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ORIGINAL PAPER

Growth-promotion of strawberry plants inoculated with *Azospirillum brasilense*

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Abstract *Azospirillum brasilense* (strains REC3, RLC1, PEC5) were root inoculated in strawberry plants of the cultivars 'Milsei', 'Selva' and 'Camarosa' to assess plant growth-promoting effects. The bacteria were able to promote plant growth (expressed as root length, root area, and dry weight of root and shoot), depending on the genotypes of plants and bacteria used, whereas the stolon production (3–4) depended only on the strawberry cultivar. To explain whether root exudates plays any role on the growth-promotion observed herein, total protein and sugar were determined, and chemotaxis properties were evaluated. The strains showed positive chemotaxis toward the root exudates, being influenced by the total sugars content, suggesting that the latter plays an important role in the chemotaxis effect and may contribute to enhance the root capacity to recruit azospirilla from rhizosphere, thus improving the growth-promoting effect exerted by these bacteria.

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Introduction

Azospirillum species are free-living N_2-fixing bacteria commonly found in soils and in association with roots of different plant species. For their capacity to promote plant growth they are considered as plant growth-promoting bacteria (PGPB; Bashan and de Bashan 2005). Field inoculation with *Azospirillum* spp. has been evaluated worldwide in different crops, demonstrating that these bacteria are capable to improve yields of important crops in different soils and climatic regions (Okon and Labandera-Gonzalez 1994). The stimulatory effect exerted by *Azospirillum* has been attributed to several mechanisms including secretion of phytohormones (e.g. auxins and gibberellins), biological nitrogen fixation, and enhancement of mineral uptake by plants (Bashan et al. 2004). However, response to plant inoculation with *Azospirillum* has not always been successful, and the factors affecting the crop response are not completely understood (Okon and Labandera-Gonzalez 1994; Bashan and Holguin 1997).

Complex interactions exist between plant genotypes and *Azospirillum* strains. It has been observed in wheat that different *A. brasilense* strains colonize differently a single cultivar, and also that a particular strain presents different colonizing capability depending on the cultivar used (Saubidet and Barneix 1998).

Azospirillum spp. attach to and colonize plant root surfaces, and this process depends on active motility and chemotaxis toward root exudates (Bashan et al. 2004). The latter constitute an important source of nutrients for the microorganisms present in the rhizosphere and participate

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in the colonization process through chemotaxis (Bais et al. 2006). Bacilio-Jiménez et al. (2003) reported that root exudates from rice induced higher chemotactic response in endophytic bacteria than in other bacterial strains present in the rice rhizosphere. It is known that chemotactic response toward amino acids, sugars, or organic acids is fundamental for bacterial behavior as observed in vitro and in situ assays (Barak et al. 1983; Bashan and Holguin 1997; Hauwaerts et al. 2002), and probably represents the first step in root colonization (Zheng and Sinclair 1996).

Although *Azospirillum* was isolated firstly from cereals and used to inoculate the same cereal crops, there are many other non-cereal species that can be successfully inoculated with *Azospirillum* (Bashan and Holguin 1997). Recently it was reported the natural occurrence of *A. brasilense* in strawberry plants grown in Tucumán, Argentina, exhibiting three conspicuous characters defining a PGPB, namely: N_2-fixation, siderophores, and indoles production (Pedraza et al. 2007). However, the plant growth promoting properties of those isolates has not been demonstrated yet.

Strawberries are cultivated in different parts of the world, including tropical, subtropical, and temperate areas. The world production of strawberry is estimated in three million tons per year and it has increased in the last two decades. Argentina produces strawberry during the 12 months of the year, being the province of Tucumán one of the most important producers (Pedraza et al. 2007; Pérez and Mazzone 2004). Considering the benefits that could arise from the interaction between *Azospirillum* and strawberry plants, and its possible application as inoculant, the aim of this work was (i) to assess the growth-promotion effect of three *A. brasilense* strains on three commercial cultivars of strawberry, and (ii) to evaluate the chemotactic effect of the root exudates toward *Azospirillum*. Implications of the chemotactic properties of root exudates on the PGPB effect exerted by *Azospirillum* on strawberry plants are discussed.

Materials and methods

Vegetal material

Three commercial cultivars of strawberry (*Fragaria ananassa*, Duch) were used: 'Milsei', 'Selva' and 'Camarosa'. Plantlets were obtained from the strawberry Active Germplasm Bank at National University of Tucumán through in vitro culture to ensure healthy and bacterial free plants.

Inoculum preparation

Three strains of *A. brasilense*, RLC1, REC3, PEC5, isolated and characterized in a previous work were used (Pedraza et al. 2007). RLC1 was isolated from washed roots, while REC3 and PEC5 from sterilized roots and stolons of strawberry, respectively. The identification of isolates was based on microbiological, biochemical, and molecular tests specifically reported for *Azospirillum* species (Tarrand et al. 1978; Döbereiner et al. 1995; Grifoni et al. 1995), using the strains *A. brasilense* Sp7 (ATCC 29145) and Sp245, *Azospirillum lipoferum* Sp59 (ATCC 29707), *Azospirillum amazonense* Y1 (ATCC 35119), *A. irakense*, and *A. halopraeferens* as references.

They were selected based on their capacity to fix nitrogen and to produce siderophores and indoles, considered as beneficial traits within the PGPB (Pedraza et al. 2007). Pure cultures of each strain were firstly grown in NFb N-free semisolid medium (Baldani and Döbereiner 1980) for 48 h at 30°C; then a loopfull of each culture was transferred separately to 100 ml NFb liquid medium without bromothymol blue, supplemented with 1% NH_4Cl (w/v) and incubated at 30°C, without shaking for 72 h. After incubation, the cells were centrifuged at 7,000×*g* for 10 min and washed twice with buffer phosphate pH 7.0 to remove any culture medium residue that may interfere on the growth promoting effect on strawberry plants. The bacterial concentration for inoculation was at 10^6 CFU ml^{-1}.

Inoculation and growing conditions

Three months old strawberry plantlets were inoculated with the different strains of *Azospirillum* by submerging their roots in a bacterial suspension (10^6 CFU ml^{-1}) for 30 min. Plant roots were drained for 5 s and immediately planted in disinfected pots containing sterile substrate (humus:perlome, 2:1). Experiments were conducted with ten plantlets of each cultivar that were inoculated with each strain of *Azospirillum*. Control consisted in a set of 10 plants that were not inoculated. Plants were then moved to growing chambers at 28°C, 70% RH, with 16 h of photoperiod (2,500 Lux). Plants were watered every other day with 50 ml of sterile distilled water.

After 54 days from inoculation, plants were removed from the pots and the substrate was detached from the roots by washing them carefully with tap water and root length (cm), shoot and roots dry weight (g), number of stolons per plant, and root surface area were evaluated. Root area was determined by immersing air-dried roots in a $Ca(NO_3)_2$ saturated solution and recording the weight of $Ca(NO_3)_2$ removed from solution (Carley and Watson 1966).

Scanning electron microscopy

Samples of roots were fixed in a 3% glutaraldehyde solution buffered with 0.1 M phosphate buffer (pH 7.4) for 3 h at

room temperature and postfixed in 1% osmium tetroxide in the same buffer overnight. Specimens were washed three times in distilled water and then treated with an aqueous solution of 2% uranyl acetate for 40 min. After fixation samples were stepwise dehydrated with increasing concentrations ethanol (30–100%), followed with acetone (100%), critical-point dried, mounted on aluminum stubs, coated with gold and examined with a scanning electron microscope (SEM) to observe *Azospirillum* colonization and hair root formation. Samples were observed and photographed in a JEOL JSM35 CF scanning electron microscope.

Chemotaxis assay

Strawberry in vitro plants were aseptically grown in 25 ml of diluted (1:2) Hoagland solution (Hoagland 1975) and maintained in a growth-chamber at 25°C, 70% of relative humidity and a photoperiod of 16 h light. The root exudates were collected from the liquid nutrient medium (Hoagland solution) used by plants after 7, 14, and 28 days of growth. The nutrient medium (25 ml) containing the root exudates was removed and sterilized by filtration (0.2 µm Millipore), lyophilized and kept at −20°C for total protein and sugar determination and chemotaxis test. Sterility of each solution was verified by plating samples in LB medium (Sambrook et al. 1989) and incubated 72 h at 30°C.

Chemotaxis was evaluated on SM medium (Reinhold et al. 1985), without malic acid, yeast extract, neither NH_4Cl, and supplemented with 0.3% agarose (w/v). Lyophilized extracts containing root exudates from each cultivar were resuspended in distilled sterile water to obtain two concentrations: 8× (d1) and 4× (d2). Root exudates (0.1 ml) was added to 7 ml of SM medium (kept at 45°C), vigorously mixed and poured into sterile Petri dishes (60 mm diameter). Once at room temperature 0.01 ml of previously grown and washed bacteria (see below) was placed in the center of the plates. Plates were incubated at 30°C and the halo diameter measured (mm^2) after 48 h.

Mobile bacteria were obtained from 48 h old cultures grown in SM medium (Reinhold et al. 1985). Cells were collected by centrifugation (15 min at 15,000×*g*) and washed three times with potassium phosphate (60 mM pH 7.0)/Na–EDTA (0.1 mM) buffer. The cells were finally resuspended in phosphate buffer without EDTA and the concentration adjusted to 10^8 cells ml^{-1} ($OD_{600} = 1.0$). Cell motility was controlled by contrast phase microscopy (Olympus BH-2). For chemotaxis test, cell suspensions were used within 1 h after washing to avoid motility loss.

Sugar and protein determination of root exudates

Total sugar was determined from lyophilized samples of root exudates suspended in sterile distilled water. 0.64 ml of phenol 80% and 2.5 ml of concentrated H_2SO_4 (80%) was added to 1 ml of each sample, mixed for 1 min with vortex and kept at 30°C for 10 min. After color development OD was measured at 490 nm and sugar content evaluated with a standard curve made with different glucose concentrations. Three determinations were performed for each sample (root exudates and glucose standards).

Protein concentration of the root exudates was determined according to Bradford (1976) using bovine-serum albumin as standard.

Experimental design and statistical analysis

Growth promoting experiments were randomized with 10 plants per strawberry genotype and per strains used, and chemotaxis assays were carried out on a complete randomized factorial design, including four factors: strains (RLC1, REC3, PEC5, Sp7), cultivars ('Milsei', 'Selva', 'Camarosa'), root exudates dilution (d1, d2), and the time the root exudates were collected (t1, t2, t3). ANOVA was performed, and the main effect of the different treatments was evaluated by the Wald Test ($P \leq 0.05$), using the software Statistix 7.0.

Results

Values of root length and root area are shown in Fig. 1a. Root length varied between 13.6 and 19.4 cm; in the case of cv 'Milsei', the highest value was obtain when it was inoculated with *A. brasilense* PEC5 (isolated from inner tissues of strawberry stolons) and the lowest with strain RLC1 (isolated from strawberry root surface), being lower than the control (without bacterial inoculation). In cv 'Selva', root length was higher than the control when using the three strains, but a better performance was observed when plants were inoculated with *A. brasilense* PEC5. In cv 'Camarosa', results of root length were similar to those observed with cv 'Milsei', and, again a reduction of root length when using strain RLC1 was observed.

In the same Fig. 1a, values of root area varied between 0.1 and 0.9 mg $Ca(NO_3)_2$ adsorbed to the roots. The highest value corresponds to the combination of cv 'Milsei' inoculated with *A. brasilense* PEC5, and the lowest to 'Milsei'-RLC1. With the cultivars 'Selva' and 'Camarosa' the bacterial inoculation produced similar response as when observing root length, including the same bacterial strains.

Figure 2 shows *A. brasilense* attached to strawberry roots and hair root formation in treatments and controls. At ultrastructural level, controls showed scarce root hairs proliferation, while plants inoculated with strains REC3 and PEC5 exhibited an important development of root

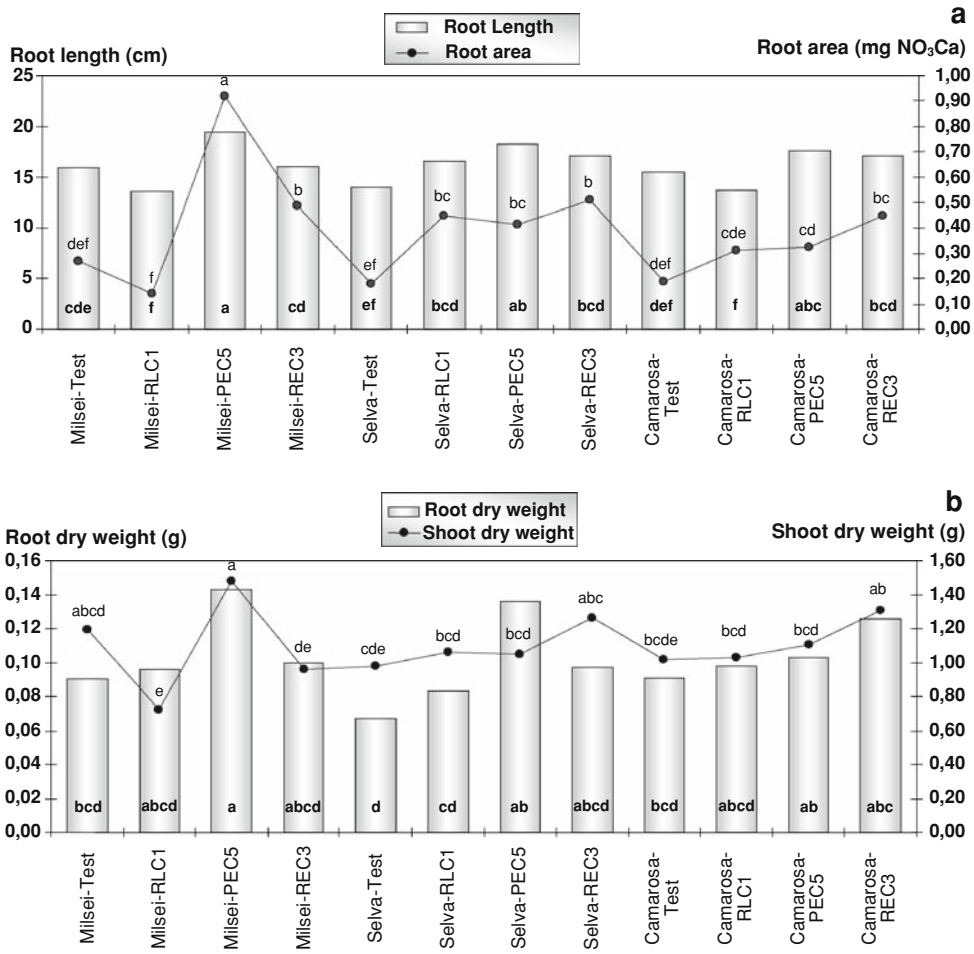

Fig. 1 Plant growth promoting effect of *A. brasilense* on strawberry plants, expressed as root length and area (**a**), and the dry weight of root and shoot (**b**). RLC1, PEC5 and REC3 correspond to local strains of *A. brasilense* inoculated onto three commercial cultivars of strawberry: 'Milsei', 'Selva' and 'Camarosa'. *Different letters* indicate significant differences at $P = 0.05$

hairs. Out of all treatments, the best colonization effect was observed in 'Camarosa' cultivar inoculated with REC3 (Fig. 3).

In Fig. 1b are shown results of root and shoot dry weight. For root dry weight, values varied between 0.09 and 0.14 g, being the values higher, although in different degree, than the controls (cultivars 'Milsei', 'Selva' and 'Camarosa' without bacterial inoculation). Regarding the shoot dry weight, different responses to the bacterial inoculation were observed. The highest value was detected when cv 'Milsei' was inoculated with *A. brasilense* PEC5 (1.48 g), but lower values were detected when using the strains RLC1 and REC3, as compared with the controls. In cv 'Selva', the best performance was achieved with *A. brasilense* REC3 (1.26 g), although the values did not differ much among the different treatments and control. A similar behavior was observed with the cv 'Camarosa', detecting the best performance with strain REC3 (1.28 g).

After concluding the bacterial inoculation assay, the number of stolons produced by the three strawberry cultivars was determined. The average ranged between three and four stolons per plant, however this parameter did not correlate with any treatment applied (with or without bacterial inoculation), but with the strawberry cultivar.

Results on the chemotactic response of the strains toward the root exudates obtained at 7, 14 and 28 days of plant growing in liquid support are showed in Fig. 4. In all cases, a positive chemotactic response was observed, with halo formation ranging between 50 and 100 mm^2, but a better response was achieved when using root exudates of the two-first weeks (7 and 14 days of plant growth).

Regarding the origin of the root exudates, halos greater than 100 mm^2 were observed in the four bacterial strains when using exudates of cv 'Selva' obtained at 28 days and with the highest dilution. Similar results were observed with cv 'Camarosa' and strain PEC5, but in this case, with the first dilution of root exudates.

The combined effects are shown in Fig. 5 where a positive chemotactic response of the four bacterial strains is observed. However, there are differences related with the origin of the strains as the two endophytic strains used showed higher number of positive chemotactic response expressed as halo formation greater than 50 mm^2 and with maximum values between 100 and 200 mm^2 with strain

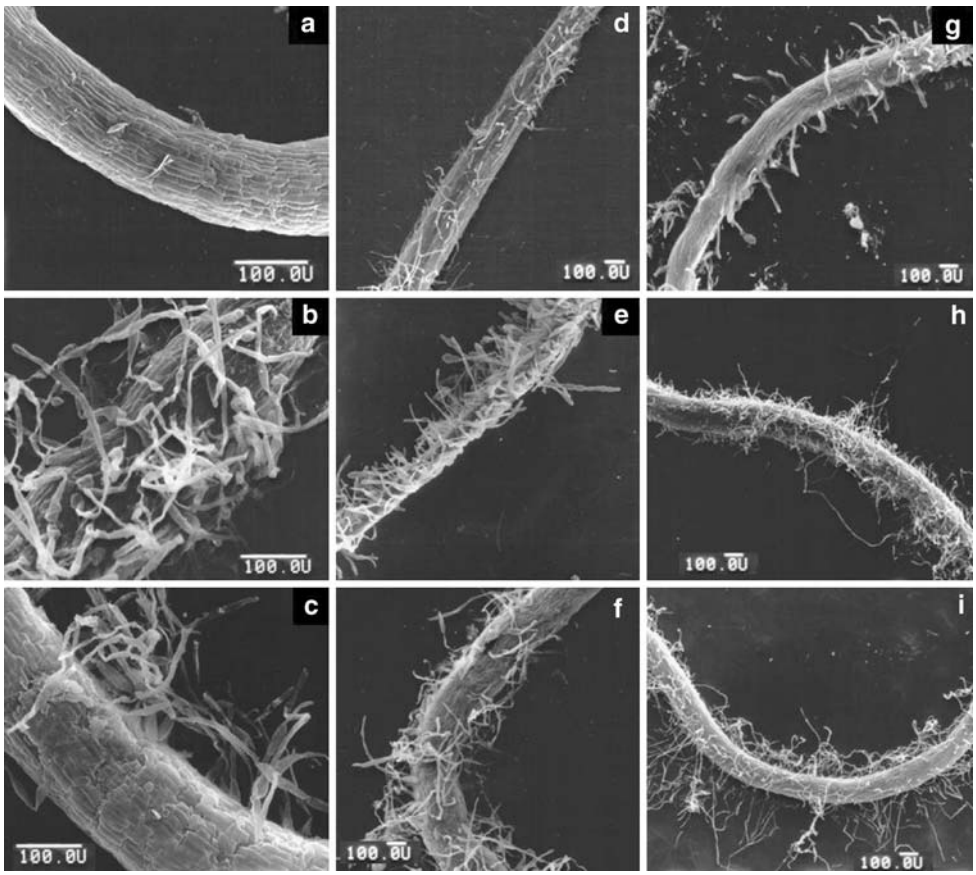

Fig. 2 *Azospirillum* colonization and hair root formation in strawberry plants, observed by SEM. **a** 'Camarosa' control (without bacterial inoculation); **b** 'Camarosa' inoculated with REC3; **c** 'Camarosa' inoculated with PEC5; **d** 'Milsei' control; **e** 'Milsei' inoculated with REC3; **f** 'Milsei' inoculated with PEC5; **g** 'Selva' control; **h** 'Selva' inoculated with REC3; **i** 'Selva' inoculated with PEC5

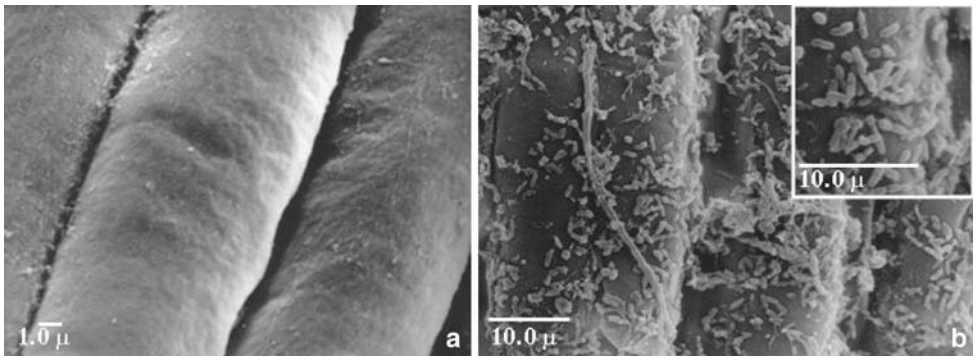

Fig. 3 *Azospirillum* colonizing strawberry roots, observed by SEM. **a** 'Camarosa' control (without bacterial inoculation); **b** 'Camarosa' inoculated with strain REC3, showing bacteria attached to root surface. *Inset*: detail at high magnification

REC3 and cv 'Camarosa'. There was also a higher chemotactic response with the higher dilution of the exudates in most of the cases. According to Wald test, the main effects observed in the factor cultivar ($P < 0.01$) and dilution ($P < 0.05$) are statistically significant as well as the interaction between strain and plant cultivar ($P < 0.05$). This indicates that the chemotactic response of strains depends on the cultivars from which root exudates were obtained.

To explain the chemotactic behavior previously observed, total sugar and protein content of root exudates was determined. The major amount of sugars was detected in the root exudates obtained at 7 days of plant growing of the three cultivars ('Milsei', 'Selva', 'Camarosa'), then, a diminution of them was observed at 14 and 28 days. The amount of total sugars varied among plant cultivars, being the cv 'Milsei' the highest producer at 7, 14 and 28 days as compared with the cvs 'Camarosa' and 'Selva' (Fig. 6). In contrast, protein content of the exudates was detected only after 28 days of plant growth, as shown in Fig. 6.

Discussion

The effect of *Azospirillum* inoculation on the growth promotion of strawberry plants was assessed on three

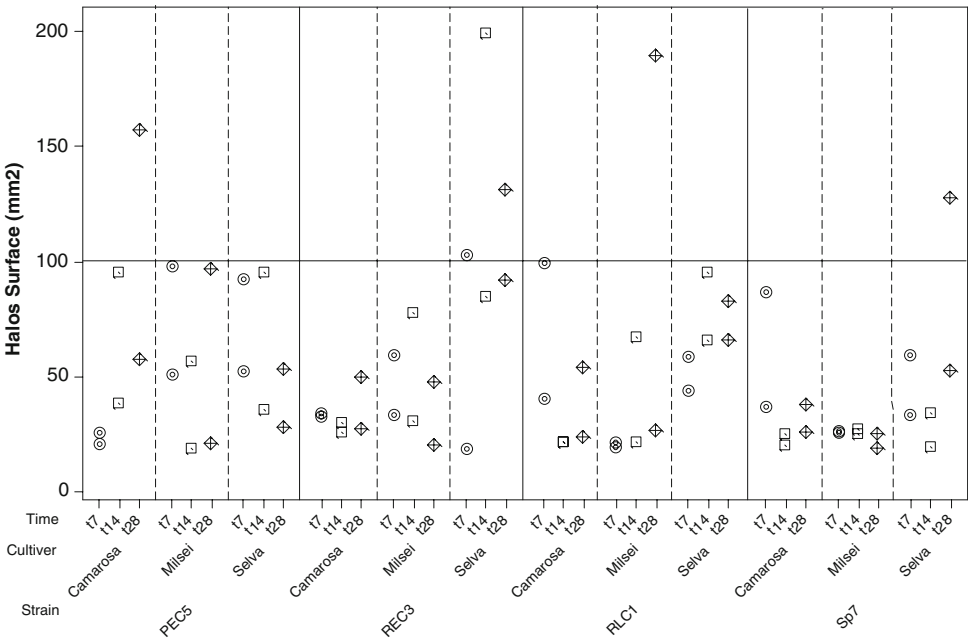

Fig. 4 Combined effects between time of extraction and dilution of the root exudates on the halo formation as a positive chemotactic response of the *A. brasilense* strains PEC5, REC3, RLC1 and Sp7 toward the root exudates. Time is expressed as days of root exudates extraction (t7, t14, t28). Each value corresponds to the mean of three replicates

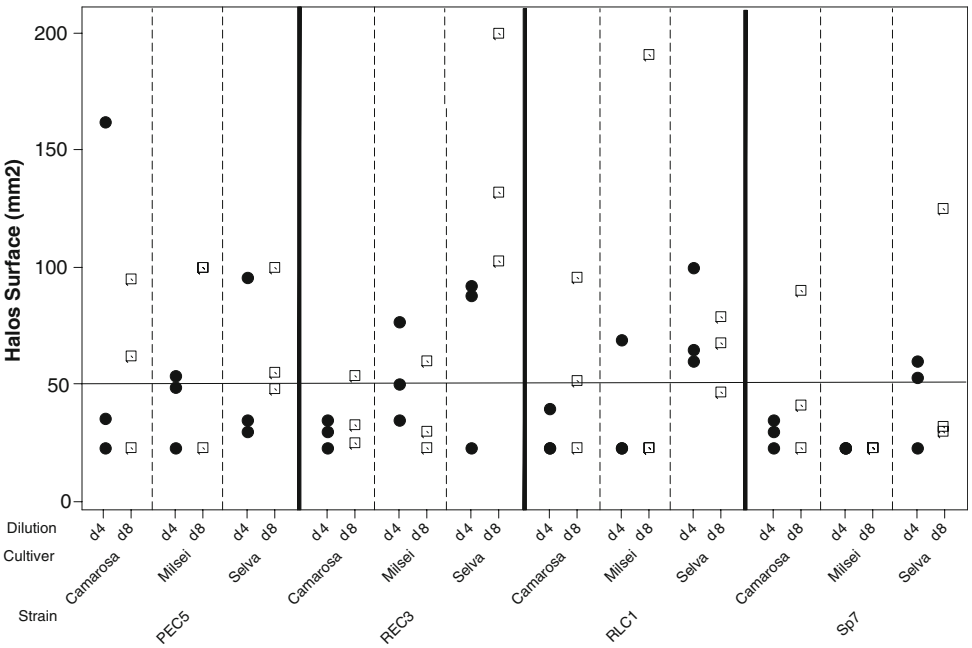

Fig. 5 Combined effects between dilution and time of root exudates extraction on the halo formation as a positive chemotactic response of the *A. brasilense* strains PEC5, REC3, RLC1 and Sp7 toward the root exudates. D4 correspond to 4-fold dilution of the exudates, and d8 to 8-fold dilution. Each value corresponds to the mean of three replicates

commercial cultivars ('Milsei', 'Selva', and 'Camarosa'). They were inoculated with three *A. brasilense* local strains: RLC1 (isolated from washed roots), REC3 and PEC5 (obtained from inner tissues of roots and stolons of strawberry plants, respectively; Pedraza et al. 2007).

As a general feature we have observed that different strains produced a positive effect on the growth promotion of strawberry plants, expressed as root length, root area, and dry root and shoot weight. But this effect strongly depends on the combination of strawberry cultivar and the bacterial strain used.

It is worthy that the best responses were observed when using strains isolated from inner tissues of strawberry roots and stolons (e.g., PEC5 and REC3; Fig. 1a, b), while a negative growth promoting effect was observed, in some cases, with the strains isolated from the root surface of strawberry plants (e.g., RLC1; Fig. 1a, b). Although bacterial inoculation did not always result in measurable positive results, we speculate that such biotized plants may exhibit an improved resistance against environmental stress after out-planting and subsequent growth in the field as suggested by Vestberg et al. (2004).

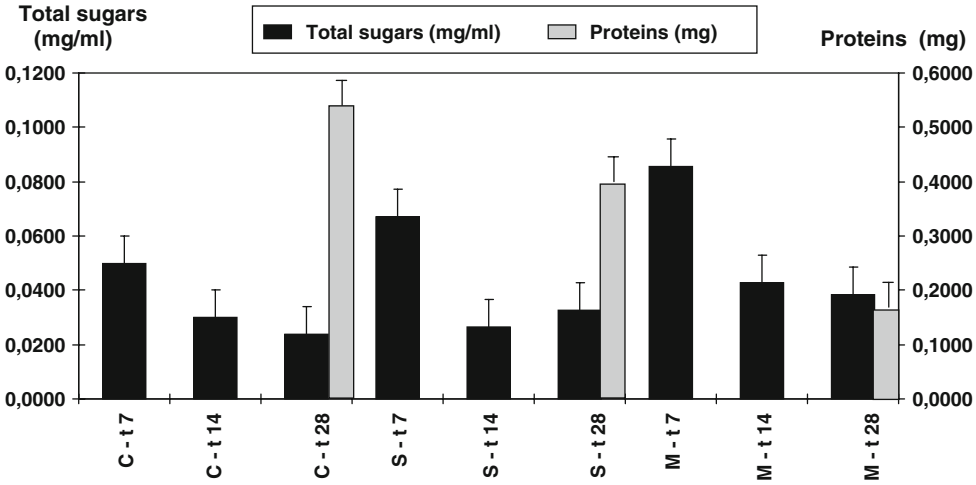

Fig. 6 Total sugars and protein content of the root exudates obtained from three commercial cultivars of strawberry at three different times (t7, t14 and t28 days of plant growth in hydroponic conditions). *C* 'Camarosa', *S* 'Selva', *M* 'Milsei'. Data are the means of three determinations and the *error bars* indicate SD

It is known that plant roots release an assortment of compounds via rhizodeposition, which includes root exudates, mucilage, sloughed cells and tissue, cell lysates and root debris (Gregory 2006; Nguyen 2003). The quantity and quality of compounds released from roots vary with plant species, cultivar, and with biotic and abiotic factors influencing root development (Dakora and Phillips 2002). Under the experimental conditions we performed the assay, which included controlled environmental conditions, therefore no abiotic factor was expected to affect the root development. But considering that soluble exudates, primarily simple sugars, account for 1–10% of C rhizodeposition (Jones et al. 2004; Paterson 2003), and that some of them can mobilize nutrients directly or indirectly by stimulating microbial activity (Paterson 2003; Wichern et al. 2007), we can infer that the growth promoting effect observed in this study was exerted by the bacterial strains used.

In order to explain, in part, whether the root exudates could play a role on the PGPB effect exerted by the different strains of *A. brasilense*, we carried out a chemotaxis assay using the same isolates and strawberry cultivars, and also determined total sugars amount of the root exudates. In all cases, a positive chemotactic response was observed, represented as halo formation, varying its area according to the root exudates-bacterial strain used. Results indicate that when using root exudates of the two-first weeks (7 and 14 days of plant growing) a better response was achieved. Furthermore, these differences are related with the origin of the strains as the two endophytic strains used (e.g., PEC5, REC3) showed higher number of positive chemotactic response. The latter is in agreement with results obtained for the PGPB effect exerted by the strains in the three strawberry cultivars used in this study, and also with Bacilio-Jiménez et al. (2003) who reported that root exudates from rice induced higher chemotactic response for endophytic bacteria than for other bacterial strains present in the rice rhizosphere.

Wood et al. (2001) observed that the incapacity of the host plant to release sufficient carbon source into the rhizosphere constituted a significant limitation on the development of the *A. brasilense*–wheat association. Accordingly, our results support the idea that, in addition to the origin of the bacteria used, the chemotactic response observed is influenced by the total sugar content of the root exudates. As protein content of the root exudates was detected only after 28 days of plant growth and the best chemotactic responses were observed at 7 and 14 days, we concluded that the chemotactic effect observed is more related to the sugar content.

We found different values regarding the origin of the root exudates and the time of obtaining. These values are in agreement with the best chemotactic responses determined with exudates obtained after 7 and 14 days of plant growing, and also, with results observed in the plant growth promotion effect after the inoculation with *A. brasilense* in the three commercial strawberry cultivars. This feature probably reflects an adaptation of the bacteria to nutrient conditions provided by the host plant and may thus play an important role in the establishment of *Azospirillum* in the association with their host. However, it is possible that increasing the bacterial number (rhizospheric and/or endophytic) by sugar content rather than protein helps colonization of bacteria which in turn somehow (other factors such as release of phytohormones, enhancement of mineral uptake, etc.) promotes the plant growth.

The wealth of previous chemotactic studies and the recent molecular data show that chemotaxis is a major force leading to colonization of roots (Bashan et al. 2004). The latter, in addition to the PGPB characteristics of these microorganisms, would constitute valuable and practical information for selecting strains as inoculants; considering

that chemotaxis on Petri dishes is easy to detect and allows for fast screening of a large collection of strains.

Concluding, in this work we present new insight about the capacity of *A. brasilense* to promote strawberry plant growth, expressed as root length, root area, and dry weight of root and shoot. This effect depends on the genotypes used in the plant–bacterial strain association. Total sugars contained in the root exudates of strawberry plants play an important role in the chemotaxis effect of *A. brasilense*, which could explain, in part, the plant growth promoting effect observed.

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