Hexanoic acid provides long-lasting protection in 'Fortune' mandarin against *Alternaria alternata*

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ABSTRACT

Alternaria brown spot disease is a serious disease in mandarins and their hybrids without effective disease control measures. In recent years, induced plant resistance has been studied as an alternative to classical pesticides, but few studies on the effectiveness of these products and their long-lasting effects in woody crops have been performed. After two inoculations with *Alternaria alternata*, citrus plants that were treated with hexanoic acid showed enhanced resistance, displaying lower levels of disease incidence associated with an activation of the jasmonic acid pathway, the accumulation of phenolic compounds and the expression of defensive genes, such as polygalacturonase-inhibiting proteins.

KEYWORDS: 'Fortune' mandarin, Induced resistance, Plant defense, Alternaria brown spot, long-lasting protection

1. INTRODUCTION

The necrotrophic fungus *Alternaria alternata* (Fr.) Keissl. pv. *citri* is the cause of *Alternaria* brown spot disease. This fungus can attack many types of tangerines and their hybrids, damaging leaves, twigs, and immature fruit [1]. The toxin that is released by the pathogen is primarily active in "Dancy" mandarin and its hybrids, such as the 'Fortune' (Clementine × Dancy) mandarin, and in tangerine/grapefruit and tangerine/sweet orange hybrids [2]. The severity and lack of control of this fungus make growing susceptible varieties unprofitable. The field usefulness of any pesticide or protective compound is directly related to the persistence of its effect. There are no curative compounds against this pest; therefore, all means of control are preventive. Classical means of controlling *A. alternata,* such as copper (Cu) application, must be sprayed several times, and the number of applications sometimes exceeds 12 per cultivation cycle [2, 3] . Recent studies have shown that covering at least 50% of leaves with Cu is necessary to achieve disease control, but at a high inoculum pressure, 75% coverage may be required [4]. Current recommendations suggest the application of Cu every 15 days in sub-tropical areas or weekly sprays year-round in tropical areas with a high risk of infection [1]. Even with an increased number of sprays, the pathogen often cannot be completely controlled, and the use of sensitive cultivars becomes impractical.

Therefore, it is necessary to find efficient control alternatives to improve natural plant defense mechanisms in response to microbial pathogens and insect herbivores. The variety of responses depends on the nature of the pathogen and its mechanism of pathogenicity. The activation of some disease responses can be detrimental to plant growth. The first means of protection against pathogens is constitutive resistance, which consists of structural defenses, such as waxes or essential oils. When these barriers fail to prevent the entry of pathogens, plants activate a second level of defense responses called pathogen-induced resistance. Generally, these responses are controlled by plant hormones. The two main pathways, which are controlled by salicylic acid

(SA) and jasmonic acid (JA), can activate the defense responses against biotrophic and necrotrophic pathogens, respectively [5]. In addition, other compounds play an important role in signal transduction. For example, methyl salicylate or pipecolic acid have recently been identified as mobile signals for systemic resistance [6].

In recent decades, there has been reported increasing evidence that more efficient activation of cellular defense responses can be induced with xenobiotic compounds. This induction is associated with enhanced resistance to various biotic or abiotic stresses. This phenomenon, which is referred to as priming the defense, has been well characterized in a wide number of species [7-9]. The majority of studies on xenobiotic compounds and their effects are performed in a laboratory setting using model plants and not using crop plants. In addition, some chemical inducers, such as beta-aminobutyric acid, acibenzolar-S-methyl and neonicotinoid-based insecticides, can induce a long-lasting induction of defenses [10, 11]. Studies in *Arabidopsis* plants showed that the primed defense state can be maintained long after the initial stimulus, indicating a form of plant immunological memory [11].

Recently, we found that hexanoic acid (Hx) can protect *Arabidopsis* and tomato plants against *Botrytis cinerea* [12-14] and *Pseudomonas syringae* pv. *tomato* [9]. This natural short-chain monocarboxylic acid displays antimicrobial activities and can also induce plant defense responses when used as a priming agent. Upon infection, the oxylipin 12-oxo-phytodienoic acid (OPDA) and the bioactive molecule jasmonate-isoleucine were significantly induced in treated plants. Additionally, callose deposition was primed, and abscisic acid (ABA) acted as a positive regulator of hexanoic acid-induced resistance (Hx-IR) by enhancing callose accumulation [14].

The effectiveness of Hx as a systemic resistance inducer in woody plants has only been tested in citrus against *A. alternata* over short time periods [15], where it was able to reduce the number and size of lesions 5 days after inoculation and stimulate the defense pathways of citrus. The aim of this work is to evaluate the long-lasting effect of Hx in 'Fortune' mandarins against *A. alternate*, which may minimize the excessive use of harmful chemical pesticides and their effects on the environment.

2. MATERIAL AND METHODS

2.1 Plant material

For all of the experiments, we used 2-year-old 'Fortune' mandarin plants that were grafted onto Carrizo citrange plants and grown in a greenhouse in 10-L pots with substrate. One month before the commencement of each experiment, the leaves were removed to encourage uniform sprouting. The leaves with a size that was suitable for inoculation (75% expanded) were labeled and infected.

One week after the first inoculation, all of the leaves were removed again to force a new flush. Four weeks later, when the new leaves achieved the correct size, a second inoculation was performed in the same plants but without a new treatment. The second inoculation was performed 6 weeks after the Hx treatment.

2.2 Chemicals and inoculation procedures

Compounds were applied in a single application as a soil drench (500 ml of solution per pot). The timing of the treatments and their rates were chosen based on previous reports [15] of the effective dosages and timing of applications. In brief, hexanoic acid was applied 4 days before inoculation as a soil drench at 1 mM. In all of the experiments, untreated and inoculated plants were included as controls.

Spores of *Alternaria alternata* were collected from 10- to 15-day-old cultures with sterile water containing 0.02% (v/v) Tween-20. The solutions were then filtered, quantified with a hemocytometer, and adjusted to 10^5 spores/mL. The leaves were infected by dispensing 5 μL of the spore solution onto each leaf surface. After 48 and 96 h, the leaves were sampled.

2.3 Gene expression

100 A gene expression analysis by real-time quantitative PCR (RT-qPCR) was performed with
101 RNA samples that were extracted from leaf tissue using the E.Z.N.A. Total RNA Kit II (Omega
102 Bio-Tek; Norcross, GA. USA; http://www.omegabiotek.com). Citrus leaf tissue samples for
103 RNA isolation were collected at 0, 48, and 96 h post-infection (hpi), and tissues were collected
104 from both treated and non-treated plants. We used the RT-qPCR conditions that were previously
105 described by Flors et al. [16]. The primers that were used in the RT-qPCR were *CALs1* as
106 described by Enrique et al. [17], PGIP as described by Llorens et al. [15] and *AOS* and *GAPDH*
107 (as an internal standard) as described by Fernandez-Crespo et al. [18]

108 *2.4 Quantification of hormones and phenolic compounds*

109 The extractions and experimental procedures that were used in the hormone analysis were
110 performed as described by Erb et al. [19]. We analyzed the levels of JA, OPDA, ABA,
111 Chlorogenic acid and Caffeic acid using prostaglandin B1, dihydrojasmonic acid, [$^{2}H_{6}$]-ABA,
112 and propylparaben as internal standards.

113 *2.5 Detection of hexanoic acid in the soil*

114 To assess the perseverance of hexanoic acid in the soil, the plants were treated as described
115 above. Three sample soils were taken per pot using a soil-sampler tube (15×0.6 cm) at 0, 24,
116 48, 96 hours and 1, 2 and 3 weeks after soil treatment.

117 An Acquity ultra-performance liquid chromatography system (UPLC) (Waters, Milford,
118 MA, USA) was interfaced to a triple quadrupole mass spectrometer (TQD, Waters, Manchester,
119 UK). The solvent flow rate (90% H2O/10% Ethanol) was 0.3 ml min−1.

120 The calibration of ESI mass spectra performed by the direct infusion of hexanoic acid
121 showed an m/z ion of 115 in the corresponding negative ESI mass spectrum. At medium-high
122 collision energies (greater than 15 eV), no ion is observed because the compound is probably
123 disintegrated (data not shown). Therefore, we proceed to a targeted LC–MS (TQD) analysis
124 using a transition of 115 > 115 and a collision energy of 5 eV. Once confirmed, samples of

125 standard compound acid were injected onto an obtaining a retention time of 4.16 for hexanoic
126 acid.

127 The procedure for an efficient extraction was performed according to the methods that are
128 described in the literature for metabolomics analysis [20]. The efficiency of extraction was
129 corroborated by a comparison of chromatograms from 0-h treated soil and a standard of
130 hexanoic acid (supplementary material 1). The MassLynx NT version 4.1 (Micromass) software
131 was used to process the quantitative data from the calibration standards and plant samples.

132

133 *2.6 Statistical analyses*

134 The treatments were analyzed by a one-way ANOVA using Statgraphics centurion XVI.I
135 software (Statistical Graphycs Corp.), and the means were separated using Fisher's least
136 significant difference (LSD) at 95%. The treatments were 1: non-inoculated untreated plants
137 (data not shown), 2: non-inoculated Hx treated plants (control), 3: inoculated untreated plants
138 (inf), and 4: inoculated Hx treated plants (Hx inf). All of the experiments were repeated three
139 times with six plants per treatment. The figures show the average of three independent
140 experiments.

141

142 **3. RESULTS AND DISCUSSION**

143 *3.1 Protection mediated by Hx lasts 6 weeks*

144 Treatment with Hx reduces the number and size of the lesions that are produced by *A. alternata*.
145 The obtained results indicate that treating citrus plants with 1 mM Hx 4 days prior to the first
146 infection clearly reduced the disease incidence and led to smaller lesions at 96 hpi after the first
147 and second challenge infections. In both inoculations, the ratio of infected leaves to inoculated
148 leaves was nearly 30% lower in the treated plants compared to that of the non-treated plants,
149 achieving a protection rate of more than 50% in inoculated leaves (Table 1). The area of the

150 lesions was also lower in the plants that were infected and treated with Hx. Compared to those
151 of the non-treated plants, the lesions of the treated plants were 25% smaller after the second
152 infection (Table 1). The results obtained from the second inoculation are similar to those that
153 were obtained from the first, suggesting that only one application of Hx is necessary to protect
154 citrus against this fungus for at least 2 months. However, the long-lasting effects of natural
155 compounds in other citrus species have not been tested yet. In citrus, Francis et al. [10]
156 demonstrated that some neonicotinoids, such as Imidacloprid, and other resistance inducer
157 chemical compounds, such as acibenzolar-S-methyl, provided long-lasting protection against
158 citrus canker [10]. Additionally, the long-lasting protective effect of resistance inductors was
159 also described in *Arabidopsis* by Luna et al. [11], who observed trans-generational effects in the
160 progeny of plants that had repeatedly been infected with the virulent strain *P. syringae* pv.
161 *tomato* DC3000. In addition, Rasmann et al. [21] demonstrated that *Arabidopsis* and tomato that
162 were treated with JA or exposed to insect herbivory produce more resistant progeny against
163 caterpillar feeding. In citrus, long-lasting antibacterial effects of some natural compounds have
164 been described [22]. However, this is the first report of a long-lasting protective effect against a
165 fungal disease that is induced by a natural priming agent in citrus.

166 *3.2 Application of Hx induces a higher accumulation of phenolic compounds*

167 Phenols play an important role in plant defenses. Some phenols occur constitutively, whereas
168 others are formed in response to pathogen ingress and associate as part of an active defense
169 response in the host. The defensive activity of phenolic compounds is due to their direct toxic
170 effects on the pathogen and to their capacity to strengthen the cell wall [23]. Studies by Suárez-
171 Quiroz et al. demonstrated a strong antifungal activity of chlorogenic acid and various
172 derivatives thereof on the growth of several *Aspergillus* species[24]. In relation to induced
173 resistance, Lavania et al. [25] observed that phenolic compounds that are induced by plant
174 growth-promoting Rhizobacteria play an important protective role in *Piper betle* against
175 *Phytophthora nicotianae*. Direct evidence for decreases in fungal growth in tomato because of
176 phenolic profile changes in response to inoculation with *Verticillium alboatrum* is available in

177 the literature [26]. Our results demonstrated that Hx treatment increased the level of caffeic acid
178 at 48 h after the first inoculation, whereas chlorogenic acid increased only in the untreated
179 plants. At 96 h, the levels of both phenolic compounds had higher concentrations in Hx-treated
180 plants compared to those in control plants, but the level in the Hx-treated plants did not show
181 significant differences compared with to that in the untreated and infected plants (Fig. 1).
182 However, after the second inoculation, the results showed a higher accumulation of caffeic acid
183 in Hx-treated and infected plants compared to that in infected but not treated plants, and
184 chlorogenic acid showed, in general, an enhancement both in the treated and untreated plants
185 compared to that in the control plants. Moreover, studies in tomato revealed that increasing
186 amounts of chlorogenic acid reduced alternariol biosynthesis in a concentration-dependent
187 manner[27], limiting the ability to colonize the host plant[28]. These observations suggest that
188 cell wall reinforcement, which is mediated by caffeic and chlorogenic acid, in the Hx-IR could
189 be implicated in disease resistance.
190

191 *3.3 Hx enhances the defensive physical barriers*

192 To perform an in-depth study regarding the physical barriers that are involved in Hx-IR, the
193 expression levels of the polygalacturonase inhibiting protein (*PGIP*) and the callose synthase 1
194 (*CALs1*) gene were determined. *PGIP* is a ubiquitous plant cell wall protein that counteracts the
195 action of fungal polygalacturonase proteins by preventing cell wall degradation and interfering
196 with invasion. In this experiment, *PGIP* gene expression in infected plants was up-regulated
197 more rapidly in the Hx-treated plants (Fig. 2). After the first and second inoculations, higher
198 expression levels of *PGIP* were observed in the Hx-treated plants compared with to those in the
199 untreated plants. However, after the first inoculation, the expression level was higher at 48 h,
200 whereas after the second inoculation, higher levels were achieved at 96 h. In addition to other
201 mechanisms, the PGIP-endoPG interaction limits the ability of endoPG to allow pathogen
202 colonization of plants. The importance of PGIPs in plant defense has been demonstrated by
203 Ridley et al. [29]. In addition, *PGIP* overexpression mutants of both *Arabidopsis* [30] and

204 tomato [31, 32] have fewer symptoms and exhibit lower levels of *B. cinerea* colonization. In
205 citrus, the constitutive expression of PGIPs was detected in fruits. In leaves, infection with
206 *Alternaria sp*. showed an induced expression of PGIPs in response to the toxin, which might
207 play a role as an elicitor[33]. Some studies suggest that the resistance of citrus may be directly
208 related to the level of PGIP gene expression because fruits with constitutive *PGIP* expression,
209 such as Clementine and Sour orange, are resistant, whereas Clementine's leaves are slightly
210 susceptible and show disease symptoms[34]. In agreement with these results, our experiment
211 suggests that the enhanced expression of the *PGIP* gene in the treated plants after both
212 inoculations could play a major role in the protection mediated by Hx against *A. alternata.*

213 Another characteristic cellular response of early post-invasive defenses that occurs on the inner
214 surface of the epidermal cell wall is papillae accumulation (Fig 3a, b). Papillae are composed
215 mostly of callose, an amorphous high-molecular-weight 1,3-glucan, with other minor
216 constituents [35]. Callose acts as a physical barrier or as a matrix that concentrates antimicrobial
217 compounds at attempted sites of fungal penetration [36]. Previous studies have highlighted the
218 implication of enhanced callose deposition in the resistance that is mediated by hexanoic
219 acid[14, 37]. In the present experiment, after the second inoculation, we did not observe
220 differences in the accumulation of callose between the treated and untreated plants (Fig. 3a, b).
221 To confirm the results that were observed in the enhancement of callose deposition, we also
222 analyzed the expression of the *CALs1* gene. Figure 3b shows the higher expression level of this
223 gene only in the Hx-treated plants after the first inoculation, whereas after the second
224 inoculation, no significant differences were observed. The non-activation of callose synthesis
225 after the second infection may be related to the promotion of other defense mechanisms, such as
226 the reinforcement of the cell wall. Induced lignification is a known active resistance mechanism
227 of plants to fungi[38]. Wall reinforcement makes difficult pathogen invasion, modifying cell
228 walls to be more resistant to cell wall-degrading enzymes and producing toxic precursors and
229 free radicals[39]. The accumulation of *PGIP* and phenolic compounds could improve the

230 resistance of the cell wall, limiting the ability of fungus to infect the plant and delaying callose
231 accumulation.

232

233 *3.4 Hormone response induced by Hx differs after the second inoculation*

234 Resistance to necrotrophic pathogens depends on complex signaling pathways, involving the
235 major plant phytohormones JA, ethylene, and ABA. However, SA does not play a major role in
236 resistance against these pathogens [40]. As previously described in our laboratory [9, 14, 15],
237 the accumulation of OPDA was higher in the Hx-treated plants than in the non-treated plants,
238 suggesting that in the first step of infection, the JA pathway plays an important role in the
239 protection of plants (Fig 4). At 48 h after the first inoculation, the levels of JA and OPDA were
240 higher compared to those in the untreated plants, indicating an enhancement of disease defenses,
241 which prepare the plants to fight infection. However, after the second inoculation, no significant
242 differences were observed between the treated and untreated plants. The results that were
243 obtained in the hormone analysis were corroborated by a study of the *AOS* gene. After the first
244 inoculation (Fig. 5), an enhancement of *AOS* expression at 48 h only in the treated plants was
245 observed. However, after the second inoculation, the expression level of this gene was higher in
246 both the treated and untreated plants than that in the control plants, showing a constant up-
247 regulation of the JA pathway.

248 Our results after the first inoculation showed a high ABA response at 48 hpi in the treated and
249 untreated plants. However, at 48 and 96 h, no difference between the treatments was observed.
250 Nevertheless, after the second inoculation, the levels of ABA increased more slowly than after
251 the first inoculation and were higher at 96 h but without significant differences between the
252 treated and untreated plants.

253 The role of the JA pathway in resistance against necrotrophs has been widely studied [41], and
254 its implication in Hx-IR was previously reported by Vicedo [14]. Our results showed a known
255 role of the JA/ABA pathway in response to necrotrophic pathogens that was enhanced after the

first inoculation by the application of Hx. The increased levels of JA and OPDA that were observed in the treated and untreated plants after the second inoculation promote a high level of protection in plants compared to the lesions that were observed after the first inoculation. However, the lower incidence of infection in the Hx-treated plants after the second inoculation compared with to that in the infected and untreated plants could be due to a synergistic combination of phenolic compound accumulation, the expression of PGIP, and the up-regulation of the JA pathway.

3.5 Hexanoic acid does not remain in the soil.

To test the persistence of hexanoic acid in the soil, we performed HPLC detection. Previous studies indicated that hexanoic acid is expected to have very high mobility in the soil based on the coefficient of partition of organic carbon (24 for agricultural soil with pH 6.7 and 1.25% organic carbon)[42]. Furthermore, the pKa of hexanoic acid is 4.88[43], indicating that this acid will exist primarily as an anion under environmental conditions, and anions generally possess greater mobility in soils than do neutral compounds[44]. Moreover, previous studies demonstrated that hexanoic acid biodegrades quickly in a variety of screening tests [45, 46]. Our results showed that this compound disappears rapidly from the soil in the first 96 hours (Fig. 6). In addition, only one week after treatment, the level of hexanoic acid in the treated soil was similar to the level measured in the untreated soil. This result suggests that the observed effect of this compound as a resistance inducer remains in the plant, discarding the possibility that long-term protection was produced by the compound remaining in the soil.

4. **CONCLUSIONS**

The obtained results indicate that the application of Hx reduces the incidence of *A. alternata* in 'Fortune' mandarin trees, and its effect is long lasting enough to protect the plants for 2 months with only one application. The observed effect of Hx after the first inoculation indicates the early enhancement of the JA pathway, leading to callose deposition. After the second inoculation, defensive hormonal pathways are up-regulated in both the treated and untreated

plants, suggesting a remaining effect of the first inoculation, which is observed in the reduction of lesions in all treatments. However, Hx remains active in the plant, leading to an enhancement of *PGIP* expression levels and phenolic compounds. This enhancement of defensive barriers provides an effective reduction in lesions, achieving a lower susceptibility against *A. alternata* than that observed in the untreated plants. The observed reduction in the rate of infection could be enough to protect citrus plants against low-to-mid inoculum pressure, providing a long-lasting alternative to classical control measures. The use of this natural compound in an integrated pest-management system could reduce the application of Cu, making this application necessary only against the threat of high inoculum pressure and optimal conditions for infection.

ACKNOWLEDGMENTS

This work was supported by the "Programa de formació del personal investigador" of the Universitat Jaume I, the Spanish National R&D Plan (AGL2010-22300-C03-01 and AGL2010-22300-C03-02), and Prometeo 2012/066. The authors are grateful to the SCIC at the Universitat Jaume I.

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420

421 TABLES

422

423 Table 1: Effect of hexanoic acid (Hx) on 'Fortune' mandarins that were infected (Inf) with *Alternaria*
424 *alternata*. The necrotic area that was measured at 96 h post-inoculation is expressed in mm^2. The number
425 of infected leaves is expressed as a percentage. The data show the average of three independent
426 experiments as obtained with 10 plants per point ± SE. An asterisk (*) in a row represents a statistically
427 significant difference ($P < 0.05$).

428 FIGURES

429 Figure 1. Phenolic compound levels in 'Fortune' mandarin controls, *Alternaria alternata* infected, and *A.*
430 *alternata* infected and hexanoic acid treated after infection. Leaves were collected at 48 and 96 h after
431 each inoculation. The a) caffeic and b) chlorogenic levels were determined in freeze-dried material by
432 HPLC-MS. The data show the average of three independent experiments of a pool of 10 plants per
433 experiment ± SE.

434

435 Figure 2. Relative levels of polygalacturonase-inhibiting protein (*PGIP*) as analyzed in 'Fortune'
436 mandarin controls, *Alternaria alternata* infected, and *A. alternata* infected and hexanoic acid treated.
437 Total RNA was isolated from the leaves at 48 and 96 h after each inoculation, converted into cDNA, and
438 subjected to an RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression that was
439 measured in the same samples. The data show the average of three independent experiments as obtained
440 with a pool of 10 plants per point ± SE.

441

442 Figure 3. a) Callose deposition detail. b) Relative levels of *CALs1* as analyzed in 'Fortune' mandarin
443 controls, *Alternaria alternata* infected, and *A. alternata* infected and hexanoic acid treated. Total RNA
444 was isolated from the leaves at 96 h post-inoculation, converted into cDNA, and subjected to an RT-
445 qPCR analysis. The results were normalized to the *GAPDH* gene expression that was measured in the

same samples. The data show the average of three independent experiments as obtained with a pool of 10 plants per point ± SE.

Figure 4. Hormone levels in 'Fortune' mandarin controls, *Alternaria alternata* infected, and *A. alternata* infected and hexanoic acid treated after infection. Leaves were collected at 48 and 96 h after each inoculation. The a) JA, b) OPDA, and c) ABA levels were determined in freeze-dried material by HPLC-MS. The data show the average of three independent experiments of a pool of 10 plants per experiment ± SE.

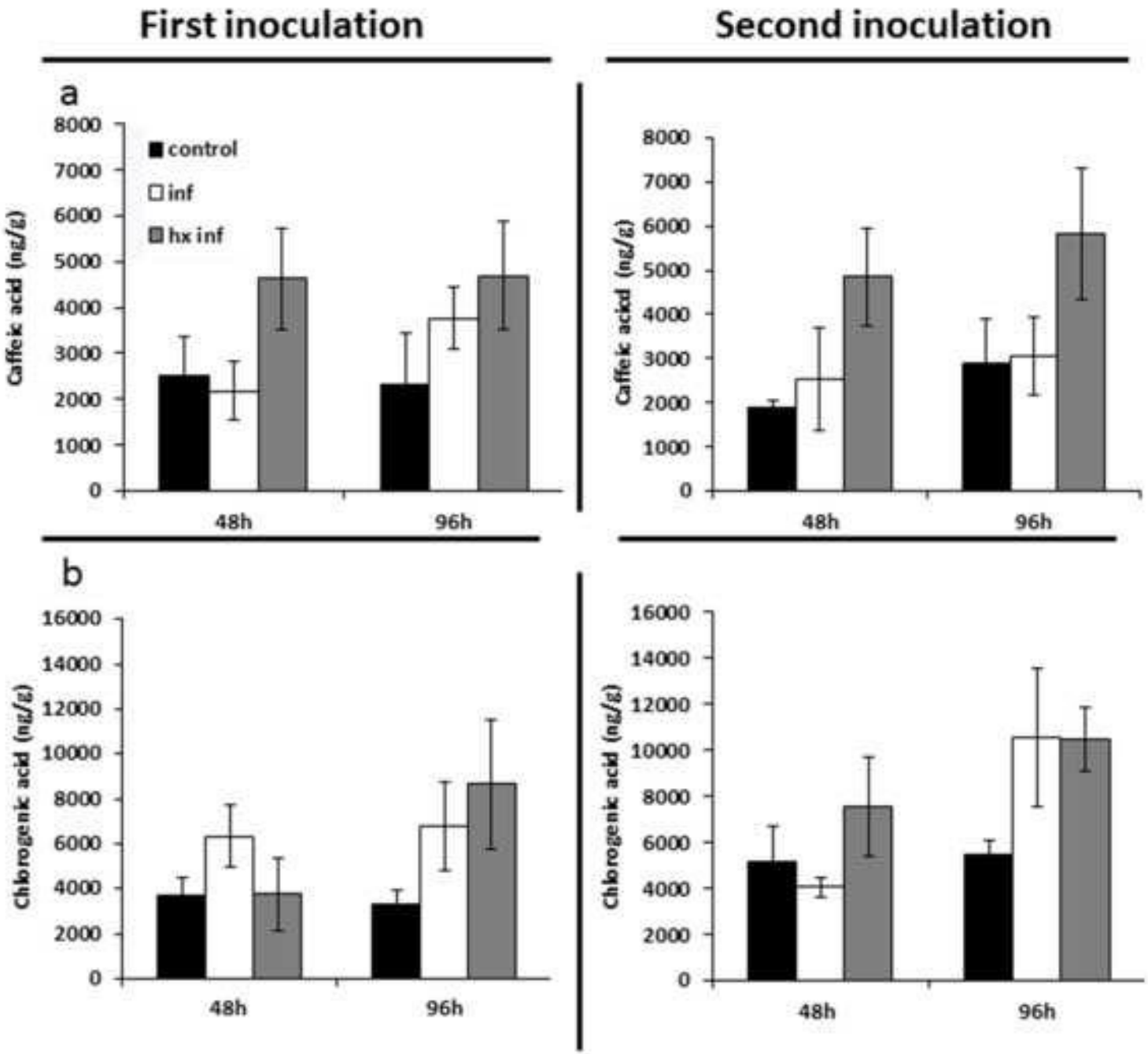
Figure 5. Relative levels of allene oxide synthase *(AOS)* as analyzed in 'Fortune' mandarin controls, *Alternaria alternata* infected, and *A. alternata* infected and hexanoic acid treated. Total RNA was isolated from the leaves at 48 and 96 h after each inoculation, converted into cDNA, and subjected to an RT-qPCR analysis. The results were normalized to the *GAPDH* gene expression that was measured in the same samples. The data show the average of three independent experiments as obtained with a pool of 10 plants per point ± SE.

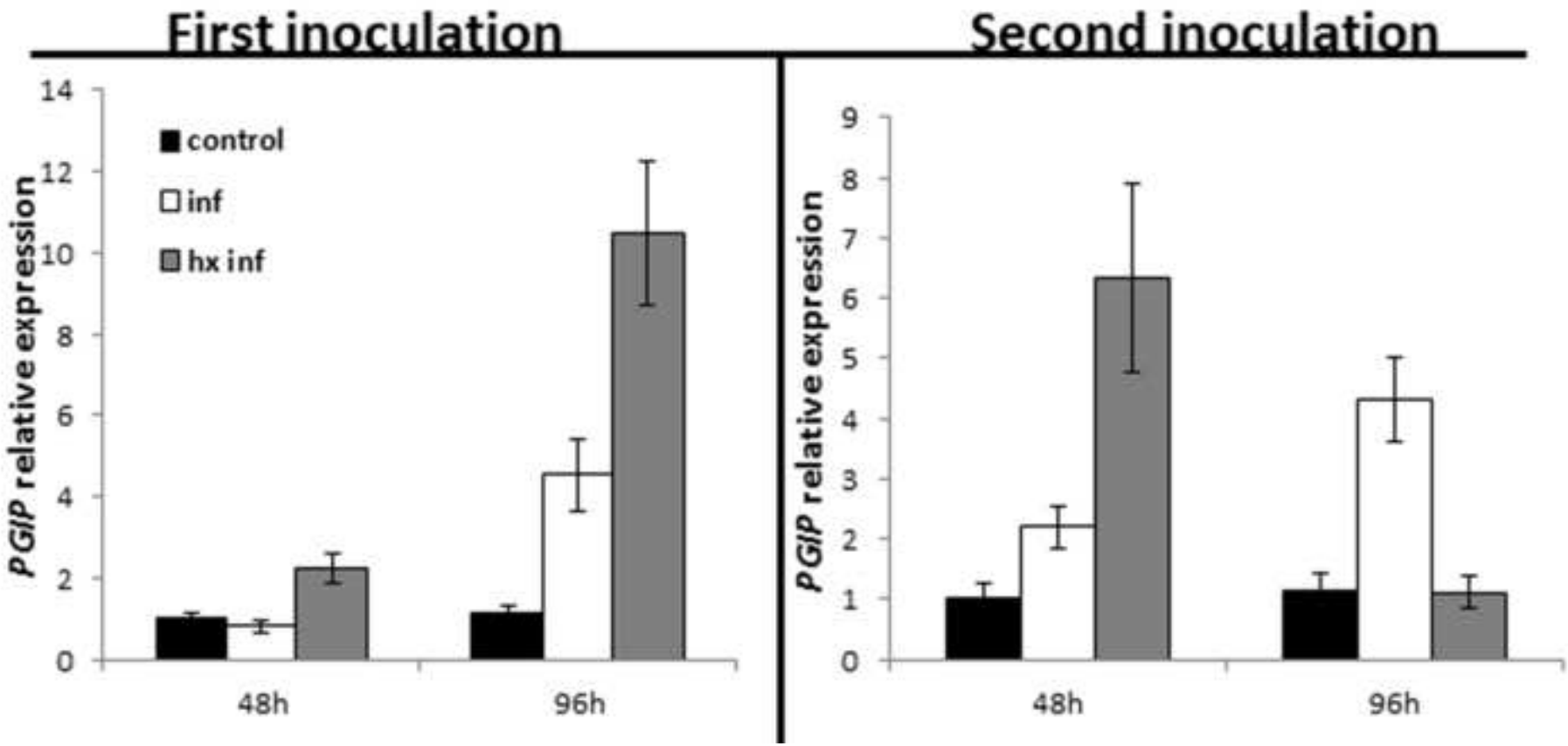
Figure 6. Hexanoic acid present in the soil after treatments. Soil samples were collected at 0, 24, 48 and 96 h and 1, 2 and 3 weeks after treatment (168, 336 and 504 h, respectively). The data show the average of three independent experiments of a pool of 10 soil samples per experiment ± SE.

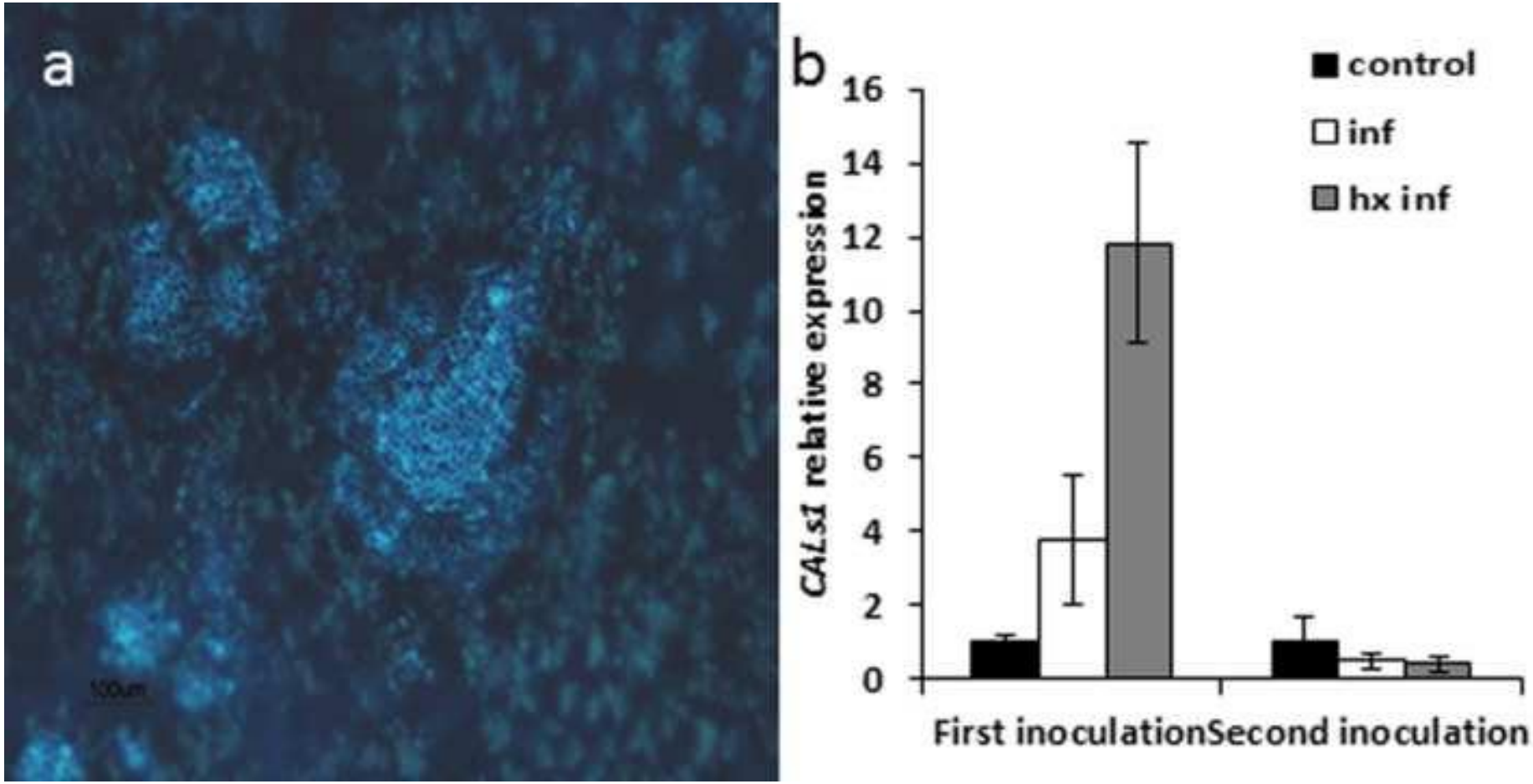
Supplementary figure 1. Comparative retention time of chromatograms of (A) hexanoic acid standard, (B) treated soil sample and (C) control soil sample.

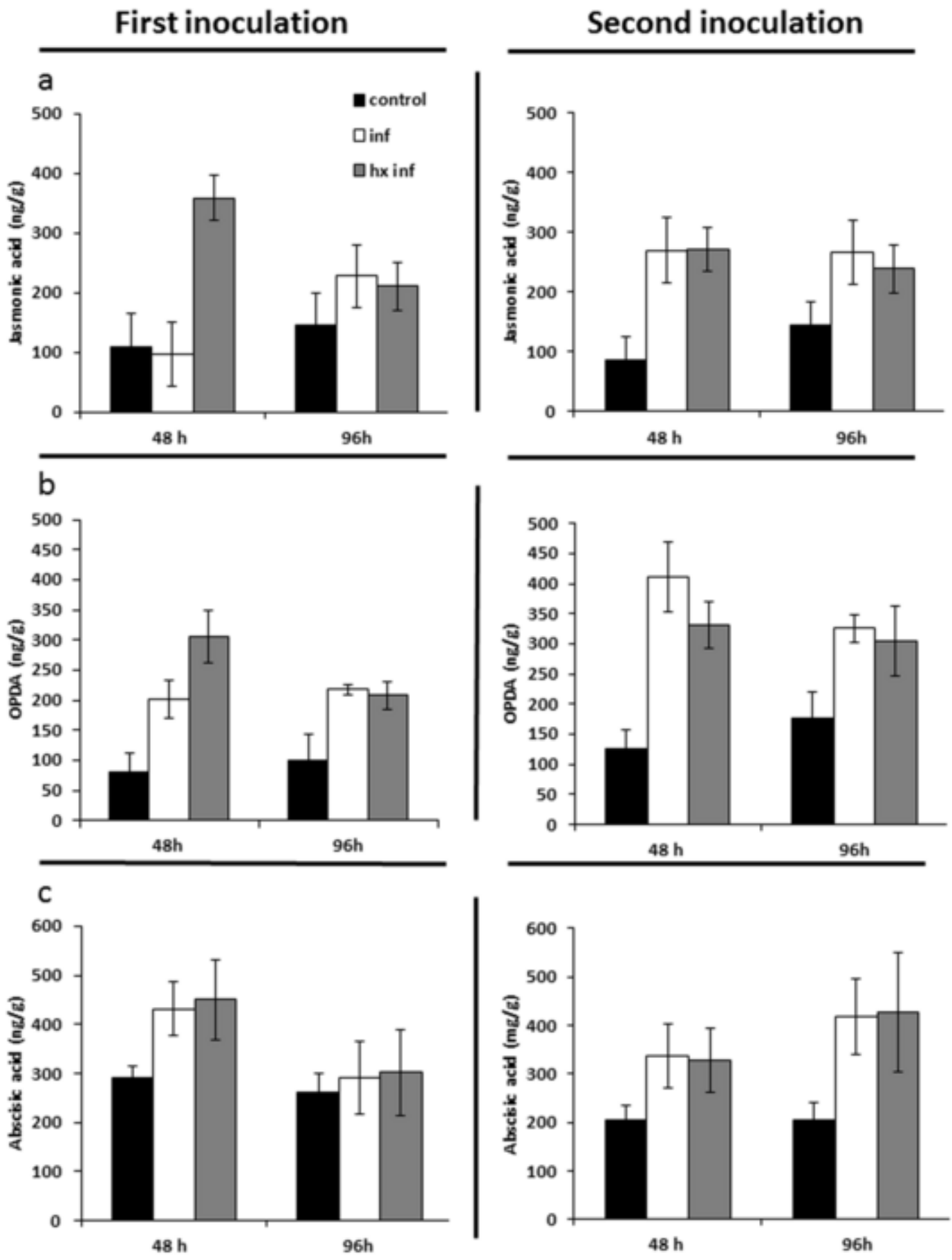
471

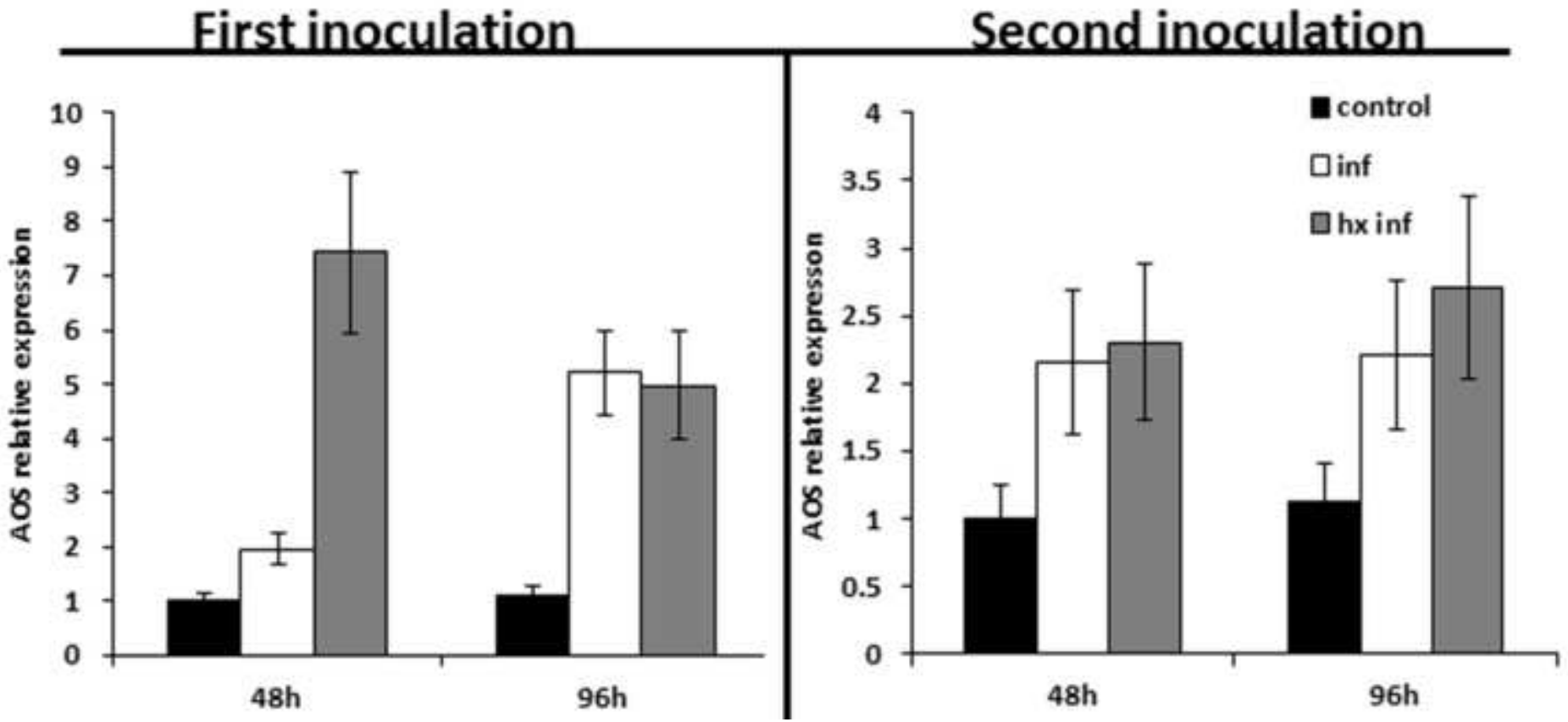
472

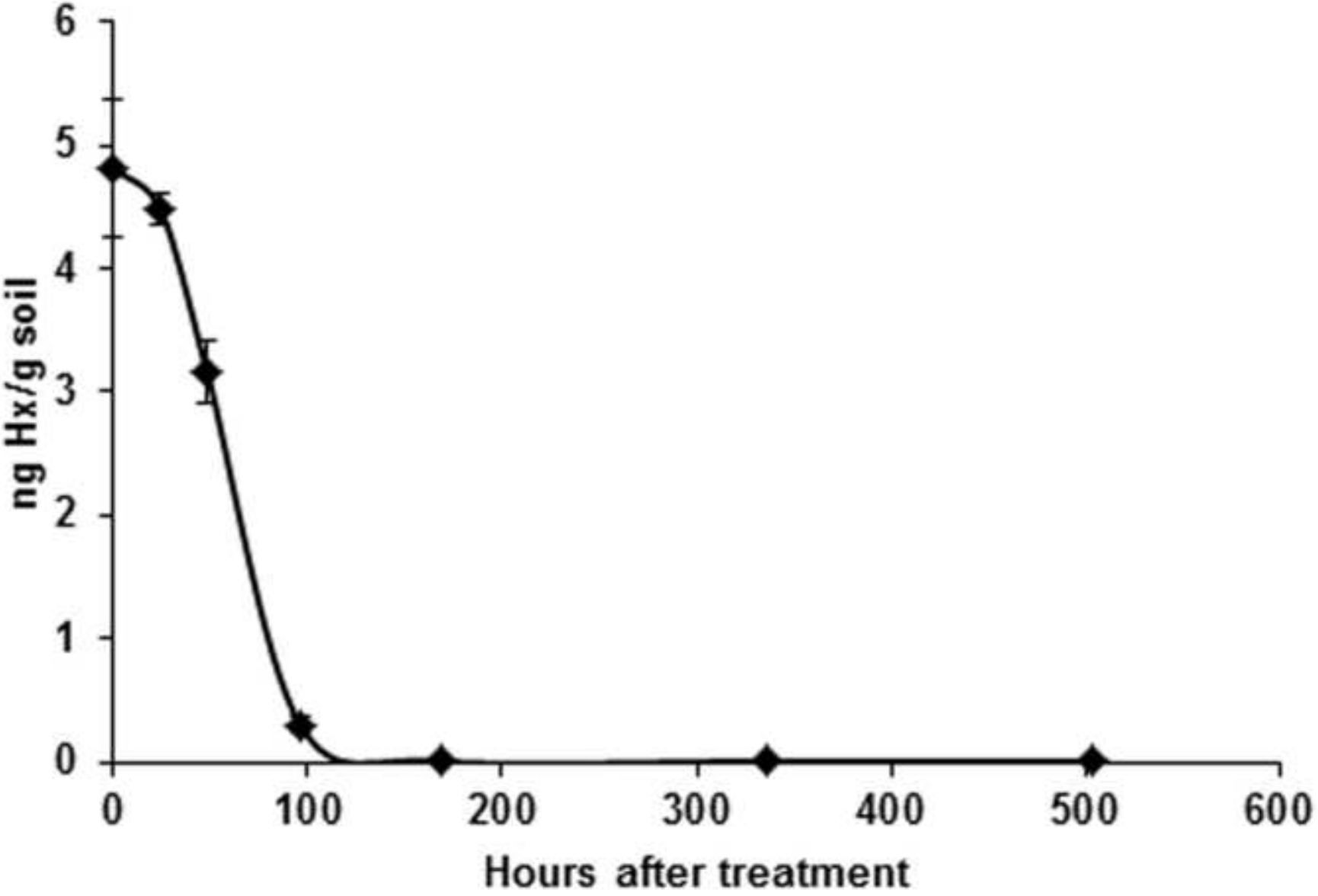

First inoculation
Second inoculation
a
b
control
inf
hx inf
Caffeic acid (ng/g)
Chlorogenic acid (ng/g)
0
1000
2000
3000
4000
5000
6000
7000
8000
10000
12000
14000
16000
48h
96h


First inoculation
Second inoculation
PGIP relative expression
control
inf
hx inf
0
2
4
6
8
10
12
14
48h
96h
PGIP relative expression
0
1
2
3
4
5
6
7
8
9
48h
96h


a
b
control
inf
hx inf
CALS1 relative expression
16
14
12
10
8
6
4
2
0
First inoculation
Second inoculation


First inoculation
Second inoculation
a
control
inf
hx inf
Jasmonic acid (ng/g)
500
400
300
200
100
0
48 h
96h
b
OPDA (ng/g)
500
450
400
350
300
250
200
150
100
50
0
48h
96h
c
Abscisic acid (ng/g)
600
500
400
300
200
100
0
48 h
96h


First inoculation
Second inoculation
AOS relative expression
10
9
8
7
6
5
4
3
2
1
0
48h
96h
AOS relative expresson
4
3.5
3
2.5
2
1.5
1
0.5
0
control
inf
hx inf
48h
96h


6
5
4
3
2
1
0
ng Hx/g soil
0
100
200
300
400
500
600
Hours after treatment

First inoculation

	Necrotic area (mm^2)	Infected leaves (%)
Hx	3.3+0.2*	45.33+4.27*
Inf	4.8+0.5	77.63+11.33

Second inoculation

	Necrotic area (mm^2)	Infected leaves (%)
Hx	1.4+0.1*	31.72+8.57*
Inf	2.1+0.3	60.83+10.61

Table 1. Effect of hexanoic acid on 'Fortune' mandarins infected (Inf) with *Alternaria alternata* and treated and infected (Hx). The necrotic area measured at 96 h post-inoculation is expressed in mm^2. The number of infected leaves is expressed as a percentage. Data show the average of three independent experiments obtained with 10 plants per point ± SE. Asterisk (*) in a row represent statistically significant differences ($P < 0.05$).