An Integrated Management of Citrus Huanglongbing in Taiwan

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ABSTRACT

Citrus Huanglongbing (HLB) was first found in China in 1943. This psyllid-borne disease caused by *Candidatus* Liberibacter asiaticus has been devastating the citrus industry in Taiwan since 1951. In order to formulate adequate control measures of HLB, etiological and epidemiological studies on HLB have been made thenceforth. So far four strains of HLB were identified in Taiwan. The HLB disease, commonly mix-infected with citrus tristeza and/or tatter leaf viruses, causes severe yellow mottling and decline. These systemic diseases are generally controlled by the integrated management measures including cultivation of pathogen-free seedlings, elimination of inoculum sources, and management of secondary spread by vector insects. The pathogen-free nursery system has been established since 1983 in Taiwan, which is primarily important for preventing HLB and virus diseases. The pathogen-free nursery has been properly managed through improved techniques of shoot-tip micrografting for obtaining HLB/virus-free citrus budwoods. The molecular diagnostic probes and polymerase chain reaction (PCR） were developed for indexing HLB trees and vectors. The ELISA, rapid diagnostic strips, RT-PCR techniques were also made for citrus virus diseases. The transmission of psyllid was studied for management of HLB spread. In chemotherapy, three applications of 1000 ppm tetracycline could reduce disease index of HLB in field trails.

Keywords: Huanglongbing, Greening, HLB management, Pathogen-free citrus seedling

INTRODUCTION

Citrus greening was first reported in 1947 from South Africa, while a similar disease known as Huanglongbing（HLB）was already found in 1943 from South China. HLB disease, locally called Likubin, was first found in Taiwan in 1951. This destructive

virus-like disease was spread throughout Southeast Asia including Philippines, Indonesia, and India, during 1960s. Eventually this disease spread to tropical and subtropical climate region such as Japan[13,15], Brazil[10], and Florida [1]. While the South African HLB organism（*Candidatus* Liberibacter africanus）is heat sensitive form and only causes severe symptoms in cool temperature climates (22-24℃）, the Asian HLB organism (*Candidatus* Liberibacter asiaticus） can cause serious symptoms in warm climates (27-32℃） and has been identified as a heat-tolerant form. The Asian form has caused greater devastation by shortening tree lifespan to less than 10 years and lowering fruit yield and quality in recent decades in tropical and subtropical Asian regions. For developing control measures, etiological and epidemiological studies on HLB have been conducted in Taiwan since 1956 up to present decade[4,6,7,8,9,11,18,19,21].

Moreover, HLB disease commonly co-infected citrus with citrus tristeza and/or tatter leaf viruses, and exocortis viroid, resulted in severe yellow mottling and tree decline, and ultimately death of citrus trees. This systemic infection is generally controlled by integrated control measures including production and cultivation of pathogen-free seedlings, elimination of inoculum sources, prevention of secondary spread by vector insects[2,3,16,17,19]. Establishment of a pathogen-free nursery system is primary measures for preventing prevalence of these diseases. The precise and rapid indexing techniques are indispensible for management of pathogen-free nursery system and health management of citrus trees in the field. Development and application of molecular diagnostic probes and polymerase chain reaction (PCR） for HLB include the followings; ELISA, rapid diagnostic strips and RT-PCR for CTV and CTLV [6,7,8,20]. In order to decrease the inoculum source and keep the old citrus trees continuously producing good quality fruit, chemotherapy was also studied to treat HLB-infected trees.

Symptoms

HLB symptoms in most susceptible mandarin cultivars are yellow mottling i.e., yellowing of the veins and adjacent tissue, followed by yellowing with pale-green mottling of entire leaf. With age, diseased leaves become harden, curling outwards, and occasionally develop vein corking. Leaf symptoms are followed by premature defoliation, dieback of branches, loss of fibrous roots and decline in vigor. In later stage, the diseased trees produce small and slender leaves along with symptoms of mineral deficiency. HLB-affected trees may become stunted, bear multiple off-season flowers, most of which fall off, and produce small irregularly shaped fruits with thick

peel of poor color-changing patterns (Fig. 1)[12].

Etiological studies

1. Causal organism

Electron microscope of HLB

The HLB bacteria causing Likubin was found in sieve tubes. Electron microscopy, using serial sections and three-dimensional assessments, confirmed the presence of various forms of HLB pathogen. The HLB bacteria were pleomorphic. The matured form of the pathogen was a rigid rod with dense cytoplasm, measuring 350-500 x 660-1500 nm in size, and surrounded by a two-layered envelop, 20-25 nm thick consisting of a cell wall and an inner cytoplasmic membrane (Fig. 2).

2. HLB strains

HLB first occurred in northern Taiwan in 1951. Previously the HLB pathogen infected main citrus cultivars in Taiwan such as Ponkan mandarins, Tankan tangors and Liucheng sweet oranges whereas it did not infect pummelos. However, HLB pathogen began to infect pummelos in 1970s implying that the evolution of pathogenicity occurred in recent decades. Four different HLB bacteria (HLBB） were identified based on the results of their pathogenicity and virulence on indicator plants. Strain I was only pathogenic on mandarins and sweet oranges by inducing typical HLB symptoms. Strain Ⅱ was pathogenic with high virulence on all differential cultivars with fast multiplication rates. Strain Ⅲ only caused intermediate symptoms on mandarins and sweet oranges, and mild symptoms on pummelos. Strain Ⅳ was a mild strain which could only infect but cause no symptoms on mandarin and sweet oranges (Fig. 3). Strain Ⅱ was prevalent in the field.

3. Diagnosis and indexing

HLB is tentatively diagnosed in the field by characteristic foliage and fruit symptoms described above. Further diagnosis requires indexing on susceptible mandarin or tangor, e.g. Ponkan and Tankan seedlings by graft-inoculation. Because of the low population and uneven distribution of HLBB within the plant, bioassay with an indicator plant requires numerous test plants and inoculum buds at least 2 buds/indicator plants, and is time-consuming[11]. HLB can be confirmed in the indicator plant by examining HLBB bodies in sieve tube of phloem section by electron microscopy[4].

The HLBB unevenly colonize the sieve tube of host plants at a low concentration. A highly sensitive and specific DNA probe, developed with DNA cloning methods has been used to detect HLBB in infected citrus hosts[6]. One of the clones containing a 0.24-kb HLBB-specific DNA fragment was labeled with biotinylated nucleotides by a PCR-labeling technique. A dot hybridization assay with the biotin-labeled DNA probe was successfully used for detecting HLBB in various citrus hosts including mandarins, tangors, sweet oranges and pummelos. This probe could specifically react with all HLBB strains from several Asian countries including Malaysia, China, Thailand, Philippines and Okinawa, Japan, but not with those from South Africa (Fig. 4). The probe developed was specific and sensitive enough to detect low levels of HLBB infection. Highly specific primer pairs for HLBB detection have been developed through HLBB-DNA cloning and sequencing. The sensitivity of HLB detection has been enhanced by the development of polymerase chain reaction (PCR) with appropriate primer pairs followed by electrophoresis analysis. The PCR detection also reacted with the foreign HLBB isolates of the Asian form, including Okinawa, Japan, China, Malaysia, Vietnam, Thailand and Saudi Arabia (Fig. 5). The technique was successfully applied for detection and ecological study of HLB pathogen. PCR has been developed and used for the detection of HLBB in host plants and vector insects[6,9]. This technology is routinely applied to indexing citrus foundation stocks and pathogen-free seedlings[9,17]. DNA primer pair used for HLBB detection via PCR of HLBB is: Forward: CAC CGA AGA TAT GGA CAA CA; Reverse: GAG GTT CTT GTG GTT TTT CTG.

Epidemiology

1. Transmission

The systemic HLB disease is transmitted mainly via vegetative propagation of citrus seedlings, and spreads by Asian citrus psyllid (*Diaphorina citri*) in persistent manner[5]. The Taiwanese biotype of the psyllids is less efficient in pathogen transmission of HLB. Less than 5% of adults acquired HLB pathogen after acquisition feeding on diseased citrus plants over a one day period, while the nymphs acquired the pathogen at a much higher rate. Transovarial passage of the pathogen by psyllid was not demonstrated. Transmission of HLB via graft propagation with infected budwood, and marcotted (layering) seedling from infected trees also plays an important role in spreading HLB.

2. Alternative hosts

Four suitable hosts of the Asian psyllid, to be considered as possible alternative hosts of HLBB, were investigated by graft-inoculation test and psyllid transmission. The multiplication of HLBB in plants was monitored by PCR. The results demonstrated that HLBB can replicate and persist in Chinese box orange (*Severinia buxifolia*) and wild lime (*Atalantia citroides*) (Fig. 6.) from Cambodia, but not in common jasmine orange (*Murraya paniculata* var. *paniculata* and *M. exocortica*) and curry leaf (*Murraya koenigii*) in Malaysia. HLBB failed to replicate in common jasmine orange and curry leaf with negative detection by PCR. HLBB could be detected in woody apple (*Limonia acidissine*) from Thailand at the sixth to tenth month after graft-inoculation. Inoculated woody apple produced yellows symptom about 6 months after graft inoculation, but plants recovered to a healthy state after one year. Accordingly, woody apple is considered a transient host of HLBB. Chinese box orange is a good host of HLBB because HLBB replicates in it as well as in citrus. Yellow-mottling symptoms developed in Chinese box oranges which can be served as donor plants of psyllid transmission as well as Valencia plants can. However, HLBB has been detected in diseased samples of jasmine orange in Brazil[(10)].

3. Ecology

For controlling the spread of the disease, production and cultivation of pathogen-free citrus seedlings have been implemented since 1983. Once the healthy trees are established in the field, the control of HLBB infection of the orchards is accomplished by controlling the psyllids. Transmission of HLBB by citrus psyllid is related to disease prevalence[(11)]. Epidemics occur when vector population is high and a reservoir of the inoculum is present. Natural spread of the disease is greatest during periods of bud-sprouting that promote the greatest psyllid feeding, reproduction and transmission[(5)] (Fig. 7.). The transmission of HLBB by citrus psyllid is related to high vector population, incidence of HLBB infection of psyllids and dispersal of the psyllid. The epidemic infection of healthy citrus trees in an orchard near HLB-affected groves showed a sigmoid progress curve of disease incidence. With the latent period set at 1.5-month after infection, the highest infection rate occurred in late February to mid-March, when HLBB infection incidence of viruliferous psyllids and the dispersal of psyllids in the field reached its peak. This time period was critical for psyllid control with insecticide sprays. The disease incidence increased up to 70% within two years

without spray and rouging of diseased trees. A survey of HLB disease progress of infection rate (%) was conducted over a 5-year period from 1999 to 2004 in southern Taiwan. The cumulative rate of HLB infection reached 17% in the psyllid control plot and 57% in the unsprayed plot[5] (Fig. 8.). Adequate insecticides such as Dimethoate and Confidor have been applied.

Disease management

1. Pathogen-free citrus nursery system

HLB disease was documented as a major threat to economic viability of citrus production in the subtropics and tropics. Since the systemic infection of HLB and viruses diseases are transmitted primarily by vegetative propagation, and then rapidly spread by vector insects in the field, integrated disease management is highly recommended for its control. Integrated disease management includes propagation of pathogen-free citrus seedling, elimination of inoculum sources, and prevention of secondary infection by insect vectors. Establishment of a pathogen-free citrus nursery system (PFNS) is fundamental to prevention of the disease epidemic. In Taiwan, a technique of modified shoot-tip micrografting in combination with heat therapy is conducted for preparing the citrus pathogen-free foundation stock. Precise and rapid indexing techniques are indispensable for management of a pathogen-free nursery system. The PFNS in Taiwan consists of the following four steps; 1) shoot-tip micrografting (STG), 2) establishment of pathogen-free citrus foundation blocks, 3) pathogen-free nurseries and 4) issue of health certificate *via* indexing. The current scheme for the PFNS and bud-wood certification program in Taiwan was initiated in 1983 and has been operated under a joint program of National Taiwan University (NTU), Agricultural Research Institute (ARI), and Council of Agriculture (COA) of Taiwan.

(1) Shoot-tip micrografting (STG) for obtaining virus-free citrus foundation stocks

STG is the most reliable method to recover pathogen-free (PF) citrus propagation material from infected parental sources. The shoot tip or meristem of auxiliary buds of infected plants is generally free of virus and HLB, and plants regenerated from the shoot tips are usually free of the systemic pathogens. The common STG method[14] was greatly improved by exchanging the inverted T cut with newly developed triangle-hole cut method [19]. The entire procedure of STG is carried out in a laminar-flow hood under sterile conditions (Fig. 9). The procedure of modified STG is described in detail

in recent publication[17]. A double grafting technique has been developed to enhance the growth of STG plants. The procedure of this technique is briefly summarized as follows : 1) The well-developed micro-grafted plants about l and half months old are removed from the test tubes. The upper part of the plant serves as the scion tissue. 2) The scion is side-grafted to a healthy and well-grown rootstock seedling and the bud was wrapped with parafilm. The grafted plant is covered by a plastic bag, with zip unsealed, and the plant placed in a greenhouse. 3) The plastic bag is removed 3 to 4 weeks after grafting. After about three months, the new shoot will grow from the shoot-tip and the new shoot is ready for use as scion-wood for further propagation. 4) Indexing for HLB and viruses is done before the STG plant is used for further propagation. For increase multiplication of pathogen-free seedlings, scion wood is harvested from double grafted STG-plants at 3 to 4 month intervals.

(2) Pathogen-free（PF） citrus foundation block

The STG-seedlings that are indexed to be free of the citrus viruses and HLB can be used as the certified pathogen-free foundation stocks. The stocks are kept in the citrus foundation block repository (Fig. 10 A) which is an insect-proof screen house constructed with a double-door entrance and surrounded by a water canal to prevent entry of ants and mites (Fig. 10 B&C). The screen house is installed with an air curtain over the first door. Concrete benches are set on gravel floor for maintaining the PF foundation trees in containers 30 cm above the floor surface (Fig. 10 D). The foundation trees are generally propagated on Troyer or Carrizo citrange from the clean STG plants. Two to four plants are maintained per cultivar, and they are pruned every year to produce a few fruits for verification of horticultural characteristics and removal of off-types. The plants are indexed periodically and inspected for fruit abnormality. The protected foundation blocks are maintained by public agencies. The national repository of pathogen-free foundation blocks is located in Chia-Yi Agricultural Experimental Station of Taiwan Agricultural Research Institute (CAES/ TARI). The citrus foundation repository is located within a rice-paddy field of CAEA/TARI.

(3) Production of pathogen-free citrus seedling

A healthy citrus orchard planted with pathogen-free seedling may outlive the grower. Healthy citrus trees have a great potential for sustainable high yield over many decades, provided appropriate horticultural and disease management practices are

followed. Accordingly, production and cultivation of pathogen-free and high-quality nursery trees are fundamentally important component of HLB management. In a screen-house nursery, effective preventive measures to control diseases caused by *Phytophthora* spp., nematodes, and bacterial canker disease have to be practiced. Budwood-propagating 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. Only a limited number of foundation trees are used for producing the budwood-propagating trees because of the stringent indexing for vector-transmitted pathogen and inspection of off-type mutation. These trees must be reindexed periodically, and used for the bud-supply up to three for avoiding re-infection and mutations during the propagated saplings. New budwood-propagating 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. Rootstock seedlings are grown from seeds of selected cultivars in seedling tubes (5 cm in diameter and 18 cm in height) or in a seedbed tank of soil containing sterile potmixture. The rootstock seedlings are transplanted to perforated plastic containers (10 cm in diameter and 30 cm in height) for further cultivation when they grow to more than 40 cm high. A rootstock cultivar should be selected in terms of the compatibility with targeted scion-cultivar, i.e., Sunki and Cleopatra mandarin for mandarin cultivars; Troyer and Carrizo citrange for sweet orange and mandarin; Swingle citrumelo and pummelo for pummelo; and Volkamer lemon for mandarin and sweet orange. The PF seedling are propagated by bud- or side grafting with wood curving knife and parafilm using bud/scions from PF budwood--propagating trees. The production of PF seedlings is accelerated by rapid propagation technique described in detail in a recent publication [17].

2. Health management of pathogen-free trees in the orchard

Rehabilitation of citrus orchards planted with PF seedlings has been showing vigorous growth and good fruit-setting. The PF citrus trees may begin fruiting as early as 2 years after orchard establishment provided appropriate health management and cultural practices such as watering, fertilization, and pruning are followed. 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 serving as inoculum sources to prevent spread of

HLB to adjacent healthy citrus trees, 2) Protection of pathogen-free trees from vector transmission by effectively spraying insecticides at critical sprouting periods, and with biological control of the vector using natural enemies including *Tamarixia radiate* introduced from Reunion by Dr. Bernard Aubert. 3) Protecting the orchards with physical barriers such as wind breaks or distance barriers. 4) Chemotherapy of HLB-infected citrus trees: Tetracycline (Achromycin) injection has been utilized by some citrus growers using the method formulated by Su and Chang in 1976[18]. Recently, the efficacy of the antibiotic injection has been improved by using an air-pressured injector (Fig. 11). Three applications (2 autumn, 1 spring) of 1,000 ppm Achromycin (2~4 L per tree) by air-pressured injector with 80-lbs pressure which improved the injection speed and had good recovery efficacy of diseased trees. The repeatedly injected trees recover well and produce normal fruits. Pruning dead branches in the upper canopy improves the therapeutic efficacy. The antibiotic injection is frequently associated with temporary phytotoxicity such as mild vein necrosis, slender leaves and defoliation, but the trees quickly recover to normal growth. The tree injection method is recommended to treat the diseased trees with diseased twigs less than half of canopy in early stage of disease development.

CONCLUSION

The Asian form of HLB has been evolving into strains with different virulence and consequently spreading over citrus-growing areas worldwide, and devastating citrus industry. The systemic disease can be well controlled by the integrated management including cultivation of PF seedlings, elimination of inoculums sources and prevention of secondary spread by vector psyllid. Establishment of pathogen-free nursery system for production and cultivation of PF seedlings is primary important for citrus rehabilitation from HLB and virus invasion. The HLB has been under control in large acreage of 200,000 hectares through prompt removal of diseased trees and adequate insecticide application in region-wide orchards in Brazil by March 2012. So far, the complete HLB management has not been well established in Asia. However, the management strategy and techniques are expected to be improved through further international collaboration in epidemiological study and disease management.

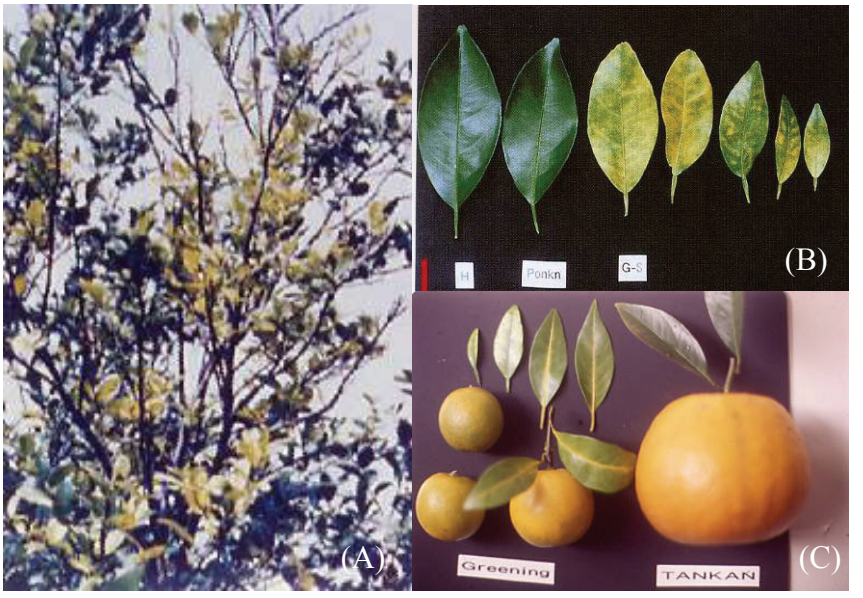


Fig. 1. Symptom expression of HLB in mandarin trees. **(A)** The diseased branch and twigs of ponkan mandarin showing yellow mottling leaves and die back. **(B)** Leaf symptoms of Ponkan leaves, showing yellow mottling of mature leaves and zinc deficiency of younger leaves（five leaves from the right）. Two leaves from the left were healthy. **(C)** HLB-disease Tankan fruits showing atrophy with greening (L) and H-ck, normal large fruit (R).

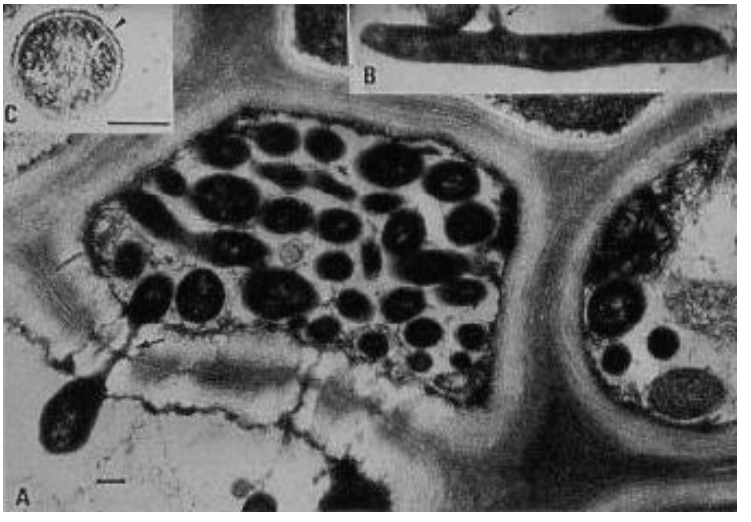


Fig. 2. Electron micrograph of citrus Huanglongbing bacteria (HLBB). A. HLBB packed in sieve tube of Ponkan mandarin. B. The rod HLBB with side-budding. C. HLBB consisted of a cell wall and inner cytoplasmic membrane from a cross-sectioned cell [(5)].

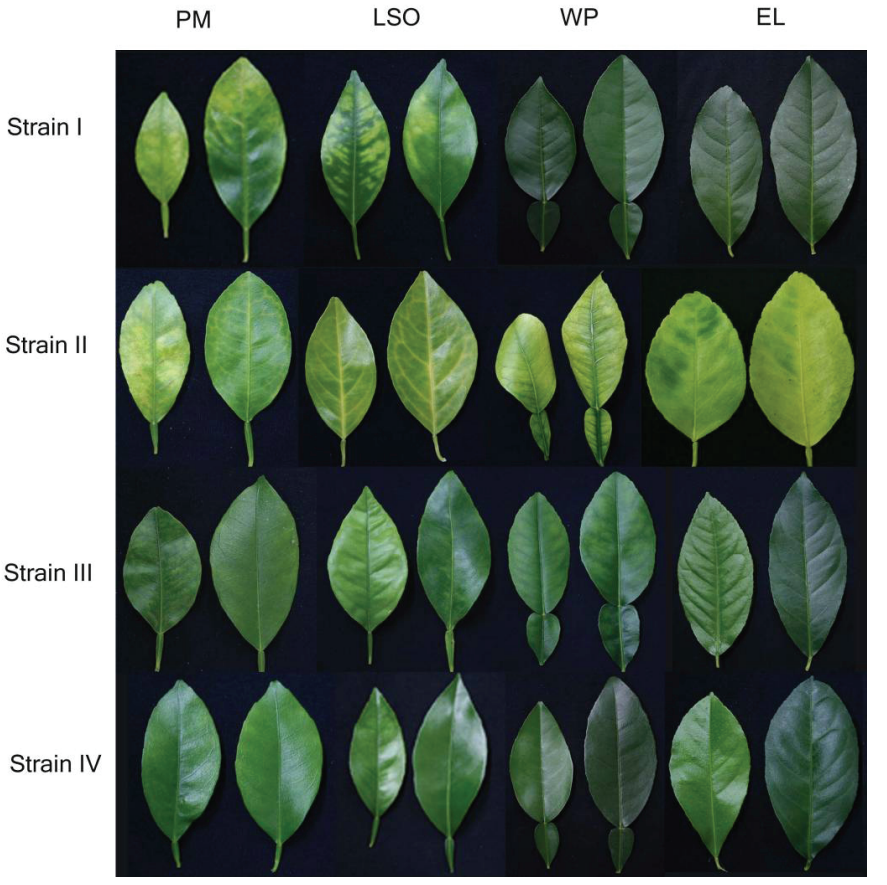
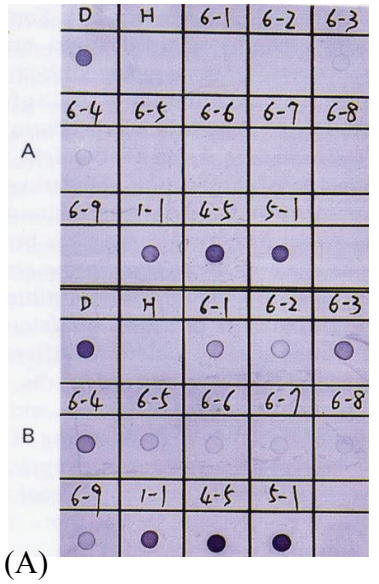
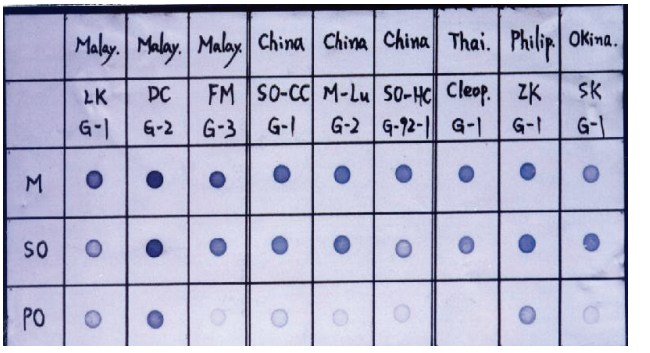


Fig. 3. Different types and severities of the symptoms induced by the 4 HLBB strains (Ⅰ~Ⅳ) in 4 differential citrus cultivars including Ponkan mandarin (PM), Liucheng sweet orange (LSO), Wentan pummelo (WP) and Eureka lemon (EL) under greenhouse conditions from 2005-2006.



(A)



(B)

Fig. 4. Detection of HLBB by dot hybridization tests, with HLBB DNA extract (A-A), and by PCR amplification of HLBB DNA followed by DH with PCR products (A-B). D, diseased sample; H, healthy sample from shoot-tip-grafted ponkan used as negative control ; 6-1~6-9, healthy-looking citrus samples collected from field; 1-1, 4-5, 5-1, citrus samples with HLB symptoms.(B) Detection of HLBB by dot hybridization including Malaysia (Duncy and Fremont mandarin), China (Chanychou, Lukan mandarin,Honchian sweet orange), Thailand (Cleopatra), Philippine (zinkom mandarin), and Okinawa Japan (Shikuashia) isolates.

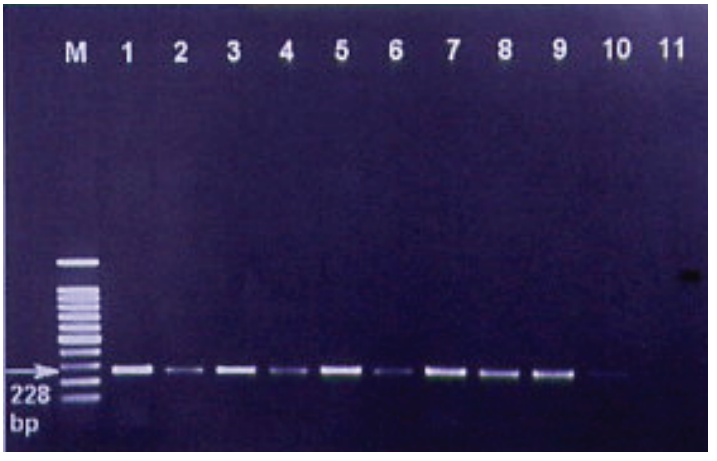


Fig. 5. Detection of HLB pathogen by polymerase chain reaction (PCR) followed with electrophoresis analysis: 1~4, Taiwan HLBB isolates; 5, Okinawa isolate, Japan; 6, Chain isolate; 7, Malaysia isolate; 8, Vietnam isolate; 9, Thailand isolate; 10, Saudi Arabia isolate; 11, Healthy CK.

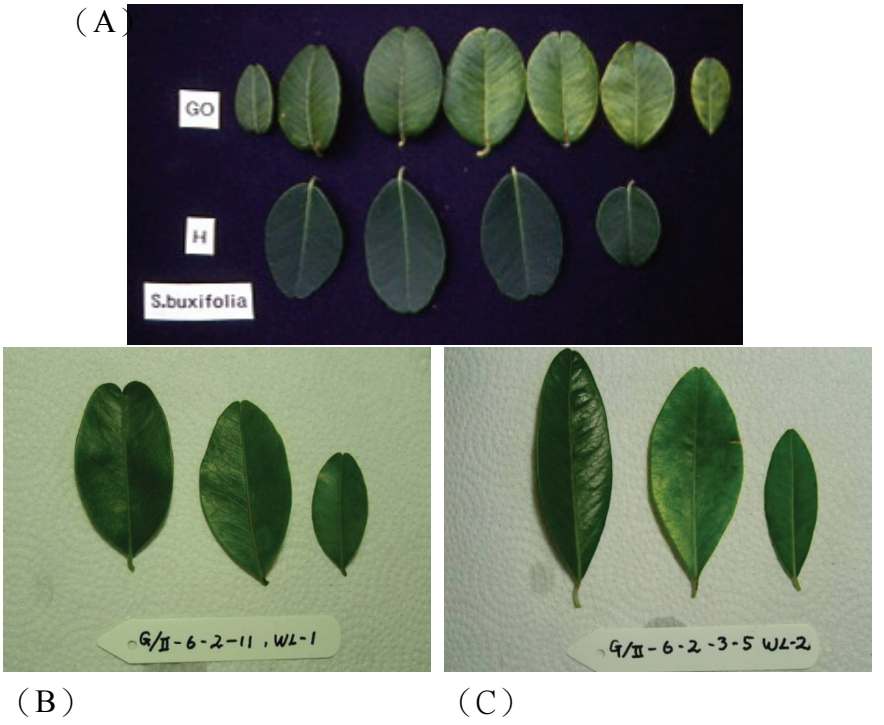


Fig. 6. **(A)** Leaves of Chinese box orange (*Severinia buxifolia*), alternative host of HLB. GO: HLB-diseased leaves showing yellow-mottling symptoms; H: healthy leaves (lower). **(B & C)** Leaf symptom on wild lime (*Atalantia* (=*Severinia*) *citroides*) infected by HLBB.

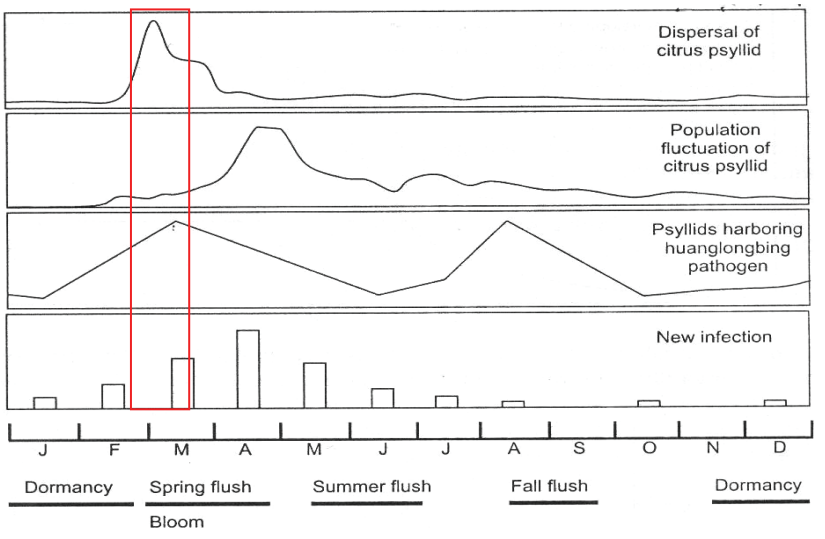


Fig. 7. Phenological patterns, population dynamic and HLBB infection of citrus psyllid in orange orchards of Southern Taiwan [6].

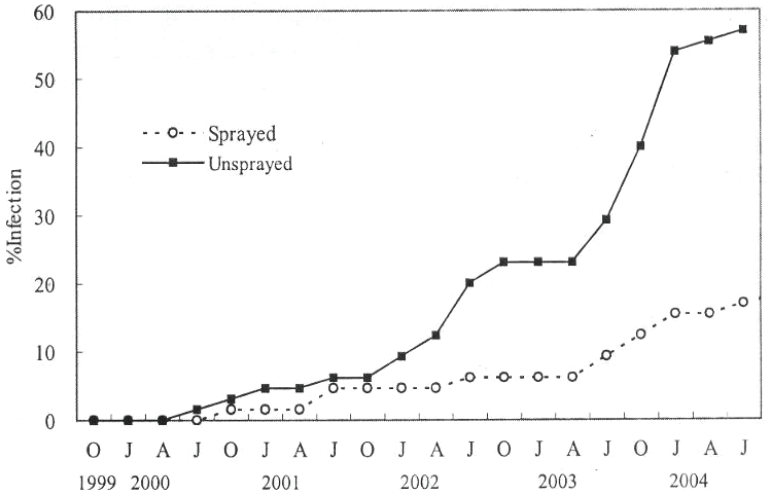


Fig. 8. HLB progresses of infection rate (%) in sprayed and unsprayed plots during a 5year period. The infection cumulative rate of HLBB infection reached 17% in sprayed plot and 57% in unsprayed plots [6].

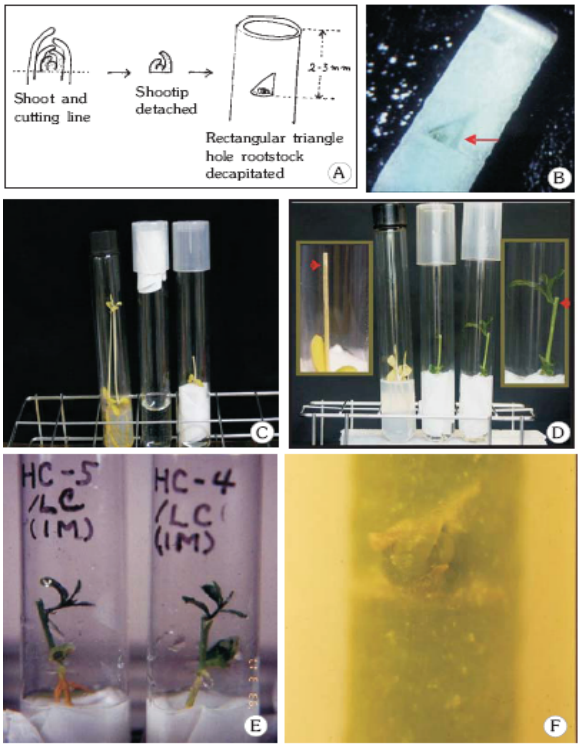


Fig. 9. Procedure for shoot tip (ST) micrografting. (A) Diagram showing excision of shoot tip with 2-leaf primodia from top of a shoot and making a rectangular triangle hole (0.3~ 0.5 mm) on a decapitated rootstock seedling by removing cortex layer with cutting edge of STG knife. (B) A shoot tip piece placed within the triangular hole of a decapitated rootstock seedling. (C) Two-week old rootstock seedlings on solid medium (left), a sterile test tube with a center-perforated filter-paper platform, containing liquid medium (center), and a test tube containing the micrografted rootstock seedling supported by filterpaper platform on liquid medium (right). (D) Different stages of STG rootstock seedlings, also showing a new sprout regenerated from the grafted shoot-tip (right). (E) Two new shoots of Hongjian sweet orange regenerated from ST in triangular hole (left) and V-shaped incision (right) of rootstock seedlings one month after micrografting. (F) A new sprout from ST in triangular hole on rootstock.

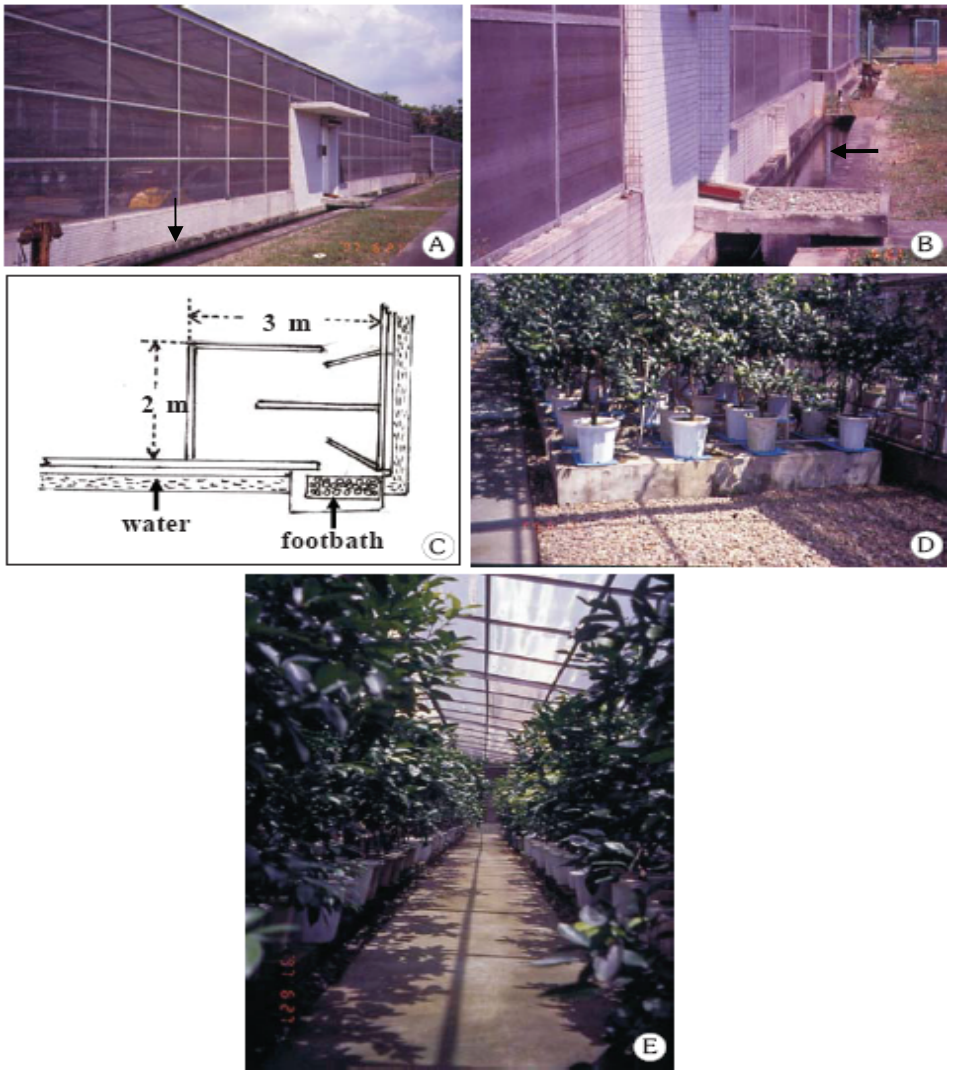


Fig.10. National repository for maintaining the certified pathogen-free citrus foundation blocks. (A) Insect-proof stainless screen-house surrounded by water canal (arrow head) and with double door entrance. (B) Close-up picture of double doors. (C) Drawing of double door, and footbath containing gravels mixed with copper sulfate solution. (D) Inside view of the screen house showing healthy foundation trees on concrete benches in gravel floor. (E) Certified PF budwood increase trees grown in a screen house attached to the foundation depository.



Fig. 11. Injection of antibiotic solution by an improved air-pressured plastic injector in the field.

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