Effect of elicitors and plant nutrition in *Candidatus* Liberibacter asiaticus in Mexican lime

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Abstract. Huanglongbing disease (HLB), caused by the bacterium '*Candidatus* Liberibacter asiaticus' (*C*Las), affects the world's citrus industry. An alternative that has been proposed for its management has been the induction of systemic resistance using elicitors and nutrition. The objective of this work was to evaluate the effect of different elicitors (biological, chemical, and organic origin) with chemical fertilization on the *C*Las concentration present in Mexican lime trees under greenhouse conditions. The elicitors used were *Bacillus subtilis* and *Funneliformis mosseae* (biological); salicylic acid (chemical) and Plasmitox®, Virus-Stop® and Blindax® (of organic origin). The chemical fertilization was applied quarterly, while the organic one, as well as the elicitors, were applied monthly, except *F. mosseae*, applied once on the roots during transplanting. After two years, the concentration of *C*Las was determined by qPCR. None of the evaluated elicitors reduced the *C*Las concentration. *Bacillus subtilis* presented a significantly higher concentration than the rest of the elicitors, and these did not show differences with chemical fertilization. These results could indicate that chemical nutrition is an important factor to evaluate when managing HLB, which should be considered when applied in conjunction with elicitors such as *B. subtilis*.

Key words: Inducer, Salicylic acid, *Bacillus subtilis, Funneliformis mosseae*

Huanglongbing disease (HLB), whose causative agent is the bacterium *Candidatus* Liberibacter asiaticus (*C*Las), is one of the main diseases affecting the citrus industry around the world (Bové, 2006). In Mexico, the presence of *Diaphorina citri*, the insect vector of the bacterium, was reported in the states of Campeche and Yucatán in 2002 (Halbert and Nuñez, 2004). Five years later (2009), symptoms of the disease were detected in Yucatan. The main strategy to prevent the spread of the disease has been the intensive use of insecticides to control the population growth and spread of the *D. citri* vector, the removal of diseased trees, and the production of HLB-free plants (Hall and Gottwald, 2011). Additional control methods include the intensive application of fertilizers and antibiotics (Gottwald *et al*., 2012; Zhang *et al*., 2011). However, since the excessive use of agrochemicals causes damage to the environment and to other organisms (Thakur and Sohal, 2013), various studies have been carried out to evaluate more efficient alternatives to control HLB in plants and reduce the use of agrochemicals.

One of the alternatives is the application of elicitors, compounds that stimulate all types of defense responses in the plant. Elicitors can be chemical, biotic, abiotic, and complex, depending on their origin and molecular structure (Thakur and Sohal, 2013). Some biological elicitors, such as the bacterium *Bacillus subtilis*, and some arbuscular mycorrhizal fungi (AMF), have been used to induce systemic resistance to various diseases in *Citrus* species, including HLB (Graham, 1986; Chen *et al*., 2020; Munir *et al*., 2020; Asad *et al*., 2021). Among the chemical elicitors used in previous studies, salicylic acid has shown good results by reducing the concentration of *C*Las and the progress of HLB in some citrus species (Olivera-Coqueiro *et al*., 2015; Hu *et al*., 2018; Trinidad *et al*., 2019). It is worth noting that the nutritional condition of the trees is an important factor that can influence their tolerance to a disease. In this regard, Shen *et al*. (2013) note that fertilization, in combination with salicylic acid, was efficient in reducing the concentration of *C*Las and mitigated the symptoms of the disease in *C. sinensis* trees.

Studies on inducers of systemic resistance in *Citrus aurantifolia* are scarce. Further research is needed not only to evaluate already known inducers, but also to search for more compounds or substances capable of inducing systemic resistance, including commercial products of organic origin. The present study aimed to evaluate the effect of systemic resistance inducers (biological, chemical and of organic origin), in combination with chemical fertilization, on the concentration of *C*Las in *C. aurantifolia* trees under greenhouse conditions.

The study was carried out on certified trees (Plant Health-SENASICA) of Mexican lime (*C. aurantifolia*) grafted onto 18-month-old *C. macrophylla* from Tecomán, Colima, Mexico. Mexican lime (Lm) trees were inoculated with *Candidatus* Liberibacter asiaticus (*C*Las) by bud grafting, using buds from scion trees symptomatic of HLB from orchards located in Tecomán, following the proposal of Coletta-Filho *et al.* (2010). The grafting was carried out eight months before transplanting the plants to 20 L pots (16 kg of substrate). Irrigation was conducted weekly at field capacity. Subsequently, the grafted plants were transplanted to a soil-based substrate: sand: agrolite: peat (Sunshine Mix® No. 3) (50: 30: 10: 10, V/V/V/V) previously sterilized (120 °C, 1.05 kg cm^{-2}, 6 h). The experiment was established under greenhouse conditions at the facilities of the Zapopan Unit of CIATEJ, in the state of Jalisco, Mexico.

The microorganisms *Funneliformis mosseae* and *Bacillus subtilis* were obtained from the strain collection of the CIATEJ Phytopathology laboratory. Salicylic acid was purchased from Sigma-Aldrich (cat. 105910) and the products of organic origin, Plasmitox®, Blindax® and Virus-Stop®, were purchased Fagro® (fagro.com.mx) through a commercial provider. Chemical fertilization was carried out by applying triple 16 (16-16-16% N-P-K) and urea (46% N) (Agrícola DASAM, México, http://agricoladasam.com.mx/).

Organic fertilization, was carried out by applying liquid vermicompost (Biojal®) (https://hidroflora.mx/producto/03OR-HUMUS20LTS) (Table 1).

The study used a completely randomized design with 10 treatments and five repetitions, as shown in Table 1. The experimental unit consisted of a pot with a Lm tree. Treatment with the arbuscular mycorrhizal fungus (AMF) *F. mosseae* was applied to the roots only once during transplanting. The rest of the treatments with elicitors, as well as the chemical and organic fertilization, began to be applied 30 days after the transplant. The elicitors and the organic fertilization were applied monthly. The chemical fertilization was applied quarterly (four times/year). The treatments with chemical control agents were applied to the phyllosphere by spraying the indicated concentrations and volumes (Table 1). The response variable was the bacterial concentration of *C*Las according to real-time quantitative DNA polymerase chain reaction (qPCR). The qPCR was performed two years after the first application of the elicitor. Coletta-Filho *et al*. (2010) reported that *C*Las concentrations were not detected before in adult trees. At the time of sampling, the age of the plants was 4 years 2 months.

For the extraction of DNA from each experimental unit, we sampled the fifth descending leaf from the apex of each of four branches located at the cardinal points in plants of approximately 4-5 months of age. These leaves were frozen at -80 ºC, lyophilized (72 h; TFD5503 IlShinBioBase, Korea), ground (60 s, 25 Hz, MM400 Retsch, Germany) and stored at room temperature until needed for DNA extraction. This was done following the CTAB (3%) protocol proposed by Zhang *et al*. (1998). DNA concentration and purity were determined at 260 and 280 nm (Nano-Drop ND-1000 UV-Vis Spectropotometer; NanoDrop Technologies, Wilmington, DE, USA).

A qPCR analysis (StepOne Applied Biosystems, USA) was performed to determine the *C*Las titer according to what was described by Li and Levy (2006). The Ct values were converted to *C*Las cell concentration values using a standard curve previously described by Lin *et al*. (2010), and with the software 7500 System SDS v. 2.0.5. The data were transformed to the square root (√x) of the bacterial concentration to satisfy the normality and homoscedasticity assumptions. These procedures were carried out using a one-way analysis of variance and LSD multiple comparison of means

Table 1. Treatments to determine the effect of elicitors and nutrients on the management of *C*Las Mexican lime plants under greenhouse conditions.

Tratamiento (T)			Condiciones de la aplicación		
Tipo de agente	(T) Agente de control	*C*Las	Concentración	Total / árbol	Periodicidad
Biológico	(1) *Funneliformis mosseae* + FQ	(+)	5 esporas g^{-1} sustrato	500 esporas	Inicio experimento
	(2) *Bacillus subtilis* + FQ	(+)	$2.5x10^8$ UFC mL^{-1}	100 mL	Mensual
Químico	(3) Ácido salicílico + FQ	(+)	500 µg L^{-1}	50 mL	Mensual
Orgánico	(4) Plasmitox® + FQ	(+)	12 mL L^{-1}	62.5 mL	Mensual
	(5) Blindax® + FQ	(+)	1.5 mL L^{-1}	62.5 mL	Mensual
	(6) Virus-Stop® + FQ	(+)	2.5 mL L^{-1}	72.5 mL	Mensual
Nutrición vegetal	(7, 8) Fertilización química (FQ)	(+) (-)	Triple 17 + urea (46 % N)	37.5-4.5-4.5 g	Trimestral
	(9, 10) Fertilización orgánica	(+) (-)	Lombricomposta líquida	100 mL	Mensual

($p \leq 0.05$) in the statistical software Statgraphics® Centurion XV v. 15.2.06 (StatPoint, 2005).

The results indicated that the biological treatment with *B. subtilis* had the highest concentration of *C*Las (73494.2 cells/100 ng DNA) and was significantly different ($p \leq 0.05$) from the rest of the treatments with elicitors (Figure 1). This result differs from other studies in which species of the genus *Bacillus* are reported to act as inducers of systemic resistance against pathogens present in citrus plants (Chen *et al*., 2020). Recently, Adsad *et al*. (2021) reported that, 60 days after the application of *B. subtilis* L1-21 + 50% Hoagland solution, there was a considerable reduction in the *C*Las concentration in asymptomatic *C. lime* trees. In symptomatic trees, the strain without Hoagland solution had better results. This suggests that the stage of disease progression influences the eliciting function of *B. subtilis*. In the present study, Lm trees with *B. subtilis* and chemical fertilization maintained a high concentration of *C*Las compared to the other treatments. This may indicate that, in addition to the stage of disease progression, the strain and the fertilization conditions influence the ability of *B. subtilis* to induce systemic resistance.

Except for the treatments with salicylic acid, Plasmitox, organic fertilization + *C*Las, and chemical fertilization + *C*Las, the bacterial concentration in the biological treatment with *F. mosseae* (32987.2 cells/100 ng DNA) showed significant differences ($p \leq 0.05$) compared to the other treatments (Figure 1). AMF are beneficial microorganisms that, in addition to providing plants with nutrients and water, also increase their resistance to diseases and pathogenic organisms (Ortas, 2012). There have been numerous studies on the ability of economically important species, such as *Solanum lycopersicum*, to induce local and systemic resistance to pathogens such as *Phytophthora parasitica*, *Clavibacter michiganensis* subsp. *michiganensis*, and *Alternaria solani* (Pozo *et al*., 2002, HoNg-Duc and

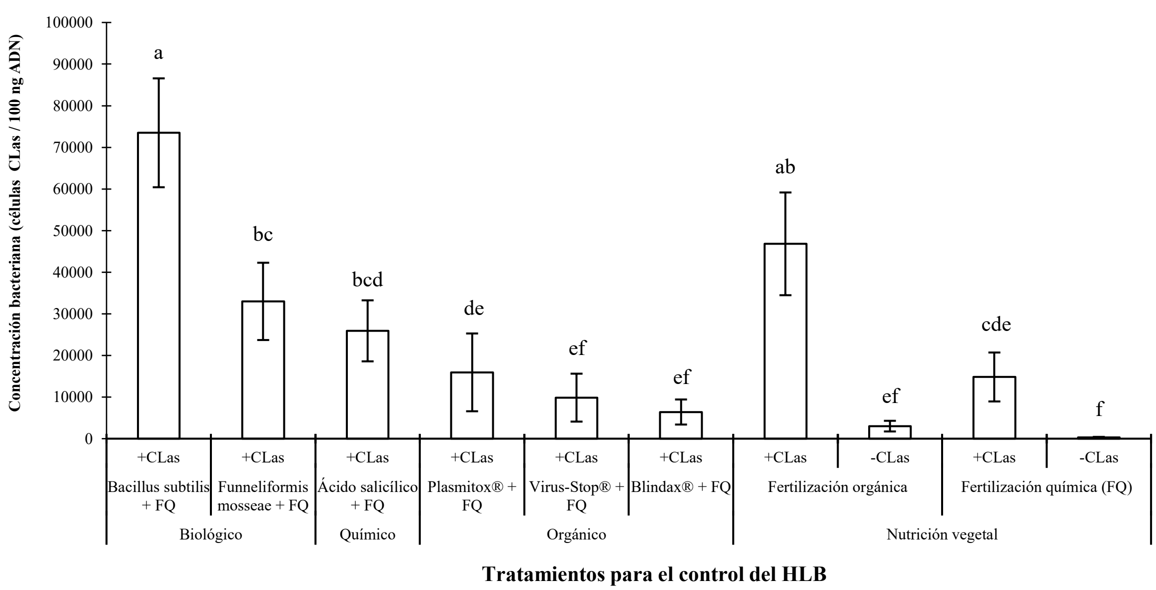

Figure 1. Effect of the application of different elicitors (biological, chemical, of organic origin) and plant nutrition on the concentration of *C*Las in Mexican lime plants under greenhouse conditions, 2 years and 8 months after infection. Bars represent ± standard error. Different letters indicate significant differences according to the LSD statistical test (*p*≤0.05). FQ = Chemical fertilization.

Posta, 2018; Song *et al*., 2015). However, the same phenomenon has been only little studied within the genus *Citrus* (Graham, 1986; Sikora, 1992; Calvet *et al*., 1995; Michelini *et al*., 1993, mentioned in Ortas, 2012). The results of the present study could differ from those reported by studies focused on tomato plants, since the species have different life cycles (annual vs perennial), which could influence the eliciting effect of AMF on the defense system of the lime tree. However, further studies are required to confirm this hypothesis.

The concentration of *C*Las cells was significantly higher ($p \leq 0.05$) with salicylic acid (25910.7 cells/100 ng DNA) than with the VirusStop and Blindax treatments but did not show any differences compared to the Plasmitox, chemical fertilization + *C*Las, and organic treatments (controls; Figure 1). Other authors have reported that although salicylic acid promotes systemic resistance in citrus species, there are biological elicitors, such as *Azospirillum brasilense* Cd or some antibiotics, that are more efficient resistance inducers (Hu *et al*., 2018; Trinidad *et al*., 2019). In contrast, Olivera-Coqueiro *et al*. (2015) pointed out that the treatment with salicylic acid applied to *C. sinensis* trees activated more genes (1425 genes) involved with the metabolic processes associated with systemic resistance than the treatment with chitosan (640 genes).

The concentrations of *C*Las cells in the organic treatments Plasmitox® (insecticide), Virus-Stop® (antiviral) and Blindax® (fungicide) (15931.5; 9856.95; 6410.26 cells/100 ng DNA, respectively) were significantly lower ($p \leq 0.05$) compared to the biological treatments with *B. subtilis* and *F. mosseae* but did not show any differences between them nor with the chemical and organic fertilization treatments without *C*Las, or with the chemical fertilization + *C*Las treatment (Figure 1). This suggests that chemical nutrition affects the decrease in *C*Las concentrations. One possible explanation is that the activation of the immune system by insecticidal, viral or fungal elicitors may interfere with the colonization of *C*Las in the phloem of lime trees. However, corroborating this explanation requires more detailed research on the possible systemic resistance induction processes associated with these products.

The concentration of *C*Las cells in the chemical fertilization treatments with and without *C*Las was significantly lower ($p \leq 0.05$) than in the organic fertilization treatment with *C*Las (14826.1 and 46812.9 cells/100 ng DNA, respectively). The late onset of HLB indicates the potential beneficial effect of chemical fertilization. There is controversy about the potential of plant nutrition to reduce the effects of HLB. Some authors mentioned that different types of improved nutrition programs for *C. sinensis* have not decreased the symptoms of the disease or the concentration of *C*Las cells (Gottwald *et al*., 2012; Bassanezi *et al*., 2021), while other authors have shown, in the same species and in *C. reticulata*, that chemical nutrition reduced the symptoms of the disease (Pustika *et al*., 2008; Shen *et al*., 2013). The results of the present work are similar to the latter since we observed that chemical fertilization by itself was associated with a significantly lower concentration of *C*Las compared to organic fertilization with *C*Las, even compared to the biological treatment with *B. subtilis*. It is worth mentioning that *C*Las was detected both in the organic and chemical fertilization treatments "without *C*Las". This indicates that despite the care taken with the Lm trees, there was some contamination, probably due to the presence of the vector (*D. citri*), which shows how difficult it is to stop the spread of the disease under the controlled conditions of a greenhouse and in the field. The required biosecurity levels are very high.

In conclusion, the biological treatment with *B. subtilis* proved to be the least efficient in reducing the concentration of *C*Las in Lm. Except for organic fertilization, the rest of the treatments presented lower concentrations of *C*Las compared to the *B. subtilis* treatment. However, none of them was different from the treatment with chemical fertilization. It is recommended to thoroughly evaluate the ability to induce systemic resistance without this type of fertilization. Treatment with chemical fertilization (without elicitor and + *C*Las) was shown to reduce the concentration of *C*Las in Lm trees under greenhouse conditions, which highlights the importance of the present study for the management of the HLB disease.

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