

Assessment of the Role of Chemotaxis and Biofilm Formation as Requirements for Colonization of Roots and Seeds of Soybean Plants by *Bacillus amyloliquefaciens* BNM339

P. M. Yaryura · M. León · O. S. Correa · N. L. Kerber · N. L. Pucheu · A. F. García

Received: 31 October 2007 / Accepted: 11 January 2008 / Published online: 12 March 2008
© Springer Science+Business Media, LLC 2008

Abstract This article correlates colonization with parameters, such as chemotaxis, biofilm formation, and bacterial growth, that are believed to be connected. We show here, by using two varieties of soybean plants that seeds axenically produced exudates, induced a chemotactic response in *Bacillus amyloliquefaciens*, whereas root exudates did not, even when the exudates, also collected under axenic conditions, were concentrated up to 200-fold. Root exudates did not support bacterial cell division, whereas seed exudates contain compounds that support active cell division and high cell biomass at stationary phase. Seed exudates of the two soybean varieties also induced biofilm formation. *B. amyloliquefaciens* colonized both seeds and roots, and plant variety significantly affected bacterial root colonization, whereas it did not affect seed colonization. Colonization of roots in *B. amyloliquefaciens* occurred despite the lack of chemotaxis and growth stimulation by root exudates. The data presented in this article suggest that soybean seed colonization, but not root colonization, by *B. amyloliquefaciens* is influenced by chemotaxis, growth, and biofilm formation and that this may be caused by qualitative changes of the composition of root exudates.

Introduction

We previously isolated a soil bacterium that was identified as *Bacillus amyloliquefaciens* strain BNM339 with biocontrol characteristics able to inhibit the growth of several fungi that are causes of crop-related diseases. It was shown previously that for a bacterium to act as a biocontrol agent, it must be accompanied by certain properties that facilitate colonization of the rhizoplane. These bacteria, commonly present in the environment, usually have positive chemotaxis toward root or seed exudates [38] and use chemical compounds present in the exudates as growth factors and/or as nutrient sources [21]. Moreover, competition for nutrients plays an important role in colonization and biocontrol activity [15], biofilm-formation capacity [25, 4], and competitive root colonization [20, 15]. Chemotaxis and biofilm formation are part of the mechanisms that determine colonization [11, 24]. The plant roots excrete an enormous variety of chemotaxis-inducing compounds to the rhizosphere [23, 13], which encompass some of the most complex chemical, physical, and biologic interactions involving the roots of the plants and microorganisms present therein [5].

Many important biologic processes, such as nodulation, in leguminous plants result from root colonization and likewise are the induction of biocontrol activities [32, 4]. It is possible that chemotaxis represents the first step in bacterial colonization of roots [8, 38], an event which, for biocontrol agents, is at the basis of their plant-protection activity [36, 9].

Colonization has been claimed to be intimately related to biofilm formation, and this phenomenon constitutes a strategy for bacteria to survive desiccation or other environmental stresses and actively participates in defense mechanisms involved in pathogenic attacks by other microorganisms [12, 24].

P. M. Yaryura · M. León · O. S. Correa ·
N. L. Kerber · N. L. Pucheu · A. F. García
Instituto de Investigaciones Bioquímicas y Fisiológicas
CONICET-FAUBA, C1417DSE Buenos Aires, Argentina

P. M. Yaryura (✉) · M. León · O. S. Correa
Cátedra de Microbiología Agrícola and Facultad de Agronomía
(UBA), C1417DSE Buenos Aires, Argentina
e-mail: yaryura@agro.uba.ar

In this study, we determined the effect of root and seed exudates of two varieties of soybean plants on the colonization process by *B. amyloliquefaciens* strain BNM339 and the relation of some relevant characteristics, such as chemotaxis, growth, and biofilm formation, with that process. Here, we consider biofilm formation and occupation of plant surfaces in terms of the potential use of the bacteria, which we isolated in our laboratory, for biocontrol purposes. In some of our previous experiments, we observed that BNM339, inoculated on seeds of the two varieties of soybean, protected plants against damping-off when they were challenged with *Pythium ultimum* in both microcosm assays using agricultural soils as substrate as well as in a growth chamber using sterile soil.

Materials and Methods

Bacterial Strains and Growth Conditions

B. amyloliquefaciens BNM339 was isolated from the rhizosphere of a soybean field in the Province of Buenos Aires, Argentina, and its antifungal activity was tested in vitro against different pathogenic fungi (*Fusarium oxysporum*, *F. solani*, *P. ultimum*, *Macrophomina phaseolina*, and *Sclerotinia sclerotiorum*). BNM339 was grown in tryptone–yeast broth [29]. *Bradyrhizobium japonicum* E109 was grown in yeast extract and glycerol or in yeast-mannitol agar (YMA) [34] as carbon source [19].

Preparation of Soybean Root and Seed Exudates

For root exudates, seeds of Ferias del Norte, FN 4.10 and FN 4.85, were surface disinfected by soaking in 3% NaOCl for 10 minutes and rinsed three times with sterile distilled water. Disinfected and pregerminated seeds (for 2 days at 25°C in the dark) were transferred to glass tubes containing 20 mL of a mineral solution as described by Murashige and Skoog (MS) [27]. A filter paper with an inverted “U” shape was used as mechanical support of the seedlings. The soybean varieties used are of the same maturity group (IV) but differ mainly in the cycle, being FN-4.10 of short and FN-4.85 of long cycle. The seedlings were then transferred to the glass tubes and grown in an orbital shaker (Shaker Pro; Vicking) at 140 rpm in a plant-growth chamber under a 16 h:8 h light-to-dark cycle. After 10 and 21 days of growth, the exudates corresponding to 15 tubes were pooled, filtered under sterile conditions to remove sloughed root caps or border cells, and lyophilized.

For seed exudates, 100 surface-disinfected seeds of each soybean variety were transferred into a 500-mL Erlenmeyer flask containing 100 mL sterile distilled water and incubated for 20 hours under continuous shaking

(150 rpm) at 30°C [6]. The corresponding exudates were filtered under sterile conditions and lyophilized. To test that both root and seed exudates had maintained sterility throughout collection, samples obtained before sterile filtration were incubated in potato–dextrose agar (PDA) and nutrient agar (NA).

Chemotaxis Assay

A modified capillary assay based on the Adler procedure [1] was used for quantitative chemotaxis measurements. *B. amyloliquefaciens* BNM339 and *B. japonicum* E109 were grown in the respective media as described previously until reaching mid-log phase (optical density at 600 nm [OD₆₀₀] of approximately of 0.4). In the case of BNM339, we used a minimal medium, thus inducing optimal motility [29, 30]. The cells were centrifuged, washed two times with chemotaxis buffer as described by Ordal and Katharine [30], and resuspended in the same buffer to OD_{600nm} of 0.4. Then chemotaxis chambers were filled with 0.35 mL of this cell suspension. Capillary tubes were loaded with seed (concentrated 0.1-, 1-, 25-, and 50-fold) or root (concentrated 1-, 25-, 50-, 100-, and 200-fold) exudates corresponding to 21 days of soybean plant growth, and the entire chambers were incubated at 37°C. After 1 hour of incubation, the capillary tubes were rinsed with sterile distilled water, and the contents of each were transferred into an Eppendorf tube containing 1.0 mL of sterile physiologic solution (PS) and serially diluted in the same solution. The colony forming units (CFU) mL⁻¹ were determined in NA plates incubated for 48 hours at 30°C. The CFU mL⁻¹ value present in the capillary tubes containing root or seed exudates divided by the corresponding control (without exudates) represents the chemotaxis ratio. All chemotaxis experiments were randomized with five replicates of each sample.

The qualitative chemotactic response to the seed as a source of exudates was determined by the method of Kadouri et al. [14]. *B. amyloliquefaciens* BNM339 was grown in the medium as described previously until logarithmic phase and resuspended in chemotaxis buffer [30]. A single surface-disinfected seed was placed in the edge of a Petri dish containing 0.06 N phosphate buffer (pH 6.8) and 0.3% agar. Twelve microliters of bacterial suspension in chemotaxis buffer were placed in the center of the plate. After 24 hours at 25°C, the visualization of bands of bacterial cells moving toward the attractant represented a positive chemotactic response.

Growth of *B. amyloliquefaciens* BNM339 on the Root and Seed Exudates

This strain was inoculated into root or seed exudates of each soybean variety either with or without 0.5% glucose

or in MS plus 0.5% glucose, as a control in the case of root, and incubated at 30°C in a rotary shaker (250 rpm). The duplication time and the total number of cells at the stationary phase of the cultures were determined through viable cell counts.

Biofilm Formation Assay

B. amyloliquefaciens BNM339 was grown in LB-plus medium until log phase as described by Hamon and Lazazzera [12], washed with PS, and finally resuspended in fresh LB-plus medium or in the seed exudates of both soybean varieties to OD₆₀₀ of 0.2. Biofilm formation was determined using 96-well microtiter plates filled with 200 µL bacterial suspension/well. The negative control contained only the corresponding culture medium. The plate was incubated at 37°C without shaking for 60 hours. Then the contents of each well was vacuum sipped, and the cell biomass attached to the surface of the well was washed five times with 250 µL PS and allowed to dry overnight at 25°C. The plate was finally stained with 200 µL/well of 1% crystal violet (CV) solution in 33% (v/v) acetic acid for approximately 20 minutes. Excess CV was then removed with water. The bound CV was solubilized with 200 µL of 33% acetic acid and measured at OD₅₉₀. To quantify biofilm accumulation, the OD₅₉₀ of each well was measured using a microtiter plate reader (Thermo Multiskan EX). Biofilm formation was normalized with respect to bacterial growth to obtain the specific biofilm formation (SBF), which was calculated by the following formula,

$$SBF = (B - NC)/BG,$$

where B is the amount of CV bound to the cells attached to the surface of the wells, NC is the negative control, and BG is the OD₆₀₀ of bacterial growth [28]. These investigators used two microscopic methods to evaluate the relevance of SBF assay, and they demonstrated the overall trends between these methods [28].

Root and Seed Colonization Assay

Seeds of FN 4.10 and FN 4.85 were surface disinfected, grown under the same conditions previously described. Before inoculation, the sterility condition of the exudates was controlled by incubating a sample in PDA and a sample in NA media. After 10 days of plant growth, 1 mL of an overnight BNM339 culture, which was grown in nutrient broth (NB), washed twice, and resuspended in sterile PS at 8×10^6 CFU mL⁻¹, was added. PS (1 mL) was added to the control tubes. The bacterial number in the root surrounding medium and that attached to the roots were determined at inoculation t₀ (0 time) and 11 days after inoculation (DAI). Plants were withdrawn from tubes,

and the roots were placed on sterile filter paper to remove excess medium. The roots were submerged in 10 mL sterile PS in Falcon tubes and vortexed for 2 minutes to extract loosely bound (rhizospheric) bacteria. To remove tightly bound (rhizoplane) bacteria, roots were sonicated (5 minutes) in a water-bath sonicator (XB2 Grant Instrumental). Each bacterial suspension was serially diluted and plated on NA. Petri dishes were incubated for 48 hours at 30°C, and bacterial counts were expressed as CFU mL⁻¹ for bacteria suspended in medium and as CFU g⁻¹ root dry weight for rhizospheric and rhizoplane bacteria.

For the seed colonization assay, the disinfected seeds as previously mentioned were inoculated with 1.5×10^6 CFU mL⁻¹ overnight culture of BNM339, which was grown in NB, washed twice with sterile PS, and placed in a Petri dish over a wet filter paper. At different times (0, 24, 48, and 72 hours), five inoculated seeds of each variety, as well as the control (seeds without inoculation), were submerged in 5 mL PS and vortexed for 2 minutes to extract bound bacteria. After serial dilution, the CFU seed⁻¹ was quantified.

Chemical Analysis of Root and Seed Exudates

The carbohydrate composition of the different samples of root or seed exudates was determined by high-performance liquid chromatography (HPLC) with a pulse amperometric detector in a Dionex DX-300 using a column CarboPac PA-10 (4 × 250 mm) with a precolumn PA-10 (Dionex). These analyses were performed at the Department of Organic Chemistry, School of Sciences, University of Buenos Aires, Argentina. For amino-acid analysis, the sample was injected on a high-performance liquid chromatographer (Hitachi L8800) and analyzed in the UTMB Protein Chemistry Laboratory, Galveston, TX, using standard analytic methods provided by the manufacturer.

Statistical Analyses

All determinations were performed at least three times, and the results were analyzed by one-way analysis of variance and Tukey's test ($p < 0.05$).

Results

Chemotactic Response to Root and Seed Exudates

Fig. 1a shows the quantitative chemotactic responses of *B. amyloliquefaciens* BNM339 challenged with seed and root exudates of both soybean varieties. Positive chemotaxis was induced by seed exudates. In contrast, no root exudates, at any concentration tested, elicited a detectable chemotactic response by this bacterium.

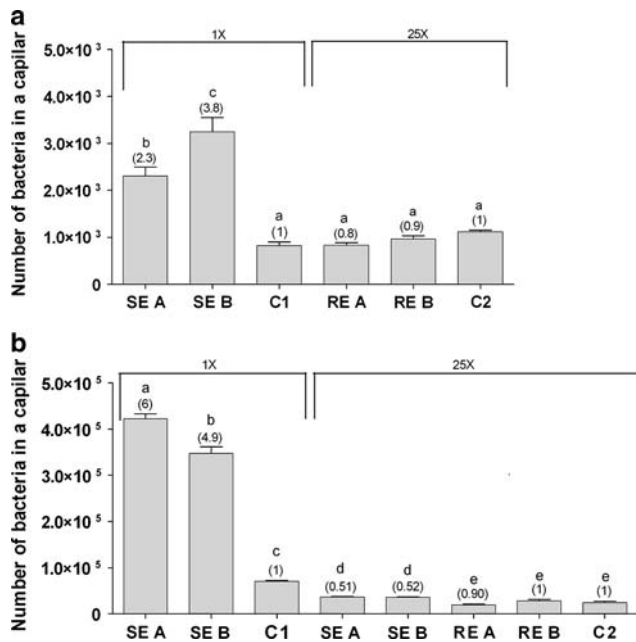


Fig. 1 Chemotactic response to soybean seed and root exudates. **(a)** *B. amyloliquefaciens* BNM339. **(b)** *B. japonicum* E109. Numbers in parentheses indicate the chemotaxis ratio (CFU mL⁻¹ present in the capillary tubes containing root or seed exudates divided by the corresponding control, C1 and C2 respectively). Different letters over the bars indicate significant differences ($p < 0.05$). SE, seed exudate; A, FN 4.10; B, FN 4.85; RE, root exudate (21 days of plant growth); C1 and C2, controls performed using water and MS, respectively, without addition of exudates. 1 × and 25 × indicate concentration of seed and root exudates used in the corresponding media. Notice that SEA and SEB concentrated 25-fold produced chemotaxis index values <1. For the bars identified as SE A and SE B (Fig. 1b), we used the same control (C1).

B. japonicum is known to be attracted to roots during nodulation [6] and is therefore used as a positive control bacterium for chemotaxis. The results presented in Fig. 1b show that *B. japonicum* was not attracted by root exudates at any concentration tested.

Seed exudates induced a positive chemotactic response in both *B. amyloliquefaciens* and *B. japonicum*. In the latter, the seed exudate produced by FN 4.10 was more attractive than that corresponding to FN 4.85. In both cases, they induced values higher than those shown by *B. amyloliquefaciens* BNM339 (approximately 2-fold greater).

We also measured chemotaxis under conditions allowing bacteria–seed interaction mediated by soluble exudates of seeds and bacteria diffusing in the agar medium. Because this experiment lasted for at least 24 hours, the bacteria underwent cell division. In this case, chemotaxis was clearly observed, and Fig. 2 shows the multiple growing and migrating bands developing toward the attractants. This observation was made earlier by other investigators who interpreted their result as being caused by swarming [33]. On one of the sides of the plate, a filter

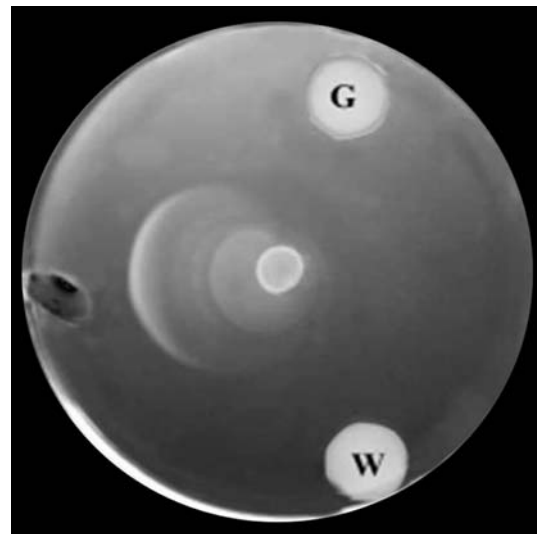


Fig. 2 Qualitative chemotactic response using seed as exudate source. Seed of FN 4.10 and *B. amyloliquefaciens* BNM339 in phosphate buffer containing 0.3% agar. G, glucose 0.5%; W, water; S, seed. Notice the preferential use of the seed exudate as the chosen attractant. Similar results were obtained with FN 4.85

paper embedded in glucose was used as attractant source. The experiment demonstrated that the bacteria were preferentially attracted to the seeds, although in other experiments we determined that 0.5% glucose acts as an attractant (data not shown).

Biofilm Formation

This experiment was performed with seed exudates because they allow cell division to occur without the addition of any external carbon source. However, because root exudates did not support growth, we could not perform similar experiments. Soybean seeds of both varieties indeed induced an SBF higher than that produced in a favorable medium, such as LB-plus, which is specially designed to induce biofilm formation in *Bacillus* [12]. Therefore, the lower index occurring in LB-plus was caused both by a higher mass of cells and by a concomitant lower formation of biofilm. The seed exudates of the variety FN 4.85 produced the highest SBF value (Fig. 3).

Effect of Root and Seed Exudates on the Growth Parameters of *B. amyloliquefaciens*

Bacterial growth curves of *B. amyloliquefaciens* BNM339 growing in the presence of root exudates plus 0.5% glucose were carried out. When glucose was not added, no growth was observed.

The addition of root exudates to one of the soybean varieties (FN 4.10) decreased duplication time in *B. amyloliquefaciens* from 15 hours to 10 hours and produced a

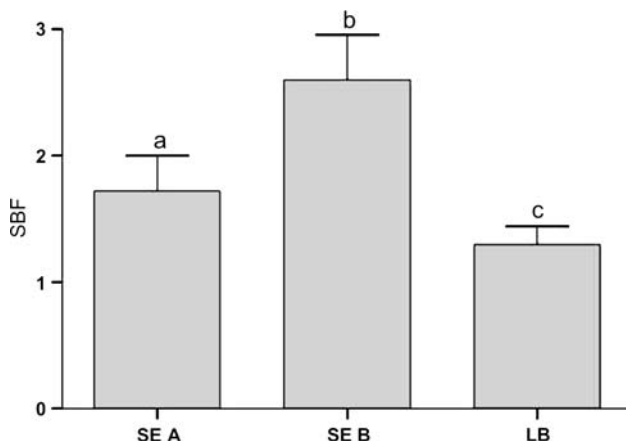


Fig. 3 Specific biofilm formation. Effects of seed exudates (SE) of different soybean varieties [(a) FN 4.10 and (b) FN 4.85] on the SBF of *B. amyloliquefaciens* BNM339 compared with LB-plus medium. Each bar shows the SD. Different letters over the bars indicate significant differences ($p < 0.05$)

slight increase in final cell mass (from 1.4×10^7 CFU mL^{-1} to 2.1×10^7 CFU mL^{-1}). In both cases, initial CFU mL^{-1} was 1.2×10^6 . The other variety did not induce variation in these growth parameters.

Seed exudates indeed produced a significant decrease in duplication time to 1.7 hours, and 0.5 % glucose did not improve this parameter. Also, maximum cell biomass in the presence of 0.5% glucose increased 5-fold, $\leq 5 \times 10^8$ CFU mL^{-1} , from an initial inoculum of 6×10^5 CFU mL^{-1} .

Root and Seed Colonization of Two Soybean Varieties by *B. amyloliquefaciens* BNM339

The initial bacterial inoculum around the roots was 5.3×10^5 CFU mL^{-1} and 11 DAI it had increased to 1.3×10^6 and 2.4×10^6 CFU mL^{-1} for FN 4.10 and FN 4.85 soybean varieties, respectively. These results show how exudates excreted from roots sustained growth at variance with axenically produced exudates.

Loosely bound bacteria were identical for both soybean varieties, whereas tightly bound bacteria were 1 order of magnitude greater for FN 4.10 than for FN 4.85 (5×10^6 and 2.7×10^5 CFU g^{-1} root dry weight, respectively) (Table 1).

Table 1 Root colonization by *B. amyloliquefaciens* BNM339^a

Soybean variety	Bacteria suspended in medium (CFU mL^{-1})		Rhizospheric bacteria (CFU g^{-1} root [dw])		Rhizoplane bacteria (CFU g^{-1} root [dw])	
	t_0	11 DAI	t_0	11 DAI	t_0	11 DAI
FN 4.10	5.3×10^5 aA	1.3×10^6 aB	ND	1.4×10^8 aA	ND	5.0×10^6 bB
FN 4.85	5.3×10^5 aA	2.4×10^6 aB	ND	1.7×10^8 aA	ND	2.7×10^5 cC

ND, not detected

^a Data represent the average of three replicates. For different sample times, values with different capital letters were significantly different ($p < 0.05$), and for each plant variety, values with different lower-case letters were significantly different ($p < 0.05$).

Table 2 Seed colonization of soybean varieties by *B. amyloliquefaciens* BNM339

Soybean variety	Days after inoculation			
	0	1	2	3
FN 4.10	3×10^3 a	1×10^4 a	4×10^5 a	1×10^6 a
FN 4.85	6×10^3 b	1.1×10^4 a	3×10^5 a	7×10^5 a

^a Data represent the average of three replicates. Values with different lower-case letters were significantly different ($p < 0.05$)

Table 2 shows that seed exudates positively induced seed colonization and that between days 0 and 3, there was a continuous increase in the number of bacteria bound to the seeds. Both seed varieties showed the same colonization capacity.

Chemical Determination of Root and Seed Exudates

Carbohydrate analysis of the root exudates (Table 3) showed that five main carbohydrates were excreted: fructose, arabinose, glucose, mannose, and galactose. Total sugar concentration increased for both soybean varieties between days 10 and 21. Arabinose, mannose, and fructose increased between days 10 and 21 of growth, whereas galactose and glucose decreased (Table 3).

In seed exudates, only three main carbohydrates, fructose, galactose, and glucose were detected after 20 hours of incubation. FN 4.85 showed higher amounts of total carbohydrates excreted than FN 4.10, but this was not statistically significant (Table 3).

Table 3 also lists the excretion of amino-acids and small peptides by root and seed exudates of both varieties. In the case of root exudates, amino-acid concentrations increased on day 21, and some of them only became detectable at this time for both soybean varieties.

Root exudates showed higher amino-acid diversity than seed exudates. However, only four main amino-acids (asparagine, α -aminobutyric acid, glutamine, and histidine) were distinguishable in roots, whereas in seeds all amino-acids were excreted in relatively equivalent amounts.

Table 3 Chemical composition of the root exudates from two soybean varieties^a

	Root exudates (nmol plant ⁻¹)				Seed exudates (nmol seed ⁻¹)	
	FN 4.10		FN 4.85		FN 4.10	FN 4.85
	10 days	21 days	10 days	21 days		
<i>Carbohydrate</i>						
Arabinose	41.41	130.58	9.11	177.84	ND	ND
Galactose	27.35	3.69	30.34	19.94	50.11	90.51
Glucose	138.32	71.10	130.48	59.83	257.42	366.59
Mannose	19.89	41.77	39.45	31.42	ND	ND
Fructose	41.03	539.66	160.82	287.78	212.00	343.78
Total nmol plant ⁻¹	268.0 a	786.80 b	370.20 a	576.81 c	519.53 a	800.88 a
<i>Amino-acid and other nitrogenated compounds</i>						
Phosphoserine	5.35	7.48	8.42	7.92	13.63	14.42
Taurine	0.27	ND	ND	ND	ND	ND
Aspartic acid	0.34	1.19	0.34	8.85	12.46	8.75
Threonine	0.30	1.10	0.22	8.62	7.60	6.31
Serine	0.93	3.84	0.71	2.91	6.46	5.05
Asparagine	2.04	144.82	2.35	55.30	8.77	4.46
Glutamine	1.23	24.33	1.50	6.98	1.81	ND
Sarcosine	1.24	1.85	1.17	1.98	ND	4.10
Glycine	0.57	3.01	1.09	2.75	7.04	3.39
Alanine	0.07	1.80	0.14	1.12	22.76	15.76
Valine	0.44	0.84	0.77	0.72	8.70	7.46
Methionine	0.11	1.07	0.14	0.72	ND	ND
Leucine	0.53	0.52	0.37	0.29	4.08	2.47
Tyrosine	0.41	0.73	0.44	0.73	3.98	1.91
Lysine	0.75	3.04	0.45	1.55	3.61	0.76
Histidine	0.91	18.33	0.04	9.97	1.83	1.02
Arginine	2.08	5.75	1.89	1.93	3.47	0.46
Proline	0.37	0.39	1.74	0.22	6.82	6.54
Citrulline	ND	0.32	ND	0.04	ND	ND
β -alanine	ND	0.47	ND	ND	3.59	1.24
Tryptophan	ND	2.79	ND	0.29	2.14	0.50
Ornithine	ND	1.25	0.19	0.76	0.25	ND
Phenylalanine	ND	0.06	1.56	0.04	3.15	1.96
Isoleucine	ND	0.20	0.12	0.16	4.41	1.89
Hydroxyproline	ND	ND	6.48	4.28	ND	ND
α -aminobutyric acid	25.43	39.05	30.57	43.25	ND	ND
β -aminoisobutyric acid	ND	3.77	ND	ND	0.48	1.24
γ -aminobutyric acid	1.52	2.66	2.63	0.30	56.74	22.91
Carnosine	11.81	8.00	21.62	12.13	ND	ND
Cystathionine	9.01	10.58	11.13	12.28	ND	ND
Total nmol plant ⁻¹	65.71 a	289.24 b	96.08 c	186.09 d	183.78 a	112.60 a

ND, not detected

^a Values denoted by different letters were significantly different ($p < 0.05$). Values are the mean of three replicates with five repetitions. SE of the mean was $< 10\%$

Discussion

The main purpose of this study was to describe the relation existing between bacterial growth, chemotaxis, biofilm formation, and colonization in a bacterium having potential biocontrol properties.

Concerning chemotaxis, we obtained results similar to those of Currier and Strobel [10], who reported that *Rhizobium* spp was not attracted by root exudates, and those of Bacilio-Gimenez et al. [3], who showed similar results for rice root exudates in chemotaxis of *Azospirillum*. Similarly, Kato and Arima [16] observed no chemotaxis induced by

root exudates of *Phaseolus vulgaris* L. Additionally, we show here that the absence of chemotactic response did not change when the exudates were concentrated up to 200-fold.

Because positive chemotaxis to roots is a postulated prerequisite for efficient root bacterial colonization [37], it is possible that in our experiments, the use of exudates excreted in the absence of bacteria did not induce positive chemotaxis because of the absence of an unknown compound(s) that was excreted by the roots in the presence of bacteria [17, 31]. We showed that seed exudates from both soybean varieties induced positive chemotaxis in strain BNM339.

B. japonicum E109 was expected to be attracted by the roots exudates because positive chemotaxis is included within the mechanism of root infection and nodulation, but we did not observe a positive chemotactic response using axenically excreted root exudates. The attraction by seed exudates observed was concentration dependent, and a 25-fold concentration induced a chemotactic index <1.0, thus indicating the occurrence of repulsion. This could be explained by the concentration of some diffusible toxins excreted by soybean seeds that inhibited *B. japonicum* growth [2].

Seed exudates sustained bacterial growth in the absence of any other external compounds. Roots, on the contrary, did not support growth unless carbon source was added. Therefore, seeds, but not root exudates, were able to provide growth factors as well as nutrient sources utilizable by these bacteria.

We also showed that specific biofilm formation by BNM339 was much higher in seed exudates than in LB-plus medium and that it was inversely correlated to the number of cells present in the stationary phase, indicating that suboptimal growth conditions favored the amount of biofilm accumulated. Coincidentally, biofilm formation by *B. subtilis* was also stimulated by nonoptimal growth conditions, and it was inhibited when a rapidly metabolizable carbon source was added [35]. The advantages represented by the possibility that seed exudates, even axenically obtained, induce biofilm formation on artificial surfaces can only be thought as a means to facilitate occupancy of the seed surface and later on be transferred to the root tips after emergence of the root. Another alternative explanation is that growing root tips move in the rhizosphere, colliding with the bacterial population present therein, and thus bring to the microorganisms the surfaces to be colonized [22].

It is well known that plant–bacteria interactions are the result of a complex exchange of chemical compounds between plants and microorganisms, governing the qualitative and quantitative changes of root exudates and perhaps also those of seeds [26]. The data presented here additionally show that some of the qualitative–quantitative composition of compounds excreted by seed and root

exudates after 21 days of plants growth were not so different and that, therefore, some other compounds not yet detected by us are most probably responsible for the different chemotactic responses observed. Therefore, we hypothesize that changes in the quality of exudates, produced when roots and bacteria are simultaneously present, allow colonization to occur and that bacterial growth and biofilm formation are also facilitated.

Acknowledgments The authors acknowledge financial support received from the Agencia Nacional de Promoción Científica y Tecnológica (Grant No. PICT 01-10892); the Centro Argentino-Brasileño de Biotecnología (Grant No. 13AR-07BR); and the Consejo Nacional de Investigaciones Científicas y Técnicas (Grant No. PIP 5003). We thank F. del Norte for kindly supplying the seeds and the phenotypic characteristics of the soybean varieties used here.

References

- Adler J (1973) A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. *J Gen Microbiol* 74:77–91
- Ali FS, Loynachan TE (1990) Inhibition of *Bradyrhizobium japonicum* by diffusates from soybean seed. *Soil Biol Biochem* 22:973–976
- Bacilio-Jiménez M, Aguilar-Flores S, Ventura-Zapata E, Pérez-Campos E, Bouquelet S, Zenteno E (2003) Chemical characterization of root exudates from rice (*Oryza sativa*) and their effects on the chemotactic response of endophytic bacteria. *Plant Soil* 249:271–277
- Bais HP, Ray F, Vivanco JM (2004) Biocontrol of *Bacillus subtilis* against infection of arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiol* 134:307–319
- Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants. *Annu Rev Plant Biol* 57:233–266
- Barbour MW, Hattermann DR, Stacey G (1991) Chemotaxis of *Bradyrhizobium japonicum* to soybean exudates. *Appl Environ Microbiol* 57:2635–2639
- Branda SS, Chu F, Kearns DB, Losick R, Kolter R (2006) A major protein component of the *Bacillus subtilis* biofilm matrix. *Mol Microbiol* 59:1229–1238
- Caetano-Anollés G, Wall LG, De Micheli AT, Macchi EM, Bauer WD, Favelukes G (1988) Role of motility and chemotaxis in efficiency of nodulation by *Rhizobium melliloti*. *Plant Physiol* 86:1228–1235
- Chin-A-Woeng TFC, Bloemberg GV, Mulders IHM, Dekkers LC, Lugtenberg BJJ (2000) Root colonization is essential for biocontrol of tomato foot and root rot by the phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391. *Mol Plant Microbe Interact* 13:1340–1345
- Currier WW, Strobel GA (1976) Chemotaxis of *Rhizobium* spp. to plant root exudates. *Plant Physiol* 57:820–823
- de Weert S, Vermeiren H, Mulders I, Kuiper I, Hendrickx N, Bloemberg GV et al (2002) Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol Plant Microbe Interact* 15:1173–1180
- Hamon MA, Lazazzera BA (2001) The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. *Mol Microbiol* 42:1199–1209

13. Hiroyuki F, Masao S, Hidenori O, Yasufumi U, Tadayoshi S, Tatsuhiko M (1998) Chemotactic response to amino acids of fluorescent *Pseudomonas* isolated from spinach roots grown in soils with different salinity levels. *Soil Sci Plant Nutr* 44:1–7
14. Kadouri D, Jurkevitch E, Okon Y (2003) Involvement of the reserve material poly- β -hydroxybutyrate in *Azospirillum brasilense* stress endurance and root colonization. *Appl Environ Microbiol* 69:3244–3250
15. Kamilova F, Validov S, Azarova T, Mulders I, Lugtenberg BJJ (2005) Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. *Environ Microbiol* 7:1809–1817
16. Kato K, Arima Y (2006) Potential of seed and root exudates of the common bean *Phaseolus vulgaris* L. for immediate induction of rhizobial chemotaxis and nod genes. *Soil Sci Plant Nutr* 52:432–437
17. Kuzyakov Y, Raskatov A, Kaupenjohann M (2003) Turnover and distribution of root exudates of *Zea mays*. *Plant Soil* 254:317–327
18. Landa BB, Hervás A, Bettiol W, Jiménez-Díaz RM (1997) Antagonistic activity of bacteria from the chickpea rhizosphere against *Fusarium oxysporum* f. sp. *ciceris*. *Phytoparasitica* 25:305–318
19. Lorda GS, Balatti AP (1996) Designing media I. Production of high cell concentrations of *Rhizobium* and *Bradyrhizobium*. In: Balatti AP, Freire, JRJ (eds) *Legume inoculants. Selection and characterization of strains, production, use and management*. Editorial Kingraf, Buenos Aires, Argentina, pp 78–93
20. Lugtenberg BJJ, Dekkers LC (1999) What make *Pseudomonas* bacteria rhizosphere competent? *Environ Microbiol* 1:9–13
21. Lugtenberg BJJ, Kravchenko LV, Simons M (1999) Tomato seed and root exudate sugars: composition, utilization by *Pseudomonas* biocontrol strains, and role in rhizosphere colonization. *Environ Microbiol* 1:439–446
22. Lugtenberg BJJ, Dekkers LC, Bloemberg GV (2002) Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annu Rev Phytopathol* 39:461–490
23. Lynch JM, Whipps JM (1990) Substrate flow in the rhizosphere. *Plant Soil* 129:1–10
24. Molina MA, Ramos JL, Espinosa-Urgel M (2003) Plant-associated biofilms. *Rev Environ Sci Biotechnol* 2:99–108
25. Morris CE, Monier JM (2003) The ecological significance of biofilm formation by plant-associated bacteria. *Annu Rev Phytopathol* 41:429–453
26. Mozafar A, Duss F, Oertli JJ (1992) Effect of *Pseudomonas fluorescens* on the root exudates of two tomato mutants differently sensitive to Fe chlorosis. *Plant Soil* 144:167–176
27. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tissue culture. *Physiol Plant* 15:473–497
28. Niu C, Gilbert ES (2004) Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. *Appl Environ Microbiol* 70:6951–6956
29. Ordal G, Goldman D (1975) Chemotaxis away from uncouplers of oxidative phosphorylation in *Bacillus subtilis*. *Science* 189:802–805
30. Ordal G, Katharine G (1977) Chemotaxis toward amino acids by *Bacillus subtilis*. *J Bacteriol* 129:151–155
31. Phillips DA, Tama CF, King MD, Bhuvanewari TV, Teuber LR (2004) Microbial products trigger amino acid exudation from plant roots. *Plant Physiol* 136:2887–2894
32. Schippers B, Baker AW, Bakker PAHM (1987) Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annu Rev Phytopathol* 25:339–358
33. Sharma M, Anand SK (2002) Swarming: a coordinated bacterial activity. *Current Sci* 83:707–715
34. Somasegaran P, Hoben HJ (1994) *Handbook for rhizobia*. Springer-Verlag, New York, NY, p 337–341
35. Stanley NR, Britton RA, Grossman AD, Lazazzera BA (2003) Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. *J Bacteriol* 185:1951–1957
36. Thomashow LS (1996) Biological control of plant root pathogens. *Curr Opin Biotechnol* 7:343–347
37. Vande Broek A, Lambrecht M, Vanderleyden J (1998) Bacterial chemotactic motility is important for the initiation of wheat root colonization by *Azospirillum brasilense*. *Microbiology* 144:2599–2606
38. Zheng XY, Sinclair JB (1996) Chemotactic response of *Bacillus megaterium* strain B153-2-2 to soybean root and seed exudates. *Physiol Mol Plant Pathol* 48:21–35