seedling rhizosphere and root-zone soils over time, and to understand the ecological relationship between onion seed bacterization and germination of sclerotia of *S. cepivorum*. The techniques and bioassays developed during this research have proven to be useful in obtaining such information.

The information obtained in the case of the bacterial strains and disease interaction studied in this thesis leads to the conclusion that the effects of bacterization are indirect. Growth effects on onion seedlings and enhanced germination of sclerotia of the fungal pathogen *S. cepivorum* in the presence of its onion seedling host were not related to the relative rhizosphere and/or root-zone soil populations of the bacterial strains evaluated. Rather they were related to the relative abilities of these strains to suppress populations of indigenous rhizosphere, root-zone soil or sclerotial mycosphere microorganisms.

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