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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.

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

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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 *Alternaria alternata*, 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 *A. 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

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

2.4. Quantification of hormones and phenolic compounds

The extractions and experimental procedures that were used in the hormone analysis were performed as described by Erb et al. [19]. We analyzed the levels of JA, OPDA, ABA, Chlorogenic acid and Caffeic acid using prostaglandin B1, dihydrojasmonic acid, [$^2H_6$]-ABA, and propylparaben as internal standards.

2.5. Detection of hexanoic acid in the soil

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

An Acquity ultra-performance liquid chromatography system (UPLC) (Waters, Milford, MA, USA) was interfaced to a triple quadrupole mass spectrometer (TQD, Waters, Manchester, UK). The solvent flow rate (90% H_2O/10% Ethanol) was 0.3 ml min^{-1}.

The calibration of ESI mass spectra performed by the direct infusion of hexanoic acid showed an m/z ion of 115 in the corresponding negative ESI mass spectrum. At medium–high collision energies (greater than 15 eV), no ion is observed because the compound is probably disintegrated (data not shown). Therefore, we proceed to a targeted LC–MS (TQD) analysis using a transition of 115 > 115 and a collision energy of 5 eV. Once confirmed, samples of standard compound acid were injected onto an obtaining a retention time of 4.16 for hexanoic acid.

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

2.6. Statistical analyses

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