Research article

Controlling Citrus Huanglongbing (HLB) for the Rehabilitation of Citrus Orchards in Cambodia

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Abstract Citrus Huanglongbing **(**HLB) has been seriously damaging Cambodian citrus industry in terms of major yield loss, poor fruit quality, and shortened average lifespan of citrus tree. HLB is mainly transmitted by vegetatively propagated citrus seedlings and spread by psyllid vector in the field. HLB pathogen is a non-cultivable bacterium "*Candidatus* Liberibacter asiaticus*"*. Major strains of HLB pathogen have been investigated and the most virulent strain Type-II was commonly observed in Pursat sweet orange, mandarin and pummelo trees in Cambodia. Currently, there are no promising technologies and cultural practices to control HLB in citrus orchards in Cambodia. New health management practices of citrus HLB have been initiated in 2006 under the expert guidance of the National Taiwan University (NTU) and the Food and Fertilizer Technology Center in the Asian and Pacific Region (FFTC/ASPAC). Pathogen-free (PF) seedlings propagated in screen houses of the Royal University of Agriculture were transplanted in the demonstration orchard of Battambang and Takeo Provinces. The supply of the PF-seedlings of major citrus species cannot currently catch up with the demand of citrus growers. According to a preliminary survey, marcotted or grafted seedlings of sweet orange propagated by farmers transplanted in major citrus production areas such as Battambang, Pursat and Siem Reap Provinces were seriously infected by HLB. Up to now, fundamental knowledge and techniques such as identification of major strains of the HLB pathogen and establishment of disease indexing laboratory and pathogen-free nursery system have been conducted. In addition, a variety of relevant techniques and cultural practices to control HLB were transferred to Cambodia, such as technologies for the production of PF-seedlings and transplanting to demonstration farms for their health management, and so forth.

Keywords huanglongbing (HLB), health management, citrus production, pathogen-free citrus seedling

INTRODUCTION

Citrus greening was first reported in 1947 from South Africa, although a similar disease known as "Huanlongbing" (HLB, yellow shoot) was already known in 1943 in Southern China (Cheng, 1943). The HLB disease, locally called "Likubin," was first identified in Taiwan in 1951, six years after the end of World War II. The HLB inoculums might have been intentionally brought into Taiwan from southern China through some infected propagation materials such as citrus scions or seedlings. HLB was first considered as a kind of physiological disorder caused by nutrient deficiency, poor drainage, and so forth. However, HLB could not be controlled by the amendment of soil properties, and has spread rapidly all over Taiwan. Matsumoto and his coworkers initiated etiological studies on HLB in 1956, and they successfully demonstrated in 1961 that the so-called HLB was not a physiological disorder of citrus tree, but was caused by a virus-like microbe transmitted through grafting of diseased scion, and named it Likubin (decline) (Matsumoto, Wang and Su, 1961). This

destructive disease spread all over Southeast Asia during the 1960s, and was locally called leaf mottle yellows, citrus vein phloem degeneration (CVPD), and citrus dieback in Philippines, Indonesia, and India, respectively. In a short period of time, HLB became one of the most devastating diseases for citrus growing areas in tropical and subtropical Asia. Miyakawa and Tsuno (1989) first found HLB in Iriomote, the southernmost island of Okinawa, Japan in 1988. Afterwards, HLB was reported in Brazil (Lopes, 2006) and Florida (Bove, 2006) in 2004 and 2005, respectively. Etiological and epidemiological studies on HLB have been conducted in Taiwan in order to develop effective and efficient management strategy of HLB since 1955. HLB commonly occurs as a mixed infection with citrus tristeza and/or tatter leaf viruses, causing severe yellow mottling and tree decline, and ultimately death of citrus trees. These diseases are generally controlled by integrated control measures. Establishment of a pathogen-free nursery system is of primary importance for reducing prevalence of these diseases in the early stage of tree development. Combination of shoot-tip micrografting (STG) technique and heat-treatment has been successful in establishing pathogen-free foundation stock of citrus cultivars. Along with the said techniques, a precise and rapid indexing technique is indispensable for health management of production and cultivation of pathogen-free citrus seedlings.

Up to present, pathogen-free citrus foundation, nursery system, and disease indexing laboratory were established in RUA through the international collaboration project among RUA, NTU and FFTC/RDF. The on-going project aims to conduct etiological and epidemiological studies of HLB, and technology transfer of health management of PF-seedlings in the demonstration farms established in major citrus production areas in Cambodia.

CITRUS PRODUCTION IN CAMBODIA

Pursat sweet orange seemed to be introduced to Cambodia in Angkor period from China. Sweet orange has been grown only in the surrounding area of houses. After the French occupation in 1954, sweet orange cultivation has been spread nationwide, thanks to technical training on modern citrus cultivation for farmers.

In Cambodia, fruit crop production is the 2nd major crop next to rice production. Within fruit crops, citrus is the second important fruit crop next to mango according to the statistic acreage of fruit crops reported by MAFF, i. e. Areas (ha) of fruit crop, 2009: Mango, 23,734; Orange, 3,553; Custard apple, 3,213; Longan, 2,376; Sapodilla, 2,052; Guava, 1,745 and Milk fruit, 1,216 has. Citrus cultivation provides year-round harvest with suitable income for increasing rural economy from 1954 to 1967 before the civil war. The total growing areas of citrus was 3976 ha (Battambang, 2391 ha; Pursat, 345 ha; Kandal, 246 ha; Kampong Cham, 331ha; and other provinces, 663 ha). During 1968-1969, around 18000 tons of citrus fruits were exported abroad. In 1987 Cambodia was able to produce 40000 tons of citrus fruits.

In recent years, areas of citrus production decreased significantly due to HLB occurrence, i.e. 2556 ha in 2007 (Table 1) decreasing from 3976 ha in 1967. Battambang province holds the highest growing area at 1865 ha, decreasing from 2391 ha in 1967, while Kampong Cham still holds 311 ha without decrease, coming to the second. Citrus areas in Pursat province decreased drastically from 345 ha in 1967 down to 59 ha in 2007 due to HLB epidemic.

Table 1 Production areas of citrus in Cambodia (2007)

Province	Area (ha)
Siem Reap	145
Battambang	1865
Pursat	59
Kampong Thom	74
Kampong Cham	311
Kampot	102
Total	2556

Source: Provincial Agriculture Department of Battambang.

The major citrus species cultivated in Cambodia are sweet orange, mandarin, pummelo, lime and Kaffir lime (Martin, 1971). Pursat sweet orange has been most commonly cultivated. The HLB disease has commonly affected all cultivars.

HLB SITUATION

Citrus trees grown in Cambodia have been affected by many different diseases which are causing considerable damage to citrus production. The most serious threat has been caused by Huanglongbing (HLB) which is known as " Slek Prak " in Khmer language. At present there is not enough technical disease control or prevention capacity for this kind of disease. The HLB disease has been commonly occurring in citrus-growing areas in Cambodia since 1980s. The common symptoms caused by HLB pathogen include yellowing of the veins and adjacent tissue, followed by yellowing with pale-green mottling of entire leaf. With ageing, the diseased leaves become hard, curling outwards and occasionally develop vein corking. The typical symptoms produced on different citrus cultivars e. g., Pursat sweet orange, mandarin and pummelo, and common root-stock cultivar, Rangpur lime, are shown in Fig. 1. However, the symptoms vary with citrus variety, strains of the pathogen, and environmental conditions.

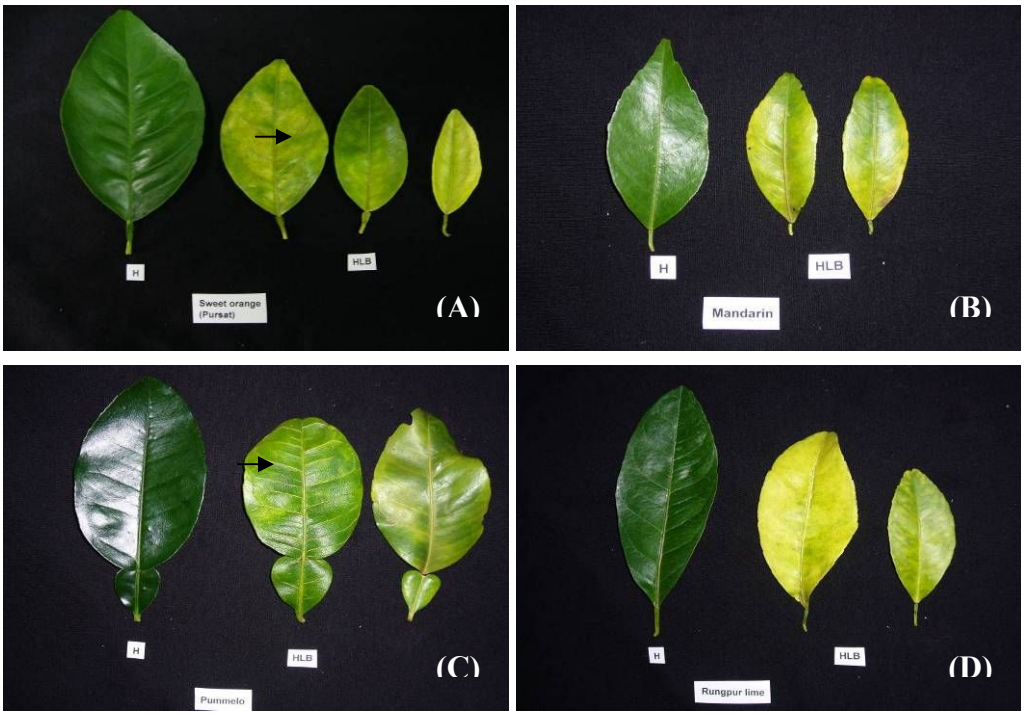

Fig. 1 Symptom expression of HLB in common citrus cultivars, and root-stock in Cambodia

(A) Leaf symptoms of HLB-affected Pursat sweet orange, showing yellowing with pale green mottling of mature leaves (second from left) with vein corking (→) and a newly grown small and slender leaf with yellow chlorosis (rightmost), and one healthy leaf (leftmost); (B) Leaf symptoms of diseased mandarin, showing yellow mottling of mature leaves (right and center) with curling; (C) Leaf symptoms of diseased pummelo, showing yellow mottling on mature leaves (right and center) with curling and severe vein corking (→) on the center leaf; (D) Leaf symptoms of diseased Rangpur leaf (right and center) showing yellow mottling, and a healthy leaf on the left.

The pathogen of the HLB disease in the above-mentioned major citrus cultivars and Rangpur root-stock were detected by polymerase chain reaction **(**PCR) test followed by electrophoresis analysis with primer pairs specific to HLB pathogen (F: CAC CGA AGA TAT GGA CAA CA; R: GAG GTT CTT GTG GTT TTT CTG). The protocol of HLB detection was described in detail in the former publications (Hung *et al.*, 1999; Su, 2008). HLB-infected leaves with typical yellow mottling symptoms, and those without any symptoms were collected from Pursat sweet orange, mandarin, pummelo and Rangpur lime trees for the infected and the control, respectively (Fig. 2A).

The entire leaf samples were subjected to PCR detection of HLB pathogen. Figure 2B presents the PCR pattern of citrus samples tested. The PCR band corresponding to the HLB pathogen (228 bp) could be observed in all the infected leaf samples while all the healthy leaves showed no band at 228 bp. This is the first report to scientifically prove that the citrus HLB disease is commonly and widely distributed in Cambodia.

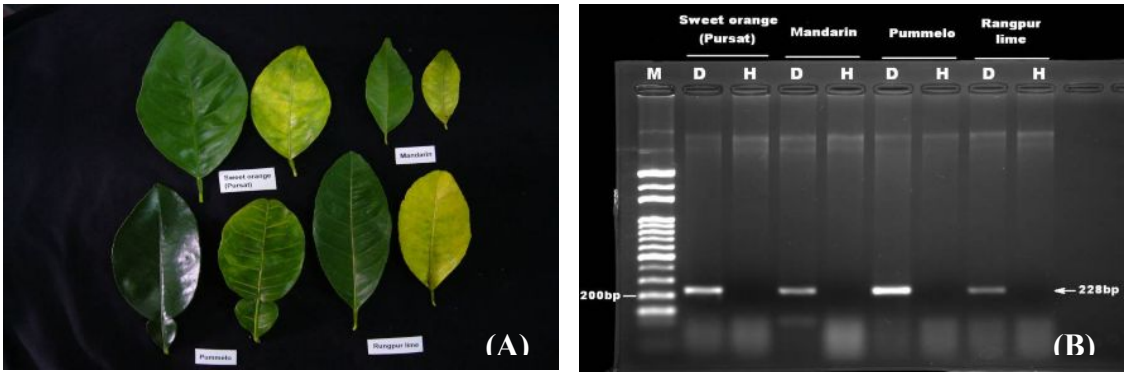

Fig. 2 Detection of HLB pathogen by polymerase chain reaction followed with electrophoresis analysis in diseased leaves of Pursat sweet orange, mandarin, pummelo and Rangpur lime each with a healthy leaf as check

(A). Photograph of PCR-electrophoresis gel showing positive detection of PCR products (228bp) (←) in each diseased leaf and negative detection with each healthy leaf (B). M, markers of molecular ladder. D, Diseased leaf and H, healthy leaf.

The high frequency of HLB is chiefly due to apparent lack of knowledge on HLB infection mechanism namely, insufficient elimination of infected citrus trees and transplanting HLB-infected seedlings. In Cambodia, citrus seedlings are commonly produced in terms of vegetative propagation by marcotting or grafting using buds taken from HLB infected trees in the open nurseries. Cambodia suffers from a serious epidemic of HLB all over major citrus orchards nationwide. Citrus growers, in principle, use uncertified citrus seedlings for their orchards, which are already infected with HLB pathogen, and the HLB symptom appears within 2 or 3 years after transplanting.

STRAIN IDENTIFICATION OF HLB PATHOGEN

The strain of HLB pathogen was precisely examined and four strains have been reported in Taiwan (Tsai *et al.*, 2008). The HLB strains were identified by bioassay and PCR analysis with four differential citrus cultivars of mandarin, sweet orange, pummel and Eureka lemon. According to the pathogenicity and virulence of the pathogen isolates, the four strains were categorized as follows: Strain I infected mandarin and sweet orange only and caused severe symptoms with high titer of pathogen multiplication; Strain II infected all four cultivars and induced severe symptoms with high pathogen titer; Strain III infected mandarin and sweet orange and induced moderate symptom with moderate pathogen titer, and infected pummel by causing mild symptoms with low titer of pathogen; Strain IV infected mandarin and sweet orange only without symptom expression.

In order to clarify the strains of HLB pathogen in Cambodia, the HLB diseased samples were collected from the suspected trees of major cultivars including Pursat sweet orange, mandarin, pummelo, Mexican lime and Rangpur lime grown in the provinces of Pursat, Battambang, Siem Reap, Koh Kong, Sihanouk ville and Kampong Speu during 2005 to 2008. The all citrus samples were subjected to the HLBB detection with PCR test, and the positive samples were selected to be the HLB pathogen isolates. The strains of the HLBB isolates were identified by bioassay of each isolate with the four differential cultivars.

The strain evolution of Cambodian isolates of HLB pathogen was investigated in RUA through international collaboration, and the results are summarized in Table 2. About 16.7% of

citrus samples (351) were found to be infected with HLB pathogen by PCR analysis. The positive diseased samples were subjected to bioassay of strain identification. Strain II could generally infect all the cultivars tested, namely, Pursat sweet orange, mandarin, pummelo, Mexican lime, Rangpur lime and Bargamot bringing about severe symptoms with high pathogen titer. Strain II (93%) was detected in all the citrus-growing areas, and showed its dominance over other strains. Strain I (3.5%) was scarcely detected in Pursat sweet orange grown in Battambang area. Strain III (3.5%) was identified from mandarin tree in Pursat province and wild lime in Siem Reap. A virulent strain IV has not been detected in Cambodia. It was the first time that HLB infection was detected in wild lime, and this means the wild lime is a new alternative host of HLBB (Hung *et al.*, 2001).

Table 2 Strain identification of HLB pathogen in Cambodia

Locality/cultivar	Number of test trees	Number of positive trees	HLBB strains			
			Ⅰ	Ⅱ	Ⅲ	Ⅳ
Ⅰ. Pursat province						
Pursat sweet orange	108	10		10		
Mandarin	19	2		1	1	
Pummelo	14	1		1		
Ⅱ. Battambang province						
Pursat sweet orange	95	9	2	7		
Pummelo	20	3		3		
Rangpur lime	10	1		1		
Ⅲ. Siem Reap province						
Pursat sweet orange	20	7		7		
Mandarin	10	5		5		
Pummelo	7	1		1		
Mexican lime	1	1		1		
Bergramot	3	2		2		
Wild lime*	1	1			1	
Ⅳ. Koh Kong province						
Pursat sweet orange	30	9		9		
Rangpur lime	1	1		1		
Ⅴ. Sihanouk ville province						
Pummelo	9	3		3		
Ⅵ. Kampong Speu province						
Pummelo	3	1		1		
Total number	351	57	2	53	2	0
Percentage (%)		(16.7%)	3.5%	93%	3.5%	0%

**Atalantia citroides, alternative host*

DESEASE CONTROL STRATEGIES

The integrated disease management, which includes propagation and cultivation of PF-seedlings, elimination of inoculum sources, and prevention of secondary infection by vector insects, is highly recommended to control the HLB disease. Establishment of PF- citrus nursery system is primarily important to prevent the disease prevalence (Su, 2008). An insect-proof screen house built in RUA has been utilized as a repository of PF citrus foundation stocks obtained through shoot-tip micrograft technique (Fig. 3). The first author, Mr. Setha, has been in charge of the PF citrus foundation program. Production of PF seedlings has been promoted by using the PF scion/bud derived from the foundation trees in RUA. Field demonstration of PF citrus seedlings which includes Pursat sweet orange, mandarin and pummelo has been conducted by Dr. Bunthan of RUA Rector in Battambang and Takae provinces. The PF citrus seedlings grow vigorously and started to bear fruits luxuriantly within 2 years after transplanting.

A healthy citrus orchard planted with PF seedlings may outlive the grower. Healthy citrus has a great potential for sustainable high yield over many decades, provided appropriate horticultural and disease management practices are followed. Accordingly, production and cultivation of PF and high-quality nursery trees are fundamentally important components of HLB management. In a screen house nursery, effective preventive measures to control disease caused by Phytophthora spp., nematodes, and bacterial canker disease have to be practiced. Budwood increase blocks are established ahead of the production of PF citrus seedlings. The blocks contain certified parent plants propagated by using budwood from foundation trees and maintained in screen houses. These trees must be reindexed periodically, and used for the bud-supply up to 3-5 years to avoid reinfection and mutations on the propagated saplings. New budwood increase blocks must be periodically established with clean buds from foundation trees. The PF citrus rootstock seedlings are produced in screen houses well-constructed with 30 mesh screen and double doors (Su, 2008).

Citrus orchards transplanted with PF-seedlings have been vigorously grown, resulting in good fruit-setting. The PF citrus trees can be fruiting as early as 2 years as long as appropriate health management and cultural practices such as watering, fertilization, and pruning are provided after the orchard establishment. Health management of pathogen-free citrus seedlings in orchards needs to be properly performed using the following strategies: 1) Prompt elimination of HLB-diseased citrus trees and alternative host plants as inoculums sources to prevent spread of HLB to adjacent healthy citrus trees, 2) Protection of pathogen-free trees from vector transmission of the HLB pathogen by effectively spraying insecticides at critical sprouting periods, and with IPM strategy such as natural enemies 3) Providing the orchards with physical barriers for avoiding the vector invasion and giving natural enemies the habitants.

Fig. 3 Pathogen-free citrus foundation stock kept in the insect-proof screen-house repository in RUA

The author (L) stood nearly the second author (R), inspecting PF foundation plants

CONCLUSION AND FUTURE PERSPECTIVES

Citrus HLB disease caused by uncultivable pathogenic bacteria was observed in most of the common citrus cultivars of Cambodia. HLB is mainly transmitted to citrus seedling by vegetative propagation and spread by vector psyllid in the field in the same manner as in other Asian countries. HLB has become a serious epidemic in many citrus growing areas in Cambodia. The integrated control measures such as elimination of inoculum sources, cultivation of PF seedling, and control of vector-insect by insecticide spraying, are generally applied for combating the disease.

Production and health management of PF seedling are primarily important for rehabilitating citrus industry. Pathogen indexing technique is indispensable for HLB management. The above mentioned facilities and technology have been already established and transferred to the Royal University of Agriculture, which is able to enhance production and healthy management of PF seedling to meet increased farmers' demand in the future. The techniques of health management have to be improved and readjusted to local conditions through more intensive epidemiological studies and well applied to protect PF trees from re-infestation of HLB and others virus diseases effectively.

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