

Citrus Huanglongbing (Greening Disease) in Egypt: Symptoms Documentation and Pathogen Detection

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Abstract: Citrus huanglongbing (HLB) or citrus greening is one of the most devastating diseases of citrus worldwide. The disease is associated with a phloem-limited fastidious α proteobacterium, ('*Candidatus Liberibacter* spp.'). which has yet to be cultured. Symptoms resembling those of huanglongbing were observed in several citrus orchards located in different areas in Egypt. Observed symptoms on infected citrus trees include: Small upright thickened chlorotic leaves, some with green islands and some resembling mineral deficiencies; leaves with asymmetric, sometimes dull, blotchy mottling across leaf veins; Yellow shoots standing out from canopy; Small, lopsided, bitter tasting green fruit with small, dark aborted seeds; leaf and fruit drop; reduced tree height; twig dieback at later stages. Early flowering is also observed. Transmission electron microscopy examination of infected and healthy leaf midribs revealed rod and pleomorphic-shaped bacteria observed in HLB-affected midribs and not observed in healthy midribs. PCR amplification using the specific primers (OI1 +OAI) / OI2c designed to amplify the a 1160 bp fragment of the 16S rDNA of '*Ca. Liberibacter asiaticus*' and '*Ca. Liberibacter africanus*', successfully yielded the expected size product from the majority of the symptomatic samples, whereas samples from asymptomatic trees did not. The disease was artificially transmitted by bud grafting from infected citrus to healthy citrus and by dodder (*Cuscuta campestris*) from infected citrus to healthy orange jasmine (*Murraya paniculata*), periwinkle (*Catharanthus roseus*), tobacco (*Nicotiana tabacum* cv. White Burley) and wild tobacco (*Nicotiana glauca*). To our knowledge, the results reported here is the first report that confirms the presence of citrus HLB in Egypt.

Key words: Huanglongbing • Egypt • Dodder • Electron microscopy • *Nicotiana glauca* • Orange jasmine

INTRODUCTION

Citrus huanglongbing (HLB) or greening is a very serious disease that affects all citrus cultivars and seriously threatening the citrus production throughout much of the citrus producing areas in the world. The disease is caused by the Gram-negative bacterium '*Candidatus Liberibacter* spp.'. This fastidious bacterium is restricted to the plant's phloem and cannot be cultured *in vitro* yet. Currently, three species of the pathogen, '*Ca. Liberibacter asiaticus*', '*Ca. Liberibacter africanus*' and '*Ca. Liberibacter americanus*', are recognized based on 16S rDNA sequence [1].

Through the movement of plant materials around the world, the disease and its insect vector have been accidentally spread throughout the citrus producing areas. The HLB bacterium is naturally vectored by the citrus psyllids *Diaphorina citri* and *Trioza erytreae*. Moreover, it is can be artificially transmitted from citrus to citrus by grafting [2, 3, 1, 4] and by dodder (*Cuscuta campestris*) from citrus to the non-rutaceous plants periwinkle (*Catharanthus roseus*) [5], tobacco (*Nicotiana tabacum* cv. Xanthi) [6] and tomato (*Solanum lycopersicon*) [7].

Symptoms of HLB on foliage of infected citrus trees vary from complete yellowing, asymmetric blotchy-mottling, or other chlorotic patterns that sometimes

resemble mineral deficiency to leaf drop, twig dieback and trees decline at later stages [8, 1]. Symptoms on fruits include small size, lopsided shape, colour inversion, aborted seeds, poor flavor and excessive fruit drop [1]. Off-season flowering is also reported in HLB pathogen-infected sweet orange [9].

The HLB bacterium was discovered in 1970 by electron microscopy [10]. To date, the HLB bacterium has not yet been grown on artificial. This explains why characterization of the HLB agent has not progressed as fast as other fastidious plant pathogenic bacteria. Molecular techniques had to become available to finally characterize the organism at the phylogenetic and taxonomic level and the nature of the HLB bacteria and their detection for HLB identification and confirmation were deeply reviewed [1].

Although, the Mediterranean basin countries are not very far from the neighbours contaminated with both psyllid vectors and HLB, the disease as well as the psyllid vectors has not reported in this region before [1, 11]. In fact, this may be quite far from reality particularly in Egypt where the climatic conditions are conducive and the intensive cultivation and production of citrus which not subject to any either plant quarantine or experimental work. There is another fact that, Egypt in addition to being one of the Mediterranean countries, it has borders on the Red Sea where the disease and psyllid vector are present in a number of countries in this region like Saudi Arabia and Yemen [1].

By the fact that, HLB is a serious threat of citrus production in Egypt, therefore the present work aimed to study the symptomology and etiology of the disease in order to determine its status in Egypt.

MATERIAL AND METHODS

Symptoms Documentation *in Situ*: Citrus trees with 3 citrus types (sweet orange, navel orange and mandarin) within several commercial groves located in different areas in Egypt were visually surveyed for symptoms of huanglongbing (HLB) during the period from April 2013 to April 2015. The trees suspected to be infected by HLB were carefully inspected and any symptoms resembling HLB within the tree were recorded and photographed. Examining of suspected trees for disease symptoms was carried out using HLB field identification guides with color photos [12, 13]. The targeted foliar symptoms were: blotchy mottling pattern on the leaves (asymmetrical blotchy mottle with light and dark green patches),

yellowing and corky raised veins, interveinal chlorosis, bright yellow shoots amongst a green canopy (yellow dragon), erect yellow new leaves, mineral nutrient deficiencies (such as those caused by zinc, iron and manganese), reduced total foliage, premature leaf drop, twig dieback. The targeted fruiting symptoms were: reduced size, misshapen and lopsided fruits with curved columella and aborted seeds, Yellow stain at base of fruit button, inversion of colour formation on fruit (stays green on the bottom), bitter tasting and excessive fruit drop. Irregular flowering was also targeted.

Pathogen Detection with Electron Microscopy: Midribs from HLB-affected and healthy citrus leaves were prepared for transmission electron microscopy (TEM) at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University. Midribs were cut into 2- to 3-mm segments. Tissues were fixed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.2, overnight at 4°C. Samples were washed in the same buffer and postfixed in 2% osmium tetroxide for 4 h at room temperature and then dehydrated in acetone and embedded in Spurr's resin. Thin sections (70 nm) were made using an ultramicrotome with diamond knife, mounted on copper grids and stained with uranyl acetate (3%) and lead citrate. Stained sections were examined using a JEOL (JEM 1010) transmission electron microscopy and images were captured and analyzed with Image-Pro software.

Pathogen Detection with PCR Analysis

Sample Preparation and DNA Extraction: Leaves were collected from trees with or without symptoms of HLB in various groves. The leaves were kept in plastic bags in cool boxes and transported to the laboratory. Upon arrival, midribs of each leaf sample were excised then processed for PCR templates following two DNA extraction methods. In the first method, the DNA was extracted using plant DNA extraction kit following the procedures (designated preparation 1) that described by [14]. The midribs (0.3 g) of leaves in each sample were chopped roughly to a fine mince with a razor blade on a clean glass slide. Approximately 0.05 g of midrib pieces were ground with a mortar and pestle then the DNA were extracted using a DNeasy plant mini kit (QIAGEN®) according to the manufacturer's instructions. The DNA solutions were adjusted to a volume of 400 µL with distilled water. In the second method, DNA extracts from citrus tissues were prepared using the CTAB method

described by [5, 16]. In this method, Leaf midribs (250 mg) were powdered in liquid nitrogen and homogenized in DNA extraction buffer (0.1M Tris-HCl [pH 8.0], 0.05M EDTA, 0.5M NaCl, 1% N-Lauroylsarcosine). Samples were incubated at 55°C for 1 h. After centrifugation at 12000 g for five min., the supernatant was treated with 1% CTAB (hexadecyl-trimethyl-ammonium-bromide) at 65°C for 10 min. DNA was precipitated with isopropanol after chloroform/isoamyl alcohol (24: 1) and phenol/chloroform/isoamyl alcohol (25 : 24 : 1) treatments. The DNA pellet was resuspended in 150 µl TE buffer (10mM Tris-HCl [pH 8.0], 1mM EDTA).

PCR Reaction: The specific primers (OI1+ OA1) /OI2c targeting 16S ribosomal DNA were used to confirm the presence of 'Ca. L. asiaticus' and/or 'Ca. L. africanus' in symptomatic leaves and the absence of bacteria in asymptomatic leaves. Both forward primers (OI1 [5'-GCG CGT ATG CAA TAC GAG CGG CA 3'] targeting *Ca. L. asiaticus* and OA1 [5'-GCG CGT ATTTTA TAC GAG CGG CA 3'] targeting *Ca. L. africanus*) were used in the reaction mixture to favor amplification of either one of the two liberibacters [17] whereas the reverse primer OI2c [59-GCC TCG CGA CTT CGC AAC CCA T-39] was the same for both liberibacters. PCR using these primers with the HLB-infected citrus was expected to amplify specific fragments of 1160 bp [17]. The PCR reaction was performed in 40µl of reaction mixture containing 1 mM of each of the primers, 200 mM of each of the four dNTP, 2 mM MgCl₂, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 U of Taq polymerase (Promega) and 1 ml of DNA preparation [18]. A mastercycle gradient thermocycler (Eppendorf) with the following program was used for DNA amplification: 9 min of predenaturation at 96°C, followed by 35 cycles of 30 s of denaturation at 96°C, 1 min of annealing at 55°C, 30 s of extension at 72°C and a single final extension of 7 min at 72°C [14]. Following amplification, 10 µl aliquots of each reaction mixture were analysed by electrophoresis on 1.2% agarose gels.

Experimental Transmission by Grafting: Budwood-grafting method was used as inoculation method to transmit the disease from infected to healthy citrus plants. The experiment was conducted from March 2013 to December 2014 and the test plants were obtained from commercial citrus propagation nurseries in Egypt. Apparently healthy young seedlings (10- to 14-month old), of sweet orange (*Citrus sinensis*), Duncan grapefruit (*Citrus paradise*) and alemow (*Citrus macrophylla*) were

used in this experiment. The sweet orange and Duncan grapefruit plants were propagated as scions on Volkamer lemon (*Citrus volkameriana*) rootstocks whereas the alemow plants were propagated as seedlings resulted from the seed planting. Five plants per each type were graft inoculated with budwood (3 budwood) that had been taken from PCR-positive HLB source trees of sweet orange. The control of each citrus type consisted both of 5 plants that were inoculated by healthy (PCR negative) budwood and 5 noninoculated plants. The grafted budwoods were left permanently in the grafted trees but the resulted shoots were cut whenever grew. The tested plants were kept in net house (Polyam 600 µ) in the experimental farm of faculty of agriculture, Al Azhar University, Cairo, Egypt. The experiment plants were observed monthly for infection initiation and progress of symptoms. The plants were sampled for PCR detection every 6 month during the experiment .

Experimental Transmission by Dodder: Ability of HLB pathogen to transmit via dodder was tested on non-citrus plant, orange jasmine (*Murraya paniculata*) and non-rutaceous plants; periwinkle (*Catharanthus roseus*), tobacco (*Nicotiana tabacum* cv. White Burley) and wild tobacco (*Nicotiana glauca*).

Dodder (*Cuscuta campestris*) seeds were germinated on pots that were previously planted by healthy seedlings of sweet orange. After the dodder had established on the seedlings, the newly developed dodder strands were attached to PCR-positive HLB infected (graft inoculated) sweet orange plants and left until the growth of dodder had developed on the infected citrus plants.

After the dodder had formed haustoria within the infected citrus plants, the strands between infected and healthy plant were cut and the newly developed dodder strands on the infected plants were attached to the tested plants to make the infection connection. The strands between the infected citrus and test plants were cut after 6 weeks. Subsequent dodder strands growing from remaining haustoria on the tested plants were removed to prevent weakening of plants by dodder. The test plants were then kept free of dodder strands and observed monthly for symptoms development and sampled for PCR detection after 3 months. A healthy dodder strands were connected to healthy seedlings of each test plants and served as a control with the same manner. The duration of the experiment was 6 months in the case of non-rutaceous plants and 15 months in the case of, orange jasmine.

RESULTS

Symptoms Documentation *In situ*: Citrus trees with 3citrus types within several commercial groves were surveyed for visual symptoms of HLB during April 2013 to April 2015. Generally, almost characteristic symptoms of the disease were recorded. The leaf yellowing symptom on a single branch or shoot (yellow shoot) was observed in a few cases, particularly in the trees with early developing stage of the disease (Fig. 1). The more typical disease symptom is what is known as asymmetrical blotchy mottle was observed on almost of the investigated trees (Fig. 2). Some infected trees displayed nutrient deficiency pattern symptoms on their leaves (Fig. 3). Vein corking symptoms what is typified by bright yellow leaf veins that are raised and have a corky appearance is also observed on several investigated trees (Fig. 4). Foliar yellowing (Fig. 5), stunting (Fig. 6), excessive fruit drop, defoliation with thin canopy, twigs dieback and tree decline were appeared particularly on elderly trees (Fig. 7).

As well, the fruit particularly on the declined trees, displayed the characteristic disease symptoms. Symptomatic fruit were commonly much smaller in size than the healthy, misshapen and appeared lopsided (Fig. 8). As they mature and ripen the styler end remains green (Fig. 9, 10 and 11). The vascular bundles in the fruit axis just below the point of stem attachment were stained yellow and the seeds appeared dark-colored and aborted (Fig. 12). Irregular flowering is also observed particularly in symptomatic sweet orange trees (Fig. 13).

Transmission Electron Microscopy Observations: Using transmission electron microscopy (TEM), cells with features consistent with '*Ca. Liberibacter*' were observed in the phloem sieve tubes of the leaf midribs obtained from symptomatic citrus trees but not from asymptomatic trees. Elongated filaments and round forms are seen in the sieve tubes (Figs. 16 and 17). In almost observed samples, the *Ca. Liberibacter*-like cells if it present, existed in small numbers and as single cells or form visible aggregates (Fig. 18).

PCR Analysis: The leaves samples from 16 visibly symptomatic citrus trees and one from healthy were prepared as PCR templates using a DNeasy plant mini kit and CTAB methods. PCR using the specific primers (OI1+ OA1) /OI2c was consistently successful in both cases amplifying the specific fragments of 1160 bp (Fig. 14

and Fig. 15). PCR with DNeasy templates was successful in 13 (i.e., 1, 2, 3, 4, 5, 7, 8, 9, 10, 13, 14, 15 and 16) symptomatic samples but failed with 3 (i.e., 6, 11 and 12) (Fig. 14). Whereas, PCR with CTAB templates was successful in 11(i.e., 1, 2, 3, 4, 7, 8, 9, 10, 14, 15 and 16) symptomatic samples and failed with 5(i.e., 5, 6, 11, 12 and 13) (Fig. 15). PCR without templates (water) and with healthy citrus samples never resulted in amplification. It's worth to be noted; the bands obtained with kit templates were more robustness than those of CTAB templates (Fig. 14 and Fig. 15).

Experimental Transmission by Grafting: Healthy citrus plants were graft-inoculated with budwood from PCR-positive HLB sweet orange trees to test the transmissibility of the disease from citrus to citrus. The symptoms expression unveiled the success of the pathogen transmission as evidenced by developing symptoms on the infected plants but not on the control plants. In general, there was no substantial variation among the citrus type in their initial response to infection but with time, the disparities appeared between types. Yellowing symptoms were shown about 4-6 months after inoculation on all infected plants. Some infected sweet orange plants exhibited yellow shoots on one or many branches randomly arranged in the canopy (Fig. 19). Characteristic leaf mottle symptoms were shown within 6-10 months after inoculation on grapefruit and alemow (Fig. 21) and within 8-12months on sweet orange (Fig. 20). Few infected plants of alemow displayed the green islands (small circular dark green dots) symptom on newly formed leaves (Fig. 22). Leaves on some affected branches were thicker and leathery with raised corky veins. On some infected branches leaves formed the "rabbit ears" symptom that is small upright shoots with compressed internodes (Fig. 23). With time, some infected trees developed acute chlorosis on the shoots with inhibited growth and flowed by defoliation and twigs dieback leading to thin canopy (Fig. 24). All inoculated plans reacted positively with PCR test where the control plants did not.

Experimental Transmission by Dodder: Transmissibility of HLB pathogen by dodder was tested on orange jasmine, periwinkle, tobacco and wild tobacco. Generally, tobacco was extremely sensitive followed by wild tobacco and periwinkle whereas, orange jasmine were less sensitive. On orange jasmine, the symptoms progressively developed as yellowing and mottling of young leaves,



Fig. 1: Yellow shoot Symptom appeared on HLB affected citrus trees



Fig. 2: Asymmetrical blotchy mottling symptom on HLB-affected sweet orange leaves



Fig. 3: Mineral nutrient deficiency patterns on HLB-affected Sweet orange leaves



Fig. 4: Yellow corky vein symptom on sweet orange leaves



Fig. 5: Foliar yellowing on HLB-affected sweet orange trees



Fig. 6: Stunted sweet orange trees affected by HLB



Fig. 7: Fully HLB affected sweet orange trees with defoliation leading to thin canopy, fruit drop and dieback.



Fig. 8: Lopsidedness symptom on sweet orange fruit



Fig. 9: Sweet orange fruit showing colour inversion (greening)



Fig. 10: Naval orange fruits showing colour inversion (greening) and is also misshapen



Fig. 11: Mandarin fruits showing colour inversion (greening)

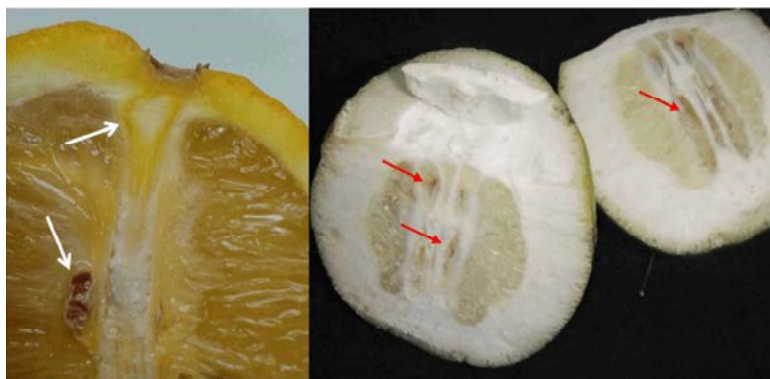


Fig. 12: Sections from HLB infected Sweet orange fruits displaying diagnostic orange-brown stain of the vascular columella and brownish-black aborted seeds



Fig. 13: Irregular flowering on HLB affected sweet orange trees

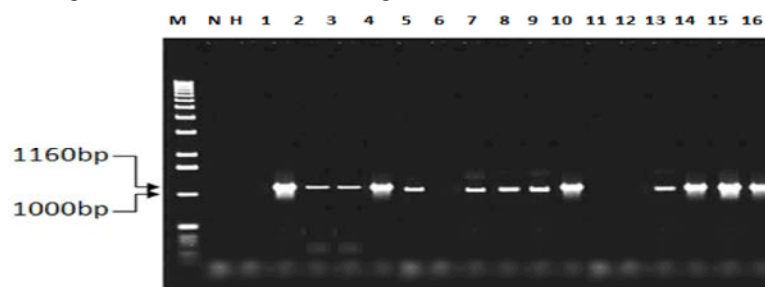


Fig. 14: Electrophoresis on 1.2% agarose gel of DNA (templates obtained using DNeasy plant mini kit method) amplified with 16S rDNA primers (OI1+ OA1)/OI2c, specific for *Ca. L. asiaticus* and *Ca. L. africanus*. lane N (water), lane H (healthy sweet orange tree), lanes: 1 – 8 (symptomatic sweet orange leaves), lanes: 9 – 12 (naval orange), lanes: 13 - 16 (mandarin). M, 1 kb ladder

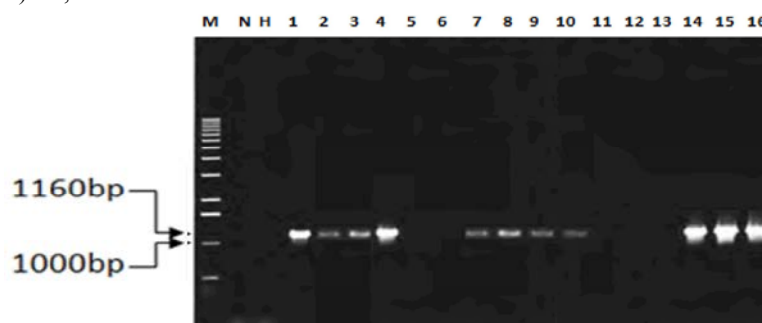


Fig. 15: Electrophoresis on 1.2% agarose gel of DNA (templates obtained using CTAB method) amplified with 16S rDNA primers (OI1+ OA1)/OI2c, specific for *Ca. L. asiaticus* and *Ca. L. africanus*. lane N (water), lane H (healthy sweet orange tree), lanes: 1 – 8 (symptomatic sweet orange leaves), lanes: 9 – 12 (naval orange), lanes: 13 - 16 (mandarin). M, 1 kb ladder

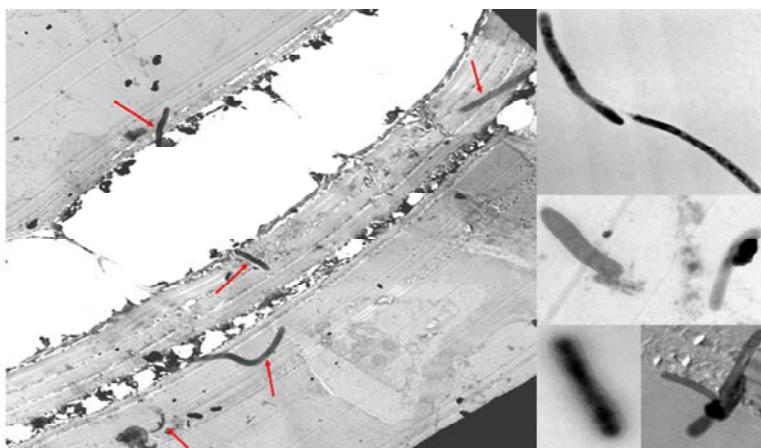


Fig. 16: Transmission electron microscopy (TEM) photomicrographs of leaf midribs obtained from symptomatic citrus trees showing presence of bacteria-like organisms as elongated filaments individual bacterial cell in the phloem sieve tubes

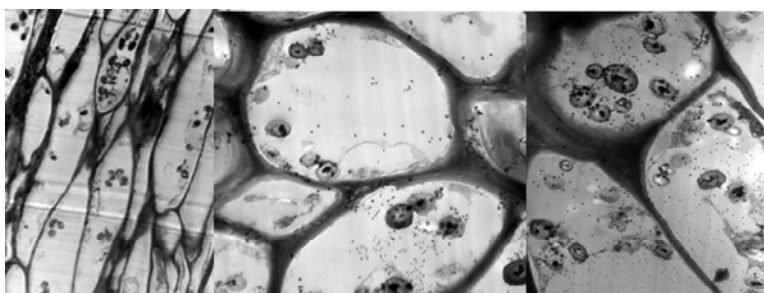


Fig. 17: Transmission electron microscopy (TEM) photomicrographs of leaf midribs obtained from symptomatic citrus trees showing presence of bacteria-like organisms as round forms of individual bacterial cell in the phloem sieve tubes

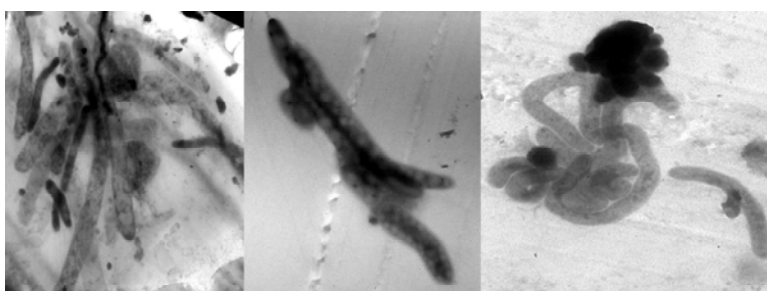


Fig. 18: Transmission electron microscopy (TEM) photomicrographs of leaf midribs obtained from symptomatic citrus trees showing presence of bacteria-like organisms as a cell aggregates in the phloem sieve tubes



Fig. 19: Yellowing and yellow shoot symptoms on budwood graft-inoculated sweet orange plants



Fig. 20: Leaves of graft-inoculated sweet orange plants displaying “blotchy-mottle” symptoms



Fig. 21: Leaves of graft-inoculated grapefruit plants displaying mottling symptoms



Fig. 22: Leaves of graft-inoculated alemow plant displaying mottling and green islands symptoms



Fig. 23: Upright shoots with compressed internodes (rabbit ears symptom) on graft-inoculated sweet orange plants



Fig. 24: Defoliation and twigs dieback with thin canopy on on graft-inoculated sweet orange (left) and alemow (right).



Fig. 25: Symptoms progress on dodder inoculated orange jasmine



Fig. 26: Symptoms progress on dodder inoculated periwinkle plants



Fig. 27: Symptoms progress on dodder inoculated tobacco plants



Fig. 28: Symptoms progress on dodder inoculated wild tobacco Plants

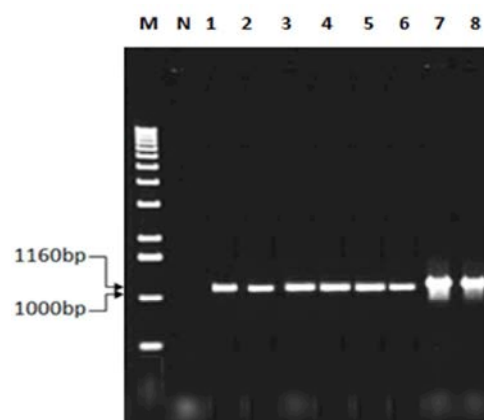


Fig. 29: PCR detection of the HLBB in dodder-inoculated plans. Lane M, 1 kp DNA ladder for size marker; lane N, water; lanes, 1 and 2 orange jasmine; lanes, 3 and 4 wild tobacco; lanes 5 and 6 tobacco; lanes 7 and 8 periwinkle. The PCR products were analyzed by electrophoresis in a 1.2% agarose gel. Samples collected 12 month after inoculation from orange jasmine and 3 months from other plants

reduced growth and eventually leaf drop and twig dieback leading to thin canopy appearance (Fig. 25). Tobacco, wild tobacco and periwinkle responded to infection with initially localized yellowing progressively developed around the secondary veins followed by yellowing of leaf margins (Figs. 26, 27, 28). Eventually, severe yellowing prevailed in the entire leaves. The plants of wild tobacco and periwinkle continue to grow whereas tobacco plants eventually died. All control plants remained healthy throughout the experiment. All inoculated plants reacted positively with PCR test where the control plants did not (Fig. 29).

DISCUSSION

Huanglongbing (HLB) has become a global issue that threatens the continued successful production of citrus. Nevertheless the disease is widely spread in Asia, Africa and not long ago in America [19, 18]; it had not been reported previously in Egypt. In spring of 2011, leaf and fruit symptoms resembling those of HLB were observed on sweet orange trees in groves located in different areas in Egypt (Tolba I. H., personal communication). The serious study of the disease started in 2013 in order to confirm that these symptoms representing the HLB disease.

Citrus groves have been surveyed for presence of HLB symptoms during the period from April 2013 to April 2015. Almost characteristic symptoms of the disease were observed. This symptomatology suggested that, the suspected trees are affected by citrus huanglongbing. According to EPPO [11], the Mediterranean region was reported to be free from huanglongbing psyllid vectors and huanglongbing liberibacters. So, the question that arises, how to the disease entered into Egypt? The answer could be clarified by several possibilities. The most likely and reinforced possibility is that, as is well known, the unintentional introduction of infected plant materials and subsequent unregulated movement establishes the disease in new areas or countries and then the disastrous results are expected. An illustration of this and in the same vein, the Egyptian citrus growers importing the planting material from South Africa where the disease is heavily present [1]. Clearly, the continued increases in international trade and travel are the most key elements which exacerbate the probability of pathogen and pest introduction to new places. Another possibility is that the disease is already accrued from undefined time, but did not have a noteworthy and studies. Based on the actual observations, the knowledge of the persons they were

assigned to citrus growing about the nature of this disease and its features were greatly deficient. As a consequence, they did not protect their seedlings and trees properly and also used infected trees as source of planting materials for new cultivation. The insect-vector and graft-transmissibility of the diseases exacerbate the problem by the use of seedlings which were thought to be healthy, but were in fact infected with disease.

Although, some quite characteristic symptoms associated with HLB, particularly the leaf blotchy mottle and the lopsided fruit with green color remaining on the stylar end [1], diagnosis based on visual symptoms can be somewhat difficult because of these symptoms are not specific to huanglongbing. For example, stubborn disease (*Spiroplasma citri*) produce substantially similar symptoms [10]. Also, severe forms of citrus tristeza virus (CTV) and species of *Phytophthora* can produce similar blotchy mottle patterns [20]. Hence, sensitive and specific methods should be followed for accurate disease diagnosis.

During the period from 1970 to 1990, the transmission electron microscopy (TEM) has been the first and only laboratory technique for indisputable identification and confirmation of HLB and has been widely used [21]. Using TEM, '*Ca. Liberibacter*'-like cells were seen in the phloem of the symptomatic citrus trees but not in asymptomatic. Both, elongated filaments and round forms are seen in the sieve tubes and appear to be related forms [22]. Laflèche and Bové [10] reported that mycoplasma-like organisms (MLOs) were present in the phloem sieve elements of infected plants but not in healthy. On close examination, these organisms were seen to have thicker envelopes than MLOs, suggesting that they were true bacteria [23]. To date, efforts to culturing the organisms in the different media used previously [6] has been so far unsuccessful, but a combination of EM and enzymatic treatments showed the cell wall to be of the Gram negative type [24].

PCR is now the main confirmatory test and is used for rapid, sensitive and specific diagnosis for HLB in the laboratory and as a prelude to disease management worldwide. PCR tests using the specific primers (OI1+ OA1)/OI2c targeting 16S ribosomal DNA of '*Ca. L. asiaticus*' and/or '*Ca. L. africanus*' [17] were conducted for 16 samples collected from symptomatic citrus trees. PCR templates resulted from two extraction methods (DNeasy kit and CTAB) were successful in respectful numbers of the tested samples with some superiority to kit method. The PCR products obtained were the 1160 bp as expected for the both form of the pathogen with these primers [17]. Regarding to these results, it is confirmed

that the citrus trees that showed varied putative HLB symptoms in the surveyed areas were infected by the causal agent of HLB disease (*Ca. L. asiaticus* and/or '*Ca. L. africanus*') and not due to other causes. [17] described a conventional PCR method to detect *Liberobacter asiaticus* and *africanus* species in citrus trees based on the amplification of an 1160 bp fragment of their 16S rDNA with primer pair OA1/OI2c for *Ca. L. africanus* and OI1/OI2c for *Ca. L. asiaticus*. They revealed that, the 1160 amplicon from the Asian *liberibacter* yields two restriction fragments (520 bp and 640 bp) when treated with XbaI and can thus be easily distinguished from the 1160 amplicon of the African *liberibacter*, which yields three fragments (520 bp, 506 bp and 130 bp). [18] used the specific primers GB1/GB3 for PCR amplification a 1027 bp of the 16S rDNA of *Ca. L. Americanus*. Recently, real time PCR (RTi-PCR) and quantitative real time PCR (q-PCR) have been applied to the detection and quantification of *liberibacters* in plants and insect vectors [25-28]. Real time PCR was 1000 times more sensitive than conventional 16SrDNA PCR [28].

All forms of HLB pathogen has been reported to be graft transmissible [1-4]. Our results showed that, the experimental transmissibility of the disease from citrus to citrus by grafting has been successful to a quite extent. HLB symptoms appeared 4 to 6 months after inoculation on infected plants but not on control plants. The initial response to infection was evenly matched among the citrus cultivars. Positive reaction with PCR is a conclusive proof on disease transmission. Virtually, the HLB bacterium can infect all citrus species, cultivars and hybrids, as well as several citrus relatives [12]. Some citrus-related plants (family Rutaceae) have been confirmed as hosts for HLB, namely *Verpris lanceolata* [29], *Limonia acidissima* [16], *Severinia buxifolia* [30], *Murraya paniculata* and *Murraya exotica* [31]. The ornamental *Murraya paniculata* and *Murraya exotica* are also attractive host plants of the Asian citrus psyllid [31]. Thus, having these ornamentals in the landscape can allow psyllid populations to build up and increase the risk of spreading the disease to other ornamental and citrus plants.

HLB can also be transmitted by dodder (*Cuscuta spp.*) [32-34, 6]. Using this method, the disease was successfully transmitted to non citrus plant, *Murraya paniculata* and to non-rutaceous plants, periwinkle, tobacco and wild tobacco. Transmission of "*Ca. Liberibacter asiaticus*" from *M. paniculata* to sweet orange (*Citrus sinensis*) was reported previously [34]. Herein, the inversely case is that, the dodder-transmission

of "*Ca. Liberibacter*" from sweet orange to *M. paniculata* has already happened. This evidenced by the progressively developed symptoms on the infected plants well as being reacted positively with PCR test. Also, citrus *liberibacters* reported to be transmitted to periwinkle (*Catharanthus roseus*) by dodder (*Cuscuta campestris*) in the early 1980s [5] and tobacco (*Nicotiana tabacum* cv. Xanthi) in the early 1990s [6]. Recently, *Ca. L. asiaticus* was dodder-transmitted to tomato [7]. To our knowledge, this is the first report about the experimental dodder-transmission of "*Ca. Liberibacter*" to wild tobacco (*Nicotiana glauca*).

The results reported here confirm the presence of HLB in Egypt and further studies are undertaken to stand on its epidemiological aspects under Egyptian conditions.

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