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GROWTH EFFECTS ASSOCIATED WITH SEED BACTERIZATION NOT CORRELATED WITH POPULATIONS OF *BACILLUS SUBTILIS* INOCULANT IN ONION SEEDLING RHIZOSPHERES

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Summary—Populations of a marked strain of the B-2 isolate of *Bacillus subtilis* (B-2) in the rhizospheres of onion seedlings grown from bacterized seeds in muck soil at various pH, moisture and temperature regimes were monitored for 14 weeks. Irrespective of regime, populations of B-2 in onion rhizospheres declined rapidly within the first 14 days after seeding, from 4.8×10^6 cfu per seed on day zero to a mean of 1.1×10^3 cfu per plant at day 14, and less rapidly during the next 12 weeks following seeding. An average population of 9.5×10^1 cfu per plant was recovered at 14 weeks following seeding. Within the pattern of general decline, survival of B-2 in the rhizosphere was favored by high temperature, high moisture and high pH regimes; temperature appeared to be the most important variable. Seed bacterization significantly increased shoot dry weight (12–94%), root dry weight (13–100%) and shoot height (12–40%) of onion seedlings over controls. Increases in shoot height and shoot weight were greatest at low temperature and high moisture, under all pH regimes. Root weight was similarly affected by temperature and moisture, but was significantly increased at pH 6.5 compared to 5.5 and 4.5. Though *B. subtilis* B-2 failed to maintain high populations in the onion rhizosphere, it nevertheless caused significant growth effects on bacterized onion seedlings. The observed growth effects were not proportional to rhizosphere populations of 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. There are many reviews available on this subject (Burr and Caesar, 1984; Curl and Truelove, 1986; Schippers *et al.*, 1987; Schroth and Hancock, 1981, 1982; Schroth *et al.*, 1984; Suslow, 1982; Whipps and Lynch, 1986). 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. Strains of *Bacillus subtilis* used as seed inoculants have enhanced growth and yield of a range of nursery, vegetable, cereal and field crops (Broadbent *et al.*, 1971, 1977; Merriman and Birkenhead, 1977; Merriman *et al.*, 1975). Utkhede and Rahe (1980, 1983) studied the control of onion white rot, caused by the fungus *Sclerotium cepivorum* Berk., afforded by seed bacterization with six different isolates of *B. subtilis* recovered from sclerotia of *S. cepivorum*. Among the six isolates, *B. subtilis* B-2 provided significant amounts of season-long control of onion white rot in 1978 (Utkhede and Rahe, 1980). Control was also obtained in a field trial in 1979 (Utkhede and Rahe, 1983), but not in field trials conducted in 1980 and 1981 (unpublished data). No information was available from these trials on either the initial degree of colonization of seeds by *B. subtilis*, or on subsequent persistence or decline of the bacteria on roots during the season.

Inconsistent effects of seed bacterization from trial-to-trial raise questions about the causes of such variation as well as the practical significance of the effects (Mishustin and Naumova, 1962; Rovira, 1965). Are effects of seed bacterization on plant growth related 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 (Howie and Echandi, 1983). We have evaluated the effects of soil pH, moisture and temperature on populations of *B. subtilis* B-2 in onion-seedling rhizospheres following sowing of bacterized seeds, and associated effects of seed bacterization on the growth of onion seedlings. We used a strain marked with antibiotic resistance because this approach allows study of the population dynamics of specific strains in the presence of indigenous populations of soil-inhabiting bacteria (Kloepper and Schroth, 1981). Fluorescent antibodies were used in an earlier study (D. C. W. Wong and J. E. Rahe, unpublished data) but these lacked strain specificity.

An abstract of the present study was published earlier (Reddy and Rahe, 1986).

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MATERIALS AND METHODS

Sources of bacterial isolate and bacterization of onion seeds

B. subtilis B-2 (B-2), isolated from sclerotia of *S. cepivorum* from naturally-infected onions grown on muck soil near Cloverdale, B.C. (Utkhede and Rahe, 1980), was the parental source of the antibiotic tolerant strain used for this study. A strain of B-2 tolerant to the combination of 300 μg ml^{-1} streptomycin sulfate (S) (Sigma, St Louis, Mo.), 100 μg ml^{-1} cycloheximide (C) (Sigma) and 30 μg ml^{-1} benomyl (B) (Plant Products, Bramalea, Ontario) in potato dextrose agar (PDA) (S_{300}CB–PDA) was obtained from the parental isolate by reselection from increasing concentrations of S in the presence of C (100 μg ml^{-1}) and B (30 μg ml^{-1}) under non-mutagenizing conditions. The marked strain was comparable to the parental isolate in morphological and biochemical characteristics, and rate of growth on unamended PDA. The ratio of colony numbers developing on S_{300}CB–PDA and unamended PDA after growth of the marked strain for 8 h in tryptic soy broth (TSB) was 0.97. There were no significant differences in survival between the marked strain and the parental isolate for at least 90 days when these were inoculated into sterilized soil and maintained axenically at 22°C and 80% moisture content. The parental and marked strains showed similar types and amounts of inhibition against *S. cepivorum* in dual culture on PDA. The marked strain was maintained on S_{300}CB–PDA until required.

Onion seeds cv. Autumn Spice (Stokes Seeds, St Catharines, Ontario) were surface sterilized in 0.1% $HgCl_2$ solution for 5 min, rinsed in 70% ethanol and then 4 times with sterile distilled water (SDW). Fresh bacterial cultures of marked B-2 were grown in TSB at 24–25°C for 8–10 h on a reciprocating shaker (75 rev min^{-1}). Bacteria were pelleted by centrifugation for 20 min at 1500 *g*, suspended in SDW and repelleted and finally suspended in sterile 0.1 M $MgSO_4$. Surface-sterilized onion seeds were bacterized by immersion in bacterial suspension for 10 min, and then dried in flowing sterile air for 1–2 h. When dry (zero time), 5 samples each containing 5 bacterized onion seeds were transferred into 10 ml aliquots of sterile 0.1 M $MgSO_4$ and magnetically stirred for 10 min. Serial dilutions of the $MgSO_4$ washing suspensions were made, and 0.1 ml aliquots of appropriate dilutions were plated onto S_{300}CB–PDA in triplicate. Plates were held for 72 h at 24–25°C. Colonies were counted and recorded as colony forming units (cfu) per seed. Surface-sterilized onion seeds treated similarly with sterile 0.1 M $MgSO_4$ served as non-bacterized controls.

Soil treatments and experimental design

Muck soil collected from an onion field near Cloverdale, B.C. was used for all experiments. Three pH (4.5, 5.5 and 6.5), two moisture (80% and 55%) and two soil temperature (22–25°C and 17–19°C) regimes were selected to evaluate the effects of these physical environmental variables on onion rhizosphere colonization by marked B-2, and on the growth of onion seedlings. The three pH regimes were obtained by adding appropriate amounts of $Ca(OH)_2$, mixing thoroughly and storing the soil out of doors in large clay pots. After 35 days the soils of each pH regime were divided into two portions and moisture contents adjusted to 80 and 55% (% moisture = g water g^{-1} dry soil × 100), and 75 cm^3 portions of the various soil treatments were placed in disposable rectangular 5.5 cm deep plastic pots of 110 cm^3 capacity (Westcan Horticultural Supply, Calgary, Alberta). Individual treatment pots were put into 28 × 50 × 5 cm plastic propagating flats without drain holes. Each flat contained six replicate pots of each of the six soil treatments (one for each sampling time), arranged in a randomized complete-block design. There were four replicate flats for each temperature. Four bacterized or non-bacterized onion seeds were sown in each pot. To maintain the low soil temperature (17–19°C), the flats were floated on a water bath maintained at about 12°C. The high soil temperature (22–25°C) occurred naturally in flats placed on a bench at ambient laboratory temperature. Sterile water was added periodically on a weight basis to the pots to maintain the desired moisture contents. The experiment ran in a laboratory plant growth room for 14 weeks with a 14 h photoperiod (55–80 μM m^{-2} s^{-1} PAR, measured at plant height with a Li-Cor Quantum Radiometer, Model LI-185A).

Rhizosphere bacterial populations and growth of seedlings

Populations of marked B-2 in the rhizospheres of seedlings growing from bacterized and non-bacterized 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 was removed from each replication 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 segments approx. 5 mm long 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 held for 72 h at 24–25°C and colonies were counted and recorded as cfu per plant. All root samples were processed within 24 h of harvesting. A backward elimination procedure (Draper and Smith, 1981; Gomez and Gomez, 1984) using GLIM (Baker and Nelder, 1978) was used to obtain a best fit regression of the colony count data on time to a linear model.

Growth of seedlings was measured at 2, 4, 6, 8, 11 and 14 weeks after seeding. Individual seedlings were removed from each treatment pot and shaken to remove excess soil, and shoot heights were recorded. Roots and shoots were separated, 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

Populations of the B-2 marked strain in onion rhizospheres declined rapidly within the first 14 days

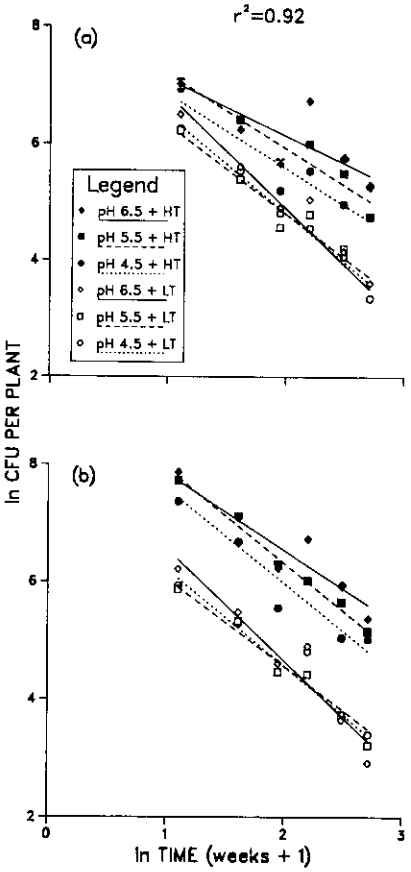

Fig. 1. Regression curves showing the effects of different soil pH and temperature regimes at two soil moisture levels on populations of a marked strain of *B. subtilis* B-2 in rhizospheres of onion seedlings grown from bacterized seeds in muck soil. (a) 55% soil moisture, (b) 80% soil moisture. HT = 22–25°C; LT = 17–19°C. Overall $r^2 = 0.92$.

after seeding, from 4.80×10^6 cfu per seed on day zero to an average of 1.06×10^3 cfu per 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 per plant was recovered at 14 weeks following seeding. Though the recovered populations were low, the marked strain persisted in onion rhizospheres throughout the 14 week duration of the experiment in all treatments. The B-2 marked strain was not recovered from rhizospheres of control onions (seedlings grown from non-bacterized seeds) from any of the treatments throughout the experiment.

Regression curves showing the effects of the moisture, temperature and pH treatments on rhizosphere populations of the marked strain of *B. subtilis* are shown in Fig. 1. Every regression model attempted substantially underestimated the observed populations at zero time. The regression of ln cfu plant^{-1} on ln time gave an overall $r^2 = 0.92$ for the data from 2 through 14 weeks following seeding. The final model used to present the data shown in Fig. 1 was comprised of the factors soil pH, temperature and moisture and interactions of temperature × moisture and temperature × pH, all of which were significant ($P \leq 0.05$). Interactions of pH × moisture and pH × moisture × temperature were not significant.

Higher populations of the marked strain of B-2 were maintained at 22–25°C than at 17–19°C (Fig. 1). The differential effect of temperature on rhizosphere populations of the marked B-2 strain 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 favorable treatment for persistence of B-2 in onion seedling rhizosphere was the high temperature, high moisture, high pH regime; temperature appeared to be the variable exerting the greatest effect.

Data on the overall effects of soil pH, moisture and temperature, and seed bacterization with the marked B-2 strain on growth of onion seedlings at 14 weeks are presented in Table 1. Seed bacterization caused significant increases in shoot height (Fig. 2), dry weight (Fig. 3) and root dry weight (Figs 4 and 5) of onion seedlings over non-bacterized controls, irrespective of the soil treatments. Increases ranged from 12 to 94% for shoot dry weights, from 13 to 100% for shoot dry weights, and from 12 to 40% for shoot heights. 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 2

Table 1. Overall effects of soil pH, moisture, temperature and seed bacterization with a marked strain of *B. subtilis* B-2 on the growth of onion seedlings at 14 weeks after seeding

Treatment factor	Level	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	Treated	25.16 ± 1.54 b	37.31 ± 4.87 b	3.82 ± 0.41 b
	Control	21.25 ± 1.41 a	31.04 ± 4.66 a	2.82 ± 0.26 a

[1]Mean ± SE of four replications, one plant each, from each treatment containing the indicated factor level.
[2]Means within a column followed by the same letter do not differ significantly according to Student Newman–Keul's test, $P = 0.05$.

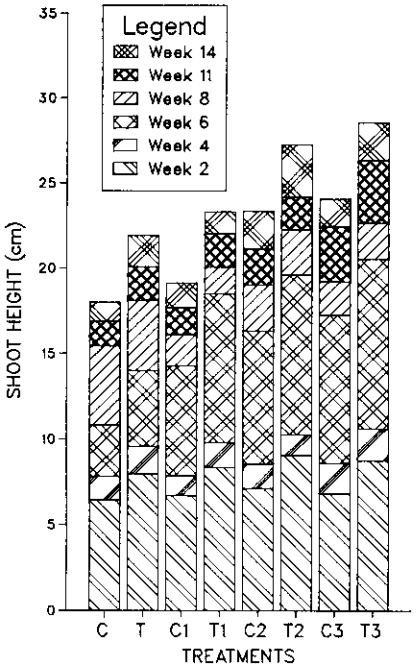

Fig. 2. Influence of seed bacterization with a marked strain of *B. subtilis* B-2 on shoot height of onion seedlings grown at various soil moisture and temperature regimes. Data presented are means of data from three pH regimes. Treatment abbreviations: C, C1, C2, C3 = controls; T, T1, T2, T3 = bacterized treatments; C, T = 55% moisture and 22–25°C; C1, T1 = 80% moisture and 22–25°C; C2, T2 = 55% moisture and 17–19°C; C3, T3 = 80% moisture and 17–19°C.

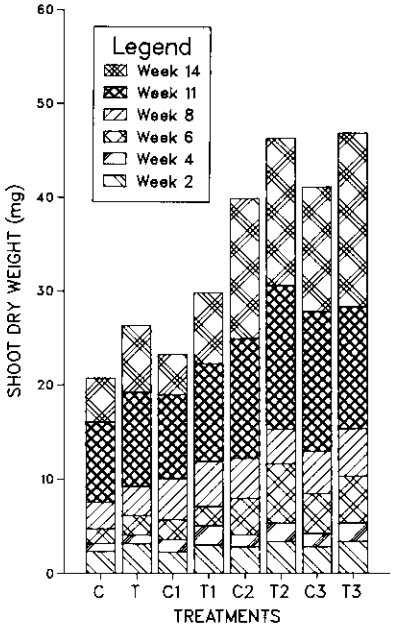

Fig. 3. Influence of seed bacterization with a marked strain of *B. subtilis* B-2 on shoot dry weight of onion seedlings grown at various soil moisture and temperature regimes. Data presented are means of data from three pH regimes. Treatment abbreviations: C, C1, C2, C3 = controls; T, T1, T2, T3 = bacterized treatments; C, T = 55% moisture and 22–25°C; C1, T1 = 80% moisture and 22–25°C; C2, T2 = 55% moisture and 17–19°C; C3, T3 = 80% moisture and 17–19°C.

and 3). Root weight was similarly affected by temperature (Fig. 5), but was significantly increased at pH 6.5 compared with 5.5 and 4.5 (Fig. 4).

DISCUSSION

Populations of a strain of *B. subtilis* selected for tolerance to the combination of 300 μg streptomycin ml^{-1}, 100 μg cycloheximide ml^{-1} and 30 μg benomyl ml^{-1} in potato dextrose agar were successfully monitored in onion seedling rhizospheres for 14 weeks after application to seeds. Populations of the marked strain in onion seedling rhizospheres as low as 10 cfu per plant were recovered without interference from indigenous soil microorganisms, thus confirming the applicability of the method for study of the population dynamics of a specific bacterial strain occurring in low numbers in a complex, biologically-active environment. Moreover, it allowed quantitative estimates of changes in populations of the marked strain within the rhizosphere associated with various soil treatments.

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 on roots for the next 12 weeks under all test conditions. High soil moisture at high temperature appeared to favor persistence of B-2 in onion rhizospheres. The

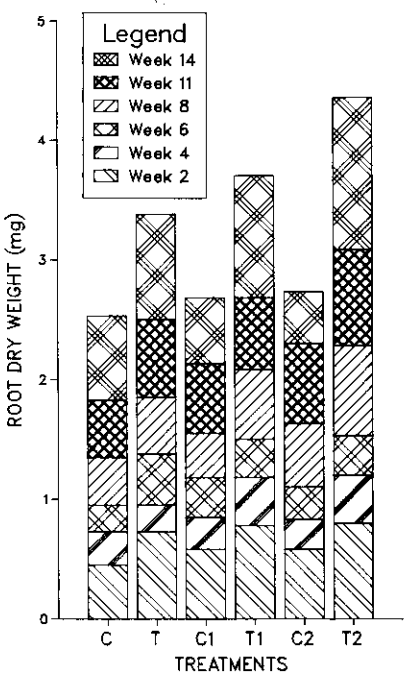

Fig. 4. Influence of seed bacterization with a marked strain of *B. subtilis* B-2 on root dry weight of onion seedlings grown at various soil pH levels. Data presented are means of data from two moisture regimes at each of two temperatures. Treatment abbreviations: C, C1, C2 = controls; T, T1, T2 = bacterized treatments; C, T = pH 4.5; C1, T1 = pH 5.5; C2, T2 = pH 6.5.

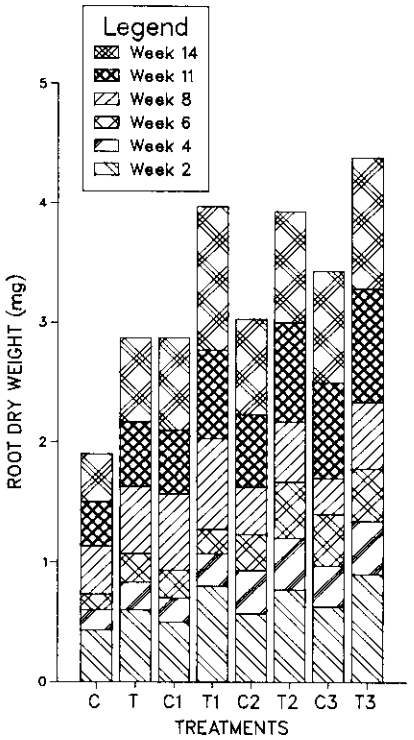

Fig. 5. Influence of seed bacterization with a marked strain of *B. subtilis* B-2 on root dry weight of onion seedlings grown at various soil moisture and temperature regimes. Data presented are means of data from three pH regimes. Treatment abbreviations: C, C1, C2, C3 = controls; T, T1, T2, T3 = bacterized treatments; C, T = 55% moisture and 22–25°C; C1, T1 = 80% moisture and 22–25°C; C2, T2 = 55% moisture and 17–19°C; C3, T3 = 80% moisture and 17–19°C.

results suggest that the effect of soil moisture is temperature dependent.

The failure of seed bacterization with B-2 to control onion white rot in field trials conducted in 1980 and 1981 (R. S. Utkhede and J. E. Rahe, unpublished data) may have been related to the abnormally cool and wet conditions that prevailed in those years. The data reported here show that 17–19°C suppressed B-2 populations in the rhizosphere relative to temperatures of 22–25°C, particularly under high soil moisture conditions. Soil temperatures in the field following seeding are typically well below 17–19°C and in cool years do not often exceed this level during the growing season. Thus, it is possible that the potential of *B. subtilis* B-2 as a biocontrol agent for onion white rot may be restricted to relatively warm and moist soil conditions.

Although 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. This effect may have been exerted by the relatively low populations of the marked B-2 strain in the seedling rhizospheres; it is also possible that the bacterium in non-rhizosphere root-zone soil may have influenced the growth of onion seedlings. The mechanism by which B-2 promoted increased onion seedling growth is not clear. The observed growth responses were not proportional to rhizosphere populations of *B. subtilis* B-2; seedling growth was greatest under the low temperature treatments whereas B-2 rhizosphere populations were most favored by the high temperature treatments.

The observed enhancement of onion seedling growth associated with seed bacterization with B-2 is consistent with published reports on stimulation of plant growth by *Bacillus* spp in various cereal and vegetable crops (Broadbent *et al.*, 1971, 1977; Merriman *et al.*, 1974; Ridge and Rovira, 1968; Rovira, 1963, 1965). Broadbent *et al.* (1977) 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 (Burr *et al.*, 1978; Rovira, 1963), *in vitro* requirements for particular amino acids (Eklund, 1970), antigenic reactions (Burr *et al.*, 1978) or biochemical reactions *in vitro* (Barea *et al.*, 1975).

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, or unfavorable effects of soil moisture, soil pH and temperature. The use of a strain of *B. subtilis* marked with tolerance to a mixture of antibiotics and fungicide that prevented growth of indigenous soil microorganisms on amended potato dextrose agar permitted its selective recovery from a biologically-complex plant–soil environment. This made possible a precise determination of the ability of this bacterium to colonize and persist in the rhizospheres of onions grown from bacterized seed in microbially-active muck soil, and of the effect of soil physical environmental variables on its persistence.

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