

## Electron Microscopy of Root Colonization of *Setaria viridis* by Deleterious Rhizobacteria as Affected by Soil Properties

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### Abstract

Root colonization of green foxtail (*Setaria viridis*) seedlings grown in three different soils inoculated with selected deleterious rhizobacteria (DRB) was studied using scanning electron microscopy. The three DRB originated from *Setaria faberi* seedlings growing in soils under different management and with different organic matter and pH. The DRB strains reduced green foxtail seedling growth 50% in agar bioassays. Green foxtail seedling shoot growth was significantly affected by the interaction of soil and DRB. Electron microscopic observations revealed the DRB strains preferentially colonized root surface crevices over ridges. Each strain densely colonized roots of green foxtail when inoculated in its native soil, probably due to adaptation to the particular chemical, physical, and biological properties embodied by each soil. Bacterial colonization and establishment on seedling roots led to growth inhibition. *Pseudomonas fluorescens* strain L2-19 and *Stenotrophomonas maltophilia* strain TFR1 significantly reduced green foxtail growth in all three soils, however, *P. putida* strain B1-7 inhibited green foxtail only in the high organic matter, high pH soil under an organic farming management system.

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All three strains significantly inhibited green foxtail shoot growth in the organically managed soil, indicating that organic matter content is an important factor affecting growth suppressive activity of DRB. In general, seedlings grown in an uncultivated prairie soil with high organic matter and low pH produced less biomass than those grown in other soils. This suggests that soil pH also may be involved in the growth suppression of host plants by DRB.

Keywords: Colonization, rhizobacteria, plant growth suppression, pseudomonads, rhizosphere, soil microcosms

## 1. Introduction

Microorganisms in the rhizosphere environment may be saprophytic, plant-growth promotive, or detrimental to plant growth (Fredrickson and Elliott, 1985). Those bacteria detrimental to plant growth known as deleterious rhizobacteria (DRB) live superficially on plants and induce damage by liberation of toxins or other detrimental substances that are absorbed by the plant (Woltz, 1978). DRB prevent seed germination, cause root distortions and lesions, retard root growth, and predispose roots to infection by fungi (Suslow and Schroth, 1982). DRB that curtail plant growth and reproduction may be useful for biological control of weeds (Souissi et al., 1997). Reports of growth inhibitory effects of DRB on several weed species illustrate the potential for biological weed control (Kennedy et al., 1991; Kremer et al., 1990).

Root colonization is an essential step for rhizobacteria to establish on rhizoplane or in rhizosphere and further exert suppressive activity on host plants. Growth suppressive effects of rhizobacteria on their host plants will depend on their ability to grow in the root environment and to colonize the root surface. Root colonization takes place at the root surface, the inside of the root and/or the rhizosphere (Weller and Thomashow, 1994) and is a series of complex events that determine the efficacy of DRB. The distribution, multiplication, and survival of DRB are profoundly affected by biotic and abiotic factors. Soil moisture, soil texture, plant species, root exudate composition, and bacterial viability can influence colonization of the rhizosphere and rhizoplane (Howie and Echandi, 1983). Fredrickson and Elliott (1985) found that colonization was similar at root zone temperatures of 5, 10, and 15°C but was dependent on soil type, with higher populations occurring in soil with lower organic matter and microbial biomass.

The biological composition of the rhizosphere dramatically influences root colonization. Introduced bacteria must establish and grow in an ecological habitat that includes indigenous microorganisms (Schroth and Becker, 1990) because root colonization is a competitive process affected by characteristics of both the DRB and the host. Investigations of root colonization by selected DRB

in the soil environment is essential for understanding of the complex phenomena influencing colonization, survival, and suppressive activity of potential biological control agents in the field.

In preliminary studies DRB originating from the economically important weed giant foxtail significantly reduced green foxtail in agar bioassays (Li and Kremer, 2000). The effectiveness of selected DRB strains in suppressing green foxtail growth may depend on their ability to adapt to the root environment, colonize the root surface, and exert deleterious effects on seedling roots. Soil environmental factors, such as soil organic matter, soil pH, and soil physical properties, greatly affect the colonization and impact on root growth by DRB. This study was conducted to understand the association of DRB with root surfaces of green foxtail and to relate these associations to effects on root growth and morphology; and to determine the effects of different soil properties on colonization and growth suppressive activity of rhizobacteria.

## 2. Materials and Methods

### *Bacterial cultures*

All rhizobacteria strains were isolated from the rhizosphere of giant foxtail seedlings growing in different ecosystems (Li and Kremer, 2000). *Pseudomonas fluorescens* strain L2-19 originated from seedlings growing in strawberry (*Fragaria virginiana*) under an organic farming system (OF) that received no synthetic chemical inputs. *Pseudomonas putida* strain B1-7 originated from a maize (*Zea mays*) monoculture under conventional tillage and chemical fertilizer and herbicide inputs (SF). *Stenotrophomonas maltophilia* strain TFR1 originated from an uncultivated prairie of native, warmseason grasses and forbs (TP). Each bacterial isolate was cultured on modified King's B agar medium (Sands and Rovira, 1970), incubated at 27°C for 48 h, and suspended in sterile 0.1 M MgSO<sub>4</sub> solution prior to inoculation of microcosms.

### *Microcosm growth experiment*

Microcosms consisting of polystyrene test tubes (17 × 100 mm) with small holes drilled in the bottom for drainage contained a bottom layer of vermiculite approximately 1 cm deep over which was dispensed approximately 12 g soil (moist weight). Soils from three different ecosystems were classified as Mexico silt loam (fine, montmorillonitic, Mesic, Aeric Vertic Epiaqualf), differed in organic matter content and pH (Table 1). Soils were exposed to microwave radiation for two minutes with full power (625W heating power, 2,450 MHz) to reduce the populations of soilborne pathogens. Microwave treatment reduces

recolonization of soil by *Fusarium* and other fungi without releasing excessive nutrients into the soil solution that would stimulate soil prokaryotes (Ferriss, 1984).

Green foxtail was used as the test weed species because germination was more consistent than giant foxtail and effects of DRB were similar on both species. Seeds were surface sterilized by immersion in 5% sodium hypochlorite for 4 min, rinsing with sterile distilled water five times, followed by immersion in 70% ethanol for 5 min and fully rinsed with sterile distilled water. The surface-sterilized seeds were pregerminated on 1% agar for 50 hr at 27°C. Five pregerminated seeds were planted in each microcosm. Seeds in each treatment were inoculated with 2 ml bacterial suspension containing log 9 cfu. Controls received 2 ml of 0.1 M MgSO<sub>4</sub> alone. Seeds were lightly covered with soil after inoculation. The microcosms were set up in the laboratory at ambient temperature (19–24°C) and supplemented with incandescent light.

Symptoms and abnormalities developing on green foxtail seedlings were recorded during the growth period. Seedlings were harvested 3 wk after inoculation. Representative seedlings from each treatment were selected for SEM observations. Fresh and dry shoot weights of remaining seedlings were determined for each treatment. At harvest, root systems of each plant were thoroughly rinsed in sterile distilled water after which they were suspended in sterile phosphate-buffered saline (PBS; 0.01 M K<sub>2</sub>HPO<sub>4</sub>, 0.14 M NaCl, pH 7.2) containing 0.01% (v/v) Tween 20 and agitated vigorously on a vortex shaker for 5 min. Bacterial populations were determined by serially diluting (10-fold steps) the root suspension in PBS and spread plating onto duplicate plates of King's B agar and incubating for 72 hr at 27°C.

#### *Scanning electron microscopy*

Roots of inoculated and uninoculated seedlings were thoroughly washed in distilled water, sectioned into 5–6 mm lengths and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (pH 7.0) for 4 hr at 4°C. Samples were washed in the same buffer over 1 hr and postfixed in 1% osmium tetroxide for 4 hr. The fixed root specimens were rinsed in distilled water, and dehydrated through a graded ethanol series (20, 40, 60, 80, 95, and 100%) and critical point dried in liquid carbon dioxide. Dried specimens were mounted on aluminum stubs and sputter coated with gold-palladium alloy.

The specimens were examined and photographed with an Amray 1600C scanning electron microscope. The parameters were as follows: beam current: 10 KeV; electron source: secondary electrons; spot size: 6; objective lens aperture: 100 µm; tilt angle: 22.8.

Table 1. Chemical properties of soils used

Site (code)	pHs	O.M. <sup>1</sup> %	P mg/kg	Ca mg/kg	Mg mg/kg	K mg/kg
Organic farm (OF)	6.6	6.2	130	2470	320	280
Maize monoculture (SF)	5.8	3.2	90	1210	80	140
Uncultivated prairie (TP)	4.9	5.3	20	1800	320	250

<sup>1</sup>O.M. = Organic matter content (%).

Table 2. Shoot fresh weight of green foxtail affected by soil and DRB inoculation<sup>1</sup>

DRB strain	Shoot fresh weight (g · microcosm <sup>-1</sup> )		
	SF	OF	TP
Control	9.0 (100) <sup>2</sup>	8.3 (100)	4.9 (100)
B1-7	8.7 (96)	3.5 (42)	5.1 (104)
L2-19	4.7 (52)	3.2 (38)	1.7 (34)
TFR1	6.2 (68)	3.1 (37)	1.9 (38)
LSD (0.05) <sup>3</sup>		0.98	

<sup>1</sup>See Table 1 for description of site codes for soils. <sup>2</sup>The number in parenthesis is the percentage of control. <sup>3</sup>LSD value for comparison across all means.

### Statistical analysis

This study was a two-factor completely randomized block design. Three soils and three isolates plus control without inoculation for each soil were tested for bacterial colonization of seedling roots in a soil microcosm. Three replications were performed for each treatment and the study was repeated once. The fresh and dry shoot weights of each treatment were subjected to two-way analysis of variance. Where F-test was significant, treatment means were separated using LSD ( $\alpha=0.05$ ).

## 3. Results

### Visual observations

Selected bacterial isolates reduced shoot growth of green foxtail in non-

sterile soil within eight days after inoculation. At 14 d after inoculation, uninoculated controls exhibited taller shoot height, and more expanded healthy leaves relative to seedlings inoculated with DRB strains. Visually, strain L2-19 dramatically reduced root growth in OF soil. Strain L2-19 also severely injured seedling roots in OF soil resulting in extremely short and discolored tap roots with no lateral roots or root hairs. Strains B1-7 and TFR1 inhibited shoot growth when inoculated in OF soil.

Strain L2-19 greatly inhibited shoot growth when inoculated in TP soil. Strain TFR1 inoculated in TP soil consistently inhibited and retarded shoot growth compared to the control. Generally, seedlings grown in TP soil had less shoot biomass compared to the other two soils. B1-7 inoculated in TP soil did not significantly reduce seedling growth.

For green foxtail grown in SF soil and inoculated with B1-7, no apparent shoot growth inhibition was observed relative to the controls. TFR1 reduced shoot biomass, without other symptoms, of seedlings grown in SF soil. Shoot growth of seedlings grown in SF soil and inoculated with L2-19 was inhibited as manifested by shorter shoot and smaller dark green leaves. Growth retardation was obvious because the control plants were in the 3-leaf stage while treated plants in 2- or 2.5-leaf stage.

The root fresh weights were not determined for each treatment due to the lack of material. Inoculated roots were 2 mm or less in length while controls were 2–3 cm. A closer examination of root systems revealed that seedlings inoculated with deleterious rhizobacteria had shorter tap roots, less lateral root development, and less root biomass in contrast to the well-developed root systems of uninoculated control.

#### *Soil effects*

Growth responses of green foxtail to DRB strains varied depending on soil (Table 2). Strain B1-7 (from SF soil) inhibited green foxtail seedling growth only in OF soil, which had the highest organic matter content (6.6%). Strain L2-19 from OF soil consistently inhibited growth of green foxtail in all soils. Shoot growth of seedlings inoculated with TFR1 (from TP soil) was significantly reduced in all soils, but most extensively in OF and TP soils. All strains inhibited shoot growth of green foxtail similarly when inoculated in OF soil. The growth inhibition caused by L2-19 and TFR1 in TP soil was not significantly different. Seedling biomass across all treatments in TP soil was lowest compared to the other soils.

#### *Electron microscopic studies*

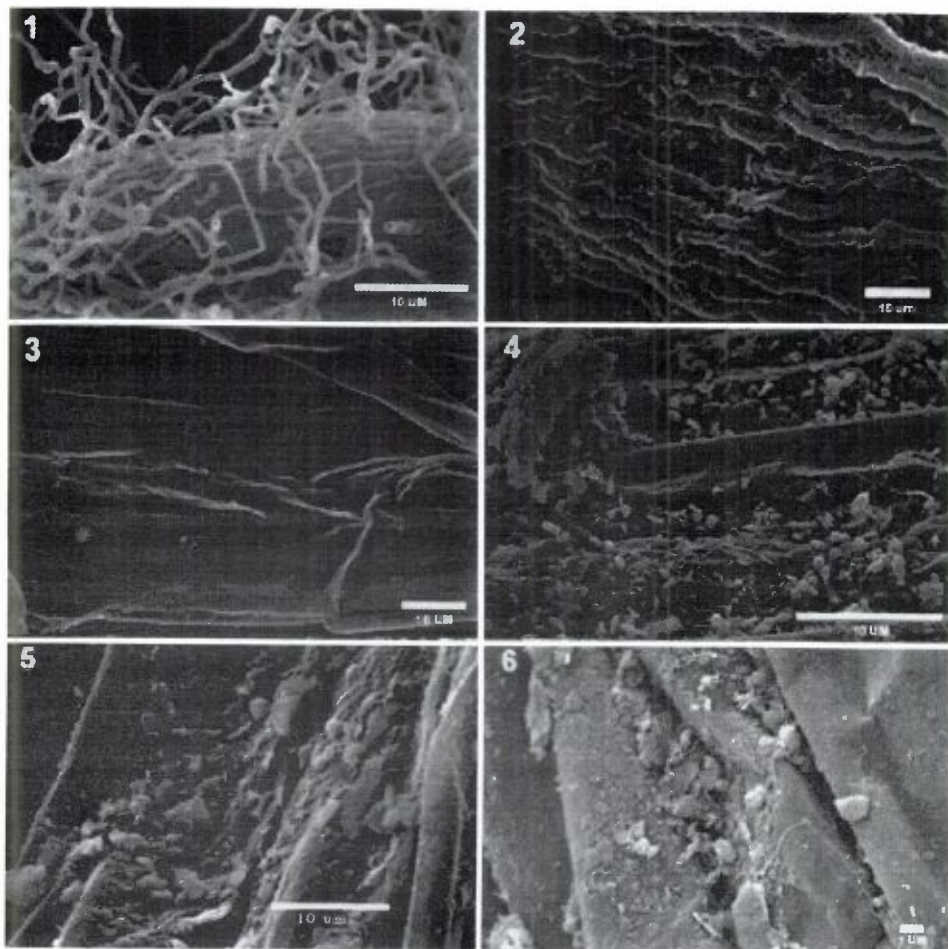
DRB associated with seedlings caused root morphological abnormalities in

contrast to healthy control seedlings grown in OF soil characterized by abundant and turgid root hairs (Fig. 1). Strain L2-19, for example, completely inhibited root hair development (Fig. 2). Surfaces of roots of uninoculated green foxtail seedlings were smooth with cells of the epidermal layer forming a series of parallel ridges and valleys (Fig. 3). No discernable bacteria were observed on the root surface. In contrast, root surfaces from DRB-inoculated seedlings exhibited large surface populations with bacteria intruding into surface crevices (Figs. 4–6). The root surfaces of inoculated seedlings were generally rough with large amounts of irregular, amorphous particles, in which bacterial cells often were embedded (i.e., Fig. 6). The aggregates may have formed after leakage of cellular substances through activity of bacteria colonizing root cells leading to increased membrane permeability due to substances secreted by rhizobacteria to facilitate root cell attachment (Souissi et al., 1997). Cells of DRB strains were typically rod-shaped, single-celled and in different growth stages, as revealed by size differences among individual bacterial cells (Figs. 4, 7, 8). The distribution of bacteria on host cells followed no specific pattern although most cells were clustered in crevices or as aggregates scattered on the ridges.

DRB strains colonized the rhizoplane of green foxtail differently in each soil. When L2-19 was inoculated in its native soil, OF, the root surface was densely colonized (Fig. 7). Colonization of the root surface by L2-19 inoculant was denser in TP soil than in OF soil (Fig. 9) and even less root colonization occurred in SF soil (Fig. 5). When TFR1 was inoculated in its native soil, TP, more colonization was in crevices adjacent to emerging root hairs and associated with large amounts of fibrils and large aggregates (Fig. 8). Fewer bacterial cells were found attached to seedling roots grown in OF soil, bacteria appeared to adhere to fibrils or aggregates, which may help the attachment and provide a protected environment for multiplication (Campbell et al., 1987). Fewer cells of TFR1 colonized the crevices of seedling roots grown in SF soil (Fig. 6). Strain B1-7 was associated with a particular colonization pattern when inoculated in SF soil noted as clustering of numerous cells in shallow crevices leaving the ridges intact and smooth (Fig. 4). Abundant colonization by B1-7 also occurred in OF soil (Fig. 10).

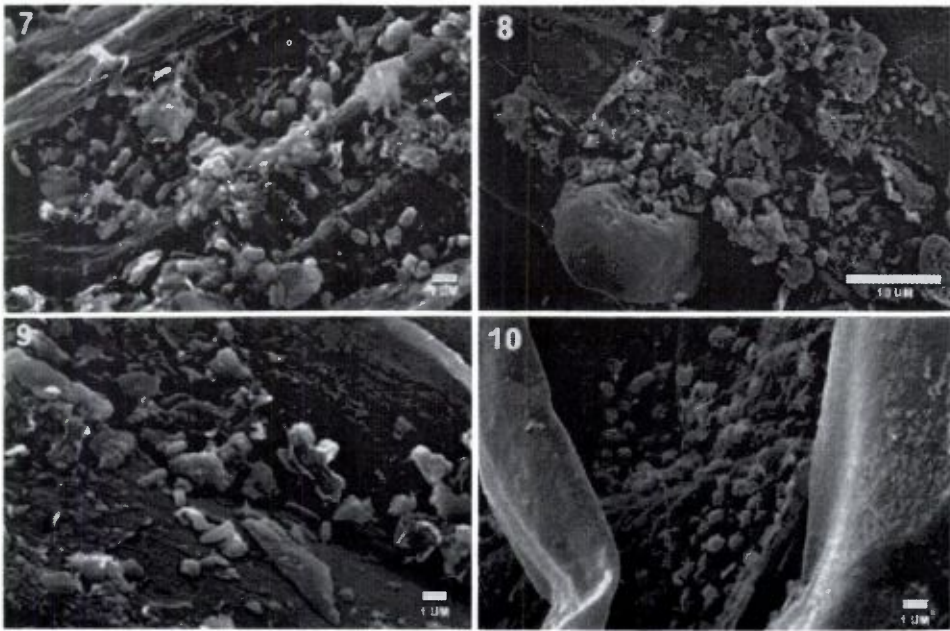
#### 4. Discussion

Following introduction in soil, DRB strains B1-7, L2-19, and TFR1 aggressively colonized roots of green foxtail seedlings. The test tube microcosm provided a soil environment for plants simulating natural conditions that bacteria encounter in the field. Scanning electron microscopy revealed the attachment and population increases of DRB on root surfaces in non-sterile



- Figure 1. Root surface of non-inoculated green foxtail seedling in soil from organic farming system (OF) showing abundant and turgid root hairs.
- Figure 2. Root surface of green foxtail seedling colonized by *P. fluorescens* L2-19 in OF soil showing lack of root hair development.
- Figure 3. Root surface of non-inoculated green foxtail seedling in soil from maize monoculture system (SF).
- Figure 4. Root surface of green foxtail seedling colonized by *Pseudomonas putida* B1-7 in SF soil abundant colonization and clustering of bacterial cells in root crevices.
- Figure 5. Root surface of green foxtail seedling colonized by *P. fluorescens* L2-19 in SF soil showing scattered bacterial cells in root crevices.
- Figure 6. Root surface of green foxtail seedling colonized by *Stenotrophomonas maltophilia* TFR1 in SF soil. Bacterial cells are embedded with amorphous particles within crevices of the root surface.





- Figure 7. Root surface of green foxtail seedling colonized by *P. fluorescens* L2-19 in OF soil showing bacterial aggregates in crevices.
- Figure 8. Root surface of green foxtail seedling colonized by *Stenotrophomonas maltophilia* TFR1 in Tucker Prairie (TP) soil.
- Figure 9. Root surface of green foxtail seedling colonized by *P. fluorescens* L2-19 in Tucker Prairie (TP) soil.
- Figure 10. Root tissue of green foxtail seedling colonized by *P. putida* B107 in OF soil.

conditions. Unlike the uniform colonization patterns on seedling roots grown in gnotobiotic systems where introduced bacteria cover much of the root surface without competition from indigenous microorganisms, DRB strains preferentially established in root crevices or root junctures when inoculated in soil. Previous research showed that rhizobacteria growing in or near infection courts and in micro-channels in the rhizosphere that provide physical access to roots are ideally positioned to limit establishment and spread of competitors (Weller and Thomashow, 1994). Crevices of roots provide a unique and protected environments for bacteria as roots grow through abrasive soil particles. Close contact or attachment to the rhizoplane likely enhances uptake and internal transport of bacterially-produced compounds into internal root tissues. Howie et al. (1987) hypothesized a two-phase process for translocation of rhizobacteria from inoculum to critical sites along the root and

in the rhizosphere. In phase I, bacteria on seeds are attracted to the emerging root tip and are passively translocated with the growing roots. During the process of root growth, some cells are left behind on older portions of the root while some cells remain associated with the root tip. Bacteria may be dislodged as the root extends through the soil or become adsorbed to soil particles (Bahme and Schroth, 1987). In phase II, bacteria deposited along the root multiply and form microcolonies in nutrient-rich microsites, compete with indigenous microflora and avoid displacement. Any bacterium applied to or near the seed can be transported into the soil with the roots but only those that are rhizosphere competent will maintain or increase their population on the root (Howie et al., 1987).

Root elongation, root biomass accumulation, and root hair development of inoculated seedlings was significantly inhibited by DRB. Root morphology of inoculated seedlings were dramatically changed compared to the uninoculated controls, suggesting that growth inhibitory effects resulted from disruption of physiological functions. DRB attached to root cells may intrude into intracellular spaces in a manner affecting cell permeability of the roots (Souissi et al., 1997) causing leakage of nutrients and other intracellular substances. This increased production of root exudates or lysates can sustain continued colonization of the rhizosphere by DRB through chemotaxis (Begonia and Kremer, 1994). Attraction to root exudates largely accounts for the high root-colonizing capacity of many rhizosphere bacteria (Scher et al., 1984).

Compared to the uninoculated controls where no microbial particles or aggregates accumulated on root surfaces, inoculated tissues contained large amounts of aggregates or exudates, probably mixed with bacterial secretions. Development of bacterial fibrils on the rhizoplane may anchor and entrap more DRB cells (Souissi et al., 1997). The elaboration of fibrils by bacteria may result from the binding of the lipopolysaccharide component of bacterial cell envelopes to receptor sites on roots (Matthyse et al., 1982). Accumulation of mucigel, derived partially from root surface cells and from root-associated microorganisms (Greaves and Darbyshire, 1972), on the root surface was promoted by the colonizing bacteria, previously demonstrated with the DRB interaction with velvetleaf (*Abutilon theophrasti*) (Begonia et al., 1990).

Plant growth responses are affected by DRB in a manner similar to phytopathogenic bacteria through the production of toxins, enzymes, or growth regulating substances (Goodman et al., 1986). Souissi et al. (1997) suggested that cell walls appeared to be dissolved or eroded at some points of contact between DRB and leafy spurge cells. Damaged root surfaces and cellular debris may be due to production of hydrolytic enzymes by the colonized rhizobacteria because many plant bacteria typically produce an array of cell-wall-degrading enzymes during pathogenesis to facilitate the penetration into and colonization of host plant tissue (Collmer and Keen, 1986; Goodman et al., 1986).

Abundant colonization of the rhizoplane does not always lead to phytotoxic effects. Although strain B1-7 appeared to densely colonize the root surface when inoculated in SF soil (Fig. 4), it did not significantly inhibit seedling growth. Factors that contribute to inconsistent effects of DRB on the host in the soil environment include variability in root colonization and inconsistent production or inactivation *in situ* of secondary metabolites such as siderophores and exopolysaccharides that contribute to phytotoxicity (Weller, 1988). For growth inhibition to occur, production of these metabolites must coincide when the plant is vulnerable to attack. In the rhizosphere, the regulation of metabolically expensive secondary metabolites is likely to be more tightly controlled and very dependent on the environment within the microsite (Weller and Thomashow, 1994). Strain B1-7 reduced shoot growth only in OF soil indicating that expression of possible phytotoxicity genes was very condition-dependent.

Strains L2-19 and TFR1 inhibited green foxtail growth to different extents in different soils. Strain L2-19 inhibited seedling growth of green foxtail to a greater extent in SF soil than did strain TFR1 indicating that isolate L2-19 more readily adapted to SF soil than strain TFR1. Strain B1-7 inhibited green foxtail growth only in OF soil reducing biomass equivalent to the other DRB strains. The high organic matter and near neutral pH of OF soil may be conducive for expression of growth suppressive activity by strain B1-7, which originated from a low organic matter, low pH soil. Araujo et al. (1994) observed a faster decline of inoculant bacteria in clay soil at pH 4.7 than at higher pH levels. Rapid death at low soil pH due to acidity may cause metabolic stress in introduced bacteria, making them less competitive. Rhizobacteria colonize roots optimally at slightly below neutral pH (Weller and Thomashow, 1994), therefore, TP soil may be too acidic for most rhizobacterial isolates despite favorable levels of soil organic matter. Strain TFR1 abundantly colonized root surfaces of green foxtail seedlings in TP soil probably because TFR1 was more adapted to those soil conditions from which it was originally isolated.

All three isolates significantly reduced green foxtail shoot/root growth in OF soil, with the highest organic matter content and pH 6.6, indicating organic matter content played an important role in the suppressive activity of DRB toward weed seedlings. Our results demonstrate that soils with high organic matter harbored high rhizosphere bacterial populations. Root inhibition was lowest for both strains L2-19 and TFR1 when inoculated in SF soil, which further indicates involvement of soil organic matter as an important factor in weed seedling growth suppressive activity by DRB.

Only limited studies have been conducted on the influence of soil properties on the root-colonizing ability and suppressive activity of DRB on plants. Our study presents evidence that soil organic matter is an important factor for expression of deleterious activity of DRB. For DRB to be effective biological

weed control agents, they must survive under field conditions, colonize root surfaces, and exert phytotoxic effects on their host plants. The work presented here is an initial step in studying relationships between environmental parameters, especially soil properties, and the performance of biological control agents. We attempted to determine favorable ecological niches for survival and establishment of introduced DRB for weed control and determine those rhizosphere properties that might be exploited for expression of phytotoxicity toward target weeds by indigenous DRB. Effective tactics using DRB to manage weeds will be devised when there is a good understanding of the many factors that affect the dynamics of the rhizosphere. Our results show that effective use of DRB or other biocontrol agents will depend on matching the microorganism of interest with the environment that favors its activities, or altering the environment to favor the microorganism.

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