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Chemotaxis of deleterious rhizobacteria to birdsfoot trefoil

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Abstract

Intact seeds and seed and seedling root exudates of birdsfoot trefoil (*Lotus corniculatus* L.) were used as chemoattractants in experiments to determine the relative importance of chemotaxis in spermosphere and rhizosphere colonization by selected rhizobacteria. Results for soft-agar, capillary tube and soil chemotaxis assays indicated that selected deleterious rhizobacteria were attracted to seed and seedling root exudates. Several sugars and phenolic fractions detected in exudates were chemoattractants for these rhizobacteria. Using soil-chemotaxis assemblies, migration of rhizobacterial isolates through 2 cm distances of soil toward birdsfoot trefoil seeds was detected within 24 h. Isolates were not detected at the same site in soils without seeds until 72 h after inoculation. These results suggest that attraction of deleterious rhizobacteria toward seeds and seedling roots mediated by exudates (chemotaxis) might be the first step in the establishment and subsequent colonization of bacteria involved in soilborne disease complexes of birdsfoot trefoil. © 1999 Elsevier Science B.V.

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1. Introduction

The presence of exudates in the rhizosphere and spermosphere environments influences microbial activity. Stimulation of microbial growth around seeds and plant roots may result from chemotaxis of micro-organisms toward seeds and plant roots. This is primarily due to exudation of compounds such as amino acids, carbohydrates, organic acids, and other components during germination and seedling development (Vande Boek and Vanderleyden, 1995). Seeds and seed extracts of certain plant species containing diverse compounds directly stimulate the growth of selected micro-organisms (Short and Lacy, 1976; Lynch, 1978; Scher et al., 1985).

Successful activity of rhizosphere bacteria, either beneficial or detrimental species, is dependent on seed and/or root colonization (Lam, 1990). Seed and root colonization are complex processes with numerous associated attributes (Weller, 1988; Lam, 1990). Migration of bacteria toward seed and seedling exudates (chemotaxis) is regarded as an important first step in colonization of seeds and roots in soil (Lynch, 1978; Scher et al., 1985). Chemotaxis, by promoting colonization of the spermosphere and rhizosphere, is therefore, important in initiating interaction of specific bacteria with their host plants. Fluorescent pseudomonads isolated from soil were chemotactic toward soybean seeds and seed exudates placed in a soil chemotaxis assembly (Scher et al., 1985). Plant

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growth-promoting fluorescent pseudomonads exhibited chemotaxis toward tomato seeds and exudates in solarized and nonsolarized soils in a similar assembly (Gamliel and Katan, 1992). Both of these studies showed that germinating seeds and developing seedling roots exuded copious amounts of various compounds to which certain bacteria were attracted. Chemotaxis of specific bacteria toward seed and seedling exudates in soil was related to attraction toward specific components detected in the exudates (Scher et al., 1985; Gamliel and Katan, 1992).

Chemotaxis may present a competitive advantage for certain detrimental micro-organisms in early establishment on the seed and root of many crop plants leading to reduced seedling vigor. Seedling vigor is critical in establishment of birdsfoot trefoil as a forage or seed crop. Birdsfoot trefoil seedlings are often susceptible to various soilborne phytopathogens that reduce vigor and result in poor field establishment. Bacteria in the rhizosphere have been shown to have primary roles in root and crown rot (Berkenkamp et al., 1972) and wilt (Lukezic et al., 1963) diseases of birdsfoot trefoil. Also, inhibition of root and shoot growth of birdsfoot trefoil by selected rhizobacteria was apparently related to colonization of the seedling root surfaces in distinctive patterns (Begonia et al., 1993). In the development of detrimental bacteria as part of the disease complex, chemotactic ability may be a key characteristic required for effectiveness in the spermosphere and/or rhizosphere of birdsfoot trefoil. The objective of this study was to determine if rhizobacteria native to the rhizosphere exhibit chemotaxis to birdsfoot trefoil seed and seedling root exudates in vitro and directly in soil.

2. Materials and methods

2.1. Bacterial strains and inocula preparation

Rhizobacterial strains were originally isolated from rhizospheres of birdsfoot trefoil seedlings collected from field plots located at the University of Missouri Agronomy Research Center (UMARC), 16 km east of Columbia. The strains varied in their effects on birdsfoot trefoil seedlings including suppressive, promotive and neutral growth effects (Begonia, 1989). Cultures were maintained on agar slants of Sands and Rovira (SR) medium (Sands and Rovira, 1970) which were overlayed with sterile mineral oil and stored at 3°C. Another set of cultures was lyophilized and stored in the freezer at −40°C.

Bacteria for chemotaxis assays were grown for 36 h in SR broth on a rotary shaker at 150 rpm at room temperature. Cells were harvested by centrifugation at $1200\times g$ for 20 min at 5°C and washed three times with phosphate buffered saline (PBS; 0.01 M K_2HPO_4–KH_2PO_4, 0.14 M NaCl, pH 7.2). Pelleted cells were resuspended in PBS and absorbance at 420 nm was adjusted to yield a concentration of at least 10^8–10^9 cells ml^{-1} estimated by optical density using a spectrophotometer. Before chemotaxis assays were performed, motility of the bacterial cells was examined microscopically.

2.2. Seed exudate

Seed exudate was prepared according to the method of Scher et al. (1985) with slight modifications. Birdsfoot trefoil seeds (‘MU 81’) were surfaced sterilized by immersion in 1.25% (w/v) sodium hypochlorite for 8 min; immersion in 70% (v/v) ethanol for 4 min and then rinsed thoroughly (5× at least) with sterile distilled water. Seventeen grams of surface-sterilized birdsfoot trefoil seeds were added to 100 ml sterile distilled water in 250 ml Erlenmeyer flasks. Flasks were incubated on a rotary shaker at 60 rpm at 27°C. After 20 h, exudates were collected and filter-sterilized through a 0.20 μm Acrodisc membrane (Gelman). Sterile exudates were kept in vials and stored in the freezer. A portion of the exudate was concentrated to 10% of its original volume by rotary evaporation at 45°C.

2.3. Seedling exudate

Seedling root exudates were prepared in a manner similar to seed exudates. Exudates from seedlings grown from surface-sterilized seeds were collected in 100 ml sterile distilled water contained in a 1 l beaker. A stainless steel wire mesh (3×3 mm) fitted inside the beaker supported the seedlings during growth for 5–7 days at room temperature under light. Seedling exudates were filter-sterilized, dispensed in vials and frozen. Portions of the collected exudates were concentrated to 10% of the original volume by

rotary evaporation at 45°C. All exudates were tested for contamination by streaking on potato dextrose agar (Difco) and any contaminated exudate was discarded.

2.4. Chemical characterization of seed and seedling root exudates

Compounds present in seed and seedling exudates were screened to determine the relationship to chemotactic activity toward the crude exudates. Free amino acids were detected and quantified using an amino acid analyzer (Beckman Model 121 M) with a 56 cm AA-15 ion exchange resin equilibrated with 0.2 N sodium citrate buffer, pH 2.2. Amino acid identity was determined using standard solutions of known amino acids and comparing elution times. Quantities of known amino acids were determined with a computing integrator attached to the amino acid analyzer. Sugars were detected by spotting 50 μl of the aqueous concentrated exudates onto silica gel TLC plates (Sigma) and developing in *n*-butanol:glacial acetic acid:water (4:1:5 v/v/v, upper phase) (Harborne, 1984). After development and drying, plates were sprayed with orcinol–ferric chloride and heated for 10 min at 100°C. Comparisons of R_f values were made to 1 mM sugar standards. Organic acids were detected using the same TLC procedure as for sugars except that dried plates were sprayed with 0.04% brom cresol green in ethanol and comparisons were made to 1 mM organic acid standards. Total phenolic contents of the exudates and extracts were determined colorimetrically using the Folin–Denis method (Horowitz, 1980). Aqueous solutions of tannic acid served as standards for this analysis.

2.5. Chemotaxis in vitro

Chemotaxis of the rhizobacteria toward seed and seedling exudates were determined using two in vitro procedures. For the soft agar assay (Adler, 1973), a heavy bacterial suspension was spotted onto 0.2% water agar containing concentrated seed or seedling exudate. Each treatment included a control which contained either PBS or sterile distilled water. Outward migration of bacterial cells as measured by the diameter of resulting swarms were determined after at least 24 h but not more than 120 h of incubation at 27°C.

A capillary tube method was carried out according to Adler (1966) as modified by Scher et al. (1985). Capillary tubes were filled with either seed or seedling root exudate, individual test compounds, or PBS and distilled water as controls. One end of each filled capillary tube was heat sealed. Individual capillaries were placed in sterile polystyrene tubes (12×75 mm) containing 0.5 ml of bacterial suspension in PBS (10^8 cells ml^{-1}) for 1 h. Capillary tubes were removed, and contents expelled into 5 ml PBS by breaking the heat sealed end. The contents were serially diluted in PBS. Bacterial concentrations in each capillary tube were determined by spread-plating onto duplicate SR agar plates. Plates were incubated at 27°C for 48 h and colonies enumerated. Results were expressed as the mean of at least 3 separate assays.

2.6. Chemotaxis of bacteria in soil

Chemotaxis of bacteria toward imbibed seeds, seed and seedling exudates in soil was carried out using the method of Scher et al. (1985). Mexico silt loam (Aeric Vertic Epiaqualf, pH 5.9) was sieved (4 mm mesh), amended with 10^9 cfu g^{-1} and held in an outer ring of a 20×100 mm Petri glass dish holding a 40 mm diameter×20 mm deep interior glass ring. Either pre-imbibed seeds (10 seeds per plate) or 5 μl of concentrated exudate was placed in the center of non-inoculated soil in the inner ring (at 1 cm depth) and covered. Total soil per plate was 70 g and was adjusted to 30% moisture by addition of sterile water to both compartments of soil. The inner ring barrier was removed and the plates were tamped lightly to seal the fissure. Subsequently, soil samples were taken from the center ('seed or exudate site'); just inside the inner ring ('2 cm site'); and from the inoculated soil in the outer ring ('inoculation site') by vertically inserting a narrow spatula and withdrawing 0.1 g soil into 9.9 ml PBS. Soil samples were serially diluted and plated on SR agar. After incubation for 3–4 days at 27°C, colonies forming on the plates were enumerated. Each treatment included a control in which neither seed nor exudate was present in the center ring. The plate assemblies were arranged in a completely randomized design and replicated three times.

Rhizobacteria in the inocula were detected by their growth patterns and distinctive colony characteristics on SR agar. Strain identification was further verified

by frequent culturing randomly selected colonies from soil platings onto SR medium containing multiple antibiotics at concentrations to which each strain was intrinsically resistant (Begonia, 1989). Antibiotic concentrations for rhizobacterial strains under study were 100 $\mu g\ ml^{-1}$ nalidixic acid, 5 $\mu g\ ml^{-1}$ rifampicin, 5 $\mu g\ ml^{-1}$ streptomycin, and 80 $\mu g\ ml^{-1}$ tetracycline for *Pseudomonas aeruginosa* Pa007; and 100 $\mu g\ ml^{-1}$ nalidixic acid, 10 $\mu g\ ml^{-1}$ rifampicin, 40 $\mu g\ ml^{-1}$ streptomycin, and 30 $\mu g\ ml^{-1}$ tetracycline for *Alcaligenes* sp. AS004. No indigenous bacteria were detected in the test soil that possessed intrinsic antibiotic resistance patterns similar to the selected strains.

2.7. Statistical analyses

Analysis of variance was conducted on all data for each experiment. Where F-values were significant at $p<0.05$ level, least significant differences (LSD) were calculated for mean separation.

3. Results

3.1. Chemotaxis: In vitro assays

Seven birdsfoot trefoil rhizobacteria and *Mesorhizobium loti* (formerly *Rhizobium loti*) strain 3078 exhibited chemotaxis toward filter-sterilized birdsfoot trefoil seed exudates in soft agar (Table 1). Chemotaxis, indicated by outward migration of cells through the agar, produced swarm diameters ranging from 5 to 11 mm when exudate was incorporated in the agar. However, when exudate was omitted from the agar (control treatment), no swarm by birdsfoot trefoil rhizobacteria was observed. Birdsfoot trefoil seedling inhibition data (Begonia, 1989) were included to indicate the relationship of chemotaxis and deleterious activity by certain isolates. *Mesorhizobium loti* 3078 (USDA strain obtained from Beltsville Rhizobium Culture Collection), a nitrogen-fixing microsymbiont and which showed strong chemotaxis in the soft agar assay, also exhibited chemotaxis toward birdsfoot trefoil seed exudate in the capillary tube assay (Fig. 1). Significant cell densities of *M. loti* 3078 in capillaries containing seed exudate were detected beginning at 60 min of incubation and were continually detected throughout 120 min of incubation when the maximum density of 2.5×10^{6} cfu was reached. Relative chemotactic ability of all rhizobacterial strains was similar for both the soft agar and capillary tube assays (data not shown). The significant response of *M. loti* and the rhizobacterial strains to birdsfoot trefoil exudates suggested that specific compounds present in exudates were responsible for the active chemotaxis.

Birdsfoot trefoil seed and seedling exudates contained eight sugars at concentrations <1 mM. Chemotactic responses to exudates and chemical components were similar among all bacterial strains, thus representative results for strain Pp007 are presented. Of the sugars present at greatest concentrations, Pp007 was significantly chemotactic to all but arabinose and

Table 1
Chemotaxis of birdsfoot trefoil rhizobacteria to concentrated filter sterilized birdsfoot trefoil seed extract after 48 h dark incubation at 27°C (soft-agar assay)

Rhizobacterial strain		Swarm diameter (mm)[a]	Birdsfoot trefoil seedling inhibition (%)[b]
Pseudomonas aureofaciens	Pa001	5b	0.0
Pseudomonas sp.	Ps002	7b	0.0
P. fluorescens	Pf003	5b	0.0
Alcaligenes sp.	As004	6b	16.5
P. putida	Pp007	8b	31.0
Pseudomonas sp.	Ps011	10a	0.0
Pseudomonas sp.	Ps012	11a	0.0
Rhizobium loti	3078	10a	0.0

[a] Values followed by a common letter within a column do not differ significantly ($p<0.05$) according to least significant difference test.
[b] Inhibition of birdsfoot trefoil seedling growth expressed as % reduction in root length=[(control root length−strain root length)/control root length]×100. Data from Begonia et al. (1993).

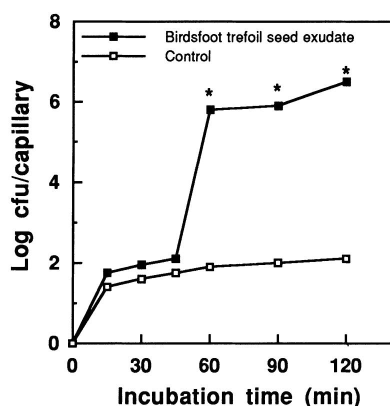

Fig. 1. Numbers of *Mesorhizobium loti* 3078 cells in capillary tubes containing birdsfoot trefoil seed exudate or PBS (control) determined after various incubation periods. Cell concentrations in tubes within times with (*) are significantly different (p<0.05) based on least significant difference test.

raffinose, compared to the PBS control (Table 2). Chemotaxis to sugars did not differ from that to either seed or seedling exudate. Pp007 was significantly attracted to the major phenolic fractions detected in the exudates except for the flavonols/flavones (Table 2). Both exudates also contained 19 amino acids at concentrations $\leq 5\times10^{-2}$ mM and the organic acids α-ketoglutarate, citrate, fumarate, malate, oxaloacetate, hydroxybutyrate, pyruvate, and succinate at <1 mM concentration. All bacterial strains tested did not exhibit significant chemotaxis to any of the individual amino acids or organic acids in the capillary assay.

3.2. Soil chemotaxis assay

Occurrence of chemotaxis in vitro does not establish the phenomenon in situ, therefore, it was necessary to assess bacterial chemotaxis to seeds and seed exudates directly in soil. Populations of rhizobacteria were maintained between log 8 and 9 cfu g^{-1} soil at the inoculation site in the soil-chemotaxis assemblies. When intact birdsfoot trefoil seeds were placed at the center of the plate containing soil, strain As004 moved from the inoculation site and was present in the 2 cm and seed sites within 24 h (Fig. 2(A)). Strain As 004 was detected at significantly lower numbers at the 2 cm site of the control plate after 24 h and was not detected at the seed site until 72 h post-inoculation (Fig. 2(B)). When strain Pp007 was tested, similar results were obtained although the rate of chemotaxis was slightly faster than strain As004 (Fig. 3(A)). Like strain As004, strain Pp007 was not detected at the seed site in the control treatment until 72 h post-inoculation (Fig. 3(B)). When seed or seedling exudates were added to the soil as chemoattractants, both strain As004 and strain Pp007 showed similar chemotactic responses as observed when seeds were used. The strains were detected in both 2 cm and exudate sites at levels similar to the inoculation site after 24 h (data not presented).

Table 2
Chemotaxis of *P. putida* Pp007 to sugars and phenolic fractions detected in birdsfoot trefoil exudates after 1 h by capillary tube assays

Compound	Log cfu per capillary[a]
Control	6.95c
Seed exudate	7.76b
Seedling exudate	7.81b
Sugars:	
Arabinose	7.30c
Fructose	7.76b
Glucose	7.75b
Lactose	7.78b
Raffinose	7.00c
Ribose	7.77b
Sucrose	7.69b
Xylose	7.73b
Phenolic fractions:	
Catechin/Leucoanthocyanidins	8.40a
Flavonoids	8.50a
Flavonols/Flavones	7.00c

Cell counts were determined after capillary samples were plated on SR medium and incubated in dark at 27°C for 48 h.
[a] Values followed by a common letter do not differ significantly (p<0.05) according to the least significant difference test.

4. Discussion

Soft agar and capillary tube assays have been used successfully in previous studies to demonstrate that chemotaxis of rhizobacteria to seeds and seedlings likely occurs in soil (Scher et al., 1985; Lifshitz et al., 1986; Gamliel and Katan, 1992). Likewise, our results showed that soft agar and capillary tube assays were useful as general in vitro screening methods to assess chemotactic activity of rhizobacteria toward seed or

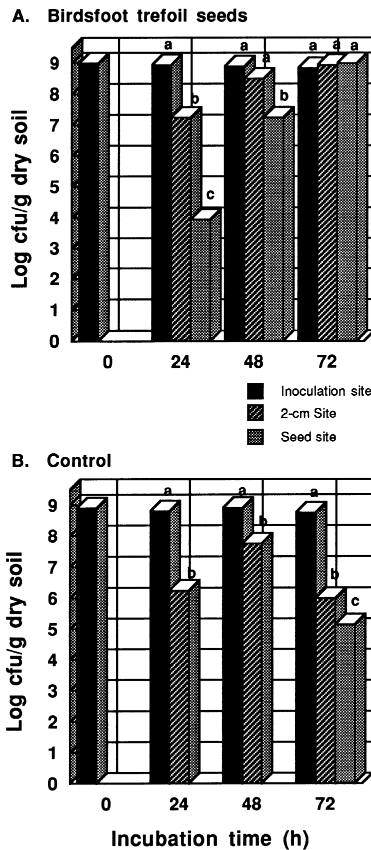

Fig. 2. Chemotaxis of *Alcaligenes* sp. As 004 cells toward birdsfoot trefoil seeds in soil. Cell concentrations in soil sites within times with the same letter are not significantly different ($p<0.05$) based on least significant difference test.

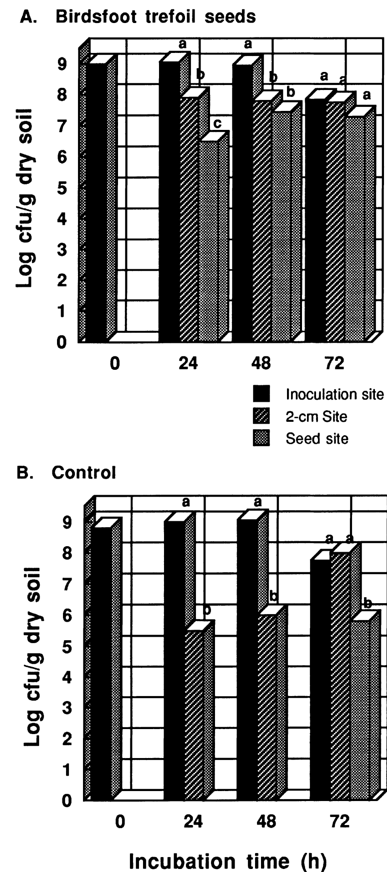

Fig. 3. Chemotaxis of *Pseudomonas putida* Pp007 cells toward birdsfoot trefoil seeds in soil. Cell concentrations in soil sites within times with the same letter are not significantly different ($p<0.05$) based on least significant difference test.

seedling root exudates of birdsfoot trefoil. The high chemotactic activity of *M. loti* in capillary tube assays compared to other rhizobacteria indicates strong attraction to certain compounds similar to those chemoattractants described in other rhizobia-legume symbioses (Vande Boek and Vanderleyden, 1995).

Chemotactic attraction of rhizobacteria to sugars and phenolic compounds detected in seed and seedling exudates indicated that these compounds are probably partially responsible for attractiveness of the exudates to specific seed- and root-colonizing bacteria. Previous characterizations of chemotactic ability of soil and rhizosphere bacteria have demonstrated the importance of chemical constituents of exudates as major factors in chemotaxis (Scher et al., 1985; Gamliel and Katan, 1992).

Chemotactic responses to sugars by strain As004 and strain Pp007 are similar to the results reported previously for other soil bacteria (Gitte et al., 1978; Klopmeyer and Ries, 1987; Chet et al., 1973). However, they contradict earlier work by Seymour and Doetsch (1973) and Scher et al. (1985) who reported that a strain of *Pseudomonas fluorescens* was not attracted in vitro to sugars. This suggests that response to sugars and ultimately exudates of different plant hosts may vary among related bacteria.

Strain Pp007 was significantly attracted to seed and seedling exudate phenolic fractions containing catechins, leucoanthocyanidins, and flavonoids, a

response similar to that toward seed or seedling exudates (Table 2). These results indicate that the phenolic compounds may act as strong attractants when present in exudates. Similar previous work with *Rhizobium* and *Bradyrhizobium* showed enhanced chemotactic response to diverse phenolic compounds (Parke et al., 1985). Other studies indicate that certain phenolic substances serve as chemoattractants for *Agrobacterium tumefaciens* (Ashby et al., 1987; Parke et al., 1987). The ability of phenolic compounds to function as chemoattractants to rhizobia appears to be closely related to their ability to stimulate the expression of the common nodulation genes (Caetano-Anolles et al., 1988). The parallel effects of specific phenolic compounds on chemotaxis and induction of vir gene expression in *Agrobacterium tumefaciens* (Parke et al., 1987) suggest that this pattern of coordinated responses may be of general importance to plant-microbe interactions. Based on results presented here, similar mechanisms may exist in rhizobacteria and host plant root and seed exudate interactions. Further research to clearly demonstrate these relationships is needed.

Bacterial population densities detected in soil at 2 cm and seed sites during chemotaxis assays were equivalent to the populations at inoculation sites and likely included bacteria which multiplies at those sites after initial chemotaxis. Population densities in soil after 72 h incubation are similar to the culturable populations of bacteria detected in rhizosphere soil of several crop species (Grayston et al., 1998). Rhizobacteria may use exudates as nutrient sources for growth and subsequent colonization of rhizosphere soil and the rhizoplane. A previous study showed that high populations ($\approx$log 5 cfu cm^{-1} root) of DRB from birdsfoot trefoil rhizosphere developed on the seedling rhizoplane (Begonia et al., 1993). Growth of rhizobacterial isolates in exudate-amended minimal culture medium is enhanced compared to growth on standard culture media (Begonia, 1989). Thus, the ability of rhizobacteria to colonize the rhizosphere may depend on seed and seedling exudates serving as chemoattractants and nutrient sources.

This study focused on testing in vitro and in situ (soil) techniques to assay chemotaxis of DRB, and did not examine various other factors known to affect chemotaxis including temperature, concentration of test compounds and incubation time. The test concentration of 1 mM for sugars, amino acids, and organic acids assayed in capillary tubes was selected based on previous studies showing that optimum chemotaxis occurred near this concentration (Scher et al., 1985; Gamliel and Katan, 1992). Actual quantities and composition of compounds in root exudates released under field conditions will vary due to influences of environment and plant factors (Grayston et al., 1998). Thus, manipulation of various factors including soil moisture, temperature and texture could lead to a description of mechanisms contributing to the observed chemotactic responses.

In this study, it was demonstrated that rhizobacterial strains were attracted to seeds, crude exudates of seeds and seedling root exudates of birdsfoot trefoil in vitro and in soil. Likewise, the observed attraction to crude exudates was reproduced by individual chemicals detected in the exudates including sugars and phenolic fractions. In most cases attraction of rhizobacteria was significantly higher in treatments containing seeds or exudates or containing certain chemicals as the chemoattractants compared to control treatments. The ability of rhizobacteria to respond to chemotactic attractants released by seeds and seedling roots of birdsfoot trefoil may result in higher populations of these bacteria in the rhizosphere. Consequently, this would provide the rhizobacteria with a competitive advantage in a stressful habitat. Chemotaxis to seed and root exudates is likely one of the several traits which constitutes a successful seed and root colonizing bacterium. A previous study presented scanning electron microscopic evidence of the intimate association of rhizobacteria on the root surface of birdsfoot trefoil, suggesting the direct involvement of these organisms in root disorders (Begonia et al., 1993). The present study strongly suggests that chemotaxis mediates the bacterial-root association and that this mechanism contributes to microbial-induced seedling diseases culminating in the observed poor seedling vigor of this plant in the field.

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