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Soil application of imidacloprid and related SAR-inducing compounds produces effective and persistent control of citrus canker

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Abstract Soil application of the systemic insecticide imidacloprid (Admire®, Bayer Crop Science) produced season-long control of citrus canker caused by *Xanthomonas citri* sbsp. *citri*. Imidacloprid is a neonicotinoid that breaks down *in planta* into 6-chloronicotinic acid, a compound closely related to the systemic acquired resistance (SAR) inducer isonicotinic acid. Potted Swingle citrumelo seedlings (*Citrus paradisi* × *Poncirus trifoliata*) were treated with imidacloprid and the SAR inducers, isonicotinic acid, and acibenzolar-s-methyl as soil drenches or with acibenzolar-s-methyl as a foliar spray 1week prior to inoculation of immature leaves with *X. citri* sbsp. *citri*. Seedlings were re-inoculated four times over a 24-week period. SAR induction was confirmed by expression of the *PR-2* gene (β-1,3 glucanase). Soil drenches of imidacloprid, isonicotinic acid, and acibenzolar-s-methyl induced a high and persistent up-regulation of *PR-2* gene expression and reduced the number of canker lesions for up to 24 weeks compared to 4 weeks for foliar acibenzolar-s-methyl. Soil applied inducers of SAR reduced canker lesions up to 70% compared with the untreated inoculated plants. Lesions on leaves were small, necrotic, and flat compared to pustular lesions on inoculated untreated plants. Populations of *X. citri* sbsp. *citri* per leaf were reduced 1–3 log units in soil-treated plants compared to inoculated untreated plants.

Keywords Gene expression · Pathogenicity-related proteins · *Xanthomonas citri*

Abbreviations

ASM	acibenzolar-s-methyl
Imid	imidacloprid
INA	isonicotinic acid
PR-2	pathogenicity-related protein 2
SAR	systemic acquired resistance
SA	salicylic acid
Xcc	*Xanthomonas citri* sbsp. *citri*

Introduction

Asiatic citrus canker is caused by the bacterial pathogen *Xanthomonas citri* sbsp. *citri* (Xcc). Canker is a serious disease of commercial citrus cultivars and some citrus relatives. The pathogen causes distinctive necrotic, erumpent lesions on leaves, stems, and fruits. Severe infections can cause a range of symptoms from defoliation, blemished fruit, premature fruit drop, and twig dieback to general tree decline. Grapefruit (*Citrus*

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paradisi) is the most important fresh fruit citrus grown in Florida and the most susceptible variety to canker. In the year 2006 the fresh citrus industry in Florida was impacted by the establishment of citrus canker, the end of the eradication programme and the interim rule that quarantined the entire state, to avoid the spread of the disease to other regions (http://www.thefederalregister.com/d.p/2006). As a consequence, the shipping of all citrus produced in Florida to citrus-producing states was prohibited whether the fruit had lesions or not. New regulations approved in 2007 allow 'asymptomatic fruit' to be shipped to other states regardless of the presence of canker in the grove (http://edocket.access.gpo.gov/2007/pdf/E7).

The experience of other citrus-producing countries with endemic canker indicates that if the incidence of infected fruit in a block is >2–5%, it will be difficult to eliminate all canker affected fruit by culling in the packing house. Thus, reduction of canker incidence on fruit in the groves is an essential first step in producing asymptomatic fruit for packing.

There are no highly effective canker disease-suppression strategies for the most susceptible cultivars of citrus such us grapefruit when the crop is grown in wet subtropical areas like Florida (Kuhara 1978; Stall et al. 1982a; Leite and Mohan 1990). Copper reduces bacterial populations on leaf surfaces, but multiple applications are needed to achieve adequate control on susceptible citrus hosts such as grapefruit and sweet orange (Graham et al. 2004; Schubert et al. 2001; Leite et al. 1987; Stall et al. 1980, 1982b). The protective activity of copper is diminished by wind-blown rain that introduces bacteria directly into stomata (Gottwald and Graham 1992; Gottwald and Timmer 1995). Therefore, copper is used in combination with wind-breaks in South America under weather conditions similar to Florida (Graham 1998; Leite 1990; Leite and Mohan 1990; Muraro et al. 2002). Disadvantages of long-term use of copper bactericides include induced copper resistance in xanthomonad populations (Marco and Stall 1983; Rinaldi and Leite 2000) and accumulation of copper in citrus soils with potential phytotoxic and adverse environmental effects (Alva et al. 1995). However, other contact bactericides are not as effective as copper because they lack sufficient residual activity to protect leaf and fruit surfaces for extended periods (Graham et al. 2006, Rinaldi and Leite 2000; McGuire 1988; Timmer 1988; Leite et al. 1987).

Systemic acquired resistance (SAR) is a mechanism of induced defence that may confer long-lasting protection against a broad spectrum of microorganisms (van Loon et al. 2006; Durrant and Dong 2004). Plants acquire an enhanced defensive capacity against subsequent pathogen attack as a result of a primary, limited pathogen infection. SAR requires the signal molecule salicylic acid (SA) and is associated with accumulation of pathogenicity-related (PR) proteins, which are thought to contribute to resistance. These pathways can be activated in the absence of pathogens by treatment of plants with chemical inducers. For example, acibenzolar-S-methyl (ASM, Actigard®, Syngenta Crop Protection) is a functional homologue of salicylic acid. Foliar application of ASM-induced systemic resistance against canker under greenhouse conditions, but repeated spraying of fruiting orange trees failed to control disease on foliage and fruit in field trials (Graham and Leite 2004).

To confirm SAR in citrus the PR protein gene, *PR-2* (β-1,3 glucanase), from citrus showed increased expression in response to SAR inducers, ASM and isonicotinic acid (INA; Dekkers et al. 2004). *PR-2* response and the disease control effect by foliar-applied INA and ASM lasted for only a few weeks (Dekkers et al. 2004). However, the longevity of activity of soil drenches of INA and ASM and the systemic insecticide imidacloprid (Imid) controlled citrus bacterial spot (CBS), caused by *X. axonopodis* pv. *citrumelo*, in potted citrus seedlings for 90 days (Graham and Leite 2007). Imid breaks down into 6-chloronicotinic acid, an analogue of INA (Beckers and Conrath 2007), so not unexpectedly, Imid induces SAR in citrus. Bacterial control activity by Imid in the field was tested against epidemics of CBS in a nursery and citrus canker in groves in Florida and Brazil (Graham and Leite 2007). Under field nursery conditions, a single drench of Imid at the beginning of the season was as effective as weekly applications of copper-mancozeb for CBS control. Based on these field results, application of compounds to soil to control bacterial infection rather than foliar application appears to play a key role in sustaining the SAR-induction necessary for control of disease in the field (Graham and Leite 2007).

In this work, we investigated the response of potted seedlings to soil-applied Imid, INA, and ASM in comparison with foliar-applied ASM. Canker control activity and longevity were evaluated as the

ability to reduce the number of lesions and populations of Xcc in lesions 1, 4, 16, and 24 weeks after applications. SAR activity was monitored by following expression of *PR-2* gene from citrus to establish the kinetics of induction after soil application in pots. The results of this greenhouse study substantiate the use of soil-applied SAR inducers for season-long control of canker epidemics on young citrus trees.

Materials and methods

Plant material and treatments

Swingle citrumelo plants (*Citrus paradisi* x *Poncirus trifoliata*) were propagated in soil-less medium (The Scotts Co., Marysville, OH, USA) contained in 3.8 l pots and maintained in the greenhouse at a temperature of between 20 to 30°C. Plants were fertilised every 2 weeks with Peters 20-10-20 (0.5g l^{-1}), and supplemented with Essential Minor Elements (5 g per pot; The Scotts Co.). Six weeks prior to the treatments, seedlings were cut back to approximately 40 cm, and only one shoot per plant was allowed to grow to approximately 20–30 cm in order to obtain 4–5 immature leaves (75% expanded) suitable for inoculation.

Compounds with SAR-inducing activity against Xcc were compared with the soil-applied systemic insecticide, imidacloprid (Imid; ADMIRE® Pro 4.6F, Bayer Crop Science). Compounds were applied in a single application as a soil drench (500ml of solution per pot) or as a spray on the foliage using an airbrush (Crown Spra-Tool, Aervoe Industries, Inc. Gardnerville, NV, USA) 1week prior to the first set of inoculations with Xcc.

The treatments and rates evaluated were as follows: (1) acibenzolar-S-methyl (ASM; Actigard® 50WG, Syngenta Crop Protection) as a foliar spray at 1mg a.i. per plant, or (2) as a soil drench at 5 mg a.i per plant, (3) imidacloprid at high rate (Imid high) at 550 mg per plant, or (4) low rate (Imid low) at 225 mg per plant, (5) isonicotinic acid (INA; 1,6-dichloronicotinic acid, Sigma-Aldrich Chemical, USA) at 10 mg per plant, (treatments 3–5 were applied as a soil drench) and (6) untreated inoculated plants (UTP). At 7 days after treatment, 8 plants per treatment were inoculated with Xcc and a buffer control was mock-inoculated with sterile saline phosphate buffer (PBS; 40 mM Na_2HPO_4 + 25 mM KH_2PO_4).

Bacterial inoculum was prepared with Xcc strain, X2002-0014 originally isolated from *C. sinensis* in Dade County, FL, cultured in nutrient broth and grown at 28°C for 24 h to log phase. Bacterial suspension was centrifuged at 10,000 *g* for 20 min, re-suspended in PBS, and adjusted to 0.1 OD at A_{620nm} equivalent to 10^8 colony-forming units (cfu) ml^{-1}. Bacterial cell density was adjusted to 10^4 or 10^5cfu ml^{-1} for inoculations.

Immature leaves (75% expanded) were inoculated using a tuberculin syringe (1.0 cm^3) with no needle as previously described (Graham and Leite 2004). In brief, Xcc inoculum was infiltrated by pressing the needleless syringe tip against the abaxial leaf surface to produce a zone of water-soaked tissue 2 mm beyond the diameter of the syringe opening. The infiltrated area of the leaf was approximately 6 mm diam and contained an estimated 2 μl of bacterial suspension. Three injection infiltrations were performed on each side of the mid-vein. At least three leaves were inoculated per plant. The inoculated shoots were covered with plastic bags for 1 day to maintain high humidity conducive for bacterial growth in the leaves. Lesions were counted at each inoculation site 14 days after inoculation. The number of lesions per leaf was analysed statistically (SAS Institute Inc., Cary, NC, 1996) using ANOVA in a nested design. Significant differences were calculated with Waller's K-ratio t-test for pair-wise comparisons. After the first set of inoculations, the shoots were allowed to grow another 2 weeks until 4–6 new leaves developed and a second set of inoculations was performed 5 weeks after treatment application.

To determine the persistence of the treatment effect over time, plants were pruned below the inoculation point (6 weeks after product application). When new single shoots produced 4–6 young leaves the third set of inoculations was performed (16 weeks after product applications). After lesions were developed and counted, plants were pruned again (20 weeks after product applications) to produce the flush for the fourth set of inoculations performed 24 weeks after the beginning of the assay. The effectiveness of control of Xcc for each treatment was calculated as the ratio of mean lesion number in the treated plants divided by the mean lesion number in the respective set of untreated, inoculated plants, and was expressed as a percentage by multiplying the ratio by 100.

Quantification of Xcc

Inoculated leaves were sampled 2 weeks after each set of inoculations and the samples were immediately frozen in liquid nitrogen and stored at −80°C until processed. Xcc populations per leaf were quantified by quantitative real-time PCR (Q-PCR; Cubero and Graham 2005). One leaf per plant was ground in liquid nitrogen and 200 mg of tissue was mixed in 1000 µl of AP1 buffer (Qiagen, MD, USA), vigorously mixed, and centrifuged at 2000 *g* for 2 min to remove the plant debris. Then 600 µl of the supernatant containing Xcc cells was used for DNA extraction with the mini DNA kit for plant tissue (Qiagen, Maryland, USA). DNA was eluted in a final volume of 100 µl of buffer and one 1.0 µl of a 10-fold dilution of the samples was assayed by Q-PCR.

Q-PCR assays were carried out using primers and probe for the *pth* gene that occurs universally in Xcc (Table 1; Cubero and Graham 2005). Samples consisted of 1 µl of template DNA in 25 µl reaction mixture containing 12.5 µl of 2 × PCR universal TaqMan master mix (Applied Biosystems, Foster City, CA, USA), 1 µl of 5 µM forward primer (*J-RTpth3*),1 µl of 5 µM reverse primer (*J-RTpth4*), and 1 µl of 10 µM TaqMan probe labelled with FAM (6-carboxy-fluorescein) at the 5' end, and a non-fluorescent quencher MGB^{TM} (Applied Biosystems, Foster City, USA) at the 3′ end. Q-PCR reactions were run in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, USA). Amplification conditions were an initial activation step of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

The Xcc population was estimated with a standard curve that related bacterial concentration to a threshold cycle (C_t) using a series of 10-fold dilutions of bacteria from 10^5 to 1 Xcc µl^{-1} included in each Q-PCR plate. One µl of each sample was run in duplicate and the Xcc concentration was estimated with the Applied Biosystems software (ABI®PRISM 7000 Sequence Detection System, Foster City, CA, USA).

Reverse transcription (RT-) PCR analysis of plant gene expression

Leaves located immediately below the inoculation point on the shoot (basal leaves) were used to monitor expression of the *PR-2* gene. Three biological repetitions per treatment were used per time period. Samples were collected weekly after each set of inoculations and frozen in liquid nitrogen and stored at −80°C until processed.

Total RNA was extracted by grinding two leaves per sample in liquid nitrogen and 200 mg of tissue was processed using the RNeasy® Mini kit for plant tissue (Qiagen, MD, USA). Genomic DNA was digested with DNase I, while bound to the silica-gel membrane and incubated at room temperature for 15 min, and RNA was eluted in 100 µl of RNase free water. RNA concentration was adjusted to 100 ng µl^{-1} and 1 µl of sample was used for real time RT-PCR relative quantitation of gene expression.

Oligonucleotide primers and probes were designed using ABI PRISM^{TM} Primer Express software version 2.0 (Applied Biosystem, Foster City, USA) (Table 1). The induction of SAR was monitored by the relative transcription levels (mRNA) of *PR-2* gene using primers designed for the β-1, 3 glucanase gene from *Citrus sinensis*. (NCBI data base accession AJ000081, PR-2) (http://www.ncbi.nlm.nih.gov).

Transcriptional levels were detected by real-time reverse transcription (RT-) PCR in a one-tube reaction using the Power SYBR® Green Reverse Transcription kit (Applied Biosystems, Foster City, USA), 250 nM of

Table 1 Primers and probe used in real time-PCR reactions

Primers	Sequence (5′→3)'	Target
J-RTpth3 forward	ACCGTCCCCTACTTCAACTCAA	pth gene from Xanthomonas citri sbsp. citri
J-RTpth4 reverse	CGCACCTCGAACGATTGC	
J-Taqpth2[a]	FAM-ATGCGCCCAGCCCAACGCMGB	
GAPDH forward	GGAAGGTCAAGATCGGAATCAA	GAPDH-C Citrus sinensis
GAPDH reverse	CGTCCCTCTGCAAGATGACTCT'	
β-glucanase forward	'TTCCACTGCCATCGAAACTG	*PR-2* (β-1,3-glucanase) *Citrus sinensis*
β-glucanase reverse	TGTAATCTTGTTTAAATGAGCCTCTTG	

[a] TaqMan probe

each primer, 10 units of Multiscribe reverse transcriptase, 8 units of RNase inhibitor , and 1μl of mRNA sample in a total reaction volume of 20 μl. The PCR conditions were 48°C for 30 min, 95°C for 10 min and 40 cycles of 15 s at 95°C and 1 min at 60°C, followed by a dissociation protocol of 1°C increase every minute up to 95°C. Threshold cycles (C_t) were determined according to the ABI-PRISM 7000 Sequence Detection Systems using a threshold of 0.40 and automatic baseline calculation according to ABI software. The relative levels of mRNA were calculated relative to the buffer control treatment at the beginning of the assay (value = 1) and normalised to the reference house keeping gene glyceraldehyde-3-phosphate dehydrogenase-C (*GAPDH-C*) sequence from *C. sinensis* (HarvEST database accession USDA-FP 120000-73; http://harvest.ucr.edu). The amount of target gene was normalised to the endogenous reference and calculated using the comparative C_t method ($\Delta\Delta C_t$ method; http://docs.appliedbiosystems.com/pebiodocs/04310251.pdf).

Results

Symptoms, lesion quantification and bacterial populations

Typical canker lesions developed on untreated plants inoculated with Xcc. Lesions were pustular, 2–3 mm diam consisting of callus tissue that became suberised, with an oily margin and a chlorotic halo (Fig. 1a). Lesion phenotype varied with the treatments. On plants treated with the SAR inducers ASM, Imid and INA applied to the soil, lesions were smaller (1–2mm) with a brown centre, little callus proliferation, a dark oily margin and minimal chlorosis (Fig. 1c–f).

Lesion number per leaf was reduced after the first set of inoculations by spray-applied ASM compared to the untreated inoculated plants (Table 2). There was no disease control by the foliar treatment after the second set of inoculations, and control activity from this treatment was not regained after third and fourth set of inoculations (Table 2). ASM foliar-treated plants had lesion numbers and bacterial populations similar to those on the untreated inoculated plants. In contrast, soil applications of ASM, Imid, and INA were most effective for reducing citrus canker lesion development after the latter sets of inoculations. Lesion numbers were reduced more by ASM soil treatment, INA, and Imid high than by Imid low. Reduced disease development by these treatments was maintained after the plants were cut back and re-inoculated at third and fourth set of inoculations (16 and 24 weeks after the single treatment application, respectively). Optimal disease control from soil applications was attained 16 weeks after application: an average of one lesion per leaf for Imid high, four lesions for ASM soil and eight lesions for INA

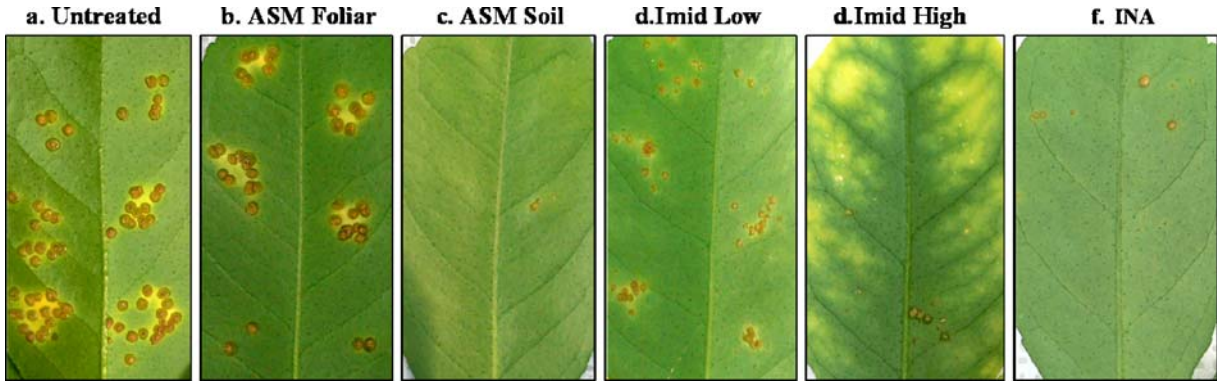

Fig. 1 Effect of a single soil drench application of acibenzolar-s-methyl (ASM), imidacloprid at a high (Imid high) and low rate (Imid low) or isonicotininic acid (INA) compared to foliar-applied ASM on development of lesions of citrus canker on Swingle citrumelo leaves inoculated 6 weeks after treatment application. Each leaf was inoculated at six sites by injection infiltration with a suspension of Xcc (10^4 cfu ml^{-1}). Lesion development was assessed 14 days after inoculation: (a) untreated inoculated plants developed typical canker lesions 1–2 mm in diam; (b) lesions on leaves of ASM foliar-treated plants were phenotypically similar to those on the untreated inoculated plants; lesions in the (c) ASM soil, (d) Imid Low, (e) Imid high and (f) INA treatments were darker brown and reduced in size (<1 mm). Leaves in the Imid high treatment showed interveinal chlorosis

Table 2 Effect of SAR inducer compounds on control of citrus canker lesions and bacterial population*

Treatment*	Set 1		Set 2		Set 3		Set 4	
	Lesions	Log bacterial population	Lesions	Log bacterial population	Lesions	Log bacterial population	Lesions	Log bacterial population
Xcc untreated	175±5a	6.65±0.03ab	102±6b	6.42±0.04b	36±3a	6.63±0.02a	809±11a	6.23±0.06ab
ASM foliar	93±10d	6.90±0.55a	119±9a	6.65±0.02a	39±4a	6.36±0.02ab	692±13b	6.48±0.22a
ASM soil	115±13c	6.20±0.11c	9±2d	5.49±0.01e	4±1cd	5.65±0.02c	249±11d	5.85±0.06c
Imid low	150±9b	6.69±0.03ab	87±5b	6.19±0.01c	24±3b	5.81±0.04bc	459±10c	6.02±0.03bc
Imid high	135±7 bc	6.42±0.04bc	43±6c	5.36±0.03f	1±0.4d	3.21±0.61d	246±12d	4.88±0.02d
INA	115±10 c	6.66±0.02ab	21±3d	5.67±0.06d	8±2c	5.73±0.02bc	211±10d	5.72±0.02c

*Persistence of the effect of a single soil drench application of acibenzolar-s-methyl (ASM), imidacloprid at a high rate (Imid high) and low rate (Imid low) or isonicotininic acid (INA) compared to foliar-applied ASM on development of lesions of citrus canker and populations of *Xanthomonas citri* spp. *citri* (Xcc) after four sets of leaf inoculations over a 24-week period. Immature leaves of Swingle citrumelo (*Citrus paradisi* × *Poncirus trifoliata*) were inoculated with Xcc suspension at time intervals of 1 week (1st set), 4 weeks (2nd set), 16 weeks (3rd set) and 24 weeks (4th set). For inoculation sets 1–3, the inoculum concentration of Xcc was 10^4 cfu ml^{-1} and for set 4 was 10^5 ml^{-1}. Number of lesions per leaf was assessed 14 days after each inoculation time. Values represent, mean and standard error calculated using 4 leaves per plant and 8 plants per treatment. Xcc population was estimated by quantitative PCR (Q-PCR), and expressed as $\log_{10}$ of bacteria per leaf. Means (n=8) followed by the same letter are not significantly different at $P \leq 0.05$ according to Waller's K-ratio t-test. See Materials and methods for treatment details

compared to 36 lesions per leaf on untreated inoculated plants (Table 2). After the third set of inoculations, there were fewer and smaller canker lesions on treated leaves compared to those on untreated plants (Fig. 1). Leaves of plants treated with Imid high and INA also showed interveinal chlorosis, and reduced leaf expansion. After subsequent cutback of shoots for the fourth set of inoculations the appearance and size of leaves resembled that of the untreated inoculated plants.

Xcc populations estimated by Q-PCR after the second set of inoculations were reduced one log unit by soil applications of ASM, INA, and Imid high. The effectiveness of soil applications in reducing Xcc populations increased with time after soil applications and was greatest after the third set of inoculations (16 weeks after treatment). The lowest bacterial population was observed in plants treated with Imid high (3.21 log units), 3 log units lower than the untreated inoculated plants (6.63 log units). At higher bacterial inoculum potential (10^5) used for the fourth set of inoculations, INA, ASM soil, and Imid high produced a 1–2 log unit reduction in bacterial population compared with the untreated inoculated plants (6.23 log units).

PR-gene response

Transcription of the *PR-2* gene was monitored weekly in the leaves below the inoculation point for each set of inoculations. Means of the relative expression per treatment calculated according to the $\Delta\Delta C_t$ at different weekly intervals post-treatment are shown in Fig. 2a–c. In the first 6 weeks after treatment, expression levels of *PR-2* in the buffer control, Xcc-inoculated, and the Imid low remained low (Fig. 2a). At the end of the first week following treatment, ASM foliar produced higher expression of *PR-2* response than any of the other treatments. In addition, that level of expression was sustained for the first 4 weeks before decreasing. Soil application of INA and ASM resulted in a gradual increase in *PR-2* expression over time. The gene expression response to the Imid high and low treatments were less consistent. By 5 weeks after treatment, the response to INA was 80-fold higher than that immediately after soil treatment whereas *PR-2* expression in plants following foliar-applied ASM was similar to that in the buffer and untreated inoculated plants.

At 16–20 weeks after treatment, plants treated with buffer, Xcc and ASM foliar had low levels of *PR-2* expression (2 to 20-fold) compared 30 to 100-fold increases obtained with ASM soil, Imid high and INA (Fig. 2b). *PR-2* expression following treatment with Imid low was intermediate varying from 5 to 40-fold increase. Among treatments, *PR-2* expression was similar from 24–27 weeks to that from 16–20 weeks (Fig. 2c). In contrast, *PR-2* expression in response to the Imid low treatment was similar to that in plants

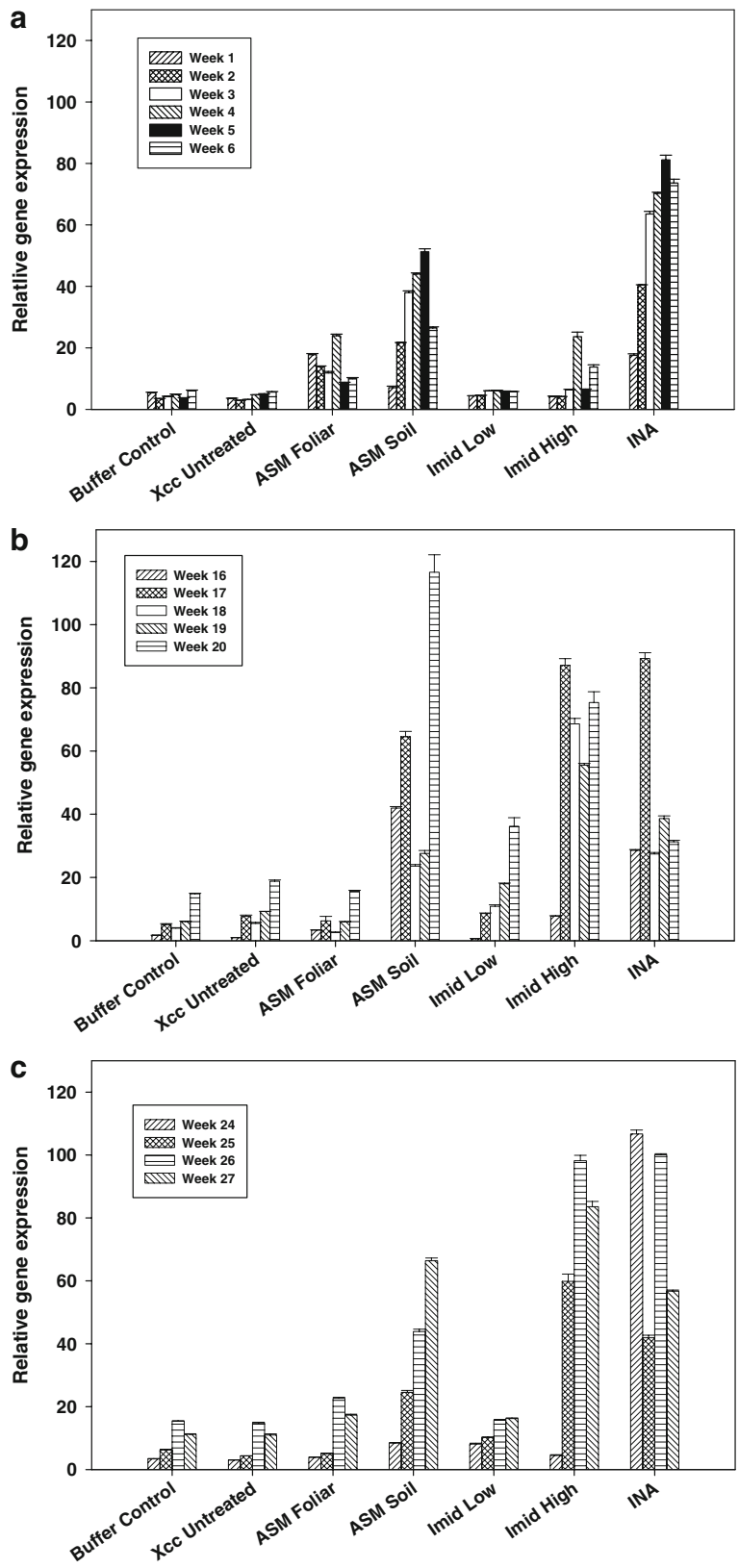

Fig. 2 Relative expression of the *PR-2* (β-1,3-glucanase gene) in Swingle citrumelo leaves after a single application of different SAR-inducer compounds. Plants were inoculated with Xcc at weeks 1 (1st set), 4 (2nd set), 16 (3rd set) and 24 (4th set) after treatment. Relative gene expression was calculated using the $\Delta\Delta C_t$ method. Values represent the mean of three biological replicates, each sample consisted of combined two leaves from two plants (a total of six plants were assayed per treatment), bars represent standard error. Treatments are: non-inoculated (buffer control), Xcc untreated inoculated (Xcc untreated); ASM foliar, ASM soil, Imid low, Imid high and INA (See Materials and methods for treatment details). *PR-2* gene expression was assessed at different time intervals: (a) 1st and 2nd set of inoculations (1–6 weeks); (b) 3rd set (16–20 weeks) and (c) 4th set of inoculations (24–27 weeks after product application)

treated with ASM foliar and buffer, and untreated inoculated plants.

Effect of SAR inducers over time

Based on the percentage reduction of lesions, the ASM foliar treatment was most immediately effective for disease control compared to the untreated inoculated treatment, but the effect was subsequently lost (Fig. 3a). On the other hand, the effectiveness of soil-applied ASM over time increased up to the third and fourth set of inoculations (16–24 weeks after treatment application). Peak disease control was obtained with Imid high at the third set of inoculations (16 weeks after soil application). The effectiveness of soil-applied SAR inducers began to drop slightly after the third set of inoculations (24 weeks after product application), (Fig. 3a).

To substantiate that Xcc control over time was related to induction of SAR, the percentage reduction of Xcc lesions for each set of inoculations (first to fourth) was regressed against *PR-2* expression obtained at 7 days post-inoculation (Fig. 3b). An exponential model explained >80% of the variation in the percentage lesion reduction to the increase in SAR response.

Discussion

SAR is one of the defence mechanisms plants employ against pathogen attacks, resulting in resistance and protection of distal tissue. SAR is characterised by the accumulation of SA and the expression of PR genes (Hammerschmidt et al. 2001). Synthetic analogues of

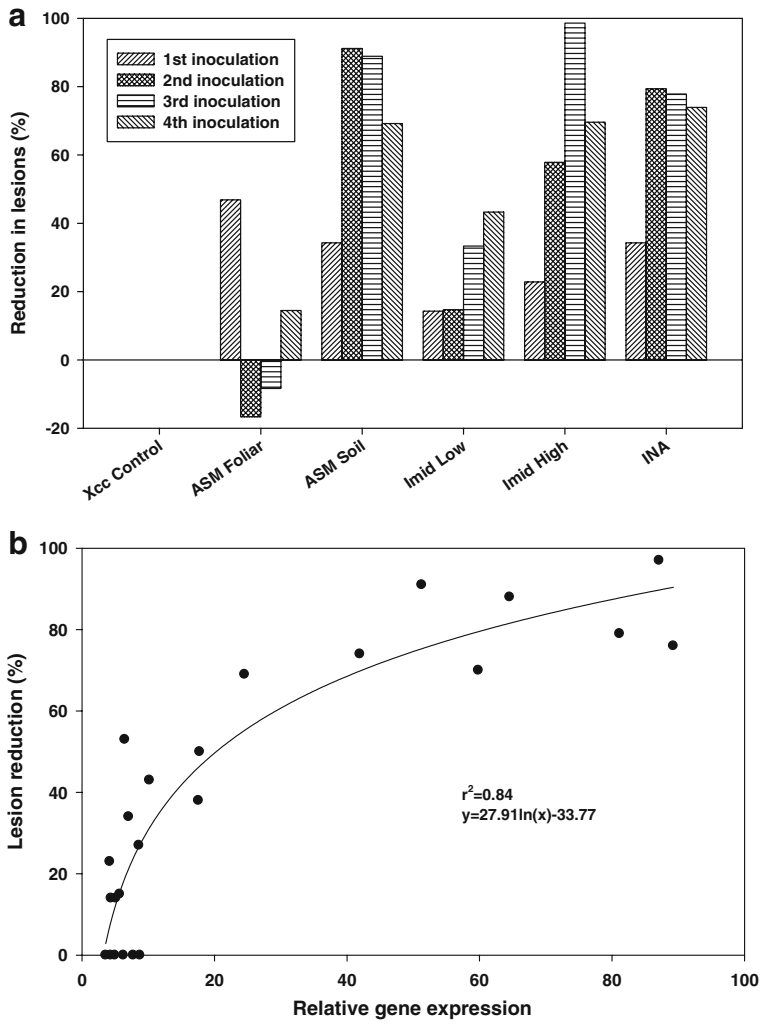

Fig. 3 Persistence of the effect of a single soil drench application of acibenzolar-s-methyl (ASM), Imidacloprid at a high (Imid high) and low rate (Imid low) or isonicotininic acid (INA) compared to foliar-applied ASM on development of lesions of citrus canker and populations after four sets of leaf inoculations with *Xanthomonas citri* spp. *citri* (Xcc) over a 24-week period. (a) Reduction of lesions is the percentage compared with the untreated, inoculated plants in each inoculation set (1^{st} - 4^{th}). Values were calculated using the average number of lesions per treatment presented in Table 2. (b) Correlation is between the percentage lesion reduction and the relative expression of *PR-2* gene. Gene expression corresponds to values of *PR-2* gene present in samples collected 1, 5, 17 and 25 weeks after treatment

SA, such as INA and ASM, can induce SAR and have been employed to control pathogens. Most evaluations have been conducted in model systems like *Arabidopsis* and tobacco (van Loon et al. 2006), or in vegetable crops like tomato but few studies have examined woody plants (da Rocha and Hammerschmidt 2005)

SAR-inducers, like ASM, used as a foliar application were effective for control of *Erwinia amylovora* in young apple seedlings (Maxson-Stein et al. 2002). However, weekly applications of foliar ASM were necessary to maintain control of fire blight. Expression of the *PR-2* gene was confirmed to be useful for monitoring the response to ASM in apple (Brisset et al. 2000; Maxson-Stein et al. 2002). *PR-2* gene expression was increased up to 100-fold compared to untreated seedlings. Bonasera et al. (2006) monitored the expression of several PR-proteins in young apples shoots and observed moderate induction of *PR-2* after sprays of INA, and that *PR-2* was a more robust indicator of SAR than *PR-1a*, *PR-5*, and *PR-8*.

In Swingle citrumelo, foliar spray applications of ASM, 3 to 7 days pre-inoculation, reduced the number of lesions produced by Xcc and *Xanthomonas axonopodis* pv. *citrumelo*, the cause of CBS, in greenhouse assays (Dekkers et al. 2004). Similar to that in apple, ASM treatment of Swingle citrumelo seedlings in the greenhouse induced a *PR-2* response 7 to 21 days after foliar application. However, *PR-2* gene response and CBS control lasted no more than 21 days post-treatment (Dekkers et al. 2004). In field trials with sweet orange varieties, multiple foliar sprays of ASM in combination with copper were no more effective than copper alone for control of canker in southern Brazil (Graham and Leite 2004).

In contrast to foliar application of ASM, a single soil drench application of Imid at the beginning of the season was as effective for controlling CBS in a citrus nursery trial as weekly applications of copper-mancozeb as a foliar spray. In subsequent field trials with canker in Brazil and Florida, a single soil application of Imid to 3 year-old citrus trees at the beginning of the season reduced the incidence of foliar canker and the number of canker-affected sweet orange fruit (Graham and Leite 2007). These results demonstrated potentially greater and more long-lived disease control activity could be obtained by soil applications of SAR inducers than by foliar applications.

In the present study, foliar application of ASM was more immediately effective than soil-applied SAR inducers for reducing canker lesions and inducing *PR-2* gene response. Disease control and *PR-2* response after ASM foliar treatment was not evident following inoculations made more than 4 weeks after treatment. These observations are consistent with the failure of foliar ASM to control canker in field evaluations in Brazil.

As opposed to the time-limited activity of foliar ASM, soil applications of Imid, ASM and INA, induced a persistent and high expression of the *PR-2* gene from 4 to 27 weeks. Control of canker by soil-applied SAR inducers far exceeded that by foliar application of ASM. Moreover, disease control was sustained at a high level (~80% lesion reduction) for several weeks after soil application.

Soil application of SAR inducers not only reduced the number of lesions but also changed the phenotype: lesions were smaller, darker and less erumpent than on untreated inoculated plants. Altered lesion phenotype reflected reductions in the population of Xcc of 1 to 3 log units compared to untreated plants. Canker symptoms and Xcc population responses produced in Swingle citrumelo treated with soil applications of Imid, INA, and ASM, were similar to the symptoms observed in resistant cultivars of the citrus relative kumquat (*Fortunella* spp.) and hybrids with susceptible acid limes (Viloria et al. 2004). This suggests that SAR as a mode of plant defence has substantial activity and benefit for control of Xcc in more susceptible citrus genotypes. Moreover, uptake of an SAR inducer via the roots and translocation throughout the plant is capable of inducing sustained resistance to repeated pathogen challenge.

From the standpoint of producing of season-long control of canker, the greenhouse pot study confirms results of field trials of young citrus plantings in that a single soil application controls the disease on new flushes for a period of up to 6 months. This long-lasting, residual effect will be very useful for the management of canker in young orange trees in Florida and Brazil where subtropical climatic conditions promote repeated leaf flushes that might otherwise require multiple applications of foliar copper to control canker-induced defoliation.

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