Physiological and nutrimental effect of *C*Las chronicity in Mexican lime (*Citrus aurantifolia*)

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Received: August 28, 2022. **Accepted:** October 30, 2022.

Mora-Aguilera G, Acevedo-Sánchez G, Hernández-Chan EC, Loeza-Kuk E, Velázquez-Monreal JJ, Cano-Medrano R and López-Arroyo JI. 2022. Physiological and nutrimental effect of *C*Las chronicity in Mexican lime (*Citrus aurantifolia*). Mexican Journal of Phytopathology 40(4).
DOI: https://doi.org/10.18781/R.MEX.FIT.2022-20

Abstract. The purpose of this was to measure NO_3, K, Ca and chlorophyll concentration in Mexican lime (*Citrus aurantifolia*) in endemic condition and chronic infection of *Candidatus* Liberibacter asiaticus (*C*Las) in Colima, Mexico. The fundamental premise was that the analysis of physiological effect caused by *C*Las on citrus allows to understand the impact on citrus production and to define effective management strategies. A total of 11 orchards were selected from Tecomán and 13 Armería in high (AT), moderate (MT) and low technology (BT) orchards management (MH) categories. Twenty-five trees were selected in each orchard, five per HLB severity-canopy class (0, 25, 50, 50, 75 and 100 %) as an estimator of infection chronicity. *In situ*, 5 symptomatic leaves (HS) and 5 asymptomatic leaves (HA)/tree were evaluated for chlorophyll concentration with a SPAD502 and for nitrate (NO_3), potassium (K) and calcium (Ca) nutrients with ionometers. All fruit were counted and weighed in 20 trees/9 orchards. By severity class, the MH effect and asymptomatic-symptomatic condition were compared by split-plot ANOVA and Tukey (p = 0.05) (SAS ver9.4). In all severity classes, NO_3 had a significant effect ($p < 0.0001$) regarding technological level, where farms with AT (113 - 126 ppm) were higher respect to MT (81 - 89 ppm) and BT (50 - 55 ppm). K and Ca nutrients did not differ statistically between MH categories. In asymptomatic *versus* symptomatic tissue, NO_3, K and Ca concentration were not significant effect (p > 0.05). The comparative severity-canopy between 25 % versus 100 % affected chlorophyll units with 24.9 % reduction in HS and 1.3% in HA. The results show an average decrease of 2, 11, 58 and 79 % in yield-production (kg) for 25, 50, 75 and 100 % severity, respectively, suggesting the importance of NO_3 in the productive management of Mexican lime affected by *C*Las. The integration of NO_3 to a regional integrated management program is a viable option for profitable orchard management in an endemic condition and high chronic *C*Las infection. This is the first report of *C*Las chronicity effect on nutrients concentration in Mexican lime.

Keywords: HLB, Nutrition, Severity, Nitrate.

The basis of the systemic and rational management of diseases lies in the biological comprehension of the interaction between host and pathogen in a regional epidemiological approach (Mora-Aguilera *et al*., 2023; 2021; Esquivel-Chávez *et al*., 2012). In the plant-pathogen subsystems interaction, planta nutrition is directly related to severity of wide range of diseases (Tripathi *et al*., 2022). The crop management via nutrition helps develop thresholds of tolerance to pests and pathogens; that is, response mechanisms can be generated against infectious processes at a structural and genic level. Overall, the management of non-systemic diseases is supported by the paradigm of suppression or mitigation of causal agent using chemical, biological and/or cultural control. However, other systemic problems caused by viruses and bacteria such as *Candidatus* Liberibacter asiaticus (*C*Las) require comprehensive strategies that incorporate nutrition, resistant varieties, genetic modification, resistance inducers, and others. Nutrients may increase or reduce the resistance or tolerance of crops to pathogens (Tripathi *et al*., 2022). It has been proven that optimal citrus physiology and nutrition can mitigate the effects of an infections by *C*Las and other pathogens (Bassanezi *et al*., 2021, 2014; Gottwald *et al*., 2012; Spann *et al*., 2011; Xia *et al*., 2011; Ahmad *et al*., 2011). However, specific regional studies related to technological practices and the use of productive resources are required to incorporate the nutrition into integrated citrus management programs (Kwakye *et al*., 2023; Uthman *et al*., 2022; Bassanezi *et al*., 2021).

Due to establishment of *C*Las, the causal agent of Huanglongbing or HLB, and the fast endemic condition with high chronic infection rates in the Pacific region (Mora-Aguilera *et al*., 2014a), a management strategies were proposed comprised by use of nutritional programs, high planting densities, genotypes with greater tolerance, and the control of *Diaphorina citri* vector using chemical and biological products (Manzanilla-Ramírez *et al*., 2019). This comprehensive approach was proposed as a mandatory alternative to the official *D. citri* Regional Control Areas program (ARCOs), which was successful in sub-endemic regions of Mexico (Flores-Sánchez *et al*., 2017; Mora-Aguilera *et al*., 2014b). Nevertheless, a comprehensive program must be supported by experimental evidence under conditions of commercial production and in specific environments (Bassanezi *et al*., 2021; Flores-Sánchez *et al*., 2016). The purpose of this study was to analyze the chronic effect of *C*Las infection in the production of Mexican lime (*Citrus aurantifolia*) associated to NO_3, K and Ca in the plants, and with chlorophyll concentration under endemic conditions of *C*Las in Colima, State, in order to support the nutritional component of orchards in the regional management of HLB.

Eleven commercial Mexican lime orchards were selected in the citrus-producing region of Tecomán y 13 in Armería, Colima. The spatial distribution of the orchard sampling was carried out based on differential criteria of epidemic inductivity, endemicity and regional *C*Las chronicity, through an interpolate map of the Relative Multivariate Index comprised by 15 plant-vector-management variables (Figure 1A and 1B) (Flores-Sánchez, 2016). Per orchard, 25 plants were selected and assessed with a 'T' sampling method which explores the edge effect and internal orchard furrow (Figure 1C). The agronomic management (MH) was determined, considering a qualitative scale of high

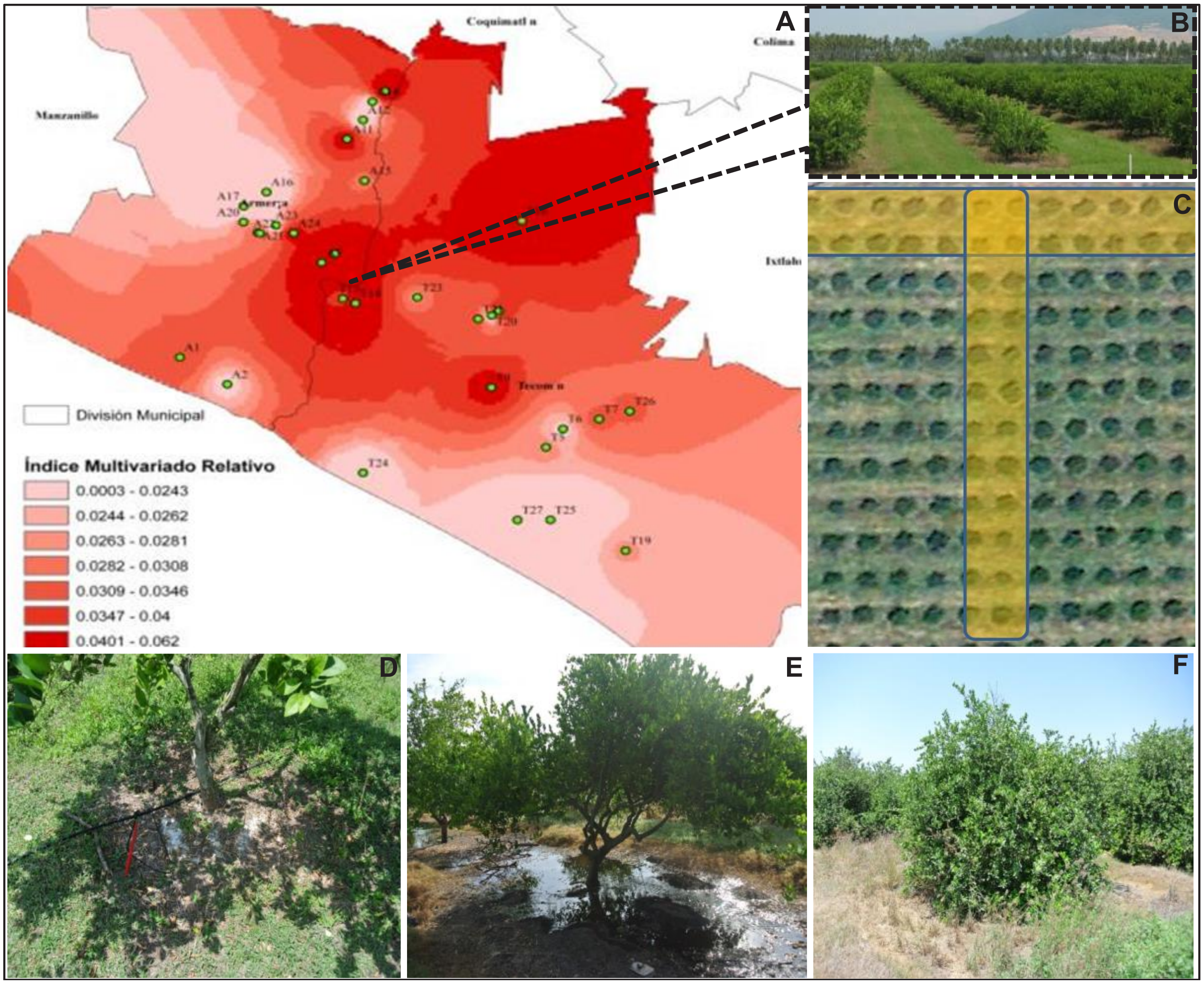

Figure 1. A. Regional distribution of 11 orchards (green dots) in Tecomán and 13 in Armería associated to an interpolated map of *C*Las chronicity, expressed by a relative multivariate index. B. Example of a Mexican lime orchard with high technology (AT) management. C. Representation of the sampling method of 25 plants in 'T'. D. Drip-sprinkler irrigation system in an orchard with high technology. E. Flood irrigation system associated with orchards with moderate technology. E. Orchard with weeds associated with low technology.

(AT), moderate (MT) and low technology (BT). MH was generated from the variables parametrization such as the general orchard condition, nutrition program, plant vigor, control of weeds and pests, and irrigation type (Figure 1D-1F).

The HLB severity was assessed in the canopy of the plant, using a five-classes (1 – 5) scale with 0, 25, 50, 75 and 100 % of canopy with HLB symptoms, as an indicator of the level of chronic infection (Flores-Sánchez *et al*., 2015) (Figure 2). Five symptomatic leaves (HS) and five asymptomatic leaves were selected per planta (the latter was feasible, even in class 5) (Figure 3B) to quantify the chlorophyll concentration (μmol) using a Minolta® SPAD-502Plus gauge and the amount of nutrients in parts per million (ppm) using portable HORIBA® ionometers, specifically for NO_3, K and Ca (Figure 3C), which measure

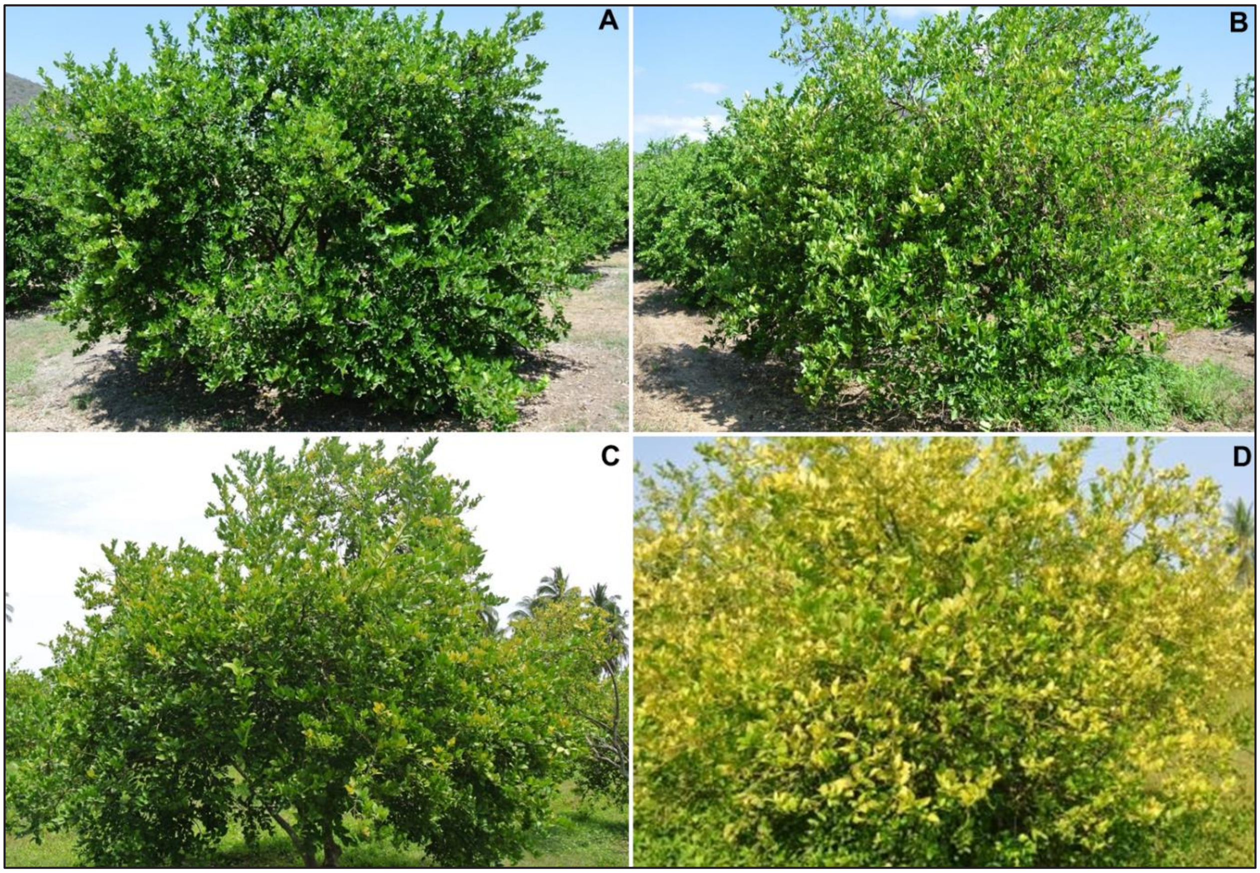

Figure 2. Diagrammatic-qualitative five-class (0-100 %) scale for assessment of HLB severity in plant canopy. A. Class 2 with at least 25 % of the plant canopy exhibiting HLB symptoms. B. Class 3 represents 50 % severity. C. Class 4 represents 75 % severity. D. Class 5 represents 100 % severity. Class 1 represents apparently healthy plants (not included). In Mexican lime, HLB exhibit symptoms of severe foliar yellowing (D).

micro volumes with a 39 to 3900 ppm range. The leaves collected were macerated and divided into three samples. The samples were mixed with distilled water and 2-3 drops were taken to make up the reading sample by nutrient. Using a non-destructive (counting) and a destructive method (harvest), all the fruit harvested from 25 plants from nine orchards, selected for phenological condition in the production stage, were counted and weighed. By severity class, the MH effect and asymptomatic *versus* symptomatic condition were compared using an analysis of variance (ANOVA) in a split-plot design and means comparison using Tukey's test ($\alpha = 0.05$). The statistical analyses were conducted using SAS v9.4.

Overall, the experimental results showed that the reduction of NO_3, K, and Ca was mainly and significantly determined by technological management of the orchards (MH) (Dong *et al*., 2021; de Bang *et al*., 2020). This reduction was greater in MH of low-technology, with maximum effects of 60, 47.5 and 34.1%, respectively. On the other hand, the HLB severity in plant canopy did not show direct significant implication on the nutrients concentration (Figure 4). The greatest reduction in concentration by HLB severity classes for NO_3, K

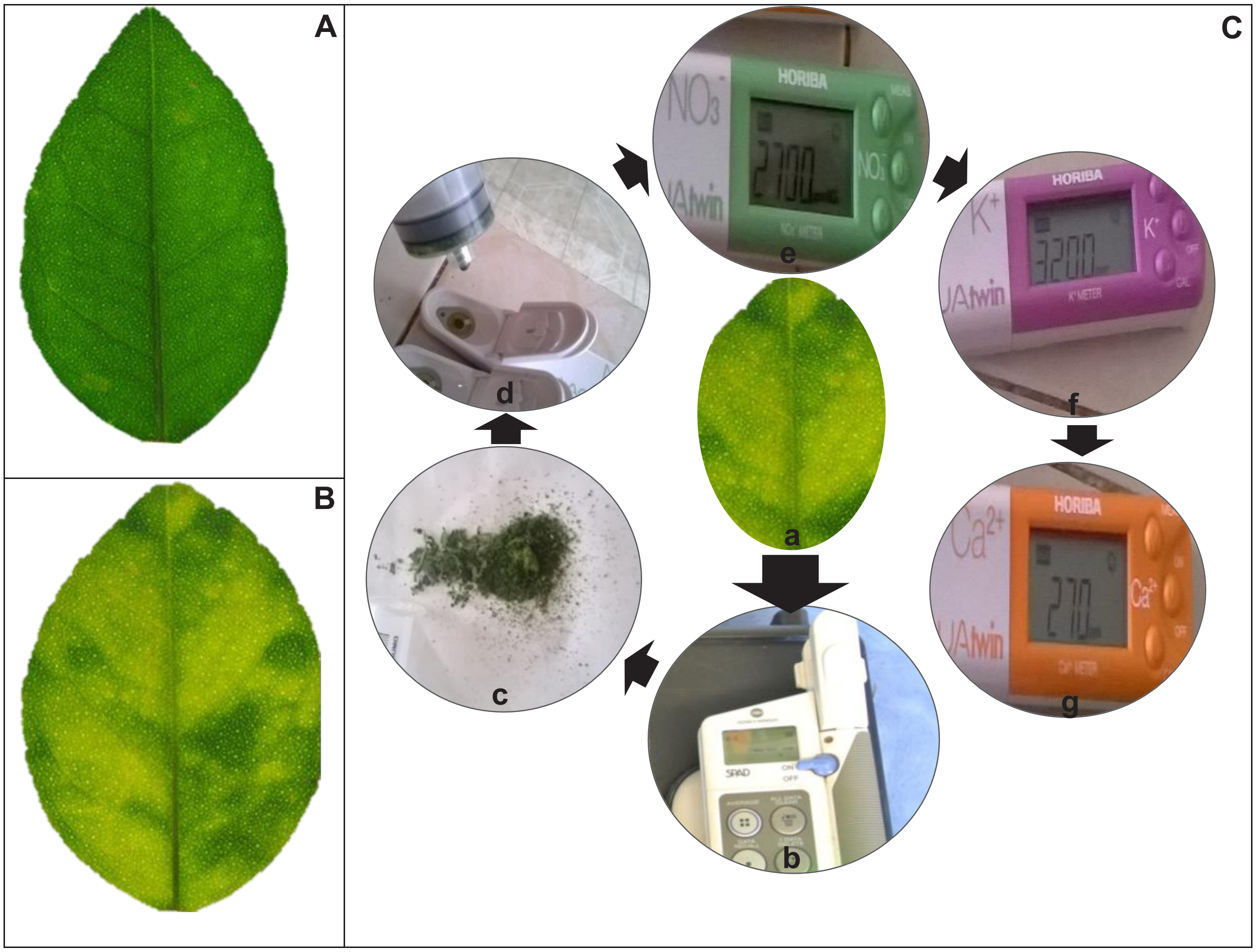

Figure 3. A. Example of asymptomatic leaves selected during sampling by orchard/plant. B. Leaf with HLB symptoms selected for the nutrients quantification. C. Measurement for the nutrition variables. The process begins with selection of an asymptomatic or symptomatic leaf (a), measurement of chlorophyll units using SPAD equipment (b), cutting and maceration of tissue (c), extraction of sample with the aid of a syringe with sterile water and deposit (d) in NO_3 ionometers (e), Potassium (f) and Calcium (g).

and Ca was 9, 28 and 25 %, respectively. The NO_3 concentration had a considerable effect regarding to the technological management of the orchards. In AT (113 – 126 ppm) NO_3 was significantly greater compared with MT (81 – 89 ppm) and BT (50 – 55 ppm) ($p < 0.0001$) in the four severity classes (25 – 100 %) (Figure 4). Nitrates, due to physiological implications during interaction with other nutrients such as Fe or P, can be associated to reduction of chlorophyll, lower activity of nitrate reductase (NO_3 reducer) and other metabolic physiological activities, leading nutritional deficiencies (Kwakye *et al*., 2022; Zhao *et al*., 2013). In plants exhibiting high severity and low NO_3 concentration, it can be associated to vascular bundle flow, particularly in the phloem, obstructed by bacterial masses or by changes in the cellular structure (Liu *et al*., 2014; Esquivel-Chávez *et al*., 2012). Although K and Ca were not statistically different between MH categories or severity classes ($p > 0.05$), they displayed a decreasing tendency in comparison to the technological management, being lower

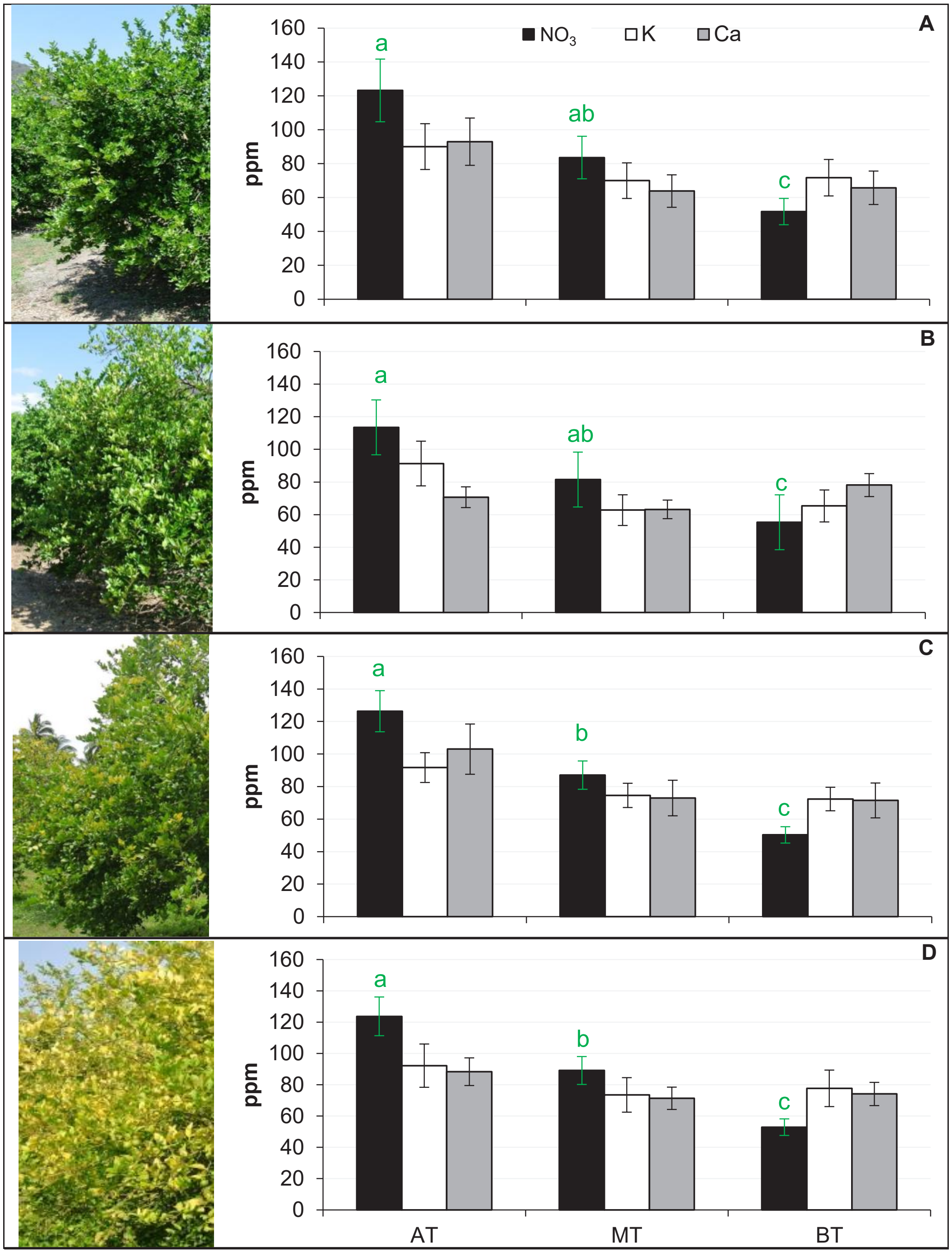

Figure 4. Effect of NO_3, K and Ca concentration expressed in parts per million (ppm), for orchards with high (AT), moderate (MT) and low technology (BT), compared by severity classes: A. 25 %, B. 50 %, C. 75 %, and D. 100 % severity. Bars with different letters show significant differences with Tukey's test *p* = 0.05. Bars without letters do not display significant differences. Class 1 was not included because no trees were found without HLB symptoms.

in MT and BT. This effect was greater in plants with severity less than 75% (class 3) (Figure 4). A similar effect of the productive technology on these nutrients was reported for sweet citrus plants (Fan *et al*., 2020). These nutrients, particularly Ca, have been proven to be important for plant photosynthetic capability, growth and cell wall composition (Eticha *et al*., 2017).

Specifically, by damage class, the plants with a severity less than 25 % (**class 2**) showed a reduction 58 % reduction in NO_3 ($p < 0.0001$) for BT orchards (66 ppm) compared to AT (123 ppm). Although not significantly, K and Ca concentration decreased 20 and 27 % (51 – 90 ppm), respectively (Figure 4A). In plants with 50 % severity (**class 3**), the NO_3 concentration in BT (55 ppm) decreased 51% in comparison with AT (113 ppm, $p < 0.001$). Orchards with MT and BT did not show a statistically different reduction in K and Ca either (Figure 4B). In 75 % severity (**class 4**), BT had up to 60 % less NO_3 concentrations than AT and MT (126 y 87 ppm; $p = 0.001$). Nutrients K and Ca in MT/AT orchards with 74 and 72 ppm, respectively, were up to 50 % lower compared to AT, although without statistical differences ($p > 0.05$) with BT, due to the high variability (Figure 4C). Plants with 100 % severity (**class 5**) maintained analogous tendencies. The NO_3 concentrations in BT orchards (52 ppm) had 57 % significant reduction ($p < 0.0001$) in comparison with AT orchards (123 ppm). The K and Ca behavior had reduction of 15 and 16 %, respectively, between MH categories (Figure 4D).

Interestingly, comparing the asymptomatic *versus* symptomatic foliar condition, NO_3, K and Ca concentration did not show significant effects due to high variability ($p > 0.05$). On the other hand, the chlorophyll units (UC) of symptomatic leaves in plants with 100 % severity decreased 17.7 ppm (27.1%) in comparison to symptomatic leaves with severity 25 % (14.9 ppm). The reduction in asymptomatic leaves was 4.2 % under same comparison (Figure 5A). These results are analogous to those reported in Colima for Mexican lime (Manzanilla-Ramírez *et al*., 2019). This effect, as previously mentioned, could be related to the nutrients reduction, mainly NO_3 and Ca, involved in the photosynthetic capacity, foliar growth and cell wall strengthening (Liu *et al*., 2014).

Regarding to *C*Las effect on the production of fruit, a 2, 11, 58, and 79 % reduction was observed for fruit weight (kg) in 25, 50, 75, 100 % severity classes, respectively. These reductions were mainly related in the BT and MT orchards. In general, production loss showed the most significant effect from 75 % severity onwards (Flores-Sánchez *et al*., 2015) (Figure 5B). The production loss (y) based on severity in the canopy as an estimator of infection chronicity was fitted to model $\boldsymbol{y} = 131.9 - 0.88$ (sev %) with 76 % accuracy ($r^2 = 0.76$). On average, using this model, the production loss of Mexican lime was 54.2 %. In 25 % severity, production ranged between 88 – 130 km/plant, which, in comparison to orchards with 100% severity, represented reductions up to 81 % (25 – 46 kg) (Figure 5C). This is in agreement with reports for Mexican and Persian limes (Robles-González *et al*., 2017; Flores-Sánchez *et al*., 2015). This suggests the importance for considering the NO_3 and other nutrients in the productive management of *C. aurantifolia* affected by *C*Las. Ca and K, in association with N, have been involved in the productive increase of several crops, therefore the reduction could be linked to loss production (Atta *et al*., 2021; Cruz-Álvarez *et al*., 2020; Fan *et al*., 2020).

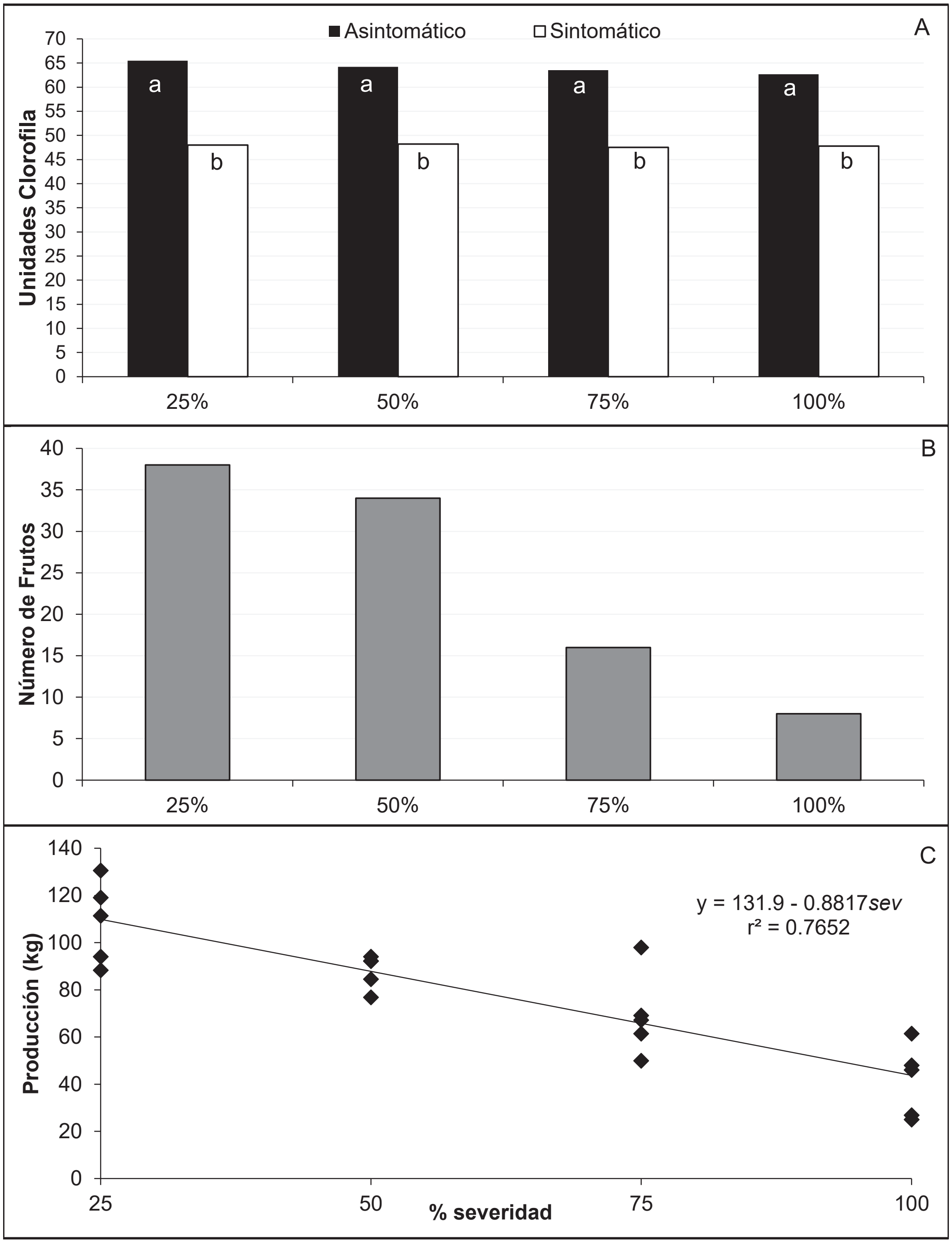

Figure 5. A. Chlorophyll units (ppm) comparison between symptomatic and asymptomatic leaves by HLB severity class-canopy (25, 50, 75 and 100 %). B. Number of fruits comparison per class of HLB severity-canopy. C. Fitting to linear production (kg) regression model based on the classes of HLB severity-canopy in Mexican lime.

CONCLUSIONS

The *C*Las chronicity, expressed through severity in *Citrus aurantifolia* canopy, had a significant detrimental effect on the nutrients concentration, mainly NO3, implying an increase in generalized chlorosis of leaves, affecting the photosynthetic capacity of the plant, and therefore influencing a reduction in production. Ca and K did not show a significant effect associated with HLB severity. Chlorophyll unit measurements decreased 27.1 % in symptomatic versus asymptomatic condition. These findings suggest that nitrates are important in the *C*Las management of Mexican lime. However, the exclusive use of nutrition excluding irrigation management, high planting densities, plant renewal programs, and regional control of the vector *Diaphorina citri*, could be limited.

Acknowledgements

To SENASICA-DGSV and the Colima State Plant Health Committee (Comité Estatal de Sanidad de Vegetal de Colima -CESAVECOL) for the logistical and operational support to the CP-LANREF. To the CONACYT for the funds INIFAP 2009-01-108591.

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