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DENSITY OF RHIZOSPHERIC MICROORGANISMS IN MEXICAN LEMON WITH HLB TREATED WITH CHEMICAL INDUCERS

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ABSTRACT

Citriculture is a highly profitable activity that is threatened by Huanglongbing (HLB), which is caused by the bacterium *Candidatus* Liberibacter *asiaticus* (CL*as*). The control of this bacterium is fundamental to the management of HLB. The objective of this study was to determine the abundance of total culturable bacteria (B) and filamentous fungi (HF) in the rhizosphere of Mexican lemon (Lm) plants infected with CL*as* and treated with chemical defense inducers based on commercial molecules (Salicylic acid, Plasmitox®, Blindax®, and Virus-Stop®). A completely randomized experimental design was used, with six treatments and five replicates: five treatments with CL*as* (three with commercial inducers, one with salicylic acid, and one without inducer), one without CL*as*, and one without inducer. The presence of CL*as* and the severity of HLB were both determined. Five rhizospheric samples were collected every 70 days. Microbial populations were determined by plate count. The presence of CL*as* was found in Lm-HLB plants, but no significant decrease (Kruscal-Wallis, $p \leq 0.05$) in HLB symptomatology was detected by the effect of the inducers. However, the population dynamics of total rhizospheric B and HF of Lm were affected by inoculation with CL*as* (Tukey; $p \leq 0.05$), being even lower with the application of the inducers Plasmitox® and Virus-stop®. This response may play an important role in the microbiome associated with the health of Mexican lemon plants.

Keywords: total rhizospheric bacteria, *Candidatus* Liberibacter *asiaticus*, elicitors, total rhizospheric fungi.

INTRODUCTION

Mexican lemon (Lm) (*Citrus aurantifolia*) and Persian lemon (*Citrus latifolia* Tan.) are plants grown in a large number of countries in the world, mainly in tropical and subtropical regions (Sunday *et al*., 2015). Lm production is affected by the disease called

Huanglongbing (HLB) or citrus yellow dragon, caused by the bacterium *Candidatus* Liberibacter *asiaticus* (CL*as*) (Mora-Aguilera *et al*., 2016). HLB has been reported to produce losses in the Persian lemon production chain of 17.6 % in Yucatán, Mexico (Flores-Sánchez *et al*., 2015). Robles-González *et al*. (2017) estimated yield losses of 46 % in Mexican lemon, while in orchards with this same citrus and low agronomic technology, yield losses amounted to 62.7 % for Colima (Mora-Aguilera *et al*., 2022) and 36.7 % for Michoacán (Mora-Aguilera *et al*., 2016). CL*as* is present in 24 states of the Mexican Republic with detections in plant material (Pimentel-González *et al*., 2018). HLB symptoms on leaves consist of mottling, asymmetric angular spots, thickening of veins, chlorosis, and defoliation of trees (Esquivel-Chávez *et al*., 2012; Mendoza-Hernández *et al*., 2015).

Several strategies have been proposed for HLB disease control, including the induction of systemic resistance (Bagio *et al*., 2016). Inducing resistance is defined as the enhancement of plant defensive capacity against a wide range of pathogens that occurs after appropriate stimulation (van Wess *et al*., 1997). Elicitors or activators are exogenous molecules, known as inducers, that can activate defense or resistance mechanisms in plants (Gómez and Reis, 2011). They can be molecules derived from insect pests, plants, microorganisms, plant-derived biological preparations, or synthetically produced analogues (Riveros-Angarita, 2001). The application of inducers in plants can trigger two main defense pathways: 1) systemic acquired resistance (SAR), and 2) induced systemic resistance (ISR) (Thakur and Sohal, 2013; Riveros-Angarita, 2001). SAR is an inherent phenomenon of the immune system to reduce disease development and spread, having potential use as a resistance inducer against viral, bacterial, and fungal diseases of a variety of biotrophic, hemibiotrophic, and necrotrophic pathogens (Heil and Bostock, 2002; Choi and Hwang, 2011).

Systemically protected leaves respond quickly and efficiently to the challenges of infection with a virulent pathogen when plants are pretreated with necrotizing pathogens or synthetic SAR inducers (Camarena-Gutierrez and de la Torre-Almaráz, 2007). Different salicylic acid (SA)-mediated signal transduction pathways are involved in SAR (Torres *et al*., 2006; Heil and Bostock, 2002). SAR can be induced when plants are sprayed or injected with SA, H_2O_2, potassium salts, methyl-2,6-dichloro-isonicotinic acid (INA), benzo (1,2,3) thiadiazole-7-carbothionic acid (BTH), acibenzolar-s-methyl (ASM), imidacloprid, and β-aminobutyric acid (BABA) (Camarena-Gutiérrez and de la Torre-Almaráz, 2007; Shen *et al*., 2013; Thakur and Sohal, 2013; Tiwari *et al*., 2013).

On the other hand, ISR can be induced by plant growth-promoting rhizobacteria (PGPR) (Mauch-Mani and Métraux, 1998). ISR uses pathways regulated by jasmonic acid (JA) and ethylene (ET) (Camarena-Gutiérrez and de la Torre-Almaráz, 2007). JA stimulates the production of molecules related to the accumulation of antimicrobial compounds (Romanazzi *et al*., 2016). Strains of the genera *Bacillus*, *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Azospirillum*, and *Paenibacillus* induce ISR (Zhu *et al*., 2022). The application of JA protects oats against *Erysiphe graminis* f. sp. *Hordej*, tomato and potato against *Phytophthora infestans*, rice against the germination of spores of

Pyricularia oryzae, and spruce seedlings against *Phytium ultimun* (Laredo-Alcalá *et al*., 2017; Eng-Sánchez, 2008).

Despite the positive effect of beneficial microorganism inoculation as inducers of systemic resistance in the control of diseases caused by phytopathogenic microorganisms or to reduce disease severity, the effect on rhizospheric microorganism populations remains unclear. The use of biotechnological tools that induce SAR and ISR signaling pathways has been proposed for HLB management. However, there are few works on Mexican lemon that evaluate the effect produced by the use of chemical resistance inducers on the increase or decrease of rhizosphere microorganisms. Therefore, in the present research, the objective was to determine the abundance of total rhizosphere culturable bacteria (B) and filamentous fungi (F) of Mexican lemon plants with Huanglongbing (*Candidatus* Liberibacter *asiaticus*) treated with chemical inducers based on commercial molecules (Salicylic acid, Plasmitox®, Blindax®, and Virus-Stop®) under greenhouse conditions.

MATERIALS AND METHODS

Experiment establishment and conditions

The experiment was conducted in a zenithal greenhouse with polycarbonate walls and roof at the CIATEJ Zapopan Unit (20° 42′ 3.099″ N, 103° 28′ 25.878″ W, 1560 m altitude) over a one-year period. It was a completely randomized design with six treatments and five replications each. The experimental unit was a pot with a Mexican lemon tree (*Citrus aurantifolia*) grafted on macrophylla (*C. macrophylla*). One and a half years old, certified disease-free Mexican lemon trees from Tecomán, Colima, were set in 5 kg bags of soil which were later transplanted into 20 L pots.

Mexican lemon plants from five of the six treatments were infected with CL*as* via bud grafting on both rootstock and graft (Mendoza-Hernández *et al*., 2017), using rods from HLB-symptomatic trees from Lm orchards in Tecomán, Colima. Treatment 6 was not grafted, so these plants were considered the healthy control (Table 1). The plants were grafted eight months prior to the beginning of the experiment and transplanted into 20 L pots. The trees were irrigated at field capacity once a week. The first application of the chemical inducer treatments was initiated 30 days after transplanting to 20 L pots. All treatments received chemical nutrition (Q) consisting of urea (46 % N) and triple 16 (16-16-16 %; N-P-K) three times a year, for a total dose of 150-18-18. The substrate used in the experiment was a mixture of soil/sand/perlite/peat (Sunshine Mix® No. 3) (50:30:10:10; v/v/v/v/v/v).

Prior to autoclaving (120 °C, 1.05 kg cm^{-2}, 6 h), total B and HF were counted on the plate in decimal dilutions (Camacho-Cruz *et al*., 2009) in order to determine the initial microbial load of the substrate and compare it with the concentrations obtained during the experiment. After substrate sterilization, the colony-forming unit (UFC) count of B and HF was zero. Black plastic containers with a capacity of 20 L were used as pots for a Mexican lemon plant; 16 kg of the sterilized mixture were placed in each pot. This

Table 1. Treatment design to determine the effect of chemical inducers on culturable bacterial and fungal rhizospheric populations during HLB management on Mexican lemon (*Citrus aurantifolia*) trees under greenhouse conditions.

Key	Treatment				Conditions for each application		
	Inducer	CL*as*	n	Total concentration (g per treatment) of inducer	Volume (mL)	Concentration (mL L^{-1})	Frequency (days)
As CL*as*	Salicylic acid	Yes	5	0.025	50.0	500.0	Every 30
Ptx CL*as*	Plasmitox®	Yes	5	750.00	62.5	12.0	Every 30
Bdx CL*as*	Blindax®	Yes	5	93.75	62.5	1.5	Every 30
Vst CL*as*	Virus-Stop®	Yes	5	181.25	72.5	2.5	Every 30
Q CL*as*	No inducer	Yes	5	NA	NA	NA	NA
Q	No inducer	No	5	NA	NA	NA	NA

CL*as*: *Candidatus* Liberibacter *asiaticus*; AsCL*as*: treatment with salicylic acid and infected with CL*as*; PtxCL*as*: treatment with Plasmitox® and infected with CLas; BdxCL*as*: treatment with Blindax® and infected with CL*as*; VstCL*as*: treatment with Virus-Stop® and infected with CL*as*; Q CL*as*: treatment infected with CL*as* (diseased control); Q: treatment without CL*as* (healthy control); NA: not applicable.

mixture presented a loam texture, neutral pH in water (6.5), organic matter content of 13.5 %, electrical conductivity of 3.3 ds m^{-1}, and N-NO_3, P, K, Ca, and Mg contents of 51, 184, 445, 3348, and 712 mg kg^{-1}, respectively; these analyses were performed by the Agricultural Analysis Laboratory of Fertilab® of Celaya, Guanajuato (fertilab.com.mx) in accordance with the procedures of the Mexican Official Standard NOM-021-RECNAT-2000 (NOM, 2002). For the production of *C. aurantifolia* in loam-textured substrates, with a content of 2.89, 61, 389, 149, 1400, and 420 mg kg^{-1} of N-NO_3, P, K, Ca, and Mg, annual applications of 1.2, 0.6 and 0.6, kg $tree^{-1}$ of N, P, and K are recommended (Pérez-Zamora and Orozco-Romero, 2004).

Rhizosphere sampling and microorganism counting

After the application of each treatment, five rhizosphere samples were taken at 70, 140, 210, 280, and 368 days after the first application of the inducers (ddpa), using a PVC tube of 1.3 cm in diameter and 35 cm high, introduced in four cardinal points around the plant at three different depths (5, 15, and 30 cm). These samples were then homogenized by treatment and stored at 4 °C until processing. For the total bacterial count, nutrient agar medium (AN, 213000-DIFCO®) was used, while for the determination of total fungi, potato and dextrose agar medium (PDA, 213400-DIFCO®) was used at a pH of 5.5, 3.3 mL L^{-1} of 1 % rose Bengal (198250-Sigma®) and 40 μg mL^{-1} of streptomycin (S9137-Sigma®).

The quantification of colony-forming units (UFC) was performed on seeded plates by means of the serial decimal dilutions method carried out in each sampling, following the method indicated by Camacho-Cruz *et al.* (2009), with some modifications: for which the dry weight of the samples to be analyzed was determined, then for each of

the treatments, 10 g of rhizospheric soil were taken and 90 mL of sterile distilled water were added to obtain the 10^{-1} dilution, followed by taking 1 mL of the 10^{-1} dilution and dissolving it in 9 mL of distilled water to obtain the 10^{-2} dilution. The above steps were repeated with the last dilution obtained up to the 10^{-6} dilution. AN boxes were incubated at 30 °C for 48 h and PDA boxes at 28 °C for seven days, seeding four Petri dishes per respective dilution.

Detection of CL*as* by PCR and determination of disease severity

Prior to the application of the treatments, leaf tissue samples were collected from healthy and CL*as*-infected plants at random (for DNA extraction and subsequent PCR). The presence of CL*as* in Mexican lemon (Lm) trees inoculated with *Candidatus* was determined using endpoint PCR with the kit DreamTaq™ DNA Polymerase (EP0702, Thermo Scientific™). DNA was extracted from leaf midribs using the 3 % hexadecyltrimethylammonium bromide (CTAB) protocol (Zhang *et al*., 1998). The partial sequence of the 16S ribosomal rDNA gene (1160 bp) was amplified using the specific oligonucleotides OI1 (5′-GCGCGCGTATGCAATACGAGCGGCA-3′) and OI2c (5′-GCCTCGCGACTTCGCAACCCAT-3′) (Jagoueix *et al*., 1996) under the following PCR conditions: 94 °C for 5 min; 35 cycles of 45 s at 94 °C, 45 s at 68 °C, 60 s at 72 °C; and a final cycle at 72 °C for 10 min (Lou *et al*., 2012). The PCR reaction product was migrated by electrophoresis (90 V for 40 min) on agarose gels (0.8 %) and observed in a transilluminator with GelRed® (1X, 41003 Biotium®). To determine the size of the PCR fragment, the amplification profile was compared visually using the 1 kb Plus Ladder marker (Invitrogen™) as a reference.

During the 280 ddpa sampling of the rhizospheric soil, the severity of HLB disease was quantified using an ordinal qualitative severity scale with seven levels (Figure 1). In each tree, three branches were considered according to tree height: apical,

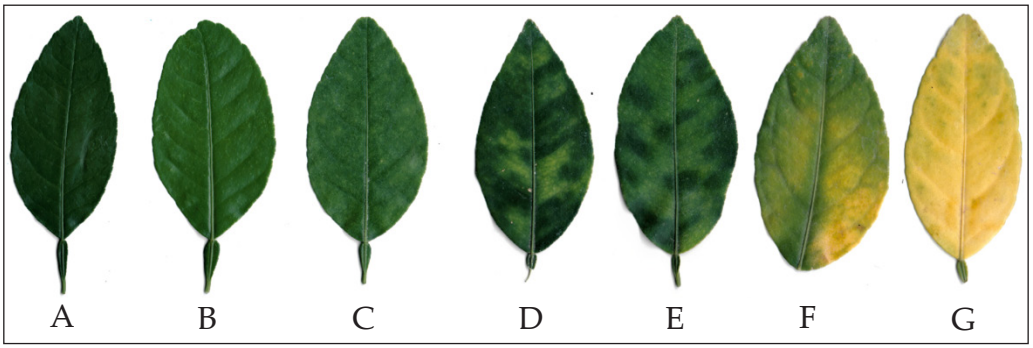

Figure 1. Ordinal severity scale of HLB trees with the goal of determining the effect of chemical defense inducers on Mexican lemon plants under greenhouse conditions. A: healthy leaves; B: less than 50 % of leaf area with clear chlorotic spots; C: more than 50 % of the leaf area with clear chlorotic spots; D: less than 50 % of leaf area with light chlorotic spots; E: more than 50 % of leaf area with light chlorotic patches; F: less than 50 % of the chlorotic leaf area yellow; G: More than 50 % of the chlorotic leaf area yellow.

intermediate, and basal. In turn, the branch was divided into three sections: apical, intermediate, and basal. The severity of each leaf of the intermediate section of the three branches per tree was quantified according to the severity scale (Figure 1).

Statistical analysis of data

Bacterial and fungal concentration data were subjected to one-way analysis of variance and Tukey multiple comparison of means, both at a 5 % significance level ($p \leq 0.05$). While for the HLB severity scale data were analyzed using a Kruskal-Wallis nonparametric test ($p \leq 0.05$) and 95 % confidence intervals at the median. All of these analyses were performed using the statistical program Statgraphics® Centurion XV version 15.2.06 (StatPoint, 2005).

RESULTS AND DISCUSSION

Infection of Mexican lemon (Lm) trees with *Candidatus* Liberibacter *asiaticus* (CL*as*) and HLB development

Lm plants were infected with CL*as* by grafting budwood from symptomatic trees from Tecomán, Colima (Figures 2A, B, and C). For the infection of the plants, a double grafting was performed, both on the rootstock (*C. macrophylla*) and on the Mexican lemon graft (*C. aurantifolia*); this allowed the development of typical HLB symptoms on both the graft and the rest of the tree (Figure 2D).

Leaf tissue samples were collected from Lm trees with and without HLB symptoms (280 ddpa). DNA was extracted from infected and uninfected plants (Figure 3A). A band of 1167 bp was obtained from partial amplification of the bacterial CL*as*-specific 16S ribosomal gene in plants with HLB symptoms (Figure 3B), indicating the presence of CL*as* in Lm trees inoculated with the phytopathogenic bacterium and demonstrating that Lm plants were infected with CL*as* through bud grafting. This allows determining an effect of chemical inducers on Lm plants with or without HLB on microbial populations at the rhizosphere level under greenhouse conditions. Healthy plants show a degree of clear chlorotic spots, which can be confused with other symptoms caused by microelement nutrient deficiencies (Robles-González *et al*., 2013); in addition, the molecular analysis in this work showed the absence of CL*as* in the samples of healthy plants (Figure 3B).

The severity of HLB disease at 280 days after the first application (ddpa) of the inducers is shown (Figure 4). The application of different chemical inducers has no effect on the degree of HLB, since severity levels 3 and 4 are typical symptoms of this disease. This result is consistent with the increase in CL*as* concentration reported by Trinidad-Cruz *et al*. (2019) when treating Mexican lemon trees with the inducer salicylic acid (SA), indicating that the AcCL*as* treatment did not result in a significant decrease inCL*as* concentration (Trinidad-Cruz *et al*., 2019) or typical HLB symptoms (Figure 4).

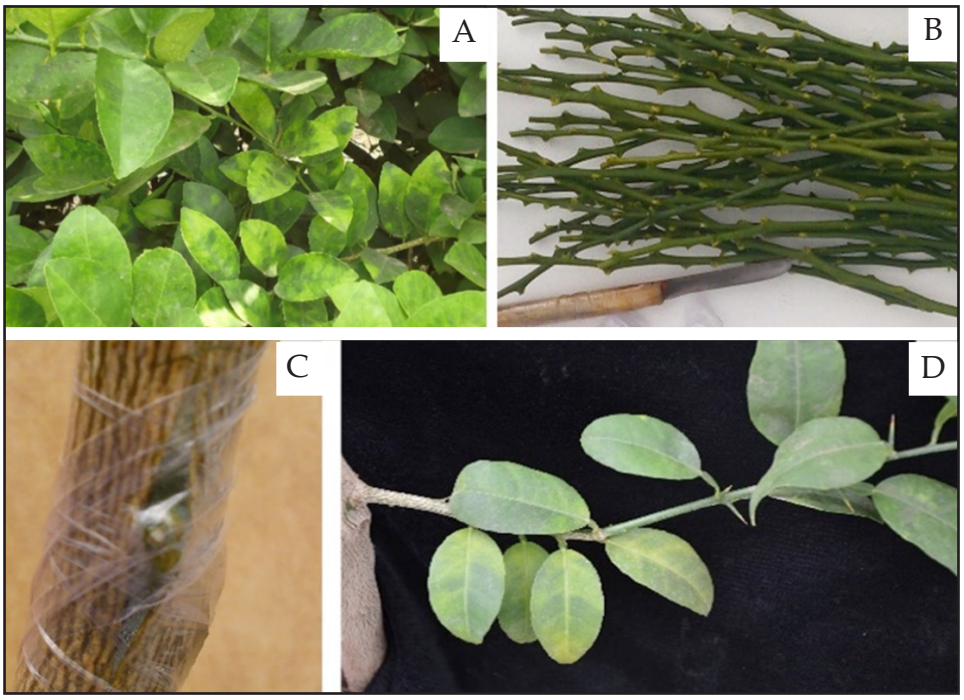

Figure 2. Details of the development of *Candidatus* Liberibacter *asiaticus* infection in Mexican lemon (*Citrus aurantifolia*) trees by bud grafting under greenhouse conditions. A: Symptomatic trees with HLB in orchards in Tecomán, Colima; B: scions of symptomatic trees with HLB from orchards in Tecomán, Colima; C: grafting of diseased buds with HLB onto certified disease-free Mexican lemon trees; D: graft development and growth with typical HLB symptoms on HLB-free certified Lm plants.

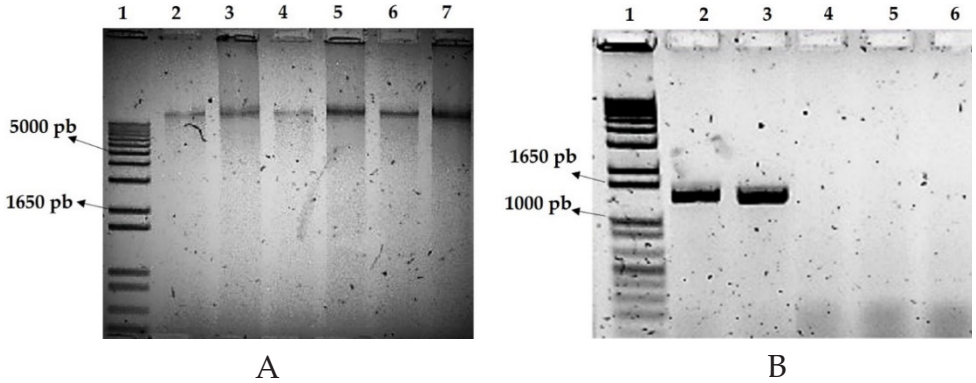

Figure 3. *Candidatus* Liberibacter *asiaticus* (CL*as*) detection in Mexican lemon (*Citrus aurantifolia*) plants inoculated with bud grafting under greenhouse conditions. A: Quality and presence of DNA extracted from Lm plants, lane 1 corresponds to 1 kb Plus Ladder marker (Invitrogen^{MR}), lanes 2–4 to CL*as*-infected plants, and lanes 5–7 to CL*as*-free plants; B: determination of the presence of CL*as* in Lm trees by endpoint PCR (1167 pb) of plants grafted with rods from HLB diseased trees, prior to the application of treatments with chemical inducers, lane 1 corresponds to 1 kb Plus Ladder (Invitrogen^{MR}), lanes 2 and 3 to CL*as*-infected plants, lanes 4 and 5 to healthy plants, and lane 6 to the PCR control reaction without DNA.

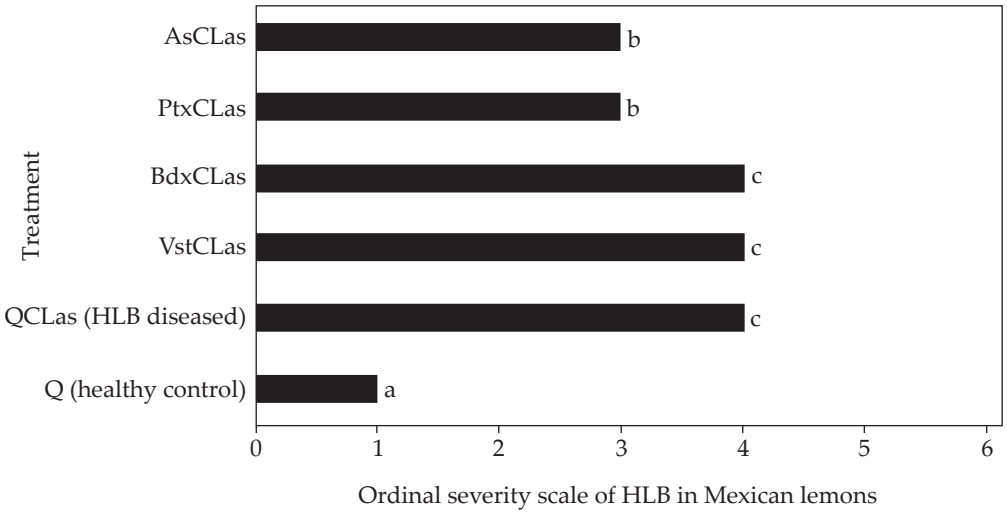

Figure 4. Effect of the application of chemical defense inducers on HLB disease severity in Mexican lemon 280 days after the first application of the treatment under greenhouse conditions. Different letters indicate significant differences according to the Kruskal-Wallis test ($p \leq 0.05$) and 95 % confidence intervals of the median. CL*as*: *Candidatus* Liberibacter *asiaticus*; AsCL*as*: treatment with salicylic acid and infected with CL*as;* PtxCL*as*: treatment with Plasmitox® and infected with CL*as*; BdxCL*as*: treatment with Blindax® and infected with CL*as*; VstCL*as*: treatment with Virus-Stop® and infected with CL*as*; QCL*as*: treatment infected with CL*as* (diseased control); Q: treatment without CL*as* (healthy control).

Effect of the application of chemical inducers of systemic resistance on the dynamics of total rhizospheric culturable bacterial populations

The initial count of total culturable bacteria in the substrate (before sterilization) was $4.2x10^6$ UFC g^{-1} rhizospheric soil. At 71 days after the first application of chemical inducers (ddpa) bacteria were found in all treatments. The total bacterial UFC count was lower at 71 ddpa when compared to the initial count prior to sterilization. However, after sterilization, the count increased. The treatment with the highest significant bacterial concentration (Tukey, $p \leq 0.05$) was BdxCL*as*, with an average of $6.1x10^5$ UFC g^{-1} rhizosphere soil, compared to the treatment with the lowest bacterial concentration (PtxCL*as*), with only an average of $0.45x10^5$ UFC g^{-1} substrate (Table 2). When comparing healthy Mexican lemon plants (Q) with plants diseased by HLB (QCL*as*) without the application of inducers, a significant decrease in total bacteria (Tukey; $p \leq 0.05$) was observed in diseased plants with respect to healthy plants in the corresponding samples of 221 and 368 ddpa, while for 146 and 280 ddpa, the opposite occurs. This behavior could be explained by the interaction between QCL*as* with their host and the environmental conditions (such as temperature) where the HLB disease develops. Genes related to abscisic acid and ethylene metabolism show twice the expression in HLB-infected plants compared to healthy plants (Arce-Leal *et al*., 2020), suggesting that infected plants activate their defense system, which may include the

Table 2. Behavior of the population dynamics of rhizospheric total culturable bacteria after the first application of chemical inducers on Mexican lemon (*Citrus aurantifolia*) trees infected with *Candidatus* Liberibacter *asiaticus* under greenhouse conditions.

Treatment	Days after the first application of the inducer (10^5 UFC g^{-1} soil)				
	71	146	221	280	368
AsCL*as*	5.10 a	7.30 c	12.70 ab	19.00 a	5.70 cd
PtxCL*as*	0.45 b	46.0 b	4.40 d	9.70 b	3.20 d
BdxCL*as*	6.10 a	9.00 c	8.30 c	2.40 bc	9.70 b
VstCL*as*	6.03 a	0.53 c	9.70 bc	3.90 bc	6.70 bc
QCL*as*	5.60 a	135.00 a	4.40 d	9.10 bc	7.40 bc
Q	5.50 a	68.70 b	15.00 a	1.60 c	27.00 a

Different letters in the same column indicate significant differences according to Tukey's test ($p \leq 0.05$). CL*as*: *Candidatus* Liberibacter *asiaticus*. As CL*as*: treatment with salicylic acid and infected with CL*as*; PtxCL*as*: treatment with Plasmitox® and infected with CL*as*; BdxCL*as*: treatment with Blindax® and infected with CL*as*; VstCL*as*: treatment with Virus-Stop® and infected with CL*as*; QCL*as*: treatment infected with CL*as* (diseased control); Q: treatment without CL*as* (healthy control).

production of antimicrobial compounds such as PR proteins, with a concomitant decrease in rhizospheric populations in HLB plants.

In general, HLB-infected plants that received chemical inducers treated with the commercial products Plasmitox®, Blindax®, and Virus-stop® showed a significant decrease in rhizosphere bacteria (Tukey; $p \leq 0.05$) when compared to HLB-diseased plants without inducer treatment. A large number of microorganisms exist in the rhizosphere, and their diversity is highly dependent on nutrient composition and concentration. Microorganisms normally compete for space and nutrients in order to survive, resulting in changes in microbial populations (Cano, 2011). Variation in bacterial concentration can be affected by factors such as temperature, ultraviolet radiation, ionizing radiation, humidity, pH, REDOX potential, organic acids, salts, and gases such as nitrogen and oxygen (Calvo *et al*., 2008).

UFC counts were found to be between $1.6x10^5$ and $2.7x10^6$ UFC g^{-1} in rhizospheric soil from 221 to 368 ddpa (Table 2). Pathogenic organisms that have adapted to their hosts can manipulate the plant defense system for their own benefit, with consequences for other microorganisms (Hacquard *et al*., 2017). CL*as* bacteria cause changes in the defense system, regulatory system, and metabolism of citrus, indirectly affecting the rhizosphere microbiota (Wang and Trivedi, 2014; Wang *et al*., 2017). In addition, bacteria such as CL*as* affect the microbiome associated with Lm, restructuring the bacterial communities of leaves, stem, root, rhizoplane, and rhizosphere in comparison to healthy citrus (Trivedi *et al*., 2011; Zhang *et al*., 2021). CL*as* bacteria have been reported to alter the development of rhizosphere microbial communities, shifting the availability of carbon sources to recalcitrant carbon sources (Trivedi *et al*., 2011).

Citrus trees infected with CL*as* classified on a 5-level severity scale (DR1=vigorous tree with mild symptoms to DR5=dead or dying trees) showed that disease severity

influences the abundance of bacteria in the root by decreasing the abundance of beneficial bacteria in DR1, followed by an enrichment of beneficial bacteria in trees surviving the disease (DR2), and an increase in the abundance of pathogenic bacteria, saprophytes, and microorganisms associated with antibiotic production in trees in DR3 to DR4 (Ginnan *et al*. , 2020). According to the results (Table 2), this phenomenon is evident when comparing the treatment of healthy plants to those infected with HLB. In the present study, the treatment that showed the lowest UFC count of total culturable bacteria during 3 of the 5 sampling times analyzed was PtxCL*as* (Table 2). Plasmitox® is an organic insecticide product made from botanical extracts for foliar application, with active ingredients allyl sulfur, eugenol, and cis-jasmona. Cis-jasmona has been associated to the induction of plant defenses indirectly through the activation of cytochrome P450 CYP81D11 (Matthes *et al*., 2011). In plants, cis-jasmona induces the production of secondary metabolites that inhibit the development of pests, diseases, and weeds (Blassioli-Moraes *et al*., 2008). The application of cis-jasmona induces the production of flavonoids, among them aglycone genistein, an antimicrobial compound with protective functions and inducer of phytoalexins (da Graça *et al*., 2016). The production of these compounds can decrease the bacterial concentration in the rhizosphere of Mexican lemon plants under greenhouse conditions.

Effect of the application of chemical inducers of systemic resistance on the population dynamics of total rhizospheric culturable filamentous fungi

The total culturable filamentous fungi (HFT) count of the initial sampling was $7x10^2$ UFC g^{-1} rhizospheric soil. HFT counts were possible at 71 ddpa in five of the six treatments (Table 3). The treatments were statistically different (Tukey; $p \leq 0.05$) and the number of UFC in the PtxCL*as* and AsCL*as* treatments was lower than the initial

Table 3. Behavior of population dynamics of total rhizospheric cultivable filamentous fungi after the first application of chemical inducers on Mexican lemon (*Citrus aurantifolia*) trees infected with *Candidatus* Liberibacter *asiaticus* under greenhouse conditions.

Treatment	Days after the first application of the inducer (10^3 UFC g^{-1} soil)				
	71	146	221	280	368
AsCL*as*	0.07 b	0 c	3.40 a	1.00 bc	0 b
PtxCL*as*	0 b	0 c	2.00 b	1.70 b	0.33 b
BdxCL*as*	1.10 a	0.01 c	2.30 b	3.30 a	0.33 b
VstCL*as*	1.60 a	0 c	0.67 c	0 d	0 b
QCL*as*	1.50 a	0.37 b	0 c	0.50 cd	1.00 ab
Q	1.50 a	0.50 a	0.73 c	1.30 b	1.70 a

Different letters in the same column indicate significant differences according to Tukey's test ($p \leq 0.05$). CL*as*: *Candidatus* Liberibacter *asiaticus*; AsCL*as*: treatment with salicylic acid and infected with CL*as*; PtxCL*as*: treatment with Plasmitox® and infected with CL*as*; BdxCL*as*: treatment with Blindax® and infected with CL*as*; VstCL*as*: treatment with Virus-Stop® and infected with CL*as*; QCL*as*: treatment infected with CL*as* (diseased control); Q: treatment without CL*as* (healthy control).

count. At 146 ddpa, the treatments with the highest concentrations of HFT occurred in healthy plants (Q) and plants with HLB (QCL*as*) (Table 3) with $0.5x10^3$ and $0.37x10^3$ UFC g^{-1} rhizospheric soil, respectively. In three of the four treatments with chemical inducers, it was not possible to identify HFT, being statistically equal to each other (Tukey; $p \leq 0.05$). At 221 ddpa, the treatment with the highest concentration of HFT was AsCL*as* ($3.4x10^3$ UFC g^{-1} soil), while the treatments with the lowest significant (Tukey; $p \leq 0.05$) concentration of total HFT were VstCL*as*, QCL*as*, and Q. Finally, at 280 and 368 ddpa, the treatment that showed the lowest UFC count was VstCL*as*.

Virus-stop® is an organic product designed to generate resistance and tolerance to viruses in agricultural crops that contains activators of systemic resistance proteins, shikimic acid precursors, terpenoids as inducers of plant defense mechanisms, and self-defense alkaloids. When plants undergo stress, they use signaling phytohormones such as ethylene, jasmonic acid, and salicylic acid, which activate SAR or ISR, producing secondary metabolites such as terpenes or alkaloids, which inhibit the growth of fungi, bacteria, and viruses (Sepúlveda-Jiménez *et al*., 2003). Among these hormones, salicylic acid has been described as an inducer of cell apoptosis (Pereira *et al*., 2007). The application of salicylic acid on *Solanum tuberosum*, *Oryza sativa* subsp. *japonica*, and *Brachypodium distachyon* plants inhibits the proliferation of *Rhizoctonia solani* and reduces the incidence and severity of *Sphaeroteca pannosa* on *Rosa* spp. var. Clasicc Cezane (Hadi and Balali, 2010; Kouzai *et al*., 2018; Torres-Velázquez *et al*., 2013).

Salicylic acid is associated with systemic acquired resistance, denoting its ability to induce the production of antimicrobial proteins such as PR3 and PR8, which are chitinases (Díaz-Puentes, 2012). This could explain the low rhizospheric culturable fungal concentrations found in HLB plant treatments where chemical inducers, particularly Virus-stop® and Plasmitox®, were applied. Fungi develop better in acid pH, and some have the ability to modify pH to ensure optimal values for their development (Pereira *et al*., 2007; Sagardoy and Mandolesi, 2004). It should be taken into consideration that the pH of the substrate at the beginning of the experiment was 6.5, with the optimum pH for citrus production ranging from 5.5 to 6.5 (González-Segnana and Tullo-Arguello, 2019). Fungi are very adept at attaching to and recognizing roots, penetrating, and resisting toxic metabolites produced by plants in response to invasion by foreign organisms (Marín, 2018).

Finally, in this case, plants with HLB had significantly lower rhizospheric HFT populations than healthy plants. Treatments with chemical inducers showed a greater negative effect (at 368 ddpa), with treatments such as AsCL*as* and VstCL*as* having values of zero (Table 3). The establishment of total filamentous fungi in the rhizosphere of Mexican lemon trees has a negative impact on the plant's rhizosphere microbiota and can harm plant health.

CONCLUSIONS

Bud grafting on healthy Mexican lemon (*Citrus aurantifolia*) plants from rods with Huanglongbing symptoms proved to be an effective method of inoculating the

bacterium *Candidatus* Liberibacter *asiaticus* (CL*as*), which was detected in this work 280 days after the first application of chemical inducers. The application of chemical defense inducers did not show a significant decrease (Kruskal-Wallis; $p \leq 0.05$) of Huanglongbing in Mexican lemon trees established under greenhouse conditions. However, the presence of the phytopathogenic bacterium *Candidatus* Liberibacter *asiaticus* significantly modified the population dynamics of rhizosphere microorganisms, and a decrease in total culturable bacteria and filamentous fungi of Mexican lemon was observed in diseased plants with Huanglongbing compared to healthy plants. In plants with HLB, the exogenous application of inducers such as Plasmitox®, which activate the jasmonic acid signaling pathway, had a negative impact on the development of total culturable bacteria in the rhizosphere in plants with HLB. Similarly, the use of inducers such as Virus-stop® and salicylic acid, which activate Systemic Acquired Resistance, reduced the development of total culturable filamentous fungi in the rhizosphere of Mexican lemon plants infected with *Candidatus* Liberibacter *asiaticus*.

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REFERENCES

Arce-Leal ÁP, Bautista R, Rodríguez-Negrete EA, Manzanilla-Ramírez MÁ, Velázquez-Monreal JJ, Santos-Cervantes ME, Méndez-Lozano J, Beuzón CR, Bejarano ER, Castillo AG *et al*. 2020. Gene expression profile of Mexican lime (*Citrus aurantifolia*) trees in response to Huanglongbing disease caused by *Candidatus* Liberibacter *asiaticus*. Microorganisms 8 (4): 528. https://doi.org/10.3390/microorganisms8040528

Bagio TZ, Canteri MG, Barreto TP, Leite Júnior RP. 2016. Activation of systemic acquired resistance in citrus to control huanglongbing disease. Ciencias Agrarias 37 (4): 1757–1766. https://doi.org/10.5433/1679-0359.2016v37n4p1757

Blassioli-Moraes MC, Birkett MA, Gordon-Weeks R, Smart LE, Martin JL, Pye BJ, Bromilow R, Pickett JA. 2008. Cis-jasmone induces accumulation of defense compounds in wheat *Triticum aestivum*. Phytochemistry 69 (1): 9–17. https://doi.org/10.1016/j.phytochem.2007.06.020

Calvo VP, Meneses LR, Zúñiga DD. 2008. Estudio de las poblaciones microbianas de la rizósfera del cultivo de papa (*Solanum tuberosum*) en zonas altoandinas. Ecología Aplicada 7 (1–2): 141–148.

Camacho-Cruz A, Giles-Gómez M, Ortegón-Ávila A, Palao-Rincón M, Serrano-López B, Velázquez-Madrazo O. 2009. Técnicas para el análisis microbiológico de alimentos (Segunda edición). Universidad Autónoma Nacional de México: Ciudad de México, México. 196 p.

Camarena-Gutiérrez G, de la Torre-Almaráz R. 2007. Resistencia sistémica adquirida en plantas: estado actual. Revista Chapingo Serie Ciencias Forestales y del Ambiente 13 (2): 157–162.

Cano MA. 2011. Interacción de microorganismos benéficos en plantas: micorrizas, *Trichoderma* spp. y *Pseudomonas* spp: una revisión. Revista U.D.C.A. Actualidad y Divulgación Científica 14 (2): 15–31.

Choi HW, Hwang BK. 2011. Systemic acquired resistance of pepper to microbial pathogens. Journal of Phytopathology 159 (6): 393–400. https://doi.org/10.1111/j.1439-0434.2010.01781.x

da Graça JP, Ueda TE, Janegitz T, Vieira SS, Salvador MC, de Oliveira MCN, Zingaretti SM, Powers SJ, Pickett JA, Birkett MA, Hoffmann-Campo CB. 2016. The natural plant stress elicitor cis-jasmone causes cultivar-dependent reduction in growth of the stink bug,

Euschistus heros and associated changes in flavonoid concentrations in soybean, *Glycine max*. Phytochemistry 131: 84–91. https://doi.org/10.1016/j.phytochem.2016.08.013

Díaz-Puentes LN. 2012. Systemic acquired resistance induced by salicylic acid. Revista Bio Agro 10: 257–267.

Eng-Sánchez F. 2008. Jasmonatos: compuestos de alto valor para la agricultura. Parte 1. Actividad biológica y ruta biosintética del ácido jasmónico en plantas. Instituto Cubano de Investigaciones de los Derivados de la Caña de Azúcar 42 (1–3): 51–59.

Esquivel-Chávez F, Valdovinos-Ponce G, Mora-Aguilera G, Gómez-Jaimes R, Velázquez-Monreal JJ, Manzanilla-Ramírez MÁ, López-Arroyo JI. 2012. Análisis histológico foliar de cítricos agrios y naranja dulce con síntomas ocasionados por *Candidatus* Liberibacter *asiaticus*. Agrociencia 46 (8): 769–782.

Flores-Sánchez JL, Loeza-Kuk E, López-Arroyo JI, Domínguez-Monge S, Acevedo-Sánchez G, Robles-García P. 2015. Pérdidas en producción inducidas por *Candidatus* Liberibacter *asiaticus* en limón persa en Yucatán, México. Revista Mexicana de Fitopatología 33 (2): 195–210.

Ginnan NA, Dang T, Bodaghi S, Ruegger PM, McCollum G, England G, Vidalakis G, Borneman J, Rolshausen PE, Caroline Roper M. 2020. Disease-induced microbial shifts in citrus indicate microbiome-derived responses to Huanglongbing across the disease severity spectrum. Phytobiomes Journal 4 (4): 375–387. https://doi.org/10.1094/PBIOMES-04-20-0027-R

Gómez DE, Reis EM. 2011. Inductores abióticos de resistencia contra fitopatógenos. Revista Química Viva 10: 6–17.

González-Segnana RL, Tullo-Arguello CC. 2019. Guía técnica cultivo de cítricos. Universidad Nacional de Asunción: San Lorenzo, Paraguay. 84 p.

Hacquard S, Spaepen S, Garrido-Oter R, Schulze-Lefert P. 2017. Interplay between innate immunity and the plant microbiota. Annual Review Phytopathology 55: 565–589. https://doi.org/10.1146/annurev-phyto-080516-035623

Hadi MR, Balali GR. 2010. The effect of salicylic acid on the reduction of *Rizoctonia solani* damage in the tubers of marfona potato cultivar. American-Eurasian Journal of Agricultural and Environmental Science 7: 492–496.

Heil M, Bostock RM. 2002. Induced systemic resistance (ISR) against pathogens in the context of induced plant defenses. Annals of Botany 89 (5): 503–512. https://doi.org/10.1093/aob/mcf076

Jagoueix S, Bové JM, Garnier M. 1996. PCR detection of the two "Candidatus" liberobacter species associated with greening disease of citrus. Molecular and Cellular Probes 10 (1): 43–50. https://doi.org/10.1006/mcpr.1996.0006

Kouzai Y, Kimura M, Watanabe M, Kusunoki K, Osaka D, Suzuki T, Matsui H, Yamamoto M, Ichinose Y, Toyoda K *et al*. 2018. Salicylic acid-dependent immunity contributes to resistance against *Rhizoctonia solani*, a necrotrophic fungal agent of sheath blight, in rice and *Brachypodium distachyon*. New Phytologist 217 (2): 771–783. https://doi.org/10.1111/nph.14849

Laredo-Alcalá EI, Martínez-Hernández JL, Iliná A, Guillen-Cisneros L, Hernández-Castillo FD. 2017. Aplicación de ácido jasmónico como inductor de resistencia vegetal frente a patógenos. Revista Mexicana de Ciencias Agrícolas 8 (3): 673–683.

Lou BH, Zhao XL, Song YQ, Bai XJ, Deng CL, Chen GP. 2012. *Candidatus* Liberibacter *asiaticus*, associated with citrus Huanglongbing, infects pollen, seed coat and endosperm of pummelo in China. The Plant Pathology Journal 93 (3): 703–705.

Marín C. 2018. Conceptos fundamentales en ecología de hongos del suelo: una propuesta pedagógica y de divulgación. Boletín Micológico 33 (1): 32–56. https://doi.org/10.22370/bolmicol.2018.33.1.1168

Matthes M, Bruce T, Chamberlain K, Pickett J, Napier J. 2011. Emerging roles in plant defense for cis-jasmone-induced cytochrome P450 CYP81D11. Plant Signaling and Behavior 6 (4): 563–565. https://doi.org/10.4161/psb.6.4.14915

Mauch-Mani B, Métraux JP. 1998. Salicylic acid and systemic acquired resistance to pathogen attack. Annals of Botany 82 (5): 535–540. https://doi.org/10.1006/anbo.1998.0726

Mendoza-Hernández CS, Quiñones-Aguilar EE, Rincón-Enríquez G. 2015. Palomas en vías de extinción por el "Dragón Amarillo". Revista Ciencia y Desarrollo 41 (280): 36–41.

Mendoza-Hernández CS, Quiñones-Aguilar EE, Rincón-Enríquez G. 2017. Técnicas de inoculación y cuantificación de *Candidatus* Liberibacter *asiaticus* en limón mexicano. Revista Biotecnología y Sustentabilidad 2 (1): 43–50.

Mora-Aguilera G, Flores-Sánchez J, Acevedo-Sánchez G, Domínguez-Monge S, Oropeza-Salin C, Flores-Olivas A, González-Gómez R, Robles-García P. 2016. Vigilancia epidemiológica y estatus actual del amarillamiento letal del cocotero, punta morada de la papa y Huanglongbing de los cítricos (HLB) en México. Revista Mexicana de Fitopatología 32 (2): 120–131.

Mora-Aguilera G, Flores-Sánchez JL, Loeza-Kuk E, Acevedo-Sánchez G, López-Arroyo JI, Velázquez-Monreal JJ, Domínguez-Monge S, Gutiérrez-Espinosa MA, Reyes González B. 2022. Implicaciones del manejo de cultivo y cronicidad de infección de *Candidatus* Liberibacter *asiaticus* en la producción de limón mexicano. Revista Mexicana de Fitopatología 40 (4): 69–85.

NOM-021-RECNAT-2000 (Norma Oficial Mexicana). 2002. Que establece las especificaciones de fertilidad, salinidad y clasificación de suelos. Estudios, muestreo y análisis. Secretaría de Medio Ambiente y Recursos Naturales. Ciudad de México, México. http://diariooficial.gob.mx/nota_detalle.php?codigo=717582&fecha=31/12/2002 (Retrieved: June 2023).

Pereira C, Camougrand N, Manon S, Sousa MJ, Côrte-Real M. 2007. ADP/ATP carrier is required for mitochondrial outer membrane permeabilization and cytochrome C release in yeast apoptosis. Molecular Microbiology 66 (3): 571–582. https://doi.org/10.1111/j.1365-2958.2007.05926.x

Pérez-Zamora O, Orozco-Romero J. 2004. Rendimiento y concentración nutrimental foliar de árboles de limón mexicano fertilizados con nitrógeno, fósforo y potasio. Terra Latinoamericana 22 (1): 99–108.

Pimentel-González CE, Ramírez-Mendoza C, García-Hernández NE, Padilla-Sánchez JA, Sánchez-Álvarez P, Robles-García PL. 2018. Quinto informe mensual: Campaña contra el Huanglonging de los cítricos. Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria. Ciudad de México, México. https://www.gob.mx/cms/uploads/attachment/file/340214/Informe_mayo_2018_HLB.pdf (Retrieved: June 2023).

Riveros-Angarita AS. 2001. Moléculas activadoras de la inducción de resistencia, incorporadas en programas de agricultura sostenible. Manejo Integrado de Plagas 61: 4–11.

Robles-González MM, Orozco-Santos M, Manzanilla-Ramírez MÁ, Velázquez-Monreal JJ, Carrillo-Medrano SH. 2017. Efecto del HLB sobre el rendimiento de limón mexicano en Colima, México. Revista Mexicana Ciencias Agrícolas 8 (5): 1101–1111. https://doi.org/10.29312/remexca.v8i5.111

Robles-González MM, Velázquez-Monreal JJ, Manzanilla-Ramírez MÁ, Orozco-Santos M, Medina-Urrutia VM, López-Arroyo JI, Flores-Virgen R. 2013. Síntomas del Huanglongbing (HLB) en árboles de limón mexicano [*Citrus aurantifolia* (Christm) Swingle] y su dispersión en el estado de colima, México. Revista Chapingo Serie Horticultura 19 (1): 15–31. https://doi.org/10.5154/r.rchsh.2012.01.005

Romanazzi G, Sanzani SM, Bi Y, Tian S, Gutierrez Martínez P, Alkan N. 2016. Induced resistance to control postharvest decay of fruit and vegetables. Postharvest Biology and Technology 122: 82–94. https://doi.org/10.1016/j.postharvbio.2016.08.003

Sagardoy M, Mandolesi M. 2004. Biología del suelo. Editorial de la Universidad Nacional del Sur: Bahía Blanca, Argentina. 105 p.

Sepúlveda-Jiménez G, Porta-Ducoing H, Rocha-Sosa M. 2003. La participación de los metabolitos secundarios en la defensa de las plantas. Revista Mexicana de Fitopatología 21: 355–363.

Shen W, Cevallos-Cevallos JM, Nunes da Rocha U, Arevalo HA, Stansly PA, Roberts PD, van Bruggen AHC. 2013. Relation between plant nutrition, hormones, insecticide applications, bacterial endophytes and *Candidatus* Liberibacter Ct values in citrus trees infected with Huanglongbing. European Journal of Plant Pathology 137 (4): 727–742. https://doi.org/10.1007/s10658-013-0283-7

StatPoint, Inc. StatGraphics Centurion XV version 15.02.06, 2005. Warrenton, Virginia, USA. www.statgraphics.com

Sunday EO, Oladejo OI, Smart BM, Sotonye OI, Musa SM, Folorunsho AS. 2015. Ethnomedical importance of *Citrus aurantifolia* (Christm) Swingle. The Pharma Innovation 4 (8): 1–6.

Thakur M, Sohal BS. 2013. Role of elicitors in inducing resistance in plants against pathogen infection: a review. International Scholarly Research Notices 2013: 762412. https://doi.org/10.1155/2013/762412

Tiwari S, Meyer WL, Stelinski LL. 2013. Induced resistance against the asian citrus psyllid, *Diaphorina citri*, by β-aminobutyric acid in citrus. Bulletin of Entomological Research 103 (5): 592–600. https://doi.org/10.1017/S0007485313000229

Torres MA, Jones JDG, Dangl JL. 2006. Reactive oxygen species signaling in response to pathogens. Plant Physiology 141 (2): 373–378. https://doi.org/10.1104/pp.106.079467

Torres-Velázquez SP, Velandia-Monsalve J, Murcia-Herrera H. 2013. Aplicación alternada de ácido acetilsalicílico con fungicidas en el control de mildeo polvoso en rosa. Ciencia y Agricultura 10: 45–51. https://doi.org/10.19053/01228420.2840

Trinidad-Cruz JR, Rincón-Enríquez G, Quiñones-Aguilar EE, Arce-Leal AP, Leyva-López NE. 2019. Inductors of plant resistance in the control of *Candidatus* Liberibacter *asiaticus* in Mexican lemon (*Citrus aurantifolia*) trees. Revista Mexicana de Fitopatología 37 (2): 304–317. https://doi.org/10.18781/r.mex.fit.1901-1

Trivedi P, He Z, Nostrand JDV, Albrigo G, Zhou J, Wang N. 2011. Huanglongbing alters the structure and functional diversity of microbial communities associated with citrus rhizosphere. The ISME Journal 6 (2): 363–383. https://doi.org/10.1038/ismej.2011.100

van Wess SCM, Pieterse CMJ, Trijssenaar A, van't Westende YAM, Hartog F, van Loon LC. 1997. Differential induction of systemic resistance in arabidopsis by biocontrol bacteria. Molecular Plant-Microbe Interactions 10 (6): 716–724.

Wang N, Stelinski LL, Pelz-Stelinski KS, Graham JH, Zhang Y. 2017. Tale of the Huanglongbing disease pyramid in the context of the citrus microbiome. Phytopathology 107 (4): 380–387. https://doi.org/10.1094/phyto-12-16-0426-rvw

Wang N, Trivedi P. 2014. Citrus Huanglongbing: a newly relevant disease presents unprecedented citrus Huanglongbing. Phytopathology 103 (7): 652–665. https://doi.org/10.1094/phyto-12-12-0331-rvw

Zhang Y, Trivedi P, Xu J, Roper MC, Wang N. 2021. The citrus microbiome: from structure and function to microbiome engineering and beyond. Phytobiomes Journal 5 (3): 249–262. https://doi.org/10.1094/pbiomes-11-20-0084-rvw

Zhang Y, Uyemoto JK, Kirkpatrick BC. 1998. A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. Journal of Virological Methods 71 (1): 45–50. https://doi.org/10.1016/S0166-0934(97)00190-0

Zhu L, Huang J, Lu X, Zhou C. 2022. Development of plant systemic resistance by beneficial rhizobacteria: Recognition, initiation, elicitation and regulation. Frontiers in Plant Science 13 (8): 1–16. https://doi.org/10.3389/fpls.2022.952397