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Effect of Root Agglutinin on Microbial Activities in the Rhizosphere

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A total of 220 bacterial isolates were obtained from pea rhizosphere and nonrhizosphere samples. Of these samples, 100 isolates were chosen randomly to test for their agglutinative reaction against pea root exudate. The percentage of positive agglutination of bacteria isolated from the nonrhizosphere sample was significantly lower than that of bacteria isolated from the rhizosphere sample. Moreover, this agglutinative reaction could not be blocked either by treating the bacterial cells or root exudate with different carbohydrates before they were mixed or by boiling the root exudate first. Bacteria that could be agglutinated by pea root exudate followed the downward growth of the pea root through the soil profile. The greater abilities of such bacteria to colonize the pea rhizosphere were indicated by their higher rhizosphere-colonizing (rhizosphere/nonrhizosphere) ratios, whether the bacteria were added alone or together with nonagglutinating bacteria. However, bacteria did show different agglutinative reactions toward root exudates obtained from different plants.

Seed treatment is a common method of adding nitrogen-fixing bacteria (i.e., *Rhizobium* spp.) to the rhizosphere. Lately, it has been used as a means of introducing biological control agents (e.g., *Trichoderma* spp.) to the soil-plant interface (5, 11, 19–21). For nodulation or control of root pathogens by means of seed inoculation, rhizobia or biocontrol agents must first migrate to or be transported from the seed coat into the rhizosphere and then colonize it. It has been reported elsewhere that neither bacteria nor fungi can move by themselves from the seed coat onto the root surface (6, 13, 15, 21); however, exceptions have been noted. When wheat seeds or potato seed pieces were treated with fluorescent pseudomonads, the entire rhizosphere of each test plant was colonized by the introduced microorganisms (5, 18–20).

Interactions between plants and microorganisms have been investigated by many workers. The adsorption of microorganisms to plant cells is suggested as a pioneer step in the process by which *Rhizobium* spp. or plant-pathogenic microorganisms infect their respective hosts (8, 12). Root surfaces of various plants are covered with mucilaginous layers rich in pectic compounds and other acidic polysaccharides (14). These compounds can cause the agglutination of various bacteria (3, 17). In addition to these pectic compounds, there is some unknown agglutinin existing on root surfaces which can be released into the rhizosphere and cause the agglutination of certain isolates of saprophytic pseudomonads (2). Furthermore, these agglutinated bacteria seem to be able to adhere to and colonize the root surfaces more readily (2). According to James et al. (10), under sterile conditions the adherence to and colonization of the radish root surface and rhizosphere by plant growth-promoting rhizobacteria (e.g., *Pseudomonas fluorescens*) are significantly more frequent than similar processes by *Escherichia coli*. The purpose of our study was to determine whether root agglutinin enhances the downward translocation and colonization of the rhizosphere by specific bacteria.

MATERIALS AND METHODS

Organisms. The bacterial strains used were isolated by first placing 0.1 ml of ground pea (*Pisum sativum* L.) rhizosphere sample or a soil sample on a one-third-strength tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.) plate. These plates were then incubated at 30°C in the dark for 48 h. Colonies were picked at random, and 220 isolates were obtained. The bacteria were purified by the streak-plate method and maintained on one-third-strength TSA slants in screw-cap tubes at 4°C.

Agglutination test. Seeds of pea, celery (*Apium graveolens* L.), and tomato (*Lycopersicum esculentum* Mill.) were grown in vermiculite at room temperature for 10, 18, and 20 days, respectively. Root agglutinin was collected by the method of Anderson (2). The roots were removed carefully from the vermiculite to prevent excessive damage, dipped in 200 ml of distilled water in a 1-liter beaker, and swirled gently by hand for 15 min. The roots were then removed from the water and allowed to drain for 10 min. The wash was clarified by first passing it through layers of cheesecloth and then centrifuging it at 10,000 × *g* for 20 min at 4°C. The supernatant was treated with 1.5 g (dry weight) of prehydrated CM–Sephadex C-50 (Pharmacia Fine Chemicals, Piscataway, N.J.) and mixed gently with a magnetic stirrer for 15 min. The slurry was filtered and again treated similarly with 1.5 g (dry weight) of prehydrated DEAE–Sephadex A-50 (Pharmacia). The filtrate was concentrated to a small volume by lyophilization, and 3 volumes of ethanol were added. The filtrate was placed at 4°C overnight, and the precipitate was collected by centrifugation at 10,000 × *g* for 30 min at 4°C. A portion (10 ml) of sterile distilled water was added to the precipitate and left at 4°C for 30 min. Insoluble substances were removed by recentrifugation at 10,000 × *g* for 15 min at 4°C.

One drop of bacterial cell suspension (around 10^8 CFU/ml), prepared by suspending a loopful of cells grown for 15 h at 30°C on a one-third-strength TSA plate in sterilized distilled water, was added to a solution consisting of 150 μl of 0.1 mM $MgCl_2$ and 350 μl of root wash. In the control set, sterile distilled water was used to replace the root wash. After gentle shaking, the suspension was allowed to stand for 30 min, and the results were read by the naked eye and confirmed by microscopic observation.

Downward translocation. To monitor the activities of isolates 84, 42, and 95 in the experimental setup, spontaneous

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mutants from medium amended with tetracycline or rifampin (100 mg/ml) were obtained. The stability of the antibiotic-resistant property of these mutants was tested by the method of Chao et al. (6). Cell suspensions of these bacteria were prepared by suspending the organisms in autoclaved distilled water. The organisms were grown on one-third-strength TSA plates at 30°C for 24 h and collected by scraping the plates with sterile spatulas. Of this cell suspension, 1 ml was subsequently mixed with an equal portion of 40% (wt/vol) gum arabic (Sigma Chemical Co., St. Louis, Mo.). Pea seeds were uniformly coated with the cell suspension. Immediately after treatment, the seeds were planted in a soil column and incubated in a moist chamber. The soil column and incubation setups have been described in detail previously (6). After incubation, the rhizosphere was sampled by cutting the soil column into 1-cm sections; the roots plus adhering soil were removed following gentle shaking. The samples were then ground in a sterile porcelain mortar with 2 ml of sterile distilled water. A series dilution was made in 0.85% (wt/vol) NaCl solution, and 0.1 ml of each sample was spread on a one-third-strength TSA plate amended with antibiotics. The plates were incubated at 30°C in the dark for 24 to 48 h. The dry weight of each sample was obtained by drying it in a 60°C oven overnight.

Rhizosphere colonization. Mollisol (silt loam) collected from the upper 10 cm of a fallow field was air dried and sieved to remove large stones and plant debris. A bacterial suspension prepared as described above was added to this air-dried soil to a final level of around 10^5 CFU/g of soil. The water content of the soil was then adjusted to 10% with distilled water. After the soil was thoroughly mixed, pea seeds were planted in it and allowed to grow for 9 days at room temperature, with 10 h of illumination per day. The plants were then removed with care, and the root samples were processed as described above. The remaining soil was again mixed, and six subsamples (5 g per sample) were taken. The numbers of the introduced organisms in both rhizosphere and nonrhizosphere samples were determined by plating on one-third-strength TSA plates amended with antibiotics. The rhizosphere/nonrhizosphere (R/S) ratio for a specific organism was calculated by dividing the CFU per gram of oven-dried rhizosphere sample by the CFU per gram of oven-dried nonrhizosphere soil sample (1).

Effect of sugars and high temperature on agglutinin activity. Eleven carbohydrates were used in the present study. Cell suspension (isolate 84) (100 ml) prepared as described above was added to the reaction mixture, which consisted of 150 μl of 1 mM $MgCl_2$, 300 μl of root exudate, and 50 μl of 1 M sugar solution. In another setup, cells of isolate 84 were first suspended in a 1 M sugar solution. Subsequently, a portion (50 μl) of this suspension was added to the solution containing 150 μl of 1 mM $MgCl_2$ and 300 μl of root exudate. Sterile distilled water was used instead of root exudate for the negative control, while a no-sugar treatment was used for the positive control.

When the effect of high temperature was studied, root exudate was placed in boiling water for 20 min before the assay was carried out.

RESULTS

A total of 100 randomly chosen isolates were tested for their agglutinative responses toward pea root exudate. Of the bacteria isolated from nonrhizosphere soil, 34% showed positive agglutination toward pea root agglutinin, whereas 82% of the bacteria isolated from the rhizosphere showed a positive response. Fourteen bacterial isolates were selected randomly to be tested against the agglutinins isolated from the roots of pea, celery, and tomato plants. A difference in response toward various agglutinins was observed (Table 1). None of the 14 bacterial isolates tested showed agglutination with tomato root exudate. On the other hand, seven isolates reacted positively against pea and celery exudates, and three showed negative responses to the root exudates from these two plants. The rest of the test organisms were agglutinated by either pea or celery root exudate.

TABLE 1. Responses of bacterial isolates to different root exudates

Bacterial isolate no.	Response to root exudate of:		
	Pea	Celery	Tomato
6	+	−	−
10	+	+	−
11	+	+	−
42	+	+	−
50	−	−	−
78	−	−	−
84	+	+	−
90	+	+	−
95	−	ND[a]	−
106	+	+	−
2-9	+	+	−
4-3	+	+	−
7-9	−	−	−
10-7	+	−	−

[a] ND, Not determined.

Bacterial isolates 84 and 42 (both agglutination positive) and isolate 95 (agglutination negative) were used to study the effect of root agglutinin on the downward movement of bacteria from the seed coat to the rhizosphere. Isolate 84 was resistant to tetracycline, and isolates 42 and 95 were resistant to rifampin. Generally, no antibiotic-resistant indigenous bacteria were observed on our antibiotic-amended plates. However, some exceptions were noted. Results showed that isolates 84 and 42 could move from the seed coat into the rhizosphere and colonize it (Table 2). On the other hand, no CFU of isolate 95 could be detected in the rhizosphere 3 cm below the seed, and colonization of the rhizosphere by isolate 84 was sometimes completely or partially inhibited. This inhibition coincided with the appearance of some indigenous tetracycline-resistant microflora.

TABLE 2. Movement of bacteria from treated seeds into the rhizosphere

Distance (cm)	10^2 CFU/cm of root for isolate[a]:		
	84	42	95
1	TNTC[b]	TNTC	27,000 ± 33,000
2	TNTC	TNTC	98 ± 127
3	49 ± 33	8 ± 5	ND[c]
4	55 ± 50	11 ± 16	ND
5	53 ± 50	11 ± 15	ND
6	46 ± 81	25 ± 38	ND
Last cm	25 ± 50	74 ± 99	ND

[a] The initial numbers were 1.7×10^7, 1.5×10^8, and 3.8×10^8 CFU per seed for isolates 84, 42, and 95, respectively. Ten rhizosphere samples were taken for isolate 84. However, in two of the samples no CFU could be detected. Therefore, these two samples were excluded from the calculation. For isolates 42 and 95, five rhizosphere samples per isolate were taken.

[b] TNTC, Too numerous to count.

[c] ND, None detectable.

TABLE 3. Rhizosphere-colonizing ability (R/S ratio) of selected microorganisms

Isolates treated	R/S ratio
Isolate 84 alone	23.3 ± 16.8
Isolate 95 alone	1.2 ± 0.8
Isolate 42 alone	27.6 ± 15.8
Isolate 84 + isolate 95	
Isolate 84	24.7 ± 12.5
Isolate 95	1.5 ± 0.4
Isolate 84 + isolate 42	
Isolate 84	2.6 ± 1.4
Isolate 42	0.9 ± 0.1

The ability of isolates 84, 42, and 95 to colonize the pea rhizosphere competitively was determined by measuring their R/S ratios. When isolates were individually added to the soil, the R/S ratios for isolates 84, 42, and 95 were 23.2 ± 16.8, 27.6 ± 15.8, and 1.2 ± 0.8, respectively (Table 3). When isolates 84 and 95 were added together to the soil, the R/S ratio was 24.7 ± 12.5 for the former and 1.5 ± 0.4 for the latter. On the other hand, when isolates 84 and 42 were both added to the soil, the R/S ratios of isolates 84 and 42 were reduced to 2.6 ± 1.4 and 0.9 ± 0.1, respectively.

When different carbohydrates [L-(−)-fucose, D-(+)-fucose, D-(+)-galactose, L-(−)-rhamnose, D-(+)-mannose, D-(+)-glucose, L-(−)-sorbose, sucrose, 2-deoxy-D-glucose, *N*-acetylglucosamine] were used to try to block this agglutinative reaction, they all produced negative results. For this test, the root exudate was treated with sugar before assay; bacterial cells were suspended in a sugar solution. Furthermore, when the agglutinin was placed in boiling water for 20 min, it still retained its activity.

Isolate 95 was identified as *Enterobacter agglomerans* by using the Enterotube II system (Roche Diagnostics, Div. Hoffmann-LaRoche Inc., Nutley, N.J.), and isolates 84 and 42 were identified as *Pseudomonas cepacia* and *Agrobacterium radiobacter*, respectively, by using API 20NE.

DISCUSSION

Several investigators have reported successful colonization of the rhizosphere by antagonistic bacteria introduced by seed coating. The organisms used were all saprophytic pseudomonads (5, 18–20). Suppression of *Gaeumannomyces graminis* var. *tritici*, which causes take-all in wheat, is known to develop after continuous cropping of a susceptible cereal (4, 7). This suppression of the disease was related to the populations of fluorescent pseudomonads (18, 20). Charigkapakorn and Sivasithamparam (7) have shown that *Pseudomonas putida* is more abundant on healthy roots than on infected roots. Howie et al. (9) proposed that colonization of the rhizosphere by bacteria introduced on seeds occurred in two phases. In phase 1, the bacteria moved downward with root extensions through the soil, and in phase 2, the population increased to the limits of the ecological niche. From our results, we propose that those bacterial isolates that can be agglutinated by the root exudate of a certain plant can and will follow the downward growth of that root into the soil. Furthermore, the large R/S ratios of these agglutination-positive bacteria also indicated their ability to colonize the rhizosphere of a specific plant more readily than agglutination-negative bacteria. Bacteria that show negative agglutinative activity toward specific plant root exudate will neither follow the root growth nor colonize the rhizosphere of that specific plant.

It is apparent that root exudate enhances the colonization of root surfaces or rhizosphere by specific microorganisms. However, it is known that the compositions of root exudates of different plants vary (16). Therefore, by understanding the relationship between rhizosphere competence and the agglutination response of each microorganism to each plant root exudate, we may be able to select proper antagonistic microorganisms to control root disease effectively.

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