Bacteriology

Foliar Applications of Microbe-Associated Molecular Pattern Molecules Enhance Citrus Resistance Against Huanglongbing

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Abstract

Citrus huanglongbing (HLB), associated with '*Candidatus* Liberibacter asiaticus' (CLas), a biotrophic pathogen, is a devastating citrus disease worldwide. Enhancing host plant resistance through elicitor treatments has been exploited for crop disease management. HLB was suggested to be a pathogen-triggered immune disease in HLB-susceptible varieties. Here, we hypothesized that boosting citrus immunity to CLas with biotic elicitors, including microbe-associated molecular patterns (MAMPs), can increase citrus resistance to HLB. In greenhouse assays, foliar spray of chitosan, harpins, or lipopolysaccharides (LPSs) activated sweet orange plant immunity in a dosage-dependent manner. Peak expression levels of citrus defense genes including *FRK1*, *MKK4*, *PAL1*, *WRKY22*, and *WRKY29* were recorded at 12 to 72 h posttreatment, and chitosan and harpins induced stronger gene expression than LPSs. Additionally, all the three MAMPs elicited reactive oxygen species (ROS) burst in treated leaves, and both chitosan and harpins induced a higher level of ROS than LPSs. Moreover, chitosan and harpins, sprayed weekly at 1.0 and 0.25 mg/ml, respectively, significantly reduced CLas growth in sweet orange trees and suppressed plant growth. In a 2-year field trial under natural CLas infection conditions, foliar spray of chitosan, harpins, or salicylic acid monthly delayed CLas infection and slowed down the increase of CLas titer without suppressing plant growth. This study demonstrated that promoting immunity can be used as a useful strategy in controlling a pathogen-triggered immune disease caused by a biotrophic pathogen.

Keywords: citrus greening, disease management, elicitor, immunity, Liberibacter, MAMPs, plant resistance

Citrus Huanglongbing (HLB), also known as citrus greening disease, is one of the most economically important citrus diseases worldwide (Graham et al. 2024; Wang et al. 2024). All commercial citrus varieties are susceptible to HLB, and the citrus industries in affected areas have suffered a decline in both production and profit (Graham et al. 2020; Wang et al. 2017). In the United States, since it was first identified in Florida in 2005 (Halbert 2005), HLB has expanded to Alabama, California, Georgia, Louisiana, South Carolina, Puerto Rico, Texas, and the U.S. Virgin Islands (USDA APHIS 2026). Particularly, in Florida, HLB is now present in all commercial citrus-producing counties and is devastating the $9 billion citrus industry (Graham et al. 2020). It was estimated that HLB played a key role in the dramatic reduction of citrus acreage in Florida from 748,555 acres (302,929 ha) in 2004 to 274,705 acres (111,169 ha) in 2024 and reduction of production from 8.5 to 0.8 million metric tons between 2005 and 2024, a 90% decline (USDA NASS 2023). HLB is also a significant threat to the citrus industries in California and Texas (Graham et al. 2020).

Globally, citrus HLB is associated with three Liberibacter species: '*Candidatus* Liberibacter asiaticus' (CLas), '*Ca*. L. africanus', and '*Ca*. L. americanus' (da Graça et al. 2016; Garnier et al. 1984; Jagoueix et al. 1994). All three species are biotrophic, phloem dwelling, and insect vectored. There are two psyllid species transmitting Liberibacters: Asian citrus psyllid (ACP, *Diaphorina citri*) in Asia and the Americas (Bové 2006; Halbert 2005) and African citrus psyllid (*Trioza erytreae*) in Africa (Bové 2006). CLas and ACP are the most prevalent and important pests throughout HLB-affected citrus-growing areas worldwide (Bové 2006). During feeding, CLas-infected psyllids transmit the bacteria into the leaf phloem, and the bacteria multiply locally and eventually spread throughout the tree, including the root, although not evenly (Bové 2006; Pandey et al. 2021), leading to a systemic infection. CLas infection of HLB-susceptible citrus varieties causes detrimental effects on host phloem tissues, including physical blockage, thickening of phloem vessel walls, and phloem cell death due to chronic immune responses, including production of reactive oxygen species (ROS) and phloem callose deposition, leading to the proposing of a pathogen-triggered immune disease model (Ma et al. 2022). Huang et al. (2026) further provided genetic evidence for such a model. The resulting phloem dysfunction is responsible for the development of HLB symptoms, including blotchy mottled leaves, reduced canopy volume and density, root dieback, lopsided and malformed fruit, increased preharvest fruit drop, lower juice quality, and a shortening of the life of the tree (Achor et al. 2026; Bové 2006; Graham et al. 2020).

Due to the absence of resistant citrus varieties, cost-effective and environmentally sustainable approaches that can successfully control HLB remain unavailable (Li et al. 2020). Currently, in citrus-producing regions in which HLB is sporadic and the disease

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pressure is low, an integrated control program, including control of psyllid vectors with insecticides, removal of inoculum sources through removing HLB-symptomatic trees, and prevention of transplanting infected trees by propagating and using pathogen-free budwood and nursery trees, is employed to control HLB (Yuan et al. 2021), whereas in regions such as Florida, where HLB has become endemic and disease pressure is high, trunk injection of the antibiotic oxytetracycline has been adopted by many growers to improve the health and yield of HLB-affected trees, although more research is needed to optimize the rate and timing of oxytetracycline application (Graham et al. 2024; Hu and Wang 2016; Li et al. 2019). However, there have been concerns about antibiotic use in citrus production, especially regarding the environmental effects and the potential impacts on public health related to antibiotic resistance (McKenna 2019). Therefore, alternative methods that are environmentally sound and harmless to humans are desired to control citrus HLB. Multiple antimicrobial peptides have shown a positive control effect against HLB. For instance, a stable antimicrobial peptide from Australian finger lime has shown dual functions of inhibiting CLas growth in HLB-positive trees and activating host immunity to prevent new infections (Huang et al. 2021). A small microbial peptide named APP3-14 was shown to effectively reduce HLB symptoms in greenhouse tests and in a field trial with a limited number of trees (Zhao et al. 2025). Overall, more research is needed to develop sustainable management strategies for citrus HLB, such as development of HLB-resistant/-tolerant citrus varieties using traditional and biotechnological approaches (Huang et al. 2023; Liu et al. 2025; Su et al. 2023).

Plants have evolved sophisticated immune systems to confront pathogen attack (Jones and Dangl 2006; Jones et al. 2024). The plant defense mechanisms are activated upon the recognition of microbe-associated molecular patterns (MAMPs)/pathogen-associated molecular patterns by the plant cell surface-located pattern recognition receptors (PRRs), triggering a complex set of responses known as pattern-triggered immunity (PTI), including production of ROS, Ca^{2+} influxes, activation of protein kinases, cell wall reinforcement, and defense-related genes (Bigeard et al. 2015). Typical MAMPs include bacterial flagellin, harpin proteins, and lipopolysaccharides (LPSs), as well as chitin, a primary component of fungal cell walls, and its closely related derivative, chitosan (i.e., deacetylated chitin) (Newman et al. 2013). These MAMPs, along with damage-associated molecular patterns including oligogalacturonides from plant cell walls, are also called elicitors and induce similar defense responses in given plant species (Jones et al. 2024). For instance, cold shock protein (csp22) (Ngou et al. 2025), flagellin (flg22), and chitin are known to induce immune responses in citrus and its relatives (Trinh et al. 2023). Chitosan also induces citrus defense responses (Coqueiro et al. 2015).

Interestingly, distinct MAMPs can activate convergent signaling pathways, leading to the observations of overlaps in the PTI-associated genes induced by different MAMPs (Couto and Zipfel 2016; Lee et al. 2024). Some of those genes are conserved in plants and are termed PTI marker genes. Examples include *FRK1*, *GST1*, *MKK4*, *PAL1*, *WRKY22*, and *WRKY29*. In Arabidopsis, *FRK1* encodes the flg22-induced receptor kinase1 and is upregulated by both flg22 and small molecular surrogates, and some surrogates induce *FRK1* expression in the *fls2* mutant, suggesting FLS2 receptor-independent signaling pathways (Lee et al. 2024). Similarly, *GST1*, encoding the glutathione S-transferase 1, is rapidly induced by elicitors of different types of plant fungal and bacterial pathogens (Malnoy et al. 2006). *MKK4*, encoding the mitogen-activated protein kinase kinase 4 and acting redundantly with *MKK5*, is a key positive regulator in PTI and is responsive to flg22 (Asai et al. 2002) and chitin (Kawasaki et al. 2017) in Arabidopsis and rice, mediating resistance against bacterial and fungal pathogens. *PAL1*, encoding the phenylalanine ammonia-lyase 1 involved in the phenylpropanoid pathway, which is responsible for biosynthesis of plant defense-related secondary metabolites such as phenolic compounds, including salicylic acid (SA) (La Camera et al. 2004), is upregulated by chitin in soybean (Li et al. 2025) and by laminarin in tobacco (Klarzynski et al. 2000). *WRKY22* and *WRKY29*, which encode WRKY family transcription factors, are induced by flg22 (Asai et al. 2002) and chitooligosaccharide (chitin fragments) (Wan et al. 2008) and function downstream of the MAPK cascade consisting of MEKK1/MTK-MKK4/MKK5-MPK3/MPK6. Considering the ability of elicitors to activate the natural defenses of plants, their use may provide a useful strategy for sustainable management of plant diseases.

Deployment of microbial elicitors to enhance plant resistance has been extensively explored for effective control of plant diseases. For example, chitin and chitosan can enhance defense responses against various plant pathogens, including bacterial and fungal pathogens on Arabidopsis (Kawasaki et al. 2017), rice (Gong et al. 2020), and lettuce and tomato (Makechemu et al. 2025); *Phakopsora pachyrhizi* on soybean (Li et al. 2025); *Fusarium* on wheat (Hao et al. 2022; Kheiri et al. 2016; Makechemu et al. 2025); and *Fusarium* on chickpea (Sravani et al. 2023). Spray of harpin (HrpN) from *Erwinia amylovora* elicited systemic resistance in Arabidopsis to *Peronospora parasitica* and *Pseudomonas syringae* pv. *tomato* (Dong et al. 1999). Harpin αβ proteins enhanced orange fruit resistance against postharvest black spot by the fungal pathogen *Guignardia citricarpa* (Lucon et al. 2010). HpaXpm from the cassava bacterial blight pathogen *Xanthomonas phaseoli* pv. *manihotis* can improve tobacco resistance against *Tobamovirus tabaci* (Liu et al. 2020). Interestingly, harpin-loaded chitosan nanoparticles could improve the ability to induce tobacco defense responses more than the harpin or chitosan alone (Kongala and Mamidala 2023). LPS has also been well documented to induce plant defense responses against various diseases, such as Pierce's disease on grapevine (Castro et al. 2023), rice blast disease (Ashajyothi et al. 2022), and tomato bacterial spot (de Almeida Halfeld-Vieira et al. 2023). Additionally, PdEIX, a pathogen-associated molecular pattern from the citrus green mold pathogen *Penicillium digitatum*, could trigger immune responses in citrus fruit and enhance the resistance of citrus and other fruit against fungal pathogens causing postharvest diseases (He et al. 2025). Collectively, those studies are primarily proof-of-concept demonstrations that application of microbe-derived elicitors could be an effective strategy for the control of various crop diseases. Additionally, chitin, chitosan, and harpins have been approved as biopesticides by the U.S. Environmental Protection Agency (https://www.epa.gov). These elicitors have been tested across various fruit crops, such as apples and grapes (Popa et al. 2026); however, relatively limited investigations were conducted with citrus.

Citrus is able to sense flagellin and multiple CLas effectors to trigger immune responses, including ROS production and phloem callose deposition (Hu et al. 2026; McClelland et al. 2026; Zou et al. 2012). On the other hand, CLas can suppress citrus immunity through the delivery of Sec translocon-dependent effectors (SDEs) into plant cells, to promote citrus HLB disease. Examples of well-characterized SDEs included SDE1 (Clark et al. 2018, 2020), SDE3 (Shi et al. 2023a), SDE5 (Zhao et al. 2025), SDE15 (Pang et al. 2020), SDE19 (Huang et al. 2024), and SDE4405 (Shi et al. 2023b). These SDEs have unique targets and facilitate CLas growth in citrus. Consequently, citrus is unable to mount an effective immune response to CLas; instead, CLas causes disease damage mainly through chronic immune responses including ROS accumulation and phloem callose deposition (Huang et al. 2026; Ma et al. 2022). Nevertheless, CLas is a biotrophic pathogen, and we hypothesize that it is plausible that MAMP treatment can activate citrus immune responses to break the status quo related to immune inducing and suppressing by CLas, thus combating CLas infection and suppressing its proliferation.

The purpose of this study was to test the aforementioned hypothesis and examine whether chitosan, harpins, or LPS treatment can elicit plant defense responses and suppress citrus HLB. The findings

provide novel information about the effect of MAMP treatment on citrus defense response to CLas and demonstrate the potential of pursuing biotic elicitors for preventing and delaying CLas infection on young citrus trees, thereby aiding in the control of citrus HLB.

Materials and Methods

Plant materials

Healthy 6-month-old plants of Valencia sweet orange (*Citrus* × *aurantium* var. *sinensis* L.), Murcott mandarin (*C. reticulata* Blanco), and Sugar Belle 'LB8-9' mandarin ('Clementine' mandarin [*C.* × *aurantium* L. var. *chrysocarpa* (Hassk.) ined.] × 'Minneola' tangelo ['Duncan' grapefruit {*C.* × *aurantium* L. var. *racemosa* (Risso) ined.} × 'Dancy' tangerine {*C.* × *aurantium* L. var. *chrysocarpa* (Hassk.) ined.}]), grafted to the rootstock X639 (Cleopatra mandarin [*C.* × *aurantium* L. var. *chrysocarpa* (Hassk.) ined.] × Flying Dragon [*C. trifoliata* L.]), were obtained from a commercial citrus nursery in Lake Wales, Florida, United States. The plants were repotted in 3.8-liter containers in "Metro-Mix" potting soil (Sun Gro Horticulture, Agawam, MA, U.S.A.) and maintained in a greenhouse at the University of Florida (UF) Citrus Research and Education Center (CREC) in Lake Alfred, Florida, at a temperature of 20 to 30°C and 50 to 60% relative humidity. Plants were watered every 2 days and fertilized biweekly with Peters Professional 20-10-20 (N: 20%, P: 10%, and K: 20%) (The Scotts Company, Marysville, OH, U.S.A.) at a rate of 0.5 g/liter in water. Two months after repotting, plants with multiple young, fully expanded leaves were used for the experiments. Valencia sweet orange plants were used for greenhouse experiments. Valencia sweet orange, Murcott, and Sugar Belle 'LB8-9' mandarins were used for field tests.

MAMP preparations

Three MAMPs—chitosan (85% deacetylated chitin) (Fisher Scientific, Pittsburgh, PA, U.S.A.), harpin proteins (AXIOM; RX Green Technologies RX, Manchester, NH, U.S.A.; active ingredient: 1.0% active harpin αβ protein), and LPS from *Pseudomonas aeruginosa* 10 (Sigma, St. Louis, MO, U.S.A.)—were used in this study. Chitosan was dissolved in 0.5 M aqueous acetic acid to prepare a 10.0 mg/ml stock, and the pH was adjusted to 5.2 with 1.0 M KOH. Harpin proteins and LPS were dissolved in deionized water (DIW) to prepare a stock at 2.0 and 10.0 mg/ml, respectively. The stocks were kept at 4°C for 1 day before use, and assay solutions were prepared by diluting the stock in DIW to the indicated concentrations. The pH of the chitosan assay solution was adjusted to 7.0 using 1.0 M KOH. DIW with the selected surfactant INDUCE nonionic spreader-sticker (Helena Agri-Enterprises, Collierville, TN, U.S.A.) (0.25%, vol/vol; see details below) served as the mock treatment for foliar spray experiments with MAMPs.

Assays for induced expression of citrus defense genes by MAMPs

Healthy Valencia sweet orange plants, with at least 15 young fully expanded, immature leaves for each plant, were used for the experiments. Prior to the assays, the two non-ionic surfactants Silwet L-77 (PhytoTech Labs, Lenexa, KS, U.S.A.) and INDUCE nonionic spreader-sticker (Helena Agri-Enterprises) were compared for their ability to facilitate the uptake of chitosan, harpins, and LPS by treated trees following foliar spray. INDUCE was selected for the experiments because of its better effectiveness of inducing the expression of citrus defense genes than Silwett L-77 at the labeled rate (0.25%, vol/vol) (Supplementary Fig. S1). Subsequently, chitosan, harpins, and LPS were separately tested at three concentrations (chitosan: 100 μg/ml, 500 μg/ml, and 1.0 mg/ml; harpins: 10, 50, and 250 μg/ml; and LPS: 50, 100, and 500 μg/ml). Briefly, chitosan, harpins, or LPS mixed with INDUCE (0.25%, vol/vol) were applied onto the trees with a handheld pump sprayer at approximately 400 kPa until runoff from the leaves. Each treatment was implemented with four plants, each as a biological replicate. DIW mixed with INDUCE was used as mock control. After spray, the plants were maintained in a greenhouse at approximately 25 (±5)°C and a relative humidity of 50 to 60%.

To examine the expression of defense genes in treated plants, leaf samples were collected at 1, 6, 12, and 18 h posttreatment (hpt) and 1, 3, 5, and 7 days posttreatment. At each time point, two leaves per plant were sampled and pooled for RNA isolation. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD, U.S.A.), according to the manufacturer's instructions. Subsequently, the extracted RNA was treated with RNase-free DNase I (Promega, Madison, WI, U.S.A.) to eliminate contaminating DNA. First-strand cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega) per the manufacturer's recommendations. Quantitative PCR (qPCR) was conducted in a QuantStudioTM3 96-Well (0.1 ml) instrument (Thermo Fisher Scientific, Hillsboro, OR, U.S.A.). Each 20-μl PCR mixture consisted of 10 μl of 2× SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, U.S.A.), 2 μl of 1:10 diluted cDNA template, 1 μl of each primer (5 μM), and 6 μl of DNase- and RNase-free water. Reaction parameters were set to an initial activation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 40 s. All cDNA samples were run in triplicate. The expression of sweet orange defense genes, including *CsFRK1* (*FLG22-INDUCED RECEPTOR KINASE 1*), *CsGST1* (*Glutathione S-transferase 1*), *CsPAL1* (*Phenylalanine ammonia-lyase 1*), *CsMKK4* (*MAP KINASE KINASE 4*), *CsWRKY22*, and *CsWRKY29*, were estimated and compared, with the glyceraldehyde-3-phosphate dehydrogenase gene *CsGAPDH* as the endogenous control for normalization of gene expression. The gene-specific primers are listed in Table 1. Gene expression levels, relative to mock samples at each time point, were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

TABLE 1. Plant defense genes in sweet orange and primers used for RT-qPCR analysis

Gene name	Annotation	Primer name and sequence (5′–3′)	Reference
FRK1	*FLG22-INDUCED RECEPTOR KINASE 1*	FRK1-F: AGTGACGTGTACAGCTTTGG; FRK1-R: TCTGATATTTCCCTCGGCAAG	Pang et al. 2020
GST1	*Glutathione S-transferase 1*	GST1-F: AGACATGAAGGCTGGTGAAC; GST1-R: AACTCCTGGGCGATGTATTG	Pang et al. 2020
PAL1	*Phenylalanine ammonia-lyase 1*	PAL1-F: TTCCTCTTTCCTACATTGCCG; PAL1-R: GCAACTCAAAGAACCCGAAAC	This study
MKK4	*MAP KINASE KINASE 4*	MKK4-F: GGATCTTAGCACAGACGATGG; MKK4-R: CTCCAATATACTCACCCCTAAGC	This study
WRKY22	*Transcription factor*	WRKY22-F: AGGAAACAGGTGGAACGAAG; WRKY22-R: GACCTTGAGCCTTTGACATTG	Pang et al. 2020
WRKY29	*Transcription factor*	WRKY29-F: ACATATACGGCAGAGCACAAC; WRKY29-R: AGTGGGTTTGGTTGAGGAAG	Pang et al. 2020
CsGAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*	GAPDH-F: GGAAGGTCAAGATCGGAATCAA; GAPDH-R: CGTCCCTCTGCAAGATGACTCT	Pang et al. 2020

Fold change ≥ 2.0 was considered as a fold-change threshold of different expression. The experiments were repeated twice.

ROS assay

ROS assays were performed using a previously described method (Li et al. 2014) with modifications. Briefly, leaf discs about 4 mm in diameter were punched using a cork borer from fully expanded, immature leaves of 8-month-old healthy Valencia sweet orange plants. A total of eight leaf discs for each treatment were collected from four leaves (two discs per leaf) of the same plant. The discs were placed in individual wells of a clear 96-well plate, with 150 μl of DIW per well. The plate was incubated in the dark at room temperature overnight. For ROS measurement, the water was replaced with a 150-μl assay solution containing 10 μg/ml luminol L012 and 10 μg/ml horseradish peroxidase (Sigma). MAMP treatments were included in the assay solution as follows: (i) 100 μg/ml chitosan, (ii) 10 μg/ml harpins, (iii) 50 μg/ml LPS, and (iv) DIW as the untreated negative control. The luminescence was recorded at room temperature over a period of 90 min using the VANTAstar microplate reader (BMG Labtech, Cary, NC, U.S.A.). All tests were performed in eight replicate wells and repeated three times.

Hydrogen peroxide (H_2O_2) quantification

Eight-month-old healthy Valencia sweet orange plants were sprayed with chitosan (100 μg/ml), harpins (10 μg/ml), and LPS (50 μg/ml), respectively, as described above for the citrus defense expression assays. Three young leaves were collected from each treated and untreated (sprayed with DIW) plant at 6 hpt for H_2O_2 quantification. H_2O_2 contents in leaves were estimated using a method described previously (Alexieva et al. 2001), with modifications. Briefly, 0.2 g of leaf samples was homogenized in liquid nitrogen, and the ground samples were extracted with 0.3 ml of ice-cold 0.1% (wt/vol) trichloroacetic acid for 10 min and centrifuged at 12,000 × *g* for 15 min at 4°C. The supernatant was collected and mixed with 1.7 ml of 1.0 M potassium phosphate buffer (pH 7.0) and 1.0 ml of 1.0 M potassium iodide solution. After incubation in darkness for 15 min, the absorbance of the resulting oxidation product was measured at 390 nm. H_2O_2 contents were determined using a standard curve established with known H_2O_2 concentrations and expressed as μmol/g fresh weight. All tests were performed with four replicate plants and repeated two times.

Greenhouse tests for the effect of MAMP treatment on CLas titer in infected plants

The experiments were performed in a quarantine greenhouse at UF-CREC in Lake Alfred, Florida, from April 2022 to January 2023. Six months prior to the experiments, 6-month-old Valencia sweet orange healthy seedlings were graft-inoculated with budwood collected from mature, CLas-infected Valencia trees, symptomatic for HLB at the UF-CREC research grove. MAMPs were sprayed to the graft-inoculated trees when leaves distant from the graft site (≥20 cm from the graft site) were first found to be positive by qPCR, as described below.

Four-tree plots were arranged in a randomized complete block design with four treatments and four replicates for each treatment. The space between trees was maintained at least at 1.5 × 1.5 m to avoid spray drift onto untargeted trees. Treatments included an untreated control (sprayed with water containing 0.25% [vol/vol] INDUCE nonionic spreader-sticker), chitosan (1.0 mg/ml) in water containing 0.25% (vol/vol) INDUCE, harpins (250 μg/ml) in water containing 0.25% (vol/vol) INDUCE, and LPS (500 μg/ml) in water containing 0.25% (vol/vol) INDUCE. Treatment applications were made weekly by spraying 200 ml of solution onto each tree with a handheld pump sprayer at approximately 400 kPa until runoff from the leaves. The trees were irrigated and fertilized as needed. The greenhouse was maintained at 25 (±5)°C with water-cooling pads and ventilation fans, and artificial light was not provided. Treatment applications continued for 4 months, then stopped due to the observation of suppression of plant growth by the MAMP treatments, as the treated plants were shorter than the untreated plants and had a smaller canopy size (Supplementary Fig. S2). CLas titers in treated plants were determined before treatment and monthly after treatment application, using qPCR as described below. Because CLas is unevenly distributed within an infected plant (Folimonova et al. 2009; Tatineni et al. 2008), two leaves per branch were randomly sampled from three different branches of each plant and pooled for the determination of CLas titer at each sampling time point.

Field tests for the effect of MAMP treatment on CLas infection and HLB progression

The experiments were conducted at the UF-CREC research grove in Lake Alfred, Polk County, Florida, from April 2023 to April 2025. This is an environment of high ACP pressure and neighboring mature HLB-diseased trees, allowing for the spread of CLas. Three citrus cultivars, Valencia sweet orange, Murcott mandarin, and Sugar Belle 'LB8-9' mandarin on "X639" rootstock, were used for the tests. These cultivars are representatives in commercial citrus production in Florida for processing and fresh orange market respectively. Valencia sweet orange and Murcott mandarin are susceptible to HLB, whereas Sugar Belle 'LB8-9' is considered HLB-tolerant (Deng et al. 2019; Stover et al. 2016). In April 2023, healthy 8-month-old trees were repotted in 15.2-liter containers in "Metro-Mix" potting soil (Sun Gro Horticulture) and were transferred to the research grove. During the study, the trees were irrigated every other day and fertilized biweekly with Peters Professional 20-10-20 (N: 20%, P: 10%, and K: 20%) (The Scotts Company) at a rate of 0.5 g/liter in water. During the experimental period, no insecticides against *D. citri* were applied to control the psyllid, allowing for natural infection of the trees with CLas. Treatments were randomly assigned to trees after they were arranged in a 20 × 3 grid pattern. For each citrus cultivar, there were five trees for each treatment and five untreated controls. The treatments included (i) water containing 0.25% (vol/vol) INDUCE nonionic spreader-sticker as the untreated control, (ii) chitosan (500 μg/ml) in water containing 0.25% (vol/vol) INDUCE, (iii) harpins (50 μg/ml) in water containing 0.25% (vol/vol) INDUCE, and (iv) SA (100 μg/ml) in water containing 0.25% (vol/vol) INDUCE as a positive control, given that our previous studies demonstrated that regular sprays of SA decreased CLas titers and slowed HLB disease progress in young citrus trees in the field (Li et al. 2016). Therefore, there were a total of 12 cultivar–treatment combinations, and each had five replicated trees. Treatment applications were implemented monthly and continued for 2 years until April 2025, by spraying 300 ml of solution onto each tree with a handheld pump sprayer at approximately 400 kPa until runoff from the leaves. The treated trees were subjected to CLas detection and quantification right after the experiment setup (0) and every 3 months post initial treatment (mpt) using qPCR assays, as described below. HLB disease severity in treated trees was assessed every 3 months after initial treatment, as described below. The tree growth performance was evaluated and compared at the end of the experiments, as described below.

qPCR for the detection and quantification of CLas

To detect and quantify CLas titer in treated trees in the field, two to four symptomatic or asymptomatic young leaves per branch, when symptomatic ones were not available, were randomly collected from three different branches of each tree. The midribs of the leaves were cut into 2-mm pieces for DNA extraction. Total DNA from 100-mg samples of the cut midribs was extracted using the Wizard Genomic DNA purification kit (Promega) following the manufacturer's recommendations. The extracted DNA was quantified using a Synergy HTX microplate reader (BioTek Instruments, Winooski, VT, U.S.A.) and adjusted to 50 ng/μl.

qPCR assays were performed with a QuantStudio3 96-Well 0.1-ml instrument (Applied Biosystems, Waltham, MA, U.S.A.),

using the CLas-specific primer/probe set "CQULA04F-CQULAP10P-CQULA04R" (Wang et al. 2006) and 2 μL of DNA in a 20-μl final reaction volume. The amplification setting was 95°C for 15 min, followed by 40 cycles at 94°C for 15 s and 60°C for 60 s, based on the instruction of the QuantiTect Probe PCR Kits (Qiagen). Samples with cycle threshold (Ct) values <38.0 were considered CLas-positive, and those with values ≥38.0 were considered CLas-negative (Graham et al. 2020). Ct values were converted to the estimated bacterial titers as described previously (Li et al. 2016). Each individual sample included three replicates, and the whole test was repeated twice.

Assessment of HLB disease severity and tree growth performance

Visual assessments of HLB disease severity were performed using the method described by Gottwald et al. (2007) with modifications. Briefly, the tree canopy was divided into four quadrants, and the incidence of leaf chlorosis (blotchy mottle or asymmetrical patches of greening and/or yellowing in the leaves) in each section was visually scored using a 0 to 5 scale that indicates the proportion of leaves showing chlorosis as follows: 0 = no leaves showing chlorosis; 1 = 1 to 20% of the leaves showing chlorosis; 2 = 21 to 40% of the leaves showing chlorosis; 3 = 41 to 60% of the leaves showing chlorosis; 4 = 61 to 80% of the leaves showing chlorosis; and 5 = 81 to 100% of the leaves showing chlorosis. This produces a single overall severity score of 0 to 20 for each tree. Additionally, the disease severity data from individual evaluations for each tree across the field trial were computed to the standardized area under the disease progress stairs (sAUDPS) as described by Simko and Piepho (2012).

Tree growth was evaluated by estimating tree height, trunk diameter, and canopy volume on both MAMP-treated and untreated control trees. Tree height was measured with a tape measure from the soil surface to the tallest living shoot. Trunk diameter was calculated as the average of two measurements (east–west and north–south orientation) taken with a digital caliper (Fowler, Newton, MA, U.S.A.) at 10 cm above the graft union. Canopy volume was estimated by taking the average of four independent measurements of the diameter of the canopy at different directions (east–west, north–south, northeast–southwest, and northwest–southeast). The canopy volume was estimated assuming a tree shape of an ellipsoid (Pons et al. 2012), using the following equation:

$$V = (2/3) \times \pi \times h \times (d/2)^2 \quad (1)$$

where "*h*" is the tree height and "*d*" is the average diameter of the canopy.

Statistical analysis

All data analyses were performed using SAS V9.4 (SAS Institute, Cary, NC, U.S.A.). The data of gene expression, H_2O_2 content, maximum ROS levels, sAUDPS, and tree growth performance traits were respectively analyzed with one-way analysis of variance, and means were compared with Tukey's honestly significant difference test ($\alpha = 0.05$). Prior to analysis, the data were $\log_{10}$ transformed to satisfy assumptions of normality and homoscedasticity. Repeated-measures analysis of variance with the MIXED procedure was performed to evaluate the effects of treatment, time since application, and the interaction between treatment and time since application on CLas titers. Before the analysis, CLas titers were $\log_{10}$ transformed to satisfy assumptions of normality and homoscedasticity. The first-order autoregressive covariance structure option was selected based on the Akaike and Bayesian information criteria. When treatment effects were significant ($P < 0.05$), pairwise comparisons were made using the LSMEANS statement with Tukey's honestly significant difference ($\alpha = 0.05$) adjustment for mean separation. A Cox proportional hazards model was used to compare the effect of treatment on the first occurrence of CLas in experimental trees in the field trial, and the significance of difference between treatments was evaluated using the log-rank test with a significance level of $P < 0.05$. Because there were no significant differences among citrus cultivars in the first occurrence of CLas within each treatment (P values > 0.05, two-sample proportions Z test at $\alpha = 0.05$) (Supplementary Fig. S3), data across the three cultivars were combined to increase sample sizes for treatment groups to improve the power of the statistical analysis with a Cox proportional hazards model.

Results

Induction of defense genes in young citrus trees sprayed with MAMPs

Foliar spray of chitosan, harpins, or LPS activated immunity in Valencia sweet orange plants in a dosage-dependent manner: usually, a higher dosage induced higher levels of expression of the defense genes tested. Peak levels of expression of citrus defense genes including *CsFRK1*, *CsMKK4*, *CsPAL1*, *CsWRKY22*, and *CsWRKY29* were mostly recorded at 12 to 72 hpt, with substantial variations (Fig. 1). Chitosan and harpins generally induced stronger expression of the genes assessed, except *CsGST1*, compared with LPS, with an average peak induction above eightfold for chitosan, above sixfold for harpins, and fourfold for LPS. *CsGST1* showed no significant induction by any of the MAMP treatments. Under chitosan treatments at three different concentrations, *CsPAL1* showed rapid induction with peak levels at 1 and 24 hpt, and *CsMKK4* peaked at 18 hpt, whereas *CsFRK1*, *CsWRKY22*, and *CsWRKY29* peaked at 24 hpt (Fig. 1A). A significant (fold change ≥ 2) induction of expression of the genes by chitosan lasted for 1 to 5 days, depending on individual genes. In response to harpin treatment, expression of *CsMKK4* peaked at 24 hpt, and *CsFRK1*, *CsWRKY22*, *CsPAL1*, and *CsWRKY29* peaked at 72 hpt (Fig. 1B). Moreover, the induction of *CsFRK1*, *CsWRKY22*, *CsPAL1*, and *CsWRKY29* showed a harpin dosage-dependent pattern, with higher levels of expression associated with higher dosage. In the case of LPS treatments, expression of *CsPAL1* peaked at 1 hpt under 50 or 100 μg/ml of LPS spray and at 6 hpt with 500 μg/ml of LPS (Fig. 1C). Induced expression of *CsMKK4* by LPS peaked at 12 hpt, and its induction was not affected by LPS dosage. *CsFRK1*, *CsWRKY22*, and *CsWRKY29* expression were induced by LPS at a relatively high concentration (≥100 μg/ml), with peak levels at 24 hpt (*CsWRKY22* and *CsWRKY29*) and 72 hpt (*CsFRK1*). Additionally, in all cases, the induced expression of most genes returned to normal within 5 days posttreatment, except *CsPAL1*. When induced by harpins at 250 μg/ml, the expression of *CsPAL1* reversed to normal at 7 days posttreatment. Overall, foliar spray of chitosan, harpins, or LPS induced rapid and strong expression of citrus defense genes, and the response lasted for approximately 3 to 5 days.

ROS induction in citrus treated with MAMPs

Chitosan, harpin, and LPS treatments induced fast ROS responses in detached leaves of healthy Valencia sweet orange trees (Fig. 2A). The ROS reached a peak level of approximately 700 RLU (relative luminescence units) for chitosan or harpin treatment at 20 and 17 min posttreatment, respectively, and 480 RLU for LPS treatment at 19 min posttreatment, then slowly declined and returned to base level after a 90-min treatment with chitosan or harpins and a 60-min treatment with LPS (Fig. 2A). These observations implied that rapid and transient ROS were induced in detached leaves treated by chitosan, harpins, or LPS, but ROS levels varied in different treatments. Additionally, the pattern of H_2O_2 contents in citrus plants triggered by foliar spray of chitosan, harpins, or LPS was consistent with that of the ROS burst observed with detached leaves. All three MAMP treatments showed significantly higher H_2O_2 contents than the untreated control at 6 hpt (Fig. 2B). Moreover, the H_2O_2 content in chitosan- or harpin-sprayed plants was significantly higher than that in LPS-treated plants. Together, these results indicate that chitosan, harpins, and LPS individually can trigger a rapid ROS response in citrus plants, but the levels of ROS induced substantially varied by MAMP treatment.

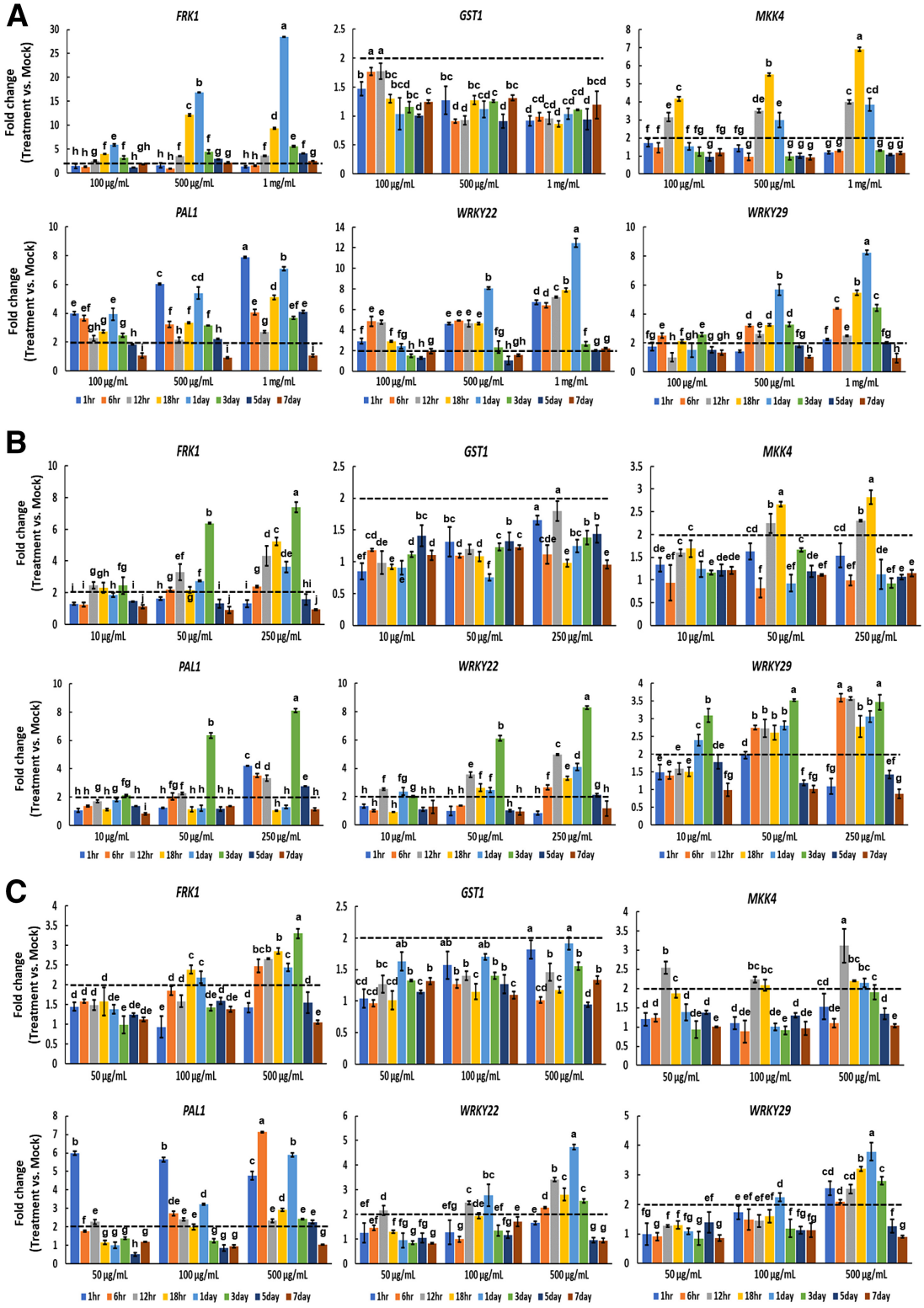


Fig. 1. Induction of defense genes in Valencia sweet orange plants triggered by chitosan, harpins, or lipopolysaccharide (LPS). Eight-month-old orange trees were sprayed with 0.25% (vol/vol) INDUCE in water containing different concentrations of **A,** chitosan (100 µg/ml, 500 µg/ml, and 1.0 mg/ml); **B,** harpins (10, 50, and 250 µg/ml); or **C,** LPS (50, 100, and 500 µg/ml). Plants treated with 0.25% (vol/vol) INDUCE surfactant in water alone served as the mock control. Each treatment contained three replicate plants. Two leaves from each plant were collected at different time points (1, 6, 12, and 18 h and 1, 3, 5, and 7 days) after treatment. Total RNA was isolated, and gene expression was estimated by RT-qPCR. The sweet orange glyceraldehyde-3-phosphate dehydrogenase gene *GAPDH* was used as an endogenous control for transcript normalization. The fold change of gene induction was calculated relative to the mock from each time point indicated. The dotted line indicates the threshold of induction at fold change = 2. Error bars represent standard deviation ($n = 3$ biological replicates). Different letters indicate significant differences at $P < 0.05$ (Tukey's honestly significant difference test at $\alpha = 0.05$). The experiments were repeated two times with similar results.

Effect of MAMP treatment on CLas titer in infected citrus plants in the greenhouse

To investigate the effect of MAMP applications to reduce CLas titer, we treated 1-year-old Valencia sweet orange with MAMPs or water by weekly spray application under greenhouse conditions and monitored CLas titers over a 4-month period. There were significant effects of treatment ($F = 23.58$; df = 3, 48; $P < 0.0001$) and time since application ($F = 63.77$; df = 4, 48; $P < 0.0001$) on CLas titers in infected plants. There was no significant interaction between treatment and time since application ($F = 1.57$; df = 12, 48; $P = 0.1252$) on CLas titers. Foliar spray with chitosan or harpins, but not LPS, significantly ($P < 0.05$) reduced CLas titers in infected trees compared with the untreated control at 3 months after treatment (Fig. 3). Prior to this time point, either chitosan, harpin, or LPS treatment did not significantly ($P > 0.05$) reduce CLas titers. Over time, the average of CLas titers in untreated control trees increased from 1.36×10^2 (i.e., 2 $\log_{10}$ units) to 6.24×10^4 (4 $\log_{10}$ units) per 100 ng plant genomic DNA (gDNA), and that in chitosan- or harpin-treated trees increased from 1.32×10^2 to 1.71×10^3 and 1.50×10^2 to 1.31×10^3 per 100 ng plant gDNA, respectively. The pattern of CLas titer in LPS-treated plants was similar to that in the untreated control, and there was no significant difference in CLas titer between LPS treatment and untreated control at the end of the experiments (Fig. 3). These results suggest that weekly applications of chitosan or harpins but not LPS over 4 months reduced CLas proliferation in infected citrus plants grown in the greenhouse.

Effect of MAMP treatment on CLas infection under field conditions

CLas infection. To investigate the effect of MAMP application on CLas infection and HLB disease progression in the field, three citrus cultivars with different susceptibility to HLB were exposed to ACPs with a high HLB disease pressure under natural field conditions and sprayed with chitosan, harpins, or SA monthly for 24 months. The establishment of CLas infection in the trees was monitored following the treatments. Citrus cultivar did not significantly influence the first occurrence of CLas within each treatment (P values > 0.05, two-sample proportions Z test at $\alpha = 0.05$) (Supplementary Fig. S3). However, there was a significant effect of MAMP treatment on the establishment of CLas infection in experimental trees ($P < 0.05$; log-rank test). Untreated control trees started to test positive for CLas at 6 mpt, and 20% of the trees were already infected; 56 and 100% of the trees were already infected by CLas at 9 and 12 mpt, respectively (Fig. 4). However, 0% of the trees treated with chitosan, harpins, or SA tested positive for CLas at 6 mpt, and approximately 4, 25, 55, 70, 96, and 100% of trees treated with chitosan, harpins, or SA were infected by CLas at 9, 12, 15, 18, 21, and 24 mpt, respectively. These results indicate that chitosan, harpin, and SA treatments delayed CLas infection in new settings of citrus.

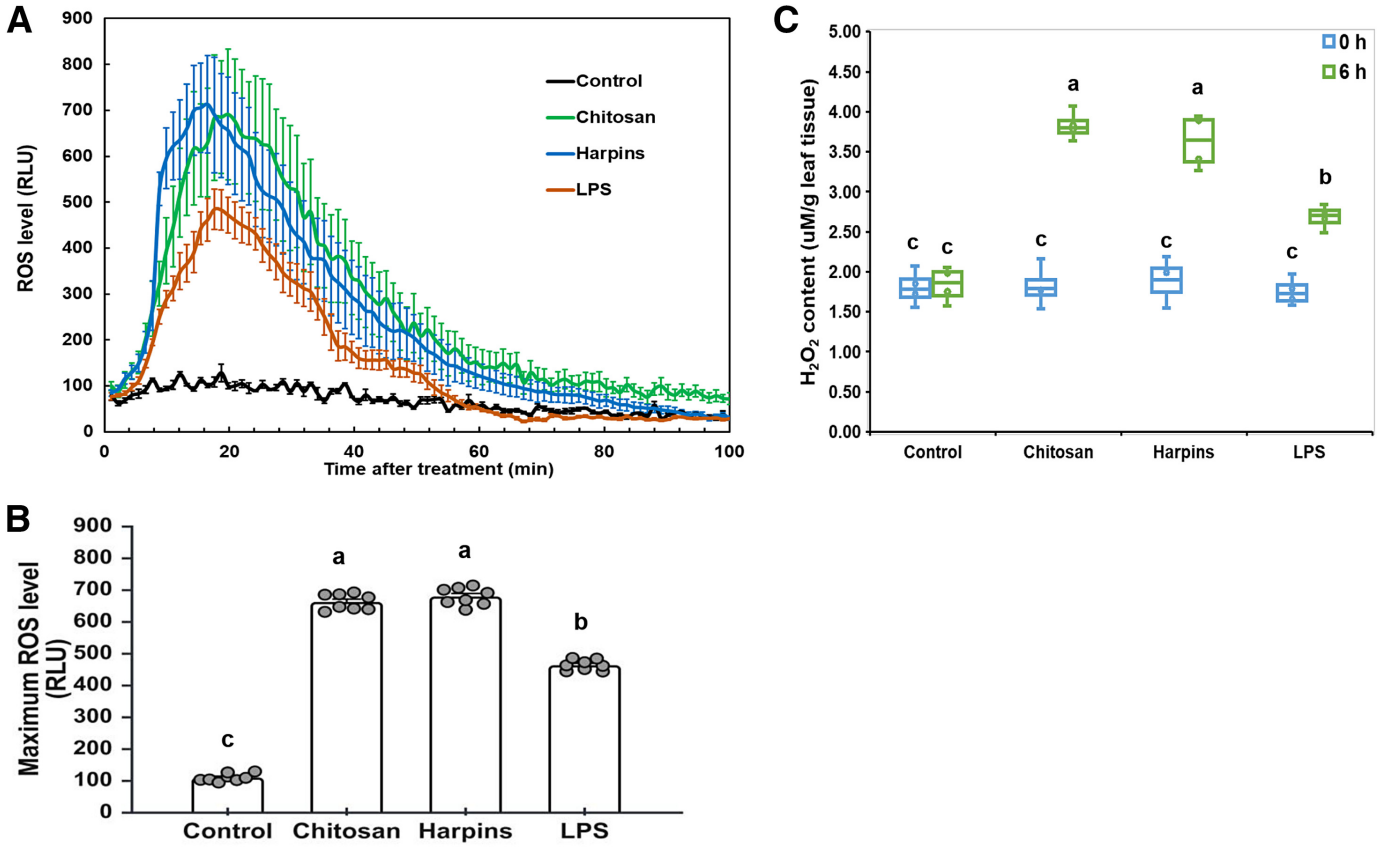


Fig. 2. Reactive oxygen species (ROS) production in Valencia sweet orange plants. **A,** ROS burst induced in leaves treated with chitosan, harpins, or lipopolysaccharide (LPS). Leaf discs (4 mm) were punched from 8-month-old healthy Valencia sweet orange plants and treated with 100 µg/ml chitosan, 50 µg/ml harpins, or 100 µg/ml LPS using a 96-well plate. Control: deionized water (DIW). ROS were measured using luminol-based chemiluminescence with L012 as a substrate using a 96-well plate reader (Synergy HT), and the signals (RLU, relative luminescence units) were recorded for about 100 min. Error bars represent standard deviation ($n = 8$ replicated discs from one plant). Experiments were repeated three times with similar results. **B,** Comparison of maximum ROS levels triggered by chitosan, harpins, or LPS in the test as described in A. Different lowercase letters indicate significant differences ($P < 0.05$; Tukey's honestly significant difference [HSD] test at $\alpha = 0.05$). **C,** H_2O_2 content in leaves of 8-month-old healthy Valencia sweet orange plants sprayed with chitosan (500 µg/ml), harpins (50 µg/ml), or LPS (100 µg/ml) in water containing 0.25% (vol/vol) INDUCE. Leaf samples were collected, and H_2O_2 content was examined right before treatment (0) and 6 h posttreatment. Plants sprayed with water containing 0.25% (vol/vol) INDUCE served as the untreated control. Data shown represent mean ± SEM ($n = 8$). Different lowercase letters indicate significant differences ($P < 0.05$; Tukey's HSD test at $\alpha = 0.05$) ($n = 4$ biological replicates). The experiments were repeated two times with similar results.

CLas titer dynamics. CLas titers in treated trees were monitored during the experimental period. For all the three cultivars (i.e., Valencia, Murcott, and Sugar Belle), there were significant effects of treatment ($F = 466.58$; df = 3, 128; $P < 0.0001$ for Valencia; $F = 482.21$; df = 3, 128; $P < 0.0001$ for Murcott; and $F = 497.24$; df = 3, 128; $P < 0.0001$ for Sugar Belle) and time since treatment application ($F = 40.76$; df = 8, 128; $P < 0.0001$ for Valencia; $F = 51.33$; df = 8, 128; $P < 0.0001$ for Murcott; and $F = 46.15$; df = 8, 128; $P < 0.0001$ for Sugar Belle) on CLas titers. There was no significant interaction between treatment and time since application ($F = 1.64$; df = 24, 128; $P = 0.1122$ for Valencia; $F = 1.55$; df = 24, 128; $P = 0.1184$ for Murcott; and $F = 1.36$; df = 24, 128; $P = 0.1295$ for Sugar Belle) on CLas titers. In Valencia, Murcott, and Sugar Belle control (water-sprayed) trees, CLas was detected as early as 6 mpt, with a titer of 0.78×10^3, 1.15×10^3, and 1.25×10^2 per 100 ng plant gDNA, respectively (Fig. 5); thereafter, CLas titers rapidly increased and reached 2.98×10^4, 3.16×10^4, and 6.71×10^3 per 100 ng plant gDNA at 24 mpt, respectively. CLas titer increased 40-, 30-, and 50-fold in infected Valencia, Murcott, and Sugar Belle control trees at the end of the experiments. However, in the MAMP- or SA-treated Valencia, Murcott, and Sugar Belle trees, CLas was detected as early as 9 mpt, 3 months later than in control trees, although infection could have occurred earlier, as the trees were sampled only at 3-month intervals.

Citrus cultivar had a significant effect on CLas titer after infection ($P < 0.05$), regardless of treatment, and infected Sugar Belle trees harbored a lower CLas titer than Valencia and Murcott within each treatment at each sampling time point. At 9 mpt, CLas titer in chitosan- or harpins-treated Valencia plants was approximately 4.00×10^2 per 100 ng plant gDNA; in SA-treated Valencia plants, it was 1.80×10^2 per 100 ng plant gDNA; and in Valencia control plants, it was 1.54×10^3 per 100 ng plant gDNA. At the end of the experiments, CLas titer in chitosan- or harpins-treated Valencia plants was approximately 3.30×10^3 per 100 ng plant gDNA, and in SA-treated Valencia plants, it was 1.42×10^3 per 100 ng plant gDNA, with a 12- and 8-fold increase, respectively. In the case of Murcott trees, at 9 mpt, CLas titer in chitosan- or harpins-treated plants was approximately 4.10×10^2 per 100 ng plant gDNA; in SA-treated plants, it was 3.82×10^2 per 100 ng plant gDNA; and in control plants, it was 1.71×10^3 per 100 ng plant gDNA. At 24 mpt, CLas titer in chitosan- or harpins-treated plants was approximately 4.00×10^3 per 100 ng plant gDNA, and in SA-treated plants, it was 2.45×10^3 per 100 ng plant gDNA, a 10- and 7-fold increase, respectively. For Sugar Belle trees, at 9 mpt, CLas titer in chitosan- or harpins-treated plants was approximately 1.30×10^2 per 100 ng plant gDNA; in SA-treated plants, it was 0.72×10^2 per 100 ng plant gDNA; and in control trees, it was 5.15×10^2 per 100 ng plant gDNA. At 24 mpt, CLas titer in chitosan- or harpins-treated plants was approximately 1.20×10^3 per 100 ng plant gDNA, and in SA-treated plants, it was 0.58×10^3 per 100 ng plant gDNA, about a 10- and 8-fold increase, respectively. These results indicate that chitosan, harpin, and SA treatments suppressed the growth of CLas in plants after CLas infection.

HLB disease severity. For all treatments, regardless of citrus cultivar, leaf chlorosis symptoms, such as blotchy mottle or asymmetrical patches of greening and/or yellowing in the leaves, were observed as early as 9 mpt and generally became more severe, showing a gradual increase in the severity score over time (Fig. 6A). However, Sugar Belle trees generally had fewer symptoms relative to Valencia and Murcott, with lower severity scores within

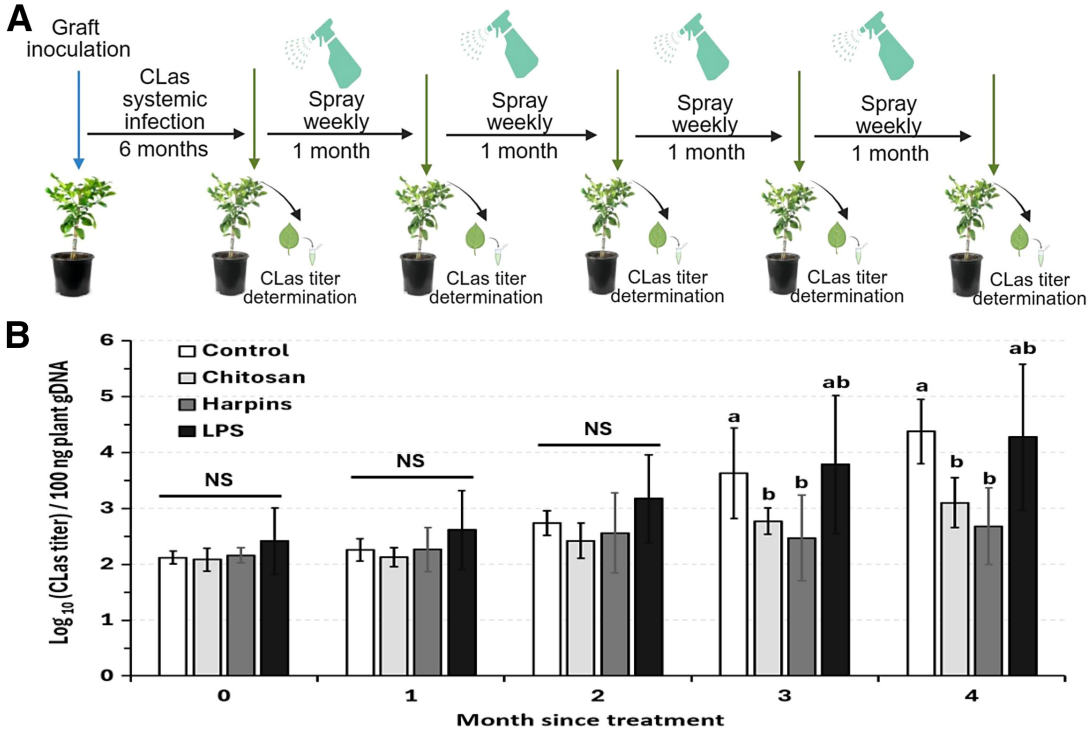


Fig. 3. Effect of microbe-associated molecular pattern (MAMP) treatment on '*Candidatus* Liberibacter asiaticus' (CLas) titer in infected citrus plants in the greenhouse. **A,** Experiment overview. Six-month-old Valencia sweet orange healthy plants were graft-inoculated with CLas. Six months later, the plants were confirmed as CLas-infected and subjected to MAMP treatment by foliar spray weekly, and CLas titers were determined monthly. The image was created with biorender.com. **B,** Dynamics of CLas titer in citrus plants treated with chitosan, harpins, or lipopolysaccharide (LPS) in the greenhouse. One-year-old CLas-infected Valencia sweet orange trees were sprayed with chitosan (1.0 mg/ml), harpins (250 µg/ml), or LPS (500 µg/ml) in water containing 0.25% (vol/vol) INDUCE weekly in the greenhouse, and the CLas titers in treated plants were measured monthly using qPCR. Trees sprayed with water containing 0.25% (vol/vol) INDUCE served as the untreated control. gDNA, genomic DNA. Data and error bars represent mean and standard deviation respectively ($n = 8$ replicated leaves from 4 trees). Different lowercase letters indicate significant differences among treatments within the indicated time point ($P < 0.05$; repeated-measures analysis of variance and Tukey's honestly significant difference test at $\alpha = 0.05$). NS, not significantly different ($P > 0.05$).

each treatment. In terms of mean chlorosis, chitosan, harpin, and SA treatments had similar averages across the seven evaluations, which were lower than those of the untreated control. There were significant differences ($P < 0.05$) in the accumulated disease severity (i.e., sAUDPS) between SA, chitosan, or harpin treatment and the untreated control at the end of the experiment (Fig. 6B). SA, chitosan, or harpin treatment resulted in approximately 42% less severe symptoms of HLB (reduction in sAUDPS) compared with the control. Although SA had a lower mean sAUDPS value than chitosan or harpins, the differences were not statistically significant (Fig. 6B). Additionally, Sugar Belle trees generally had lower mean sAUDPS values than Valencia and Murcott, especially for untreated control trees. Together, these results indicate that chitosan, harpin, and SA treatments delayed the development of HLB symptoms after the establishment of systemic CLas infection.

Tree growth performance. Tree growth traits with treatment groups were measured and compared at the beginning (0 mpt) and end of the field trial (24 mpt). For all three citrus cultivars, at both 0 and 24 mpt, chitosan (0.5 mg/ml), harpins (50 μg/ml), or SA (100 μg/ml) treated and water-sprayed control trees were not significantly different ($P > 0.05$) in tree height, trunk diameter, or canopy volume (Table 2). These observations suggest that chitosan (0.5 mg/ml), harpins (50 μg/ml), or SA (100 μg/ml) sprayed monthly did not negatively impact the development and growth of young citrus trees.

Discussion

A variety of biotic elicitors, including MAMPs, were explored to enhance plant disease resistance. Chitin, chitosan, harpins, and LPS have been shown to induce ROS production and defense gene expression in a range of crops and thereby suppress various plant diseases (Hao et al. 2022; Li et al. 2025; Makechemu et al. 2025; Wanke et al. 2020). In the present study, we showed that chitosan, harpins, and LPS activated immune responses in citrus plants, triggering the production of ROS and expression of defense genes. Furthermore, foliar spray of chitosan or harpins at an appropriate dosage enhanced citrus resistance to HLB under field conditions, delaying CLas infection under the sampling scheme used and suppressing its population growth in infected plants. Therefore, even though CLas-triggered chronic immune responses are potential causes of HLB disease damage, boosting immune responses with defense inducers can exert a positive control effect toward CLas by changing the status quo.

Chitosan, harpin, or LPS treatment rapidly induced robust ROS production, which might restrict the colonization of CLas in cit-

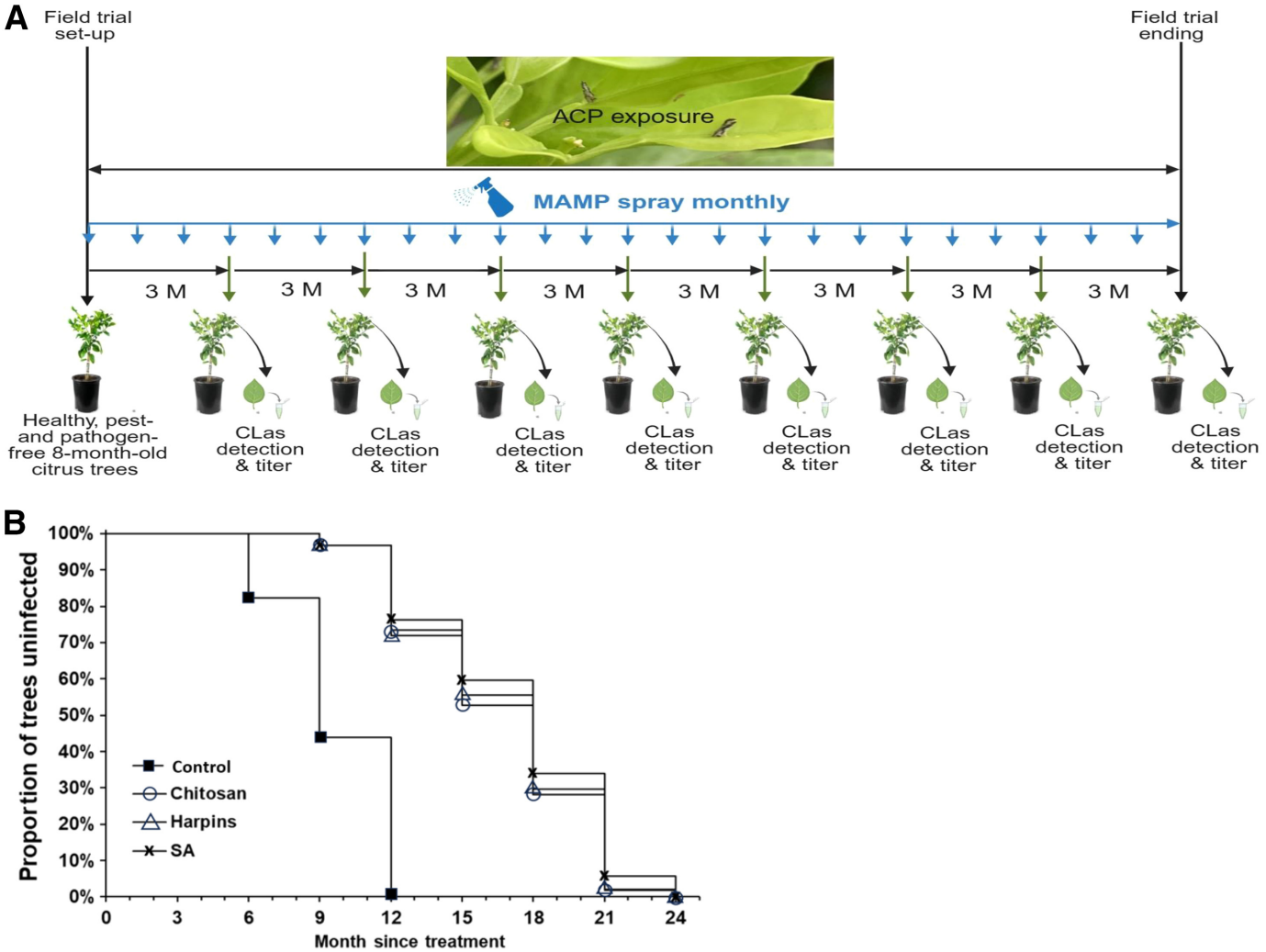


Fig. 4. Field tests of microbe-associated molecular patterns (MAMPs) for the effect on '*Candidatus* Liberibacter asiaticus' (CLas) infection and Huanglongbing progression. **A,** Experiment overview. Healthy 8-month-old citrus trees were exposed to Asian citrus psyllid (ACP) in the field and sprayed with MAMPs monthly for 24 months. CLas detection and titer determination in the trees were conducted every 3 months. **B,** Survival curves showing the proportion of citrus trees within each elicitor treatment that were infected over time by CLas under natural conditions. The trees (including Valencia sweet orange, Murcott, and Sugar Belle mandarin) were sprayed with chitosan (500 μg/ml), harpins (50 μg/ml), or salicylic acid (SA, 100 μg/ml) in water containing 0.25% (vol/vol) INDUCE monthly in the field. Each treatment contained 15 plants. The untreated control (UTC) was sprayed with water containing 0.25% (vol/vol) INDUCE only.

rus plants transmitted by psyllids feeding in the field. ROS have been shown to have toxigenic effects on plant pathogens (Grant and Loake 2000). Specifically, H_2O_2 has demonstrated antibacterial activity against *Liberibacter crescens*, a cultured surrogate species of CLas (Jain et al. 2018). CLas infection triggers chronic immune responses in citrus phloem tissue, including the production of ROS, such as H_2O_2 (Huang et al. 2026; Ma et al. 2022). CLas also encodes detoxification enzymes to mitigate the damage by ROS and thereby establishes colonization in citrus (Jain et al. 2018). It is possible that if the ROS accumulated due to MAMP treatment reach a level over the threshold that CLas can scavenge, its colonization in citrus will be reduced, leading to lower bacterial populations.

ROS induced by chitin or its derivatives, harpins, or LPS have been documented in various plant species, such as Arabidopsis (Kawasaki et al. 2017), rice (Kawasaki et al. 2017), soybean (Li et al. 2025), and wheat (Hao et al. 2022). In addition, ROS function as cellular signaling molecules that participate in multiple plant signaling pathways in response to biotic and abiotic stresses (Castro et al. 2021; Grant and Loake 2000). Multiple studies have demonstrated that rapid production of ROS can activate the defense-related gene expression and regulate local and systemic immunity, suppressing pathogen growth and disease spread (Li et al. 2025; Singh et al. 2021). Our findings that CLas infection and growth were reduced by MAMP treatments agree with those prior studies. However, chronic ROS accumulation caused by CLas infection con-

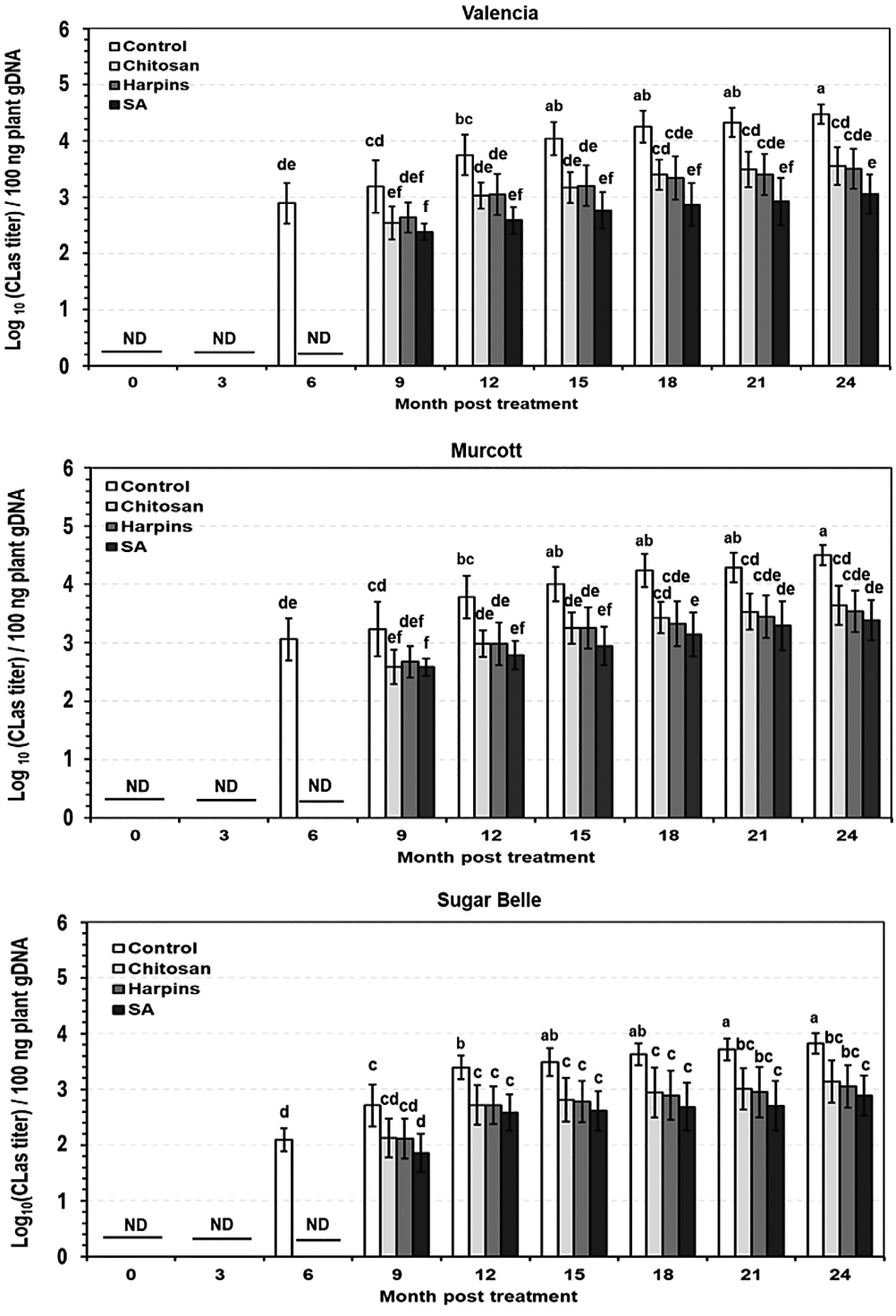


Fig. 5. '*Candidatus* Liberibacter asiaticus' (CLas) titers in citrus trees treated with elicitors via foliar spray in the field. Valencia sweet orange, Murcott, and Sugar Belle mandarin trees were sprayed with chitosan (500 µg/ml), harpins (50 µg/ml), or salicylic acid (SA, 100 µg/ml) in water containing 0.25% (vol/vol) INDUCE monthly in the field. Each treatment contained five plants. The CLas titers in leaves of treated plants were measured using qPCR every 3 months. Trees sprayed with water containing 0.25% (vol/vol) INDUCE served as the control. gDNA, genomic DNA. Data and error bars represent mean and standard deviation, respectively ($n = 5$ replicates). Different lowercase letters indicate significant differences among treatments over time ($P < 0.05$; repeated-measures analysis of variance and Tukey's honestly significant difference test at $\alpha = 0.05$). ND, not detected for CLas by qPCR.

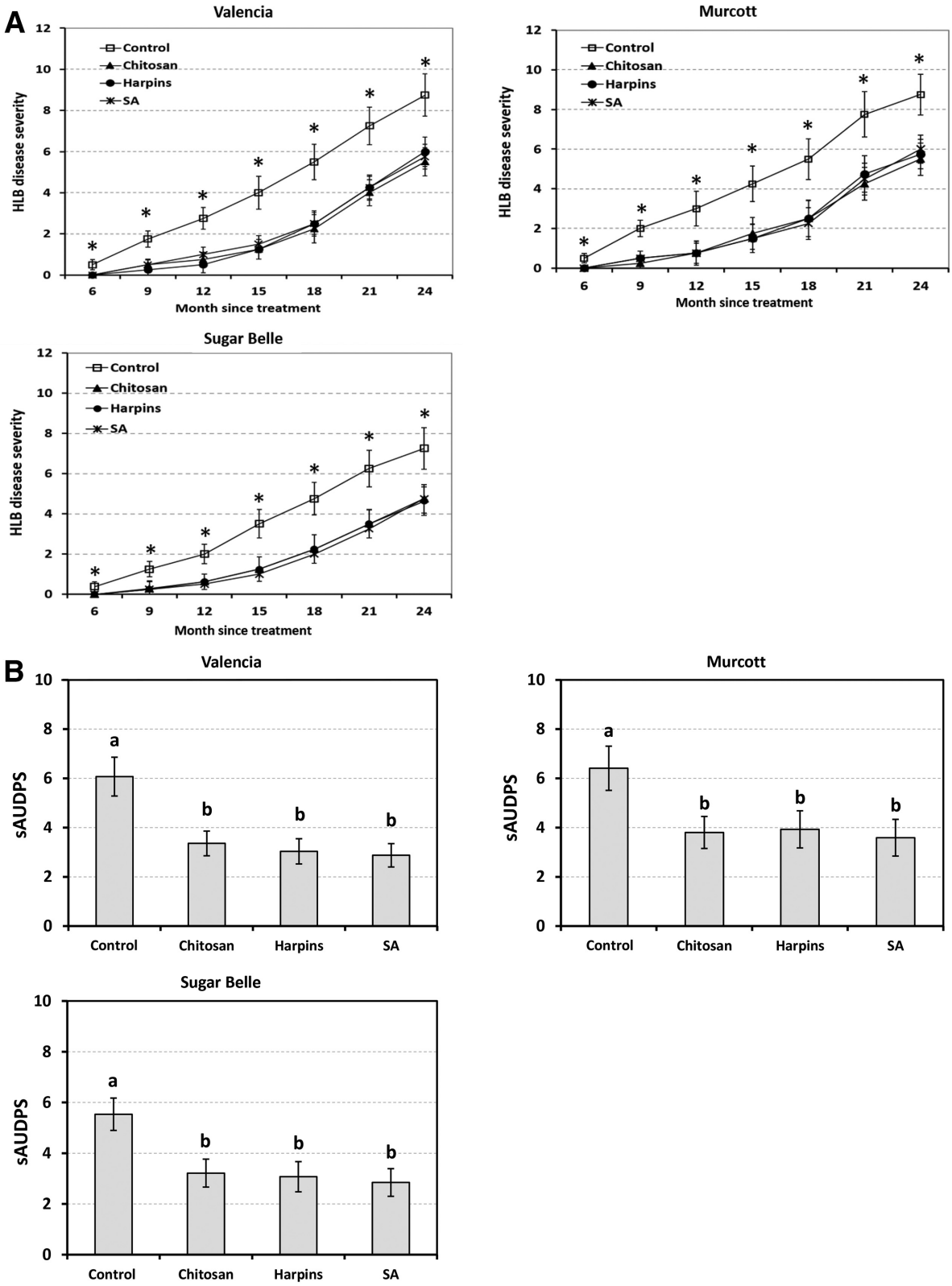


Fig. 6. Suppression of Huanglongbing (HLB) disease progression by foliar spray of elicitors under natural infection conditions. **A,** HLB severity progression of citrus plants following treatments under natural infection conditions. Valencia sweet orange, Murcott, and Sugar Belle mandarin trees were sprayed with chitosan (500 μg/ml), harpins (50 μg/ml), or salicylic acid (SA, 100 μg/ml) in water containing 0.25% (vol/vol) INDUCE monthly in the field. The mean disease severity score was calculated from evaluations of the treated trees. Disease severity scores were shown as means and error bars (standard deviation) ($n = 5$ replicated plants). Asterisks (*) on the bars indicate significant differences ($P < 0.05$) between the untreated control (UTC) and chitosan, harpin, or SA treatment within indicated time points based on Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$). The UTC was sprayed with water containing 0.25% (vol/vol) INDUCE. **B,** HLB disease severity, expressed as standardized area under the disease progress stairs (sAUDPS), achieved by various treatments at the end of a 2-year field trial (2023 to 2025). Different letters on the bars indicate significant differences ($P < 0.05$) among the treatments based on Tukey's HSD test ($\alpha = 0.05$).

tributes to phloem cell death and subsequent HLB disease damage (Huang et al. 2026; Ma et al. 2022). To avoid potential ROS-associated cell damage due to MAMP applications, the application frequency of MAMPs must be fine-tuned to achieve a limited period of ROS accumulation, considering that PTI activated by MAMPs leads to transient ROS production, pathogen effector-triggered immunity results in stronger and sustained ROS accumulation (Torres et al. 2006), and ROS-associated gene-edited citrus lines remain to be characterized to mitigate the damage caused by CLas infection for HLB tolerance (Huang et al. 2026).

Following MAMP treatments, the expressions of PTI marker genes, including *FRK1*, *GST1*, *WRKY22*, and *WRKY29*, and downstream signaling and defense genes (*MKK4* and *PAL1*) were rapidly upregulated, mostly peaking within 1 to 3 days. These observations provide further evidence that chitosan, harpin, or LPS treatment activates PTI and downstream defense responses in citrus plants. Our findings are consistent with a previous report that infiltration of hexaacetyl-chitohexaose, an oligosaccharide from chitin, into citrus leaves upregulated the expression of defense genes, although the effect was transient (Shi et al. 2019). Similarly, chitin (200 μg/ml) triggered significant expression of PTI-related and downstream defense genes in soybean plants at 3 to 6 h after spray treatment (Li et al. 2025). Specifically, *PAL1* exhibited a significant induction in soybean treated with chitin (Li et al. 2025) and in citrus treated with chitosan or harpins (this study). *PAL1* encodes the phenylalanine ammonia-lyase 1, which is involved in the phenylpropanoid pathway responsible for biosynthesis and/or accumulation of plant defense-related secondary metabolites such as phenolic compounds, including SA (La Camera et al. 2004). Multiple studies have shown that high PAL activity is important for pathogen-induced SA accumulation in plants (Coquoz et al. 1998; Huang et al. 2010; Kim and Hwang 2014; Pallas et al. 1996). In addition to being a signaling molecule for systemic acquired resistance in plants, SA demonstrated antibacterial activity at relatively low concentrations (5 to 25 mM = 0.69 to 3.45 μg/ml) against *Agrobacterium tumefaciens* (Anand et al. 2008; Yuan et al. 2007) and *Rhizobium meliloti* (Martínez-Abarca et al. 1998), and both bacteria are close relatives of CLas (Duan et al. 2009). Applications of MAMPs might induce the accumulation of SA in citrus. Our previous investigations (Li et al. 2017, 2021) and the present study have shown that multiple foliar sprays of SA at an appropriate rate reduced CLas infection and HLB disease severity, although CLas encodes a functional SA hydroxylase to degrade SA for survival in citrus plants (Li et al. 2017). Together, the present study and others discussed above demonstrate that MAMP treatments can induce plant defense responses that enhance citrus disease resistance.

CLas inoculation assays further demonstrated that chitosan and harpin treatments enhance citrus resistance against CLas, with implications for management of citrus HLB. Our data showed that chitosan or harpin treatments not only delayed the establishment of systemic infection of CLas transmitted by naturally infested psyllids under field conditions but also suppressed CLas population growth after establishment of systemic infection. Globally, management of citrus HLB has been largely based on the use of insecticides and/or antibiotics (oxytetracycline), and there are concerns regarding their impact on human health and the environment; therefore, alternative strategies are desired. Elicitors such as chitin, chitosan, and harpins can activate the natural defenses of plants and have been increasingly used to enhance plant resistance against diseases and insect pests. Specifically, chitosan and harpins are biodegradable and nontoxic and have demonstrated their benefits as biofertilizers or biopesticides on crop production (Boamah et al. 2023; Popa et al. 2026). Therefore, chitosan and harpin treatments may be an environmentally sound approach for the control of citrus HLB, especially in young plantings. It is critical to protect young citrus trees because they are more vulnerable than older trees to CLas infection (Gottwald 2010). As an alternative strategy, individual protective covers (IPCs) are widely used to protect newly planted citrus trees from HLB, especially in Florida (Gaire et al. 2024). However, IPCs may protect the trees for a maximum of 3 years after their initial setting only if there is no damage that leads to exposure to psyllids, because the trees grow to a size over the capacity of the IPCs, and they are removed ultimately (Gaire et al. 2021). Once IPCs are removed, measures must be taken to treat the trees to prevent and control CLas infection as they are exposed to psyllids. Insecticides and antibiotics are usually the choice, and chitosan and harpins may be an alternative based on the findings in this study. However, further investigations are needed to determine whether and how chitosan and harpins, alone or in rotation with or in combination with insecticides and/or antibiotics, can effectively control CLas infection in young plantings and mitigate HLB damage on mature trees in the field, especially the longer-term effects on yield and fruit quality. Additionally, the economic feasibility of monthly foliar applications of these materials under commercial citrus production conditions needs to be evaluated, with the consideration of material and labor cost and compatibility with existing HLB management approaches and instruments.

TABLE 2. Growth performance of citrus trees treated with microbe-associated molecular patterns via foliar spray over the duration of the field experiments from April 2023 to April 2025[z]

	0 days since treatment			24 months since treatment		
Treatment	Tree height (cm)	Trunk diameter (mm)	Canopy volume (m^3)	Tree height (cm)	Trunk diameter (mm)	Canopy volume (m^3)
Valencia						
UTC	65.6 ± 3.9 a	8.1 ± 0.5 a	0.006 ± 0.002 a	129.5 ± 5.6 a	29.2 ± 3.6 a	0.34 ± 0.05 a
Chitosan	66.1 ± 4.2 a	7.8 ± 0.7 a	0.005 ± 0.003 a	132.3 ± 5.8 a	28.8 ± 3.8 a	0.36 ± 0.06 a
Harpins	65.9 ± 3.5 a	8.3 ± 0.6 a	0.006 ± 0.002 a	127.6 ± 6.3 a	27.9 ± 4.3 a	0.33 ± 0.06 a
SA	67.5 ± 4.1 a	8.0 ± 0.5 a	0.006 ± 0.003 a	130.4 ± 5.9 a	28.4 ± 4.1 a	0.36 ± 0.05 a
Murcott						
UTC	69.4 ± 4.3 a	9.1 ± 0.5 a	0.008 ± 0.002 a	135.4 ± 4.8 a	30.6 ± 3.9 a	0.39 ± 0.04 a
Chitosan	68.8 ± 3.8 a	8.7 ± 0.6 a	0.007 ± 0.003 a	133.8 ± 5.1 a	29.5 ± 4.2 a	0.38 ± 0.05 a
Harpins	67.6 ± 4.6 a	8.9 ± 0.5 a	0.007 ± 0.003 a	134.6 ± 5.3 a	29.7 ± 4.5 a	0.39 ± 0.06 a
SA	70.2 ± 4.2 a	8.8 ± 0.6 a	0.009 ± 0.003 a	136.2 ± 4.7 a	31.2 ± 4.4 a	0.40 ± 0.05 a
Sugar Belle						
UTC	73.4 ± 4.5 a	8.9 ± 0.7 a	0.010 ± 0.003 a	139.8 ± 4.6 a	32.1 ± 4.2 a	0.42 ± 0.06 a
Chitosan	71.8 ± 4.7 a	9.3 ± 0.6 a	0.009 ± 0.003 a	138.5 ± 5.2 a	30.9 ± 4.5 a	0.40 ± 0.05 a
Harpins	72.5 ± 4.4 a	9.2 ± 0.6 a	0.009 ± 0.002 a	140.1 ± 5.5 a	31.6 ± 4.4 a	0.41 ± 0.06 a
SA	73.7 ± 4.8 a	9.1 ± 0.7 a	0.011 ± 0.003 a	141.9 ± 4.8 a	32.3 ± 4.3 a	0.43 ± 0.07 a

[z] Data shown are mean ± SD of five replicate trees. Values within a column followed by the same letter indicate no significant differences ($P > 0.05$; Tukey's honestly significant difference test at $\alpha = 0.05$). Valencia sweet orange, Murcott, and Sugar Belle mandarin trees were sprayed with chitosan (500 μg/ml), harpins (50 μg/ml), or salicylic acid (SA, 100 μg/ml) in water containing 0.25% (vol/vol) INDUCE monthly in the field. UTC, untreated control (sprayed with water containing 0.25% [vol/vol] INDUCE).

Our data showed that LPS activated weaker PTI response in citrus plants, relative to chitosan and harpins, and was not effective in reducing CLas titer in infected trees. One possible explanation might be that there lacks a citrus PRR that can ideally perceive and recognize the LPS tested, from *Pseudomonas aeruginosa* 10, for which citrus is not likely to be a host, resulting in difficulty regarding strong PTI and downstream defense responses. Thus far, only the PRRs that can recognize flg22 from the bacterial canker pathogen *Xanthomonas citri* subsp. *citri* and trigger rapid ROS production and induction of PTI marker genes have been identified in several resistant citrus genotypes (Shi et al. 2015, 2016), and these PRRs also occur in susceptible citrus genotypes but trigger weak PTI responses. Whether these PRRs can recognize the LPS from *X. citri* or other bacterial species remains unclear. The LPS from *Liberibacter crescens* could trigger systemic acquired resistance in sweet orange plants against *X.* subsp. *citri*, probably through nitric oxide-mediated systemic acquired resistance activation (Jain et al. 2022), but the corresponding PRR remains to be identified. Another probable reason might be because the dosage of LPS tested in the present study was not adequate for the corresponding citrus PRR to perceive and recognize, thereby hampering robust PTI and downstream defense responses. Additionally, we cannot exclude the possibility that the potential citrus LPS receptor recognized something being a contaminant in the LPS tested but not the LPS itself and thus triggered relatively weak PTI response, as the assumed LPS receptor in Arabidopsis (LORE) does not recognize LPS but a fatty acid named medium-chain 3-hydroxy fatty acid (mc-3-OH-FA) (Kutschera et al. 2019). Therefore, more investigations are needed to determine whether and how LPS can enhance citrus resistance against HLB.

Interestingly, our results indicated that Sugar Belle 'LB8-9' mandarin did not outperform Valencia sweet orange and Murcott mandarin in the tests for delaying CLas infection under field conditions but did in restricting the growth of CLas and development of HLB symptoms after infection. These observations are consistent with previous reports that HLB rating is strongly correlated with CLas titer in multiple citrus cultivars (Stover and McCollum 2011), and symptomatic leaves have higher CLas titers than asymptomatic leaves (Pandey et al. 2021). Our results agree that Sugar Belle 'LB8-9' is more tolerant/resistant to HLB relative to Valencia sweet orange and Murcott mandarin, as pathogen titer is a commonly used indicator for disease resistance (Pagán and García-Arenal 2018), and infected Sugar Belle trees generally harbored lower CLas titers than Valencia and Murcott.

The present study evaluated the effect of a single application of MAMPs on citrus defense gene expression and ROS production. Repeated elicitor exposure can result in immune priming in some plant–elicitor systems (Mauch-Mani et al. 2017); however, immune desensitization or altered responsiveness cannot be excluded in citrus because responses after repeated applications of elicitors were not measured in this study. Whether and how repeated applications of MAMPs may affect citrus immune responses remains to be investigated.

Utilizing induced resistance for plant disease management could adversely affect crop biomass and yield production due to the metabolic competitions among biological processes for vegetative growth, reproductive development, and the synthesis of defense-related compounds (Cipollini et al. 2003). This issue seems possible to overcome by adjusting the dosage and frequency for application of chitosan and harpins for control of citrus HLB. Our data showed that application of chitosan and harpins at an appropriate dosage and frequency had no suppressive effect on the development and growth of young citrus trees. Additionally, consistent with our findings, harpins have been shown to not only enhance disease resistance but also improve yield in a diversity of fruit and vegetable crops (Popa et al. 2026). However, further estimations of the possible plant fitness cost of repeated PTI activation in citrus trees by chitosan and harpins are needed, especially the influence on fruit bearing, yield, and quality.

Taken together, this study demonstrates that foliar spray of chitosan or harpins has positive control effects on delaying CLas infection under the sampling scheme used and suppressing CLas population growth in infected plants. Therefore, it may be a promising effective and sustainable strategy for control and management of citrus HLB, especially to protect young flushes after IPC removal from new plantings. However, more studies are needed to optimize the rate, frequency, and timing of application of chitosan and/or harpins to maximize their benefits when used for the control of citrus HLB, with a consideration of economic feasibility in citrus production. This study showed that interrupting the status quo related to immune responses triggered by CLas by boosting immunity can be used as a useful strategy in controlling a pathogen-triggered immune disease caused by a biotrophic pathogen.

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