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Chemical characterization of root exudates from rice (*Oryza sativa*) and their effects on the chemotactic response of endophytic bacteria

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Abstract

Root exudates represent an important source of nutrients for microorganisms in the rhizosphere and seem to participate in early colonization inducing chemotactic responses of rhizospheric bacteria. We characterized the root exudates collected from rice plantlets cultured under hydroponic conditions and assessed their effects on the chemotaxis of two strains of endophytic bacteria, *Corynebacterium flavescens* and *Bacillus pumilus*, collected from the rice rhizosphere. We compared these chemotactic effects on endophytic bacteria with those on two strains of plant-growth-promoting bacteria, *Azospirillum brasilense* (isolated from the corn rhizosphere) and *Bacillus* sp. (from the rice rhizosphere). The root exudates were collected at different time intervals. The highest concentration and diversity of amino acids and carbohydrates were found during the first 2 weeks after seeding. Histidine, proline, valine, alanine, and glycine were the main amino acid residues identified during the 4 weeks of culture. The main carbohydrates identified were glucose, arabinose, mannose, galactose, and glucuronic acid. The chemotactic responses of the analyzed endophytic bacteria to root exudates were 3.9 to 5.1 times higher than those of *A. brasilense* and 2.2 to 2.8 times higher than *Bacillus* sp. Our results indicate that rice exudates may induce a higher chemotactic response for endophytic bacteria than for other bacterial strains present in the rice rhizosphere.

Introduction

Stimulation of microorganisms present in the rhizosphere seems to be due to the presence of organic compounds released by the roots and representing up to 20% of the plant dry weight. This material includes flaked cells of the root cap, mucilage, and soluble and non-soluble exudates, which may contain free amino acids, proteins, carbohydrates, alcohols, vitamins, or hormones (Hawes and Pueppke, 1986). In cereals, it has been estimated that 4–29% of the photosynthates can be transferred to the rhizosphere, readily available for consumption by microbes (Lynch and Whipps, 1990). Root exudates are an important source of nutrients for the microorganisms present in the rhizosphere and participate in the colonization process through chemotaxis of soil microorganisms (Campbell and Greaves, 1990; Hiroyuki et al., 1998; Lynch and Whipps, 1990). Bacterial chemotaxis is a primitive sensing mechanism by which bacteria swim toward high concentrations of attractant (Caetano-Anolles et al., 1988; De Weger, 1987; Zhulin and Armitage,

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1992). This mechanism is activated by changes in pH, temperature, osmolarity, viscosity, and chemicals, some of them are nutrients or related compounds, such as secondary metabolites (Blair, 1995). The capacity to colonize the rhizosphere of a host plant could be favored and even increased by several components of the root exudates, which could, in turn, induce some temporary modifications in the structure of bacterial lipopolysaccharides (Begonia and Kremer, 1999; Dekkers et al., 1998; Song and Lin, 1999).

Up to date, the exact mechanisms that allow to colonize as well as to identify the specific host organisms have not been clearly identified. Chemotactic response towards amino acids, sugars, or organic acids is fundamental for bacterial behavior both *in vitro* and *in situ* (Barak et al., 1983; Bashan and Holguin, 1994) and represents, very probably, the first step in root colonization (Zheng and Sinclair, 1996). Once bacteria are in the vicinity of the root, attachment to target cells on the plant surface can be mediated by a network of fibrillar material (Bashan et al., 1991; Vande Broek and Vanderleyden, 1995). Colonization of the roots is a complex phenomenon in which one of the first steps is the migration of microorganisms towards the roots. Other characteristics that participate are movement along the root (Schippers et al., 1987); agglutinability by root exudates (Chao et al., 1988) and adherence (De Weger et al., 1987; Vesper, 1987).

Endophytic bacteria seem to be ubiquitous in most plant species, inducing beneficial effects, as inferred from their ability to promote plant growth (Bacilio-Jimenez et al., 2001) and to confer resistance against plant pathogens (Benhamou et al., 1996; Hallmann et al., 1997; Pleban et al., 1995). The aim of this study was to identify the amino acids and sugars liberated by rice plants cultivated hydroponically during the first 4 weeks of growth. Later, to characterize the effects of these root exudates on the chemotactic response of rice endophytes, such as *Bacillus pumilus* and *Corynebacterium flavescens*. These endophytes were compared to plant–growth-promoting bacteria, *Azospirillum brasilense* (isolated from the corn rhizosphere) and *Bacillus* spp. (isolated from the rice rhizosphere).

Materials and methods

Seeds and bacteria

Rice, *Oryza sativa* L., seeds, var. Morelos A-88 were from INIFAP-Zacatepec, Morelos. *Azospirillum brasilense* strain 6-81, hyperproducing indolacetic acid (IAA) phenotype, derived from the wild strain UAP-154, originally isolated from maize roots, was donated by the University of Puebla, Mexico. *Bacillus* sp. strain 709, producing indolacetic acid, originally isolated from rice cultivation soil in Zacatepec, Mexico, was obtained from the collection of the Plant Physiology Laboratory from the National School of Biological Sciences, Instituto Politecnico Nacional (IPN), Mexico. *Corynebacterium flavescens* and *Bacillus pumilus* strains were isolated from rice var Morelos A-88 seeds, as endophytic bacteria (Bacilio-Jimenez et al., 2001). The four bacteria were cultivated separately in Py culture medium (5 g of yeast extract, 10 g of casein peptone, and 10 mL of 0.7 *M* $CaCl_2$ in 1000 mL of distilled water). Exponential-phase cultures were harvested by centrifugation at 4000 × *g* for 20 min, washed three times with 60 m*M* phosphate buffer (pH 7.0), and adjusted to a density of 10^4 cells mL^{-1} for the experiments.

Germination of rice seeds

Rice seeds without glumes were surface-sterilized by soaking in a solution of 0.5% mercury chloride for 7 min and then rinsed with sterile distilled water five times until all traces of the disinfectant were removed. The seeds were soaked in a solution of 150 mg nalidixic acid L^{-1} (Sigma, St Louis, MO, USA) under constant agitation, 100 rpm, at 28±2 °C for 24 h, and rinsed again several times with sterile distilled water. To allow germination and to detect the presence of microorganisms, seeds were aseptically transferred to agar plates containing a medium with the following composition L^{-1}:250 g potato (boiled and filtered); 10 g D-glucose; 10 g peptone, and 15 g agar and cultured at room temperature of 22±2 °C.

Cultivation of rice seedlings

Twelve axenic rice plantlets were cultivated in a sterile hydroponic system based on the one described by Prikryl and Vancura (1980). The system consists of a tube with nutrient medium and a gauze on which the seedlings were placed. Glass tube diameter and length were modified (40 and 450 mm, respectively) to allow sufficient space for the plants to grow for 30 days. Roots were kept in half-strength complete Hoagland's solution (Hoagland, 1975) containing 50 mg L^{-1} nalidixic acid to avoid development of microorganisms.

Plants were kept at a 14 h light:10 h dark cycle at 25–35 °C for 7, 14, 21, and 28 days, adding nutrient solution periodically, under axenic conditions, in a laminar flow chamber to maintain the roots in an aqueous medium and permit aeration of systems.

Root exudates

Root exudates were obtained from the cultivated plants. The root exudates were collected every 7 days, cultivated plants were then rinsed with distilled water, and the nutritient solution renewed every 7 days. From each cultivation exudate, sterility of the solution was verified by placing samples in the medium described before under seed germination; those showing microbial growth after 2 days of incubation at 28 °C were discarded. The nutrient solution of each plant growth system was filtered through a 0.2-μm Millipore filter, lyophilized and kept at −20 °C for later identification of amino acids and sugars. The root dry weight of each hydroponic system was evaluated weekly from six hydroponic systems, each containing 12 plants. Root dry weight was obtained by keeping roots in an oven at 60 °C until reaching constant weight.

Bacterial chemotaxis

We evaluated the chemotactic responses of the four bacterial species towards rice plant root exudates using a modification of Adler's technique (1973). Briefly, an acrylic chamber provided with two holes and a well was used. In the holes, two capillary tubes were introduced, one containing the sample of root exudates and the other, as a control, Py liquid medium. The exudates used for chemotaxis assays were reconstituted after lyophilization with distilled water and the amino acid concentration was adjusted to 25 μM mL^{-1} (as optimal dose, identified previously with dose–response assays) (Heinrich and Hess, 1985). In the well, 0.5 ml of adjusted bacterial suspension (1×10^4 cells mL^{-1}) was placed; the well was covered with a coverglass and incubated for 40 min at 25 °C. After bacterial migration, the capillaries were rinsed with 0.85% sterile saline solution and their contents was serially diluted with 60 mM phosphate buffer (pH 7.0). We plated 100-μL aliquots on Py medium supplemented with 1% agar. The plates were incubated at 28 °C for 48 h and colony forming units (CFU) were quantified. Control experiments were performed using Py culture medium. Relative responses were calculated as the number of CFU per capillary containing root exudates, divided by the number of CFU in capillaries containing the control (Py liquid medium) (Barbour et al., 1991). The experiments were repeated three times with three replications of each bacterium in the chemotactic assay. The results were analyzed and compared using one-way ANOVA.

Analytical methods

Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as the standard. Carbohydrate composition was determined with the heptafluorobutyrate derivatives of *O*-methylglycosides from the root exudates obtained after methanolysis in 0.5 M methanol–HCl for 24 h at 80 °C; lysine (Sigma) was used as the internal standard. The samples were analyzed by gas chromatography using a capillary column (25 × 0.32 mm) of 5% Silicone OV 210 (Applied Science Lab., Buffalo, NY), in a Varian 2100 gas chromatograph (Orsay, France), equipped with a flame detector and a glass solid injector; the carrier gas was helium, and the oven temperature programmed from 150 to 250 °C at 3 °C/min as described by Zanetta et al. (1999). For amino acid analysis, a 100-μg sample was hydrolyzed under vacuum with 2 mL 6 M HCl at 110 °C, with a drop of phenol to avoid degradation of tyrosine residues, in sealed tubes for 24, 48, and 72 h. The samples were analyzed in an automatic amino acid analyzer Durrum 500, according to Bidlingmeyer et al. (1984), using norleucine as internal standard. Analyses were performed in triplicate.

Results

Chemical characterization of root exudates

The concentration of amino acids in the root exudates was higher ($P < 0.05$) during the first and second weeks of rice cultivation than during the third and fourth weeks. We obtained 7.84 and 2.01 μmol amino acids g^{-1} root dry weight, at 1 and 4 weeks, respectively. In the first week, we observed the highest exudation of amino acids. Exudation decreased more than 50% from week 1 to week 2, but was still significantly higher in week 2 than at 3 and 4 weeks of culture. In the first and second weeks of culture we obtained 16 different amino acids, whereas only 15 and 12 amino acids were obtained during the third and fourth weeks of culture, respectively. The main amino

Table 1. Amino acid composition (μmol g^{-1} root dry weight) of root exudates from rice *(Oryza* sativa) cultivated during 7, 14, 21 and 28 days in an axenic hydroponic system

Amino acid	Culture (days)			
	7	14	21	28
Asx	0.12	0.03	0.01	0.04
Glx	0.22	0.09	0.03	0.07
Ser	0.27	0.11	0.03	0.09
Gly	0.74	0.30	0.08	0.25
His	1.58	1.38	1.21	ND
Arg	0.11	0.08	ND	ND
Thr	0.24	0.10	0.03	0.09
Ala	0.48	0.17	0.04	0.16
Pro	1.33	0.20	0.06	0.24
Tyr	0.04	0.01	0.001	ND
Val	1.73	0.57	0.26	0.83
Met	0.02	0.02	0.01	ND
Cys	ND	ND	ND	ND
ILe	0.21	0.08	0.02	0.06
Leu	0.28	0.11	0.03	0.09
Phe	0.18	0.06	0.02	0.04
Lys	0.29	0.07	0.01	0.05
Total	7.84[a]	3.38[b]	1.84[c]	2.01[c]

Values denoted by different letters differ significantly $P < 0.05$ using one-way ANOVA. Values are the mean of six replicates with 12 seedlings in each culture system. Standard error of the mean was lower than 10%. ND – not detected.

Table 2. Carbohydrate composition (μmol g^{-1} root dry weight) of root exudates from rice (*Oryza sativa*) cultivated during 7, 14, 21 and 28 days in an axenic hydroponic system

Carbohydrate	Culture (days)			
	7	14	21	28
Arabinose	0.2	0.5	ND	0.3
Mannose	3.4	0.6	3	ND
Galactose	3.0	5.0	0.8	1.1
Glucose	270.4	211.8	45.6	41.4
Glucuronic acid	6.5	10.7	3.9	1.0
Xylose	ND	0.5	ND	ND
Total	283.5[a]	219.1[b]	50.6[c]	43.8[c]

Values denoted by different letters differ significantly $P < 0.05$ using one-way ANOVA. Values are the mean of six replicates with 12 seedlings in each culture system. Standard error of the mean was lower than 10%. ND – not detected.

acids identified were histidine, proline, valine, alanine, and glycine; minor amino acids were aspartic, arginine, tyrosine and methionine residues; methionine was present only in the first and second weeks of culture (Table 1).

Similar changes were observed for sugars (Table 2). The root exudates contained higher concentrations and a larger diversity of carbohydrates during the first 2 weeks of culture: 283.4 and 219.0 μmol of carbohydrates g^{-1} of root dry weight compared to 50.6 and 43.8 μmol g^{-1} root dry weight at the third and fourth weeks of culture. Exudation of sugars was significantly higher in the first than in the second week and, as for amino acids, the concentration of sugars in the second week was higher than during the third or fourth week of culture (Table 2). The main carbohydrates identified were mannose, galactose, glucose, and glucuronic acid. Xylose was observed only at the second week of culture; arabinose residues were not present in the third week, and root exudates lacked mannose by the fourth week of culture. Glucose was the main sugar derivative identified in the rice exudates, and its concentration increased on the fourth week (from 91 to 97%), when compared with the first and second weeks of culture. Glucuronic acid decreased concentration in exudates with culture time and its concentration was 6-fold lower at the end than during the initial days of culture. The dry root biomass production at 7,14, 21 and 28 days was 0.66, 1.17, 1.32, and 1.36 mg pl^{-1}, respectively; similarly the dry shoot biomass production was 6.3, 8.5, 9.8 and 10.7 mg pl^{-1}. This means that the highest grown rate was attained during the first 2 weeks and that the biomass production decreased significantly in the third and fourth weeks.

Chemotactic response

Chemotactic response toward root exudates obtained at 7, 14, 21 and 28 days was compared among bacterial strains coming from different sources (plant growth promoters and endophytic bacteria). Results indicated positive chemotactic response by the four bacterial strains tested; however, the relative responses showed differences that correlated with the source of the bacterial strain. The highest chemotactic responses were obtained from the endophytic bacteria *C. flavescens* and *B. pumilus,* whereas the lowest chemotactic response was from *A. brasilense* (from the corn rhizosphere). *Bacillus* sp., from soil in which rice had been cultured, showed an intermediate response but significantly higher than *A. brasilense* (Figure 1). The highest chemotactic response of the endophyte *C. flavescens* was observed to exudates from 7- and 14-

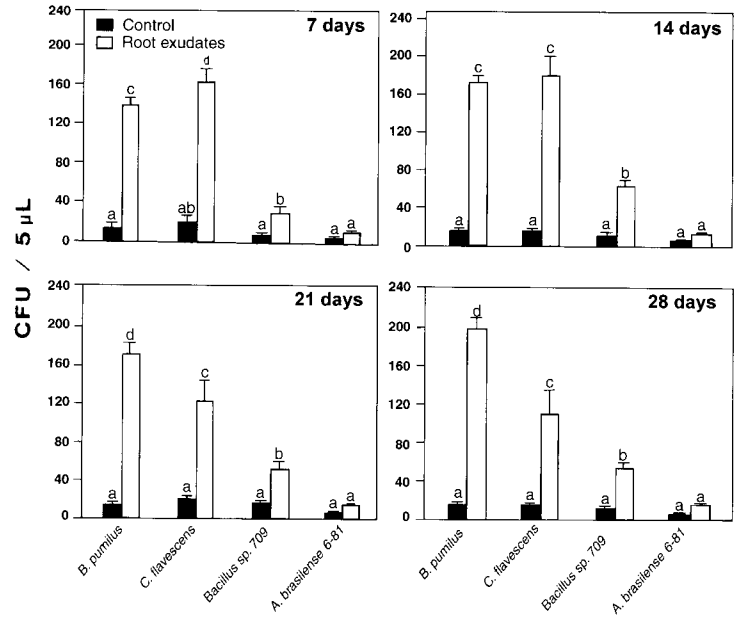

Figure 1. Comparative chemotactic response of endophytes (*Bacillus pumilus* and *Corynebacterium flavescens*) and rhizobacteria (*Bacillus* sp. 709 and *Azospirillum brasilense* 6-81) to rice root exudates obtained at 7, 14, 21, and 28 days of culture. The exudates' amino acids concentration was adjusted to 25 μM mL^{-1} (as optimal dose). Results represent the mean ± standard error of three experiments with three replicates each one. Lines represent the standard error of the mean. Different letters indicate significant differences at $P < 0.05$ using one-way ANOVA.

day cultures; for *B. pumilus*, the optimal response was found with exudates from 21- and 28-day cultures. *A. brasilense* showed no significant activity, regardless of exudates collection time (Figure 1).

Discussion

The root exudates of rice cultured in hydroponic conditions showed higher concentrations of carbohydrates of amino acids and carbohydrates in the first 2 weeks than later on. In the root exudates, glucose represented over 90% of the carbohydrates, indicating that this sugar residue represents the main source of carbon in the rhizosphere of young rice plants. Similar findings have been observed during the growth of wheat seedlings, which at the first week after seeding showed eight times higher concentrations of hydrocarbon compounds than at 2 to 4 weeks (Jones and Darrah, 1993; Prikryl and Vancura, 1980). The decrease in exudation rate of plants in hydroponic culture systems is probably caused by at least two potential mechanisms: (i) accumulation of high levels of organic substances in the vicinity of the root, repressing the release of more organic compounds (Jones and Darrah, 1993; Prikryl and Vancura, 1980), and (ii) by reabsorption of the organic compounds by the plant (Guckert et al., 1991; Jones and Darrah, 1993). Although the plants are capable of regulating the net amount of soluble exudates released into a root-bathing solution, the culture conditions employed in the collection of exudates determine the amount and composition of exudates recovered in solution culture experiments (Jones and Darrah, 1993). Lack of aeration in the tubes may also contribute to the decline in root exudation over time. The root exudates from rice caused chemotaxis toward the four bacterial strains investigated; however, rice exudates showed significant attraction for endophytic bacteria *C. flavescens* and *B. pumilus* ($P < 0.05$); *Bacillus* sp. 709 was less attracted than endophytes and *A. brasilense* showed no significant chemotactic capacity. These differential responses can be due to their origin, since *A. brasilense* was isolated from maize rhizosphere and *Bacillus* sp. from soil in which rice had been grown, whereas *Corynebacterium flavescens* and *Bacillus pumilus* are rice endophytes (Bacilio-Jimenez et al., 2001).

Studies on attraction and migration of beneficial rhizosphere bacteria provide important information about ecological traits for root colonization. Most studies, including those with the genus *Azospirillum*, have been conducted *in vitro*. Similar responses have

been found to occur under soil conditions: the migration of two rhizosphere beneficial bacteria in the soil towards living wheat plants or towards synthetic attractants known to be produced *in vivo* (Rovira, 1969); and the migration of the *A. brasilense* and *Pseudomonas fluorescens* towards wheat roots in the soil (Bashan, 1986). Furthermore, results from soft-agar, capillary tube, and soil chemotaxis assays indicate that rhizobacteria are attracted to seed and seedling root exudates (Begonia and Kremer, 1999). *A. brasilense* exhibits positive chemotaxis towards a large number of organic compounds such as amino acids, saccharides, organic acids, typical for plant roots exudates. Malate, succinate, and fructose have been the most effective attractants for *A. brasilense* (Zhulin et al., 1988). Our results suggest that endophytic bacteria respond stronger than plant growth promoter bacteria *A. brasilense* and *Bacillus* sp., to chemical effectors, which might favor root colonization. The capacity of root exudates to attract bacteria could be attributed to some of their individual components. In this study, we found that the composition and concentration of sugars and amino acids from rice root exudates, during the first month of life of the plants, exerts great attraction for endophytic strains. This may provide them with a clear advantage over *A. brasilense* and *Bacillus* sp., allowing them to compete better with plant growth promoting bacteria (Bacilio-Jimenez et al., 2001), such as the rice rhizosphere bacterium *Bacillus sp.* These results strongly suggest that the chemo-attractant characteristics probably favor endophytes and could induce exclusion of other colonizing microorganisms from the ecological niche (Bacilio-Jimenez et al., 2001; Hozore and Alexander, 1991).

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