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RESEARCH PAPER

Physiological, structural and molecular traits activated in strawberry plants after inoculation with the plant growth-promoting bacterium *Azospirillum brasilense* REC3

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Keywords
Azospirillum brasilense; callose deposition; cell wall fortification; *Fragaria ananassa*; gene expression; lipid peroxidation; phenolic compounds.

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ABSTRACT

The plant growth-promoting strain REC3 of *Azospirillum brasilense*, isolated from strawberry roots, prompts growth promotion and systemic protection against anthracnose disease in this crop. Hence, we hypothesised that *A. brasilense* REC3 can induce different physiological, structural and molecular responses in strawberry plants. Therefore, the aim of this work was to study these traits activated in *Azospirillum*-colonised strawberry plants, which have not been assessed until now. Healthy, *in vitro* micropropagated plants were root-inoculated with REC3 under hydroponic conditions; root and leaf tissues were sampled at different times, and oxidative burst, phenolic compound content, malondialdehyde (MDA) concentration, callose deposition, cell wall fortification and gene expression were evaluated. *Azospirillum* inoculation enhanced levels of soluble phenolic compounds after 12 h post-inoculation (hpi), while amounts of cell wall bound phenolics were similar in inoculated and control plants. Other early responses activated by REC3 (at 24 hpi) were a decline of lipid peroxidation and up-regulation of strawberry genes involved in defence (*FaPR1*), bacterial recognition (*FaFLS2*) and H_2O_2 depuration (*FaCAT* and *FaAPXc*). The last may explain the apparent absence of oxidative burst in leaves after bacterial inoculation. Also, REC3 inoculation induced delayed structural responses such as callose deposition and cell wall fortification (at 72 hpi). Results showed that *A. brasilense* REC3 is capable of exerting beneficial effects on strawberry plants, reinforcing their physiological and cellular characteristics, which in turns contribute to improve plant performance.

INTRODUCTION

Strawberry is a small fruit crop cultivated in different parts of the world, and due to the organoleptic properties and health beneficial properties its consumption has greatly increased in recent years. According to the Food and Agriculture Organization of the United Nations (FAOSTAT 2014), in the year 2012 annual worldwide strawberry production was estimated at over 4.5 million tonnes.

Presently, there is an increasing worldwide interest in the development and use of new, more accurate and environmentally safer alternatives for crop nutrition and protection against phytopathogens. Many plant growth-promoting bacteria (PGPB), *e.g. Azospirillum brasilense*, are able to promote plant growth of different crops and induce disease resistance in plants by activating genetically programmed defence pathways (Vleesschauwer *et al.* 2006; Tortora *et al.* 2012). *Azospirillum* induces plant growth promotion and disease control through numerous mechanism, such as the augmentation of root biomass, nitrogen fixation, phosphate solubilisation, synthesis of phytohormones, siderophores, enzymes, vitamins, and the possibility of more than one mechanism being involved at the same time (Bashan & de-Bashan 2010; Bashan *et al.* 2014). Root inoculation with selected PGPB can elicit a type of systemic immunity in plants called induced systemic resistance (ISR; Van Loon *et al.* 1998; Pieterse *et al.* 2009). When plants recognise different beneficial microbe-associated molecular patterns (MAMP), a mild but effective immune response is activated in systemic tissues (Pieterse *et al.* 2009). Bacterial flagellin is found among MAMPs because it is an evolutionary conserved molecule that can be sensed by plants *via* a specific receptor, flagellin-sensing 2 (FLS2), which directly binds to flg22 (a highly conserved 22-amino acid epitope of flagellin protein) and this contributes to recognition specificity (Zipfel 2008).

One of the earliest events in systemic defence response is a burst of oxidative metabolism leading to the generation of superoxide anion radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2; Pieterse *et al.* 2009). Due to their chemical reactivity, these reactive oxygen species (ROS) can be directly protective against invading microorganisms, activate phytoalexin biosynthesis, drive oxidative cross-linking of cell wall components and induce an array of protective genes, such as *PR* (*Pathogene-*

sis Related) genes (Mittler *et al.* 2004; Foyer & Noctor 2005, 2009; Møller *et al.* 2007). However, accumulation of ROS can seriously disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids (Møller *et al.* 2007). A common indicator of membrane lipid peroxidation is malondialdehyde (MDA) content, a secondary end product of the oxidation of polyunsaturated fatty acids (PUFA), generally increased under stress conditions such as pathogen infection (Møller *et al.* 2007).

In order to regulate ROS levels, plant cells can activate scavenging enzymes like catalases, superoxide dismutases, alternative oxidases and ascorbate peroxidases among others, as well as non-enzymatic mechanism such as numerous classes of reducing metabolites and antioxidants (Mittler *et al.* 2004; Møller *et al.* 2007). Polyphenols are antioxidants crucial for plant functioning and development. They play important roles in different supporting or protective tissues, are involved in defence strategies and have signalling properties, particularly in the interactions between plants and their environment (Boudet 2007; Huda-Faujan *et al.* 2009). These antioxidant secondary metabolites are especially important because of their high redox potentials, allowing them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Kähkönen *et al.* 1999). Enhancement of plants phenolic metabolism has been often associated with the induced resistance phenomenon against several bacterial, fungal and viral diseases (Boudet 2007). Hence, phenolics may act as soluble antimicrobial or anti-feeding compounds or they may cross-link with callose, proteins and polysaccharides into the cell walls, thus inhibiting pathogen penetration and the absorption of nutrients by the invading pathogen (Hukkanen *et al.* 2007).

Besides physiological modifications, plants may also exhibit structural changes in cell walls in response to biotic stress. The synthesis of callose can be induced through mechanical wounding, physiological stress and phytopathogen infection (Tortora *et al.* 2012). In strawberry seedlings, the avirulent fungal strain *Colletotrichum fragariae* F7 was able to induce defence responses shortly after infection, showing cell wall thickening, ROS production and accumulation of pigments and amorphous crystals (Salazar *et al.* 2007).

As mentioned, plants have evolved sophisticated strategies to perceive and defend themselves against different types of pathogen by initiating diverse downstream signals as well as complex defence mechanisms. However, PGPB can also elicit plant-mediated resistance responses against soil-borne pathogens, and the mechanisms by which beneficial and pathogen microorganisms activate the host immune response are similar, sharing many signalling events but also displaying crucial differences (Van Wees *et al.* 2008). Hence, plants have the capacity to finely regulate immune responses depending on the invader encountered (Pieterse *et al.* 2009). Previous studies have demonstrated that the isolate REC3 of *A. brasilense* was able to effectively colonise strawberry roots and stolons, promote plant growth, have active chemotaxis toward strawberry root exudates, fix nitrogen and produce indole compounds (Pedraza *et al.* 2010; Guerrero-Molina *et al.* 2012). It was also demonstrated that the REC3 strain exerted a biocontrol effect against anthracnose disease caused by a virulent isolate of *Colletotrichum acutatum*, and this was accompanied by the production of siderophores, increasing salicylic acid (SA) production and PR expression (Tortora *et al.* 2011, 2012). However, the physiological, structural and molecular mechanisms activated in the *Azospirillum*–strawberry interaction remains largely unclear. Therefore, the aim of this work was to further investigate the plant defence mechanisms activated by the interaction with *Azospirillum*, even when plants are not challenged with a pathogen. This was investigated by analysing callose deposition, cell wall fortification, accumulation of phenolic compounds, ROS production, lipid peroxidation and the expression of genes usually associated with defence responses.

MATERIAL AND METHODS

Vegetal material

In vitro micropropagated plants of strawberry (*Fragaria ananassa*, Duch) cv. 'Camarosa', obtained from the Strawberry Active Germplasm Bank at the National University of Tucumán, were used. Plants were first grown in disinfected pots containing sterile substrate (humus:perlome, 2:1), then 3-month-old plants were washed with deionised water and placed in sterile plastic trays containing sterile modified Hoagland nutrient solution (Epstein 1972) with constant aeration, and maintained in a growth chamber at 28 °C, 70% relative humidity (RH) and a 16-h photoperiod (250 μmol·photons·m^{-2}·s^{-1}). Hydroponic medium volume was periodically measured and maintained constant by adding distilled water to retain the optimum nutrient concentration.

Inoculum

A pure culture of *Azospirillum brasilense* REC3 (FJ012319.1) grown for 48 h at 30 °C was used. Bacterial suspension containing about 10^6 CFU·ml^{-1} (DO_{600} 0.2) was prepared in NFb liquid medium as described in Pedraza *et al.* (2010). This strain was previously isolated from strawberry roots and has been characterised (Pedraza *et al.* 2007).

Inoculation and sampling

Hydroponic strawberry plants were inoculated with *A. brasilense* REC3 by submerging the roots in the bacterial suspension (10^6 CFU·ml^{-1}) for 20 min, and then placed back in plastic trays with the sterile hydroponic solution. Control plants were submerged for 20 min in sterile distilled water.

Roots and leaves of five plants per treatment were randomly selected at every sampling time (0, 12, 24, 48, 72, 96 and 120 h post-inoculation; hpi) to evaluate oxidative burst, phenolic compound content and MDA concentration. Leaf tissues were sampled at 24, 48, 72 and 96 hpi for callose deposition and cell wall fortification analysis, and also at 24 and 48 hpi for gene expression analysis. All sampled plants were 3 months old, and leaves of the third petiole from the crown were harvested and pooled to perform all the determinations. Each plant was sampled only once to avoid eventual cutting effects.

Histochemical determination of superoxide and hydrogen peroxide

To assess whether *Azospirillum* was able to produce an oxidative burst locally or systemically, roots and leaves of strawberry plants were sampled and stained with nitro-blue tetrazolium

(NBT; Sigma-Aldrich, Bornem, Belgium) for superoxide detection (Grellet-Bournonville & Díaz-Ricci 2011) and 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) to evaluate H_2O_2 accumulation (Vleesschauwer *et al.* 2006). Roots were rinsed with deionised water and subsequently incubated in the dark at room temperature, in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1% (w/v) NBT and 10 mM sodium azide for 8 h; or in water with 0.1% (w/v) DAB and 0.01% Triton-X100 for 12 h. Detached leaves were equally treated, except that Triton-X100 (Sigma-Aldrich) was not used. Superoxide production was visualised as a purple formazan deposit, and a brownish-red precipitate was produced by the peroxidase-catalysed reaction of DAB with H_2O_2. Positive controls for DAB were prepared by submerging plant tissue for 30 min in 10 mM H_2O_2 or in 10 mM ascorbic acid as negative control. For NBT positive reaction, plant tissues were submerged in 50 mM potassium phosphate buffer (pH 7.8) containing 27 mM riboflavin and 17 mM methionine for 1 h under clear light.

Extraction and analysis of phenolic compounds

For analysis of total soluble phenolics, 1.0 g of fresh leaves were weighed and homogenised with 80:20 (v/v) ethanol:water. The extracts were then centrifuged at 3000 *g* for 10 min and the alcoholic phase sonicated for 30 min. The concentration of NaOH-hydrolysable cell wall-bound phenolics was determined according to Hukkanen *et al.* (2007). The remaining pellet was washed once with 100% (v/v) methanol followed by addition of 1.0 ml 1 M NaOH per each 20 mg fresh plant tissue; and then it was hydrolysed at 70 °C for 1 h and centrifuged at 10,000 *g* for 10 min. The supernatant was neutralised with a volume of 1 M HCl, vacuum evaporated at room temperature, and resuspended in 1.0 ml 80:20 (v/v) ethanol:water. Positive controls consisting of plants UV-irradiated for 1 h and plants submerged for 30 min in 10 mM H_2O_2 were included. Total content of both soluble phenolic and cell wall-bound phenolic extracts were determined with the Folin-Ciocalteu reagent (Anedra, Argentina) according to the method of Singleton & Rossi (1965). Three replicates of each sample (100 µl) were mixed with 2.5 ml 0.2 N Folin-Ciocalteu reagent and 2.0 ml 7.5% (w/v) Na_2CO_3. The absorbance of all samples was measured at 765 nm after incubating at 25 °C for 10 min. Results are expressed as milligrams of gallic acid equivalent (GAE) per gram fresh weight of leaves or roots.

Lipid peroxidation analysis

Lipidic peroxidation was determined by measuring malondialdehyde (MDA) concentration according to the method of thiobarbituric acid reactive substances (TBARS) described in Hodges *et al.* (1999). Approximately 1.0 g (fresh weight) of root or leaf tissue was homogenised in 20 ml 96% ethanol: water (80:20 v/v), followed by centrifugation at 3000 *g* for 10 min. Two 0.5-ml aliquot of the alcoholic extract were taken, one was mixed with 0.5 ml (i) +TBA solution containing 20% trichloroacetic acid, 0.01% butylated hydroxytoluene (BHT) and 0.65% thiobarbituric acid (TBA), and the other was mixed with (ii) -TBA solution that had the same composition as solution (i) but without TBA. The mixture was heated at 95 °C for 25 min, cooled and then centrifuged at 4000 *g* for 10 min. Absorbance was measured at 450, 532 and 600 nm in a Beckman spectrophotometer (Model DU #640; Beckman Coulter, Inc., USA). MDA equivalents were calculated according to the following formulae (Hodges *et al.* 1999):

1 $A = [(Abs\ 532_{+TBA} - Abs\ 600_{+TBA}) - (Abs\ 532_{-TBA} - Abs\ 600_{-TBA})]$
2 $B = [(Abs\ 440_{+TBA} - Abs\ 600_{+TBA})\ 0.0571]$
3 MDA equivalents $(nmol \cdot ml^{-1}) = [(A\text{-}B/157000)10^6]$

Absorbance at 532 nm represents the maximum absorbance of the TBA-MDA complex, 600 nm the correction for nonspecific turbidity, 440 nm the absorbance of sucrose, 0.0571 the molar absorbance ratio of sucrose at 532 and 440 nm, and 157,000 the molar extinction coefficient for MDA. Plants UV-irradiated for 1 h and plants submerged for 30 min in 10 mM H_2O_2 were also assayed as positive controls.

Callose staining

Callose cell wall deposition was analysed in strawberry leaves of control and bacteria-inoculated plants at 24, 48, 72 and 96 hpi according to methods described in Currier & Strugger (1956). After clearing and dehydrating leaves for 12 h with 100% absolute ethanol, they were rehydrated sequentially through increasing concentrations (0, 25, 50, 75 and 100%) of buffer, 67 mM K_2HPO_4 (pH 12.0). Hydrated samples were then 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 25 °C. The stained material was mounted on slides with 30% glycerol and examined under UV light using an Olympus BX51 wide-field fluorescence digital imaging microscope (Olympus Optical Co., Tokyo, Japan). Callose deposition was identified as bright-green spots under fluorescent light (UV filter U-MWU2, 330–385 nm; Olympus). All experiments shown in this study were performed at least three times with similar results, and the images shown are representative of the observation of at least three leaves of each plant.

Cell wall fortification

To evaluate fortification of cell walls, leaves were sampled at 24, 48, 72, 96 hpi, fixed in FAA (formalin–acetic acid–alcohol) and clarified. For this, they were decolourised in boiling 96% ethanol, rehydrated in two steps of 30 min each, first with 10% (w/v) KOH: 96% ethanol (50:50) and subsequently with 10% (w/v) KOH, and finally cleared with 50% (v/v) HClO for 1 min. Samples were mounted on slides with 30% glycerol, observed and photographed with an Olympus BX51 microscope (Olympus).

RNA isolation and qRT-PCR analysis

Quantitative reverse transcription PCR (qRT-PCR) was used to evaluate expression of genes encoding PR1 protein (*FaPR1*), cytosolic ascorbate peroxidase (*FaAPXc*), catalase enzyme (*FaCAT*) and flagellin-sensing 2 receptor (*FaFLS2*) from *Azospirillum*-inoculated strawberry plants and non-inoculated controls sampled at 24 and 48 hpi.

Total RNA was isolated from 100 mg leaf tissue using RNAqueous-4PCR kit (Ambion, Austin, TX, USA). Crude RNA samples were treated with DNase I (Ambion) to remove genomic DNA contamination before reverse transcription reaction. The integrity and quality of total RNA was confirmed

spectrophotometrically and by electrophoresis (1% agarose gel). An aliquot of 1 μg DNA-free total RNA samples was reverse-transcribed using 1 μl Oligo(dT)$_{18}$ primer (Thermo Scientific, Rockford, IL, USA) and 1.5 μl M-MulV reverse transcriptase (Thermo Scientific). cDNA amplification was done in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Primers were designed with Primer Express 3.0 software (Applied Biosystems) using sequences of *F. ananassa* (*FaPR1,* AB462752; *FaAPXc*, AF022213; *FaG-APDH-1,* AB363963) and *F. vesca (FLS2*, LOC101315061; *CAT*, LOC101298322) available at the GenBank database of the National Center for Biotechnology Information (NCBI). Gene-specific primers were: *FaPR1-Fw*: 5′-TGGCCCTTATGGT-GAAAACC-3′ and *FaPR1-Rv*: 5′-CAC AGCAGATGTGCCTGATAAGT-3′; *FaAPXc-Fw*: 5′-CGCTCA CGGCGCTAACA-3′ and *FaAPXc-Rv*: 5′-GCTCCTTGATCGG CTCCAA-3′; *FaFLS2-Fw*: 5′-TGAGTTAAGCGGGACAATAC CA-3′ and *FaFLS2-Rv*: 5′-CAGGGTCAACATTTGCAAT GA-3′; *FaCAT-Fw*: 5′-TTTTCCCACCATCCAGAAAGTC-3′ and *FaCAT-Rv*: 5′-TGGAATTCCCAGGTCATCAAA-3′. To normalise gene expression, transcript levels of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase gene *FaGAPDH-1* (primers *FaGAPDH-1-Fw*: 5′-GCCGAG GAGCTGCTCAGA-3′ and *FaGAPDH-1-Rv*: 5′-GAACTTT TCCAACAGCCTTTGC-3′) were measured. Relative gene expression (RQ) was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001).

Experimental design and statistical analysis

A factorial design with completely randomised blocks of two treatments (REC3-inoculated and non-inoculated controls) with 35 plants per treatment and seven sampling times (0, 12, 24, 48, 72, 96 and 120 hpi) were used. Thus, there were five plants per time per treatment. The whole experiment was performed twice. Statistical analysis of data was processed using one-way ANOVA, and the statistical significance of the means was assessed using LSD multiple comparison test ($P \leq 0.05$) with the software InfoStat (version 2008; Córdoba, Argentina). Only gene expression data were analysed by applying a non-parametric Kruskal-Wallis test ($P \leq 0.05$) and, in this case, for each sample there were three technical qRT-PCR replicates and three biological repeats, as described in Vargas *et al.* (2012).

RESULTS

Oxidative burst analysis

The levels of superoxide and peroxide after NBT and DAB staining in both roots and leaves of hydroponically grown strawberry plants were assessed at different times. Leaves of root-inoculated and non-inoculated strawberry seedlings did not show DAB or NBT staining. Similarly, bacteria-inoculated roots showed the same amount of DAB and NBT precipitates as control roots, confirming the inability of REC3 to induce enhanced levels of H_2O_2 or $O_2^{\cdot-}$. However, strong accumulation of H_2O_2 and $O_2^{\cdot-}$ was observed in both plant tissues previously treated with H_2O_2 and riboflavin-methionine solution (positive controls), which confirmed the specificity of the staining (Fig. S1). A negative control of the technique was also included using ascorbic acid as H_2O_2 scavenger, where precipitates of the dyes were not observed (data not shown).

Determination of soluble and cell wall-bound phenolic compounds

The accumulation of phenolics in strawberry leaves and roots was examined at 0, 12, 24, 48, 72, 96 and 120 hpi. LSD multiple comparison tests showed that *A. brasilense* REC3 inoculation increased levels of soluble phenolic compounds in strawberry leaves and roots after 12 hpi in comparison with non-inoculated plants ($P < 0.05$; Fig. 1). But there were no differences among the means in effect of time, nor in the interaction (treatment-time). Values of positive controls, corresponding to UV-irradiated and H_2O_2-treated plants were, for leaves 3.83 ± 0.24 and 5.89 ± 0.16 mg·GAE·g^{-1} fresh leaves, and for roots 2.03 ± 0.13 and 2.49 ± 0.15 mg·GAE·g^{-1} fresh root.

Compared to soluble compounds, concentrations of NaOH hydrolysable phenolics were ten-fold lower. Qualitatively, there was a fall in cell wall phenolics of REC3-treated plants during the first 96 hpi, but this behaviour switched at 120 hpi in both tissues. However, ANOVA of cell wall-bound phenolics of roots and leaves indicated that there was no significant difference between the means of inoculated and control tissues ($P < 0.05$; Fig. 2).

Analysis of lipid peroxidation

The content of MDA is an indicator of lipid peroxidation and oxidative damage to biological membranes. Figure 3 shows the

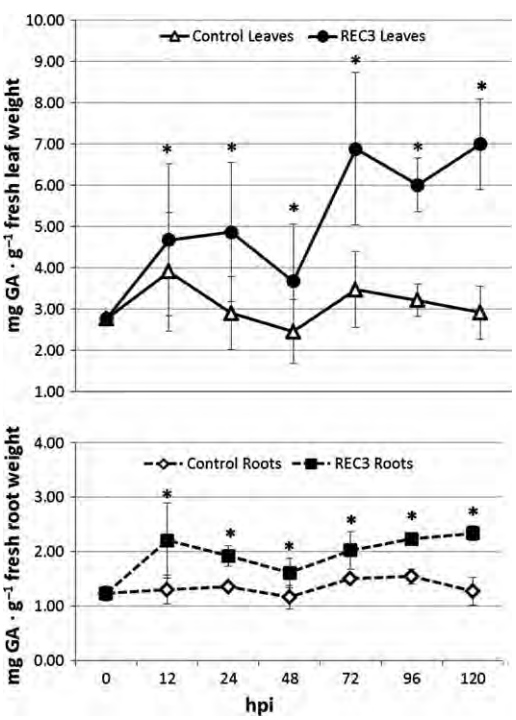

Fig. 1. Total soluble phenolics in strawberry leaves and roots after inoculation with *A. brasilense* REC3 (10^6 CFU·ml^{-1}) and in non-inoculated controls. Measurements were made at 0, 12, 24, 48, 72, 96 and 120 hpi and concentrations of phenolics expressed as mg gallic acid (GA) per g fresh weight. Data are means ± SD of five replicates. ANOVA (significance *$P < 0.05$) and LSD tests were done separately for each plant tissue.

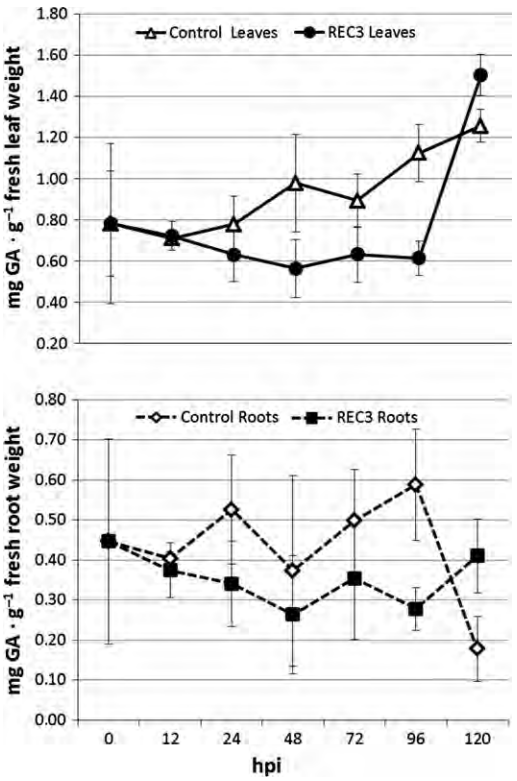

Fig. 2. Cell wall-bound (NaOH-hydrolysable) phenolics in strawberry leaves and roots of plants inoculated with *A. brasilense* REC3 (10^6 CFU·ml^{-1}) and in non-inoculated controls. Measurements were made at 0, 12, 24, 48, 72, 96 and 120 hpi and concentrations of phenolics expressed as mg gallic acid (GA) per g fresh weight. Data are mean ± SD of five replicates. ANOVA ($P < 0.05$) and LSD tests were done separately for each plant tissue.

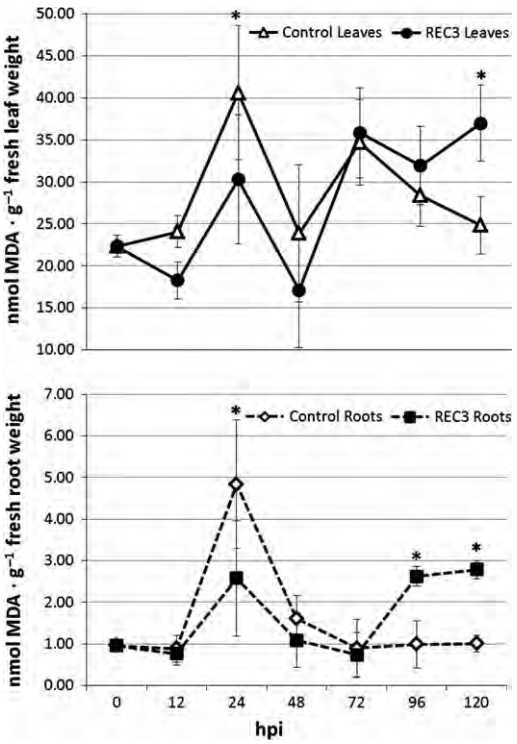

Fig. 3. Lipid peroxidation expressed as nmol malondialdehyde (MDA) per g fresh weight of strawberry roots and leaves determined at 0, 12, 24, 48, 72, 96 and 120 hpi with *A. brasilense* REC3 (10^6 CFU·ml^{-1}). Control plants were not inoculated. Data are mean ± SD of five replicates. ANOVA (significance $*P < 0.05$) and LSD tests were done separately for each plant tissue.

TBARS values of control (not-inoculated) and *A. brasilense* REC3-inoculated strawberry roots and leaves at 0, 12, 24, 48, 72, 96 and 120 hpi. Statistical analysis showed that REC3 inoculation significantly lowered lipid peroxidation of leaves and roots at 24 hpi in comparison to control plants ($P < 0.05$; Fig 3). However, MDA content of inoculated roots and leaves increased significantly with respect to control tissues at 96 and 120 hpi, respectively ($P < 0.05$; Fig 3).

Morphological analyses of strawberry leaves

Macroscopic and microscopic observations of control and REC3-colonised plants did not show any disease symptoms or tissue damage during the experiments, as observed in previous works (Pedraza *et al.* 2010; Guerrero-Molina *et al.* 2014). Microscopy studies were carried out in order to confirm any structural changes caused by *Azospirillum* inoculation in strawberry plants. Results showed that *A. brasilense* REC3 induced callose deposition in strawberry leaves after 72 hpi (Fig. 4b) as well as at 96 hpi (Fig. S2) in contrast to control leaves of non-inoculated plants (Fig. 4a). Also, paradermal microscopy analysis of leaves of REC3-inoculated plants showed a significant increment in cell wall size caused through the thickening of the semi-lamina at 72 hpi (Fig. 4d) compared to control non-inoculated plants (Fig. 4c). Considering that the tested plants were root-inoculated and that structural modifications were observed in leaves, these results suggest that REC3 has the ability to trigger systemic responses in strawberry plants.

Gene expression analyses

In order to analyse whether inoculation with REC3 caused molecular responses in strawberry plants, we designed primers and studied expression of the genes *FaPR1*, *FaAPXc*, *FaCAT* and *FaFLS2* using qRT-PCR at 24 and 48 hpi (Fig. 5). *FaPR1*, a defence-related gene encoding PR1 protein, and the cytosolic ascorbate peroxidase gene, *FaAPXc*, were up-regulated at 24 hpi. Meanwhile, *FaCAT*, encoding catalase, and *FaFLS2*, a flagellin receptor, showed strong induction in expression at 24 and 48 h after inoculation with *A. brasilense* in relation to control plants sampled at the same times ($P < 0.05$; Fig 5). The stronger up-regulation of all tested genes at 24 hpi showed that molecular responses occurred rapidly after bacterial inoculation, suggesting that *FaPR1*, *FaAPXc*, *FaCAT* and *FaFLS2* are early response genes to bacterial inoculation.

DISCUSSION

In this work we showed that strawberry plants inoculated with *A. brasilense* REC3 induced activation of different biochemical, structural and molecular mechanisms. We observed increased accumulation of soluble phenolic compounds in roots and leaves of REC3-inoculated plants from 12 to 120 hpi; similar to results observed in betelvine (*Piper betle* L.) plants after inoculation with a PGPB strain of *Serratia*

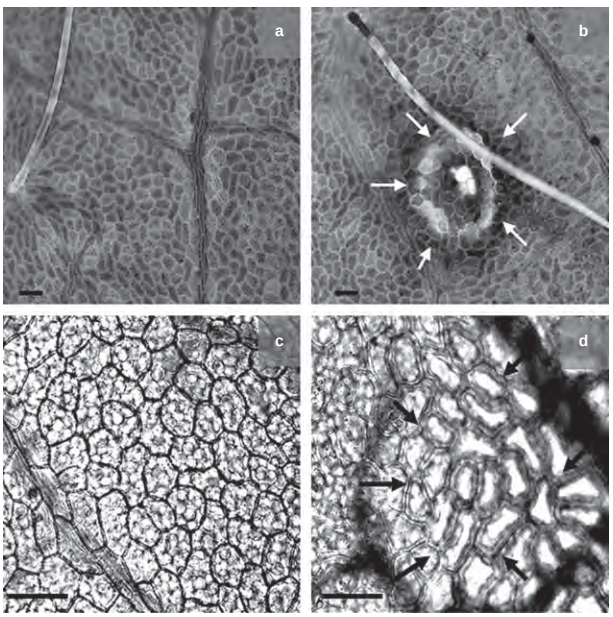

Fig. 4. Microscopic observations of callose depositions (b; indicated by white arrows) and cell wall fortification (d; indicated by black arrows) observed in *A. brasilense* REC3-inoculated strawberry leaves at 72 hpi. (a, c) Control non-inoculated strawberry plants. Scale bars = 100 μm.

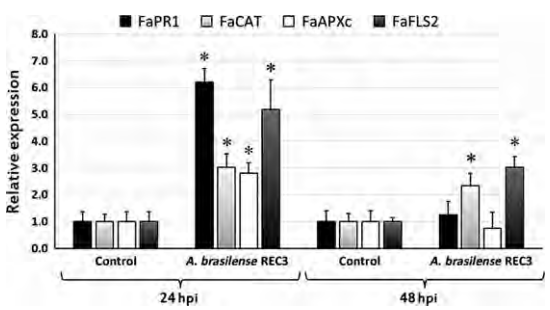

Fig. 5. Relative expression of the strawberry genes *FaPR1*, *FaCAT*, *FaAPXc* and *FaFLS2* determined with qRT-PCR at 24 and 48 hpi with *A. brasilense* REC3. Transcript levels of these genes were normalised to *FaGAPDH-1* levels and to non-inoculated control plants. Data are mean ± SD of three biological repeats with three technical replicates each. Asterisks (*) indicate significant differences in expression between control and REC3-inoculated plants determined by Kruskal-Wallis test at $P < 0.05$.

marcescens, where not only the quantity but also the diversity of phenolics was increased (Lavania *et al.* 2006). In strawberry plants, treatment with benzothiadiazole (BTH) enhanced accumulation of soluble and cell wall-bound phenolics in leaves, improving the resistance to powdery mildew infection under greenhouse conditions (Hukkanen *et al.* 2007). High amounts of soluble phenolics in biotised plants may act as an antimicrobial barrier, leading to enhanced resistance to many plant pathogens (Hukkanen *et al.* 2007; Tortora *et al.* 2012). Some authors have highlighted the correlation between increases in the corresponding phenylalanine ammonia lyase (PAL) gene/protein expression/activity and increases in phenolic compounds in response to different stimuli (Boudet 2007; Ruíz-Sánchez *et al.* 2011). Hence, the role of polyphenolics as signal molecules should be considered as they may mediate activation of PAL, which could lead to synthesis of a variety of defence-related plant secondary metabolites, such as salicylic acid (SA), phytoalexins and lignin-like polymers (Hahlbrock & Scheel 1989). However, failure to observe differences in the concentrations of phenolics bound to the cell wall between inoculated and control plants may be due to the short testing time (120 h) as it not may have been sufficient for synthesising new compounds that fortify the cell wall.

Histochemical analysis of leaves from strawberry plants inoculated with *A. brasilense* REC3 revealed that there was no H_2O_2 or $O_2^{\cdot-}$ accumulation at any assayed time, neither locally nor systemically. The positive staining reaction in controls to DAB and NBT in plants treated with ROS-inducing substances, such as H_2O_2 and riboflanvin/methionine, respectively, proved that the inability to detect an oxidative burst could not be attributed to inaccuracy of the method used. In this work, plants were not cultivated under stress conditions, but because strawberry plants can sense the inoculated bacteria, as demonstrated by up-regulation of the flagellin-receptor gene, it was expected that this molecular recognition would induce an oxidative burst at some point as one of the many signals activated during the plant–microorganism interaction. Thus, regarding the lack of redox signalling, we can infer that if there are any ROS generated by *Azospirillum* colonisation, enhanced levels of phenolics and activation of catalase and ascorbate peroxidase genes determined in REC3-inoculated plants would scavenge them, thus avoiding a higher oxidative burst in strawberry leaves. The fact that a high oxidative burst was not detected might support the hypothesis that the ROS scavenging effect could be a specific host mechanism that would allow *A. brasilense* to colonise plant tissues, evading activation of the plant innate immune system.

Although we did not observe ROS accumulation, we detected lipid peroxidation in leaves and roots. It was expected that there would be a fall in the content of MDA (a secondary end product of oxidative lipid degradation) in bacteria-inoculated plants due to an increase in soluble phenolics and their antioxidant action; however, this was true only during the first 48 hpi, when lipid peroxidation values of REC3-treated plants were lower than in control plants. This fall in oxidative damage (MDA) also correlates with increased gene expression of the antioxidant enzymes catalase and cytosolic ascorbate peroxidase (at 24 and 48 hpi) and the apparent absence of ROS accumulation. Our data also showed that strawberry seedlings increase their MDA content after 96 hpi in plants inoculated with *Azospirillum* as compared to the non-inoculated plants. Similarly, Ruíz-Sánchez *et al.* (2011) also observed MDA increases in rice plants inoculated with *A. brasilense* 30 days after inoculation, but they assumed that this effect was due to plant size. The increases in MDA content observed in this study could be explained considering that this may be advantageous to the plant cell from an energetic point of view, since a damaged lipid or PUFA molecule is cheaper to remove and replace than a protein molecule (Møller *et al.* 2007).

Deposition of the 1,3-β-glucan (callose) is one of the biochemical markers used to indicate that a defence mechanism is activated, as it can contribute to disease resistance through reinforcing the plant cell wall around fungal penetration sites (Kauss 1992), and is often associated with the priming effect induced by PGPB (Benhamou *et al.* 1997; Tortora *et al.* 2012). Our studies showed callose accumulation in leaves of straw-

berry plants inoculated with REC3 at 72 hpi, as reported in Tortora *et al.* (2012). This structural modification of cell walls might constitute an important mechanism of the systemic acquired resistance (SAR) and priming of plants, as reported in Kohler *et al.* (2002), who observed that pre-treatment with benzothiadiazole (BTH) or salicylic acid (SA) primes *Arabidopsis* plants and induces callose accumulation.

Another morphological trait observed here was the fortification of cell walls in plants that were inoculated with bacteria. Our results showed that *A. brasilense* REC3 induced an increase in cell wall thickening at the same time as callose deposition reached a maximum (72 hpi). In agreement with this, strawberry seedlings challenged with an avirulent fungal strain of *Colletotrichum fragariae* triggered cell wall fortification as a mechanism involved in the defence response against a virulent strain of *Colletotrichum accutatum* (Salazar *et al.* 2007). The reinforcement of cell walls observed in this study was probably produced through the crosslinking of pectin, lignin and other insoluble cell wall components, unlike callose deposition, which results in the polymerisation of sugar residues (Kauss 1992).

Even when cell walls of REC3-colonised plants were strengthened, the cell wall-bound phenolics were low at all assayed times. This unpredicted result might be explained by considering the method used for determination and the chemical nature of cell wall compounds. The technique described in Hukkanen *et al.* (2007), used in this work, is based on detection of phenolics bound with ester bonds to polysaccharides in the cell wall and not on tightly bound polymers such as lignin. The compounds determined with this method are considered to derive from ester-linked material since they were released during the first hour of hydrolysis with a weak base (1 M NaOH) and moderate temperature (70 °C); whereas lignin and other ether-linked compounds are not easily released from cell wall material and stronger hydrolysis conditions should be used for their extraction (von Röpenack *et al.* 1998; Martens 2002).

Primers for *FaGAPDH-1, FaPR1* and *FaAPXc* genes were designed using *F. ananassa* sequences, while *F. vesca* sequences (Shulaev *et al.* 2011) were used to design primers to specific genes, such as *FaCAT* and *FaFLS2*, that were not previously reported in *F. ananassa* according to Merchante *et al.* (2013).

In this work we used qRT-PCR to successfully measure expression of catalase and flagellin-receptor genes using *F. vesca* sequences as template for primer design. The data corroborate the assumption made by Merchante *et al.* (2013) based on a previous report that the diploid genome of wild strawberry *F. vesca* presents extremely high sequence identity to the octoploid genome of *F. ananassa* (Rousseau-Gueutin *et al.* 2009; Bombarely *et al.* 2010). The induction of the flagellin sensing-2 receptor, *FaFLS2,* was observed at both sampled times (24 and 48 hpi) as expected considering *A. brasilense* colonisation of roots is mediated through the flagella (Steenhoudt & Vanderleyden 2000; Guerrero-Molina *et al.* 2012); thus induction of this receptor indicates that strawberry plants do sense bacteria in their proximity. The up-regulation of catalase and ascorbate peroxidase genes, *FaCAT* and *FaAPXc*, suggests higher activity of peroxide scavengers, causing a decrease in H_2O_2 and thus avoiding an oxidative burst. We consider that recognition and signalling are activated shortly after bacterial inoculation because all the *F. ananassa* genes studied here were activated at 24 hpi. Subsequently some of these signals might be turned off in order to allow endophytic colonisation of REC3. Hence, further evidence is provided by the early induction of the defence-related genes *FaPR1* and *FaAPXc* at 24 hpi, and their subsequent down-regulation at 48 hpi, suggesting that plants repress the outset of their innate defence responses upon recognition of the PGPB strain. Also, overexpression of the tested genes at such short times after inoculation (24 h) indicates that they may be early response genes to bacteria inoculation.

Overall, the early strawberry plant responses to *Azospirillum* inoculation consist of increased levels of phenolic compounds, reduced lipid peroxidation and activation of genes related to defence (*FaPR1)*, ROS removal (*FaCAT* and *FaAPXc*) and a flagellin receptor (*FaFLS2*), while structural modifications (*e.g.* callose deposition and cell wall fortification) occurred later and can be considered as delayed responses.

In conclusion, the results presented in here indicate that the plant growth-promoting bacterium *A. brasilense* REC3 is capable of inducing beneficial traits in strawberry plants by inducing physiological, structural and molecular modifications. Since all of these signals play important roles in defence responses, they may provide strawberry plants with long-lasting and wide-spectrum resistance to pathogenic organisms, which will ultimately enhance plant health and performance.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. DAB and NBT staining of leaves of strawberry plants root-inoculated with *Azospirillum brasilense* REC3 at 12, 24, 48, 72, 96 and 120 hpi and non-inoculated controls. Positive controls for DAB were treated with 10 mM H_2O_2 for 30 min; for NBT positive reaction, they were treated with 50 mM potassium phosphate buffer (pH 7.8) containing 27 mM riboflavin and 17 mM methionine for 1 h under clear light.

Figure S2. Micrographs of callose deposits in leaves of strawberry plants root-inoculated with *Azospirillum brasilense* REC3 and control non-inoculated plants at 12, 24, 48, 72 and 96 hpi. Scale bars = 100 μm.

REFERENCES

Bashan Y., de-Bashan L.E. (2010) How the plant growth-promoting bacterium *Azospirillum* promotes plant growth – A critical assessment. *Advances in Agronomy*, **108**, 77–136.

Bashan Y., de-Bashan L.E., Prabhu S.R., Hernandez J.P. (2014) Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and Soil*, **378**, 1–33.

Benhamou N., Rey P., Chérif M., Hockenhull J., Tirilly Y. (1997) Treatment with the mycoparasite *Pythium*

oligandrum triggers induction of defense-related reactions in tomato roots when challenged with *Fusarium oxysporum* sp. *radicis-lycopersici*. *Phytopathology*, **87**, 108–122.

Bombarely A., Merchante C., Csukasi F., Cruz-Rus E., Caballero J.L., Medina-Escobar N., Blanco-Portales R., Botella M.A., Muñoz-Blanco J., Sánchez-Sevilla J.F., Valpuesta V. (2010) Generation and analysis of ESTs from strawberry (*Fragaria×ananassa*) fruits and evaluation of their utility in genetic and molecular studies. *BMC Genomics*, **11**, 503.

Boudet A.M. (2007) Evolution and current status of research in phenolic compounds. *Phytochemistry*, **68**, 2722–2735.

Currier H., Strugger S. (1956) Aniline blue and fluorescence microscopy of callose in bulb scales of *Allium L. Protoplasma*, **45**, 552–559.

Epstein E. (1972) *Mineral nutrition of plants: principles and perspectives*. Wiley, New York, NY, USA.

FAOSTAT (2014) Available from http://faostat3.fao.org/faostat-gateway (accessed 25 March 2014).

Foyer C.H., Noctor G. (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell*, **17**, 1866–1875.

Foyer C.H., Noctor G. (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxidants and Redox Signaling*, **11**, 861–905.

Grellet-Bournonville C.F., Díaz-Ricci J.C. (2011) Quantitative determination of superoxide in plant leaves using a modified NBT staining method. *Phytochemical Analysis*, **22**, 268–271.

Guerrero-Molina M.F., Winik B.C., Pedraza R.O. (2012) More than rhizosphere colonization of strawberry plants by *Azospirillum brasilense*. *Applied Soil Ecology*, **61**, 205–212.

Guerrero-Molina M.F., Lovaisa N.C., Salazar S.M., Díaz-Ricci J.C., Pedraza R.O. (2014) Elemental composition of strawberry plants inoculated with the plant growth-promoting bacterium *Azospirillum brasilense* REC3, assessed with scanning electron microscopy and energy dispersive X-ray analysis. *Plant Biology*, **16**, 726–731.

Hahlbrock K., Scheel D. (1989) Physiology and molecular biology of phenylpropanoid metabolism. *Annual Reviews Plant Physiology and Plant Molecular Biology*, **40**, 347–369.

Hodges D.M., DeLong J.M., Forney C.F., Prange R.K. (1999) Improving the thiobarbituric acid-reactive substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta*, **207**, 604–611.

Huda-Faujan N., Noriham A., Norrakiah A., Babji A. (2009) Antioxidant activity of plant extracts containing phenolic compounds. *African Journal of Biotechnology*, **8**, 484–489.

Hukkanen A.T., Kokko H.I., Buchala A.J., McDougall G.J., Stewart D., Kärenlampi S.O., Karjalainen R.O. (2007) Benzothiadiazole induces the accumulation of phenolics and improves resistance to powdery mildew in strawberries. *Journal of Agricultural and Food Chemistry*, **55**, 1862–1870.

Kähkönen M.P., Hopia A.I., Vuorela H.J., Rauha J.P., Pihlaja K., Kujala T.S., Heinonen M. (1999) Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry*, **47**, 3954–3962.

Kauss H. (1992) Callose and callose synthase. In: Gurr S.J., McPherson M.J., Bowles D.J. (Eds), *Molecular Plant Pathology*, Oxford University Press, Oxford, UK, Vol. 2, pp. 1–8.

Kohler A., Schwindling S., Conrath U. (2002) Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the *NPR1/NIM1* gene in *Arabidopsis*. *Plant Physiology*, **128**, 1046–1056.

Lavania M., Singh Chauhan P., Chauhan S.V.S., Singh H.B., Nautiyal C.S. (2006) Induction of plant defense enzymes and phenolics by treatment with plant growth-promoting rhizobacteria *Serratia marcescens* NBRI1213. *Current Microbiology*, **52**, 363–368.

Livak K.J., Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$. *Methods*, **25**, 402–408.

Martens D.A. (2002) Identification of phenolic acid composition of alkali-extracted plants and soils. *Soil Science Society of America Journal*, **66**, 1240–1248.

Merchante C., Vallarino J.G., Osorio S., Aragüez I., Villarreal N., Ariza M.T., Martínez G.A., Medina-Escobar N., Civello M.P., Fernie A.R., Botella M.A., Valpuesta V. (2013) Ethylene is involved in strawberry fruit ripening in an organ-specific manner. *Journal of Experimental Botany*, **64**, 4421–4439.

Mittler R., Vanderauwera S., Gollery M., Van Breusegem F. (2004) Reactive oxygen gene network of plants. *Trends in Plant Science*, **9**, 490–498.

Møller I.M., Jensen P.E., Hansson A. (2007) Oxidative modifications to cellular components in plants. *Annual Review of Plant Biology*, **58**, 459–481.

Pedraza R.O., Motok J., Tortora M.L., Salazar S.M., Díaz-Ricci J.C. (2007) Natural occurrence of *Azospirillum brasilense* in strawberry plants. *Plant and Soil*, **295**, 169–178.

Pedraza R.O., Motok J., Salazar S.M., Ragout A.L., Mentel M.I., Tortora M.L., Guerrero-Molina M.F., Winik B.C., Díaz-Ricci J.C. (2010) Growth-promotion of strawberry plants inoculated with *Azospirillum brasilense*. *World Journal of Microbiology and Biotechnology*, **26**, 265–272.

Pieterse C.M.J., Leon-Reyes A., Van der Ent S., Van Wees S.C.M. (2009) Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology*, **5**, 308–316.

von Röpenack E., Parr A., Schulze-Lefert P. (1998) Structural analyses and dynamics of soluble and cell wall-bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. *Journal of Biological Chemistry*, **273**, 9013–9022.

Rousseau-Gueutin M., Gaston A., Aïnouche A., Aïnouche M.L., Olbricht K., Staudt G., Richard L., Denoyes-Rothan B. (2009) Tracking the evolutionary history of polyploidy in *Fragaria* L. (strawberry): new insights from phylogenetic analyses of low-copy nuclear genes. *Molecular Phylogenetics and Evolution*, **51**, 515–530.

Ruíz-Sánchez M., Armada E., Muñoz Y., García de Salamone I.E., Aroca R., Ruíz-Lozano J.M., Azcón R. (2011) *Azospirillum* and arbuscular mycorrhizal colonization enhance rice growth and physiological traits under well-watered and drought conditions. *Journal of Plant Physiology*, **168**, 1031–1037.

Salazar S.M., Castagnaro A.P., Arias M.E., Chalfoun N.R., Tonello U., Díaz-Ricci J.C. (2007) Induction of a defense response in strawberry mediated by an avirulent strain of *Colletotrichum*. *European Journal of Plant Pathology*, **117**, 109–122.

Shulaev V., Sargent D.J., Crowhurst R.N., Mockler T.C., Folkerts O., Delcher A.L., Jaiswal P., Mockaitis K., Liston A., Mane S.P., Burns P., Davis T.M., Slovin J.P., Bassil N., Hellens R.P., Evans C., Harkins T., Kodira C., Desany B., Crasta O.R., Jensen R.V., Allan A.C., Michael T.P., Setubal J.C., Celton J.-M., Rees D.J.G., Williams K.P., Holt S.H., Ruiz Rojas J.J., Chatterjee M., Liu B., Silva H., Meisel L., Adato A., Filichkin S.A., Troggio M., Viola R., Ashman T.-L., Wang H., Dharmawardhana P., Elser J., Raja R., Priest H.D., Bryant D.W. Jr, Fox S.E., Givan S.A., Wilhelm L.J., Naithani S., Christoffels A., Salama D.Y., Carter J., Lopez Girona E., Zdepski A., Wang W., Kerstetter R.A., Schwab W., Korban S.S., Davik J., Monfort A., Denoyes-Rothan B., Arus P., Mittler R., Flinn B., Aharoni A., Bennetzen J.L., Salzberg S.L., Dickerman A.W., Velasco R., Borodovsky M., Veilleux R.E., Folta K.M. (2011) The genome of woodland strawberry (*Fragaria vesca*). *Nature Genetics*, **43**, 109–116.

Singleton V.L., Rossi J. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphothungstic acid reagents. *American Journal of Enology and Viticulture*, **16**, 144–158.

Steenhoudt O., Vanderleyden J. (2000) *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiology Reviews*, **24**, 487–506.

Tortora M.L., Díaz-Ricci J.C., Pedraza R.O. (2011) *Azospirillum brasilense* siderophores with antifungal activity against *Colletotrichum acutatum*. *Archives of Microbiology*, **193**, 275–286.

Tortora M.L., Díaz-Ricci J.C., Pedraza R.O. (2012) Protection of strawberry plants (*Fragaria ananassa* Duch.) against anthracnose disease induced by *Azospirillum brasilense*. *Plant and Soil*, **356**, 279–290.

Van Loon L.C., Bakker P.A.H.M., Pieterse C.M.J. (1998) Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology*, **36**, 453–483.

Van Wees S., Van der Ent S., Pieterse C.M.J. (2008) Plant immune responses triggered by beneficial microbes. *Current Opinion in Plant Biology*, **11**, 443–448.

Vargas L., de-Carvalho T.L.G., Ferreira P.C.G., Baldani V.L.D., Baldani J.I., Hemerly A.S. (2012) Early responses of rice (*Oryza sativa* L.) seedlings to inoculation with beneficial diazotrophic bacteria are dependent on plant and bacterial genotypes. *Plant and Soil*, **356**, 127–137.

Vleesschauwer D.D., Cornelis P., Höfte M. (2006) Redox-active pyocyanin secreted by *Pseudomonas aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe grisea* but enhances *Rhizoctonia solani* susceptibility in rice. *Molecular Plant-Microbe Interactions*, **19**, 1406–1419.

Zipfel C. (2008) Pattern-recognition receptors in plant innate immunity. *Current Opinion in Immunology*, **20**, 10–16.