

Protection of strawberry plants (*Fragaria ananassa* Duch.) against anthracnose disease induced by *Azospirillum brasilense*

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Abstract

Background and aims *Azospirillum brasilense* REC3 is a plant growth-promoting and siderophore-producing bacterium isolated from strawberry. *Colletotrichum acutatum* M11 is the causal agent of anthracnose, an important disease in strawberry crop. The aim of this study was to characterize at the biochemical and molecular level, the systemic resistance induced by *A. brasilense* on pathogen-challenged strawberry plants. **Methods** Phytopathological tests were performed; the content of phenolic compounds was determined spectrophotometrically; callose depositions in leaves by aniline blue staining; salicylic acid (SA) content in leaves by HPLC; and defense-related gene expression [pathogenesis-related proteins (FaPR1), chitinases (FaChi2-1; FaChi2-2) and glucanase (FaBG2-2)] by RT-PCR.

Results *A. brasilense* REC3 reduced anthracnose symptoms on pathogen-challenged plants, and the effect became greater as the elapsed time between bacterial inoculation and fungal infection increased. Biochemical and transcriptional studies revealed a transient accumulation of SA and the induction of defense-related genes, suggesting further that this response is related to structural cell wall modifications as consequence of the observed increase in phenolic compounds and callose deposition. **Conclusions** The plant growth-promoting bacterium *A. brasilense* REC3 participates actively in the induction of systemic protection on strawberry plants against anthracnose disease caused by *C. acutatum* M11.

Keywords Anthracnose · *Azospirillum brasilense* · *Colletotrichum acutatum* · Induced protection · Strawberry plant

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Introduction

Azospirillum is one of the most studied associative plant growth-promoting bacteria (PGPB), due to its capacity to improve yields of different plant species of agronomic and ecological significance (Bashan et al. 2004), including strawberry (Pedraza et al. 2010). Different mechanisms, such as nitrogen fixation, phytohormone production, nitrate reduction and phosphate solubilization and mobilization, have been proposed to explain the plant growth enhancement

observed after inoculation (Bashan and de-Bashan 2010; Bashan and Holguin 1997; Bashan et al. 2004; Bothe et al. 1992; Okon 1985; Steenhoudt and Vanderleyden 2000). Although *Azospirillum* is not known as a typical biocontrol agent, it has been reported that some strains are able to stimulate plant growth indirectly by suppressing soil-borne pathogens and other deleterious microorganisms in various systems; e.g., crown gall disease (Bakanchikova et al. 1993); *Rhizoctonia solani* (Gupta et al. 1995); biocontrol of soil-borne plant pathogens attacking *Cucumis sativus* (Hassouna et al. 1998); bacterial leaf blight of mulberry (Sudhakar et al. 2000); bacterial leaf tomato disease caused by *Pseudomonas syringae* pv. tomato (Bashan and de-Bashan 2002a; b); development of bacterial disease on fresh-market and cherry tomato (Romero et al. 2003); biocontrol effect on *Prunus cerasifera* (Russo et al. 2008); and disease resistance in rice (Yasuda et al. 2009).

Strawberries are cultivated in different parts of the world, including tropical, subtropical, and temperate areas. The world production of strawberries has been increasing over the last two decades and is now estimated at 3 million tons per year. Argentina produces strawberries throughout the 12 months of the year, and is one of the most important producers in South America (Pedraza et al. 2007). Strawberry anthracnose, caused by different species of the fungus *Colletotrichum*, is one of the most serious diseases affecting the strawberry crop (Salazar et al. 2007). This disease affects almost all plant tissues, producing important economic losses on both strawberry fruit and plant production, under both greenhouse and field conditions. Nowadays, disease control is based on the intensive use of chemicals, which causes environmental pollution, pathogen resistance, increase in production costs and serious risks to the environment and human health. The increasing world demand for sustainable agriculture promoting food safety is leading to the development of alternative approaches for crop protection, including the use of biocontrol agents, such as PGPB, that are able to suppress soil-borne pathogens in addition to promoting plant growth. Numerous investigations have been conducted to find biological control agents for anthracnose in different crops, and several potential candidates have been reported, including *Trichoderma* sp. (Freeman et al. 2004; Verma et al. 2006), *Bacillus* spp. (Mahadnanapuk et al. 2007), *Candida oleophila* (Wharton and Diéguez-Urbeondo 2004), *Emericella*

nidulans (Talubnak and Soyong 2010), *Gliocladium catenulatum* (Verma et al. 2006) and some PGPR strains (Bardas et al. 2009; Karnataka 2005).

In previous works, we have reported that strawberry plants are natural hosts of *A. brasilense*, and different strains exhibiting plant growth-promoting effects and positive chemotaxis properties for this crop have been isolated (Pedraza et al. 2007; 2010).

Some strains were also able to produce catechol-type siderophores, including salicylic acid (SA) under iron-starved conditions (Tortora et al. 2011). Besides being a compound with siderophore activity (Meyer et al. 1992; Visca et al. 1993) and a precursor in the biosynthesis of microbial catechol type siderophores (Jones et al. 2007; Serino et al. 1995), SA can play a crucial role as an endogenous regulator of localized and systemic acquired resistance (SAR) against pathogen infection in many plant species (Delaney et al. 1994). We have demonstrated that *A. brasilense* siderophores have antifungal activity in vitro against isolate *Colletotrichum acutatum* M11—the causal agent of anthracnose in strawberry plants. This finding was consistent with the protection observed in different strawberry cultivars that were challenged with the pathogen prior to bacterial inoculation (Tortora et al. 2011). However, until now the biochemical and molecular features of the induced resistance have not been characterized. Consequently, the working hypothesis of this paper is that *A. brasilense*, in addition to promoting plant growth, is able to protect strawberry plants from anthracnose disease caused by *C. acutatum*. Hence, the aim of this study was to characterize, at the biochemical and molecular levels, the systemic resistance induced by *A. brasilense* in pathogen-challenged strawberry plants. This was done by determining the content of phenolic compounds and callose depositions in leaves, and by evaluating the SA levels associated with modifications in defense-related genes expression, such as those encoding pathogenesis-related proteins (PRs) such as PR1, chitinases and glucanase.

Materials and methods

Bacterial inoculation

The strain *A. brasilense* REC3 used in inoculation experiments was isolated from strawberry inner root tissues and characterized in a previous work (Pedraza

et al. 2007). Its capacity to promote strawberry plant growth was reported recently (Pedraza et al. 2010).

For plant inoculation, bacterial inoculum was prepared on N-free malate liquid medium (NFb) (Baldani and Döbereiner 1980) without bromothymol blue, containing 0.1% NH₄Cl (w/v), and incubated at 30°C for 72 h without shaking. Cultures were centrifuged at 6,000 g for 10 min and washed twice with buffer phosphate (pH 7.0) to remove any culture medium residue. The inoculation of strawberry plant roots was performed by watering the pots with 30 ml bacterial suspension of about 10⁶ CFU ml⁻¹ (OD₅₆₀ 0.2).

A. brasilense REC3 tagged with green fluorescent protein

To confirm root colonization of bacterial inoculated strawberry plants, the wild-type strain *A. brasilense* REC3 rifampicin (Rf^R) resistance mutant obtained in our laboratory, was transformed with pHRGFPTC transconjugant plasmid (Tc^R) containing the *gfpmut3* gene encoding the green fluorescent protein (*gfp*), under the constitutive *pgen* promoter (Ramos et al. 2002). The plasmid was transferred from *Escherichia coli* DH5α to *Azospirillum* by triparental matting, using the plasmid pRK2013 (Km^R) in *E. coli* HB101 as helper (Figurski and Helinski 1979). *E. coli* strains were grown on LB medium (Sambrook et al. 1989) supplemented with 10 μg ml⁻¹ tetracycline (Tc) for pHRGFPTC, and with 50 μg ml⁻¹ kanamycin (Km) for pRK2013. *A. brasilense* REC3 transconjugants were selected on agar plates containing NFb solid medium supplemented with Tc 10 μg ml⁻¹ and Rf 20 μg ml⁻¹, and confirmed by colony PCR analysis using *gfp*-specific primers (forward: 5' ATGC CATGTGTAATCCCA-3', and reverse: 5'-ATGAG TAAAGGAGAAGAAC-3').

For plant inoculation, bacterial inoculum was prepared as described above, except that liquid NFb medium was supplemented with 10 μg ml⁻¹ Tc, and 20 μg ml⁻¹ Rf.

Fungal inoculation

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 about 1.5 × 10⁶ conidia ml⁻¹ and applied to plants by spraying the leaves up to runoff using a hand pump sprayer (Smith and Black 1990).

Vegetal material

Strawberry (*Fragaria ananassa*, Duch) cv. 'Camarosa' plants were used in this work. Plantlets were obtained from the Strawberry Active Germplasm Bank at National University of Tucumán and propagated in vitro to ensure healthy and bacteria-free plants. To evaluate bacterial localization on roots, plants were grown under hydroponic conditions, using diluted (1:2) Hoagland solution (Hoagland 1975) as nutrient medium, and maintained in a growth chamber at 28°C, 70% relative humidity (RH) with a light cycle of 16 h day⁻¹ (250 μmol photons m² s⁻¹). For phytopathological tests, plants were planted in disinfected plastic pots containing sterile substrate (peat and perlome; 1:1 v/v) and maintained in a growth chamber. The plants were watered every other day with 50 ml distilled water except 4 days before and after bacterial inoculation to favor bacteria–plant association.

Bacterial localization on roots

To confirm *Azospirillum* colonization of root inner tissues, an inoculation assay using the strain *A. brasilense* REC3-*gfp* (Rf^R) was performed. Plants grown under hydroponic conditions were inoculated by submerging their roots in a bacterial suspension (10⁶ CFU ml⁻¹) for 30 min, and then drained for 5 s. Non-inoculated plants (treated only with water) were considered as controls. Root samples were collected at different times from 2 to 20 days after bacterial inoculation, and processed immediately for fluorescence microscopy. Root samples were cut longitudinally under sterile conditions into pieces of 1-cm long using a sterile razor blade and immediately mounted in glycerol 30% (v/v) for microscopic observations using an Olympus system microscope, model BX51 equipped with the U-LH100HG reflective fluorescent system (Olympus, <http://olympusamerica.com>).

Phytopathological tests

Plant susceptibility to anthracnose was evaluated on infected plants (as described above). Uninoculated plants (treated only with water) were used as control. Immediately after infection, plants were placed in an infection chamber at 100% RH and 28°C for 24 h in the dark, and then returned to the growth chamber.

The disease severity rating (DRS) of anthracnose was assessed according to Delp and Milholland (1980) 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. The DSR was evaluated 9, 21, 30 and 40 days post infection (dpi) with the fungus. Results were subjected to ANOVA and LSD ($P=0.05$) analysis with the Statistix Analytical Software 1996 for Windows (<http://www.statistix.com/>).

Induced resistance experiments

Strawberry plants were inoculated first with *A. brasilense* REC3 and then with *C. acutatum* M11 at 2, 4, 5 and 15 days after bacterial inoculation (described above).

The DSR was evaluated 9, 21, 30 and 40 dpi, and results were subjected to ANOVA and LSD ($P=0.05$) analysis with Statistix Analytical Software 1996 for Windows.

Biochemical analysis

Total phenolic content

Total soluble phenolic concentration was determined in leaves of plants inoculated with bacteria (as described above). At different times after bacterial inoculation (4 and 5 days), leaves were excised and ground in liquid nitrogen. Phenolic compounds were extracted from 200 mg ground powder by two sequential extractions using methanol 80% (v/v). Each extraction was performed shaking the suspension at 100 rpm for 3 h at 30°C in the dark. Supernatants were recovered by centrifugation at 7,000 g for 30 min at 4°C. Thereafter, 1 ml of each supernatant was dried by lyophilization to determinate dry weight, and resuspended in 1 ml ethanol 20% (v/v). Phenolic compound quantification was performed

using the Folin-Ciocalteu technique (Singleton et al. 1999), measuring the absorbance at 765 nm and using gallic acid (PA grade) (Sigma-Aldrich, St. Louis, MO) as a standard. The increase in total soluble phenolics (%TSP) after REC3 strain inoculation, was determined as $[(T2-T1)/T1]*100$, where T1 and T2 represent the total soluble phenolic contents of uninoculated and REC3 inoculated plants, respectively.

Callose deposition assay

Callose cell wall depositions in strawberry leaves were analyzed in plants inoculated with bacteria before and after challenging with the pathogen *C. acutatum* M11 (explained above). Callose staining was performed on leaves excised 2 days after fungal infection according to Currier and Strugger (1956). Briefly, leaves were cleared and dehydrated with absolute ethanol for 12 h. They were then transferred sequentially from 100% ethanol to 67 mM K_2HPO_4 (pH 12.0) through solutions of gradually decreasing ethanol concentration (e.g., 100%, 75%, 50%, 25% and 0%). Finally, the leaves were stained with 0.01% (w/v) aniline blue (molecular grade; Sigma-Aldrich) in 67 mM K_2HPO_4 (pH 12.0) for 1 h at room temperature. The stained material was mounted in glycerol 30% (v/v) and examined using UV fluorescence in an Olympus system microscope model BX51, equipped with a U-LH 100HG reflected fluorescence system (Olympus). Callose depositions were observed as bright green spots under fluorescent light.

Phloematic salicylic acid

The SA content of strawberry plants induced by inoculation at different times (0, 2, 3, 4 and 5 days) with strain REC3 of *A. brasilense* was analyzed. The endogenous level of total SA was measured in phloem exudates collected from three petioles of each plant with a micropipette. To precipitate the sugars, the exudates were collected immediately into 500 ml of ice-cold absolute ethanol (pH 2.0 with HCl) in the dark. The supernatants were recovered by centrifugation at 10,000 g for 10 min and dried by evaporation under vacuum to determinate the dry weight. Finally, the dried residues were resuspended in methanol 20% (v/v) and analyzed by HPLC (Gilson, <http://www.gilson.com>) using a C18-column (Phenomenex, <http://>

www.phenomenex.com/) with a flow rate of 0.5 ml min⁻¹ and a linear gradient of 20 to 100% of methanol as the mobile phase containing 0.01% (v/v) of trifluoroacetic acid (TFA; Sigma-Aldrich). SA eluted from the column was detected at 303 nm using a UV- detector (Gilson). SA (PA grade) at 10 µg ml⁻¹ (Fluka, Buchs, Switzerland) was used as the standard. Fractions with the same retention time (RT) as the standard were collected and analyzed for quantification by fluorescence spectroscopy using an ISS Multidimensional Fluorescence Spectrometer with the VINCI software (VINCI, <http://www.iss.com/fluorescence/software/vinci.html>). Emission spectra of samples were evaluated at 406 nm with excitation at 296 nm, using SA PA grade as the standard.

Molecular analysis

Semiquantitative RT-PCR

Expression of FaPR1 (PR protein 1), FaBG2-2 (β-1,3-glucanase), FaChi2-1 (class II chitinase) and FaChi2-2 (chitinase) was evaluated from total RNA extracted from leaves of strawberry plants inoculated with REC3 strain at different times before and after challenging with the M11 isolate.

Approximately 1 g frozen leaves was ground in liquid nitrogen. The ground powder was transferred to a polypropylene tube, and the RNA was extracted according to the technique described by Iandolino et al. (2004). RNA samples were treated with DNaseI (2U µl⁻¹) (Ambion, <http://ambion.com/>) and used for the first-strand cDNA synthesis in a 25 µl mix reaction, using GoScript reverse transcriptase (1U µl⁻¹) (Promega, Madison, WI) and oligo (dT)₁₅ primer (GenBiotech, Buenos Aires, Argentina). RNA concentration was estimated spectrophotometrically by measuring absorbances at 230 nm, 260 nm and

280 nm (Sambrook et al. 1989), and the expression level of each sample was normalized to that of glyceraldehyde-3P dehydrogenase (GAPDH-1). PCR reactions were carried out in 25 µl volume containing 1x Green GoTaq Reaction Buffer (Promega; pH 8.5), 1.5 unit of GoTaq DNA polymerase (Promega), 1.5 mmol l⁻¹ MgCl₂; 400 µmol l⁻¹ dNTPs, 0.4 µmol l⁻¹ of each primer and 0.2 µg template DNA. The reaction mixtures were subjected to PCR using an Apollo ATC-201 thermocycler (Continental Laboratory Products, San Diego, CA) with the following conditions: 10 min at 95°C, 25 cycles of 30 s at 95°C, 1 min at the optimal annealing temperature (45–60°C), and 1 min at 72°C, followed by 5 min at 72°C. The gene-specific primers used are shown in Table 1. Three independent PCR reactions were performed using the same RNA.

Experimental design and statistical analysis

For the phytopathological assays as well as for callose determination and molecular analysis, the experimental design was randomized with five plantlets per treatment, and each treatment consisted of: (1) plants treated only with sterile distilled water, (2) inoculated only with REC3 strain, (3) plants infected only with *C. acutatum* M11, and (4) plants inoculated with *A. brasilense* REC3 strain and then infected with *C. acutatum* M11. For quantification of SA and phenolic compounds, the experimental design was randomized with five plantlets per treatment; each treatment consisted of: (1) plants treated only with sterile distilled water, and (2) inoculated only with REC3 strain. Results were analyzed with the Statistix program (Analytical Software, 1996). LSD test was used to determine the arithmetic mean (significance level, 0.05) and the analysis of variance test (ANOVA) was used to evaluate data dispersion with respect to

Table 1 Primers used in semi-quantitative RT-PCR analysis

Gene	GenBank accession number	Amplicon size (bp)	Forward primers (5' to 3')	Reverse primers (5' to 3')
GAPDH-1	AB363963	400	CTACAGCAACACAGAAAACAG	AACTAAGTGCTAATCCAGCC
FaPR1	AB462752	147	TGCTAATTCACATTATGGCG	GTTAGAGTTGTAATTATAGTAGG
FaBG2-2	AY989818	350	CTCCATTGTTGCCCAA	AACCCTACTCGGCTGA
FaChi2-1	AF147091	851	TCGTCACCTGCAACTCCTAA	GGACTTCTGATTTTCACAGTCT
FaChi2-2	AF320111	781	CAAGTCAGATAACAATGGAGAC	TTGTAACAGTCCAAGTGTCC

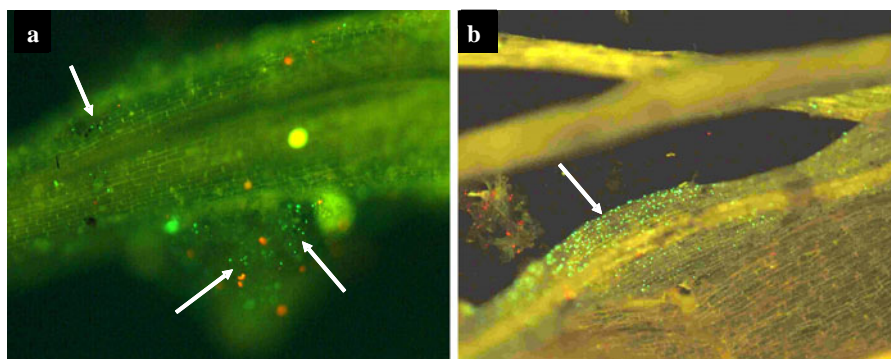


Fig. 1 Endophytic root colonization of strawberry plants cv. ‘Camarosa’ by *Azospirillum brasilense* REC3 strain tagged with green fluorescent protein (*gfp*)-expressing plasmid. Fluorescence microscope images (10 \times) of strawberry roots analyzed

a 10 days and **b** 20 days after bacterial inoculation. Arrows indicate the position of single fluorescent bacteria and root colonization zones

the mean value. All experiments were repeated three times and the results presented are the average of the three repetitions of each experiment.

Results

To confirm *A. brasilense* REC3 endophytic colonization of roots, strawberry plants of the cv. ‘Camarosa’ were inoculated with *A. brasilense* REC3 strain tagged with GFP. At different times after inoculation, from 1 to 20 days, single bacteria and small cell aggregates of *Azospirillum* were visualized under fluorescence microscope. After the fifth day of inoculation, fluorescent cells were found inside root tissues localized in young root zones such as emerging lateral roots, and became progressively more pronounced. Figure 1 shows strawberry root colonization at 10 and 20 days after bacterial inoculation.

A. brasilense strain REC3 was tested for its ability to protect strawberry plants from *C. acutatum* M11, a casual agent of anthracnose disease. Plants inoculated with REC3 strain alone displayed no significant difference in disease severity as compared with those treated only with distilled water (DSR=1, T1 and T2 in Fig. 2). Plants infected only with M11 strain exhibited anthracnose symptoms from the 1st week of fungal infection and were dead within 3 weeks (DSR=5, T3 in Fig. 2). When plants were treated with REC3 strain prior to infection with *C. acutatum* M11, they showed an increased tolerance to the disease, characterized by low values of DSR, in

comparison with untreated plants (T4 in Fig. 2). The difference in DSR values between treated and untreated plants after M11 infection increased with the elapsed time between bacterial inoculation and fungal infection.

The potential ability of *A. brasilense* REC3 strain to activate the innate immunity resistance in strawberry plants against *C. acutatum* M11 was evaluated

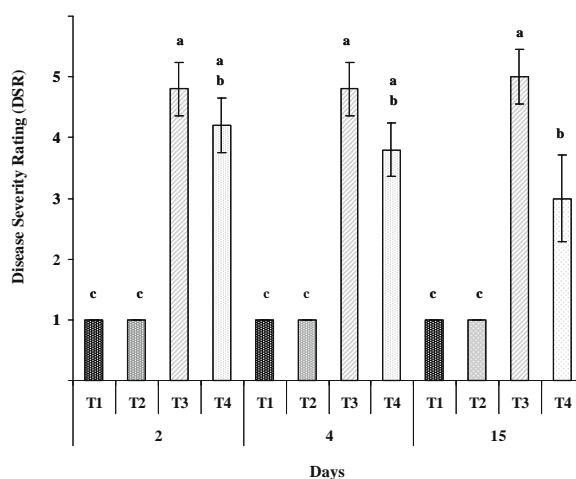


Fig. 2 Influence of the amount of time between *A. brasilense* REC3 inoculation and *Colletotrichum acutatum* M11 infection on the susceptibility of strawberry plants cv. ‘Camarosa’ to M11. T1 Control plants treated with distilled water; T2 plants inoculated only with REC3 strain; T3 plants infected only with M11; T4 plants inoculated with *A. brasilense* REC3 strain 2, 4 and 15 days prior to M11 infection. The disease severity rating (DSR) was evaluated 40 days post inoculation (dpi) with M11 strain. Each value represents the mean of five determinations and the error bars indicate SE. DSR values with different letters indicate statistically significant differences at $P=0.05$

at the biochemical and molecular level. The biochemical analysis consisted of determination of total soluble phenolic compounds (%TSP), callose and SA. The results showed an increment in the %TSP in the leaves of REC3-inoculated plants in comparison with non-inoculated control plants. It was also observed that, 5 days after bacterial inoculation, the amount of phenolic compounds in inoculated plants was 25% higher than in control plants (Fig. 3).

Resistance induced by PGPB is often associated with priming for enhanced deposition of callose, which would contribute to the reinforcement of cell wall papillae at the sites of the pathogen attack (Benhamou et al. 1996, 1998). Considering that some virulent pathogens are able to suppress basal defense associated with callose depositions, the accumulation of callose in REC3-treated and untreated plants with and without pathogen challenge was evaluated. As shown in Fig. 4, in non-pathogen challenged plants that were inoculated only with the REC3 strain, an increase in callose deposition was observed, reaching a maximum level on the 3rd day after bacterial inoculation. A strong induction of callose deposition was observed in pathogen-challenged plants that were previously inoculated with the REC3 strain, while no callose accumulation was observed in plants treated only with M11. These results suggest that the amount of callose deposition was dependent on the time that had elapsed between bacterial inoculation and fungal infection.

To investigate if SA is involved in the systemic protection against anthracnose disease induced by

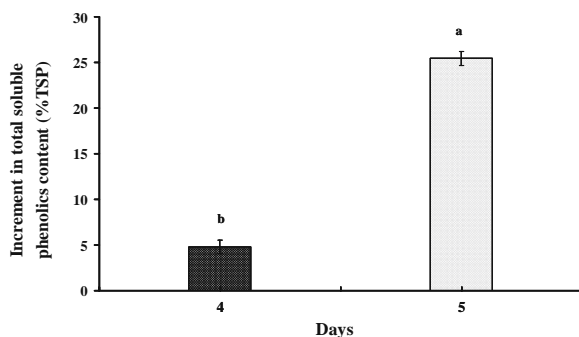


Fig. 3 Increment of total soluble phenolic (%TSP) amount in leaves of strawberry plants cv. 'Camarosa' inoculated with *A. brasilense* REC3 in comparison with uninoculated plants. Results were obtained at 4 and 5 days after bacterial inoculation. Different letters indicate significant differences at $P=0.05$

strain REC3 in strawberry plants, the endogenous level of SA was investigated in plants inoculated with bacteria. The results showed that inoculation with strain REC3 led to significant accumulation of SA at the 4th day after bacterial inoculation, while no accumulation was observed in the non-treated control plants (Fig. 5). Interestingly, the level of SA dropped to the level seen in untreated plants after 5 days (Fig. 5). This could be explained by the fact that SA acts as an endogenous signal triggering both local and systemic responses, and its accumulation precedes the onset of SAR and ISR (Malamy et al. 1990; Métraux et al. 1990; Zhang et al. 2002).

It is well known that SA plays an important signaling role in plant defense against pathogens, and that an increase in endogenous SA levels in plants is associated with the induction of many defense-related genes. To determine the molecular changes that occur in plants inoculated with strain REC3 and in non-inoculated plants before and after pathogen challenge, the expression of strawberry pathogenesis-related (FaPR1) and other (FaChi2-1, FaChi2-2, and FaBG2-2) genes was analyzed by semiquantitative RT-PCR. As shown in Fig. 6, in non-pathogen challenged plants, inoculation with REC3 strain caused the induction of defense related genes such as FaPR1, FaChi2-1 and FaChi2-2 at different times after bacterial inoculation (T2; Fig. 6a) in comparison with non-inoculated control plants (T1; Fig. 6a). When plants were challenged with pathogen after bacterial inoculation (Fig. 6b), a strong induction of FaPR1, FaChi2-1, FaChi2-2 and FaBG2-2 expression was observed in comparison with plants treated only with REC3. Confirming the results obtained previously, we observed that REC3 inoculation resulted in augmented expression of defense genes in comparison with non-inoculated plants (T2; Fig. 6b). The level of defense gene expression in plants that were infected only with the pathogen was not significantly different from water-treated plants.

Discussion

This paper reports a protection effect on strawberry plants exerted by *A. brasilense* REC3 against anthracnose disease caused by *C. acutatum* M11 through activation of plant defense responses, studied at the biochemical and transcriptional levels. The localiza-

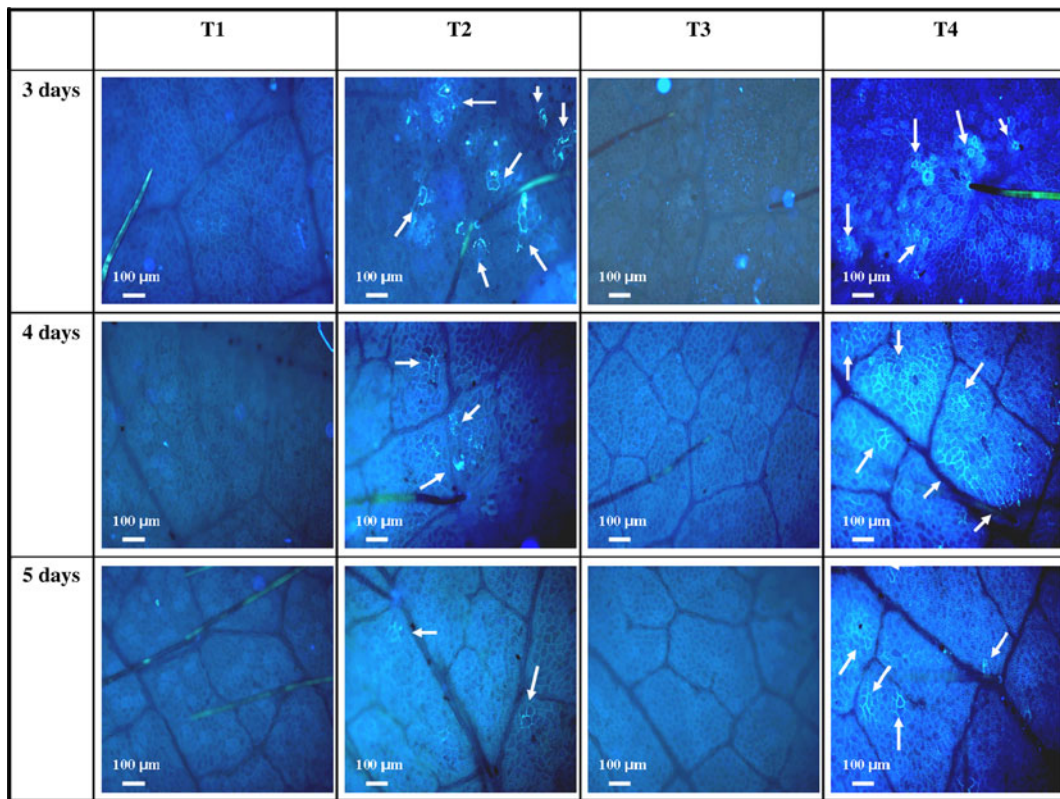


Fig. 4 Callose deposition in leaves of strawberry plants inoculated with *A. brasilense* REC3 before and after challenge with *C. acutatum* M11. Plants were infected with M11 strain at 3, 4 and 5 days after bacterial inoculation, and leaves were assessed 2 days after fungal infection. *T1* Control plants treated

with distilled water; *T2* plants inoculated only with REC3 strain; *T3* plants infected only with M11; *T4* plants challenged with M11 strain after bacterial inoculation. *Arrows* indicate the site of callose depositions

tion of REC3-*gfp* strain in inner tissues of strawberry roots after bacterial inoculation confirmed the endophytic nature of this strain that had been reported previously (Pedraza et al. 2007; 2010; Winik et al. 2009). Endophytic bacteria have also been reported to have important advantages as potential biocontrol agents due to their natural and intimate association with plants, influencing plant physiology directly and increasing resistance to pathogen attack (Benhamou et al. 1996; Ramamoorthy et al. 2001). Our results showed that when strawberry plants were first inoculated with REC3 strain, the disease symptoms produced by M11 decreased as the time between bacterial and fungal inoculations increased, reaching a maximum protection level at a time between inoculations of 15 days. This effect may be explained by the plant growth-promotion effect exerted by strain REC3 on strawberry plants (Pedraza et al. 2010), which would produce a stronger plant that could

better tolerate fungal infection. The latter is similar to what has been reported by Bashan and de-Bashan (2002a) for tomato plants that were inoculated with *Azospirillum* and then challenged by the epiphytic bacterial pathogen *Pseudomonas syringae* pv. tomato. However, the REC3 strain also reduced anthracnose symptoms in pathogen-challenged plants at 2 and 4 days after bacterial inoculation. A criterion for the systemic resistance induced by PGPB is the spatial separation between the tested bacteria and the challenging pathogen (Yan et al. 2002). Considering that *A. brasilense* REC3-*gfp* could not be detected on strawberry leaves at the time of the pathogen infection, we infer that the protection observed in this study was due to a systemic resistance.

Our results showed that *A. brasilense* strain REC3 increased the content of total phenolic compounds and callose deposition in the leaves of inoculated plants prior to infection with the pathogen, indicating

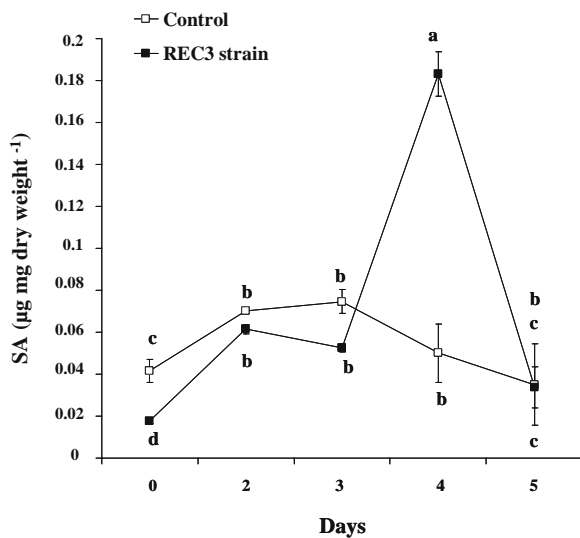


Fig. 5 Salicylic acid (SA) accumulation in strawberry plants of the cultivar ‘Camarosa’ inoculated with *A. brasilense* REC3 evaluated at different times (0, 2, 3, 4, and 5 days) after bacterial inoculation. Data correspond to the mean values of three independent determinations; error bars SD. Different letters indicate significant differences at $P=0.05$

that the presence of *A. brasilense* REC3 strain on strawberry roots could be perceived systemically in leaves. Although these results are not typically detected in PGPB treated plants before pathogen challenge, we propose that plant inoculation with endophytic REC3 strain causes physiological changes in plant metabolism that might not affect bacterial root colonization but that could contribute to a rapid defense reaction following pathogen attack. Supporting this, we observed that, in REC3-treated plants, the pathogen challenge triggered higher accumulation of callose depositions in comparison with unchallenged plants. However, plants infected only with M11 strain were unable to accumulate callose, coinciding with a previous report by Salazar et al. (2007) who found that the ability of *C. fragariae* F7 (an avirulent strain) to interfere with defense mechanisms is necessary for the development of resistance against anthracnose in strawberry plants.

Although most previous studies on SA have been focused on interactions between plants and virulent or avirulent pathogens (Bari and Jones 2009; Delaney et al. 1994; Gaffney et al. 1993; Tarchevsky et al. 2010), it has been demonstrated that some PGPB, including *A. brasilense* (Bashan and De-Bashan 2002b; Ramos Solano et al. 2008), can stimulate plants to accumu-

late SA either locally in roots (Chen et al. 1999) or systemically in leaves (De Meyer et al. 1999; Maurhofer et al. 1994; Zhang et al. 2002). However, depending on the PGPB-plant interaction, SA does not always accumulate to a large extent to produce modifications in plant defense responses. Our results showed that *A. brasilense* REC3 strain inoculation on strawberry roots caused a systemic increase in SA in leaves 4 days after bacterial inoculation.

The fact that callose deposition is a SA-dependent defense response (DeRoy et al. 2004) could explain the higher amount of callose depositions observed when plants were challenged with the pathogen 4 days after bacterial inoculation. In a previous study, we reported that REC3 strain was able to produce SA under iron-limited conditions (Tortora et al. 2011). However, as reported by Maurhofer et al. (1994), it is

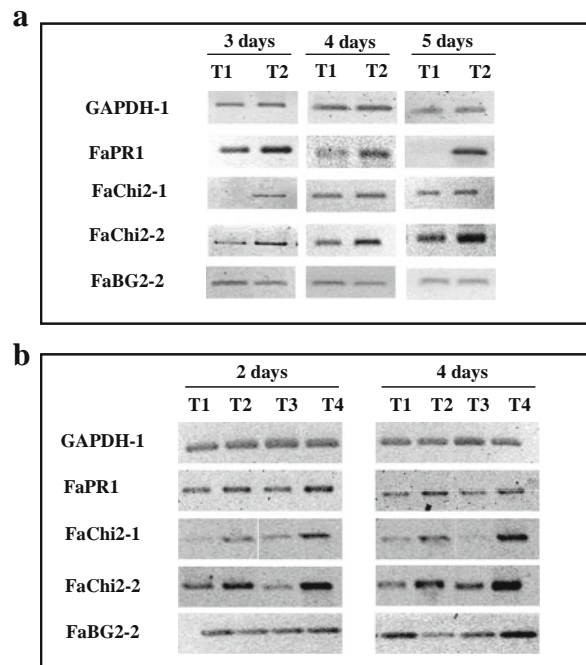


Fig. 6 Expression of defense related genes in strawberry plants evaluated by semiquantitative RT-PCR. **a** Plants treated with *A. brasilense* strain REC3 analyzed for gene expression at 3, 4 and 5 days after bacterial inoculation. *T1* Plants treated with sterile distilled water, *T2* plants inoculated with REC3 strain. **b** Plants infected with *C. acutatum* M11 after bacterial inoculation. *T1* Plants treated with sterile distilled water, *T2* plants inoculated with REC3 strain, *T3* plants infected only with M11, *T4* plants inoculated with REC3 strain 2 and 4 days prior to the infection with M11. Results are from plants evaluated at 2 days after fungal infection

not clear if the bacteria induced the production of SA in plants or if plants up take bacterial SA and translocate it to the leaves.

Since an increase in plant endogenous SA levels is usually associated with the induction of defense-related genes, we analyzed the gene expression of a PR-protein (FaPR1), two chitinases (FaChi2-1; FaChi 2–2) and a glucanase (FaBG2-2) when plants were primed with REC3 before and after challenge with the M11 strain, and analyzed the results, taking into account the kinetics of SA accumulation when plants were inoculated only with *A. brasilense* REC3. Although a priming state is a common feature of the resistance responses induced by beneficial microorganisms (Van Loon et al. 1998), and represents an enormous advantage in terms of energy costs for the plant because defense responses are expressed only when they are needed (Conrath et al. 2002), our results showed that REC3-treated plants exhibited a significant increase in FaPR1 gene expression that remained high until 5 days after bacterial inoculation.

The increase in FaPR1 gene expression was also observed in REC3-primed plants after a challenge with the M11 strain, whereas no modification was observed when plants were treated only with M11. Considering that PR1 protein is a molecular marker for the SAR response, these results, together with those obtained for SA, confirm that REC3 strain in some way involves SA in the transduction of defense signals prior to pathogen challenge. Increased expression of the chitinases genes (FaChi2-1 and FaChi2-2) was observed on REC3-treated plants, especially after pathogen challenge, while up regulation of the glucanase (FaBG2-2) gene was observed only on REC3-primed plants after infection with M11. The induction of plant chitinases and glucanases has been associated with the systemic resistance against fungal diseases induced by some PGPB strains (Benhamou et al. 1996; Maurhofer et al. 1994; M'Piga et al. 1997; Saravanakumar et al. 2007). Hence, the accumulation of these hydrolytic enzymes at the site of penetration of fungal hyphae can result in the degradation of fungal cell walls (Benhamou et al. 1996). Two studies have been conducted in *Azospirillum* to increase its antifungal activity by cloning and expressing chitinase genes of rice plant and *Bacillus cereus* origin (El-Hamshary et al. 2010; Jayara et al. 2004); however, there are no studies to date on the ability

of this bacterium to induce the expression of plant chitinase genes and to reduce indirectly the damage caused by phytopathogens.

In conclusion, in this paper we provide evidence that endophytic root colonization of strawberry plants with *A. brasilense* strain REC3 confers systemic protection against *C. acutatum* M11 by the direct activation of some plant defense reactions, and also primes the plant for a stronger defense reaction when exposed to further infection.

Defense mechanisms induced by *A. brasilense* REC3 include the reinforcement of plant cell wall by increasing the content of total soluble phenolic compounds and callose depositions, and the transient accumulation of SA. The latter brings about the upregulation of defense-related genes, such as those encoding pathogenesis-related proteins like PR1, chitinases and glucanase. We propose therefore, that the activation of a systemic defense response, together with the plant growth-promoting effect exerted by *A. brasilense* REC3 strain could, in part, explain the increase of strawberry plants' tolerance to anthracnose disease caused by *C. acutatum* M11.

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