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ORIGINAL ARTICLE

Revisiting seed transmission of '*Candidatus* Liberibacter asiaticus' in different citrus species and hybrids

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Abstract
Huanglongbing (HLB) caused by '*Candidatus* Liberibacter spp.' is the most destructive disease for citrus production. These bacteria are disseminated naturally by insect vectors, such as the psyllid *Diaphorina citri*. However, the transmission of the bacteria vertically from seeds to seedlings has been controversial. Therefore, the objective of this study was to re-investigate the vertical transmission of the bacterium '*Ca.* Liberibacter asiaticus' (CLas) from seeds to seedlings. Seeds of 'Rangpur' lime, 'Swingle' citrumelo, 'Ponkan' mandarin and the sweet oranges 'Folha Murcha', 'IAPAR-73', 'Pera Rio', 'Shamouti' and 'Valencia' were included in the study. Besides the seeds, 3493 citrus seedlings were also examined for the presence of the bacterium and for the development of HLB symptoms. The presence of the bacterium in different parts of the seeds and in citrus seedlings was examined by standard PCR. CLas was detected in all parts of the seeds, including seed coat or testa, tegmen, and endosperm plus embryos of all citrus cultivars, except for 'Shamouti' sweet orange. Although transient, the presence of the HLB bacterium was also detected in seedlings of 'Ponkan' mandarin and 'Folha Murcha' sweet orange obtained from seeds of HLB-infected trees up to 18 months after seedlings transplanting. However, no typical symptoms of HLB were observed in the citrus seedlings. Although CLas may infect the citrus seeds, the results of this study do not support the vertical transmission of viable cells of CLas from seeds to seedlings.

Keywords Bacteria · *Citrus* spp. · Huanglongbing · Vertical transmission

Introduction

Huanglongbing (HLB) is the most destructive disease for citrus production in the major citrus producing areas around the world (Bové 2006). The disease is associated with the fastidious, Gram negative and phloem-inhabiting bacteria '*Candidatus* Liberibacter asiaticus', '*Ca.* Liberibacter africanus' and '*Ca.* Liberibacter americanus' (Bové 2006; Coletta-Filho et al. 2004; Teixeira et al. 2005). In Brazil, HLB was first reported in citrus growing areas in the state of São Paulo in 2004 (Coletta-Filho et al. 2004; Teixeira et al. 2005). Further, the species '*Ca. L. asiaticus*' and '*Ca. L. americanus*' have been reported in Brazil (Coletta-Filho et al. 2004; Teixeira et al. 2005). Currently, the disease is also present in the Brazilian states of Mato Grosso do Sul, Minas Gerais and Paraná (Belasque Júnior et al. 2009; Agência Estadual de Defesa Sanitária Animal e Vegeta 2020).

The HLB bacteria are transmitted by contaminated propagative material and by insect vectors, such as the Asian citrus psyllid *Diaphorina citri*, Kuwayama (Bové 2006). Vertical transmission of '*Ca.* Liberibacter spp.' through seeds and the consequent development of HLB symptoms in the citrus trees has been controversial. Citrus seedlings obtained from seeds of fruits from diseased trees may develop symptoms similar to HLB, supporting the possible transmission of '*Ca. L. asiaticus*' through seeds, but with no detection of bacteria, probably due to the absence of sensible enough diagnosis tools (Tirtawidjaja 1981). Graham et al. (2008) also reported the potential presence of '*Ca. L. asiaticus*' in citrus seedlings produced from seeds obtained from HLB symptomatic trees, but the results were inconclusive. Although the HLB bacteria have been detected in seedlings obtained from seeds extracted

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from symptomatic fruits, the presence of the bacterium in the seedlings did not necessarily correspond to the development of HLB-like symptoms in those seedlings (Albrecht and Bowman 2009; Benyon et al. 2009; Hartung et al. 2008). In addition, a true transmission of the HLB bacterium from seeds to seedlings implies the multiplication and colonization of the host tissue by the bacterium. However, neither the presence of '*Ca. L. asiaticus*' nor the development of typical symptoms of HLB were observed in seedlings produced from seeds infected with the bacterium (Hartung et al. 2010). A few studies have been carried out to evaluate the vertical transmission of '*Ca. L. asiaticus*' in citrus species and hybrids used as rootstocks, but the number of seedlings examined in these studies was relatively small (Albrecht and Bowman 2009; Benyon et al. 2009; Hartung et al. 2010).

The vertical transmission of '*Ca.* Liberibacter' spp. through seeds has also been controversial for some other crops. The potential transmission of '*Ca.* Liberibacter solanacearum', associated with several diseases on solanaceous and apiaceous crops through true seeds has been subject of several studies. Whereas Bertolini et al. (2015) reported the transmission of the bacterium by seeds after cultivation of carrots under greenhouse conditions after five months with transmission rates up to 42%, Loiseau et al. (2017a) did not find evidence of bacterial transmission by seeds. Moreover, additional studies also did not support the transmission of '*Ca.* Liberibacter solanacearum' by carrot seeds (Loiseau et al. 2017b).

The planting of citrus nursery trees free from '*Ca. L. asiaticus*' is of upmost importance in the management of HLB (Belasque Júnior et al. 2009; Bové 2006). Furthermore, the citrus nursery trees have been produced commercially under a protected environment to avoid the presence of the Asian citrus psyllid and to guarantee the healthiness of these citrus trees (Belasque Júnior et al. 2009). In contrast to the scion propagative materials that are also produced under screenhouse conditions, the citrus rootstocks are usually obtained from seeds extracted from fruits produced by trees maintained under unprotected conditions. Thus, if these rootstock seed source trees are infected with '*Ca. L. asiaticus*' and if this bacterium is transmitted through seeds, all efforts to prevent HLB will be endangered (Belasque Júnior et al. 2009; Bové 2006). In addition, the commercialization of citrus fruits in the fresh fruit market may also have implications for the dissemination of the HLB bacterium to new areas still considered free of this pathogen.

Therefore, the objectives of this study were to reexamine the presence of '*Ca. L. asiaticus*' in different parts of the citrus seeds and to reassess the possibility of the vertical transmission of the bacterium through seeds of citrus rootstock and scion cultivars. This information may have crucial implications for the adoption of measures for management of HLB in regions where the bacterium is already present and for avoiding the introduction of the pathogen in new areas that are free of the disease.

Materials and methods

Seed samples

Seeds of the citrus rootstocks 'Rangpur' lime (*Citrus limonia* Osbeck) and 'Swingle' citrumelo (*Citrus paradisi* Macf. cv. 'Duncan' x *Poncirus trifoliata* L. Raf.), and scions 'Folha Murcha', 'IAPAR 73', 'Pera Rio',' Shamouti' and 'Valencia' sweet oranges (*Citrus sinensis* (L.) Osbeck), and 'Ponkan' mandarin (*Citrus reticulata* Blanco) were included in the study. Trees at different ages of all these citrus cultivars were at productive stage and had typical HLB symptoms on leaves and fruits. The infection of the citrus trees by '*Ca. L. asiaticus*' was confirmed by standard polymerase chain reaction (PCR) (Hocquellet et al. 1999). Asymptomatic HLB fruits of all the citrus cultivars for seed extraction, except for the 'IAPAR 73' sweet orange, were harvested randomly from the tree at the ripening stage. These seeds were considered as the whole tree samples used as the source of the fruits. With regard to the sweet oranges 'IAPAR 73','Pera Rio' and 'Valencia', and 'Ponkan' mandarin, the seeds were also extracted and processed separately, from asymptomatic fruits collected in HLB symptomatic branches and HLB asymptomatic branches. The seeds were manually extracted, washed and then dried in the shade, treated with fungicide (carbendazim + thiram at the equivalent dose of 45 and 105 g a.i., per 100 kg of seeds, respectively) and stored in a cold room at 5 °C, until use. These seeds were used in the experiments aimed at HLB detection in different parts of the seed and in the vertical transmission of '*Ca.* Liberibacter asiaticus' from seeds to seedlings.

The rootstock trees were from experimental orchards planted in the Citrus Experimental Station of Bebedouro (EECB), Bebedouro, SP, Brazil. The scion cultivar trees were from field experimental plots planted in the Experimental Station of Londrina of the Agronomic Institute of Paraná (IAPAR), Londrina, PR, Brazil. Seeds of the scion cultivars were also obtained from fruits of diseased trees of commercial citrus orchards planted in the Northern region of the state of Paraná, Brazil.

Citrus seedlings

The seeds were sown in perforated plastic trays (20-cm wide, 40-cm long and 10-cm deep) containing sterilized sand. Thirty-five days after sowing, the seedlings were transplanted to 4 l capacity plastic pots, containing commercial substrate (Plantmax®, Paulínia, SP, Brazil), and 8 g of slow release fertilizer Osmocote® 20 N-08P-04 K (The Scotts Co., Marysville, OH, USA). The seedlings were kept in an insect-proof greenhouse with partially controlled environment, with average temperature around 24 °C and with daily watering. Pest management was carried out with regular

applications of insecticides and mitecides, according to the recommendations for citrus cultivation in the state of Paraná, Brazil (IAPAR, 1992). Neither adults nor juveniles of *Diaphorina citri*, the '*Ca. L. asiaticus*' psyllid vector, were detected in the greenhouse during the study.

DNA extraction from citrus seeds and seedlings

The presence of '*Ca. L. asiaticus*' was examined in the different parts of the seeds of all the different citrus species and hybrids, including the seed coat or testa, tegmen, and the endosperm plus embryo. The different parts of the seeds were separated manually and individually processed for extraction of total DNA using the procedure described by Ausubel et al. (1987), with minor modifications. The different parts of the seeds were cut into approx. 2-mm pieces and placed in 2 mL plastic tubes. A volume of 40 μL of phosphate buffered saline was added to each tube, which were then shaken at 130 rpm at 28 °C for 24 h. The tubes were centrifuged at 5000 rpm for 10 min and the pellet was resuspended in sterilized Milli-Q water. One milliliter of resuspended pellets was transferred to new 2 mL tubes and subjected to centrifugation at 14,000 rpm for 2 min. The pellets were resuspended in 567 μL of TE lysis buffer (10 mM Tris HCl, 1 mM EDTA, pH 8). Then, 30 μL of 10% SDS and 3 μL of proteinase K (20 mg mL^{-1}) were added and the tubes maintained for at 37 °C 1 h. Next, 100 μL of 5 M NaCl and 80 μL of 10% CTAB (cetyltrimethylammonium bromide) in 0.7 M NaCl were added and the tubes kept in a water bath at 65 °C for 10 min. Subsequently, 780 μL chloroform:isoamyl alcohol (24:1) was added, the mix homogenized and centrifuged at 14,000 rpm for 10 min. Then, 600 μL of the supernatants were transferred to new tubes, mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and the tubes centrifuged at 14,000 rpm for 5 min. The supernatants were transferred to new tubes and 0.6 volumes of ice-cold isopropanol were added and the tubes maintained at −20 °C for 1 h. The pellets were washed with 70% ethanol, resuspended in 100 μL of TE buffer (10 mM Tris HCl, pH 8 and 1 mM EDTA, pH 8) and the DNA solutions were stored at −20 °C until used.

The citrus seedlings were tested for the presence of '*Ca. L. asiaticus*' at 5, 18, 24 and 36 months after transplanting. Initially, each sample consisted of leaves of five seedlings, with one mature leaf being removed from each plant. Subsequently, the samples with positive results for the HLB bacterium were reexamined, with each seedling being sampled individually. Each sample consisted of five mature leaves taken from a single plant. In both cases, 500 mg of leaf tissue comprising the central leaf veins were used for total DNA extraction using the procedure described by Murray and Thompson (1980), with minor modifications. Leaf tissues were macerated in a mortar containing CTAB extraction buffer plus 0.2% β-mercaptoethanol. The macerated tissue was transferred to 2 mL microfuge tubes, which were kept in a water bath at 65 °C for 30 min. The tubes were centrifuged at 10,000 rpm for 5 min. Aliquots of 1 mL of the supernatant were transferred to new tubes and an equivalent volume of chloroform:isoamyl alcohol (24:1) was added. The mixes were homogenized and centrifuged at 14,000 rpm for 10 min. A volume of 0.9 mL of the aqueous phases was transferred to new tubes and 0.6 volumes of ice-cold isopropanol were added. The tubes were maintained at −20 °C for 30 min, and then centrifuged again at 14,000 rpm for 20 min. The pellet was washed twice with 1 mL of 70% ethanol and resuspended in 100 μL of TE buffer solution (10 mM Tris HCl, pH 8, 1 mM EDTA, pH 8). The DNA solution was stored at −20 °C until used.

Detection of '*Ca. L. asiaticus*' in citrus seeds and seedlings

All samples of the different parts of the seeds and the citrus seedlings were tested for presence of '*Ca. L. asiaticus*' by standard PCR. The primers A2 and J5 were used to amplify a 703-bp DNA fragment of the bacterial genome (Hocquellet et al. 1999). PCR was conducted in a 20 μL mix containing 2 μL of 10X Tris-HCl buffer pH 8.4, 50 mM KCl, 0.8 μL of 50 mM $MgCl_2$, 0.8 μL of each deoxynucleotide, 1 μL of 1 μM Taq DNA polymerase (Invitrogen; Carlsbad, CA, USA), 0.1 μL of 50 mM A2 and J5 primers, 1 μL of DNA and 14.2 μL of Ultrapure Milli-Q water. The reactions were conducted with the following temperature cycles: one cycle at 92 °C for 40 s for initial denaturation, followed by 35 cycles of amplification comprising 92 °C for 40 s for denaturation, 62 °C for 20 s for hybridization and 72 °C for 45 s for extension, and final extension at 72 °C for 45 s in a Veriti 96 well model thermocycler (Applied Biosystems; Waltham, Mas., USA). PCR products were stained with ethidium bromide (5 μg mL^{-1}) for 15 min and analyzed in a 1% agarose gel using SB buffer (1X) (sodium borate) subjected to 80 V for 40 min. Bands in the gel were visualized and photographed in a L-PIX EX photodocumentation system (Loccus do Brasil Ltda.; Cotia, SP, Brazil). The molecular marker 1 Kb DNA Ladder (Life Technologies; Carlsbad, CA, USA) was used to determine the size of the amplified fragments.

Results

Detection of the presence '*Ca. L. asiaticus*' in citrus seeds

'*Candidatus* Liberibacter asiaticus' was detected in most of the seeds of the different citrus rootstock and scion cultivars included in this study Table 1, *i.e.* seeds of 'Valencia' sweet orange (Fig. 1). The only exception was 'Shamouti' sweet orange, as

the presence of the HLB bacterium was not detected in any of the seed samples (Table 1). In addition, the presence of the HLB bacterium was observed in all three different parts of the seeds examined, seed coat or testa, tegmen, and endosperm plus embryo, regardless of the presence or absence of disease symptoms in the tree branch used as source of the fruits from where the seeds were extracted (Table 1). It should also be pointed out that no strong relationship was observed between the frequency of the presence of the bacterium and the tissues of the seeds or citrus cultivar (Table 1). However, a slightly higher frequency of positive results for the presence of the HLB bacterium was observed in seeds obtained from fruits collected from disease symptomatic branches compared to seeds extracted from fruits collected from asymptomatic ones (Table 1). With regard to rootstocks, '*Ca. L. asiaticus*' was detected in all three tissues of 'Swingle' citrumelo seeds (Table 1). In contrast, the HLB bacterium was found only in the seed coat or testa and tegmen tissues of 'Rangpur' lime seed (Table 1).

A higher frequency of seeds positive for the presence of the HLB bacterium was detected in tissues of seeds from fruits harvested in symptomatic branches, although '*Ca. L. asiaticus*' was detected in tissues of seeds from fruits collected from asymptomatic branches as well (Table 1). In relation to the citrus cultivars, seeds of 'Folha Murcha' and 'Pera Rio' sweet oranges had the highest frequency of the bacterium, with positive results in 100% of the samples examined, for the three kinds of tissues (Table 1). On the other hand, '*Ca. L. asiaticus*' was not detected in any of the samples of 'Shamouti' sweet orange seeds (Table 1).

Transmission of '*Ca.* Liberibacter asiaticus' from citrus seeds to seedlings

In the first PCR test performed five months after transplanting, the presence of '*Ca. L. asiaticus*' was not detected in any of the seedlings of the seven citrus cultivars included in the study (Table 2). However, two seedlings of 'Folha Murcha' sweet orange and 40 of 'Ponkan' mandarin tested positive for the bacterium in the PCR test performed 18 months after transplanting (Table 2; Fig. 2). These results indicate a higher number of 'Ponkan' mandarin seedlings infected with '*Ca. L. asiaticus*' (Table 2; Fig. 2). In the following tests, at 24 and 36 months after transplanting, the bacterium was not detected in any of the seedlings examined, including those that showed positive results previously in the PCR test (Table 2). It should also be noted that no relationship was established between citrus seedlings infected by the HLB bacterium and the presence of disease symptoms in the branch used as source of the

Table 1 Summary of the PCR results for the presence of *'Candidatus* Liberibacter asiaticus' in different parts of the seeds of citrus cultivars and hybrids

Citrus cultivar	Source of the fruit[a]									Total
	Whole tree			HLB symptomatic branch			HLB asymptomatic branch			
	Seed coat[b]	Tegmen	Endosperm + embryo	Seed coat	Tegmen	Endosperm + embryo	Seed coat	Tegmen	Endosperm + embryo	
Rootstock										
'Swingle' citrumelo	1/2[c]	1/2	2/2	nd	nd	nd	nd	nd	nd	4/6
'Rangpur" lime	1/2	1/2	0/2	nd	nd	nd	nd	nd	nd	2/6
Scion										
'Folha Murcha' sweet orange	2/2	2/2	2/2	nd	nd	nd	nd	nd	nd	6/6
'IAPAR-73' sweet orange	nd	nd	nd	0/2	2/2	0/2	1/2	2/2	0/2	5/12
'Pera Rio' sweet orange	4/4	4/4	4/4	2/2	2/2	2/2	2/2	2/2	2/2	24/24
'Shamouti' sweet orange	0/2	0/2	0/2	nd	nd	nd	nd	nd	nd	0/6
'Valência' sweet orange	2/2	2/2	2/2	2/2	2/2	2/2	1/2	2/2	2/2	17/18
'Ponkan' mandarin	2/2	2/2	2/2	1/2	2/2	2/2	0/2	0/2	2/2	13/18
Total[d]	12/16	12/16	12/16	5/8	8/8	6/8	4/8	6/8	6/8	71/96

[a] All citrus trees had HLB symptoms and the presence of '*Ca.* Liberibacter asiaticus' was confirmed by PCR test. The seeds were grouped based on the presence of HLB symptoms in the branch; Whole tree, fruits collected randomly from the tree; HLB symptomatic branch, fruits collected from branches with HLB symptoms; HLB asymptomatic branch, fruits collected from branches with no HLB symptoms

[b] Seed tissue

[c] Number of positive samples/number of samples tested; nd = not determined

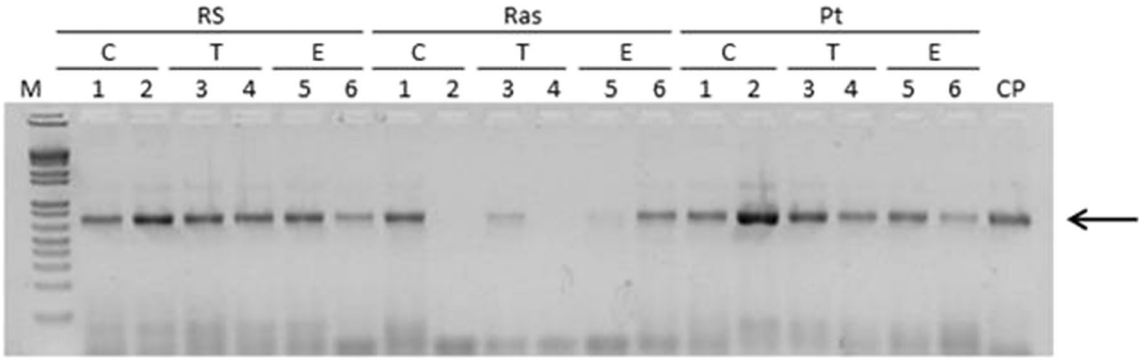


Fig. 1 PCR results for the presence of '*Ca.* Liberibacter asiaticus' in different parts of the seeds of 'Valencia' sweet orange. Source of the fruits: RS, fruits from branches with symptoms of the disease; Ras, fruits from asymptomatic branches; Pt, fruits of the whole tree. Parts of the seed: C, seed coat or testa; T, tegmen; and E, endosperm plus embryo. Lines, M, molecular marker 1 kb; 1 and 2, duplicates of seed coat or testa samples; 3 and 4, duplicates of tegmen samples; 5 and 6, duplicates of endosperm plus embryo samples; CP, positive control

fruits from where the seeds were extracted. In addition, no symptoms of HLB or any other abnormalities were observed in any of the citrus seedlings included in this study, including the seedlings of 'Folha Murcha' sweet orange and 'Ponkan' mandarin, which tested positive for '*Ca. L. asiaticus*' by PCR at 18 months after transplanting.

Discussion

'Candidatus Liberibacter asiaticus', the causal agent of HLB, colonizes the phloem vessels and spreads systemically through the entire host plant, with rapid downward movement (Shokrollah et al. 2010; Tatineni et al. 2008). It should be noted that the root colonization by the HLB bacterium has been considered an important factor for the rapid and severe evolution of the disease symptoms in citrus trees (Johnson et al. 2014). However, in addition to the roots, the bacterium may be present in all other organs of the citrus trees, such as the central vein and peduncle of the leaves, the fruit columella, the tissues of the seeds and trunk, and in different floral parts, including the petals, pistils and stamens of the flowers in symptomatic branches (Hilf 2011; Tatineni et al. 2008). Although the HLB bacterium may be detected in the seed coat or testa, the embryonic and endosperm tissues are apparently free from this pathogen (Hilf 2011). Zhou et al. (2008) reported the transmission of '*Ca. L. asiaticus*' by seeds in vinca (*Catharantus roseus*) artificially inoculated with *Cuscuta*

Table 2 Summary of the PCR results for the presence of *'Candidatus* Liberibacter asiaticus' in seedlings of citrus cultivars and hybrids

Citrus cultivar	Total number of seedlings tested	Source of the fruit[a]			Number of seedlings positive for HLB bacterium			
		Whole tree	HLB symptomatic branch	HLB asymptomatic branch	5[b]	18	24	36
Rootstock								
'Swingle' citrumelo	420	420[c]	nd	nd	0	0	0	nd
'Rangpur' lime	420	420	nd	nd	0	0	0	nd
Scion								
'Folha Murcha' sweet orange	240	240	nd	nd	0	2	0	0
'IAPAR-73' sweet orange	277	nd	11	266	0	0	0	nd
'Pera Rio' sweet orange	1126	128	581	417	0	0	0	nd
'Shamouti' sweet orange	290	290	nd	nd	0	0	0	nd
'Ponkan' mandarin	720	nd	400	320	nd	40	0	0
Total	3493	1498	992	1003	0	42	0	0

[a] All citrus trees source of the fruits for seed extraction had HLB symptoms and the presence of '*Ca*. Liberibacter asiaticus' was confirmed by PCR test. The seeds were grouped based on the presence of HLB symptoms in the branch. Whole tree, fruits collected randomly from the tree; HLB symptomatic branch, fruits collected from branches with HLB symptoms; HLB asymptomatic branch, fruits collected from branches with no HLB symptoms.

[b] Months after seedling transplanting.

[c] Number of seedlings tested by kind of branch source of the fruit for seed extraction. nd = not determined.

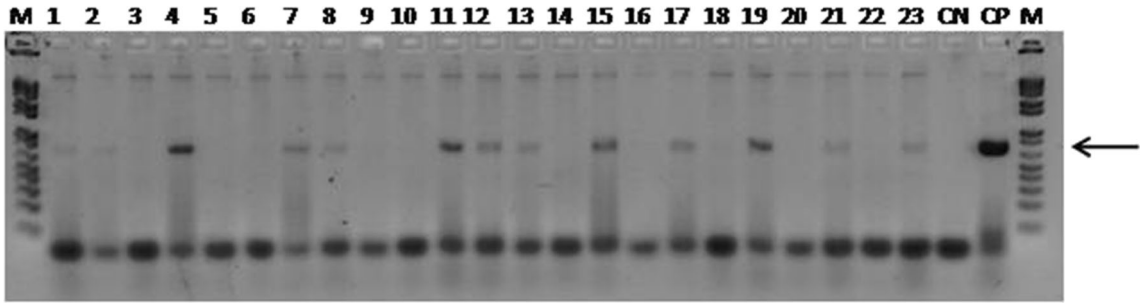


Fig. 2 PCR results for the presence of '*Ca*. Liberibacter asiaticus' in 'Ponkan' mandarin seedlings at 18 months after transplanting. Lines, M, molecular marker 1 kb; 1 to 23, individual samples of Ponkan mandarin seedlings; CN, negative control; CP, positive control

pentagona. '*Ca. L. asiaticus*' was also detected in seedlings produced from seeds obtained from diseased citrus fruits (Albrecht and Bowman 2009; Benyon et al. 2009). In some cases, these seedlings exhibited symptoms similar to those of HLB, suggesting a possible vertical transmission of the pathogen from seeds to seedlings in citrus (Benyon et al. 2009; Tirtawidjaja 1981). It is also important to note that the HLB bacterium detected in seedlings obtained from contaminated seeds have remained at very low titers and most seedlings did not develop any typical HLB disease symptoms (Benyon et al. 2009).

As reported in other studies (Hartung et al. 2010; Hilf et al. 2013; Tatineni et al. 2008), '*Ca. L. asiaticus*' was also detected here by standard PCR. Besides the seed coat or testa tissue, tegmen and endosperm plus embryo of the seeds of the two rootstocks, 'Rangpur' lime and 'Swingle' citrumelo, the HLB bacterium was also detected in different seed tissues of the other citrus scion cultivars included in this study, except for 'Shamouti' orange. However, no independent analysis was carried out for the nonzygotic nucellar and zygotic embryo tissue in the present study. It should be pointed out that these two kinds of embryos have quite different origins, as the nonzygotic nucellar embryos are originated from the maternal nucellus tissue and the zygotic embryo originates from filial tissues (Koltunow et al. 1995).

There are physical, structural and physiological possibilities for contamination of the different tissues of maternal origin of the seeds by material translocating through the phloem. The movement of material in the phloem occurs as a function of the structure and trafficking of macromolecules through the vessels, which have cells connected to each other, forming the sieve elements in the extremities (Imlau et al. 1999; Stadler et al. 2005). The pores of the sieve elements form open channels that allow the direct transport of fluids between cells, a movement referred to as the symplastic pathway (Imlau et al. 1999; Stadler et al. 2005). This conformation of the phloem cells gives rise to a structure which is a specialized tube for the transport of the phloem content at long distances, without the presence of physical barriers (Canny 1973). In addition, this movement is facilitated by the bidirectionality of the transport in the phloem, that is, from the aerial part to the root and from the root to the aerial part, unlike the xylem transport that is unidirectional (Eschrich 1975). Secondly, during the formation of the seeds there is the establishment of an intense vascularization of several tissues of the seed, linking, for instance, the nucellus tissue to the vascular system of the chalaza and from that to the funicular phloem (Koltunow et al. 1995). The nucellus apparently receives the discharging of the phloem content by the symplastic pathway (Imlau et al. 1999), through the plasmodesma that has pores with diameters usually above 1.0 μm in angiosperms plants (Liesche et al. 2015). From the nucellus tissue, the content of the phloem also migrates symplastically from cell to cell and into the tissues of the seed coat or testa (Imlau et al. 1999). It should be noted that the presence of '*Ca. L. asiaticus*' in the seed coat or testa has been reported in several studies (Hilf 2011; Hilf et al. 2013; Tatineni et al. 2008). Furthermore, citrus also have embryos developed from the nucellus cells, the nonzygotic nucellar embryos (Koltunow et al. 1995). In this way, the infection of the nucellar embryo cells by the HLB bacterium cannot be ruled out, but this was not confirmed in the present study. Finally, similarly to what occurs with plant pathogenic viruses and some other endophytic bacteria (Johansen et al. 1996; Truyens et al. 2015), the HLB bacterium remains exclusively in the symplast compartments of their hosts and, for movement it may require the symplastic continuity created by the cell-to-cell connections, the plasmodesmata, and the long-distance translocation system of the phloem (Lemoine et al. 2013).

The vertical transmission of '*Ca. L. asiaticus*' from seeds to seedlings have been reported based on the detection of the bacterium in the citrus seedlings obtained from naturally infected seeds (Albrecht and Bowman 2009; Benyon et al. 2009; Graham et al. 2008) and on the presence in the seedlings of HLB-like symptoms or abnormal growth including stunting, defoliation and chlorosis (Benyon et al. 2009; Hartung et al. 2008; Tirtawidjaja 1981). However, the percentage of citrus seedlings infected with the bacterium has usually been very low (Albrecht and Bowman 2009; Graham et al. 2008; Tirtawidjaja 1981). Exceptionally, Benyon et al. (2009) detected the presence of the bacterium in up to 41.7% of the citrus seedlings obtained from seeds of trees with HLB. High

transmission rates of '*Ca. L. asiaticus*' through seeds was also observed in vinca (*C. roseus*) artificially inoculated by using *C. pentagona* (Zhou et al. 2008). Furthermore, the presence of the bacterium in the citrus seedlings due to potential vertical transmission from seeds has been transient (Albrecht and Bowman 2009; Benyon et al. 2009; Graham et al. 2008; Hartung et al. 2008). In citrus seedlings, '*Ca. L. asiaticus*' has been detected between 4 and 5 months after sowing, but in subsequent samplings the bacterium was no longer observed (Albrecht and Bowman 2009). Here, unlike other studies (Hartung et al. 2010), the infection of 'Folha Murcha' sweet orange and 'Ponkan' mandarin seedlings by the HLB bacterium was detected as long as 18 months after transplanting. However, subsequent analyses performed at 24 and 36 months after transplanting gave negative results for the presence of the bacterium in these same citrus seedlings. Therefore, the results obtained in our studies do not support the vertical transmission of intact and viable cells of the bacterium '*Ca. L. asiaticus*' from seeds to seedlings.

In this study, comprising approximately 3500 seedlings of seven citrus cultivars of rootstocks and scions, no symptoms characteristic of HLB or any other abnormality was observed in the citrus seedlings throughout the experimental period, which was extended to 36 months after transplanting. In some investigations, typical HLB symptoms were not observed in citrus seedlings produced from seeds harvested from diseased trees (Albrecht and Bowman 2009; Hartung et al. 2010). On the other hand, HLB-like symptoms or some abnormality in the citrus seedlings produced from seeds of diseased trees were reported in other studies (Benyon et al. 2009; Hartung et al. 2008, 2010; Tirtawidjaja 1981; Zhou et al. 2008). However, these HLB-like symptoms apparently did not evolve (Benyon et al. 2009). Furthermore, there was not a clear correlation between bacterial presence and HLB-like symptoms in the seedlings (Hartung et al. 2008; Zhou et al. 2008). The populations of the bacterium in these citrus seedlings apparently did not increase, based on PCR tests (Zhou et al. 2008). In this way, it was not surprising that the citrus seedlings that at some time tested positive for '*Ca. L. asiaticus*' in standard PCR tests did not develop typical HLB symptoms.

In conclusion, this study comprising seven cultivars of citrus rootstocks and scions, revealed the presence of the bacterium '*Ca. L. asiaticus*', the HLB agent, in different parts of the seed, including the seed coat or testa, the tegmen, and the endosperm plus embryo. Furthermore, the transient infection of the citrus seedlings obtained from seeds of diseased trees by the HLB bacterium was also detected by standard PCR tests. However, none of these citrus seedlings developed yellow branches, leaf mottling, dieback, or any other typical HLB symptoms. Thus, these results do not support the possibility of vertical transmission of viable cells of '*Ca. L. asiaticus*' from seeds to seedlings in citrus. Yet, some questions remain unanswered, such as how transient contamination by this bacterium may occur in the citrus seedlings and why the bacterium is not able to establish and consequently cause the development of typical HLB symptoms. As discussed above, there are no physical, structural or physiological impediments for the HLB bacteria to reach the different tissues of the seed, including the nucellus that gives rise to the nonzygotic nucellar embryos. Based on the results of this and other studies, there is no support for the vertical transmission of viable cells of the bacterium '*Ca. L. asiaticus*' from citrus seeds to seedlings.

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