Induced resistance in sweet orange against *Xanthomonas citri* subsp. *citri* by hexanoic acid

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ABSTRACT

Citrus canker, caused by *Xanthomonas citri* subsp. *citri*, is a serious and wide-spread disease of citrus, causing losses in fruit yield and quality. There are no highly effective citrus canker disease control measures. Repeated spray applications of copper are often employed to protect fruit from bacterial infection with consequences for copper phytotoxicity and accumulation in the soil. Alternatively, innate plant defense mechanisms can be enhanced by plant treatments with specific natural and synthetic inducers for control of bacterial diseases. In this study, hexanoic acid applied as a soil drench or foliar spray on 9-month-old potted citrus trees reduced lesions on leaves by 50% compared with control plants. Disease-reducing activity lasted up to 50 days after application. Induction of resistance mediated by hexanoic acid was demonstrated by enhanced expression of

Pathogenesis-related *(PR)* genes and callose deposition in treated and infected plants. These findings indicated that hexanoic acid applications trigger a defensive response in the plants. The application of this natural compound may have potential for management of citrus canker in conjunction with other disease control measures and may reduce the frequency or rate of copper bactericides.

Keywords: hexanoic acid, citrus, *Xanthomonas citri,* induced resistance.

1. INTRODUCTION

Citrus canker, caused by the bacterial pathogen, *Xanthomonas citri* subsp. *citri* (Xcc; syn. *X. axonopodis* pv. *citri*), is a serious disease of commercial citrus cultivars including grapefruit (*Citrus paradisi* Macf.) and early mid-season oranges (*C. sinensis* (L.) Osb) for juice processing. The pathogen causes necrotic, erumpent lesions on leaves, stems, and fruits that create a range of symptoms including defoliation, blemished fruit, premature fruit drop, and twig dieback to general tree decline (Graham and Leite, 2004)

Currently, citrus canker is present in wet subtropical citrus-producing regions of the world, but it has not been reported from areas dominated by Mediterranean climates such as southern Europe, where it is considered a quarantine pathogen (EPPO, 2012). Rapidly expanding leaves and continuously growing fruit tissues are most vulnerable to infection, and field resistance of trees to Xcc is directly related to tissue juvenility (Graham et al., 1992). Citrus cultivars and species with greater frequency, size, and duration of leaf flushes and duration of fruit growth are more field-susceptible to Xcc than less vigorous cultivars or those whose foliage matures more rapidly (Favaro et al., 2014; Gottwald et al., 1993).

There are no highly effective canker disease treatments when susceptible cultivars are growing in areas with favorable conditions for bacterial multiplication. Copper reduces bacterial populations on leaf surface, but multiple applications are needed to protect fruit of susceptible citrus varieties. Disadvantages of long-term use of copper bactericides include induced copper resistance in xanthomonad populations (Behlau et al., 2012) and accumulation in soils with potential phytotoxic and adverse environmental effects. The protective activity of copper is diminished by wind-blown rain that introduces bacteria directly into stomata (Behlau et al., 2008; Serizawa and Inoue, 1974). However, other bactericides such as antibiotic-based products are not as effective as copper because they lack sufficient residual activity to protect leaf and fruit surfaces for extended periods (Behlau et al., 2008; Graham et al., 2006). On the other hand, the lack of effective means of control of this bacterium promoted new lines of investigation focused on the development of transgenic plants with genes that confer resistance to Xcc (Cardoso et al., 2010; Mendes et al., 2010).

Plant-pathogen interaction during infection induces signal cascades which activate a cellular response to minimize lesions. Depending on the pathogen that activates the response we can divide the defensive models in systemic acquired resistance (SAR), herbivore-induced resistance (HIR) and induced systemic resistance (ISR). ISR is defined as the induced resistance independent of Salicilic acid and mediated by Jasmonic acid, which could include the resistance against herbivores, resistance against necrotrophic pathogens and resistance induced by beneficial microbes. The SAR term defines the resistance that is dependent of salicylic acid (SA) accumulation and resistance against biotrophic pathogens (Pieterse et al., 2012).

Once the pathogen attack is recognized by the plant, it activates cell wall-associated defense such as the callose deposition and the rapid accumulation of reactive oxygen species (ROS) which is the principal initial barrier against *Xanthomonas* infection in citrus plants (Enrique et al., 2011). In addition to these physical barriers, innate plant defenses trigger a broad spectrum of metabolic and hormonal responses (Durrant and Dong, 2004; Lorenzo et al., 2004). Natural induced resistance can be activated by pathogens, beneficial microbes and herbivores. Is widely known that the exposure of plants to some stresses can induce a state of sensitization of the whole plant for enhanced defense; characterized by a faster and stronger activation of cellular defenses upon invasion. This state is known as Priming of defense (Conrath, 2009; Goellner and Conrath, 2008; Jung et al., 2009; Pastor et al., 2012).

In the recent years, various synthetic compounds including β-aminobutyric acid (BABA) and acibenzolar-S-methyl (ASM) have been investigated for systemic disease control without expression of a direct toxic effect on the pathogen (Jakab et al., 2001). In addition, plant extracts of neem (*Azadirachta indica*), ginger (*Zingiber officinale* Roscoe) and curcuma rhizomes (*Curcuma longa* L.) (Vechet et al., 2009) have been reported to be capable of controlling plant disease without directly inhibiting the pathogen.

Acibenzolar- *S*-methyl (ASM; Actigard or Bion; Syngenta Crop Protection), a functional homolog of salicylic acid (SA), is the most widely known commercial resistance inducer (Tally et al., 1999). Although ASM has been extensively evaluated as a component for plant disease control in the field, its effectiveness in disease management has been questioned due to variability of control (Walters and Fountaine, 2009). Field studies showing promise for control of bacterial diseases have been conducted with foliar sprays of

ASM either alone or in combination with copper on tomato and pepper (Huang et al., 2012; Louws et al., 2001; Ortuno et al., 2008; Romero et al., 2001). Recently, reductions in foliar infection and canker-induced defoliation on young non-bearing grapefruit trees were measured after soil applications with the neonicotinoids (imidacloprid and thiamethoxam) and ASM (Graham and Myers, 2011). Expression of the PR (β-1,3 glucanase) gene, *PR2*, in citrus increased in response to soil drenches of ASM and neonicotinoids (Francis et al., 2009). Moreover, reduction of lesions was sustained for weeks with soil drenches, whereas after a foliar spray of ASM, *PR2* activity and disease control lasted only weeks.

Similarly, our research group has demonstrated the efficacy of soil applications of carboxylic acids for protecting tomato plants against *Alternaria solani* and *Phytophthora citrophthora* (Flors et al., 2003). More recently, we found that hexanoic acid (Hx) can protect Arabidopsis and tomato plants against *Botrytis cinerea* (Kravchuk et al., 2011; Vicedo et al., 2009) and citrus plants against *Alternaria alternata* (Llorens et al., 2013). This natural short-chain monocarboxylic acid displays antimicrobial activity and can also induce plant defense responses when used as a priming agent. Post-infection, oxylipin (1,2-oxo-phytodienoic acid; OPDA) and the bioactive molecule jasmonate-isoleucine (JA-Ile) were significantly induced in treated plants. Additionally, abscisic acid (ABA) acted as a positive regulator of Hx-induced resistance (Hx-IR) by enhancing callose accumulation (Vicedo et al., 2009).

Plant disease control based on systemic resistance induced by a compound like Hx with low toxicity could potentially be integrated with copper for citrus canker control as recently proposed for other inducers of systemic acquired resistance (Graham and Myers, 2013). Hence, the aim of this work was to evaluate the efficacy of Hx as an inducer of resistance

in citrus against Xcc and to compare the disease control and resistance responses with those obtained after treatment of ASM. In addition, method of application, and longevity of the systemic activity was assessed to determine whether Hx as an inducer of resistance has potential for sustained control of citrus canker in the field.

2. MATERIAL AND METHODS

2.1 Bacterial strain, culture media and growth conditions

The Xcc strain X2002-0014 used in this study was isolated in 2002 from sweet orange in Dade County, FL and was routinely grown on Luria Bertani broth (LB) (10 g tryptone, 5 g yeast extract and 5 g sodium chloride per liter) or on LB plates (1.5% bacteriological agar) at 27ºC for 48 h.

2.2 Bacterial growth assay

Growth of Xcc was measured in LB broth adjusted to pH 7 with addition of MES buffer and amended with Hx (Sigma-Aldrich, St. Louis, MO ref.153745) at 0, 0.06, 0.6, 1.5, 3, 6, 10, and 20 mM. Xcc was grown in LB broth overnight and the bacterial suspension was centrifuged, washed and resuspended in 10 mM $MgSO_4$. The growth assay was carried out in a total volume of 300 µL in microtiter wells using an initial bacterial density of 1.5×10^3 colony-forming units (cfu) mL^{-1} adjusted with a spectrophotometer set at A_{600nm}. Bacteria were incubated on a rotary shaker for 96 h at 26℃ and optical density of the suspension was measured every 10 min using a Bioscreen C Reader (Labsystems Oy, Helsinki, Finland) set at A_{600nm}. After the growth assay, a LIVE/DEAD® (Life Technologies Corp, Carlsbad, CA, USA) test was performed to assess cell mortality caused by Hx acid.

2.3 Inoculum preparation, plant treatment and inoculation procedures

Xcc inoculum was prepared in nutrient broth and grown at 28°C for 24 h to log phase. Bacterial suspension was centrifuged at 10,000g for 20 min, re-suspended in phosphate buffer saline (PBS; 40 mM Na_2HPO_4 + 25 mM KH_2PO_4), and adjusted to 10^4 cfu mL^{-1} for attached leaf inoculations and 10^5 cfu mL^{-1} for detached leaf inoculations as previously described (Francis et al., 2010).

Nine-month-old 'Pineapple' sweet orange plants growing in 2.5-L containers of a general purpose peat-based soil (Pro-Mix BX; Premier Horticulture, Red Hill, PA) were maintained in a greenhouse located at the Citrus Research and Education Center in Lake Alfred, FL. Four 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 treatment and/or inoculation.

Test compounds were applied in a single application as a soil drench (500 ml of solution per pot) or as a foliar spray (100 mL of solution per plant) using an airbrush (Crown Spra-Tool, Aervoe Industries, Inc.). The timing of treatments and their rates were chosen based in previous reports (Francis et al., 2009; Llorens et al., 2013) of effective dosages and timing of applications. In brief: acibenzolar-S-methyl (ASM; Actigard® 50WG, Syngenta Crop Protection) used as a positive control was applied as a foliar spray at 1 mM 4 days before inoculation or as a soil drench applied 7 days before inoculation; Hx was applied as a foliar spray at 1mM or 3 mM one day before inoculation or as a soil drench at 1 mM 4 days before inoculation. In all the experiments, nontreated and inoculated plants were included as nontreated checks (NTC). Inoculation of all plants was made on the same day.

For Xcc inoculation, immature leaves (75% expanded) were injection-infiltrated in the abaxial side with 10^4 or 10^5 colony forming units (cfu) mL^{-1} respectively for attached leaf or detached leaf assay using a tuberculin syringe (1 cm^3) with no needle as previously described (Francis et al., 2009). A 6-mm diameter area of the leaf was infiltrated with approximately 2 µL of bacterial suspension. Three injections were performed on each side of the leaf mid-vein.

2.4 Detached leaf assay

After spray applications, five leaves per treatment were collected from greenhouse plants in the morning, rinsed and disinfested as previously described (Francis et al., 2010). Leaves were rinsed three times with sterile distilled water in the same plastic bags to remove any debris or spray residues, dipped in 70% ethanol for 30 s, immersed in 0.5% sodium hypochlorite for 30 s, and then immediately rinsed three times with sterile distilled water. Leaves handled by the petiole end placed on a sterile paper towel and inoculated with 10^5 cfu ml^{-1} as described above. Excess inoculum was wiped from the leaf surface with a sterile paper towel. Inoculated leaves were placed on the surface of soft water agar (0.5%) with the abaxial side up. The petiole was removed and the leaf pressed onto the agar surface with a plastic spreader to obtain as much contact as possible. Petri dishes were sealed with Parafilm and incubated in an environmentally controlled growth chamber under fluorescent light at 60 µmol m^{-2} s^{-1} for 12 h photoperiod at 28°C. Symptoms on the inoculated detached leaves were assessed 7, 10 and 14 days post inoculation (dpi). The experiment was conducted three times with six replications per treatment.

2.5 Attached leaf assay

After spray or soil drench treatments, 4 leaves per plant were injection-infiltrated with inoculum on the abaxial surface of the leaf. Four plants per treatment were inoculated with Xcc and a buffer control was mock inoculated with sterile phosphate buffer saline (PBS).

Greenhouse inoculations were performed from 9:00 to 12:00 h when stomata were fully open with 10^4 cfu ml^{-1} as described above. Inoculated shoots were immediately enclosed in plastic bags for 48 h. After removal of the bags, plants were rotated on the greenhouse bench twice weekly. Leaf samples were taken at 10 and 20 dpi. Lesions on the inoculated leaves were counted under a hand lens (×10) at 20 dpi.

To determine the persistence of the treatment effect over time, after the first set of inoculations, all the leaves were removed to force a new flush. Shoots were allowed to grow another 2 weeks until 4–6 new leaves developed. When the leaves achieved the requisite size, a second set of inoculations was performed (5 weeks after initial treatment) without an additional chemical application. The experiment was conducted three times with six replications per treatment.

2.6 Quantification of Xcc

Viable bacteria in the inoculated areas were estimated at 14 dpi for detached leaf assay and 20 for attached leaf assay. Leaf disks (6 mm diameter) were excised from three infiltrated sites and ground in 1 mL of PBS buffer using a glass homogenizer. Serial dilutions of bacterial suspension were plated on KCC medium (nutrient agar plus kasugamycin 16 mg L^{-1}, cephalexin 16 mg L^{-1}, and chlorothalonil 12 mg L^{-1}) as previously decribed (Francis et al., 2010). Total bacterial colonies were expressed as log cfu per inoculation site. Total Xcc populations per inoculation site were quantified by quantitative real-time PCR (Q-PCR) as previously described (Francis et al., 2009). In brief,

leaf disks (6 mm diameter) were excised from three infiltrated sites and processed for DNA extraction with the mini DNA kit for plant tissue (QIAGEN Sciences Inc., Germantown, MD). Q-PCR assays were carried out using primers and probe for the Xanthomonas pathogenesis gene (*pth* gene) that occurs universally in Xcc (Francis et al., 2009).

2.7 Reverse transcription and real-time quantitative PCR analysis of plant gene expression

Three discs of 6-mm diameter per plant and treatment were collected 10 and 20 days after first and 10 and 20 days after second, inoculations and frozen in liquid nitrogen and stored at −80°C until processed. Each plant was processed as a different biological sample. RNA was extracted using an RNeasy® Plant Mini Kit (QIAGEN) following the manufacturer's instructions. Reverse transcription and real-time quantitative PCR (RT-qPCR) were performed as previously described (Francis et al., 2009). The primers used in the RT-qPCR were *pth* and *PR2* previously described by Francis et al. (2009), Callose synthase 1 (CALs1) described by Enrique et al. (2011) and Allene oxide synthase (*AOS*) and *PR5* described by Fernández-Crespo et al. (2012). Actin and 18S gene expression were used as an internal standard (Yan et al., 2012) (supplementary table 1: primer sequences).

2.8 Callose deposition

Callose deposition was determined as described by Flors et al. (2007) in control and infected leaves at 20 days after first and 20 days after second inoculation. Leaves were collected at the time-points indicated and incubated in 95% ethanol at room temperature. De-stained leaves were washed in 0.07 M phosphate buffer (pH 7), incubated for 15 min in 0.07 mM phosphate buffer containing 0.01% aniline blue at room temperature, and then incubated in 0.1% aniline blue one week at room temperature. Observations were

performed with an epifluorescense microscope. Callose deposition was quantified from digital photographs of aniline blue-stained leaves. Fluorescence emitted by stained callose was observed under UV light as bright yellow spots and were analysed for number of pixels using ADOBE PHOTOSHOP CS4 software. Callose intensity was expressed as the average of yellow pixels/million pixels on digital photography.

2.9 Data analysis

Data were analyzed with the Kolmogorov-Smirnov test prior to the statistical analysis. Data not following a normal distribution (bacterial populations) were log-transformed before analysis. Treatments were analyzed by one-way ANOVA using Statgraphics centurion XVI.I software (Statistical Graphycs Corp.), and means were separated using Fisher's least significant difference (LSD) at 95%. Treatments were 1) non-inoculated nontreated plants, 2) inoculated nontreated plants, 3) inoculated Hx soil drench treated plants 4) inoculated Hx spray treated plants 5) inoculated ASM soil drench treated plants and 6) inoculated ASM spray treated plants. All experiments were repeated three times with six plants per treatment. Figures show the average of three independent experiments.

3. RESULTS

3.1 Characterisation of antibacterial activity of hexanoic acid

To characterise the effect of Hx on Xcc *in vitro*, bacterial growth was measured for 96 h in LB medium amended with increasing concentrations of Hx (0.06, 0.6, 1.5, 3, 6, 10 and 20 mM). Hx at 0.06 mM did not affect the growth of Xcc compared with the non-amended control whereas 0.6, 1.5 and 3 mM Hx inhibited bacterial growth observed in a reduction of optical density by 8%, 11% and 16%, respectively (Fig. 1). At these concentrations, Hx

reduced the rate of bacterial growth because entry into the lag phase was significantly ($P<0.05$) delayed compared with the control and 0.06 mM treatments. Concentrations greater than 3 mM completely inhibited Xcc growth. Hx at 0.6, 1.5 and 3 mM apparently had a temporary bacteriostatic effect which may explain the reduction in the rate of bacterial growth. Assay with the Live/Dead Cell Viability kit indicated that concentrations greater than 3 mM Hx did not kill Xcc cells.

3.2 Lesion development and bacterial populations in a detached leaf assay

Treatments with Hx and ASM of detached leaves inoculated with Xcc almost completely prevented development of lesions compared with nontreated checks (NTC) at 15 days post-inoculation (dpi) (Fig. 2) Based on quantification of total Xcc populations at 15 dpi by RTq-PCR population was reduced by 1.41 log units with 1mM Hx and 0.91 log units with Hx 3 mM, compared to the NTC (7.99 log), whereas ASM reduced the populations 1.57 log units (Table 1)."

Recovery of viable bacteria was 2.32 log units lower in the 1.0 mM Hx-treated leaves (3.97 log), 1.81 log units lower in the 3.0 mM Hx-treated leaves (4.48 log) and 2.51 log units lower in the ASM-treated leaves (3.78 log) compared with the NTC leaves (6.29 log) (Table 1).

For subsequent experiments, 1.0 mM Hx was chosen because, *in vitro*, this concentration significantly reduced infection and bacterial population development without causing direct toxicity to Xcc.

3.3 Lesion development and bacterial populations in the attached leaf assay

271 At 20 days after the initial inoculation of attached leaves with Xcc, soil drench and foliar
272 spray treatments with Hx reduced lesions by 50.7% and 47.4%, respectively, compared to
273 the NTC (Table 2). Hx treatments also significantly reduced the viable Xcc populations in
274 leaves when compared with the NTC plants (Table 2). Hx treatments reduced the number
275 of lesions to similar levels to those obtained in plants treated with the ASM in spray.
276 However, this reduction was lower than that produced by the ASM soil drench. After the
277 second inoculation of the treated plants, Hx soil drench and foliar treatments reduced
278 lesions by 68.5% and 65.5%, respectively, compared to the NTC. Soil drench and foliar Hx
279 reduced Xcc populations by more than 50%. The magnitude of bacterial population control
280 was similar to that obtained after treatment with ASM. After the second inoculation, the
281 reductions produced by Hx treatments were greater than that for ASM spray, but less than
282 that for ASM soil drench. The Xcc populations estimated by Q-PCR in the treated leaves
283 after the first and second inoculations did not differ from the NTC (Table 2).

284 ***3.4 Expression analysis of defense-related genes after Xcc inoculation***

285 Transcription of the *PR2*, *PR5* and allene oxide synthase (*AOS*) genes was monitored in
286 leaves located immediately below the inoculation point on the shoot (basal leaves). After
287 the first inoculation of plants at 10 dpi, the expression of *PR5* was unaffected, and *AOS* and
288 *PR2* slightly increased. At 20 dpi, expression of both of the *PR* genes was significantly
289 increased by Hx and ASM applied as spray or in soil drench compared to the nontreated
290 and infected control (Fig. 3a and c), However, *AOS* gene only showed significant induction
291 with soil drench treatment.

292 After the second inoculation at 10 dpi, *AOS* expression was promoted by all the
293 treatments (Fig. 3b), but a lesser effect was observed for the Hx soil drench treatment.

294 Expression of *PR2* (Fig. 3d) was induced by Hx and ASM. This gene, after the second
295 inoculation, showed an expression much greater than observed after the initial inoculation
296 with both compounds, and the induced expression was also much greater for ASM as
297 compared to Hx. Unlike the *AOS* and *PR2* gene responses, *PR5* only responded at 20 days
298 after the first inoculation but showed no significant differences from NTC after the second
299 inoculation (Fig. 3e and f). The treated non-inoculated control did not show changes in the
300 gene expression compared with the nontreated non-inoculated plants (Fig. 3).

301 ***3.5 Callose deposition***

302 To assess the mechanism of resistance induced by Hx, callose formation was observed at
303 the Xcc infection site. Callose accumulation significantly increased upon infection in the
304 plants treated with soil drench-applied Hx after the first inoculation (Fig. 4a), whereas all
305 the treatments produced significant differences after the second inoculation. Callose
306 accumulation was 4 times higher than the other treatments. A higher *CALS1* gene
307 expression in the Hx-soil drench treated plants confirmed the *in situ* callose response (Fig.
308 4b). In contrast, Hx applied with spray and the ASM treatments did not enhance callose.

309

310 **4. DISCUSSION**

311 Hx is a natural monocarboxylic acid produced by several plants including strawberry
312 (Zabetakis et al., 2000) and *Arbutus unedo* (Soufleros et al., 2005). Hx is also detected in
313 butter and butter oil (Peterson and Reineccius, 2003) and in cheeses (Morales et al., 2006),
314 and contributes to their aromatic character. This acid has been tested as a resistance inducer
315 in crop plants such as tomato against *B. cinerea* (Vicedo et al., 2009), *P. syringae* (Scalschi

316 et al., 2013), and in citrus against the fungus *Alternaria alternata* (Llorens et al., 2013). In
317 the present work we evaluated the effectiveness of Hx for host-mediated resistance against
318 Xcc, the cause of citrus canker, the direct activity against the bacterium, and the longevity
319 of the protective effect.

320 *In vitro*, Hx concentrations over 0.6 mM in LB medium delay Xcc growth. Results
321 indicated that Hx delayed entry of the bacterium into the stationary phase, but did not
322 reduce the size of bacterial population. The LIVE/DEAD® assay demonstrated that the
323 growth inhibition was a bacteriostatic effect. In detached citrus leaf inoculations, Hx at 1.0
324 and 3.0 mM reduced lesion number and viable and total Xcc populations. In attached leaf
325 inoculation of plants, both soil drench and foliar Hx spray applications reduced the number
326 of lesions produced by Xcc. Hx produced a similar reduction in lesions and the bacterial
327 population as ASM. These disease control effects are consistent with those reported by
328 Francis et al. (2009), who demonstrated that soil drenches with ASM reduced lesions and
329 Xcc populations in leaves and differences in response between the spray and soil
330 applications. Hx and ASM significantly reduced canker symptoms for up to 45 days after
331 treatment, but disease control activity was longer-lasting after soil drenching.

332 Activation of defense pathways was confirmed by elevated expression of PR genes.
333 Wang et al. (2012) demonstrated that the exogenous application of SA promoted the
334 expression of *PR* genes and reduced the occurrence of canker disease. Moreover, Francis et
335 al. (2009) observed a correlation between the expression of *PR2* and lesion reduction. Gene
336 expression responses suggest that Hx activates both the *AOS* and PR responses, but *AOS*
337 induction was signigicant only at 20 dpi after the first inoculation in soil drench. Similar
338 results were obtained by Fu et al. (2011) using transgenic sweet orange plants

339 overexpressing spermidine synthase, which induced high constitutive levels of *PR* gene
340 expression and resistance to Xcc. These authors (Fu et al., 2011) observed an induction of
341 *AOS* after Xcc infection, which implies that jasmonic acid (JA) synthesis is involved in
342 resistance. A relationship between the inductions of JA/SA pathways is supported by
343 previous results for Hx activity in tomato against *P. syringae* (Scalschi et al., 2013).

344 The activation of defense pathways also shows differences in accordance with the mode
345 of application and the time that elapsed after treatment. Hx and ASM produced a quicker
346 *PR2* response in the spray-treated plants, but gene expression was higher in the soil drench-
347 treated plants after the second inoculation. This elevated *PR2* expression after the ASM
348 application was previously described by Francis et al. (2009).

349 Enhanced callose deposition in Hx soil drench-treated plants after the first inoculation
350 was correlated with the *CALS1* gene expression. This accumulation was not observed in the
351 Hx spray- and ASM-treated plants. Callose acts as a physical barrier that concentrates
352 antimicrobial compounds at fungal penetration sites (Huang et al., 2006). Lee et al. (2009)
353 proposed that callose also contributes to resistance against invading bacterial pathogens by
354 providing a physical barrier. Recently, Enrique et al. (2011) demonstrated in callose-
355 silenced citrus plants, that reducing callose levels weakened this barrier and led to enhanced
356 Xcc susceptibility compared to wild-type plants. Moreover, Yun et al. (2006) found that
357 callose is required for resistance to Xcc and that suppressing callose deposition induces
358 susceptibility to Xcc in *N. benthamiana* and *Arabidopsis*. In contrast, no accumulation of
359 callose in the Hx spray-treated plants was detected after the first inoculation or in any of the
360 treatments after the second inoculation. This lack of callose accumulation correlated with a

higher *PR2* expression, as previously found by Kaliff et al. (2007) for the *Arabidopsis abi 1-1* mutant.

In conclusion, Hx applications by spray or soil drench systemically reduced citrus canker lesions and viable Xcc populations with similar effectiveness to the commercial inducer ASM. Hx is a natural plant product with low phytotoxicity that has been demonstrated to be effective under greenhouse conditions, and now should be tested in field trials as previously reported for ASM (Graham and Myers, 2011, 2013). At this time, Hx has been patented and commercialized in Spain (Induct, Salquisa; Patent nº 200501535/0) for systemic resistance in tomato against Botrytis. The recommended doses of commercial compound in tomato are 2-3 L/Ha, which supposes a cost lower than 50$/Ha. Similar doses of application in citrus would suppose an effective protection with not excessively high prices. The toxicological data about this compound (http://pubchem.ncbi.nlm.nih.gov/) indicates low environmental risk, since in soil hexanoic acid was shown to biodegrade quickly in a variety of screening tests (Dore et al., 1975; Gaffney and Heukelekian, 1961; Mackay and Boethling, 2010), which suggest that the application of this compound in the field would not have side effects to the environment. Based on our results, the application of hexanoic acid could be used to augment current chemical inducers for citrus canker control to possibly reduce the number frequency of applications of copper bactericides.

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542

543 **Table 1.** Effect of a single spray application of hexanoic acid (Hx) and
544 acibenzolar-S-methyl (ASM) on bacterial population at 15 days after
545 inoculation with *Xanthomonas citri* subsp. *citri* (Xcc) at 10^5 cfu mL^{-1} in the
546 detached leaf assay. Means followed by the same letter are not significantly
547 different at P≤0.05 according to LSD test. P value lower than 0.05 indicates
548 differences between groups

	Log viable Xcc*	**Log total Xcc****
NTC	6.29a	7.99a
Hx 1 mM	3.97b	6.58b
Hx 3 mM	4.48b	7.08ab
ASM	3.78b	6.42b
P-value	0.0001	0.047

549 *Xcc recovered per inoculation site on KCC semi-selective medium

550 **Xcc detected per inoculation site by RT-qPCR

551 **Table 2.** Effect of spray or soil drench application of hexanoic acid (Hx) and
552 acibenzolar-S-methyl (ASM) on development of citrus canker lesions or
553 bacterial populations on sweet orange leaves inoculated with Xanthomonas citri
554 subsp. citri (Xcc) at 104 cfu mL−1 in the greenhouse assay. (A) 20 days after
555 first inoculation and (B) 20 days after second inoculation. Values represent the
556 average of three experiments. Numbers followed by the same letter are not
557 significantly different at P≤0.05 according to the LSD test. P value lower than
558 0.05 indicates differences between groups

A	Day 20 after first inoculation (4 weeks after treatments)		
	Lesion number	**Log viable Xcc***	**Log Total Xcc****
NTC	116a	6.96a	8.42a
Hx soil drench	57b	6.27a	8.85a
Hx spray	61b	6.67a	8.75a
ASM soil drench	36c	5.55a	8.85a
ASM spray	40bc	6.71a	8.43a
P value	0.0158	0.4465	0.7360

559

B	Day 20 after second inoculation (9 weeks after treatment)		
	Lesion number	**Log viable Xcc***	**Log Total Xcc****
NTC	138a	4.276a	8.33a
Hx soil drench	67b	3.67b	8.26a
Hx spray	71b	3.60b	8.39a
ASM soil drench	38c	3.48b	8.30a
ASM spray	60b	3.66b	8.35a
P value	0.0248	0.046	0.7091

560

561 *Xcc recovered per inoculation site on KCB semi-selective medium

562 **Xcc detected per inoculation site by RT-qPCR

563

564

Figure Captions

Figure 1. Growth of *Xanthomonas citri* subsp. *citri* (Xcc) in Luria Bertani broth amended with different concentrations of hexanoic acid in mM along 96 hours.

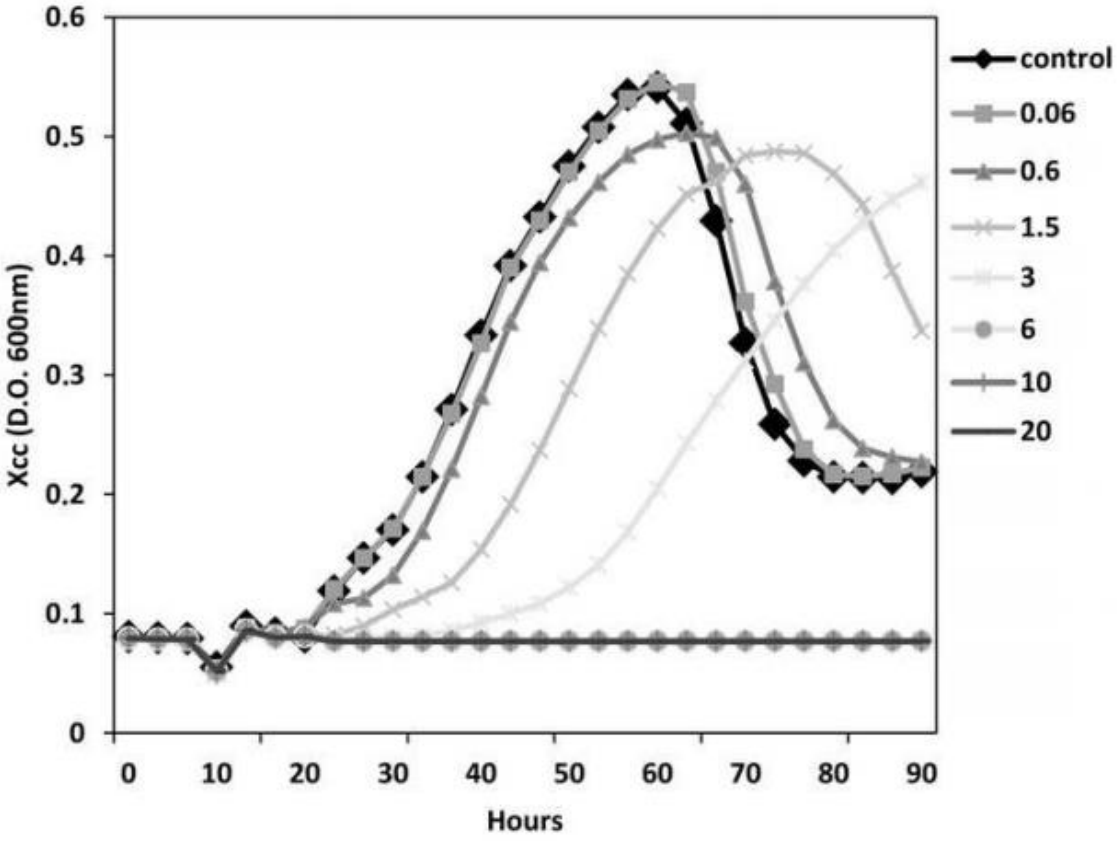

Figure 2. Effect of a single spray application of hexanoic acid (Hx) and acibenzolar-S-methyl (ASM) on development of citrus canker lesions in detached sweet orange leaves at 15 days after inoculation with *Xanthomonas citri* subsp. *citri* at 10^5 cfu mL^{-1} in the detached leaf assay. a) Hx at 1.0 mM, b) Hx at 3.0 mM, c) ASM, 1 mM and d) nontreated control.

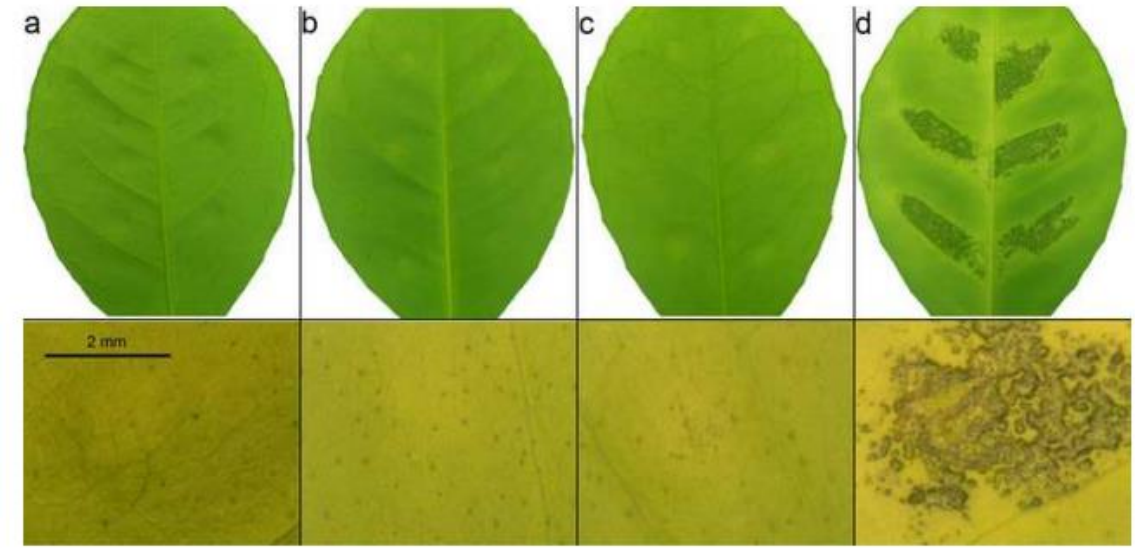

Figure 3. Effect on *AOS*, *PR2* and *PR5* gene expression relative to Actin and *18S* gene expression of spray or soil drench application of hexanoic acid (Hx) and acibenzolar-S-methyl (ASM) in sweet orange leaves inoculated with *Xanthomonas citri* subsp. *citri* at 10^4 cfu mL^{-1} in the greenhouse assay. Values represent the average of three experiments at 10 days and 20 days after first and second inoculations (4 and 9 weeks after treatment respectively) bars represent standard error of the mean values, and different letters represent significant differences for each time point at $P \leq 0.05$ according to LSD test. Relative expression lower than 2 (without letter) is considered not differential expression

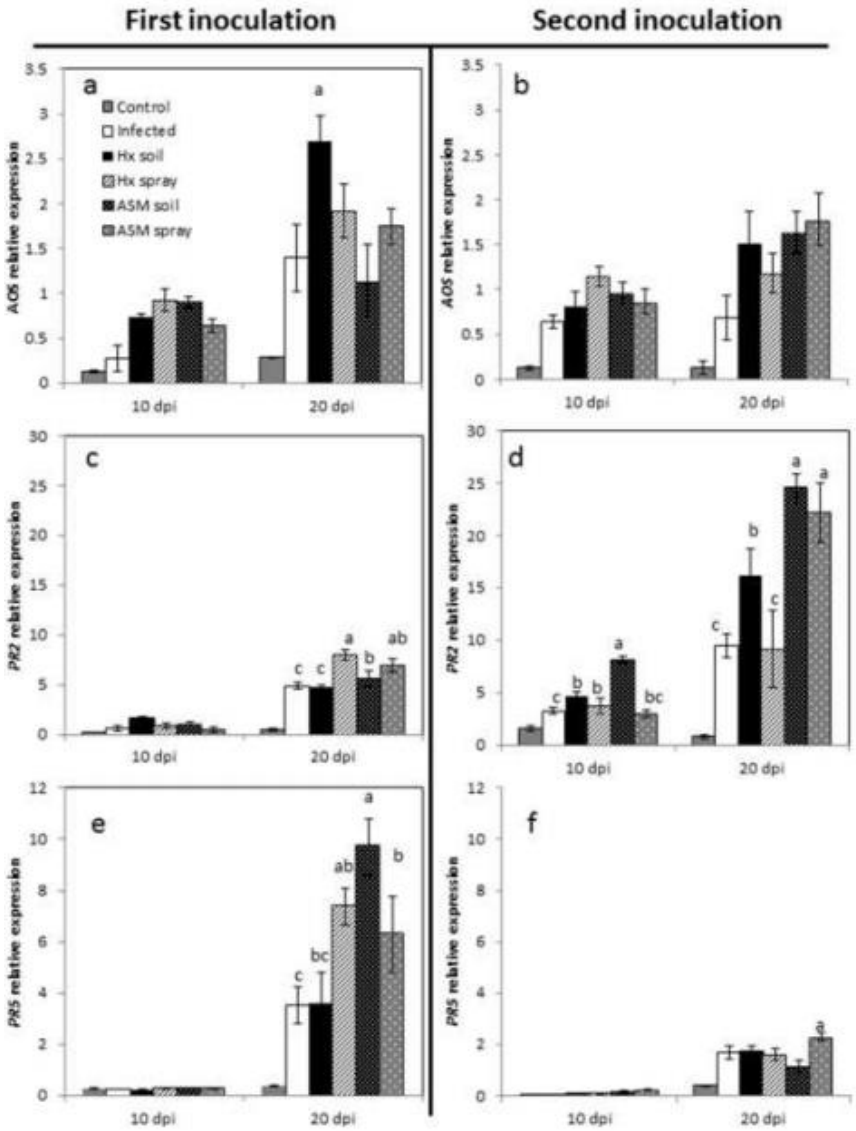

Figure 4. Effect of spray or soil drench application of hexanoic acid (Hx) and acibenzolar-S-methyl (ASM) at 20 days after first and second inoculation (4 and 9 weeks after treatment respectively) on (a) callose accumulation (Readings were calculated from the average number of blue pixels in the images), (b) *CALS1* gene expression relative to actin and *18S* gene expression of in sweet orange leaves inoculated with *Xanthomonas citri* subsp. *citri* at 10^4 cfu mL^{-1} in the greenhouse assay. Values represent the average of three experiments, bars represent standard error of the mean values, and different letters represent significant differences in each time point at $P \leq 0.05$ according to LSD test. Relative expression lower than 2 (without letter) is considered not differential expression