

BACILLUS SUBTILIS B-2 AND SELECTED ONION RHIZOBACTERIA IN ONION SEEDLING RHIZOSPHERES: EFFECTS ON SEEDLING GROWTH AND INDIGENOUS RHIZOSPHERE MICROFLORA

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Summary—Marked strains of *Bacillus subtilis* B-2 and four selected rhizobacteria were introduced into onion rhizospheres by seed bacterization. Their effects on plant growth and their populations in root surface and root zone soil environments of field-grown onions were estimated 30 days following sowing. Populations of indigenous rhizosphere bacteria and fungi were also assessed by dilution plating. Seed bacterization with B-2 UI-1 and B caused significant increases in shoot height and shoot dry weights of onion seedlings over controls. Only UI-2 caused a significant increase in root dry weight. All of the strains survived in seedling rhizospheres in substantial numbers, but there were marked differences among the strains. Overall, UI-2 and B were comparatively good colonizers, UI-1 and W were intermediate and B-2 was poor. Seed bacterization treatments significantly reduced indigenous bacterial and fungal populations in seedling rhizospheres. Promotion of onion seedling growth by seed bacterization was not correlated with the relative persistence of the introduced bacterium, but may be related to the ability of the introduced bacterium to reduce components of the indigenous rhizosphere microflora.

INTRODUCTION

In natural environments the growth and yield of plants depends on the quantity and balance of water, mineral nutrients, air, light and heat, but is also subject to positive and negative influences of various rhizosphere microorganisms. Both direct and indirect mechanisms have been suggested to explain the positive influence of certain bacteria on plant growth (Schroth *et al.*, 1984). Hypothesized direct mechanisms are that bacteria elaborate substances that stimulate plant growth, such as nitrogen, plant growth hormones and compounds that promote the availability of phosphates in the root zone. A popular hypothesis for an indirect mechanism is that populations of various pathogenic and deleterious microorganisms that affect the root system are reduced by the bacteria introduced via seed or root bacterization (Burr and Caesar, 1984; Schippers *et al.*, 1987; Suslow and Schroth, 1982). Each of these hypotheses suffers from insufficient supportive data. Direct information about the activities and interactions of microorganisms in natural soil and plant root environments is technically difficult to obtain due to the complexity and variability of these environments.

The ability to assess populations of selected microorganisms relative to those of indigenous microorganisms in an environment of interest can give evidence for interactions among organisms. We showed that a strain of *Bacillus subtilis* introduced as a seed treatment had a significant positive influence on the growth of onion seedlings under controlled conditions even though it was not preferentially associated with

onion root surfaces (Reddy and Rahe, 1989). We used a bacterial strain marked with tolerance to a combination of antibiotics and fungicides that prevented growth of indigenous soil microflora, and showed that the marked strain could be recovered from biologically-active root surfaces and soil at low population levels for at least 14 weeks following its introduction. These results encouraged us to evaluate the population dynamics and effects of this and other marked strains of selected onion rhizobacteria on onion seedling growth and indigenous rhizosphere microflora under field conditions following their introduction by seed bacterization.

MATERIALS AND METHODS

Marked strains of *B. subtilis* B-2 (B-2) (Utkhede and Rahe, 1983) and four rhizobacteria were used. The four rhizobacteria were isolated from the rhizospheres of commercial onions (cv. Autumn Spice) growing in muck soil near Cloverdale, B.C. during the summer of 1983. They were selected from a large number of bacterial isolates obtained during a season-long study of the indigenous rhizosphere bacterial microflora of field-grown onions and were representative of the colony types recovered in greatest numbers. The parental isolates of the marked strains of rhizobacteria used were designated UI-1, UI-2, B and W. From these, strains tolerant of the combination of 300 µg streptomycin sulfate ml⁻¹ (S), 100 µg cycloheximide ml⁻¹ (C) and 30 µg benomyl ml⁻¹ (B) in potato dextrose agar (PDA) (S₃₀₀CB-PDA) were selected according to the method described for B-2 (Reddy and Rahe, 1989).

Standard procedures (Harrigan and MacCance, 1966) were followed for biochemical tests (starch

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hydrolysis, nitrate reduction, indole and sulfide production) used to compare the parental strains with their respective marked strains. The abilities of the parental and marked strains to inhibit mycelial growth of *Sclerotium cepivorum* Berk. in dual culture were compared. This was done by streaking a test strain approx. 2.5 cm from the edge of a 9 cm dia Petri plate containing PDA, 24 h before placing a 5 mm dia core of PDA containing actively-growing mycelium of *S. cepivorum* 2.5 cm from the edge on the opposite side of the plate. The plates were kept at 24–25°C in the dark for several days and evaluated subjectively for possible differences in the nature and magnitude of the inhibition zones between the test bacterial strains and *S. cepivorum*.

A field trial was conducted in the summer of 1985 near Cloverdale, B.C. to compare the growth and indigenous rhizosphere microflora of onions grown from non-bacterized seeds and from seeds bacterized with S_{300} CB-tolerant strains of B-2, UI-1, UI-2, B and W. Procedures described in Reddy and Rahe (1989) were followed for surface sterilization and bacterization of seeds (cv. Autumn Spice purchased from Stokes Seeds, St Catharines, Ontario), and for estimating the zero time populations of bacteria on the seeds. The trial was seeded on 19 May. Individual treatment plots contained five rows 2 m long, spaced 25 cm apart. The outside two rows were seeded with the cv. Taurus using a tractor-mounted precision seeder and served as guard rows. The interior three rows were double seeded by hand with bacterized or non-bacterized seeds at 7 cm spacing. The plots were arranged in a randomized complete-block design with five replications. Soil pH in the trial field was approx. 4.5 (determined using a thick suspension of soil in 10 mM CaCl_2). Air temperatures (30 cm above ground) during the trial ranged from 15 to 30°C. Soil temperatures (10 cm below the surface) ranged from 10 to 26°C.

Due to poor germination and emergence, only one sampling was possible; this was done 30 days following seeding. Samples for assessing rhizosphere microflora 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 h of collection. Roots and adhering soil were detached from the five seedlings, 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 by Reddy and Rahe (1989) was followed for estimating populations of the marked bacterial strains obtained from root surfaces. Composite soil samples consisting of at least five cores (2.5 × 10 cm depth) taken at random from the region 3–7 cm around where the onion seedlings had been removed were designated root zone soil. Samples were placed in polyethylene bags and kept at about 4°C until processed. All samples were processed within 48 h of collection. Each sample was mixed thoroughly and then sieved (<2 mm). Moisture contents of the samples were estimated by drying 10 g subsamples overnight at 105°C. The population of a marked bacterial strain in a sample was estimated by a placing a 10 g subsample

into a 500 ml flask containing 100 ml of sterile 0.1 M MgSO_4 and then following the method for estimating populations of the marked bacterial strains in the seedling rhizospheres (Reddy and Rahe, 1989).

Populations of naturally-occurring bacteria and fungi in the root surface and root-zone soil samples were estimated by dilution plating (Johnson and Curl, 1972). Thornton's agar was used for bacteria and rose Bengal agar for fungi. Triplicate plates were spread with 0.1 ml aliquots of appropriate dilutions of the various samples and the plates were placed at 24–25°C. Colonies of bacteria and fungi were counted after 3 and 7 days respectively, and converted to colony forming units (cfu) per plant and g^{-1} dry weight of soil for root surface and root zone soil populations, respectively. No attempt was made to further identify the bacteria and fungi. The data were analyzed by ANOVAR and Duncan's multiple range test at 5% level of significance.

Growth measurements for onions from the various treatments were made on separate samples of five plants taken from each treatment plot. Seedlings were dug at random from the middle three rows and shoot heights were recorded. Roots and shoots were separated, washed, air dried and weighed.

RESULTS

The proportions of tolerant phenotypes (ratios of colony numbers developing on unamended and S_{300} CB-PDA) in populations of the marked strains after growth for 8 h in tryptic soy broth were 0.97, 0.96, 0.93, 0.80 and 0.98 for B-2, B, W, UI-1 and UI-2, respectively. The marked strains retained colony morphologies, biochemical cultural characteristics and antagonistic activity against mycelial growth of *S. cepivorum* in dual culture typical of the respective parental wild types. Strains B-2, B and UI-2 produced strong and comparable zones of inhibition of mycelial growth of *S. cepivorum* on PDA, whereas UI-1 and W did not inhibit mycelial growth of *S. cepivorum*.

The low percentage of emergence that occurred in most of the treatment plots is not attributed to the applied bacteria *per se* because it occurred in both control and bacterized treatments. Rather, it appeared to be due to a combination of factors: low vigor of the Autumn Spice seed used, wetting or MgSO_4 associated with bacterization treatments, poor physical condition of the soil in several of the plots, non-uniformity of seed placement by hand planting, and extreme drying conditions that immediately followed seeding. Good emergence occurred in the guard rows of mechanically seeded cv. Taurus.

The estimated populations of the marked strains occurring on bacterized seeds, on root surfaces and in root zone soil of field-grown onion seedlings 30 days after seeding are shown in Table 1. 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 root-surface and root-zone soil environments. No marked bacteria were detected from either environment of non-bacterized

Table 1. Populations of marked strains of *B. subtilis* B-2 and four onion rhizobacteria applied to seeds and recovered from onion root surfaces and onion root zone soil 30 days after seeding

Treatment*	Before seeding	30 Days after seeding	
	cfu Seed ⁻¹	cfu Plant ⁻¹	cfu g ⁻¹ dry soil
B-2	7.2 × 10 ⁵	7 × 10 ³ a†	9 × 10 ⁴ a
B	16.5 × 10 ⁵	185 × 10 ³ d	98 × 10 ⁴ c
W	8.5 × 10 ⁵	22 × 10 ³ a	22 × 10 ⁴ a
UI-1	5.4 × 10 ⁵	78 × 10 ³ b	15 × 10 ⁴ a
UI-2	4.8 × 10 ⁵	129 × 10 ³ c	52 × 10 ⁴ b
Control	0	0	0

*Parental B-2 is an isolate of *B. subtilis* obtained from sclerotia of *S. cepivorum*; parental isolates of all other bacteria were obtained from rhizospheres of field grown onions.

†Non zero means within a column followed by the same letter do not differ significantly (*P* = 0.05) according to Duncan's multiple range test.

control seedlings. Overall, UI-2 and B were comparatively good colonizers of both root surface and root zone soil, UI-1 and W were intermediate and B-2 was poor.

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 onion seedling root-surface and root-zone soil environments 30 days following seeding (Table 2). Reductions of indigenous bacteria by the five strains used for seed bacterization ranged from 21 to 71% on root surfaces, and 10 to 44% in 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. Reductions in estimated populations of indigenous fungi associated with seed bacterization ranged from 63 to 91% on root surfaces and from 59 to 86% in root-zone soil. 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 environments.

Seed bacterization with B-2, UI-2 and B caused significant increases in shoot height, and shoot dry weight of onion seedlings compared with control treatment and seed bacterization with UI-1 and W (Table 3). Only UI-2 caused a significant increase in root dry weight.

DISCUSSION

The data showing that B-2 persisted in a lower proportion of its introduced population than did any of strains UI-2, UI-1, B and W in both the root surface and the root zone soil environments suggest that B-2 may be less well adapted to the onion rhizosphere environment than are the other four strains. This possibility is consistent with the fact that B-2 was obtained from a sclerotium of *S. cepivorum* (Utkhede and Rahe, 1983) whereas the other four bacteria were obtained from the rhizospheres of commercial onions growing in muck soil.

Each of the introduced bacteria consistently reduced the populations of indigenous bacteria and fungi recovered from onion seedling root surfaces and root zone soils 30 days after seeding; only in the case of strain UI-1 for indigenous bacteria in root zone soil was the effect not significant (Table 2). The magnitude of suppression of indigenous fungal and bacterial populations by the introduced strains was not proportional to their populations in the two environments at 30 days. Of the five strains evaluated, B-2 and UI-2 were the most effective in suppressing the recovery of indigenous microflora; these two strains

Table 2. Effect of seed bacterization with marked strains of *B. subtilis* B-2 and four onion rhizobacteria on populations of indigenous bacteria and fungi detected by dilution plating from onion seedling root surfaces and root zone soil 30 days after seeding in the field

Treatment*	Root surfaces (cfu plant ⁻¹)		Root zone soil (cfu g ⁻¹ dry soil)	
	Bacteria	Fungi	Bacteria	Fungi
B-2	1.74 × 10 ⁵ b†	1.24 × 10 ³ a	2.12 × 10 ⁶ a	2.34 × 10 ⁴ a
B	2.21 × 10 ⁵ c	2.04 × 10 ³ a	2.54 × 10 ⁶ b	2.72 × 10 ⁴ a
W	3.37 × 10 ⁵ d	5.25 × 10 ³ b	2.92 × 10 ⁶ c	6.94 × 10 ⁴ c
UI-1	3.35 × 10 ⁵ d	4.73 × 10 ³ b	3.39 × 10 ⁶ d	4.71 × 10 ⁴ b
UI-2	1.25 × 10 ⁵ a	1.95 × 10 ³ b	2.12 × 10 ⁶ a	2.29 × 10 ⁴ a
Control	4.24 × 10 ⁵ e	14.1 × 10 ³ c	3.77 × 10 ⁶ d	16.9 × 10 ⁴ d

*Parental B-2 is an isolate of *B. subtilis* obtained from sclerotia of *S. cepivorum*; parental isolates of all other bacteria used for seed treatment were obtained from rhizospheres of field grown onions.

†Means within a column followed by the same letter do not differ significantly (*P* = 0.05) according to Duncan's multiple range test.

Table 3. Effect of seed bacterization with marked strains of *B. subtilis* B-2 and four onion rhizobacteria on shoot height and shoot and root dry weights of onion seedlings 30 days after seeding

Treatment*	Shoot height		Shoot dry weight		Root dry weight	
	(cm)†	% of control	(mg)†	% of control	(mg)†	% of control
B-2	8.0 c	138	8.6 b	134	0.79 bc	132
B	7.0 b	122	8.0 b	124	0.62 ab	103
W	5.4 a	93	6.3 a	98	0.60 a	99
UI-1	5.8 a	100	6.1 a	94	0.60 ab	100
UI-2	8.0 c	138	8.4 b	130	0.86 c	143
Control	5.8 a	100	6.4 a	100	0.60 ab	100

*Parental B-2 is an isolate of *B. subtilis* obtained from sclerotia of *S. cepivorum*; parental isolates of all other bacteria were obtained from rhizospheres of field grown onions.

†Means within a column followed by the same letter do not differ significantly (*P* = 0.05) according to Duncan's multiple range test.

were recovered in the lowest and highest proportions, respectively, of the five strains. The relative degree of suppression of indigenous root surface and root zone soil microflora caused by the various strains was correlated with their qualitative ability to inhibit mycelial growth of *S. cepivorum* in dual culture, however. Strains UI-1 and W did not inhibit *S. cepivorum* in dual culture; these two strains consistently showed significantly less suppression of indigenous microflora than did the other three strains (Table 2).

The value of these conclusions is tempered by the fact that estimates of populations of soil microflora by dilution plating on selective media gives a biased picture of the total indigenous microflora in these environments. The bias is likely to be in favor of those competitive saprophytes that exploit and convert available nutrients to large numbers of spores or cells capable of rapid germination or growth. Nevertheless, the definitive result of this study is that the introduction of approx. 1×10^6 bacterial cells per seed significantly affected populations of several components of the indigenous rhizosphere and root zone microflora over a period of at least 30 days under field conditions. We conclude that seed bacterization has the potential to significantly affect populations of at least some indigenous soil microorganisms over relatively long periods under field conditions.

Enhanced growth of bacterized onion seedlings compared with control seedlings was not correlated with relative populations of the various strains recovered from the root surface or root zone soil at 30 days. Seed bacterization with strains B-2 and UI-2, the strains showing the least and greatest persistence, respectively, caused significant increases in seedling growth (dry weight); seedling growth was unaffected by bacterization with strains W and UI-1 (Table 3). The relative effects of the five strains on seedling growth were similar to their relative effects on indigenous rhizosphere and root zone microflora. These results partially support the hypothesis that the mechanism of growth enhancement in the case of the five strains evaluated in this study was indirect and resulted from effects of the introduced bacteria on indigenous root zone microflora. However, the fact inconsistent with this conclusion is that generally significant reductions of indigenous rhizosphere and root zone microflora were also given by the non-growth promoting strains W and UI-1 as well as by the growth promoting strains B-2 and UI-2.

Kloepper and Schroth (1981) reported that inoculation of potato seed pieces with plant growth-promoting rhizobacteria (PGPR) resulted in generally decreased populations of indigenous Gram-positive bacteria and fungi in the root zone soil. Suslow (1982) observed both a reduction in the total population density of root colonizing fungi and a shift in the populations 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 pathogens that damage roots and reduce plant growth (Suslow *et al.*, 1979). Parasitic and non-parasitic bacteria and fungi colonizing plant roots can cause disease or reduced plant vigor (Salt, 1979; Woltz, 1978). Other workers have speculated that reduction of parasitic and non-

parasitic rhizosphere microorganisms by fumigation or chemical seed treatments contributes in part to the generally associated enhanced plant growth (Bowen, 1979, 1980; Merriman *et al.*, 1974; Salt, 1979).

The use of marked bacterial strains tolerant to combinations of antibiotics that exclude growth of indigenous soil microflora 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-2 and UI-2 to significantly modify the indigenous microflora reveals the possibilities that seed bacterization offers for affecting the rhizosphere microflora and plant growth and health under field conditions. The selection of efficient strains for various purposes and the elucidation of the mechanisms of action of these various strains pose immense and exciting challenges.

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