Induced Systemic Resistance Against Citrus Canker Disease by Rhizobacteria

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ABSTRACT

Citrus canker, caused by *Xanthomonas citri* subsp. *citri*, is an important citrus disease that causes significant economic losses worldwide. All commercial citrus varieties are susceptible to citrus canker. Currently, chemical control with copper based products is the main approach to control *X. citri* subsp. *citri* dispersal and plant colonization. However, extensive use of copper compounds can result in copper-resistant strains and cause adverse effects on the environment. Alternatives to chemical control involve the activation of citrus immunity to control the disease. Here, we investigated the ability of multiple rhizobacteria to induce a systemic defense response in cultivar Duncan grapefruit. *Burkholderia territorii* strain A63, *Burkholderia metallica* strain A53, and *Pseudomonas geniculata* strain 95 were found to effectively activate plant defense and significantly reduce symptom development in leaves challenged with *X. citri* subsp. *citri*. In the priming phase, root application of *P. geniculata* induced the expression of salicylic

acid (SA)-signaling pathway marker genes (PR1, PR2, PR5, and salicylic acid carboxyl methyltransferase [SAM-SACM]). Gene expression analyses after *X. citri* subsp. *citri* challenge showed that root inoculation with *P. geniculata* strain 95 increased the relative levels of phenylalanine ammonia lyase 1 and SAM-SACM, two genes involved in the phenylpropanoid pathway as well as the biosynthesis of SA and methyl salicylate (MeSA), respectively. However, hormone analyses by UPLC-MS/MS showed no significant difference between SA in *P. geniculata*-treated plants and control plants at 8 days post-beneficial bacteria root inoculation. Moreover, *P. geniculata* root-treated plants contained higher reactive oxygen species levels in aerial tissues than control plants 8 days post-treatment application. This study demonstrates that rhizobacteria can modulate citrus immunity resulting in a systemic defense response against *X. citri* subsp. *citri* under greenhouse conditions.

Citrus canker is a globally distributed disease that affects all commercial citrus varieties. The causal agent, *Xanthomonas citri* subsp. *citri*, infects plants through stomata or wounds, multiplies in the intercellular spaces, and causes characteristic necrosis and raised lesions in fruit, leaves, and twigs (Ryan et al. 2011). Chemical control based on copper compounds is currently the most frequent treatment for this disease. However, use of copper compounds may cause phytotoxicity in the fruit peel (Graham et al. 2008) and have a negative impact on the environment (Alva et al. 1995). Specifically, prolonged use of copper may lead to copper accumulation in the soils (Zhang et al. 2003b). Copper at concentrations of >100 mg/kg can cause phytotoxicity with subsequent decreased citrus productivity (Alva 1993). In addition, copper accumulation has been reported to reduce microbial biomass and diversity in the copper affected soils (Zhou et al. 2011). Aquatic organisms are more susceptible to copper contamination than terrestrial plants (Eisler 1998), and the transport of copper from land to surface waters has been identified as an environmental issue (Zhang et al. 2003a). Copper-based compounds

have no systemic activity in the plant and the control effect relies on their multiple applications throughout the year and their protective effect decreases with heavy winds and rains (Graham and Myers 2016; Stall et al. 1980). Moreover, copper resistant *X. citri* subsp. *citri* strains have been identified in Argentina and more recently in Reunion Island (Behlau et al. 2012; Richard et al. 2016). The adverse side effect of copper-based chemical applications calls for development of other canker control strategies.

A possible alternative for chemical control is the activation of the plant immune system systemically, called “priming” (Balmer et al. 2015). Systemic resistance occurs naturally in plants upon proper stimulation by certain pathogens, nonpathogens, herbivory and/or some chemical treatments (Kuč 1982). After the stimulus is recognized, the plant immune system is systemically activated in distal aerial tissues to better protect them from future pathogen attack. In citrus, most studies on priming against biotic stresses have focused their attentions on the chemical elicitors (Francis et al. 2009; Graham and Myers 2013; Hu et al. 2018; Li et al. 2016, 2017). For instance, acibenzolar-S-methyl (ASM), isonicotinic acid (INA), and DL-3-aminobutyric acid (BABA) have been evaluated for their protective effect against citrus canker, *Diaphorina citri* development, and Huanglongbing disease (Francis et al. 2009; Graham and Myers 2013; Li et al. 2016; Tiwari et al. 2013). Application of ASM as well as INA as a soil drench resulted in reduced disease development in small plants inoculated with *X. citri* subsp. *citri* under greenhouse conditions (Francis et al. 2009).

The activation of systemic resistance in plants can also be triggered by plant growth promoting rhizobacteria (PGPR) or bacteria (PGPB) (van Loon et al. 1998). Different PGPR strains

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have been reported to induce plant defense against a broad spectrum of pests including oomycetes, fungi, bacteria, insects, and nematodes (Walters and Bennett 2014). Microbial-mediated priming has been linked with differential expression of defense related genes, changes in phytohormone content, changes in reactive oxygen species (ROS) levels, sugars as well as amino acids and tricarboxylic acid (TCA) components (Balmer et al. 2015). PGPR induction of defense has been mostly associated with the jasmonate/ethylene (JA/ET) pathways of plant defense (Zamioudis and Pieterse 2012). Additionally, SA-dependent PGPR induction of resistance has been reported (Barriuso et al. 2008; Niu et al. 2016; van de Mortel et al. 2012). Use of PGPR in the field has been suggested as an alternative strategy for sustainable disease control. Different PGPR promoting a protective effect in plants have been used in field conditions as part of crop protection strategies against chili wilt disease and blast disease in rice (Lucas et al. 2009; Raguchander et al. 2012).

In this work, we investigated the effect of application of multiple PGPR in inducing plant defense against citrus canker. Three rhizobacteria, *Burkholderia territorii* strain A63, *Burkholderia metallica* strain A53, and *Pseudomonas geniculata* strain 95, were found to evoke a plant defense response against citrus canker. For *P. geniculata* strain 95, we also investigated the putative modes of action associated with the induction of defense responses against citrus canker.

MATERIALS AND METHODS

Bacterial cultures and growth conditions. Bacterial isolates were cultured in nutrient broth (NB) or on nutrient agar (NA) at 28°C for 24 h. For plant inoculation, bacterial strains were grown overnight in NB at 28°C with agitation at 200 rpm. Cells were centrifuged at 10,000 × *g* for 20 min and then suspended in sterile distilled water to a final concentration of 10⁸ CFU/ml. *X. citri* subsp. *citri* strain 306 (Rybak et al. 2009) was grown in NB or on NA medium at 28°C. Seven bacterial strains were selected to screen for their ability to induce systemic resistance in cultivar Duncan grapefruit against *X. citri* subsp. *citri*. The bacterial isolates were previously isolated from the rhizosphere of citrus escape trees (healthy looking trees in severely Huanglongbing [HLB]-diseased groves) in Florida (Table 1).

Plant materials and treatments. For disease severity studies, 2-year-old Duncan grapefruit plants were used for *X. citri* subsp. *citri* inoculation or PGPR treatments. Plants were grown in small pots of 500 ml with sterile soil. All plants were selected with the same age and with leaves of similar growth stage. Experiments were performed in a quarantine greenhouse facility (Citrus Research and Education Center, Lake Alfred, FL) under controlled temperature (28 to 35°C) and relative humidity of 80%.

Five treatments were applied as soil drench to the root system 7 days before pathogen challenge in the leaves. The five treatments consisted in 15 ml of suspension of (i) *P. geniculata* strain 95 (10⁸ CFU/ml), (ii) *B. territorii* strain A63 (10⁸ CFU/ml), (iii) *B. metallica* strain A53 (10⁸ CFU/ml), (iv) 5 mg of acibenzolar-S-methyl (ASM) (active ingredient of Actigard 50WG in the United States and Bion in Europe), and (v) sterile distilled water as a negative control. ASM is a functional analog of SA and was used as a positive control of plant defense activation (Francis et al. 2009; Graham and Leite 2004). The experiment was repeated in January

2015 with five plants per treatment and in May 2015 with seven plants. Data from all 12 plants were combined for statistical analyses.

Eight-month-old Duncan grapefruit plants grown in 500-ml pots in sterile soil were used for gene expression analyses. Three treatments were applied in the same way as for disease severity, consisting of (i) *P. geniculata*, (ii) ASM, and (iii) sterile distilled water-inoculated plants. Five plants were inoculated per treatment during October 2017.

Reactive oxygen species and hormone analyses were conducted with 12 2-year-old Duncan grapefruit plants in October 2015. Treatments and soil drench application were conducted as described for gene expression analyses.

***X. citri* subsp. *citri* inoculation and disease severity estimation.** Duncan grapefruit plants were challenged with *X. citri* subsp. *citri* strain 306 (10⁸ CFU/ml) by spray inoculation post-root treatment with PGPR. Plants used for disease severity estimation and relative gene expression were challenge at day 7. For each plant, at least three fully expanded immature leaves were labeled and sprayed until fully covered with bacterial suspension. Plants were then covered with a plastic bag for 24 h to maintain humidity. Symptoms were monitored at 6 weeks post-pathogen inoculation. Labeled leaves were collected and pictures were taken immediately. Pictures were uniformly taken by setting the camera (Nikon D80) on a tripod and using a blank background at 30 cm. To estimate disease severity, pictures were used for manual lesion count and image processing analysis.

Image processing analysis. The normalized area of disease was estimated using ImageJ (version 1.49). Recorded images from individual leaves were transformed to the HSB color space and further analyzed using two different macros. In the first step, the background was subtracted using the saturation channel and total leaf area was recorded. Secondly, two masks were applied to the hue channel, around the yellow color to specifically identify the lesions. Finally, the total area of lesions was recorded. The normalized area of disease per leaf was calculated as follows: Normalized area = Area_{lesions}/Area_{leaf}.

RNA extraction and relative gene expression analysis. One leaf was collected for gene expression analysis from each of five different plants at the following time points: 3, 5, 8, and 10 days post-treatment application in the root system. Days 8 and 10 represent the plant response after pathogen challenge at day 7. Individual leaves were then ground in liquid nitrogen using a mortar and pestle and then 100 mg of leaf samples was used for RNA extraction. RNA was extracted using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. Samples were then treated with Ambion DNA-free DNase Treatment and Removal Reagents. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), the final concentration was normalized to 50 ng/μl for quantitative reverse-transcription (qRT-PCR) analysis.

Relative expression of eleven defense-related genes was determined by qRT-PCR. The primer sequences are presented in Table 2. The housekeeping gene encoding a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control as previously described (Francis et al. 2009). qRT-PCR was performed using Verso 1-step RT-qPCR Kit (ThermoFisher). The relative fold change of target gene expression in *P. geniculata*- or

TABLE 1. Beneficial bacteria used for screening of induced systemic resistance ability

Strain name	Origin	Host plant	Isolation year	Source or reference
<i>Burkholderia territorii</i> strain A63	Root-associated bacteria	Cleopatra mandarin	2013	(Riera et al. 2017)
<i>Burkholderia metallica</i> strain A53	Root-associated bacteria	Cleopatra mandarin	2013	(Riera et al. 2017)
<i>Pseudomonas geniculata</i> strain 95	Root-associated bacteria	Valencia orange	2010	(Trivedi et al. 2011)
<i>Rhodococcus jialingiae</i> strain 108	Root-associated bacteria	Valencia orange	2010	(Trivedi et al. 2011)
<i>Bacillus pumilus</i> strain 104	Root-associated bacteria	Valencia orange	2010	(Trivedi et al. 2011)
<i>Pseudomonas granadensis</i> strain 100	Root-associated bacteria	Valencia orange	2010	(Trivedi et al. 2011)

ASM-treated plants was calculated and normalized against its expression levels in water-treated plants, using the $\Delta\Delta C_t$ method as previously described (Livak and Schmittgen 2001).

Plant hormones quantification and ROS determination. Plant hormones were quantified in the Duncan grapefruit plants at 0, 3, and 8 days post-treatment application in the root system. Three leaves from different plants were pooled together as one repeat and four repeats were tested. Plant tissue was kept in liquid nitrogen immediately after detaching from the plants. Tissue was ground into powder in a mortar and pestle in liquid nitrogen. The frozen powder was weighed and 100 mg of frozen powder was kept in 2-ml screw-cap tubes. Samples were kept in -80°C for further analyses.

Hormones were extracted as previously described (Pan et al. 2010) and samples were cleaned up using solid phase extraction cartridge and quantified using UPLC-MS/MS. For phytohormone extraction and quantification the internal standard mixture working solution (d_2 -IAA, d_6 -ABA, d_6 -SA, and d_5 -JA) was added to each 2-ml tube containing the frozen plant tissue powder. Then, 500 μl of extraction solvent was added (2-propanol/ H_2O /concentrated HCl [2:1:0.002, vol/vol/vol]). The samples were shaken at a speed of 100 rpm at 4°C in a cold room for 30 min. Finally, 1 ml of dichloromethane was added to the tubes and the tubes were further shaken at 4°C at 100 rpm for 30 min. Samples were then centrifuged at $13,000 \times g$ for 10 min at 4°C . The lower phase solvent was transferred to a screw-cap tube and gently evaporated with nitrogen flow. Concentrated samples were dissolved in 1 ml of methanol. Samples were cleaned in a solid phase extraction column (Oasis HLB 1 cc 30 mg extraction cartridge, waters). Cartridges were equilibrated with 1 ml water and loaded with 1 ml of sample. Samples were eluted with 1 ml methanol and evaporated with nitrogen flow. Concentrated samples were dissolved in methanol and filtered through 0.2 μm filter before analysis. For each sample, 50 μl of sample solution was injected into the reverse-phase C_{18} HPLC column Synergi for UPLC-ESI-MS/MS analysis using an Ultimate 3000 UPLC with a TSQ Quantiva MS. For each treatment, the relative hormone levels were normalized with the mean value for day 0 prior to treatment application.

For ROS determination, the fluorescent dye DHR 123 (dihydro-rhodamine) was used as previously described with modifications

(Qin et al. 2008). Briefly, extraction buffer (50 mM Tris pH = 7.8, 5 mM EDTA, 0.2% Triton 100X, 10% glycerol, 5 mM DTT, and 2% PVPP) was added to 100 mg of ground tissue. Samples were then centrifuged at 15,000 rpm for 30 min. Following centrifugation, 900 μl of dihydrorhodamine 123 probe (dihydrorhodamine 123, Sigma-Aldrich, Inc.) was added to 100 μl of supernatant. The obtained solution was incubated at room temperature for 1 h in the dark and fluorescence was determined in three technical repeats at 488 nm excitation and 534 nm emission in a Synergy HT Multi-Mode Microplate Reader (Biotek Instruments Inc.). Each tube was kept at 60°C in a stove and dry weight was determined for further calculations.

Statistical analysis. Disease severity (normalized area of disease and lesion count) data were analyzed using R Studio (Version 0.98.1049, 2009-2013 R Studio, Inc.) by applying Dunn's test (package: dunn.test) with a P value of 0.05 or less. Hormone levels data were analyzed with R Studio using analysis of variance and Tukey's honest significant difference test posthoc with a confidence interval of 95% and a P value of 0.05 or less (ANOVA, Tukey's HSD). ROS production on leaves data were analyzed with Microsoft Excel using Student's t tests on all pairs with a confidence interval of 95% and $P < 0.05$.

RESULTS

Bacteria application in the root reduced citrus canker development. To screen for beneficial bacteria capable of activating citrus immunity, we inoculated seven bacterial isolates (Table 1) in the root system via soil drench and allowed the plant to respond for 7 days. After 1 week, young leaves of Duncan grapefruit plants were spray inoculated with *X. citri* subsp. *citri* strain 306. In addition to bacterial treatments we included the chemical treatment ASM as a positive control of defense activation. ASM has been previously reported to induce citrus systemic resistance against canker (Francis et al. 2009).

Disease severity was quantified using two independent measurements: (i) by counting the number of lesions per leaf and (ii) by estimating the total area of disease. Estimation of lesion count when the pathogen is sprayed on the leaves has two major limitations: the

TABLE 2. Primers used for expression analysis of plant defense genes

Short name	Protein encoded	Primers (5'-3')	Reference	Proposed pathway
HPL1	Fatty acid hydroperoxide lyase	F-AGTGTGCCGGAAGGATTAC	This study	Production of C6 volatiles or green leaf volatiles, derived from LOX pathway
GPX	Phospholipid hydroperoxide glutathione peroxidase	R-GCCGTGATCGAAGATGAGTT F-TGTGCAAGTCGTTACCTTCTTA	This study	ROS response
NPR1	Transcriptional regulator of defense genes	R-TCTCAACAGGGTTTGCTTACA F-GTAGGCCGGCTGTTGATTT	This study	SA and JA/ET pathway
MYC	Transcriptional regulator MYC2	R-GTCTAGGAGGTGCCTCTGATAA F-GCGATGGGTATTACAAAGGAGA	This study	JA/ET pathway
PR1	Pathogenesis-related protein 1	R-TTCGCGCAGTACCTTCTTAC F-CAGGGTCTCCAAGCAACTATG	(Zhang et al. 2017)	SA pathway
PR2	Pathogenesis-related protein 2	R-CCACCTCGCGTATTCTCTAA F-TTCCACTGCCATCGAAACTG	(Francis et al. 2009)	SA pathway
PR5	Pathogenesis-related protein 5	R-TGTAATCTTGTTAAATGAGCCTCTTG F-CACCATTGCCAATAACCCCTAATG	This study	SA pathway
PAL	Phenylalanine ammonia lyase	R-GGGACAGTTACCGTTAAGATCAG F-GGAGGGTTTGTGCAAGAATAAC	This study	SA and JA/ET pathway
SAM-SACM	S-adenosyl-L-methionine-salicylic acid carboxyl methyltransferase	R-CACCATAACGCTTCACTTCCT F-GGACGCATCTTCTTGGGATAA	(Zhang et al. 2017)	MeSA synthesis
MPK4	Mitogen-activated protein kinase 4	R-CGTGACAGTTTCCTTGACGA F-GTTGGTTGCATACTTGGTGAAA	This study	SA pathway
CAT	Catalase	R-GCATCATCGGGAGAACCTATT F-GGAAACCAACTTGTGGAGTTAAG	This study	
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	R-TGAGTCGTAGAGATCCTGAGTAG F-GGAAGGTCAAGATCGGAATCAA	(Francis et al. 2009)	Housekeeping gene
		R-CGTCCCTCTGCAAGATGACTCT		

variability of the lesion size in different leaves and the aggregation of some lesions that makes it hard to discriminate individual counts. To improve this estimation, we used the same pictures and estimated the area of disease with image processing analysis using ImageJ software. According to both measurements, three bacterial strains, *B. territorii* strain A63, *B. metallica* strain A53, and *P. geniculata* strain 95, significantly reduced the canker disease severity in Duncan grapefruit (Fig. 1B and C). No significant differences were observed in plants inoculated with *P. granadensis* strain 100, *Rhodococcus jialingiae* strain 108, or *Bacillus pumilus* strain 104 compared with nontreated control plants (data not shown).

Beneficial bacteria trigger changes in expression of defense-related genes. Activation of citrus systemic immunity has been linked to the overexpression of certain defense-related genes (Distefano et al. 2008; Dutt et al. 2015; Francis et al. 2009; Li et al. 2016). We evaluated if the application of beneficial bacteria in the root system could activate expression of defense-related genes systemically. We collected leaf tissues during the first week after beneficial bacteria inoculation (days 3 and 5) prior to *X. citri* subsp. *citri* challenge and after (days 8 and 10). We monitored the expression of 11 selected genes and compared the relative expression to nontreated plants. The expression of ASM-treated plants was also included as a positive control of plant defense activation. Among

the selected genes we selected markers from both SA and lipoxygenase pathways of plant immunity (Table 2).

Since both *Burkholderia* strains used in this study belong to the *B. cepacia* complex (BCC), a group with known opportunistic human pathogens, we decided to focus on *P. geniculata* for further study. Bacterial treatments with *P. geniculata* strain 95 and the chemical ASM caused differential expression of defense-related genes from nontreated control plants. The bacterial treatment with *P. geniculata* strain 95 up-regulated the expression of the salicylic acid carboxyl methyltransferase (SAM-SACM) and the pathogenesis-related proteins 1, 2, and 5 (PR1, PR2, and PR5) during the priming phase (days 3 and 5) (Table 3). Similarly, ASM application up-regulated the expression of PR2 and PR5 in the plants before pathogen challenge (Table 3). Specifically, *P. geniculata* strain 95 induced stronger gene expression of SAM-SACM and PR1 than ASM during the priming phase (days 3 and 5). PR proteins and SAM-SACM are normally related to the activation of the SA pathway. In addition, MAPK4 levels were down-regulated at day 8 for ASM (0.53 ± 0.097) and *P. geniculata* (0.42 ± 0.22) treated plants. MAPK4 is a negative regulator of SA pathway (Loake and Grant 2007), further suggesting that a SA-mediated response is being activated. Additionally, we found SAM-SACM with a 25-fold increase in ASM-treated plants at day 10 post-treatment application. The enzyme is involved in the synthesis of methyl salicylate, an important signaling molecule in

Fig. 1. Beneficial bacteria reduce citrus canker caused by *Xanthomonas citri* subsp. *citri*. **A**, Effect of beneficial bacteria soil drench treatment of cultivar Duncan grapefruit plants on canker development. **B**, Boxplot represents disease severity of Duncan grapefruit at 6 weeks post-*X. citri* subsp. *citri* spray inoculation. Severity was estimated by counting the number of lesions of at least three different leaves per plant. Boxes represent data from all individual leaves by combining the two repeats from a total of 12 plants. **C**, Boxplot represents the mean area of disease relative to total leaf area (described in Methods) for individual leaves from the same 12 plants combining the two repeats. Dots above the boxes are outliers calculated by default using ggplot2 package. Statistical differences (data represents at least three leaves collected from 12 plants, $P < 0.05$, Dunn's test) were observed for *Pseudomonas geniculata* strain 95 (95), *Burkholderia territorii* strain A63 (A63), *Burkholderia metallica* strain A53 (A53), and acibenzolar-S-methyl (ASM; Actigard)-treated plants.

plant defense (Park et al. 2007). After spray inoculation with the pathogen at day 8, *P. geniculata* strain 95-treated plants showed increased levels of phenylalanine ammonia lyase 1 (PAL) and SAM-SACM genes. Higher expression of SAM-SACM and PR2 was also observed in the ASM-treated plants during the post-*X. citri* subsp. *citri* inoculation phase.

***P. geniculata* modulates hormone and ROS levels in grapefruit.** Plants stimulated with a proper signal enter a different physiological state named priming phase (Balmer et al. 2015). In this state, plants can respond faster to pathogen attack. We investigated two putative mechanisms involved in plant defense activation: the production of reactive oxygen species and hormone in the leaf tissue. In both cases, we studied the aerial response of *P. geniculata*-inoculated plants during the 1-week period post-root stimulation. For hormone and ROS analyses, we included ASM-treated plants in the comparison. We observed that plants inoculated with *P. geniculata* had significantly higher levels of ROS than nontreated control plants at 8 days post-beneficial bacteria root inoculation (Fig. 2). Plants inoculated with ASM showed similar ROS levels with those treated by *P. geniculata*, but not significantly higher than the control at 8 days post-treatment inoculation. However, the ASM-treated plants had significantly higher levels of ROS than the control at 5 days post-treatment application (Fig. 2).

Nontreated control plants contained lower levels of IAA compared with plants treated with ASM or *P. geniculata* at 3 and 8 days after treatment (Fig. 3). The levels of ABA at 8 days post-stimuli application were significantly lower in *P. geniculata*-treated plants but significantly higher in ASM-treated plants than nontreated control plants. The plants treated with ASM showed lower levels of SA 3 days post-application than nontreated control plants. SA is the substrate for the S-adenosyl-L-methionine-salicylic acid carboxyl methyltransferase (SAM-SACM) an enzyme that converts the hormone in its conjugate form methyl salicylate (MeSA).

DISCUSSION

Inducing systemic plant defense response by beneficial bacteria in citrus plants remains largely unexplored. Agostini et al. (2003) evaluated the effect of Serenade (biological fungicide based on *Bacillus subtilis* strain QST 713) inoculation in reducing disease severity of citrus scab on rough lemon, melanose on grapefruit, and Alternaria brown spot in mandarin under greenhouse conditions (Agostini et al. 2003). However, it is unclear whether the control effect was due to the activation of systemic resistance or direct antagonism of the bacterial strain. In this study, we inoculated the beneficial bacteria in the root system as soil drench and evaluated the effect on leaf pathogen *X. citri* subsp. *citri* to avoid any direct competition between beneficial and pathogenic bacteria. Treatments with *B. metallica*, *B. territorii*, and *P. geniculata* have

significantly reduced canker development on Duncan grapefruit plants, suggesting an activation of plant defense responses.

The expression profile of Duncan grapefruit plants changed upon root stimulation with the beneficial bacteria *P. geniculata* as well as the chemical elicitor ASM. *P. geniculata* stimulated an up-regulation of SAM-SACM and PR proteins genes. PR1, PR2, and PR5 are usually considered as markers associated with SA signaling pathway. In this work, the relative gene expression of PAL and SAM-SACM genes increased after pathogen challenge (day 8) in *P. geniculata*-treated plants 4.8 and 2.5 times compared with control plants. For ASM-treated plants in the post-challenge prime state at day 10, SAM-SACM and PR2 genes were up-regulated 25.8 and 3.7 times, respectively. The increased relative levels of SAM-SACM, PAL, or PR2 transcripts post-pathogen inoculation suggest that the priming state is achieved by ASM and *P. geniculata* root application. In comparison, the changes in gene expression after pathogen challenge in *P. geniculata*-treated plants was less than that observed for ASM-treated plants at 8 and 10 days. It is worth noting that changes in disease severity were comparable 6 weeks post-*X. citri* subsp. *citri* challenge, suggesting that bacteria application may be slower but as effective as that induced by the SA analog ASM. A possible explanation could be the ability of rhizospheric bacteria to proliferate in the rhizosphere in contrast with a chemical compound (such as ASM). The relative expression of PAL gene was

Fig. 2. Reactive oxygen species (ROS) levels in leaves treated with *Pseudomonas geniculata* strain 95, acibenzolar-S-methyl (ASM), or nontreated control. The three facets represent ROS levels at days 3, 5, and 8 per root treatment application using DHR123 probe. Bars represent the mean relative fluorescence value and error bars (standard error). Letters indicate significant differences between treatments ($n = 4$, Student's t tests on all pairs with a 95% confidence interval and $P < 0.05$).

TABLE 3. The relative gene expression of target genes in *Pseudomonas geniculata* or acibenzolar-S-methyl (ASM)-treated grapefruit plants^z

Gene	Day 3		Day 5		Day 8		Day 10	
	<i>P. geniculata</i>	ASM	<i>P. geniculata</i>	ASM	<i>P. geniculata</i>	ASM	<i>P. geniculata</i>	ASM
CAT	1.27(0.225) a	1.11(0.068) a	1.12(0.096) b	1.51(0.132) a	0.75(0.0863) b	1.19(0.068) a	0.53(0.120) b	0.82(0.090) a
GPX	1.01(0.239) a	0.61(0.038) b	0.56(0.065) b	0.78(0.106) a	0.58(0.096) b	0.85(0.059) a	0.77(0.070) b	1.06(0.039) a
MPK4	1.29(0.318) a	0.75(0.120) b	0.88(0.279) a	0.59(0.075) a	0.42(0.220) a	0.53(0.097) a	0.81(0.189) a	0.74(0.132) a
MYC	1.31(0.332) a	0.69(0.135) b	1.46(0.092) a	0.58(0.146) b	0.98(0.241) a	0.80(0.101) a	0.83(0.047) a	0.86(0.066) a
NPR1	1.05(0.127) a	1.06(0.020) a	0.94(0.232) a	1.21(0.082) a	0.55(0.041) b	0.67(0.062) a	0.87(0.220) b	1.69(0.103) a
SAM-SACM	2.82(0.206) a	2.50(0.098) a	2.09(0.234) a	1.42(0.157) b	2.50(0.235) a	0.53(0.081) b	1.46(0.341) b	25.84(0.269) a
HPL	0.47(0.210) b	0.85(0.018) a	0.98(0.176) a	1.24(0.399) a	0.67(0.235) b	1.85(0.254) a	1.46(0.100) a	1.51(0.085) a
PAL	1.66(0.124) a	0.75(0.038) b	0.82(0.107) a	0.22(0.147) b	4.79(0.175) a	0.61(0.049) b	0.75(0.062) a	0.64(0.029) a
PR1	2.22(0.167) a	1.08(0.027) b	2.29(0.118) a	0.24(0.270) b	0.32(0.079) a	0.45(0.121) a	1.15(0.054) a	0.50(0.064) b
PR2	2.14(0.049) b	4.24(0.141) a	1.83(0.141) b	6.06(0.132) a	1.94(0.185) b	4.11(0.095) a	1.55(0.132) b	3.72(0.099) a
PR5	2.78(0.380) a	2.18(0.250) a	0.29(0.011) a	0.05(0.010) b	0.34(0.030) a	0.27(0.023) a	1.59(0.052) a	0.27(0.036) b

^z The relative fold change of target gene expression in *P. geniculata*- or ASM-treated plants was calculated and normalized against its expression levels in water-treated plants. Values were shown as mean fold change (standard error) ($n = 5$). Different letters in the same row indicate significant differences between treatments at the same time points (Student's t test, $P < 0.05$). Time points selected were before (days 3 and 5) and after (days 8 and 10) pathogen challenge.

4.8-fold upregulated at 8 days post-treatment with *P. geniculata*. The PAL gene encodes a phenylalanine ammonia lyase, which is the entry point enzyme in the phenylpropanoid pathway. Phenylpropanoids play a key role in the protection of plants against biotic and abiotic stress (Mierziak et al. 2014). For example, PAL catalyzes the production of *trans*-cinnamic acid from L-phenylalanine (Hahlbrock and Scheel 1989). *Trans*-cinnamic acid is a precursor in the phenylpropanoid pathways of plant defense and is believed to be activated by JA/ET signaling pathway (Diallinas and Kanellis 1994). In addition, *trans*-cinnamic acid can also serve as a precursor in the synthesis of SA, and it has been reported to have a role in the accumulation of SA in response to pathogen recognition in some plants (Mauch-Mani and Slusarenko 1996; Pallas et al. 1996; Shine et al. 2016). It has long been considered that SA accumulation upon biotic and abiotic stress in plants was produced mainly through the isochlorismate synthase pathway. In citrus, how much each pathway contributes to total SA remains unknown (Catinot et al. 2008; Garcion et al. 2008; Wildermuth et al. 2001). However, we did not observe major changes in SA content in treated versus nontreated plants. The mechanisms by which beneficial bacteria activate systemic resistance in plants are diverse. Some bacteria are able to induce systemic resistance through activating the SA-dependent defense pathway (van de Mortel et al. 2012), some through the ET/JA signaling pathway (Pieterse et al. 1998), whereas some bacteria induce both (Niu et al. 2011). Recently, the induced systemic resistance (ISR)-eliciting beneficial strain *Bacillus cereus* AR156 has been shown to induce PR1 protein expression through SA-pathway through NPR1, inducing ROS levels compared with nontreated plants (Niu et al. 2016). In our study, we did not find any change of NPR1 relative expression in beneficial bacteria or ASM-treated plants.

After the recognition of a proper priming stimulus, plants undergo physiological changes that allow for the stronger and faster defense response upon pathogen challenge (reviewed in Balmer et al. 2015). Typically, induction of the physiological state involves changes in primary metabolism including sugars, amino acids, tricarboxylic acid metabolites as well as a burst in ROS and up-regulation of defense related genes (Balmer et al. 2015; Pastor et al. 2014). ROS species are produced as part of the plant defense response. In SA-dependent defense, it has been proposed that ROS is involved in the production of AzA, a signal molecule associated with triggering the systemic acquired resistance (SAR) response (Wang et al. 2014; Wendehenne et al. 2014). We observed that root application of ASM and *P. geniculata* in grapefruit plants results in an increase in ROS levels in aerial tissues compared with nontreated control plants. Activation of ROS by beneficial bacteria has been reported in *Nicotiana tabacum* agroinfiltrated with *Agrobacterium tumefaciens* GV3101 and in barley treated with *P. fluorescens*. ROS pathway was proposed in both cases to be part of the plant defense mechanism resulting in defense activation against *P. syringae* and *Fusarium culmorum* (Petti et al. 2010; Sheikh et al. 2014).

P. geniculata application on grapefruit roots altered the levels of plant hormones in the leaves. *P. geniculata* application resulted in an increase of IAA compared with noninoculated control plants. IAA accumulation was also reported in *Arabidopsis thaliana* treated with *P. syringae* (Pastor et al. 2014). In ASM-treated plants, we observed that the IAA levels decreased at day 3 but significantly lesser than the decrease observed in nontreated control plants. IAA levels in priming events have been associated with changes in primary metabolism and tryptophan pathway. Additionally, we found that ABA levels were significantly lower in grapefruit at

Fig. 3. Boxplot with the normalized hormone content in leaf tissue of cultivar Duncan grapefruit plants treated with *Pseudomonas geniculata* strain 95 (95), acibenzolar-S-methyl (ASM; Actigard) and nontreated control. Hormones were extracted and quantified by UPLC-ESI-MS/MS analysis at days 0, 3, and 8 per root treatment application. Dots represent outliers calculated by default with ggplot2 package. Samples were analyzed with an analysis of variance test followed with a Tukey honest significant differences posthoc (letters indicate a *P* value <0.05, *n* = 4, confidence interval 95%).

8 days post-*P. geniculata* inoculation. In contrast, ABA levels showed an opposite effect in ASM-treated plants compared with the nontreated counterparts. ABA is a key stress hormone in plants that has a dual activity in plant–pathogen interactions that range from promoting resistance to increasing susceptibility. ABA may have an active role in the early stages of defense but a suppressive role in late stages. Overall, it has been proposed that the effect of ABA in defense against bacterial pathogens is mostly negative (Ton et al. 2009). For instance, callose deposition, an important defense mechanism in plants, was shown to be suppressed by ABA in *A. thaliana* (Clay et al. 2009). *P. geniculata* inoculation in grapefruit plants results in over expression of PR-protein and SAM-SACM genes which are dependent on the SA pathway. However, we noted that the levels of SA were not significantly different from those of control plants. The same pattern was observed for ASM-treated plants, with a decrease in SA observed at 3 days post-treatment application. Additionally, the increased transcript levels of SAM-SACM in ASM-treated plants at 3 days may suggest that the observed decrease in SA may be due to the production of MeSA, an important systemic signal in plant immune response (Park et al. 2007). However, results are not strictly comparable and should be treated with caution because gene expression data were from plants aged 8 months while hormonal analyses was from plants aged 2 years.

The plant response evoked in the initial stages of priming is thought to be mild compared with the post-challenge phase after pathogen encounter (Balmer et al. 2015). *P. putida* KT2440 inoculation in the roots evokes few changes in gene expression in both local and distal tissues in maize (Planchamp et al. 2015). Similarly, application of the ISR-eliciting strain *Trichoderma asperellum* in *Arabidopsis thaliana* roots results in very few changes in the priming phase. Based on the transcriptomic profiling using qRT-PCR, only eight genes were significantly different out of 137 tested defense-related genes compared with nontreated control plants (Brotman et al. 2012). Here, we show that some root-associated bacteria are able to change citrus defense response by a series of physiological changes and that these can be effective against *X. citri* subsp. *citri*. Based on the current results, our selected ISR-eliciting strains could be triggering SA pathway of plant defense. Based on our current data, the activation of ET/JA pathway cannot be excluded as PAL1 may be activated by this pathway in the context of induced plant defense (Diallinas and Kanellis 1994). Further studies should be made to confirm whether ET/JA pathway is actually involved. In light of these results, it would be worth exploring the role of beneficial bacteria inoculation in other diseases caused by other bacteria and pathogenic fungi. Because of the lack of literature regarding citrus systemic resistance induced by beneficial bacteria, this work presents a first step in a largely unexplored field.

Conclusions. In this study, the ability of rhizospheric beneficial bacteria to activate citrus plant immunity and prime them against canker was explored. Three bacteria, *B. metallica* strain A53, *B. territorii* strain A63, and *P. geniculata* strain 95, were able to significantly decrease canker symptoms of Duncan grapefruit when inoculated via soil drench. *P. geniculata* inoculation induces changes of expression of defense-related genes, phytohormones IAA and ABA, and ROS levels, suggesting that a physiological effect is induced. Due to the adverse environmental effects of chemical control of citrus canker, the exploitation of environmentally friendly strategies represents an attractive approach to develop new control measures. The bacteria presented in this study could be potentially used to manage citrus canker disease and other bacterial diseases.

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