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ORIGINAL PAPER

Azospirillum brasilense siderophores with antifungal activity against *Colletotrichum acutatum*

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Abstract Anthracnose, caused by the fungus *Colletotrichum acutatum* is one of the most important diseases in strawberry crop. Due to environmental pollution and resistance produced by chemical fungicides, nowadays biological control is considered a good alternative for crop protection. Among biocontrol agents, there are plant growth-promoting bacteria, such as members of the genus *Azospirillum.* In this work, we demonstrate that under iron limiting conditions different strains of *A. brasilense* produce siderophores, exhibiting different yields and rates of production according to their origin. Chemical assays revealed that strains REC2 and REC3 secrete catechol type siderophores, including salicylic acid, detected by thin layer chromatography coupled with fluorescence spectroscopy and gas chromatography–mass spectrometry analysis. Siderophores produced by them showed in vitro antifungal activity against *C. acutatum* M11. Furthermore, this latter coincided with results obtained from phytopathological tests performed in planta, where a reduction of anthracnose symptoms on strawberry plants previously inoculated with *A. brasilense* was observed. These outcomes suggest that some strains of *A. brasilense* could act as biocontrol agent preventing anthracnose disease in strawberry.

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Introduction

Strawberry anthracnose is one of the major diseases that affect strawberry (*Fragaria ananassa*) crops (Freeman and Katan 1997). Three species of *Colletotrichum* have been identified as the casual agents of strawberry anthracnose: *C. acutatum*, *C. fragariae* and *C. gloeosporioides* (Adaskaveg and Hartin 1997; Smith and Black 1990; Xiao et al. 2004).

Nowadays, control of anthracnose is performed by the intensive use of chemicals that cause environmental pollution, pathogen resistance, increase in production costs and serious risks to the environment and human health. An alternative of crop protection against pathogens is the biological control exerted by some plant growth-promoting bacteria (PGPB).

Azospirillum is a well-studied PGPB capable of naturally colonizing many plant species, several of agronomic and ecological significance (Bashan et al. 2004), including strawberry (Pedraza et al. 2007, 2010). A variety of mechanisms, such as nitrogen fixation, phytohormone production, nitrate reduction and phosphate solubilization, have been proposed to explain improvement in plant growth following inoculation (Bashan and Holguin 1997; Bashan and Levanony 1990; Bothe et al. 1992; Holguin et al. 1999; Okon 1985; Oliveira and Drozdowicz 1987; Steenhoudt and Vanderleyden 2000).

However, some studies have been conducted on *Azospirillum* as biocontrol agent to promote plant growth indirectly; e.g., by limiting the proliferation of phytopathogens such as the agent of crown gall disease (Bakanchikova et al. 1993), bacterial leaf blight of mulberry (Sudhakar et al.

2000) soil borne plant pathogens that attack *Cucumis sativus* (Hassouna et al. 1998); imposing energy stress on *Rhizoctonia solani* and promoting growth of tomato plants (Gupta et al. 1995); controlling bacterial leaf tomato diseases caused by *Pseudomonas syringae* pv. *tomato* (Bashan and de-Bashan 2002a, b); and, also inhibiting development of bacterial diseases on fresh-market and cherry tomato (Romero et al. 2003), on *Prunus cerasifera* (Russo et al. 2008) or promoting disease resistance on rice crops (Yasuda et al. 2009).

Siderophores are low-molecular mass compounds (<1,500 Da) with high iron affinity (Guerinot 1994; Neilands 1995) that allows soil microorganisms to sequester and solubilize ferric iron in iron poor environments. Bacterial plant growth-promotion can result from direct or indirect mechanisms, including siderophores production. They can improve vegetal growth by increasing plant nutrient availability through iron uptake and preventing the growth of soil borne pathogens due to iron limitation (Chaiharn et al. 2009; Miethke and Marahiel 2007; O'Sullivan and O'Gara 1992; Sayyed and Chincholkar 2009). Although siderophores vary greatly in chemical structure they were classified in two main groups: catechol and hydroxamate, according to the chemical group involved in iron (III) chelation.

Bacterial siderophores production is a biocontrol mechanism that has been scarcely studied in *Azospirillum* (Bachhawaat and Ghogh 1989; Saxena et al. 1986; Shah et al. 1992; Tapia-Hernández et al. 1990). Saxena et al. (1986) and Shah et al. (1992) reported the production of salicylic acid (SA) among siderophores produced by *Azospirillum lipoferum* under iron starved conditions. SA besides being a compound with siderophore activity (Meyer et al. 1992; Visca et al. 1993) is a precursor in the biosynthesis of microbial catechol type siderophores, such as yersiniabactin, pyoverdin and pyochelin (Cox et al. 1981; Jones et al. 2007; Serino et al. 1995). Moreover, it was demonstrated to play a crucial role as an endogenous regulator of localized and systemic acquired resistance against pathogen infection in many plants (Delaney et al. 1994). Therefore, SA producing strains may increase defense mechanisms in plants. However, bacterial SA participation on plants induced systemic resistance (ISR) is still controversial (Cornelis and Matthisj 2007; Siddiqui and Shaukat 2005). It was hypothesized that bacterial SA excreted to the medium was recognized by plant roots inducing signals for systemic resistance (Buysens et al. 1996; De Meyer and Höfte 1997; Maurhofer et al. 1998), although in some interactions it has been proposed that SA may not be the primary signal for ISR induction (Press et al. 1997) but others siderophores could be implicated (Leeman et al. 1996; Siddiqui and Shaukat 2004).

In previous works, we reported the isolation of siderophores producing strains of *A. brasilense* from strawberry plants with plant growth-promotion effects over this crop (Pedraza et al. 2007, 2010). However, quantification and chemical characterization of these compounds has not been done until now. Considering the potential ability of some *Azospirillum* strains in promoting plant growth and their capacity to produce siderophores, the working hypothesis is that they can also exert biocontrol activity against soil borne pathogens on strawberry plants. Accordingly, the aim of this study was to quantify the siderophores production of previously characterized strains of *A. brasilense,* characterize these compounds nature to investigate the presence of SA, and also evaluate (in vitro and in planta) its antifungal activity against *C. acutatum* isolate M11, the casual agent of anthracnose disease in strawberry plants.

Materials and methods

Bacterial isolates and inoculum production

Root (REC) and stolon (PEC) endophytic strains and root rhizosphere (RLC) strains isolated and characterized in a previous work (Pedraza et al. 2007) were used in this work. *A. brasilense* Sp7 (ATCC 29145) was used as reference strain.

Bacterial strains were cultured on semisolid N-free malate medium (NFb) (Baldani and Döbereiner 1980) containing: 5 g malic acid, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0,1 g NaCl, 0.02 g $CaCl_2 \cdot 2H_2O$, 4.5 g KOH, 1 g NH_4Cl, 1.75 g agar and distilled water to 1,000 ml, pH 6.8, adjusted with KOH. Bacteria colonies isolation was performed on solid NFb medium containing yeast extract (0.5 g l^{-1}) and Congo Red (CR) (15 ml of 1:400 aqueous solution). CR was added to facilitate the identification of dark red colonies produced by *Azospirillum*, as suggested before (Bashan and Levanony 1985; Rodríguez Cáceres 1982). For in vitro experiments, liquid cultures were prepared on liquid NFb medium without bromothymol blue, containing 0.1% NH_4Cl (w v^{-1}) and incubated 72 h at 30°C, without shaking.

For plants inoculation, REC3 liquid culture was centrifuged at 8000×*g* for 10 min and washed twice with buffer phosphate pH 7.0 to remove any culture medium residue. The bacterial concentration for inoculation was adjusted at 10^6 CFU ml^{-1} (OD_{560} 0.2), as this is the common and recommended level of *Azospirillum* inoculation of plants (Bashan 1986).

Fungal isolate and inoculum production

The strain M11 of *Colletotrichum acutatum*, (Salazar et al. 2007) was grown on potato glucose agar medium (PGA) for 7 days under continuous fluorescent light at 28°C to

induce conidial formation (Smith and Black 1990). The culture surface was scraped with a Pasteur pipette to remove conidia and then suspended in sterile distilled water. The conidial suspension was filtered through sterile gauze to remove mycelial debris. The suspension was then diluted with sterile distilled water to a final concentration of 1.5×10^6 conidia ml^{-1} and applied to plants by spraying the leaves up to runoff using a hand pump sprayer (Smith and Black 1990).

Biological tests were performed in vitro using PGA medium amended to allow bacteria and fungi to grow simultaneously. The PGA medium was added with 5 g malic acid and 2 ml of micronutrients solution (0.04 g $CuSO_45H_2O$, 1.20 g $ZnSO_47H_2O$, 1.40 g H_2BO_3, 1.00 g $NaMoO_42H_2O$ per litre), and distilled water to 1,000 ml (pH 6.8, adjusted with KOH).

Siderophores assays

Glassware preparation

All glassware used was cleaned with HCl 1 M to remove iron traces and then rinsed with bidistilled water pH 7.0.

Chrome azurol sulphonate agar plate assay

Siderophores were detected by using the universal Chrome Azurol Sulphonate (CAS, Sigma–Aldrich, USA) assay (Schwyn and Neilands 1987). This detects color change of CAS-iron complex (from blue to orange) after the iron chelation by siderophores. The CAS agar plate assay was performed as follows: 60.5 mg CAS was dissolved in 50 ml deionised water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3{\cdot}6H_2O$ in 10 mM HCl) by stirring. This solution was slowly mixed with 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA; Sigma–Aldrich, USA) dissolved in 40 ml of water. The resultant dark blue solution was autoclaved (20 min, 121°C), cooled down and mixed with a sterile mixture of 900 ml NFb solid medium, 30.24 g 1.4-piperazine diethane sulfonic acid (PIPES; Sigma–Aldrich, USA) and 12 g of 50% NaOH (w v^{-1}) to raise the pH up to the pKa of PIPES (pH 6.8). This medium was poured on sterile Petri dishes; then spot inoculated with 10 µl of each bacterial strain and incubated 7 days at 30°C. For the spot inoculation, bacteria were previously grown overnight in NFb liquid medium at 30°C. Cells were collected by centrifuging at 8,000×*g* 10 min, the pellet was washed three times with sterile bidistilled water, and the cell concentration adjusted to 10^6 CFU ml^{-1}. Positive results were indicated by the formation of an orange halo around the colonies.

The yield of siderophores production (%Ys) was determined as [(halo diameter−colony diameter)/colony diameter] × 100. Each assay was performed by triplicate and results were subjected to ANOVA and LSD ($P = 0.05$) analysis by using the Statistix Analytical Software 1996 for Windows (USA).

To study the effect of iron concentration on siderophores production 5, 10 and 30 mM of $FeCl_3{\cdot}6H_2O$ was added to the agar CAS plates. The plates were inoculated with bacterial strains as described above and incubated 7 days at 30°C to analyze siderophores production.

Chrome azurol sulphonate liquid assay

The CAS solution assay (Schwyn and Neilands 1987) was used to quantify siderophores activity in culture supernatant extracts by measuring the decrease in the absorbance of blue color at 630 nm. It was prepared as follows: 6 ml of HDTMA 10 mM was diluted with water and mixed with 1.5 ml of iron (III) solution (1 mM $FeCl_3{\cdot}6H_2O$ in HCl 10 mM) and 7.5 ml 2 mM aqueous CAS solution under stirring conditions. Separately, 4.307 g of PIPES was dissolved in water and 6.75 ml of 12 M HCl was added. This buffer solution (pH 5.6) was slowly mixed with CAS solution and completed up to 100 ml with bidistilled water. A solution of 5-sulfosalicylic acid 0.2 M (Sigma–Aldrich, USA) was used as CAS shuttle solution to facilitate transfer of iron from the CAS complex to bacterial siderophores. For quantitative assay, 150 µl of an overnight culture in NFb liquid medium was adjusted to a final concentration of 10^6 CFU ml^{-1} and inoculated in 150 ml of the same medium and incubated 7 days at 30°C. To increase the sensitivity of the CAS assay, the cell-free medium was concentrated 30 fold by lyophilization, resuspended in 5 ml of distilled water and desalted by using a Sephadex G-25 column (Sigma–Aldrich, USA). The fraction containing siderophores was added to CAS assay solution (1:1) and mixed with shuttle solution (100:1). After 1 h at room temperature the absorbance ($A_{630\ nm}$) was measured. The percentage of siderophore units was estimated as the proportion of CAS color shift using the formula $[(A_r - A_s)/A_r] \times 100$, where A_r is the $A_{630\ nm}$ of the reference sample (medium plus CAS assay solution plus shuttle solution) and A_s is the $A_{630\ nm}$ of the sample (supernatant plus CAS assay solution plus shuttle solution).

Siderophore structure determination

Chemical assays

The chemical nature of siderophores was investigated by analyzing the absorption spectra in a Beckman DU 7500 Spectrophotometer. A peak between 420 and 450 nm and another at 495 nm after the addition of 1 ml 2% aqueous

$FeCl_3$ to 1 ml of cell free culture filtrate indicated the presence of ferric hydroxamates and ferric catecholate, respectively (Neilands 1981). The presence of catechol and hydroxamate type phenolates were assayed on ethyl acetate extracts of the culture supernatants. The extracts were prepared by extracting 150 ml supernatant twice with an equal volume of solvent at pH 2.0. Catechol siderophores were assayed according to Arnow (1937). Briefly, 1 ml of each bacterial supernatant was mixed with 1 ml of HCl 0.5 N and 1 ml of nitrite molybdate reagent (10 g $NaNO_2$ and 10 g $NaMoO_4$, dissolved in 100 ml of distilled H_2O), and then mixed with 1 ml NaOH 1 N. After 15 min at room temperature the absorbance ($A_{510\ nm}$) was measured.

Hydroxamate siderophores were analyzed in culture supernatants according to the ferric perchlorate assay (Atkin et al. 1970). Catechin and hydroxylamine hydrochloride were used as the standards. Each assay was performed by triplicate.

The fraction containing catechol type siderophores was prepared by extraction of acidified (pH 2.0) supernatants with an equal volume of ethyl acetate; the solvent was eliminated by vacuum rotary evaporator at 37°C and the residue was resuspended in methanol for TLC analysis and in ethyl acetate for GC–MS assay.

Thin layer chromatography (TLC) coupled with fluorescence spectroscopy

The methanol-soluble siderophore fraction was analyzed by TLC using silica gel plates (Merck, $SiO_2 \times H_2O$ 20; 20 cm; G 60 $F_{254\ nm}$), and a mobile phase consisting in a solvent mixture: butanol/acetic acid/water [4: 0.5: 5.5 (v/v/v)]. The plate was dried at room temperature and observed under long-wave UV light (365 nm) and short wave UV (254 nm). Salicylic acid PA grade (Fluka AG, Switzerland) was used as standard. Bands with the same Rf as the standard were scrapped out from the plate, dissolved in methanol 100% (v v^{-1}), centrifuged and concentrated by lyophilization. The residue was resuspended in methanol 20% (v v^{-1}) and analyzed by fluorescence spectroscopy using Multidimensional Fluorescence Spectrometer with VINCI software (ISS, USA). Samples emission spectra were evaluated at λ excitation of 296 nm.

Gas chromatography-mass spectrometry

To confirm the presence of SA in bacterial supernatants, cultures were analyzed by GC–MS using a Thermo Electron equipment Polaris Q model associated to an electron impact ionization source and an Ion Trap Mass Analyzer (Thermo Finnigan, USA). Separations were carried out on a DB-5 column (Agilent JW, USA) using He as carrier gas at 0.3 ml min^{-1} and an injection volume of 0.1 µl. The ion source was maintained at 200°C; the GC oven was programmed with a temperature gradient starting at 50°C for 1 min to 350°C at 10° min^{-1}. MS analysis was carried out in the electron-impact mode at an ionizing potential of 70 eV. SA PA grade (1 µg ml^{-1}) was used as standard.

Bioassays

In vitro

Siderophores producer strains, REC2 and REC3, were tested for their ability to inhibit the growth of *C. acutatum* isolate M11 in vitro. Assays were performed in sterile Petri dishes containing iron limited PGA medium supplemented with CAS solution. Bacterial cultures in NFb liquid medium were adjusted to a final concentration of 10^6 CFU ml^{-1} and 10 µl of each suspension was spotted at the centre of the plate. After incubating the plates at 30°C for 48 h, a suspension of $1.5x10^6$ conidia ml^{-1} of *C. acutatum* M11 was sprayed over the plates. Bacterial and fungal cultures were also plated separately as controls. The plates were incubated at 30°C for 7 days, and the antifungal activity of bacteria was assessed for the presence of growth inhibition halos surrounding the bacterial colony. The test was carried out by duplicate and repeated three times using independent Petri dishes for the growth of each bacterial strain and M11 isolate.

In planta

Vegetal material Strawberry (*Fragaria ananassa*, Duch) cultivars 'Camarosa' and 'Milsei' were used in phytopathologic tests. Plantlets, obtained from in vitro culture to ensure healthy and bacterial free material, were kindly provided by the strawberry Active Germplasm Bank at National University of Tucuman. Plants were maintained in growth chambers at 28°C, 70% relative humidity (RH) with a light cycle of 16 h day^{-1} (33 µmol photons m^2 s^{-1}) and watered every other day with 50 ml of distilled water except 4 days before and after bacterial inoculation to favour bacteria–plant association.

Plant inoculation and evaluation of anthracnose Three months old strawberry plantlets of the cv. 'Camarosa' and 'Milsei' were inoculated with the strain REC3 of *Azospirillum* by watering with 30 ml of bacterial suspension (10^6 CFU ml^{-1}). After 15 days of inoculation, plants were sprayed with conidial suspension (1.5×10^6 conidia ml^{-1}) as mentioned above. Immediately after infection, plants were moved to an infection chamber at 100% RH, 28°C for 24 h in the dark. Then plants were returned to the growth chamber.

Experiments were conducted with five plantlets of each cultivar for each treatment that were: (1) plants inoculated

with strain REC 3 and infected with *C. acutatum* M11; (2) only infected with *C. acutatum* M11; (3) only inoculated with *A. brasilense* REC3; and (4) a set of 5 plants that were only treated with sterile distilled water.

Disease Severity Rating (DSR) of anthracnose was assessed using the following scale: 1, healthy petiole without lesions; 2, petiole with lesions <3 mm; 3, petiole with lesions from 3 to 10 mm; 4, petiole with lesions from 10 to 20 mm and girdling of petiole; 5, entirely necrotic petiole and dead plant (Delp and Milholland 1980). The DSR was evaluated 9, 21, 30 and 40 days post infection with the fungus (dpi). Results were subjected to ANOVA and LSD ($P = 0.05$) analysis with the Statistix Analytical Software 1996 for Windows (USA).

Results

Endophytic (REC, PEC) and rhizosphere (RLC) strains of *A. brasilense* were CAS positive for siderophores production as evidence by clear halos surrounding the bacterial colonies growing under iron-limiting conditions. However, the yields of siderophores production (%Ys) were different according to the origin of the strains. For instance, isolates from inner root tissues (REC strains) and stolons (PEC strains) showed a better performance on %Ys than the root rhizosphere strains (RLC strains) after 7 days of incubation (Fig. 1).

In order to explain these differences, the time-course of siderophores production of all the strains was analyzed using CAS agar assay. As a general feature, it was observed that endophytic strains (REC, PEC) began producing siderophores on the third day of incubation at 30°C reaching to the maximum siderophore yield on the fourth day. Conversely, rhizosphere strains (RLC) began producing siderophores on the fifth day of incubation and the production remained low until the seventh day of incubation (data not shown).

In an attempt to correlate siderophores production in solid and liquid media and to quantify them, two root endophytic strains (REC2 and REC3) were selected and assayed by using CAS-liquid assay (Schwyn and Neilands 1987). The type strain *A. brasilense* Sp7, a commonly used strain, was included as reference positive control. Results showed a similar profile when using solid or liquid culture media, being the strain REC3 the highest siderophores producer among strains assessed (Table 1 and Fig. 1).

Evaluation of siderophores chemical nature indicated that REC2, REC3 and Sp7 strains produced catechol type siderophores as evidence by the maximal absorbance (λ max) at 495 nm with the $FeCl_3$ assay and positive Arnow's test (Arnow 1937) using catechin as standard (Table 1). Absorbance values at λ 420–450 were not detected, besides it was not observed any color reaction with ferric perchlorate assay indicating that these strains do not produce hydroxamate type siderophores.

As the synthesis of siderophores was reported to be strictly regulated by the presence of iron, we evaluated the ability of the strains REC3 and Sp7 to produce siderophores in solid medium with and without the addition of iron. Results showed that with 5 mM $FeCl_3.6H_2O$ the efficiency of siderophores production decreased almost four times and with 10 and 30 mM of iron the strains did not show any CAS positive reaction (Fig. 2).

Catechol type siderophores previously determined were analyzed for the presence of SA by TLC, fluorescent spectroscopy and GC–MS. On silica plates, supernatants from REC2 and REC3 showed a spot with the same R_f value than the standard (data not shown), while no spot was found for the strain Sp7. These results were confirmed using the bands from silica plate to determine the fluorescence emission spectra generated by an excitation

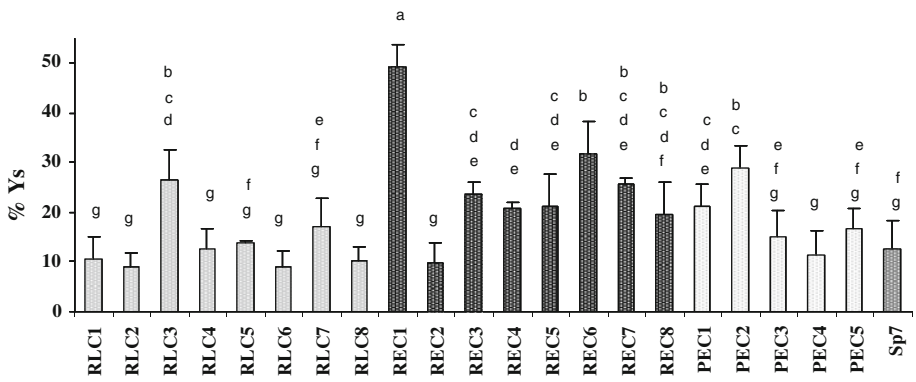

Fig. 1 Yield of siderophores production (%Ys) of different *A. brasilense* strains isolated from strawberry plants using CAS agar plates assay. Type strain *A. brasilense* Sp7 was used as the control for comparison of *A. brasilense* isolates tested. The control and rhizosphere (RLC), root endophytic (REC) and first stolon endophytic (PEC) strains were evaluated after seven days of incubation at 30°C. Data are the means of three determinations and the *error bars* indicate SD. *Different letters* indicate significant differences at $P = 0.05$

Table 1 Evaluation of siderophores production by different strains of *A. brasilense* using five different assays

Strain	CAS agar assay	CAS liquid assay (% siderophore units)[a]	$FeCl_3$ 2% test[b]		Ferric perchlorate assay[b] (hydroxamate type)	Arnow test[b] (catechol type)
			420–450 nm (hydroxamate type)	495 nm (catechol type)		
Sp7		50.63 ± 0.74	−	+	−	+
REC2		61.56 ± 0.24	−	+++	−	+++
REC3		74.15 ± 0.53	−	++	−	++

[a] Average of three determination and SD

[b] The symbols represent the intensity of siderophores production: (−) none, (+) low, (++) medium, (+++) high

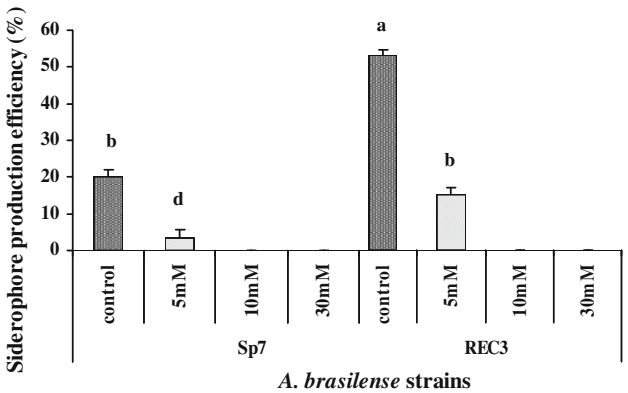

Fig. 2 Effect of iron concentration on the siderophores production by REC3 and Sp7 strains using the CAS-agar plate assay

wavelength of 496 nm, using pure SA as standard (data not shown). The presence of SA in the supernatants was finally identified by GC–MS analysis. Total ion chromatogram (TIC) for SA showed a single peak at 10.97 min retention time (Fig. 3a) that was fragmented in three characteristics peaks of 92, 120 and 138 mass to charge ratio (*m/z*). These peak patterns and values were used as internal markers during GC–MS analysis (Fig. 3b). REC2 and REC3 selected ion chromatograms (SICs) for *m/z* 138, showed a peak with almost the same retention time than pure SA (Fig. 4a, b) with a fragmentation pattern that matched exactly with that for the standard (Fig. 5a, b). Conversely, TIC and mass fragmentation analysis for Sp7 strain did not show presence of SA, although a great variety of indole-like compounds appeared after a retention time of 15 min (Fig. 6a, b).

To analyze whether the strains REC2 and REC3 were able to inhibit the growth of *C. acutatum* isolate M11, in vitro bioassays were performed using PGA medium supplemented with CAS solution. As shown in Fig. 7, after 2 days post infection (dpi) with *C. acutatum* M11, a clear and complete inhibition halo was observed around REC2 and REC3 spots. The size of inhibition zone increased with incubation time until 3 dpi reaching to the maximum values of 15.2 ± 0.8 mm and 11.8 ± 0.3 mm for REC3 and REC2, respectively. The inhibition halos matched with the orange halos of siderophores produced, suggesting that these compounds may be responsible for the inhibitory effect observed. The latter was partially confirmed when the antifungal effect was tested in the presence of iron. Results showed that the effect exerted by REC2 and REC3 against *C. acutatum* M11 was supressed by the addition of iron to the medium. Control CAS plate inoculated with

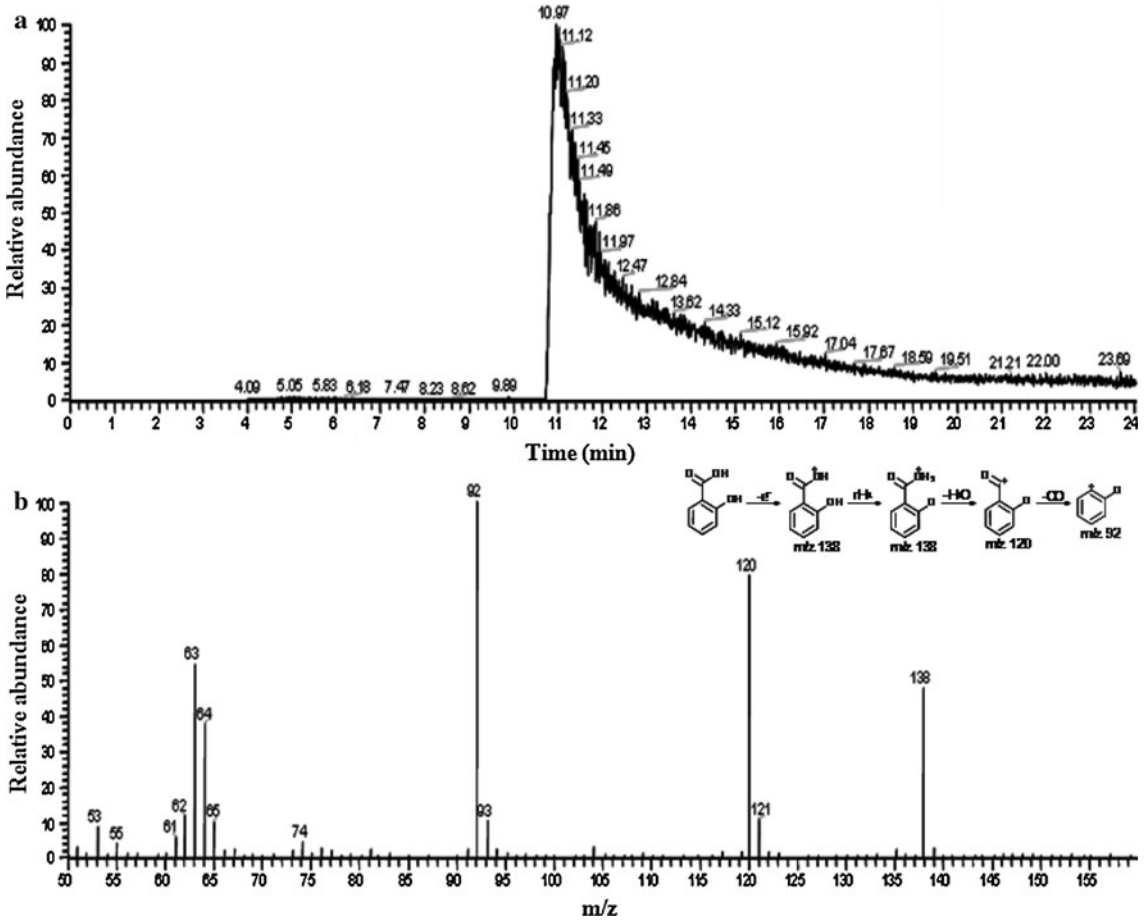

Fig. 3 GC-MS analysis of SA mass fragmentation spectra detected using pure SA as internal standard. **a** SA total ion chromatogram (TIC) and **b** mass fragmentation spectra detected at 10.97 min

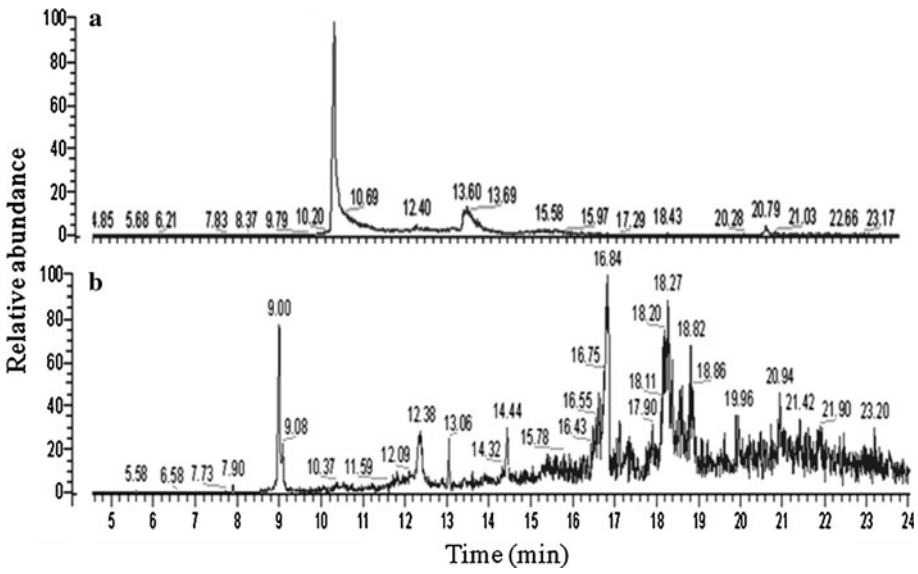

Fig. 4 GC-MS analysis of *A. brasilense* REC2 and REC3 supernatants. **a** REC2 selected ion chromatogram (SIC) for $m/z = 138$; **b** REC3 selected ion chromatogram (SIC) for $m/z = 138$ mass fragmentation spectra detected at 10.43 min

C. acutatum M11, exhibited a colour change from blue to orange 7 dpi, indicating that the fungus produced siderophores but in a minor rate than bacteria.

The antifungal activity exerted by *A. brasilense* REC3 strain against *C. acutatum* M11 was also observed *in planta* bioassays. Strawberry plants cv. 'Camarosa' and 'Milsei'

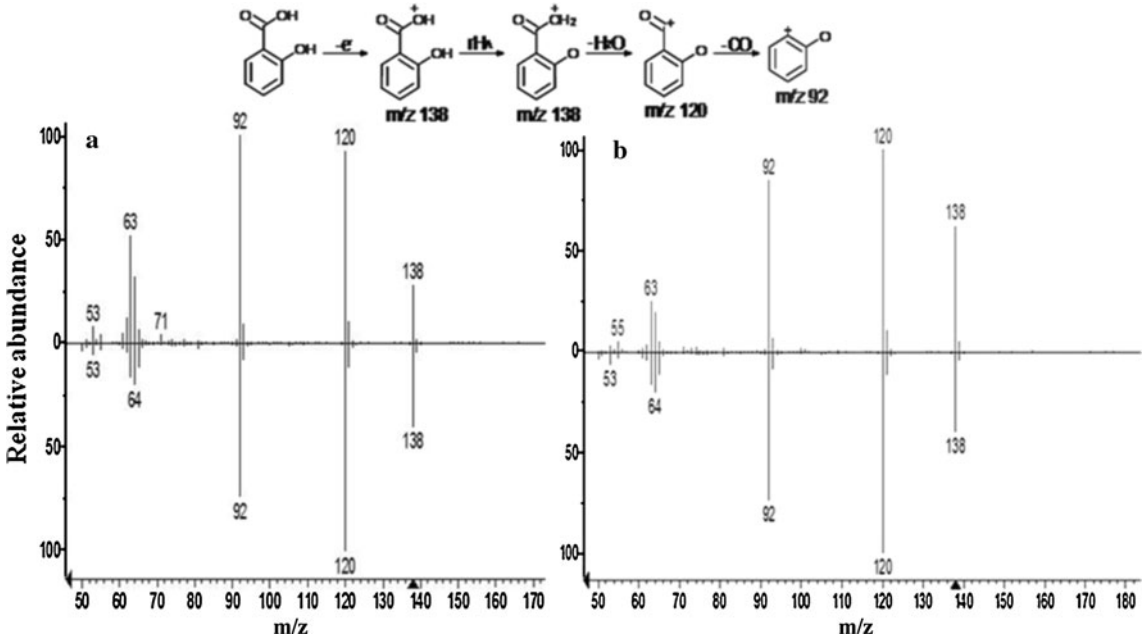

Fig. 5 GC-MS analysis of *A. brasilense* REC2 and REC3 supernatants. **a** salicylic acid ion mass fragmentation and comparison between mass spectra obtained for REC2 (*up*) and mass spectra obtained for SA used as standard (*down*); **b** salicylic acid ion mass fragmentation and comparison between mass spectra obtained for REC3 (*up*) and mass spectra obtained for salicylic acid used as standard (*down*)

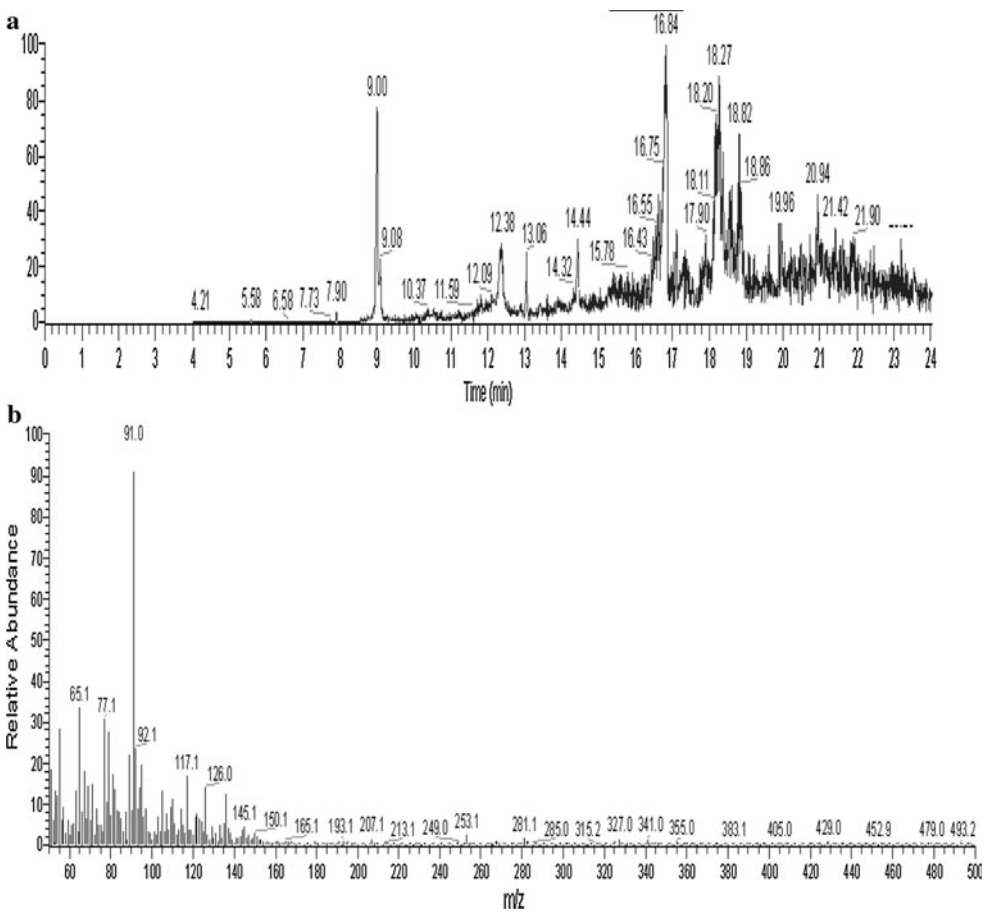

Fig. 6 GC-MS analysis of *A. brasilense* Sp7 supernatants. **a** Sp7 total ion chromatogram (TIC); **b** mass fragmentation spectra detected from 10.2 to 10.9 min

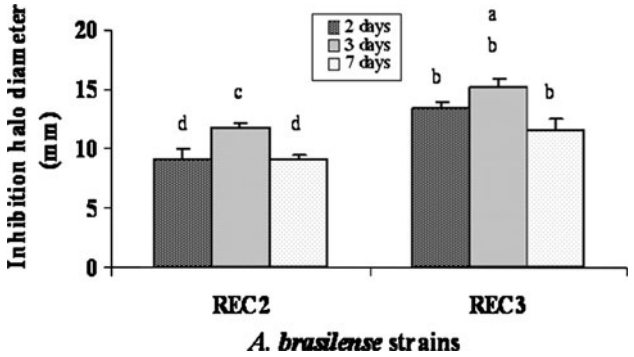

Fig. 7 Antimicrobial activity of *A. brasilense* strains REC2 and REC3 against *C. acutatum* M11 grown under condition of iron limitation on PGA medium added with CAS solution. Each value represents the mean of three determinations and the *error bars* indicate SD. *Different letters* indicate significant differences at $P = 0.05$

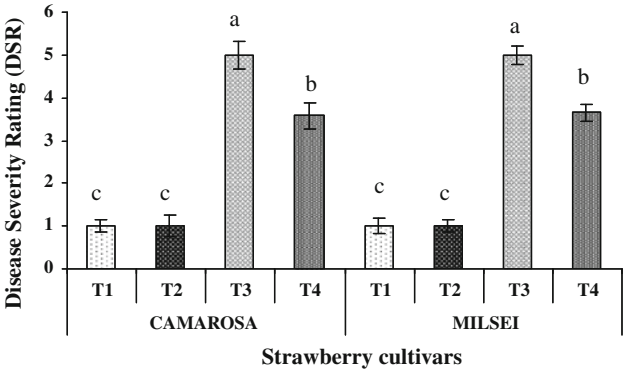

Fig. 8 Influence of *A. brasilense* REC3 strain inoculation on antrachnose susceptibility (DSR) of cultivars 'Camarosa' and 'Milsei' to *C. acutatum* M11. T1 and T2 indicate plants treated with sterile distilled water and plants inoculated only with REC3 strain, T3 indicates plants only infected with M11 strain, and T4 indicates plants inoculated with *A. brasilense* REC3 strain 15 days prior to the infection with M11 strain. Results are for plants evaluated 40 dpi with M11 strain. Each value represents the mean of five determinations and the *error bars* indicate SD. DSR values with *different letters* indicate statistically significant differences at $P = 0.05$

were inoculated with strain REC3 and 15 days after the treatment they were infected with *C. acutatum* M11. Results obtained from plants inoculated only with strain REC3 showed no difference in DSR values with those only treated with distilled water (DSR = 1; healthy petioles without lesions). Plants infected only with M11 strain exhibited anthracnose symptoms since the second day of infection and caused the death of all plants within 3 weeks after infection (DSR = 5; entirely necrotic petioles and dead plants), indicating that strain M11 produced a strong compatible interaction with both strawberry cultivars (T3 in Fig. 8). However, when plants were prior inoculated with *A. brasilense* REC3 and then infected with strain M11, both cultivars showed an increased tolerance to anthracnose disease with DSR value of 3.6 (petioles with lesions from 3 to 10 mm; T4 in Fig. 8).

Discussion

In this work we demonstrate that under iron-limiting conditions, endophytic and rhizosphere strains of *A. brasilense* isolated from strawberry plants were able to produce siderophores and that this production depends on the origin of the strains.

Siderophores quantity and rate of production were higher for endophytic than for rhizosphere strains, which could be explained by the fact that inside host plant, iron availability is probably lower than on rhizosphere environments, therefore bacterial mechanisms for iron chelation must be more efficient to ensure iron assimilation for their physiological processes. Taking into account these considerations, siderophores production by endophytic strains REC2 and REC3 was studied in detail.

Although iron availability is an important signal that regulates siderophores production, Lewenza et al. (1999) and Boyer et al. (2008) reported that cell density acts as a second signal that limits siderophores production. They demonstrated that quorum sensing mediated by N-acyl-homoserine lactones negatively regulates siderophores production in some strains of *Burkholderia cepacia* and *Azospirillum lipoferum* during stationary phase. Accordingly, we observed that both rhizosphere and endophytic strains of *A. brasilense* reached to a maximum yield of siderophores production on the fourth and fifth day of incubation respectively, but after that, when cells reached a high cellular density, siderophores production stopped and the yield remained constant.

As a general feature, we observed that strains REC2 and REC3 secrete catechol type siderophores, including SA, with antifungal activity against *C. acutatum*, one of the casual agents of anthracnose disease in strawberry crops. Using the universal CAS agar plate assay as a preliminary semiquantitative technique for siderophores determination, and CAS liquid assay to quantify them, we could observe that REC2 and REC3 strains produced siderophores as evidence by halo formation, being REC3 the main producer strain (74.15% siderophore units).

As it was mentioned above, the synthesis of siderophores is strictly regulated in response to external iron availability (Bachhawaat and Ghogh 1989; Dave and Dube 2000). Accordingly, we observed that the addition of iron (5 mM $FeCl_3 \cdot 6H_2O$) to the culture medium resulted in a decrease of almost four times the siderophores production and with 10 and 30 mM of iron the strains did not present any CAS positive reaction, although it was reported that *A. lipoferum* M decreased (but did not stop) siderophores

production at 10 mM and 100 mM $FeCl_3$ (Shah et al. 1992).

Unlike fungi, that are able to produce only hydroxamate type siderophores, bacteria can synthesize both catechol and hydroxamate, hence in order to find if REC2 and REC3 siderophores belong to one group or another, we carried out chemical assays. Results demonstrated that siderophores belong to the catechol type being in agreement with previous reports for many PGPB genera such as *Pseudomonas*, *Azotobacter, Rhizobium*, *Bacillus,* including *Azospirillum* (Bachhawaat and Ghogh 1987; Cornelis and Matthisj 2007; Crowley 2006; Glick et al. 1999; Saxena et al. 1986; Shah et al. 1992).

Among siderophores, salicylic acid (SA) has been intensely studied because it may exert a dual biocontrol activity; on one hand, acting as a siderophore, decreasing the availability of iron on iron poor environments (Meyer et al. 1992), and on the other, as a signal elicitor triggering a systemic resistance response in plants against pathogens (Delaney et al. 1994).

The presence of SA on *Azospirillum* supernatants was usually detected by using silica TLC (Saxena et al. 1986; Shah et al. 1992). However, this technique is not entirely suitable for SA determination, as it is not possible to completely separate complex mixtures of catechol-type siderophores included in bacterial supernatants. Thus, in this work, we employed silica TLC coupled with fluorescence spectroscopy and GC–MS analysis in order to confirm SA production by different *A. brasilense* strains under iron starved conditions. The application of these approaches allowed us to isolate and detect SA from others catechols, also produced by bacteria. In contrast, Sp7 strain did not exhibit SA production, although GC–MS showed a great variety of indole-like compounds, accordingly with Spaepen et al. (2007).

Considering siderophores production as a biocontrol mechanism used by some PGPB to limit the growth of phytopathogens, we analyzed whether siderophores excreted by the strains under study, including SA, were able to inhibit the growth of *C. acutatum* strain M11, using an in vitro assay. Both strains REC 2 and REC3 produced complete fungal growth inhibition zones around colonies, coinciding with the orange halo of CAS positive reaction. However, the orange halo was absent when iron was added to the medium, suggesting that the inhibition zones resulted from the activity of siderophores. The CAS color change on fungal control plates after 7 days of incubation indicated siderophores production but with a minor rate and affinity for iron than the bacterial ones. This was consistent with the fact that fungi produce hydroxamate type siderophores which have, in some cases, lower affinity for iron than catechols (Liu et al. 2002).

The antifungal effect exerted in vitro by *A. brasilense* REC3 strain against *C. acutatum* M11 was in agreement with results obtained from tests performed *in planta*. The infection with the pathogen was a challenge to *Azospirillum* inoculation that had 15 days to produce a stronger plant that might help to resist the fungal infection. The latter is similar to what has been reported for tomato plants when challenged with *P. syringae* pv. *tomato* but previously inoculated with *Azospirillum* (Bashan and de-Bashan 2002a, b). Although M11 strain produced a strong compatible interaction producing the death of both strawberry cultivars within 3 weeks after infection, when plants were previously inoculated with bacteria, both cultivars exhibited a decrease on disease susceptibility of almost 30% in comparison with controls uninoculated plants. So far, there is no direct evidence from this study that the capacity to produce siderophores in vitro also operates in vivo and perhaps the observed effect is due to a stronger plant.

The fact that the increase of plant tolerance to anthracnose disease when previously inoculated with strain REC3 was independent of the cultivar, is a relevant feature to consider this strain as a possible biocontrol agent of the anthracnose disease.

Although most bacteria synthesize siderophores in response to low iron availability, only few are able to produce them in high concentrations and with high iron affinity so that they would cause the antagonism with plant pathogens. For this reason, most studies on bacterial siderophores production have been conducted on *Pseudomonas* spp. Studies carried out with iron metabolism of *Pseudomonas* allowed to know the siderophore structure, biosynthesis, biocontrol activity and systemic resistance induction (De Meyer and Höfte 1997; Leeman et al. 1996; Loper and Buyer 1992), excluding nevertheless others PGPB such as *Azospirillum.* Thus, further studies are necessary to obtain practical information for extending the spectrum of potential biocontrol strains for plants inoculation.

In conclusion, in this work it was demonstrated that strains REC2 and REC3 of *A. brasilense*, besides exerting direct mechanisms to improve strawberry plant growth such as nitrogen fixation, indoles production, and positive chemotaxis toward strawberry root exudates (Pedraza et al. 2007, 2010), they are further able to indirectly promote plant growth, producing siderophores with antifungal activity against *C. acutatum* M11. As proposed by Bashan and Holguin (1997) in their revision, some strains of *A. brasilense*, like the ones assessed in this work, could be considered as Biocontrol PGPB and raises the possibility of using them as an alternative in the strawberry anthracnose disease management.

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