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RESEARCH PAPER

Role of ethylene and related gene expression in the interaction between strawberry plants and the plant growth-promoting bacterium *Azospirillum brasilense*

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Keywords

Azospirillum brasilense; *Fragaria ananassa*; ethylene; ISR; PGPB.

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ABSTRACT

- Induced systemic resistance (ISR) is one of the indirect mechanisms of growth promotion exerted by plant growth-promoting bacteria, and can be mediated by ethylene (ET). We assessed ET production and the expression of related genes in the *Azospirillum*–strawberry plant interaction.
- Ethylene production was evaluated by gas chromatography in plants inoculated or not with *A. brasilense* REC3. Also, plants were treated with $AgNO_3$, an inhibitor of ET biosynthesis; with 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ET biosynthesis; and with indole acetic acid (IAA). Plant dry biomass and the growth index were determined to assess the growth-promoting effect of *A. brasilense* REC3 in strawberry plants. Quantitative real time PCR (qRT-PCR) was performed to analyse relative expression of the genes *Faetr1*, *Faers1* and *Faein4*, which encode ET receptors; *Factr1* and *Faein2*, involved in the ET signalling pathway; *Faacs1* encoding ACC synthase; *Faaco1* encoding ACC oxidase; and *Faaux1* and *Faami1* for IAA synthesis enzymes.
- Results showed that ET acts as a rapid and transient signal in the first 12 h post-treatment. *A. brasilense* REC3-inoculated plants had a significantly higher growth index compared to control plants. Modulation of the genes *Faetr1*, *Faers1*, *Faein4*, *Factr1*, *Faein2* and *Faaco1* indicated activation of ET synthesis and signalling pathways. The up-regulation of *Faaux1* and *Faami1* involved in IAA synthesis suggested that inoculation with *A. brasilense* REC3 induces production of this auxin, modulating ET signalling.
- Ethylene production and up-regulation of genes associated with ET signalling in strawberry plants inoculated with *A. brasilense* REC3 support the priming activation characteristic of ISR. This type of resistance and the activation of systemic acquired resistance previously observed in this interaction indicate that both are present in strawberry plants, could act synergistically and increase protection against pathogens.

INTRODUCTION

Azospirillum brasilense is a plant growth-promoting bacterium capable of increasing yields in many crops of agricultural interest when associated with roots of different plant species, allowing a decrease in the use of fertilisers and pesticides (Okon & Labandera-Gonzalez 1994; Steenhoudt & Vanderleyden 2000; Bashan & de-Bashan 2010; Massena-Reis *et al.* 2011). Once *Azospirillum* has colonised the roots, it can affect plant metabolism directly or indirectly, providing nutrients and phytohormones, mainly auxins such as indole acetic acid (IAA), and inducing defence responses against phytopathogens (Bashan & de-Bashan 2010; Glick 2012; Tortora *et al.* 2012).

Plants recognise beneficial microorganisms or their MAMPs (microbe-associated molecular patterns) through specific receptors, initiating a signal response that activates basal resistance (Pieterse *et al.* 2009). The defence response triggered can be systemic acquired resistance (SAR) or induced systemic resistance (ISR), both of which depend on hormones acting as signal molecules, such as salicylic acid (SA) in the case of SAR and ethylene (ET) for ISR (Alonso & Stepanova 2004; Choudhary *et al.* 2007; Pieterse *et al.* 2009; Wen *et al.* 2015). Normally, when plants interact with beneficial bacteria, ISR is induced, although some authors provide evidence that SAR can also be triggered (Tortora *et al.* 2012).

Ethylene is a gaseous phytohormone produced in plant tissues, mainly in leaves, and is involved in seed germination, flowering, cell elongation, nodulation, fruit ripening, senescence, *etc.* (Abeles *et al.* 1992; Bleecker & Kende 2000; Alonso & Stepanova 2004). Its biosynthesis begins with methionine as

a precursor, which is converted to S-adenosyl-L-methionine (SAM). Through action of the enzyme ACC synthase (ACS), SAM is converted into 1-aminocyclopropane-1-carboxylic acid (ACC) and finally, in the presence of oxygen, ACC oxidase (ACO) transforms ACC into ET (Yang & Hoffman 1984). ET can diffuse easily through the cell membrane. Once inside the cell, it is recognised by specific receptors located in the endoplasmic reticulum: ETR1 and ETR2 (for ethylene receptor), ERS1 and ERS2 (for ethylene response sensor) and EIN4 (for ethylene insensitivity). These receptors are regulators of the signalling pathway. In the absence of ET, they repress hormone signalling by interacting with the protein kinase CTR1, a negative regulator. This complex receptor–CTR1 inactivates EIN2, a positive regulator of the pathway, by phosphorylating its C-terminus and suppressing ET response. In the presence of ET, the receptors bind the hormone, which inactivates them. A change in their conformation inactivates CTR1, thus preventing EIN2 phosphorylation and repression. EIN2 is cleaved by unknown mechanisms and translocated to the nucleus where it stabilises the transcription factors EIN3/EIL (ethylene-Insensitive3/Ethylene-Insensitive3-like) involved in the ET signal transduction pathway in plants. The latter promotes *erf1* (ethylene response factor 1) transcription, producing an ERF1 protein and resulting in the activation or repression of diverse genes regulated by ET (Alonso & Stepanova 2004; Pierik *et al.* 2006; Pieterse *et al.* 2009; Bisson & Groth 2011; Merchante *et al.* 2013).

It is known that plant hormones connect different parts of the plant, allowing rapid adaptation to environmental stimuli. Auxins intervene mainly in developmental processes (Pieterse *et al.* 2012). Indole-3-acetic acid (IAA), the most abundant auxin in plants (Woodward & Bartel 2005; Teale *et al.* 2006; Di *et al.* 2015), is synthesised by different pathways dependent or not on tryptophan; in one of them, the genes *aux1* and *ami1* participate in the biosynthetic pathway that converts L-tryptophan into IAA (Casanova *et al.* 2005; Zhao 2010; Mano & Nemoto 2012; Di *et al.* 2015). Auxins are also produced by microorganisms, such as *Azospirillum brasilense* (Spaepen *et al.* 2007; Spaepen & Vanderleyden 2011; Pieterse *et al.* 2012). It was reported that plant auxins stimulate ET biosynthesis through ACS gene regulation (Yu & Yang 1979; Yoshii & Imaseki 1981; Abeles *et al.* 1992; Kende 1993; Woodward & Bartel 2005; Ribaudo *et al.* 2006) and this has also been described for IAA produced by *Azospirillum* (Spaepen *et al.* 2007).

Strawberry is an intensive crop that requires high inputs of fertilisers and pesticides to enhance fruit yield. However, the intensive use of agrochemicals may be hazardous to the environment and to human health when applied incorrectly (Ajwa *et al.* 2003). Therefore, the use of plant growth-promoting bacteria (PGPB), such as *Azospirillum* as an inoculant, provides a good and environmentally sound biotechnological alternative for plant nutrition and biocontrol of phytopathogens (Bashan & de-Bashan 2010; Tortora *et al.* 2011).

We isolated and characterised the REC3 strain of *A. brasilense* from strawberry roots (Pedraza *et al.* 2007). *A. brasilense* REC3 fixes nitrogen from the atmosphere, produces indoles and siderophores, and contributes to strawberry plant mineral nutrition, among other characteristics (Tortora *et al.* 2011; Guerrero-Molina *et al.* 2014). Using the *gfp*-tagged gene, we demonstrated that strain REC3 is capable of colonising strawberry roots, promoting its growth under environmentally controlled and field conditions, and colonising new plants *via* stolons (Pedraza *et al.* 2010; Guerrero-Molina *et al.* 2012; Salazar *et al.* 2012).

In a previous work (Guerrero-Molina *et al.* 2015) we reported that *A. brasilense* REC3 is capable of exerting beneficial effects on strawberry plants, reinforcing their physiological and cellular characteristics, which in turn contributes to improve plant performance. For instance, early responses such as enhanced levels of soluble phenolic compounds, a decline in lipid peroxidation and up-regulation of strawberry genes involved in defence (*FaPR1*), bacterial recognition (*FaFLS2*) and H_2O_2 depuration (*FaCAT* and *FaAPXc*). Also, *A. brasilense* REC3 inoculation induced delayed structural responses, such as callose deposition and cell wall fortification (at 72 h post-inoculation). In another work, we also reported that *A. brasilense* REC3 participates actively in the induction of systemic protection of strawberry plants against anthracnose disease caused by a virulent isolate of *Colletotrichum acutatum* M11 (Tortora *et al.* 2012). *A. brasilense* REC3 reduced anthracnose symptoms on pathogen-challenged plants, and the effect increased as the elapsed time between bacterial inoculation and fungal infection increased. Biochemical and transcriptional studies revealed transient accumulation of SA, related to SAR, and the induction of defence-related genes, suggesting further that this response is related to structural cell wall modifications as a consequence of the observed increase in phenolic compounds and callose deposition (Tortora *et al.* 2012). However, until now the role of ET and the expression of related genes in the *Azospirillum*–strawberry interaction has not been analysed, an important marker of ISR activation previously observed in strawberry plants inoculated with *A. brasilense* REC3 and then challenged with *C. acutatum* M11. Thus, our working hypothesis is that *A. brasilense* REC3 can enhance ET production of strawberry plants as a consequence of the expression of ET-related genes, being an additional trait supporting ISR activation previously observed in this interaction. Consequently, the aim of this work was to assess ET production and expression of related genes in the *Azospirillum*–strawberry plant interaction.

MATERIAL AND METHODS

Plant material

In vitro micropropagated plants of strawberry (*Fragaria ananassa*, Duch) cv. Pájaro were used. They were obtained from the Strawberry Active Germplasm Bank, INSIBIO (CONICET-UNT). Plants were grown individually in 50-ml vials containing 15 ml sterilised modified Hoagland nutrient solution: 6 mM KNO_3, 4 mM $Ca(NO_3)_2.4H_2O$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4.7H_2O$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4.H_2O$, 2 μM $ZnSO_4.7H_2O$, 0.5 μM $CuSO_4.5H_2O$, 0.5 μM H_2MoO_4 and 26 μM FeEDTA (Epstein 1972). Plants were maintained in a growth chamber at 24 °C, 70% relative humidity, and a 16-h photoperiod (250 μmol·photons m^{-2} s^{-1}) for 15 days before the assay. The plantlets were tested to corroborate the absence of microbes by plating root and leaf macerates in trypticase soy agar medium (TSA; Difco-BBL, Sparks, MD, USA) and incubating for 120 h at 30 °C. Only plantlets free of microbes were used for the experiments.

Inoculum preparation

The strain REC3 of *A. brasilense*, previously isolated from roots of strawberry plants and characterised in our laboratory (Pedraza *et al.* 2007), was used. A pure culture of *A. brasilense* REC3 was grown in NFb medium (Baldani *et al.* 2014) for 24 h at 30 °C and 120 rpm. The cells were then centrifuged at 2000 × *g* for 10 min and washed twice with sterile bi-distilled water pH 7.0 to remove culture medium residue that might interfere with the plant assay. The cells were then resuspended in sterile bi-distilled water. The bacterial concentration for plant inoculation was 10^6 CFU·ml^{-1} ($OD_{560\ nm}$ 0.2).

Plant assay

To evaluate the ET production, the strawberry plants (total 30) received the following treatments: (i) root inoculation with 500 µl *A. brasilense* REC3; (ii) no bacterial inoculation; (iii) addition of 500 µl 1 µM $AgNO_3$, an inhibitor of ET biosynthesis (negative control); (iv) addition of 500 µl 10 nM 1-aminocyclopropane-1-carboxylic-acid (ACC), a precursor of ET biosynthesis (positive control); (*v*) 500 µl 200 nM IAA; and (*vi*) 500 µl 1 µM IAA. ACC, AgNO3 and IAA were also added to roots. The experimental design was completely randomised and each treatment included five replicates.

In parallel, to evaluate gene expression, an additional set of 20 plantlets was root-inoculated with 500 µl *A. brasilense* REC3, and 20 plantlets were kept without bacterial inoculation as control. Plants were grown under the same conditions as described above.

The experiments were performed three times. Initially, each experiment was statistically analysed separately; however, data from the experiments were combined since the results were very similar.

Ethylene quantification

Ethylene was quantified from *in vitro* strawberry plants using gas chromatography in airtight 50-ml vials. Samples of 1 ml of each treatment were taken from the headspace of the vials at different hours post-treatment (hpt): 12, 24, 48 and 72 h. Gas chromatography was carried on in an AGILENT 6890N Network GC System with a GS-Alumina column 30 m × 0.53 mm (Agilent, Santa Clara, CA, USA), FID detector (240 °C, 30:300 H_2:air), oven temperature 100 °C, injector temperature 120 °C, N_2 as carrier gas with 40 ml·min^{-1} flow rate. Pure ET was used as standard. Results were expressed as nmol ET·g^{-1} shoot dry mass. Additionally, vials containing just nutrient Hoagland solution, and vials with Hoagland solution inoculated with 500 µl *A. brasilense* REC3 were included for ET quantification.

Growth promotion assay

To evaluate the plant growth-promoting effect exerted by *A. brasilense* REC3 in the variety of strawberry used to determine ET production, plant dry mass and the growth index were determined. A set of five plants was root-inoculated with 500 µl *A. brasilense* REC3 and five other plants were not inoculated (control), only receiving 500 µl sterile distilled water. After 15 days of growth under the same conditions as described above the plants were removed from the vials and roots and shoots oven-dried at 65 °C for 72 h (constant weight) and dry weight of each tissue recorded. Total biomass was calculated as the sum of root and shoot dry weights, and the growth index as: (final dry biomass – initial dry biomass)/initial dry biomass.

Gene expression

Quantitative real time PCR was performed to analyse the relative expression of the genes *Faetr1*, *Faers1* and *Faein4*, which encode ET receptors; *Factr1*, *Faein2*, *Faacs1* (encoding ACC synthase), *Faaco1* (encoding ACC oxidase), *Faaux1* and *Faami1* (for IAA synthesis enzymes). Total RNA was extracted with RNAqueous-4PCR kit (Ambion, Austin, TX, USA) from 80 mg foliar tissue from strawberry plants inoculated with *A. brasilense* REC3 and non-inoculated plants, at 12, 24 and 48 hpt. To remove contaminant DNA, total RNA was treated with DNase (Thermo-Fisher, Waltham, MA, USA). The quality and purity of RNA was determined spectrophotometrically and by electrophoresis. A total of 5 µg RNA was retro-transcribed using 1 µl Oligo$(dT)_{18}$ primer and 1 µl RevertAid H Minus reverse transcriptase (Thermo-Fisher). cDNA amplification was done in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using iQ SYBR Green Supermix (Roche, Basel, Switzerland). Primers were designed with Primer Express 3.0 (Life Technology, Carlsbad, CA, USA) software. Gene-specific primers are shown in Table 1. Gene expression was measured as the $\log_2$ of the ratio between

Table 1. Primers used for gene expression of strawberry plants inoculated with *A. brasilense* REC3, expressed as forward (Fw) and reverse (Rv).

Gene	Primer sequences	
Faetr1	Fw 5′-GGTTGCTGACCAGGTAGCAGTT-3′	Rv 5′-TGCCCTCCTTGACTCTTCTAAGA-3′
Faers1	Fw 5′-TGTGCAACAGCACTGATGCTT-3′	Rv 5′-TCCCGGGTCTTGACAGACA-3′
Faein4	Fw 5′-TGCTCACTCGTTCCAATTGATG-3′	Rv 5′-CCAAGGCCGTGAGGAGTTT-3′
Factr1	Fw 5′-CCAGGTCCCCGACAATCA-3′	Rv 5′-CCCCTCCCCCTCGTAAAA-3′
Faein2	Fw 5′-GCAGTTGGTAGTAGAGCAGGTT CTG-3′	Rv 5′-TGCCTCTGAATCTGCAACTGA-3′
Faacs1	Fw 5′-AAAGTGAGAGGAAATCGAGTC ACA-3′	Rv 5′-TGCGCCTCCGCTCATAA-3′
Faaco1	Fw 5′-AGCACCTTCTACCTCAAACAC CTT-3′	Rv 5′-CGTCGAGATCTGGGACTTCTG-3′
Faaux1	Fw 5′-CGGCGCCACCAATATACTAT ACA-3′	Rv 5′-GGCATGCATGATTTCCACTGT-3′
Faami1	Fw 5′-GCACACCGGTAAATCCATGTG-3′	Rv 5′-AGCAGAGCCACTAGACGATCCT-3′

A. brasilense REC3-inoculated plants and control plant expression levels. Transcript levels of genes were normalised to the constitutively expressed elongation factor gene *FaEF1* (Fw 5′-CCCCCACTTGGTCGTTTTG-3′ and Rv 5′-TGATGACTCCCACAGCAACAG-3′; GenBank accession number: BK009992).

Statistical analysis

For ethylene quantification and growth promotion assay, statistical analysis of data (one-way ANOVA) was carried out with the software Infostat (version 2008 for Windows; Graph-Pad, La Jolla, CA, USA; Di Rienzo *et al.* 2013) and significant differences are reported at $P \leq 0.05$ using LSD multiple comparison test. Data are expressed as mean ± SD.

For gene expression analysis, amplification efficiencies and Ct (threshold cycle) values, which is the cycle were fluorescence of the sample reaches the detection threshold, were determined for each gene with the slope of a linear regression model using LinRegPCR software (Ruijter *et al.* 2009). These profiles were estimated in relation to the *FaEF1* reference gene using fgStatistics software (Di Rienzo 2011), based on previously published algorithms (Pfaffl 2001).

RESULTS

Ethylene quantification

Results of ET quantification are shown in Fig. 1A. Major ET production was observed in *A. brasilense* REC3-inoculated plants in contrast to non-inoculated control plants, with a maximum value (94.4 nmol ET·g^{-1} shoot dry mass) at 72 hpt. In control plants, ET production remained almost constant from 12 hpt to 72 hpt. ET production rate, defined as nmol ET.g^{-1} shoot dry biomass·h^{-1}, was also determined (Fig. 1B). The values obtained in *A. brasilense* REC3-treated plants were higher than in control plants at all times assessed, with a maximum (7.4 nmol ET·g^{-1} shoot dry mass·h^{-1}) at 12 hpt for both treatments, being significantly higher in bacterial-inoculated plants ($P \leq 0.05$). The increase in ET production rate in REC3-treated plants over control plants was 48.5%. ET was not detected in vials containing Hoagland solution with or without *A. brasilense* REC3, indicating that neither the medium nor the bacteria in Hoagland nutrient solution produced ET.

Plants treated with 10 mM ACC, as positive control for ET biosynthesis, had higher values of ET production than bacterial-inoculated and non-inoculated plants (Fig. 2A). The opposite situation was observed when plants were treated with 1 μM $AgNO_3$, an inhibitor of ET biosynthesis (negative control; Fig. 2A).

In addition, ET production was evaluated in plants treated with 200 nM IAA and 1 μM IAA (Fig. 2B). Higher ET production was observed in plants treated with 200 nM IAA, compared with REC3-treated plants and non-inoculated plants. ET produced by plants treated with 1 μM IAA was very similar to that produced in bacterial-inoculated plants at all times assessed.

Growth promotion assay

The growth promotion effect exerted by *A. brasilense* REC3 on strawberry plants was evaluated as biomass dry weight and growth index. As a general feature, *A. brasilense* REC3-inoculated plants had a significantly higher growth index

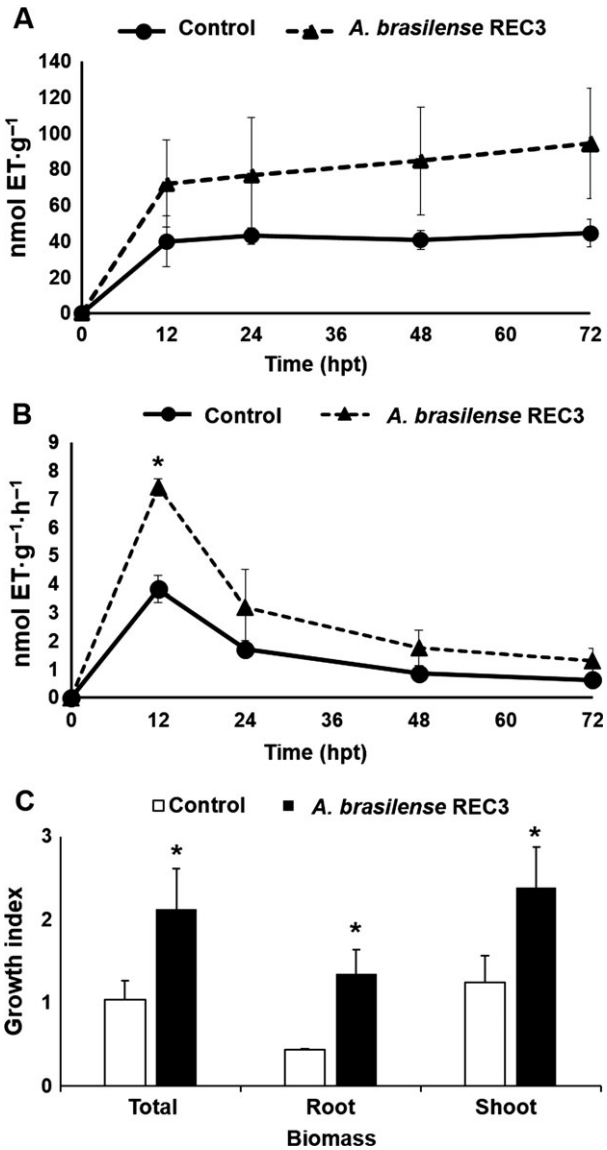

Fig. 1. (A) ET production of strawberry plants inoculated with *A. brasilense* REC3 and control plants, quantified using gas chromatography at different hpt. The values correspond to nmol ET·g^{-1} shoot dry mass. (B) ET production rate of bacterial-inoculated and control strawberry plants at different hpt. Values correspond to nmol ET·g^{-1} shoot dry mass·h^{-1}. (C) Growth index of total, root and shoot biomass of strawberry plants inoculated with *A brasilense* REC3 and treated with distilled water as control. Data are mean ± SD of 15 replicates. ANOVA (significance $*P \leq 0.05$) and LSD tests were done separately for each plant.

15 days after inoculation, compared to control plants that received only sterile distilled water. As shown in Fig. 1C, the growth index of roots, shoots and total biomass of bacterial-inoculated plants were 68%, 48% and 51% higher than control plants, respectively.

Gene expression

The molecular response to inoculation with *A. brasilense* REC3 was evaluated using qRT-PCR, studying expression levels of genes related to the ET synthesis and signalling pathway. There

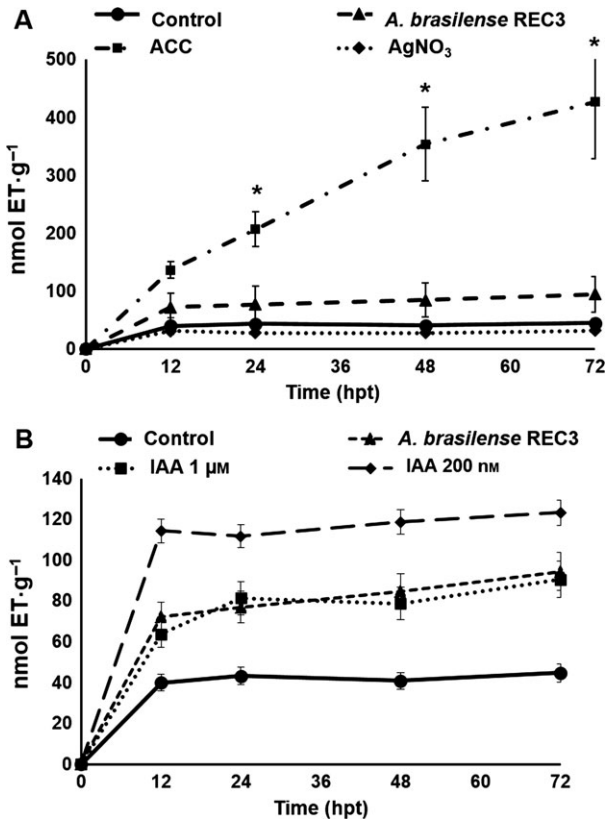

Fig. 2. (A) ET production of strawberry plants treated with 10 nM ACC, 1 µM $AgNO_3$, 10^6 CFU·ml^{-1} *A. brasilense* REC3, and treated with distilled water as control, quantified using gas chromatography at different hpt. The values correspond to nmol ET·g^{-1} shoot dry mass. (B) ET production of strawberry plants treated with 200 nM IAA, 1 µM IAA, 10^6 CFU·ml^{-1} *A. brasilense* REC3, and without treatment (control). Values correspond to nmol ET·g^{-1} shoot dry mass. Data are mean ± SD of 15 replicates. ANOVA (significance *$P \leq 0.05$) and LSD tests were done separately for each plant.

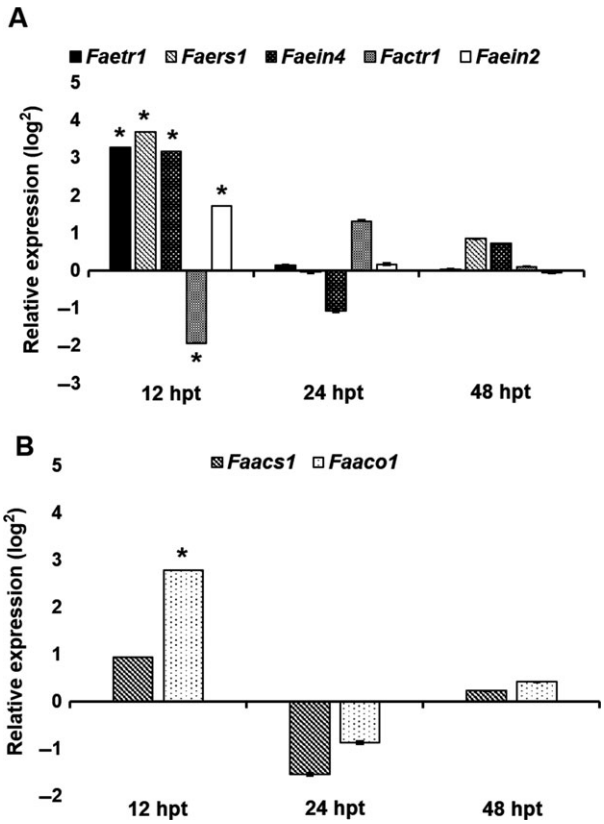

Fig. 3. (A) Relative expression of the strawberry genes *Faetr1*, *Faers1*, *Faein4*, *Factr1* and *Faein2* determined with qRT-PCR in plants inoculated with *A. brasilense* REC3 and not inoculated (controls) at 12, 24 and 48 hpt. (B) Relative expression of the strawberry genes *Faacs1* and *Faaco1* determined with qRT-PCR in plants inoculated with *A. brasilense* REC3 and not inoculated (controls) at 12, 24 and 48 hpt. All expression values of genes were normalised to *Faef1* levels and to non-inoculated control plants. Data are mean ± SD of three biological repeats with three technical replicates each. Asterisks (*) indicate significant differences in expression between control and REC3-inoculated plants determined with fgStatistics software at $P \leq 0.05$.

was a significant difference ($P \leq 0.05$) at 12 hpt: genes encoding ET receptors, *Faetr1*, *Faers1* and *Faein4*, as well as the pathway modulator *Faein2*, were up-regulated at 12 hpt in inoculated plants. In contrast, *Factr1*, encoding a negative regulator, was suppressed at 12 hpt (Fig. 3A). At 24 hpt, expression level of the receptors decreased while *Factr1* was up-regulated, suggesting that ET signal transduction diminish with time. Expression of the gene encoding ET synthesis enzymes ACC oxidase, *Faaco1*, was up-regulated at 12 hpt (Fig. 3B). At 24 and 48 hpt no significant difference was observed ($P \leq 0.05$); however, gene encoding ACC synthase, *Faacs1*, did not differ at any time (Fig. 3B).

Analysis of the genes *Faaux1* and *Faami1* showed that they were up-regulated at 12 hpt in inoculated plants. At 24 hpt only *Faami1* was significantly different, while no differences were observed at 48 hpt (Fig. 4).

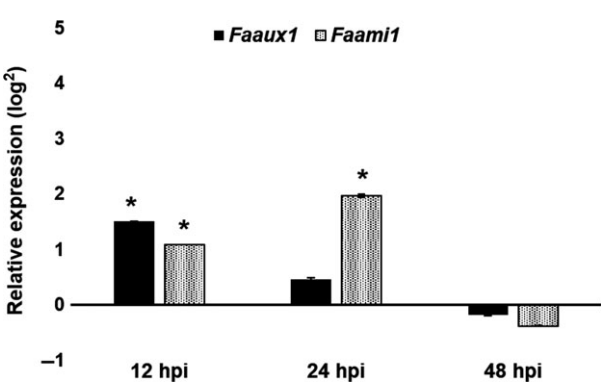

Fig. 4. Relative expression of the strawberry genes *Faaux1* and *Faami1* determined with qRT-PCR in plants inoculated with *A. brasilense* REC3 and not inoculated (controls) at 12, 24 and 48 hpt. All expression values of genes were normalised to *Faef1* levels and to non-inoculated control plants. Data are mean ± SD of three biological repeats with three technical replicates each. Asterisks (*) indicate significant differences in expression between control and REC3-inoculated plants determined with fgStatistics software at $P \leq 0.05$.

DISCUSSION

Ethylene is a phytohormone with dual behaviour. A biphasic model proposed by Pierik *et al.* (2006) suggests that lower concentrations induce plant growth and development, and higher concentrations inhibit it, leading to plant senescence. High levels of ET can induce seed germination, fruit ripening and

then senescence; in contrast, low levels of ET stimulate cell expansion, promoting root hair development (Bleecker & Kende 2000; Wang *et al.* 2002; Stepanova & Alonso 2005; Wen *et al.* 2015) and can participate in signalling processes leading to ISR activation (Choudhary *et al.* 2007; Pieterse *et al.* 2009). From the latter, major ET production in inoculated strawberry plants and changes observed in ET production rate a few hours after treatments (12 hpt) suggest that this phytohormone acts as a rapid and transient signal. As expected, ET production decreased over time, preventing the senescence that higher quantities might have caused. As part of the methodological adjustment, ET production was also assessed in the presence of $AgNO_3$, an inhibitor of ET biosynthesis, and ACC, as precursor. The results were as expected and also reported by others (Glazer *et al.* 1985; Songstad *et al.* 1988). Moreover, ET was not detected in vials containing Hoagland solution with and without *A. brasilense* REC3, indicating that neither the medium nor the bacteria living in Hoagland nutrient solution produced ET. Thus, the ET assessed was produced only by the strawberry plants.

Regarding the growth-promoting effect exerted by *A. brasilense* REC3 on strawberry plants, the growth index determined in roots, shoots and total biomass of inoculated plants was higher than that of the control plants. This is in agreement with our previous works, where REC3 also increased strawberry dry weights, but with different cultivars of strawberry (Pedraza *et al.* 2010; Guerrero-Molina *et al.* 2014). As expected, these results are due to the plant growth-promoting characteristics reported for REC3, *i.e.* the auxin-producing capacity, the most important for promoting plant growth (Pedraza *et al.* 2007).

Analysis of gene expression through qRT-PCR shows that *Faaco1*, encoding the ET synthesis enzyme ACO, was up-regulated at 12 hpt in REC3-treated plants, with an increase in ET production. Likewise, ET receptor genes, *Faetr1*, *Faers1* and *Faein4*, as well as the gene encoding a positive regulator of the signalling pathway, *Faein2*, were also up-regulated. In contrast, the negative regulator *Factr1* was down-regulated at the same time. The increase in expression level of receptor genes, as well as the positive regulatory gene, and the fall in negative regulatory gene expression, proves that strawberry plants inoculated with *A. brasilense* REC3 effectively activate the signalling pathway in response to ET. It has been suggested that ISR activation is due to an increase in plant sensitivity to ET, reflected in up-regulation of ET receptors, and not to major production of this hormone (Pieterse *et al.* 2000). This is consistent with the results observed in our work, where the increase in ET production rate was not a sustained effect, but transient, indicating that ET acts as a signal that turns off briefly. Furthermore, this modulation was not due to the action of the bacterial enzyme ACC deaminase, because *A. brasilense* REC3 does not possess it (Pedraza *et al.* 2007).

The interaction between phytohormones is of great importance in the coordination of plant growth and metabolism (Santner & Estelle 2009). As previously described, *A. brasilense* REC3 is capable of producing indoles, including IAA, when colonising strawberry plants (Pedraza *et al.* 2007). In this work, we also analysed the link between ET and IAA. It is known that there is a synergic effect between ET and IAA, and that this auxin acts as a positive regulator of ET synthesis through induction of ACS (Yu & Yang 1979; Yoshii & Imaseki 1981; Abeles *et al.* 1992; Kende 1993; Ribaudo *et al.* 2006). In this work, the effect of two different concentrations of exogenous IAA was compared with the effect of inoculation with *A. brasilense* REC3. In plants treated with 1 μM IAA ET production was similar to that in plants inoculated with *A. brasilense* REC3. In both situations, an increase in ET production was observed in comparison with control plants. We therefore infer that *A. brasilense* REC3 stimulates ET production in plants through auxin synthesis, mimicking the effect of exogenous IAA.

Sequential regulation of ET synthesis enzymes by auxins has been described. Auxins affect ACS, whose product exerts positive feedback regulation over ACO, to finally produce ET (Peck & Kende 1995). This correlates with the positive regulation observed in genes encoding ACS in different plant species, in which expression was induced through the influence of auxins (Peck & Kende 1995; Wang & Arteca 1995; Yoon *et al.* 1997; Lee *et al.* 2017). In our analysis of relative expression of genes related to IAA synthesis, *Faaux1* and *Faami1* were up-regulated at 12 hpt, showing early induction of IAA production, and at the same time stimulating early ET synthesis. However, we observed possible positive regulation of ET over IAA synthesis in roots, suggesting that auxins act synergically, stimulating at the same time both synthesis pathways, as reported previously (Pierik *et al.* 2006; Swarup *et al.* 2007; Stepanova *et al.* 2008).

From all of the above, we conclude that inoculation of strawberry plants with *A. brasilense* REC3 induced enhanced ET production, as a rapid and transient signal. This was a consequence of expression of ET-related genes in the bacterial-inoculated plants. This feature constitutes an additional trait supporting ISR activation previously observed in strawberry plants inoculated with *A. brasilense* REC3 and then challenged with the virulent isolate M11 of *C. acutatum* (Tortora *et al.* 2012). Therefore, ET production and the positive regulation of molecular markers of ET-related genes allow us to infer the priming activation characteristic of ISR. This type of resistance and the activation of SAR observed by Tortora *et al.* (2012) indicate that both are present in strawberry plants and could act synergistically, increasing protection against pathogens. However, it is important to stress that no single plant hormone is responsible for mediating all plant immune responses to varying biotic stresses, as this is a complex and interconnected combination of hormonal interactions that modulate plant immunity, referred to as hormonal crosstalk (Shigenaga & Argueso 2016).

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