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SHORT COMMUNICATION

Impact of HLB on the physiological quality of citrus rootstock seeds and non-vertical '*Candidatus* Liberibacter asiaticus' transmission

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Abstract

Huanglongbing (HLB) is the main disease of citriculture worldwide, being mainly caused by the bacterium known as '*Candidatus* Liberibacter asiaticus' (CLas), limited to the phloem and naturally transmitted by vector insects known as psyllids. For the production of citrus seedlings, it is necessary to use rootstocks that are propagated by seeds from fruits produced by mother plants grown in field, however being submitted to natural HLB transmission. Although there is no evidence of CLas transmission from seeds to seedlings, little is known about the effects of this bacterium on the physiological quality of seeds obtained from CLas-infected plants. Therefore, the aim of this work was to evaluate the physiological quality of seeds from 'Rangpur' lime and 'Swingle' citrumelo rootstocks, from CLas-infected plants, with symptomatic and asymptomatic branches. The parameters evaluated, in addition to fruit development in mother plants, were germination rate, tetrazolium test, electrical conductivity, seedling emergence in greenhouse, and vegetative development and molecular analysis *via* qPCR for the detection of bacteria (leaf petiole, fruit peduncles, roots, seed coats, and seed endosperm). The presence of CLas in plants of both rootstocks has negative impact on fruit development, as well as on the number of normal seeds. In addition, there is reduced germination and seed emergence. Although CLas was detected in different tissues (leaf petiole, fruit peduncles and seed coat, and seed embryo), no vertical bacteria transmission was detected. Results have shown that seeds from CLas-infected branches have impaired physiological quality, but without bacterial transmission from seeds to seedlings.

Keywords Bacterium · Citrus · Greening · 'Rangpur' lime · 'Swingle' citrumelo · Seeds

'*Candidatus* Liberibacter' spp. bacterium is the causal agent of disease known as Huanglongbing (HLB) or citrus greening which has been spreading rapidly causing concern on citrus growers worldwide. Nowadays, HLB is classified as the most important and destructive disease for the citrus industry around the world (Gottwald et al. 2012). Among the three species of bacteria associated with the disease, '*Candidatus* Liberibacter asiaticus' (CLas) is present in most citrus-producing regions where the disease was reported, including in Brazil (Lopes et al. 2009). CLas transmission under natural conditions occurs through a vector insect, citrus psyllid (*Diaphorina citri* Kuwayama), and experimentally may occur by dodder (*Cuscuta* spp.) and by grafting with diseased buds (Halbert and Manjunath 2004).

Studies about the systemic distribution of CLas in plants have shown the presence of the pathogen in seed coats and other fruit parts (Tatineni et al. 2008). However, there is no evidence that '*Candidatus* Liberibacter spp.' could be transmitted through seeds of fruits harvested from HLB disease plants like *Citrus reticulata* Blanco (Shokrollah et al. 2009), *C. sinensis* (L.) Osbeck (Hartung et al. 2010), *Citrus reticulata* × *C. paradisi* Mcf., *C. limon* (L.) Burm and *Poncirus trifoliata* (L) Raf. × *C. sinensis* (van Vuuren and Cook 2011) and *C. paradisi* (Hilf 2011).

Although there is no systemic transmission of CLas from seeds to seedlings, the presence of HLB-causing bacteria can affect seed quality mainly in varieties more susceptible to the bacteria (Albrecht and Bowman 2009). This can be attributed to the imbalance in carbohydrate flow from source organs (leaves) to sink organs, among them are seeds, being one of the main mechanisms of CLas pathogenicity in citrus plants

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(Etxeberria et al. 2009; Zheng et al. 2018). Negative effects of CLas on the quality of seeds obtained from fruits with CLas were observed for Cleopatra mandarin (*C. reshni* ex Hort. Tan) and Sun Chu Sha (*C. reticulata*) varieties (Albrecht and Bowman 2009), both used as rootstock. In Brazil, the main citrus rootstocks used are 'Rangpur' lime (*Citrus limonia* Osbeck) and 'Swingle' citrumelo (*C. paradisi* Macfad. cv. Duncan x *Poncirus trifoliata*) (Agricultural Defense Coordination, State of São Paulo, Brazil, unpublished) and there are no reports of the impact of CLas on the seeds physiology of these rootstocks.

Rootstocks as component of citrus production have been used since the fifth century. But the main inducer of citrus transition from ungrafted to grafted plants was the emergence of *Phytophthora* root rot in the island of the Azores in 1842 which was controlled by resistant rootstocks, as also bringing innumerable horticultural advantages compared to ungrafted plants (Chapot 1975). Regardless of crop, the use of seeds with high genetic and physiological quality is directly related to the initial vigor of plants and consequently to performance against different stressors (França-Neto et al. 2011). For citrus rootstock, the quality of seeds is also directly related to initial vigor of plants (Zucoloto et al. 2011).

The aim of the present study was to evaluate the impact of CLas bacteria and consequently the disease, HLB, on the physiological quality of seeds from the two main rootstocks used in Brazil, 'Rangpur' lime and 'Swingle' citrumelo. In addition, we generate more evidences strengthening the hypothesis that the bacterium is not vertically transmitted from seeds to seedlings.

Ripe 'Rangpur' lime and 'Swingle' citrumelo fruits were collected on May 2013 and June 2014 under the following conditions: from plants without HLB symptoms and negative detection of CLas (HLB−); from branches without HLB symptoms of plants with HLB symptoms and positive detection of CLas (HLB+ R−); and from branches with HLB symptoms of plants with positive detection of CLas (HLB+ R+) (Fig. 1). Three plants of each phytosanitary condition were sampled in field, collecting a total of thirty fruits per plant. Fruit mass (in grams), height, and diameter were evaluated using a digital caliper.

Seeds were removed from fruits, washed in running water, and dried at room temperature until reaching 30% water content and classified as normal (full and intact seed coat) and aborted (small size and malformed). Tests were performed to evaluate the physiological quality of normal seeds as describe. *Germination*: Two batches of fifty seeds from each treatment were sown on two water-moistened paper towel equivalent to 2.5 times the dry substrate mass, according to Seed Analysis Rules (Brasil 2009) and kept in germinator (27 °C ± 2 °C, 12-h photoperiod). Evaluations were performed on the 30th day after test setup, recording the percentage of germinated seedlings. *Tetrazolium*: Two batches of fifty seeds from each treatment were pre-soaked in water at 30 °C for 18 h, after which testa and internal tegument (seed coat) were removed and immersed in 0.5% tetrazolium salt solution (chloride 2-3-5 triphenyl tetrazolium) kept in the dark for 6 h at 30 °C. After color development, seeds were washed in running water, individually evaluated and classified with the aid of stereoscopic microscope. Evaluation criterion followed recommendations obtained from the Seed Analysis Rules (Brasil 2009) and expressed as percentage of viable seeds. *Seedling dry mass*: after germination evaluation, seedlings from seeds were placed in oven at 60 °C for 48 h and weighed to obtain the germinated seedling dry mass. *Electrical conductivity test*: the mass method (AOSA 1983), using two batches of 50 seeds for each treatment, was used. Seeds were weighed and immersed in 75 ml distilled water, remaining in Biochemical oxygen demand incubator at 25 °C for a 24 h soaking period. After this period, the electrical conductivity was read using mass conductivity meter. *Emergence Test*: the test was performed in greenhouse; seeds were individually placed in tubes with pine bark substrates. Two lots of 50 seeds per treatment were sown. The final evaluation of seedlings was performed until stabilization of their emergence (60 days after sowing). *Seedling height*: 3 months after sowing, the height of emerged seedlings and dry mass were evaluated, considering from the stem to the apex of the first leaf. All trials followed a completely randomized design in 2 (rootstock varieties) × 3 (phytosanitary conditions) factorial scheme with three replicates.

Four and 7 months after sowing (emergence test), four leaves per seedling were collected (two from the bottom and two from the top) for detection of CLas *via* quantitative real-time PCR (qPCR). In addition to the presence of CLas in leaves, analyses were also performed in seeds classified as small, large, and aborted, separating seed coat (testa + internal tegument) and endosperm (embryo + cotyledons). For DNA extraction from both plant tissues, methodology described by Murray and Thompson (1980) was followed. QPCR was performed in the ABI 7500 fast equipment (Life Technology) using primers and probes described by Lin et al. (2010) following the conditions used by Beloti et al. (2018), including for C_T values < 36.0 for positive samples.

Fruits from HLB + treatment plants presented lower height, diameter, and mass, *i.e.*, lower development (Table 1). The same results were reported for sweet orange fruits harvested from plants infected with '*Candidatus* Liberibacter spp'., which also showed lower yield, soluble solids content, and higher juice acidity (Bassanezi et al. 2009). According to these authors, reducing the flow of sap to fruits as consequence of phloem dysfunction by the bacterium causing HLB was the main hypothesis for the deleterious effects on fruits.

Regardless of presence of HLB, 'Swingle' citrumelo produced larger fruits (height, diameter, and mass) and also presented higher average number of seeds (normal and aborted),

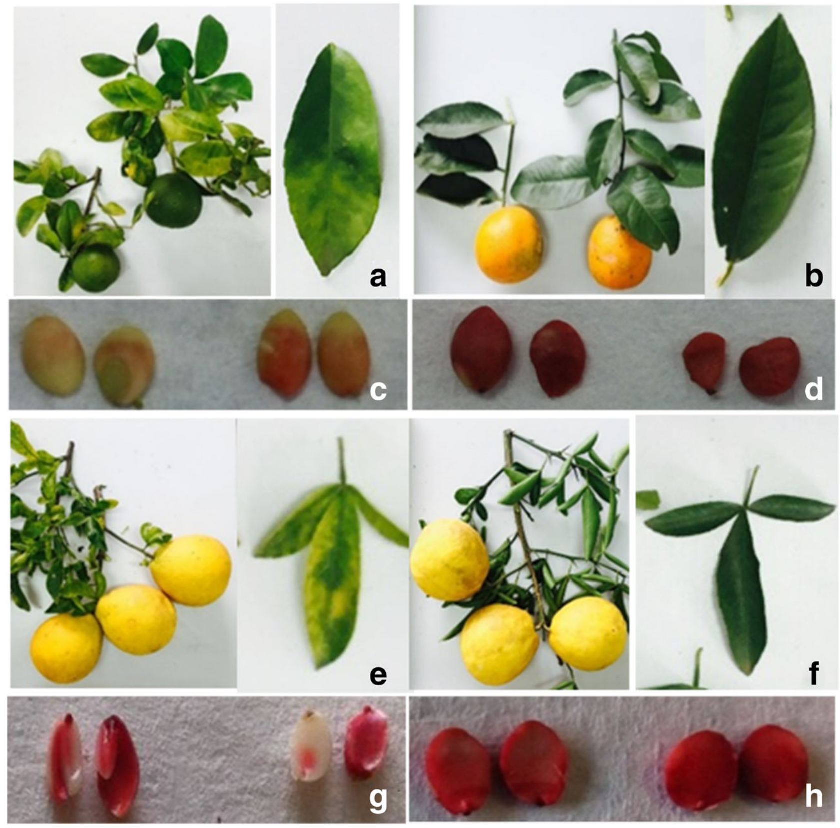

Fig. 1 Branches, leaves, and fruits and seeds after tetrazolium test of 'Rangpur' lime – LC and 'Swingle' citrumelo - CS [LC, HLB+ (a, c) and HLB− (b, d); CS, HLB+ (e, g) and HLB− (f, h)]

which were thicker and shown larger mass compared to 'Rangpur' lime (Table 1). Regularly, these are intrinsic characteristics of 'Swingle' variety (Moreira et al. 2010; Duarte et al. 2013). However, the presence of CLas in both rootstocks plants ('Rangpur' lime and 'Swingle' citrumelo) reduced the number of normal seeds and increased the number of aborted seeds compared to fruits from plants without infection (Table 1). Abnormal seed formation is one of the most evident symptoms related to CLas infection (Wang and Trivedi 2013). Less number of seeds per fruit was also reported in Valencia orange with HLB symptoms (Albrecht and Bowman 2009).

Table 1 Height (A), diameter (D), mass (M), average number of normal seeds (NNS), aborted seeds (NA_S) per fruit, and seed thickness (ST) and one thousand seed mass (TSM) in 'Rangpur' lime and 'Swingle' citrumelo fruits collected from plants with and without HLB symptoms

Variables	A (cm)	D (cm)	M (cm)	NNS (n°)	NAS (n°)	ST (mm)	TSM (g)
Rootstock (A)	**	**	**	**	**	**	**
'Rangpur' lime	5.5 b[1]	5.6 b	98.5 b	12.6 b	1.4 b	4.0 b	51.5 b
'Swingle' citrumelo	6.7 a	6.9 a	149.7 a	20.5 a	14.9 a	4.8 a	131.7 a
Phytosanitary conditions (B)	**	**	**	*	*	**	**
HLB−	6.6 a	6.7 a	146.3 a	17.2 a	8.2 a	5.0 a	110.2 a
HLB+ R−	6.4 b	6.5 a	131.3 a	17.3 a	8.3 a	4.7 a	102.1 a
HLB+ R+	5.6 c	5.7 b	94.7 b	15.3 b	7.9 b	3.6 b	58.1 b
(A)×(B)	ns	ns	ns	ns	ns	ns	ns
CV	3.7	5.1	11.5	10.7	33.7	10.5	14.9

[1] Averages followed by the same letter in column do not differ from each other (Tukey 5%)

NS, not significant; *CV*, coefficient of variation

HLB−, fruits from plants without HLB symptoms and negative detection for '*Ca.* Liberibacter asiaticus'

HLB + R−, fruits from branches without HLB symptoms in plants with HLB symptoms and positive detection for '*Ca.* Liberibacter asiaticus'

HLB + R +, fruits from branches with HLB symptoms and positive detection for '*Ca.* Liberibacter asiaticus'

The presence of aborted seeds in fruits from HLB symptomatic plants is very common (Gottwald et al. 2007; Bové 2016), suggesting a deficit of carbohydrate flow (Fan et al. 2010). In addition, CLas is able to colonize the seed parts, with reports in seed coats, cotyledons, and embryos (Tatineni et al. 2008; Lou et al. 2012). Despite this systemic colonization in seeds, no transmission to seedlings was observed, which supposes that the pathogen would have no success to pass through the vascular system of seeds, present in the chalaza, region that separates the embryo from the vascular system of the mother plant. Water and all nutrients necessary for the embryo development pass through this cell layer, but there are no differentiated vascular cells linking the two vascular systems, seeds and plant (Schneider 1968).

There was reduction in the physiological quality of seeds from plants infected with CLas (HLB +) when compared to those from healthy plants (HLB−), which can be identified by the reduction in germination and viability (tetrazolium), plant emergence, and height, in addition to increase in electrical conductivity (Table 2, Fig. 1a–h). Albrecht and Bowman (2009) also report reduced germination of Valencia orange seeds from CLas-infected plants. Blockade of translocation flow in the phloem as a result of CLas infection may be the possible reason due to the poor quality of seeds. Chen et al. (2009) also observed loss of quality of citrus seeds from plants with HLB, which generated low-vigor seedlings.

Higher electrical conductivity values were observed in seeds of fruits obtained from HLB+ branches (Table 2). Increases in electrical conductivity are directly related to the degradation of cell membranes, greater disorganization in the structure of cell membranes, allowing for increased leaching of solutes to the external environment, and, consequently, loss of permeability control, negatively affecting seed germination and vigor (Barbosa et al. 2012). On the other hand, less release of exudates into the imbibition solution was observed in seeds from the HLB− treatment, showing greater seed vigor and quality resulting in advantages on germination and emergence (Table 2).

Highest values for seedling dry mass were observed at 30 days after germination of ‘Swingle’ citrumelo, which can be explained by the larger seed mass (reserve) present for this rootstock, generating more vigorous seedlings (Table 2). Carvalho and Nakagawa (2000) reported that since larger seeds were better nourished during their development, they have well-formed embryos and larger reserves, and are therefore more vigorous, resulting in more developed seedlings. On the other hand, lower dry mass was observed in seedlings from ‘Swingle’ citrumelo fruit harvest of CLas-infected branches, showing the negative impact of the pathogen on the physiological quality of these seeds (Table 2).

Seeds harvested from fruits from branches infected with CLas (HLB +) shown the lowest emergence rates when compared to healthy plants, as well as the lowest plant height 3 months after sowing, independently of variety (Table 2). The negative effect caused by CLas infection may be results of inadequate nutrition caused by phloem dysfunction reducing translocation of photosynthates produced in leaves (source) to growing organs such as embryos (Schneider 1968; Albrecht and Bowman 2009).

In the present study, CLas was detected in various tissues in symptomatic and asymptomatic plants (HLB+ and HLB+ R−),

Table 2 Germination (G), tetrazolium, germinated seedling dry mass (DM), electrical conductivity (EC), emergence (E), and plant height (PH) emerged in ‘Rangpur’ lime and ‘Swingle’ citrumelo fruits collected from plants with and without HLB symptoms

Variables	G (%)	Tetrazolium (%)	EC (µS g^{-1})	DM (mg $plant^{-1}$)	E (%)	PH (cm)
Rootstock (A)	**	ns	**	**	**	**
‘Rangpur’ lime	94.3 a[1]	87.0 a	54.0 b	14.5 b	70.8 b	3.3 b
‘Swingle’ citrumelo	83.9 b	90.5 a	24.5 a	24.3 a	85.2 a	4.0 a
Phytosanitary conditions (B)	**	**	**	*	**	*
HLB−	92.7 a	99.3 a	28.3 c	24.1 a	78.8 a	4.3 a
HLB+ R−	90.8 a	84.2 ab	36.5 b	20.9 a	70.4 a	3.9 a
HLB+	83.8 b	78.4 b	53.0 a	14.7 b	55.1 b	2.8 b
(A) × (B)	ns	ns	ns	ns	ns	ns
CV	4.5	14.3	25.0	12.0	20.0	17.7

[1] Averages followed by the same letter in column do not differ from each other (Tukey 5%)

NS, not significant; *CV*, coefficient of variation

HLB −, fruits from plants without HLB symptoms and negative detection for ‘*Ca.* Liberibacter asiaticus’

HLB + R−, fruits from branches without HLB symptoms in plants with HLB symptoms and positive detection for ‘*Ca.* Liberibacter asiaticus’

HLB + R +, fruits from branches with HLB symptoms and positive detection for ‘*Ca.* Liberibacter asiaticus’ but at different frequencies in petiole (67%), peduncle (67%), and roots (58%) (Table 3). Tatineni et al. (2008) investigated the distribution of CLas in different tissues of infected citrus trees and found high concentrations of bacteria in fruit peduncle, suggesting that the systemic distribution of bacteria extends to reproductive structures of the host plant. Similar results were observed for many species of vascular bacteria in various crops like *Xylella fastidiosa*, causing variegated chlorosis in citrus (Coletta-Filho et al. 2014), *Clavibacter michiganensis* subsp. *michiganensis* in tomato (Xu et al. 2010), *Curtollobacterium flaccumfaciens* pv. *flaccumaciens* in dry bean (Camara et al. 2009) and '*Candidatus* Liberibacter asiaticus' in sweet orange and grapefruit (Hilf 2011).

In addition, CLas was also detected in high percentage in the seed coat (75%) and endosperm (42%) taken in batches of aborted seeds (Table 3). Also 50% in the seed coat and undetected in the endosperm (cotyledons + embryo) of normal seeds, independently if seeds came from symptomatic and asymptomatic plants (HLB + and HLB + R -) (Table 3). Li et al. (2003) and Tatineni et al. (2008) demonstrated the presence of CLas in the tegument of seeds from fruit harvest of plants infected with the bacteria, but no detection in endosperm and embryos, although only few samples were analyzed in these reported studies.

In addition to the presence of the pathogen in different plant tissues (seeds, roots, petioles, *etc.*), it is important to obtain conclusive information about the potential of bacterial transmission from seed to seedlings. In the present study, no evidence of CLas transmission from seed to seedling was obtained, even the seeds parts were colonized by the bacteria. Hartung et al. (2010) and Hilf (2011) also found no evidence of transmission in hundreds of seedlings in seeds harvested from symptomatic fruits.

Despite the possibility that CLas and other bacteria may remain within the seed coat, it seems unlikely that they can enter the vascular system and spread systemically, because there is no vascular continuity between maternal tissue and embryo development (Albrecht and Bowman 2009). Several studies on the transmission of CLas *via* seeds in citrus have found the detection of a small percentage of pathogen-positive seedlings using quantitative real-time PCR, but similar to observations in this paper, the presence of the bacterium was not confirmed in further tests (Graham et al. 2008).

Hilf (2011) studied the vertical transmission of CLas in 'Sanguinea' sweet orange seedlings germinated from seeds obtained from infected trees and detected the bacteria in 21 of the 275 seedlings germinated in greenhouse. However, a second trial with seven seedlings considered positive at the first sampling did not confirm the initial detection, suggesting that vertical transmission occurred in these seedlings, but the infection and colonization not be sustained.

Citrus plants are usually produced using rootstocks from seeds collected in field. Although CLas transmission from seeds to seedlings has not been confirmed, the presence of bacteria in 'Rangpur' lime and 'Swingle' citrumelo plants lead to reduced fruit height, diameter and mass, increased number of aborted seeds, and decreased seed mass; resulting in lower germination rate and seedling dry mass. There is also reduction of emergence, thus negatively impacting the physical and physiological quality of seeds from both HLB-disease rootstocks varieties. However, even the seeds to seedling transmission of CLas could not be proved, as already shown in previous paper, negative effect of disease was evidenced on seedlings from seeds produced on CLas-infected plants.

Table 3 Detection of '*Ca.* Liberibacter asiaticus' (CLas) by qPCR in leaf petioles, fruit peduncle, plant roots, and in the seed coat and endosperm (endosp.) of 'Rangpur' lime and 'Swingle' citrumelo

Rootstock	Sanity	No. CLas positive/no. tested samples						
		Leaf petiole	Fruit peduncle	Roots	Normal seeds		Aborted seeds	
					Seed coat	Endosp	Seed coat	Endosp.
'Rangpur' lime	HLB− [(1)]	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)
	HLB+ R−	(0/3)	(1/3)	(3/3)	(0/3)	(0/3)	(1/3)	(1/3)
	HLB+	(3/3)	(2/3)	(3/3)	(2/3)	(0/3)	(3/3)	(1/3)
'Swingle' Citrumelo	HLB−	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)
	HLB+ R−	(2/3)	(2/3)	(0/3)	(2/3)	(0/3)	(3/3)	(1/3)
	HLB+	(3/3)	(3/3)	(1/3)	(2/3)	(0/3)	(2/3)	(2/3)

[(1)] HLB−, fruits from plants without HLB symptoms and negative detection for '*Ca.* Liberibacter asiaticus'

HLB+ R−, fruits from branches without HLB symptoms in plants with HLB symptoms and positive detection for '*Ca.* Liberibacter asiaticus'

HLB + R +, fruit from branches with HLB symptoms in plants with positive detection for '*Ca.* Liberibacter asiaticus'

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