Article

Physiological changes in Mexican lemon trees in production infected with HLB

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Abstract

Huanglongbing (HLB) is the most devastating citrus disease in the world and has been present in the state of Colima since 2010, the area producing Mexican lemon (*Citrus aurantifolia*). This disease causes an imbalance in the distribution of carbohydrates and inhibits photosynthesis. It also affects the concentration of nutrients in leaf. The objective of this work was to determine the physiological changes in trees in Mexican lemon production infected with HLB. The study was carried out in Colima in 2017. The concentration of starch, sucrose and glucose was determined in the central leaf veins and sheet of leaves of different ages (120 and 30 days) and root starch. The concentration of chlorophylls by analytical method and SPAD index in leaves with different levels of HLB symptoms and foliar analysis of mineral nutrients. The leaves with HLB had a higher concentration of starch in the 120-day leaf. Sucrose was concentrated in the leaf veins of 120 days. While glucose was increased in the leaf veins of 30 and 120 days. The roots of trees infected with HLB showed 40% lower concentration of starch compared to healthy trees. Decrease in chlorophyll content and SPAD index depending on the degree of involvement. As well as lower concentration of K, Ca, Mg, Cu, Fe, Zn and B. Knowing the changes caused by HLB in Mexican lemon will allow to develop practices that reduce its impact.

Keywords: *Citrus aurantifolia*, chlorophyll, huanglongbing, sugars.

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Introduction

Huanglongbing (HLB) in Mexico, they have been associated with the presence of the α-proteobacteria '*Candidatus Liberibacter* asiaticus' (CLas), commonly known in the Pacific region of Mexico as a yellow dragon, is the most devastating citrus disease, It affects all species and varieties grown in the world (Bove, 2006). HLB is one of the most complex diseases, due to interactions between the pathogen, vector, hosts and environment (da Graça *et al*., 2015). There are no reports of any cure for trees infected with HLB, which in a short time become decadent and unproductive (Bove, 2006).

In citrus fruits, the first visible symptoms associated with HLB are yellowing of the veins on mature leaves and diffuse asymmetric mottling (Cimo *et al*., 2013), which occurs due to the high accumulation of starches and the disintegration of the thylakoid system of the chloroplast in mature leaves (Etxeberria *et al*., 2009; González *et al*., 2011). These chlorotic patterns are similar to deficiencies of minerals such as zinc, iron and manganese which may complicate the diagnosis (Etxeberria *et al*., 2009; Spann and Schumann, 2009; Spann *et al*., 2011). These mottles are also the result of low water absorption due to loss of roots due to lack of carbohydrates (Jagoueix *et al*., 1994; Spann *et al*., 2011; Cimo *et al*., 2013).

HLB causes metabolic imbalances in the host due to nutrient depletion and transport interference (Duan *et al*., 2009). What results in a decrease in production, which is the most striking symptom in the citrus producing areas of the world (Bove, 2006; Li *et al*., 2009; González *et al*., 2012). In 2010, HLB was detected in the Mexican lemon producing zone (*Citrus aurantifolia*) in the state of Colima. From its presence, there has been a 50% decrease in production (Robles-González *et al*., 2013; Robles-González *et al*., 2017).

This decrease caused the fall in employment, generating social problems and depressing the demand for goods and services in the Mexican lemon producing areas (Avalos and González, 2013). The symptoms of this disease in Mexico are associated with the presence of CLas, which is an obligate parasite, restricted to phloem and which is efficiently disseminated by the Asian citrus psyllid (*Diaphorina citri*) when it feeds on the phloem, this bacterium once within the tree, a specific volatile chemical is released from methyl salicylate, which attracts the population of vector insects to feed on the infected tree and thus, it is how the pathogen enters a greater number of insects, CLas can also be transmitted from sick to healthy citrus plants by grafting (Mann *et al*., 2012; Munir *et al*., 2017).

In Mexican lemon, there has been a decrease in production from early stages of infection to more than 50% in trees that show symptoms in more than 75% of their crown, a decrease in the size of the harvested fruit was also reported (Robles-Gonzalez *et al*., 2013; Robles-González *et al*., 2017). In Colima, the presence of *Candidatus Phytoplasma* asteris (CPa) has also been reported together with CLas (Arratia-Castro *et al*., 2014; Arratia-Castro *et al*., 2016) in Mexican lemon trees that showed the same symptoms of Huanglongbing (HLB). Symptoms in Mexican lemon leaves include chlorotic spots, angular spots, plating and thickening of veins, mottling and diffuse chlorosis until generalized yellowing on the leaf blade and defoliation.

Irregular fruit ripening has been observed, which begins with yellowing in the basal part and eventually ends in the fall of fruits (Robles-González *et al*., 2017). Histologically, there is an increase in starch in the leaf mesophyll and hyperplasia occurs, which causes phloem collapse (Esquivel-Chávez *et al*., 2012). The distribution of the pathogen is not uniform, and the highest concentration of bacteria has been found in the veins and outbreaks (Li *et al*., 2006).

Once inside the CLas plant, it migrates to the phloem, causing the formation and accumulation of hot plugs in the sieve tubes (Da Graça and Korsten, 2004; Koh *et al*., 2012). Phloem blockage causes starch accumulation in the source organs and depletion in the demand or storage organs (Etxeberria *et al*., 2009; Rosales and Burns, 2011).

Affecting nutrient transport and cell collapse (Garnier and Bove, 1996; Bové, 2006; Kim *et al*., 2009; Cimo *et al*., 2013) suffering from root damage due to the interruption of photosynthesis transport of outbreaks to the root, as a consequence the roots are poorly developed and growth is suppressed (Etxeberria *et al*., 2009; Cimo *et al*., 2013) can also be infected with secondary pathogens (Pereira and Milori, 2010).

Therefore, the minerals and water that are transported from the root to the sprouts decreases (Zhang *et al*., 2011). Another symptom present is the regressive death of twigs and branches, with a decrease in production (Etxeberria *et al*., 2009; Cimo *et al*., 2013). As the time of HLB infection increases, nutrient deficiencies and carbohydrate imbalances in leaves and roots increase (Etxeberria *et al*., 2009; Rosales and Burns, 2011), stopping plant development and finally can come to death

HLB infection has different impacts on the reabsorption of nutrients depending on the genotype. Therefore, keeping the internal nutrient cycle efficient can be a strategy for citrus species tolerant to HLB (Cao *et al*., 2015). The present work has as objective to determine the physiological changes of leaves and roots of trees in production of Mexican lemon variety Colimex infected with HLB. Because the identification of host responses to the pathogen is critical to understanding the development of the disease and can be used in the formulation of efficient management practices.

Materials and methods

The investigation was carried out from April to June 2017 in the Mexican lemon producing area of Tecoman, Colima, Mexico, characterized by having dry and semi-dry tropical climate, with an average temperature of 26 °C and an average rainfall of 750 mm. The variety of Mexican lemon “Colimex” grafted in Macrofila (*Citrus macrophylla*) with 5 years of age was used because this combination is the most used in the producing areas of this citrus. Trees with HLB were inoculated by barbed grafting and corroborated by PCR. Healthy and HLB plants were established in April 2012.

In containers with capacity 60 liters, density of 2 x 3 m. The substrate composed of 1:1:1 (V/V/V parts of coconut powder, earth and vermicompost). The rest of the cultivation work was carried out according to the technological package recommended by INIFAP (Manzanilla, 2018). This study was carried out within a 250 m^2 anti-aphids mesh structure in the facilities of the Tecoman

Experimental Field of INIFAP. Two treatments trees infected with HLB and healthy trees were evaluated. A completely randomized experimental design was used. The experimental plot consisted of 8 trees per treatment.

Carbohydrate concentration in different Mexican lemon tissues

To assess starch concentrations, leaf and tree roots with HLB and healthy trees were selected. Fifty leaves per treatment separated veins and leaf laminae of mature leaves (120 days) and immature leaves (30 days). Starch content in secondary roots was also evaluated.

Starch extraction was performed separately for leaf and root from 20 mg of lyophilized and ground tissues of each sample. 500 µL of distilled water was added and incubated at 100 °C for 10 min. Subsequently, the mixture was centrifuged at 10 774 rpm for 2 min. 300 µL of supernatant was recovered in an Eppendorf tube and 900 µL of pure alcohol was added and centrifuged at 9 449 rpm for 5 min.

The supernatant was discarded and the precipitated starch was resuspended in 1 mL of sterile double-distilled water (Milli-Q) and stirred in a vortex for 3 min (González *et al*., 2011). For quantification of starch, 30 µ0L of the starch extract and 50 µL of iodine solution was added to a 96-well optical plate and the absorbance at 595 nm was read in triplicate on a spectrophotometer (Thermo Scientific ™ Multiskan GO, USA). A standard curve was made from pure rice starch (Sigma-Aldrich, USA).

Because the main sugars present in the citrus phloem are sucrose and glucose with 64 and 20% respectively (Hijaz and Killiny, 2014). The concentrations of these were determined in leaf tissues in rib and leaf lamina of 120 and 30 days. Quantified with commercial kits (Sigma-Aldrich, USA).

Chlorophyll concentration in leaves

The concentration of chlorophyll in Mexican lemon leaves with different degrees of HLB involvement was determined (Figure 1). Five stages of disease development in mature leaves were chosen and chlorophyll a, chlorophyll b and total chlorophyll were quantified according to (Warren, 2008).

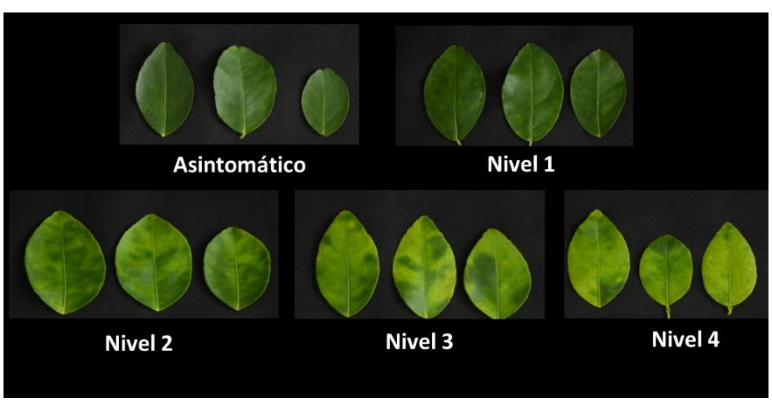

Figure 1. Levels of HLB symptoms in Colimex lemon leaves of trees in production.

The SPAD index was measured on the same leaf due to its high correlation with the actual chlorophyll content in the leaves (Intrigliolo *et al*., 2000). The readings were made with a portable chlorophyll meter SPAD 502 (Soil Plant Analysis Development, Minolta).

Foliar nutrient analysis

Concentrations of macro- and micronutrients in mature leaf (120 days) were evaluated. From healthy trees and with HLB. 100 mature leaves of each treatment were collected. For each sample a compound mixture and three repetitions were performed. The samples were dried in a Felisa® brand oven at a temperature between 65 to 70 °C for 72 h. Subsequently, they were dried at constant weight and processed in Thomas Scientific® brand electric mill, with 40 cavity mesh, finally obtaining fine powder, which was used for digestions.

The elements analyzed were total nitrogen (N), phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg); and the microelements iron (Fe), copper (Cu), zinc (Zn), manganese (Mn) and boron (B). Data were collected and analyzed by analysis of variance (Anova), the comparisons of means were made using the Tukey test ($\alpha \geq 95\%$) with the statistical analysis system InfoStat Version 2018e.

Results and discussion

Concentration of carbohydrates in veins and leaf blade of Mexican lemon

In the test of means the leaves of trees of lemon Colimex infected with CLas were statistically superior in concentration of starch in leaf of leaves of 120 days in comparison with leaves without HLB. 30 and 120 day veins showed no statistical differences between healthy and diseased trees with HLB (Figure 2). In sweet orange trees infected with CLas, the accumulation of starch in leaves has been described compared to healthy leaves (Rosales and Burns, 2011).

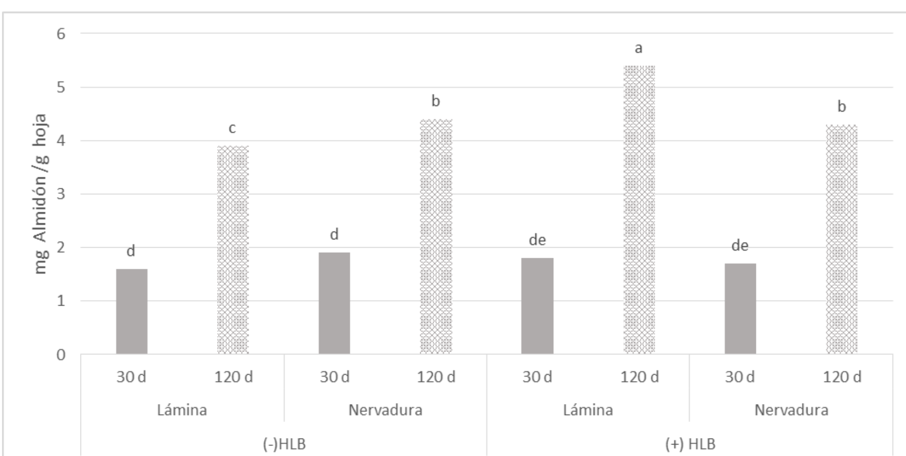

Figure 2. Effect of CLas on the concentration of starch in Mexican lemon tissue in production. 30 and 120 day leafs. Without the presence of HLB (-HLB) and infected with HLB (+HLB). Different letters on the bars indicate significant statistical differences ($p < 0.05$).

Schneider (1968), observed high accumulation of starch in leaves and petioles compared to healthy tissues. This accumulation of starch is caused by the obstruction of the transport of photoassimilates in the foliar vascular system and accelerated rates of leaf starch synthesis. In orange trees infected with HLB, the plugging of sieve tubes by deposition of calose was reported (Etxeberria *et al*., 2009; Kim *et al*., 2009). Which has an important role in the citrus defense response to the HLB (da Graça *et al*., 2015), thus affecting the transport of carbohydrates and causing massive accumulation of starch in leaf plastids (Schneider, 1968).

Also, accumulation of leaf starch due to Zn deficiency has been reported (Sagaram and Burns, 2009). These results agree with Esquivel-Chávez *et al*. (2012) that found a higher concentration of starch in Mexican lemon leaves with HLB in the greenhouse. However, the effect of HLB on starch concentration was mainly observed in the 120-day leaf blade of Colimex lemon in production, due to the higher concentration of chloroplasts in this tissue.

Figure 3 shows the comparison of means where the leaf veins presents a higher concentration of sucrose and is statistically different from the leaf blades in the two stages of development evaluated in both infected and healthy tissues. The highest concentration of sucrose occurred in the leaf veins of 120 days with HLB. In contrast, the lowest concentration was obtained in the leaf blade of healthy 30-day leaves. These results are due to CLas causing imbalance in the distribution of carbohydrates and accumulation of sucrose in leaf (Fan *et al*., 2010).

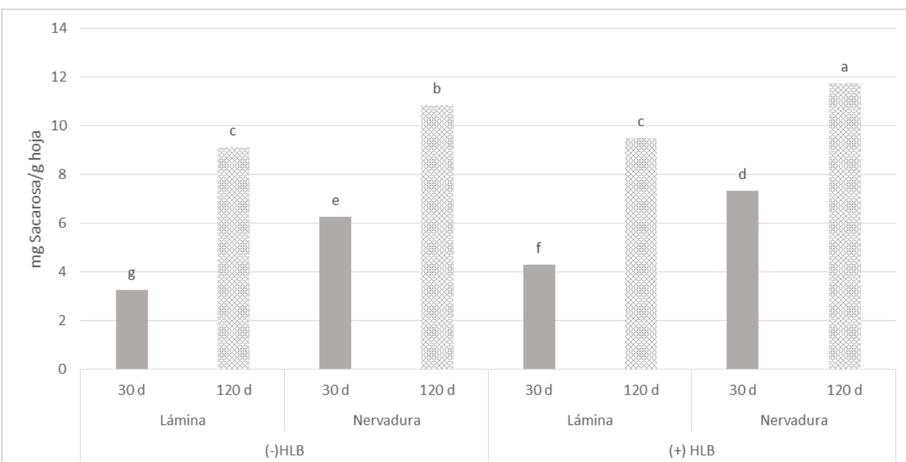

Figure 3. Effect of CLas on the concentration of sucrose in Mexican lemon tissue in production. 30 and 120 day leafs. Without the presence of HLB (-HLB) and infected with HLB (+HLB). Different letters on the bars indicate significant statistical differences ($p < 0.05$).

Due to the concentration of CLas in veins and the decrease in cellular activity within the cambium and radial parenchyma in mature leaf petioles infected with HLB, which results in a significant failure in the carbohydrate transport route (Brodersen *et al*., 2014). In lemon Colimex with HLB sucrose accumulates mainly in leaf veins of 120 days.

Glucose concentrations based on the comparison of means have significant statistical differences, showing higher concentrations in tissues with HLB. These results show the highest accumulation of glucose in 30-day veins with HLB (Figure 4). This agrees with Fan *et al*. (2010) who described that HLB causes imbalance in carbohydrate distribution and glucose accumulation. Because CLas behaves like *Spiroplasma citri*, preferably using fructose increasing the activity of invertase and consequently accumulate glucose.

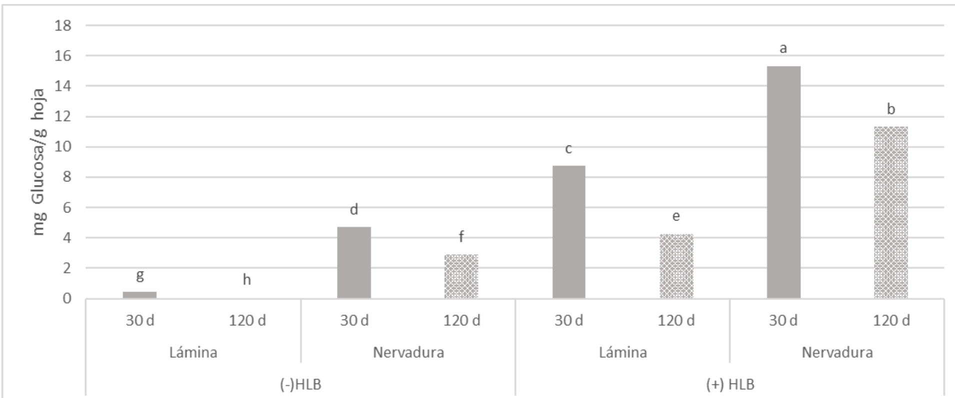

Figure 4. Effect of CLas on the concentration of glucose in Mexican lemon tissue in production. 30 and 120 day leafs. Without the presence of HLB (-HLB) and infected with HLB (+HLB). Different letters on the bars indicate significant statistical differences ($p < 0.05$).

In complement Li *et al*. (2009) reported a higher concentration of CLas in the veins of infected leaves compared to the leaf blade. HLB causes glucose accumulation mainly in the 30-day rib of lemon Colimex.

As observed in Figure 5, the roots of trees infected with HLB show significant statistical differences with healthy roots in starch concentration. With a decrease of 40%. Lemon Colimex shows a decrease in the concentration of starch in tree roots with HLB. Because they suffer from a nutritional disorder due to the interruption of photosynthesis transport from root outbreaks (Zhang *et al*., 2011). Causing a decrease in starch reserves in the roots of trees infected with CLas. This also causes them not to develop and suppress the growth of new roots (Etxeberria *et al*., 2009).

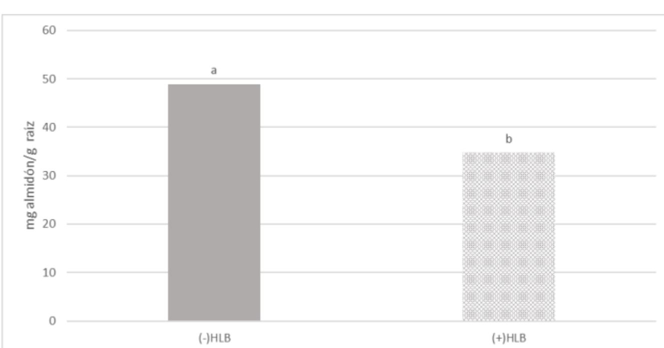

Figure 5. Effect of CLas on starch concentration in Mexican lemon tree roots in production. Without the presence of HLB (-HLB) and infected with HLB (+HLB). Different letters on the bars indicate significant statistical differences ($p< 0.05$).

Chlorophyll concentration in leaves

The levels of involvement from asymptomatic leaves to level 4 of HLB symptoms showed significant statistical differences with regard to the total chlorophyll concentration as shown in Figure 6. Due to the progressive accumulation of starches that cause the disintegration of the chloroplast thylakoid system (Etxeberria *et al*., 2009; González *et al*., 2011) and damages photosynthetic membranes by decreasing the concentration of chlorophyll (Sagaram and Burns, 2009). Although, the starch inside the chloroplast does not break the outer membranes, but the structure of the internal grana is destroyed which causes chlorosis (Achor *et al*., 2010).

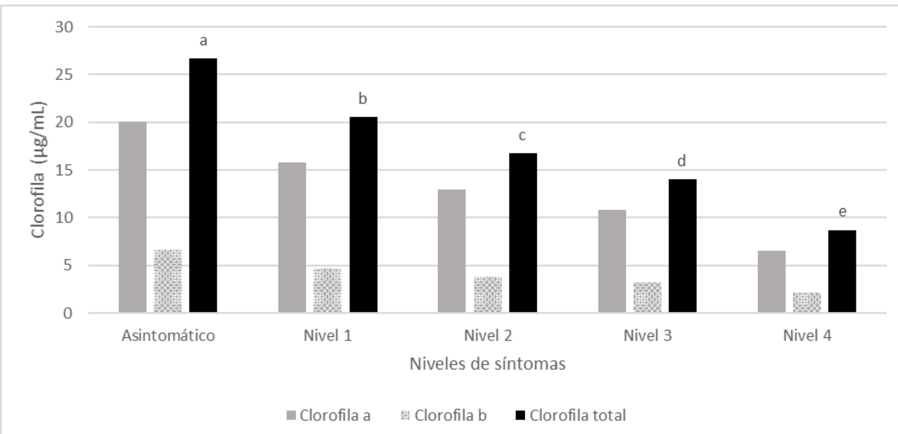

Figure 6. Concentration of chlorophyll (Chlorophyll a, Chlorophyll b and total Chlorophyll) in Mexican lemon tree leaves in production with different levels of HLB involvement. Different letters on the bars indicate significant statistical differences ($p< 0.05$).

It has also been described that increasing sucrose/glucose levels can lead to repression of genes involved in photosynthesis and cause chlorophyll decrease (Fan *et al*., 2010). Therefore, in Colimex lemon leaves the decrease in the concentration of chlorophyll a, b and total in leaf was observed depending on the degree of involvement by HLB.

When performing the SAPD readings at the HLB involvement levels evaluated, no significant statistical differences were found between the asymptomatic leaves, level 1 and level 2. In contrast, these were statistically different at levels 3 and 4 (Figure 7). The SPAD reading is only taken from a partial part of the leaf so that the destruction of chloroplast is not uniform (Achor *et al*., 2010), the sensor can capture areas with different concentrations of chlorophyll on the same leaf.

So, the statistical analysis only detects the differences in leaves with marked chlorosis in most of the leaf. In Lemon Colimex SPAD only detects statistical differences in the degrees of greatest involvement by HLB with respect to asymptomatic leaves.

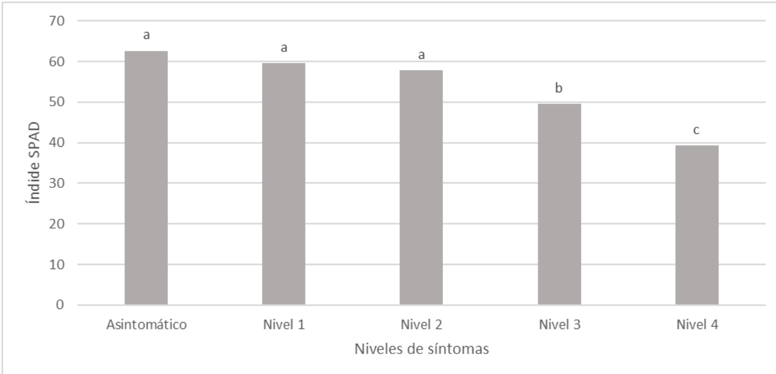

Figure 7. SPAD index in Mexican lemon tree leaves in production with different levels of HLB involvement. Different letters on the bars indicate significant statistical differences ($p< 0.05$).

Foliar nutrient analysis

Table 1 shows the nutrient data that presented significant statistical differences between leaves of trees with HLB and healthy leaves (potassium, calcium, magnesium, copper, iron, zinc and boron). The same table shows the percentage of decrease, highlighting the copper with more than 57%. Nitrogen, phosphorus and manganese did not show significant statistical differences.

Table 1. Effect of HLB on the nutrient content of Mexican lemon tree leaves in production.

Treatment	K (mg g^{-1})	Ca (mg g^{-1})	Mg (mg g^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Zn (mg kg^{-1})	B (mg kg^{-1})
Colimex (-HLB)	22.37 a	29.78 a	0.52 a	6.72 a	101.41 a	33.44 a	158 a
Colimex (+HLB)	18.83 b	25.94 b	0.47 b	2.85 b	66.8 b	24.2 b	135.06 b
Reduction %	15.8	12.9	9.6	57.6	34.1	27.6	14.5

Means with a common letter are not significantly different ($p> 0.05$).

There are works in several regions of the world with different citrus fruits that show contrasting results. The decrease of Ca and Zn in trees infected with HLB. Like Razi *et al*. (2011) showed lower foliar concentrations of Mg and Fe in trees with HLB and no difference in K, P and Zn. Etxeberria *et al*. (2009) described deficiencies of Zn and Fe in symptomatic trees. Like Tian *et al*. (2014) describe Zn deficiency in leaves with HLB. In grapefruit infected with HLB, concentrations of Ca, Mg, Fe, Zn, Mn and Cu decreased. Agreeing with this work with the exception of Mn (Nwugo *et al*., 2013).

The HLB in lemon Colimex affected the concentrations of K, Ca, Mg, Cu, Zn and B due to the loss or restriction of the root, which causes drought stress and changes in the mineral status of the outbreaks (Spann and Schumann, 2009). The results differ from what was reported, possibly to be different species and the use of containers and fertigation in this experiment. Since differences in the management of nutrition, soil, climate, variety/rootstock combination, state of plant development and nutrient reabsorption efficiency during citrus experiments infected with HLB have an effect on the data obtained (Razi *et al*., 2011; Cao *et al*., 2015).

Conclusions

Due to the HLB infection, Mexican lemon trees in production (5 years with the infection) undergo physiological changes in the distribution and concentration of carbohydrates, both in leaf and root. As well, the mineral concentration of K, Ca, Mg, Cu Fe Zn and B. decreases, while as the leaf symptoms increase, the chlorophyll concentration decreases. Because this change does not lead to the collapse of trees, it is necessary to continue with studies that allow us to know and potentiate the routes that lead the Mexican lemon to continue its productive life despite being infected with CLas.

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