Production and Cultivation of Virus-free Citrus Saplings for Citrus Rehabilitation in Taiwan

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PRODUCTION AND CULTIVATION OF VIRUS-FREE CITRUS SAPLINGS FOR CITRUS REHABILITATION IN TAIWAN

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Cover page: *Top row (left to right):* HLB-diseased Tankan fruits showing atrophy with green-coloring (left) and healthy normal fruit (right). Different stages of STG showing rootstock seedlings and new sprouts regenerated from the grafted shoot-tip.

Bottom row (left to right): Fixing a bud on graft site of rootstock with stretched band of parafilm. Acromycin treated HLB-affected pummelo tree showing normal growth.

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FOREWORD

Citrus is one of the most popular fruit crops of the world. It is cultivated in around 140 countries, including many in the Asia-Pacific. During recent years, there has been significant increase in the citrus production mainly on account of its increased use as nutritious and healthy drink.

Citrus is infected by more than 15 virus or virus-like diseases, including citrus greening, citrus tristeza, citrus tatter leaf and citrus exocortis, that severely affect its production as well as quality. In India, the Philippines, Thailand and Taiwan, the reduction in yield due to these diseases could range up to 60 percent, sometimes wiping out the whole orchard. Control of these diseases has become possible through the use of disease-free nursery seedlings, removing diseased trees, eliminating insect vectors and rejuvenating infected trees by chemotherapy. Use of pathogen-free planting material is of primary importance in raising healthy trees for which the technique of shoot-tip micrografting (STG) has proved very successful. Taiwan has made significant progress in rehabilitating citrus plantations using an integrated approach of STG-based disease-free seedling production and clean field maintenance. Wide dissemination of the STG technique, along with the complete technology for green-house propagation, disease indexing and prevention of secondary infection, can help tremendously the citrus growers in other countries of Asia-Pacific.

The Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB) was established in 2003, under the umbrella of Asia-Pacific Association of Agricultural Research Institutions (APAARI). It has been promoting appropriate use of emerging agri-technologies and tools in the region. One of the activities of APCoAB is to bring out reports on biotechnological tools and applications, both GM and non-GM, that have proved useful to the farmers and other stakeholders.

This technical bulletin entitled “Production and Cultivation of Virus-Free Citrus Saplings for Citrus Rehabilitation in Taiwan” is the fifth such publication in the current series. It provides detailed methods and protocols for the production of disease-free planting material in citrus, including disease indexing, production of virus-free nursery, cultivation and health management of saplings in orchards. APAARI is grateful to Dr. Hong-Ji Su, Professor at the National Taiwan University, for writing this

manuscript based on his vast experience and expertise. It is our expectation that this publication will equally be useful to all citrus growers, scientists and others engaged in citrus production and processing in the Asia-Pacific region.

(Dr. Raj Paroda)
Executive Secretary
APAARI

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I am grateful to Food and Fertilizer Technology Center, Rural Development Foundation and Council of Agriculture, Taiwan for their encouragement and financial supports which have made it possible for me to provide several years of technical assistance to a number of the Asian countries in establishing disease indexing laboratories and pathogen-free nursery system for citrus rehabilitation.

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Hong-Ji Su
Author

APCoAB acknowledges the help of Ms. Neha Gujaria, Research Fellow in editing the manuscript.

J.L. Karihaloo
Coordinator
APCoAB

CONTENTS

ACRONYMS AND ABBREVIATIONS

µl	Microliter
ARI	Agricultural Research Institute
CAES/TARI	Chia-Yi Agricultural Experimental Station of Taiwan Agricultural Research Institute
cDNA	Complementary DNA
CEVd	*Citrus exocortis viroid*
cm	Centimeter
COA	Council of Agriculture
CTAB	Cetyltrimethylammonium bromide
CTLV	*Citrus tatter leaf capillovirus*
CTV	*Citrus tristeza closterovirus*
DAS-ELISA	Double antibody sandwich-ELISA
ddH_2O	Double distilled water
DH	Dot hybridization
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FFTC	Food and Fertilizer Technology Center
g	Grams
HCl	Hydrochloric acid
HLB	Huanglongbing
HLBB	HLB bacteria
hr	Hour
IDM	Integrated disease management
IgG	Immunoglobulin G
KCl	Potassium chloride
KH_2PO_4	Dihydrogen potassium phosphate
M	Molar
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter

mM	Millimolar
mAb	Monoclonal antibody
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
Na-DIECA	Sodium N, N-diethyldithiocarbamate
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium trinitride
nm	Nanometer
NTU	National Taiwan University
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline Tween 20
PCR	Polymerase chain reaction
PF	Pathogen-free
pmol	Picomole
ppm	Parts per million
rpm	Revolutions per minute
RT-PCR	Reverse-transcription polymerase chain reaction
STG	Shoot-tip micrografting technique
TAE buffer	Tris acetate EDTA buffer
TE buffer	Tris-EDTA buffer
VF	Virus-free
VFNS	Virus-free nursery system
w/v	Weight/volume

1. INTRODUCTION

In Asia, citrus has been cultivated as one of the important fruit crops providing local people with valuable nutrition, and as an indispensable cash crop for both domestic consumption and export. The citrus fruit is consumed world wide as fresh fruit and processed product such as fruit juice. A variety of citrus species are cultivated in a wide range of climatic zones from temperate to subtropics, and further to tropics. In the past couple of decades, however, Asian citrus plantations have been suffering from a number of systemic virus and virus-like diseases, which have brought about serious yield loss and deterioration of fruit quality (Whiteside *et al.,* 1988). Thus, the disease has come to be one of the most serious constraints for the citrus industry in the region.

Citrus greening, a virus-like disease was first reported in 1947 in South Africa. A similar disease, locally known as Huanglongbing (HLB, yellow shoot) was reported in China as early as 1943. The HLB, locally called as Likubing, was first identified in Taiwan in 1951. HLB inocula were presumably brought into Taiwan from southern China immediately after World War II through some infected propagating materials, such as citrus seedlings. Psyllid-borne HLB has become epidemic and devastated Taiwan citrus industry since 1951 (Matsumoto *et al.,* 1961). This destructive disease has become prevalent in major citrus production areas of the subtropical and tropical Asia and Africa since 1960's.

HLB has kept on spreading worldwide, it was first identified in Iriomote Island, Okinawa, Japan in 1988 (Miyakawa and Tsuno, 1989), and by 2003 had spread north up to Tokunoshima Island, Kagoshima Pref., Japan. HLB was reported in Brazil in 2004 (Lopes *et al.,* 2005) and in Florida in 2005 (Bove, 2006).

Citrus tristeza (*Citrus tristeza closterovirus*, CTV) is assumed to have come from China a long time ago. It did not develop any prominent symptoms such as tree dwarfing and fruit atrophy in Taiwan or other countries of Asia. However, in 1920s CTV devastated the citrus industry in South America and Africa and has since rapidly spread world wide. A new strain, CTV-D, was first identified in Taiwan in 1981 which has seriously damaged pummelo industry causing tree dwarfing. The CTV virus was considered not to damage the pummelo badly under warm temperature in the tropics. However, there have been some reports of virulent strains attacking sweet orange and/or mandarin in South America and South East Asia. These strains cause

severe stem pitting and stunting, and result in deterioration of fruit yield and quality (Su and Tsai, 1991).

Citrus tatter leaf (*Citrus tatter leaf capillovirus*, CTLV) was first found in 1962, in Mayer lemon introduced to California from China. Later it was confirmed that several lines of Satsuma mandarin cultivated in Japan were infected by CTLV since 1975 (Miyakawa, 1980b). This virus may spread rapidly when trifoliate orange and its hybrid are used as rootstock of grafted seedlings. Fortunately, CTLV does not infect most citrus species in Taiwan.

Exocortis, caused by *Citrus exocortis viroid* (CEVd), is a disease with a common symptom of bark-scaling and tree-stunting. The disease was first reported as "scaly butt" in 1948 in Australia. CEVd occurs in many countries, and its infection becomes commercially important when the rootstock of trifoliate orange, its hybrid or Rangpur lime are used for the seedling multiplication. CEVs is scarcely found in sweet orange and Eureka lemon grafted onto Rangpur lemon in Taiwan.

The above mentioned systemic diseases are transmitted via vegetative propagation of seedlings through cuttings, using infected plants and bud-scions, and by insect transmission. These diseases can be well controlled by integrated management such as introduction of pathogen-free seedlings, elimination of inoculum sources, and prevention of secondary infection transmitted by vector insects. Establishment of pathogen-free nursery is of primary importance to prevent prevalence of the diseases (Su and Chen, 1991). Combination of shoot-tip micrografting (STG) technique and heat-treatment has been very successful in establishing virus-free foundation stock of citrus seedlings in Taiwan (Su and Chu, 1984). Along with, precise and rapid indexing techniques are indispensable for maintenance of pathogen-free nursery stock. Virus and virus-like diseases are, conventionally diagnosed by their of characteristic symptoms and bioassay with indicator plants. Recently, more rapid and precise molecular diagnosis methods such as monoclonal antibodies based enzyme linked immunosorbent assay (ELISA), DNA probes and polymerase chain reaction (PCR) based reverse-transcription PCR (RT-PCR) have been developed and added to indexing techniques (Su and Tsai, 1991; Hung *et al.,* 1999).

2. CAUSAL ORGANISMS AND EPIDEMIOLOGY

2.1. Citrus Huanglongbing

2.1.1. Causal agent

Fastidious bacteria (*Candidatus* Liberibacter asiaticus) causing HLB, is a submicroscopic walled prokaryote existing in plant sieve tubes, but unculturable on artificial medium. The fully matured form of the pathogen can be observed under electron microscope with its rigid rod shape measuring 350 nm-550 nm x 600 nm-1500 nm and surrounded by a two-layered envelope, 20 nm-25 nm in thickness (Fig. 1). The current dominant pathogen belongs to the heat-tolerant Asian form which can develop typical symptoms above 30 °C.

Most of the citrus species are susceptible to the Asian form the pathogen. Pummelo which was resistant has become susceptible to the Asian pathogen in Taiwan since 1971. Recently, susceptible pummelo trees have been found in the Philippines, Malaysia, Vietnam, southern China, Thailand and Cambodia. The Asian HLB bacteria (HLBB) isolates from mandarin trees induce severe greening symptoms in mandarin and sweet orange, but only mild symptoms in pummelo. The isolates collected from pummelo severely attack pummelo, but develop only slight symptoms in both mandarin and sweet orange. Some HLBB isolates from sweet orange and

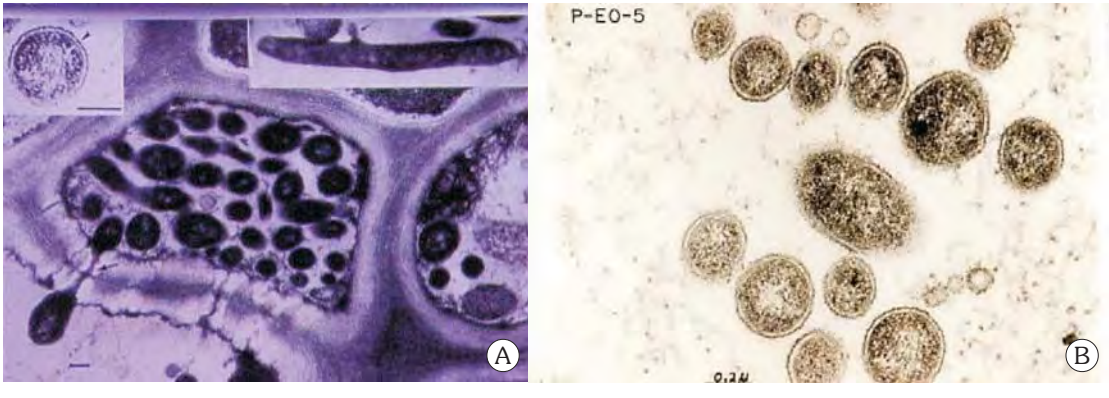

Fig. 1. Electron microscopy of HLB pathogen: Candidatus *Liberibacter asiaticus. (A) HLBB in sieve tube. (B) Cross-section of HLBB rod with double layered envelope comprising outer cell wall and inner cytoplasmic membrane.*

mandarin trees attack both mandarin and pummelo severely. These facts imply that the pathogenicity of HLBB strains has evolved in the past decades.

The Asian HLBB have been categorized into four different strains (Fig. 2):

Strain I: Shows pathogenicity to mandarins and sweet oranges in terms of induction of typical HLB symptoms;

Strain II: Shows pathogenicity and high virulence to all the citrus species tested, and can multiply rapidly in all the varieties/strains;

Strain III: Develops an intermediate symptom of HLB in mandarins and sweet oranges, and only mild symptoms in pummelo, but no infection is observed in Eureka lemons; and

Strain IV: A mild strain, can infect both mandarins and sweet oranges without any symptoms.

Strain II commonly infects all the citrus species in Taiwan, and totally dominates Strain III and I in the field.

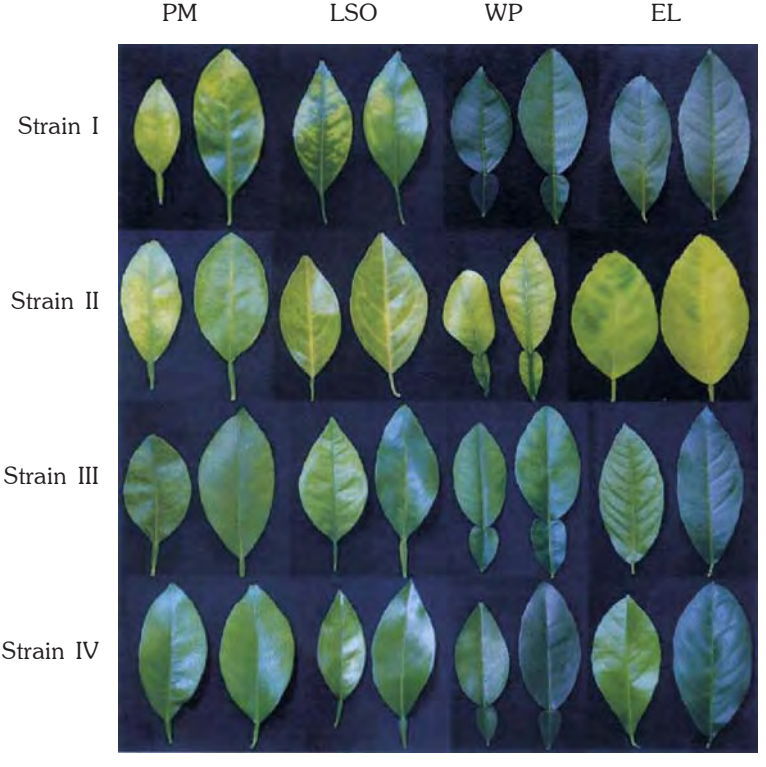

Fig. 2. Different types and severities of the symptoms induced by four HLBB strains (I-IV) in four differential citrus cultivars, Ponkan mandarin (PM), Liucheng sweet orange (LSO), Wentan pummelo (WP) and Eureka lemon (EL) under greenhouse conditions.

2.1.2. Symptoms

No commercial citrus cultivars in Taiwan possess resistance to HLB. Although the disease symptoms differ to some extent among cultivars, these commonly start with yellowing of leaf veins and adjacent tissue, followed by yellowing or mottling of the entire leaf, and occasional corking of the veins. Premature defoliation will follow, and dieback of twigs, decay of feeder rootlets and lateral roots are the next stages of symptom development. Finally, the tree gradually declines in vigor and the entire plant withers.

The diseased leaves become hardened and curled upward, while young leaves that emerge after the premature defoliation of old leaves remain small and slender with symptoms similar to zinc deficiency. Trees infected with HLB become stunted and bear multiple off-season flowers which mostly fall off. The tree ends up bearing small off-shaped fruits with thick and pale green peel (Figs. 3 and 4).

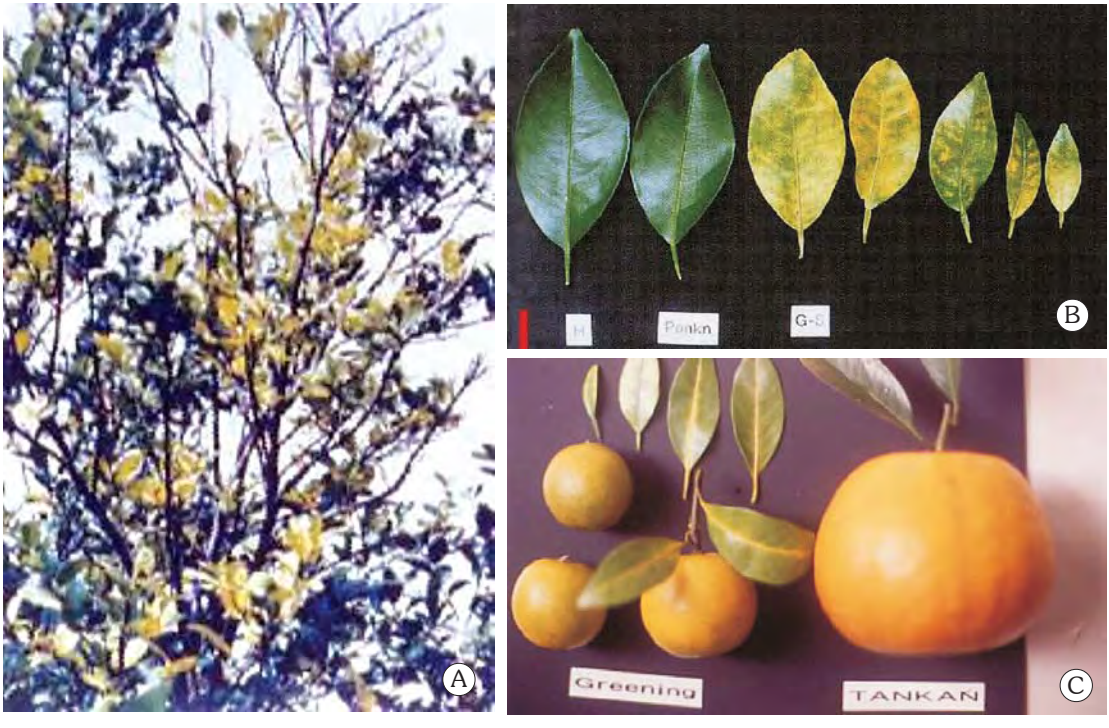

Fig. 3. Symptoms of HLB in mandarin. (A) Diseased branch and twigs of Ponkan mandarin showing yellow mottling of leaves and die back. (B) Symptoms on Ponkan mandarin leaves showing yellow mottling of mature leaves (middle two) and atrophy of younger leaves with interveinous chlorosis and deficiency symptom (right three), healthy leaves (left two). (C) HLB-diseased Tankan fruits showing atrophy with green-coloring (left) and healthy fruit (right).

Fig. 4. HLB symptoms on pummelo. (A) HLBB (pummelo strain II) infected Wentan pummelo. (B) Leaf symptoms of HLB-affected Wentan pummelo showing yellow mottle and vein corking. Healthy leaf on right. (C) HLB diseased 5-Rai pummelo growing in Vietnam showing yellow mottling on leaves and fruit atrophy (left), and healthy large fruit and green leaves (right).

2.1.3. Host range and epidemiology

The Asian form of HLB pathogen develops the symptom at either warm temperatures (27 °C-32 °C) in tropical areas or at cool temperatures (22 °C-24 °C) in subtropical areas. Strain II dominates the major citrus-growing areas and exerts considerable damage to various citrus cultivars such as Ponkan mandarin, Tankan tangor, Liucheng sweet orange, Tahitian lime, and pummelo, but does not effect Eureka lemon and Kumquat.

The Asian-form of pathogen is transmitted and is rapidly spread by Asian citrus psylla/Asian citrus psyllid (*Diaphorina citri*) (Fig. 5), while the African-form of HLBB is transmitted by African citrus psyllid (*Trioza erytreae*) in persistent manner. Taiwanese biotype of the psyllids is less efficient in pathogen transmission of HLB. Its epidemic occurs only if vector population is high and extensive reservoir of the inoculum is present (Huang *et al.*, 1990). Natural spread of the disease most likely occurs during bud-sprouting period (Fig. 6). Transmission of HLBB via infected bud-wood also plays an important role in spreading HLB (Fig. 7).

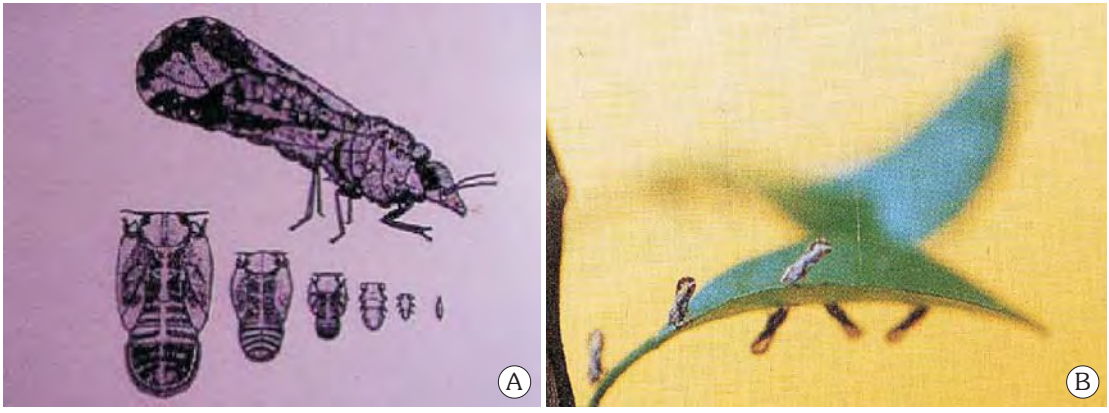

Fig. 5. Asian citrus psylla/psyllid (Diaphorina citri). *(A) life cycle showing adult (above) and egg, and five nymphal instars (below). (B) Adults feeding on citrus leaves.*

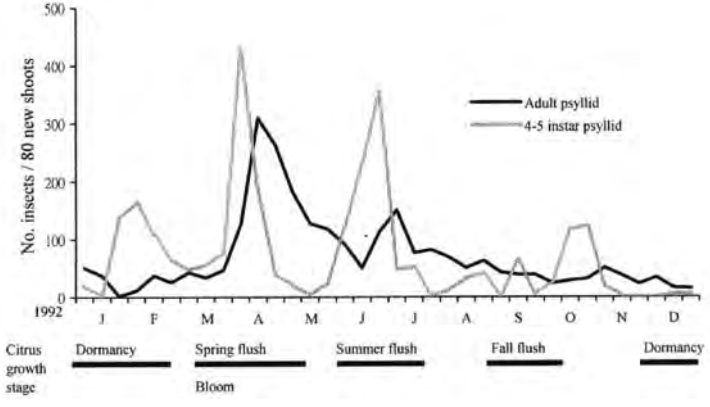

Fig. 6. Population fluctuation of citrus psyllid in a non-sprayed citrus orchard (Liucheng sweet orange) in Chai-Yi (Hung et al., *2003).*

In case such citrus varieties as sunki, pummelo, trifoliate, and their hybrids are used for rootstocks, they have no effect on the extent of HLB resistance of the grafted scion regardless of its variety. Although multiplication of HLBB is known in both graft-inoculated wood apple (*Lemonia acidissima*), and Chinese box orange (*Severinia buxifolia*), which are considered to be suitable host plants for the vector psylla, the infection is non-persistent in wood apple. No HLB infection is reported in Jasmine orange (*Murraya paniculata*) and curly leaf (*Murraya euchrestifolia*). Chinese box orange, a wild shrub commonly distributed in Taiwan, Southern China and Southeast Asia, harbors HLB persistently and hence might be an alternative host of the HLBB (Fig. 8).

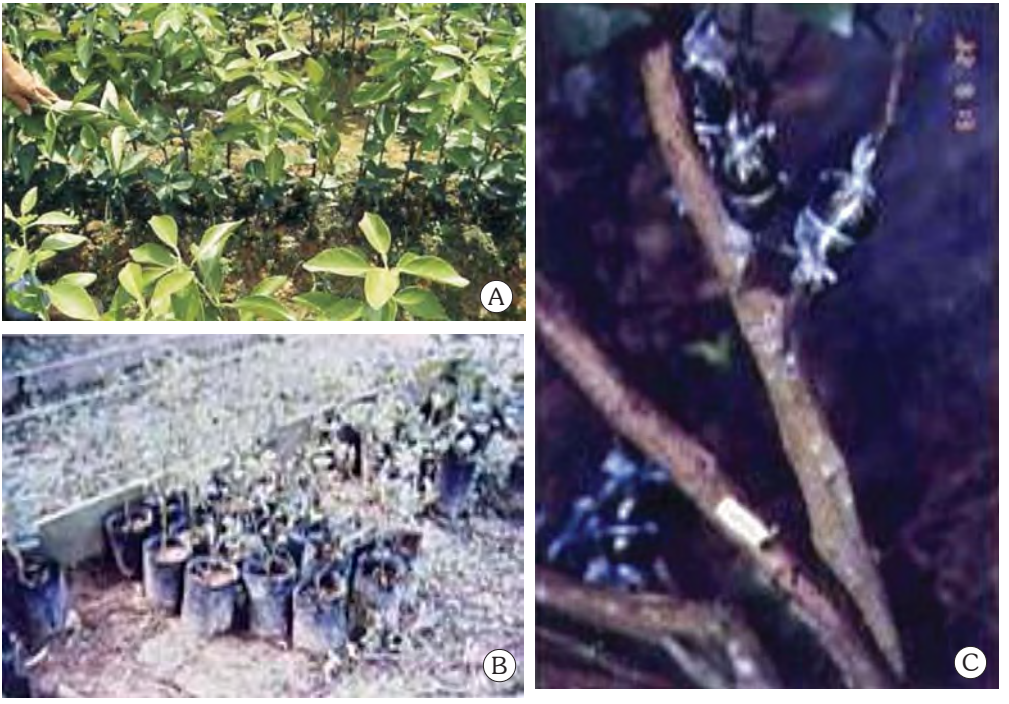

Fig. 7. HLBB transmission via *scion buds. (A) Citrus graft propagation with HLBB-infected scions showing yellow mottling and stunt symptoms. (B) Citrus seedlings propagated by layering. (C) Layering seedling showing HLB symptoms.*

Fig. 8. Leaves of Chinese box orange (Severinia buxifolia), *alternative host of HLBB. GO: HLB-diseased leaves showing yellow mottling symptoms; H: healthy leaves.*

2.2. Citrus tristeza

2.2.1. Causal agent

This disease is caused by *Citrus tristeza closterovirus* (CTV), a flexuous rod-shaped particle approximately 12 nm x 2,000 nm (Fig. 9). The virions contain a single-strand RNA with a molecular weight of 6.5 x 10^6. CTV is distributed worldwide and consists of a complex of virus strains. The strains show great variation in their pathogenicity ranging from mild to severe, and to destructive (Roistacher and Moreno, 1991; Tsai *et al.,* 1993).

2.2.2. Symptoms

The virus causes a variety of disease symptom on citrus depending upon virus strain, citrus variety and scion-rootstock combination. In Taiwan, all the citrus cultivars are propagated as graft susceptible scions onto CTV-tolerant rootstocks. The citrus trees, in general, show no symptoms, even if they are infected by the virus. Most common isolates of CTV in Taiwan are categorized as seedling yellow strain with a typical symptom of seedling yellowing in the bioassay with Eureka lemon (Fig. 10). The destructive isolates causing dwarfing and severe stem pitting (Fig. 11 A) caused by CTV-D in Wentan pummelo trees were first found in Taiwan in 1981 (Su, 1981). Pummelo cultivars available in Taiwan are hypersensitively resistant to the virus except the pummelo stem pitting strain (CTV-D). The isolate causing stem pitting on Valencia sweet orange in Indonesia, mainland China and Taiwan is the same strain as described by Musharam and Whittle (1991). The isolates causing dwarf and severe stem pitting on Cala mandarin in the Philippines, and on Langkat

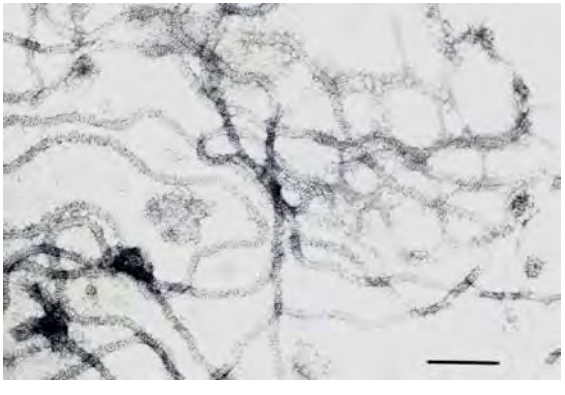

Fig. 9. Electron microscopic picture showing Citrus tristeza closterovirus *particles. Bar represents 100 nm.*

Fig. 10. Yellowing symptom on younger leaves of Eureka lemon seedlings caused by seedling yellows strain of CTV on left; healthy seedling on right.

mandarin (Fig. 11 B) in Sarawak, belong to the mandarin stem pitting strain, although mandarins are generally tolerant to CTV infection. Sweet orange or grapefruit bud-grafted on sour orange rootstock is particularly susceptible to CTV showing symptoms of dwarfing, vein clearing, and fruit atrophy and deformation (Fig. 12 A, B and C).

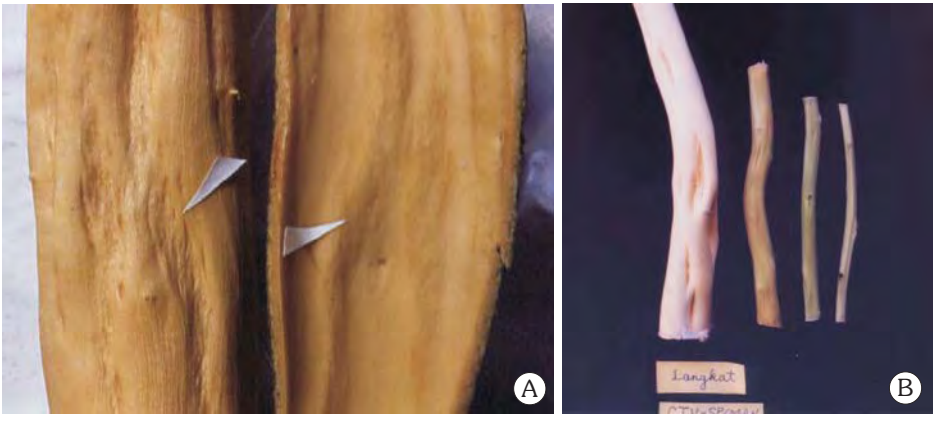

Fig. 11. (A) Stem pitting symptoms consisting of pits on xylem surface (left), and pegs on inner surface of bark (right) of pummelo stem infected by CTV-D (pummelo stem pitting strain). (B) Stem pitting symptoms on xylem surface of Langkat stem infected by mandarin stem pitting strain.

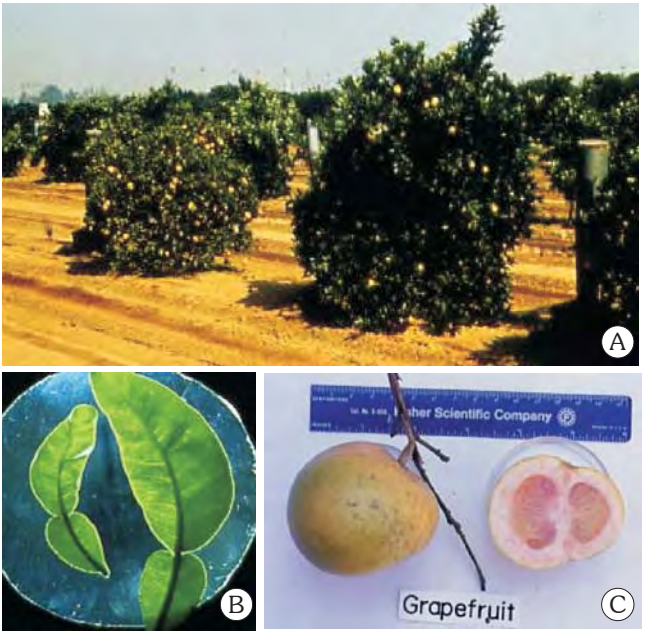

Fig. 12. Common symptoms induce by CTV. (A) Dwarfing in sweet orange. (B) Vein clearing on younger pummelo leaves. (C) Fruit atrophy and deformation of grapefruit.

2.2.3. Host range and epidemiology

CTV infects almost all the citrus species, their varieties and hybrids, except trifoliate orange (*Poncirus trifoliata*). The virus is transmitted by aphids in a semi-persistent manner and via infected bud-woods. The efficiency of virus transmission varies with the aphid species, virus isolates, and the donor and receptor hosts. *Toxoptera citricida* (Fig. 13) is the most efficient vector in virus transmission followed by *Aphis gossypii* for some virus isolates. *A. citricola* is a less efficient vector.

Fig. 13. Vector aphids (Toxoptera citricida) with ants serving as carrier of the aphids.

2.3. Citrus tatter leaf

2.3.1. Causal agent

Citrus tatter leaf capillovirus (CTLV) has flexuous virion, 600 nm x 15 nm (Fig. 14). CTLV was first found in 1962 in Mayer lemon introduced to California from China. Later, the same virus was detected in Mayer lemon trees in Australia and South Africa. Miyakawa (1980) confirmed the occurrence of CTLV infection in several lines of Satsuma mandarin grown in Japan since 1975. Many citrus species such as Ponkan mandarin, Tunkan tangor, and Luchen and Honjan sweet orange cultivated in the mainland China and Taiwan are considered to be infected with CTLV virus.

2.3.2. Symptoms and epidemiology

Most of the citrus species and their cultivars are latently infected with this virus, and show no symptoms in the susceptible scions grafted onto such tolerant mandarin rootstocks as Sunki and Cleopatra cultivars. CTLV is mainly spread by vegetative propagules from infected mother trees, and also transmitted mechanically by grafting tools such as knives. Obviously detectable symptoms of this infection are crease,

grooving and yellowing of the bud-union, which can be observed in citrus species grafted onto such trifoliate orange and its hybrids as citrange and citrimelo (Fig. 15). The virus potentially becomes prevalent in case such trifoliate orange and its hybrids as Troyer and Carrizo citrange and Swingle citrimelo are used as rootstock. So far, no vectors have been found to transmit this virus.

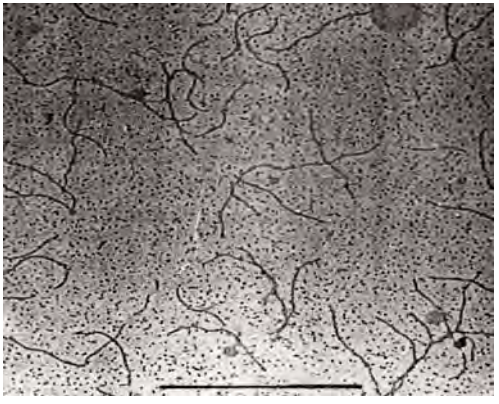

Fig. 14. Electron microscopic photograph of flexuous rod-shaped particles of Citrus tatter leaf capillovirus *(15 nm × 600 nm).*

Fig. 15. Symptoms of Citrus tatter leaf capillovirus. *(A) Yellow ring (gumming) of bud union. (B) Cleavage and swelling of bud union rooted on trifoliate or citrange stock.*

2.4. Citrus exocortis

2.4.1. Causal agent

Citrus exocortis viroid (CEVd) is a 5 nm long rod-like structure comprised of single-stranded RNA with 371 nucleotides and without a protein-coat.

2.4.2. Symptoms and epidemiology

Exocortis is caused by grafting various citrus species on the rootstock of trifoliate orange and its hybrid (citrange), and Rangpur lime. CEVd brings about bark-scaling on the rootstock (Fig. 16) followed by stunting of the entire tree. CEVd can infect not only citrus species and their cultivars, but also some citrus relatives and non-citrus hosts. However, the symptom is latent in most of the citrus species. The citrus species susceptible to CEVd include trifoliate orange, Rangpur lime and some citron and lemon which develop characteristic symptom such as stem-blotching or bark-splitting. The CEVd infection in sweet orange, mandarin and grapefruit proceeds without any distinctive symptoms. CEVd is commonly transmitted by grafting infected scions which are propagated by using symptomless but pathogen-rich budwoods. The viroid is also transmitted mechanically by contaminated cutting and pruning tools.

Fig. 16. Symptom of scaly bark on trifoliate rootstock of mandarin tree infected by Citrus exocortis viroid.

3. DIAGNOSIS AND INDEXING

3.1. Detection of HLB

HLB is tentatively identified in the field by the naked eye observation of typical symptoms developed on foliage and fruit. However, more detailed diagnosis is required for pathogen-indexing, for instance, on susceptible mandarin seedlings by grafting-inoculation method. Because of low HLBB population and its uneven distribution, the bioassay using indicator plants requires numerous test plants and inoculum buds, and therefore, is considerably time-consuming.

The citrus DNA extraction procedure for diagnosis of citrus viriods has been standardized by Nakahara *et al.* (1998). Highly specific primer pairs for HLBB detection have been developed by HLBB-DNA cloning and sequencing. Dot hybridization (DH) using selected DNA probes provides a highly specific, sensitive, and stable reaction with the DNA extract from HLBB infected citrus plants. The technique was successfully applied for detection and ecological study of HLB pathogen (Su *et al*., 1991, 1995). PCR has been developed and successfully used for the detection of HLBB in host plants and vector insects (Hung *et al.*, 1999, 2003, 2004) (Fig.17). This technology is routinely applied to indexing citrus foundation stocks and pathogen-free seedlings (Hung *et al.,* 1999).

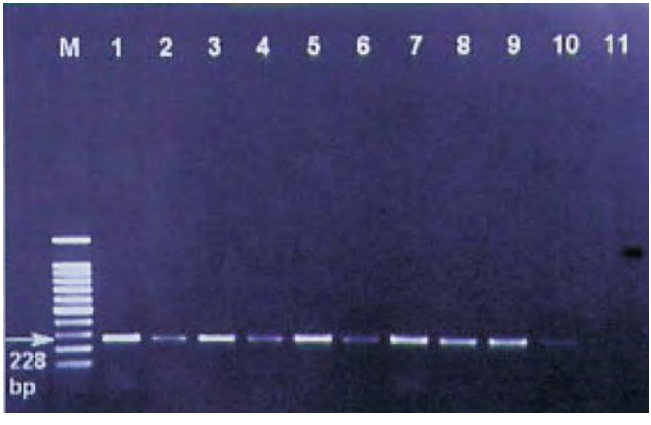

Fig. 17. Detection of HLB pathogen by polymerase chain reaction (PCR). Lanes 1~4. Taiwan HLBB isolates; 5. Okinawa, Japan isolate; 6. China isolate; 7. Malaysia isolate; 8. Vietnam isolate; 9. Thailand isolate; 10. Saudi Arabia isolate; 11. Healthy.

3.1.1. PCR detection

A.1. DNA extraction from infected plants

1. Take leaf and twig samples of the plant. Cut off the midrib from the leaf or peel the bark from twig. Air-dry the midrib and/or bark or use fresh tissues directly.
2. Freeze 0.5 g of the chopped fresh tissue in liquid nitrogen and grind to a fine powder using mortar and pestle.
3. Add 2.7 ml of **DNA-extraction buffer**[1] and 0.3 ml Sarkosyl (10%). Stir the powdered tissue suspension. Alternatively, grind fresh tissue with the 2.7 ml of DNA-extraction buffer.
4. Transfer the suspension to a 1.5 ml Eppendorf tube and incubate at 55 °C for 1 hr.
5. Centrifuge the suspension in an Eppendorf tube at 6,000 rpm for 10 min.
6. Save 0.8 ml of the supernatant, and add 100 μl of 5 M NaCl and 100 μl of CTAB/NaCl (10% CTAB in 0.7 M NaCl), then incubate at 65 °C for 10 min.
7. Add 0.5 ml of chloroform/isoamyl alcohol mixture (24:1), and mix thoroughly. Spin at 12,000 rpm (10,000 g) for 10 min, and save 0.8 ml of aqueous layer of the supernatant.
8. Add 0.6 ml of phenol/chloroform/isoamyl alcohol at the ratio of 25:24:1. Mix thoroughly and spin at 12,000 rpm for 10 min and save 0.6 ml of aqueous portion of the supernatant.
9. Add 0.6 volume (0.36 ml) of isopropanol to precipitate the nucleic acid. Spin at 12,000 rpm for 15 min at 4 °C, or keep at -20 °C for 30 min before centrifugation at room temperature.
10. Wash the pellet with 70% ethanol to remove the residual CTAB. Dry the pellet briefly and resuspend in 100 μl of **TE buffer**[2]. Store at -20 °C.

[1]**DNA-extraction buffer** (pH 8.0): 100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl

[2]**TE buffer** (pH 8.0): 10 mM Tris, 1 mM EDTA

A.2. DNA extraction from psylla/psyllid vector

1. Place one psylla/psyllid in an Eppendorf tube containing 70 μl of DNA extraction buffer.

2. Homogenize the tissue in the tube by a plastic pestle with cooling on ice.
3. Add 200 μl of DNA extraction buffer and 30 μl of 10% Sarkosyl.
4. Incubate at 55 °C for 1 hr.
5. Add 200 μl of phenol/chloroform/isoamyl alcohol at the ratio of 25:24:1.
6. Mix vigorously and centrifuge at 12,000 rpm (10,000 g) for 10 min.
7. Take 200 μl of the supernatant, and add 500 μl of 95~100% EtOH.
8. Mix gently and keep at -20 °C for 30 min.
9. Centrifuge at 12,000 rpm (10,000 g) for 15 min at 4 °C, or keep at -20 °C for 30 min before centrifugation at room temperature.
10. Vacuum-dry the DNA pellets for about 10 min.
11. Dissolve DNA pellets in 50 μl of TE buffer.

A.3. PCR

Reaction mixture (for 25 μl)

PCR reagents	**amount/tube**
ddH_2O	14.75 μl
10 X Taq buffer	2.5 μl
50 mM $MgCl_2$	2 μl
2.5 mM dNTPs	2 μl
Primers	1 μl
Taq polymerase	0.25 μl
Template DNA	2.5 μl

PCR cycles

Step 1: 94 °C 3 min

Step 2: 94 °C 1 min; 60 °C 1 min; 72 °C 2 min (30 cycles)

Step 3: 72 °C 10 min

Step 4: 4 °C soaking

Primer pair

Forward: CAC CGA AGA TAT GGA CAA CA

Reverse: GAG GTT CTT GTG GTT TTT CTG

Electrophoresis of PCR products

1. Prepare 1.4% agarose gel in **0.5 X TAE buffer**[3].
2. Load 10 μl of PCR products in **loading buffer**[4] into each well, and run the gel in an electrophoresis chamber at 100 volts for about 28 min in **running buffer**[5].
3. Stain the gel with ethidium bromide (0.5 μg/ml) solution for 3-5 min and later soak in water for several minutes.
4. Observe the amplified HLB-DNA band under UV-light.

[3]**0.5 X TAE buffer** (per liter): 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0)

[4]**Loading buffer**: 0.25% bromophenol blue, 30% glycerol at the ratio of 1:4

[5]**Running buffer**: 0.5 X TAE buffer, pH 8.0

3.1.2. Iodine kit for rapid diagnosis

Iodine Kit[6] can detect citrus trees infected by HLB based on the principle that starch tends to be accumulated in the infected leaf tissue because of a decrease in amylase activity. Iodine reacts with the accumulated starch to develop dark brown color. The Iodine Kit has been successfully applied for on-site diagnosis of HLB in citrus trees, and has proved to be very promising as a rapid diagnosis tool with relatively high accuracy (author's unpublished data). The protocol of HLB diagnosis using Iodine Kit (Fig. 18) is as follow:

1. Collect mature citrus leaves showing symptoms in a polythene bag.
2. Scratch the upper surface of infected leaf about 40 times with a piece of sand paper (120 mesh).
3. Put the sand-paper piece harboring tissue debris in a small polythene bag with a sealing mouth and add 1 ml of pure water.
4. Rub the sand-paper piece in water thoroughly for washing off the tissue debris into water.
5. Add a drop of iodine solution into the suspension in the bag and mix the solution by shaking.
6. Watch the solution for color-change: black or dark brown color shows positive reaction while no color change (retaining yellow color) shows negative reaction.

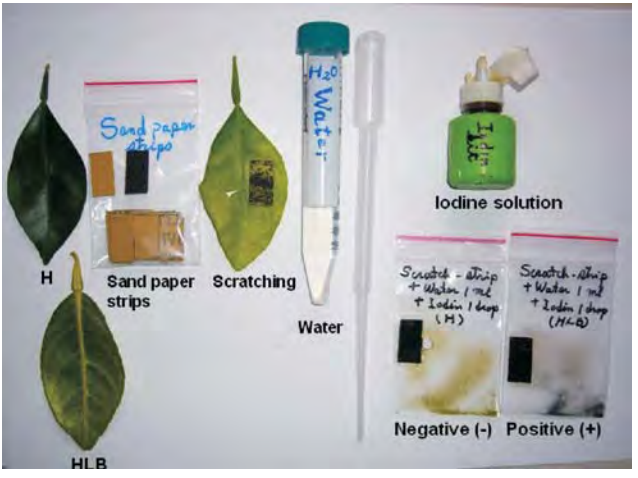

Fig. 18. Indexing of HLB infection with Iodine Kit including iodine solution, piece of sand paper, polythene bag and pure water: Positive reaction (+) showing color turning to dark brown to black; Negative reaction (-) showing yellow.

[6]**Iodine Kit**: Iodine solution: KI 3%; I_2 1.5%
Sand paper (120 C): 1×2 cm
Plastic bag with sealing mouth (5 cm × 8.5 cm)
Pure water

3.2. Detection of *Citrus tristeza closterovirus* (CTV)

3.2.1. Bioassay of CTV with indicator plants

Mexican lime (also known as Key lime) is the best indicator plant for indexing CTV. Infected lime leaves develop distinct vein clearing. The vein becomes corky, giving rise to chlorosis and cupping of the entire leaf and then stunting when the lime leaves are inoculated with severe CTV strains. Stem pitting commonly occurs in association with CTV infection although the extent of the symptom development varies with different virus strains. Eureka lemon, sour orange and grapefruit are commonly used for the indexing of seedling yellows strain of CTV. This strain easily develops the symptom of yellowing and atrophy on upper younger leaves (Fig. 10). Sweet orange, mandarin and pummelo seedlings are used for differentiating the stem pitting strains of the three citrus species (Fig. 11 A and B).

3.2.2. Enzyme-linked immunosorbent assay (ELISA)

The CTV is effectively detected by ELISA by using polyclonal and monoclonal antibodies. Monoclonal antibodies were produced by hybridoma technology and successfully applied for differentiation of certain strains of CTV (Tsai and Su, 1991). The protocol for citrus CTV detection using direct DAS-ELISA with AP-conjugate of mAb 3E10 is summarized as follows:

1. Microplates are coated with purified immunoglobulinG of monoclonal antibody against CTV. Add 100 µl of mAb (1 ppm) in **coating buffer**[7] to each well, and incubate plates in a moist box at 37 °C for 2 hours.
2. Wash the plates with **PBST**[8] 3 to 5 times.
3. Add 100 µl/well of CTV **sample extracts**[9] in **Tris-DIECA extraction buffer**[10] (pH 7.5) at 1/10 (W/V) dilution, then keep the microplates in a moist box at 37 °C for 2 hours or at 4 °C overnight.
4. Wash the plates with PBST 3 to 5 times.
5. Add 100 µl/well of alkaline phosphatase (AP)-conjugate in PBST at 3000X (400~5,000X) dilution. Incubate the plates in a moist box at 37 °C for 2 hours.
6. Wash the plates with PBST 3 to 5 times.
7. Add 100 µl of p-nitrophenyl phosphate (1 mg/ml) dissolved in the **substrate buffer**[11] to the well and incubate the plates in a moist box at 37 °C for 30 to 60 min. If the sample is CTV positive, the solution-color will turn yellow.
8. Read the absorption intensity at 405 nm for a relative concentration of CTV in ELISA.

[7]**Coating buffer** (50 mM bicarbonate buffer)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g
Distilled water	1 L

Adjust pH to 9.6, the store at 4 °C

[8]**PBST** (phosphate-buffered saline with Tween 20)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.44 g

KH_2PO_4	0.24 g
Tween 20	0.50 ml
NaN_3	0.2 g
Distilled water	1 L

[9]Sample extract

1. Collect 0.5 g of citrus tissue sample (bark or leaf) and chop it in 5 ml of the extraction buffer with scissors.
2. Homogenize the chopped sample in a homogenizer at 20,000 rpm for 20 sec, or with a mortar and pestle.
3. Centrifuge the sample at 3,000 rpm for 5 min. Decant the supernatant for testing.

   The entire procedure of the sample extraction should be carried out in ice bath.

[10]Extraction buffer

Tris base	1.18 g
Tris-HCl	6.35 g
Na-DIECA	1 g
(Sodium N, N-diethyldithiocarbamate trihydrate)	
Distilled water	1 L
Adjust pH to 7.5, then store at 4 °C	
Adjust pH to 7.4, then store at 4 °C	

[11]Substrate buffer

Diethanol amine	97 ml
$MgCl_2 \cdot 6H_2O$	100 mg
NaN_3	0.2 g
Distilled water	1 L
Adjust pH to 9.8, then store at 4 °C	

3.2.3. Diagnostic strips

Recently, rapid and accurate diagnostic strips have been developed for the detection of *Citrus tristeza closterovirus* (CTV). Monoclonal antibodies against CTV were prepared by the Plant Virology Laboratory, National Taiwan University. Based on these, diagnostic strips have been developed in cooperation with Meikang Biological Project Company (E-mail:hjsu@ntu.edu.tw). Globulins labeled with colloidal gold are used for the strips. Virus antibodies and secondary anti-mouse antibody are fixed in the lower and upper parts of a chromatographic paper strip, respectively. A kit of the CTV-strip consists of protective pouch or polythene bag containing strips and desiccants, **extraction buffer**[12] solution in bottle, Eppendorf tube, scissors and wooden stick (Fig. 19). The protocol for diagnostic strip based on this kit is summarized below and in Figs. 20 and 21.

[12]CTV-extraction buffer for strip

Tris base	1.18 g
Tris HCl	6.35 g
Na-DIECA (Sodium N,N-diethyldithiocarbamate trihydrate)	0.1 %
Tween-20	0.2 %
NaCl	0.4 M
Distilled water	1 L

Adjust pH to 8.0, then store at 4 °C

1. Collect 0.2-0.3 g of tissue specimen from several leaves and chop into an Eppendorf tube.
2. Crush the chopped specimen together with 0.8 ml of extraction buffer in the Eppendorf tube by using bamboo/wooden stick.
3. Take out a test strip from the protective pouch (should return the kit to room temperature before opening the pouch to avoid condensation of moisture on the membrane).
4. Immerse the strip into the Eppendorf tube containing the extraction buffer solution in the direction of the arrow. Do not immerse the strip over the MAX line (Fig. 20 A).
5. Wait for the development of pink-colored bands. Although the band-development time depends on the concentration of CTV, positive reactions, is usually obtained

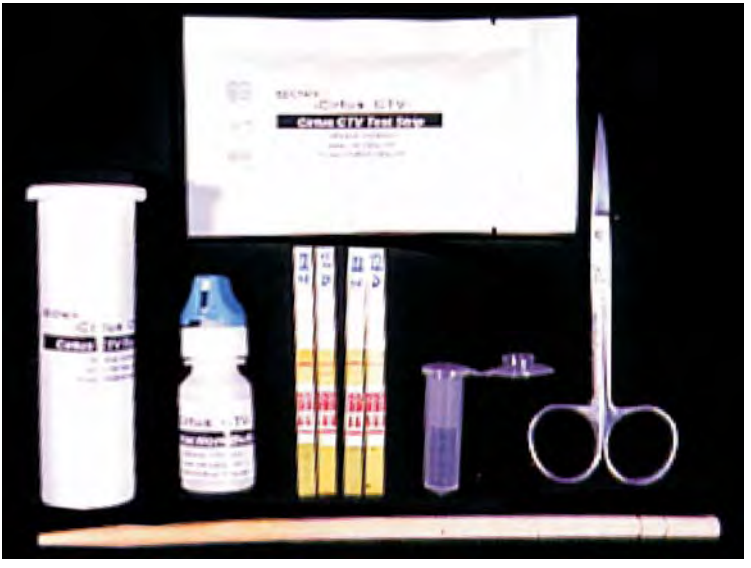

Fig. 19. Diagnosis strips for CTV detection, produced by Meikang Biological Project Co., Taiwan in collaboration with Plant Virology Laboratory, NTU.

in 2 to 3 min. However, it takes more than 10 min to complete the negative reaction, so that it is highly recommended to wait for 20 min for the confirmation of CTV infection.

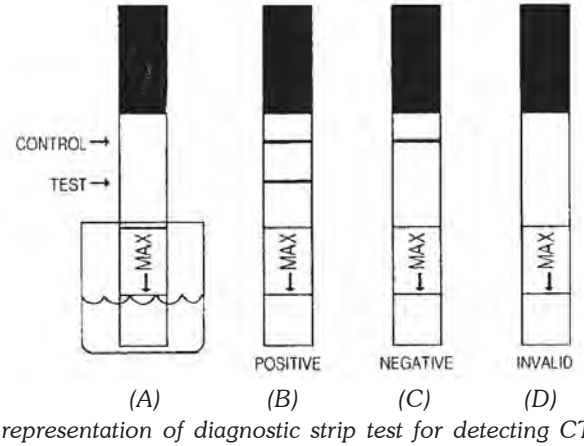

Fig. 20. Diagrammatic representation of diagnostic strip test for detecting CTV.

Interpretation of results

1. POSITIVE: Two distinct pink-colored bands will appear, one in the plant test region and one in the control region (Fig. 20 B).

2. NEGATIVE: Only one pink-colored band appears in the control region. No clear pink band appears in the plant test region (Fig. 20 C).
3. INVALID: Complete absence of pink-colored bands in both regions. If there is neither the test band nor the control band on the membrane, the test should be considered invalid due to either improper use of testing procedure or reagent deterioration (Fig. 20 D).

3.3. Detection of *Citrus tatter leaf capillovirus* (CTLV)

CTLV is indexed using Rusk and Troyer citrange as indictor plant with typical symptoms of chlorotic blotching, irregular mottling and distortion in leaves. Although ELISA using monoclonal antibodies has been developed for the CTLV indexing, the sensitivity of the virus detection is not high enough. A reverse transcription PCR (RT-PCR) based on CTLV cDNA has proved to be more rapid and accurate for detecting this virus (Ohira *et al.*, 1995).

3.3.1. Bioassay of CTLV with indicator plants

Rusk citrange is a popular indexing plant of CTLV. Two buds of infection-suspected citrus plant are graft-inoculated to the lower part of a receptor seedling

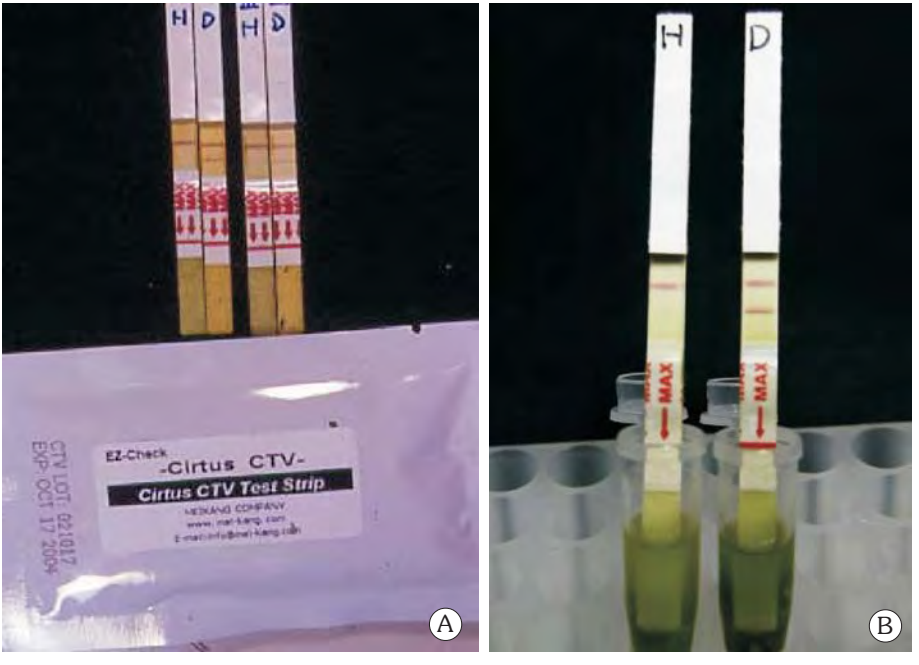

Fig. 21. Rapid diagnostic strips for detecting CTV in infected citrus tissues. (A) Diagnostic kit. (B) CTV strips with the one on right, showing two bands with lower band indicating positive reaction of CTV.

such as sweet orange, and bud or scion of Rusk citrange is grafted onto the upper part of the receptor seedling. The typical symptoms of CTLV infection are chlorotic blotchy spotting and ragged or deformed tatter on newly developing leaves of the grafted indicator plant (Fig. 22). Some strains of the virus can be also detected by mechanical inoculation in such herbaceous indicator plant as *Chenopodium quinoa*. These seedlings mechanically inoculated by using leaf sap in phosphate buffer (pH 7), produce chlorotic spots on the inoculated leaves and systemic chlorosis and deformation of upper young leaves (Fig. 23).

Fig. 22. Bioassay of Citrus tatter leaf capillovirus *with Rusk citrange bud as indicator cultivar. (A) Two buds of suspected plant grafted on lower part of receptor plant sweet orange (arrow). (B) Chlorotic spotting and tatter leaf symptoms on Rusk leaves.*

Fig. 23. Bioassay of CTLV on Chenopodium quinoa *by mechanical inoculation. (A) Chlorotic spots in initial stage and enlarging to pinkish spots at later stage on inoculated leaves. (B) Chlorosis and distortion of upper leaves.*

3.3.2. RT-PCR detection

A. Extraction of nucleic acids from citrus tissue

1. Grind 0.3 g each of tissue sample in liquid nitrogen and homogenize in 3 ml **TRIzol reagent buffer[13]**.
2. Centrifuge the suspension at 12,000 x g at room temperature for 10 min.
3. Transfer the supernatant (about 1 ml) into a new tube.
4. Add 200 ml of chloroform to the supernatant.
5. Shake tubes vigorously by vortexing for 30 sec.
6. Centrifuge the suspension at 12,000 g at room temperature for 10 min.
7. Pipette aqueous phase (about 600 ml) into a new tube.
8. Add isopropanol (about 300 ml), 0.8 M sodium citrate and 1.2 M NaCl (about 300 ml). Mix by gentle inversion.
9. Centrifuge at 12,000 g at 4 °C for 15 min.
10. Discard the supernatant. Wash pellet with 500 µl of 75% ethanol.
11. Dry the pellet for 10-12 min.
12. Add 80 ml of distilled H_2O.

> **[13]TRIzol reagent buffer (pH 5.0)**: 38% phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate, 5% glycerol

B. RT-PCR

Reaction mixture (for 25 µl)

PCR reagents	**amount/tube**
ddH_2O	11.5 µl
5X Superscript II buffer	2.5 µl
10X Taq buffer	2.5 µl
100 mM DTT	1.25 µl
25 mM $MgCl_2$	1.25 µl
10 mM dNTPs	2. µl
Taq polymerase (BRL or BerTaq)	0.25 µl

Superscript II polymerase (BRL)	0.25 μl
CTLV primers (10 pmol/μl each)	1. μl
RNA template	2.5 μl

PCR

Step 1: 50 °C 35 min; 94 °C 2 min

Step 2: 94 °C 30 sec; 56 °C 30 sec; 68 °C 45 sec (10 cycles)

Step 3: 94 °C 30 sec; 56 °C 30 sec; 68 °C 45 sec* (25 cycles)

(*: 5 sec increasing ramp)

Step 4: 68 °C 7 min

cDNA primer pair

Forward: GGA AGA CTC ACA TAG ACC CG

Reverse: TAC TCT CCG AAC CTG CCT C

The RT-PCR products are separated by gel-electrophoresis as stated in the protocol of HLB PCR. The RT-PCR analysis using the above cDNA primer pair has shown positive amplification with CTLV isolates collected from Taiwan, Korea, and China (Fig. 24).

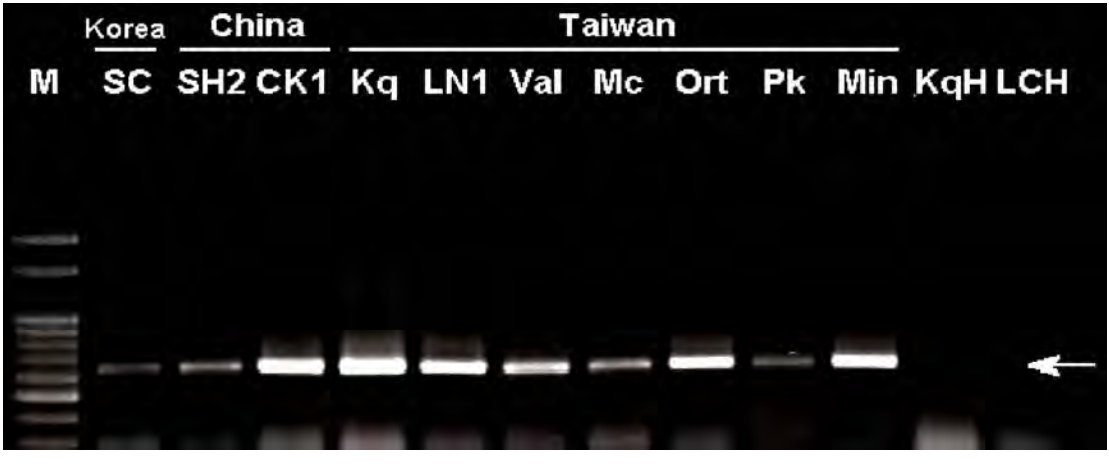

Fig. 24. CTLV detection by RT-PCR: virus infected material from Korea (SC. Satsuma orange), China (SH. Satsuma orange, Che-Chiang; CK. Calamondin, Kuantong) and Taiwan (Kq. Kanqua; LN. Lime; Val. Valensia; MC. Murcott; Ort. Ortanique; Pk. Ponkan mandarin; Min. Minneola tangelo). Healthy: KqH. Kanqua; LCH. Lucheng orange.

3.4. Detection of *Citrus exocortis viroid* (CEVd)

3.4.1. Bioassay of CEVd

The CEVd infection is identified in the field by the typical symptoms of bark-scaling on susceptible rootstock and stunting of aged trees. The stunting, however, is not obvious unless the comparison is made with virus-free plants. CEVd indexing is commonly done by using the graft-inoculation technique against CEVd-sensitive clones of Etrog vitron (861) which can easily develop leaf epinasty and curling, and vein necrosis (Fig. 25).

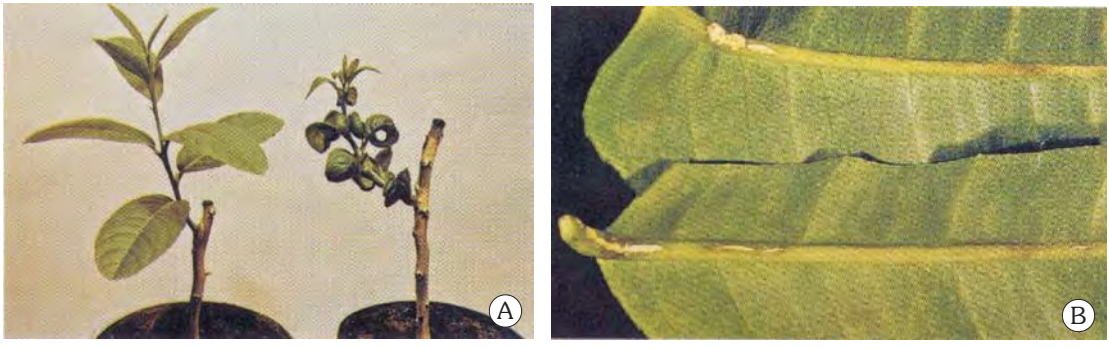

Fig. 25. Bioassay of Citrus exocortis viroid *with Etrog citron 816 bud as indicator plant grafted on receptor plant (sweet orange) and buds of suspected plant grafted on lower part. (A) Positive CEV infection, inducing epinasty and leaf curling (right); negative reaction showing normal leaves (left). (B) Vein necrosis on midrib of leaves with curring.*

3.4.2. RT-PCR detection (Fig. 26)

A. Extraction of nucleic acids from citrus tissue

1. Collect leaf samples.
2. Freeze the chopped tissue sample (0.5 g) in liquid nitrogen and grind to fine powder with a mortar and pestle.
3. Add 2.7 ml of **nucleic acid-extraction buffer**[14] and re-suspend the powdered samples.
4. Add 0.3 ml of 10% Sarkosyl and allow to stand at 55 °C for 1 hr.
5. Centrifuge at a low speed of 4,000 g for 5 min and decant 0.8 ml of the supernatant.
6. Add 100 μl of 5 M NaCl and 100 μl of 10% CTAB/NaCl, then allow to stand at 65 °C for 10 min.

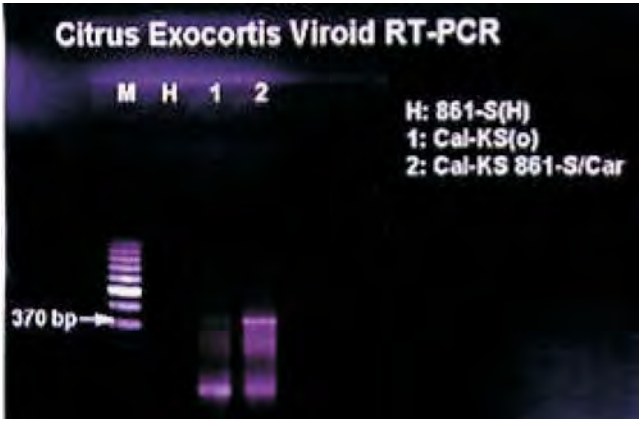

Fig. 26. Detection of Citrus exocortis viroid *by RT-PCR. H. healthy, citron 861-s; 1. Calamondin plant, negative reaction. 2. Citron 861/Carrizo, positive reaction.*

7. Add 0.6 ml of chloroform/isoamyl alcohol mixture (24:1) and then mix thoroughly.
8. Centrifuge at 10,000 g for 5 min and collect the supernatant.
9. Add 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1), then mix thoroughly.
10. Centrifuge at 10,000 g for 10 min and decant 0.66 ml of the supernatant.
11. Add 0.4 ml of isopropanol and gently mix the supernatant.
12. Centrifuge at 10,000 g for 10 min at 4 °C and collect pellet.
13. Wash the pellets with 0.5 ml of 70% ETOH.
14. Dry the pellet under vacuum.
15. Dissolve the pellets with 100 μl TE buffer.

[14]Nucleic acid-extraction buffer (pH 8.0)
100 mM Tris-HCl; 100 mM EDTA; 250 mM NaCl.
100 μl/ml proteinase K (optional).

RT-PCR

Reaction mixture (for 50 μl)

Reverse transcription

Reagents	**amount/tube**
5X Superscript II buffer	2 μl
100 mM DTT	1 μl

10 mM dNTPs	2 μl
Random primer	0.33 μl
Superscript II polymerase (BRL)	0.25 μl
RNA template make up to 10 μl	

Incubate at 42 °C for 50 min.

PCR Reaction mixture

Reagents	**amount/tube**
ddH_2O	32.8 μl
10X Taq buffer	5 μl
10 mM dNTPs	1 μl
Taq polymerase (BRL of BerTaq)	0.2 μl
Primers (10 pmol/μl each)	1 μl

(1) + (2)

PCR cycles

Step 1: 94 °C 5 min
Step 2: 94 °C 30 Sec; 50 °C 30 sec; 72 °C 2 min (40 cycles)
Step 3: 72 °C 8 min

cDNA primer pair for RT-PCR
Forward: GCT CCA CAT CCG ATC GTC
Reverse: TGG ACG CCA GTG ATC CGC

4. ESTABLISHMENT OF VIRUS-FREE CITRUS NURSERY SYSTEM

Currently, HLB and systemic virus diseases detailed in this document are a major threat to economical viability of citrus production in the subtropics and tropics. Since these diseases are transmitted by not only vector insects but also during the process of vegetative propagation, integrated disease management (IDM) is highly recommended to control them. IDM includes propagation of virus-free (VF) saplings, elimination of inoculum sources, and prevention of secondary infection by vector insects. Establishment of virus-free citrus nursery system is primarily important to prevent the disease prevalence. In Taiwan, a shoot-tip micrografting technique (STG) and the associated heat therapy are combined to prepare citrus VF foundation stock. The precise and rapid indexing techniques described in previous chapters are indispensable for management of pathogen-free nursery system. Virus-free nursery system (VFNS) in Taiwan consists of the following four steps: 1. STG, 2. Establishment of pathogen-free citrus foundation blocks, 3. VF nurseries, and 4. Issue of health certificate via indexing.

The current national scheme of citrus VFNS and bud-wood certification program in Taiwan was initiated in 1983 and is being operated under a joint program of National Taiwan University (NTU), Agricultural Research Institute (ARI), and Council of Agriculture (COA), Taiwan.

4.1. Shoot-tip micrografting (STG)

STG is the most reliable method to recover pathogen-free (PF) citrus saplings from infected parental sources. The shoot tip or meristem of axiliary bud of infected plants is generally free of virus and HLBB, and saplings regenerated from the shoot tips are usually free of these pathogens. The common STG method (Murashige *et al.*, 1972) was greatly improved by replacing their inverted T cut with newly developed triangle-hole cut method (Su and Chu, 1984). The entire procedure of STG should be carried out in a laminar-flow clean bench under sterile conditions.

4.1.1. Preparation of rootstock seedling

Troyer of Carrizo citrange is the commonly used rootstock for STG. However, other citrus cultivars such us pummelo, lemon and sweet orange are also used.

1. Seeds are extracted from fresh fruits just before culturing stock seedlings *in vitro*.
2. Skin of the seeds is removed.
3. Naked seeds are sterilized for 5 min in 1% sodium hypochlorite which is prepared by diluting one part of bleach (Chlorex) with four parts of distilled water.
4. Sterilized seeds are washed with sterile water three times, and transferred onto solid medium in a test tube.
5. Rootstock seedlings are cultured on **STG Medium**[15] in an incubator at 28 °C for two weeks. Etiolated seedlings are ready to be used for STG (Fig. 27 A).

[15]Preparation of Citrus STG Medium (pH 5.7)

1. Solid medium for growing rootstock seedling: MS salt mixture (Gibco BRL), 2.5 g; sucrose, 20 g; ddH_2O, 1 L; Agar, 1% (10 g)

2. Liquid medium for growing STG seedling: MS salt mixture, 2.5 g; sucrose, 30 g; growth factors (1 L/100X stock; i-inositol, 100 mg; thiamine-HCl, 0.2 mg; pyridoxine-HCl, 1 mg; nicotine acid, 1 mg); ddH_2O, 1 L

4.1.2. Preparation of citrus shoot

1. Young shoots are collected from infected citrus trees in orchards (Fig. 27 B). Alternatively potted citrus plants in green house are forced to sprout by defoliating and/or pruning. Young shoots of adequate size (0.5 cm~3 cm) are as scion collected for STG.
2. The shoots are kept in a plastic bag to keep them clean and moist.

4.1.3. Micrografting of shoot-tip

1. Young leaves of shoots are pruned off and shortened to less than 0.5 cm.
2. Pruned shoots are sterilized for 5 min in 0.5% sodium hypochlorite solution.
3. A sharp knife is used for STG preparation. The cutting edge (3 mm × 15 mm) of STG knife is made from a razor-blade using pliers and fixed in edge holder (Fig. 27 C). The cutting edge is flame-sterilized and cooled in sterile water in a beaker before use.
4. Sterilized shoots are washed three times in sterile water and transferred to a sterile Petri dish containing a filter-paper. A black rectangle is marked on the filter paper to facilitate working on the rootstock seedlings (Fig. 27 D).

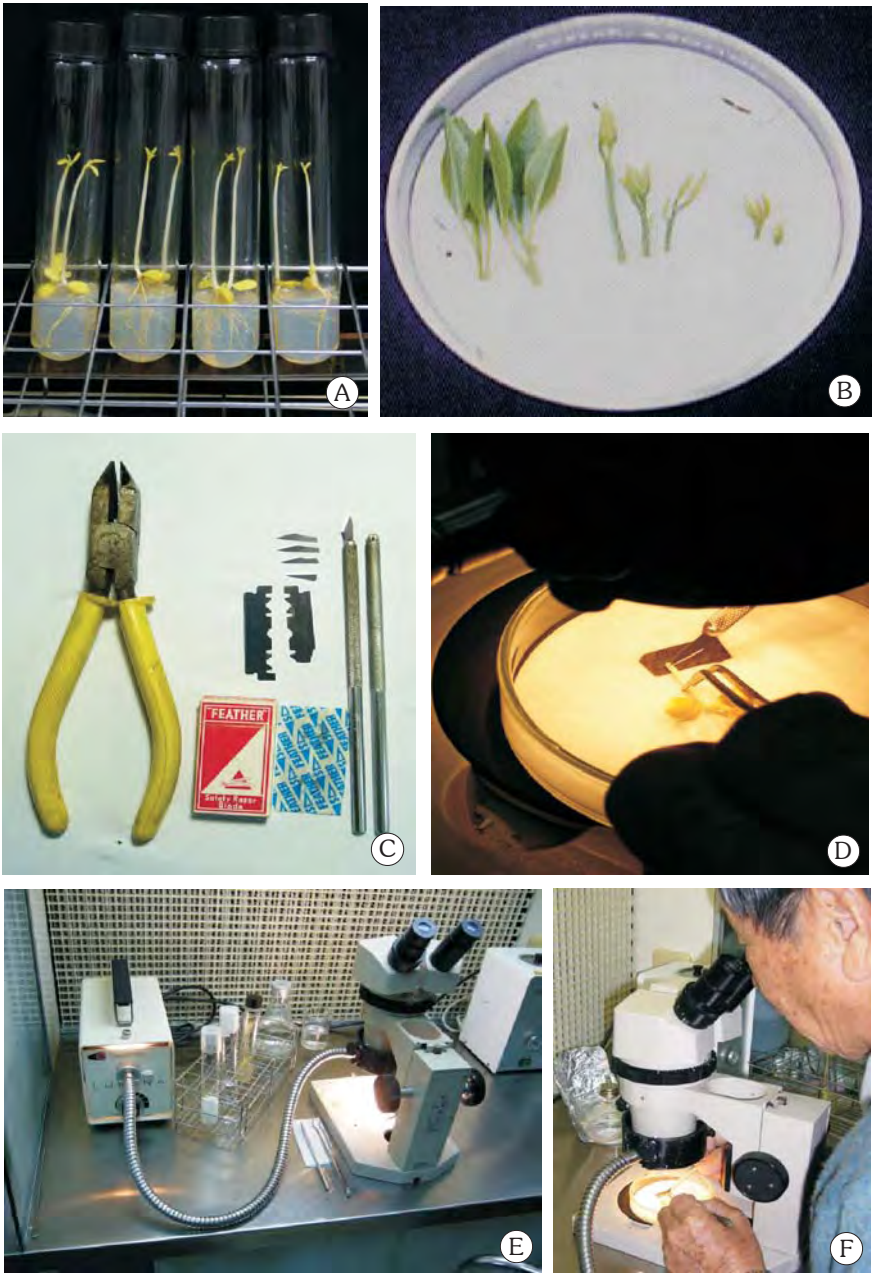

Fig. 27. Procedure of shoot-tip micrografting. (A) Culture of rootstock in solid medium. (B) Citrus shoots of different sizes for STG. (C) Cutting knife consists of cutting edge of razor blade and edge holder; pliers for making blade edge. (D) Making STG incision on upper top of rootstock seedling with tip-bended forceps and cutting knife. (E) Cold beam illuminator, binocular microscope and accessories within a laminar-flow bench. (F) Making STG incision on upper top of rootstock seedling with tip-bended forceps and cutting knife.

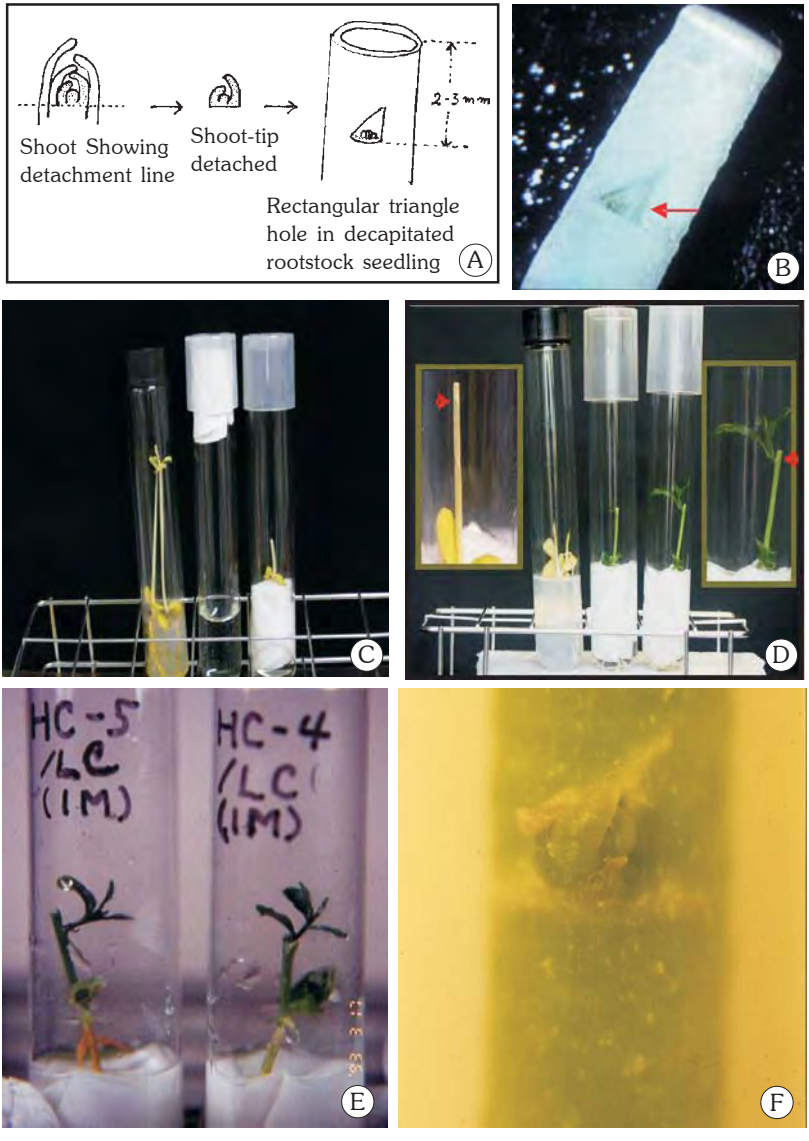

Fig. 28. Procedure for shoot-tip micrografting. (A) Diagram showing excision of shoot-tip with 2-leaf primordia and rectangular triangle hole (0.3~0.5 mm) on a decapitated rootstock seedling by removing cortex layer with cutting edge of STG knife. (B) A shoot tip placed in the hole on a decapitated rootstock seedling. (C) Two-week old rootstock seedlings in 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 filter paper 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 Hong China sweet orange regenerated from ST in rectangular hole (left) and in V-shaped incision (right) of rootstock seedlings one month after micrografting. (F) A new sprout from ST in rectangular hole on rootstock.

5. Both terminal and axiliary bud are used for STG. Apical meristem with two or three leaf primordia (0.1 mm-0.2 mm long from the cut end to the tip of largest leaf primordia, Fig. 28 A) is excised under cold beam of light illuminator with the aid of a binocular microscope (Fig. 27 E and F).
6. Two to three week old rootstocks are suitable for STG. A rectangular triangle hole (0.3~0.5 mm) is hollowed out on the side of rootstock at 2~3 mm from the top. The shoot tip is placed into the hole and covered with a small piece of tissue excised from rootstock seedling (Fig. 28 A and B). Another method of STG is to make a V-shaped incision on the top of decapitated rootstock seedling and placing the shoot-tip inside it.
7. Micrografted seedlings are transferred onto a filter paper platform in a test tube containing liquid medium (Fig. 28 C and D). The culture test tubes are kept in a dark incubator for one day.
8. The test tubes are moved to a growth chamber at 26 °C with shading by one-layer gauze and exposed to illumination at 400 Lux for 16 hours daily.
9. Shading gauze is removed after 2 week incubation. About one-month later, new shoot-buds grow and two or three leaves develop from the scion (Fig. 28 E and F).

4.1.4. Double grafting

A double grafting technique has been developed to enhance the growth of STG-plants. The procedure of this technique is briefly summarized below:

1. Micrografted plant is taken out from the test tube, its upper part serves as scion (Fig. 29 A and B).
2. The scion is side-grafted to a healthy and vigorously growing rootstock seedling with the aid of parafilm. The graft is wrapped in a plastic bag with its mouth sealed and the plant placed in a green house (Fig. 29 C and D).
3. The plastic bag is taken off 3 to 4 weeks after grafting. It takes about three months for the new shoot to fully mature and fit for use as scion-wood for further grafting (Fig. 29 E).
4. Health indexing for HLBB and viruses should be done before STG plant in used for further propagation. For an increased multiplication of pathogen-free seedlings, scion woods are harvested from STG-plants at 3 to 4 month intervals.

The healthy plants free of systemic pathogens can be regenerated from nucellar plants which have long juvenility. STG has several advantages, including quick production of healthy plants, and absence of juvenile characteristics. STG can

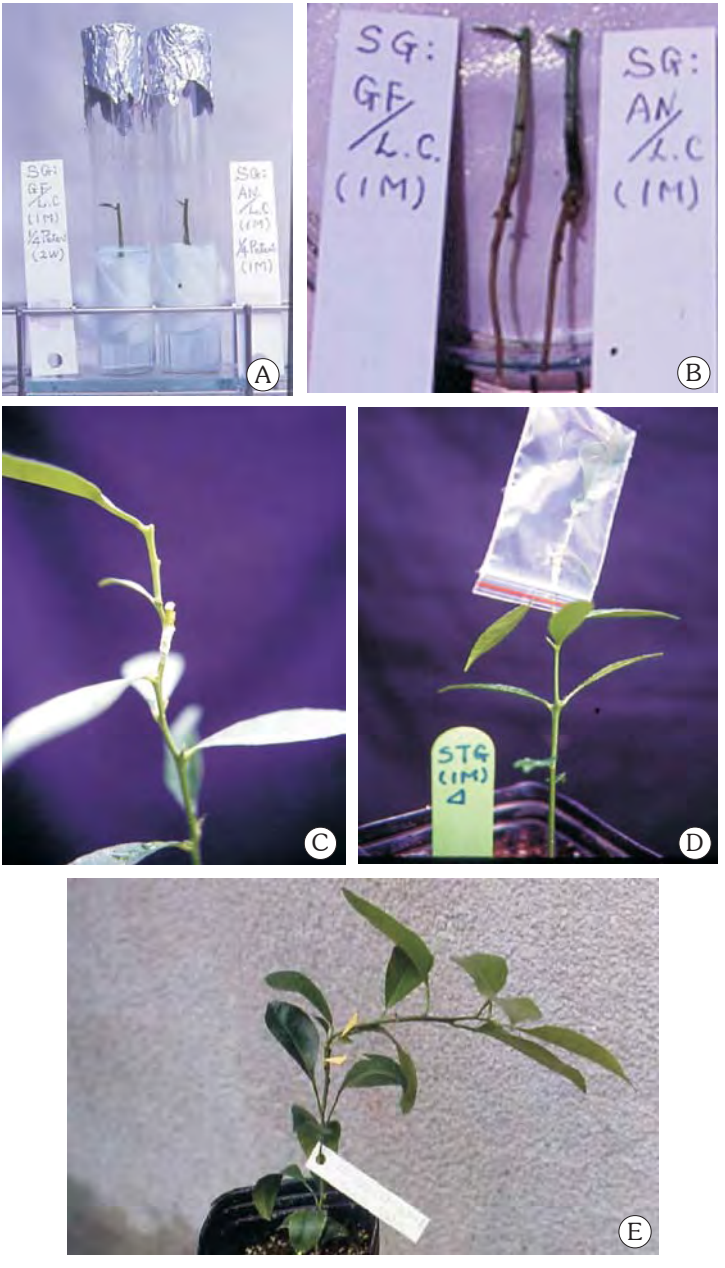

Fig. 29. Procedure of double grafting with micrografted rootstock seedlings as scion. (A) Sprouting of micrografted seedlings in test-tube culture. (B) Two STG seedlings with sprouts taken out from test tubes for secondary grafting. (C) A potted vigorous rootstock seedling side-grafted with a scion from the STG-seedling. (D) The grafted part of rootstock seedling covered with a mouth-sealed plastic bug. (E) A new mature twig grown from the double grafted rootstock three months after double grafting.

provide citrus producers with healthy plants free of such viruses as *Citrus tatter leaf capillovirus* and *Citrus exocortis viroid* that cannot be eliminated by heat therapy.

4.2. Pathogen-free foundation block

The STG-seedlings indexed to be free of the citrus viruses and HLBB serve as the certified pathogen-free foundation block and are located in the citrus foundation block repository (Fig. 30 A). An insect-proof screen-house, enclosed with stainless screen of 36 mesh is constructed with a double-door entrance and is surrounded by water canal to prevent entry of ants and mites (Fig. 30 A, B and C). The screen house is installed with air curtain on the first door. Concrete benches (200 cm length×100 cm width and 30 cm height) are constructed on gravel floor for keeping the PF foundation trees in containers (Fig. 30 D). The plants should be indexed periodically for their health status and fruit quality.

The foundation trees are propagated from the clean STG plants. Two to four plants are maintained per cultivar in general and propagated onto Troyer or Carrizor citrange. They are pruned every year and allowed to produce a few fruits for verification of horticultural characteristics and removal of off-types.

In Taiwan, the protected foundation blocks are maintained by a public agency. The National Repository of Pathogen-free Foundation Blocks is built in Chia-Yi Agricultural Experimental Station of Taiwan Agricultural Research Institute (CAES/TARI) (Fig. 30 A). The citrus foundation repository is located within paddy field of CAEA/TARI.

4.3. Production of pathogen-free saplings

A healthy citrus planting with pathogen-free (PF) saplings may outlive the grower. Healthy citrus trees have a great potential for sustainable high yield over many decades, provided appropriate health management is followed. Accordingly, production and cultivation of pathogen-free and high-quality nursery trees is considerably important. In a screen-house nursery, effective preventive measures to control root diseases caused by *Phytophthora* spp., nematodes, and bacterial canker disease also needs to be implemented.

Budwood increase blocks are established ahead of the production of PF citrus saplings. The blocks contain certified parent plants propagated by using budwoods from foundation trees and maintained in screen-houses (Fig. 30 E). Only a limited number of foundation trees are used for production of the budwood trees because of better indexing for vector-transmitted pathogen and inspection of off-types. These trees must be reindexed periodically, and used for the bud-supply up to three years to avoid reinfection and mutations in the propagated saplings. New budwood

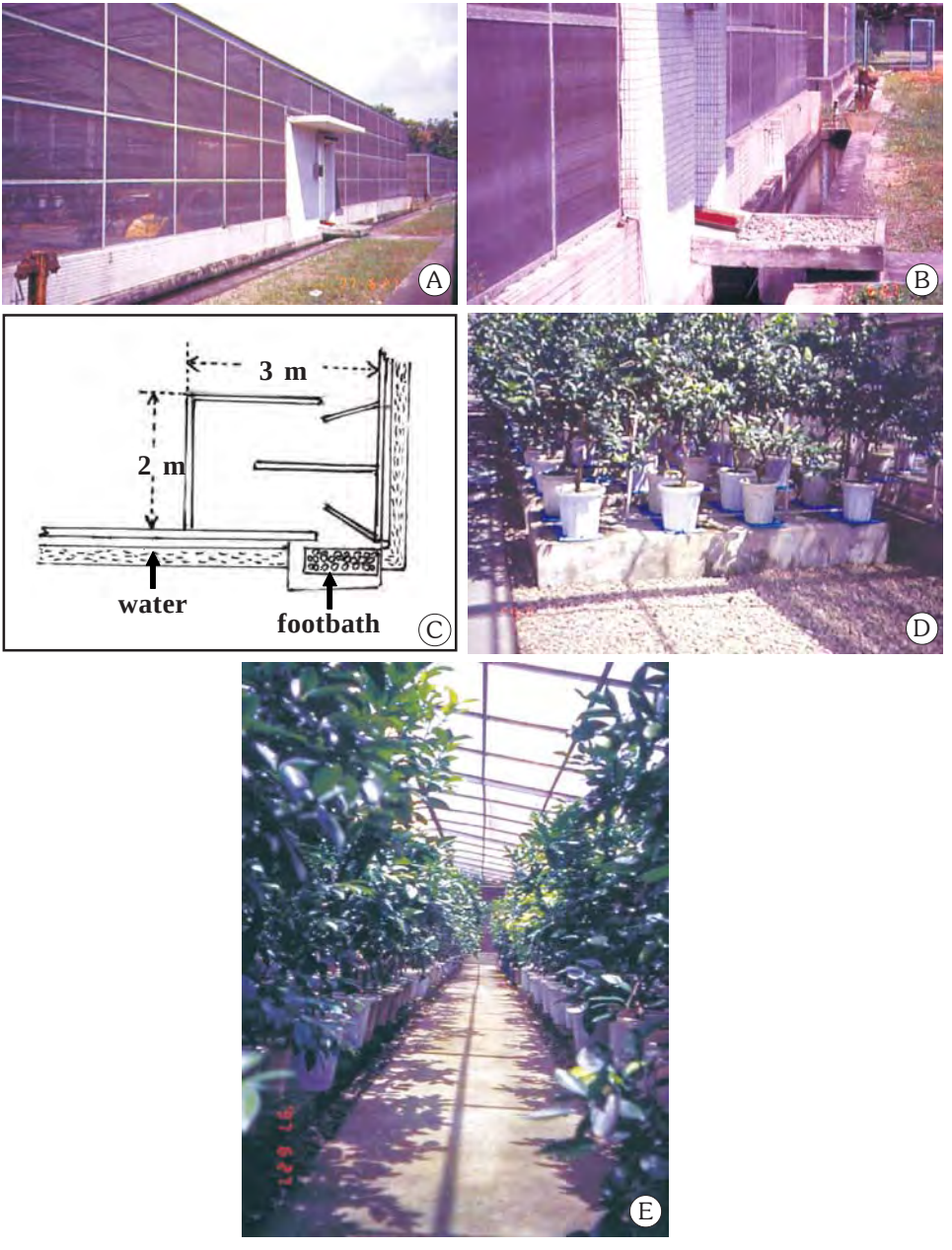

Fig. 30. National repository for maintaining certified pathogen-free citrus foundation blocks. (A) Insect-proof screen-house surrounded by water canal and with double doors. (B) Close-up picture of the entrance with double doors. (C) Drawing of an entrance with double door and footbath containing gravel mixed with copper sulfate solution. (D) Inside view of the screen-house showing healthy foundation trees on concrete benches on gravel floor. (E) Certified budwood increase trees grown in a screen-house attached to the foundation depository.

increase blocks must be periodically established with clean buds from foundation trees.

The PF citrus seedlings are produced in screen houses using budwoods from budwood increase blocks. Rootstock seedlings are grown from seeds of selected cultivars in seedling tubes (5 cm in diameter and 18 cm in height) or on seedbed of soil tank containing sterile pot-mixture. The rootstock seedlings are transplanted to perforated plastic containers (10 cm in diameter and 30 cm in height) for further cultivation when they grow up 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; Swingle citrumelo and pummelo for pummelo; and Valkamer lemon for mandarin and sweet orange.

4.3.1. Rapid propagation of pathogen-free saplings

The rapid propagation technique developed for producing PF citrus seedlings is detailed below and in Fig. 31.

1. Less than one-year old budwoods are collected from the budwood increase trees, defoliated and kept in a polythene bag to avoid drying.
2. Budwoods are sterilized in 1% sodium hypochlorite for 5 min, washed with tap water and kept in a sealed polythene bag after wrapping with tissue paper (Fig. 31 B). The packed budwoods may be stored in a refrigerator for two weeks for a later use.
3. Buds or scions are excised (Fig. 31 C and D) and grafted onto the rootstock at a height of about 25 cm-30 cm (Fig. 31, E-H).
4. The upper part of budded rootstock seedling is bent from just above the bud-union toward the opposite side to enforce sprouting of scion/bud (Fig. 31 I). Axiliary sprouts have to be removed periodically after grafting for enhancing rapid growth of the grafted bud or scions (Fig. 31 J and K).
5. It takes about 4 to 5 months after grafting to transplant the budded seedlings to the field (Fig. 31 L). The PF certified nursery trees are released to citrus growers after certification for freedom from diseases based on seedling samples randomly collected from each budline.

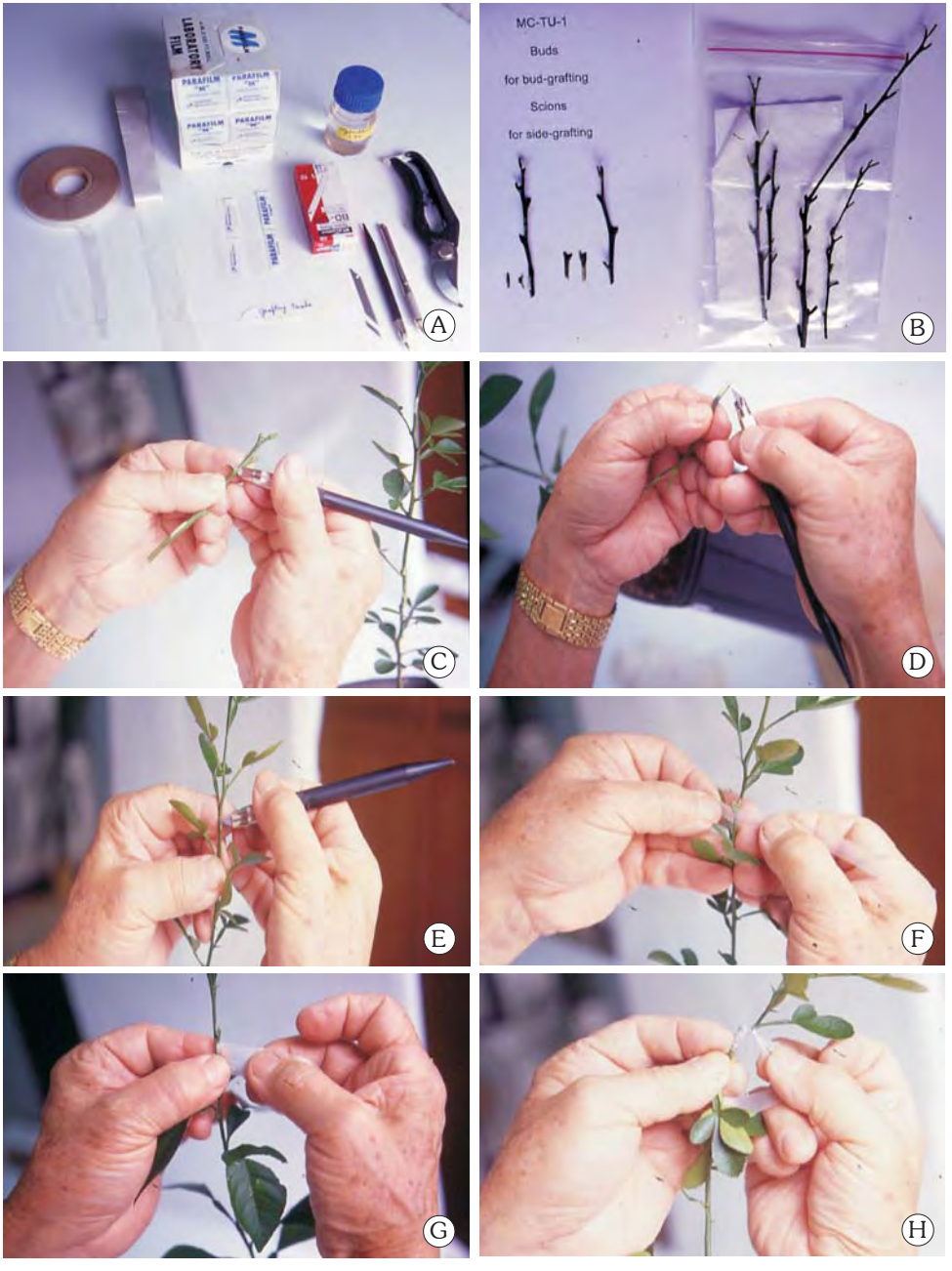

Fig. 31. Procedure of rapid propagation of PF citrus seedlings. (A) Tools of grafting including grafting knife (cutting blade and holder), parafilm, plastic band, pruning scissors and 1% Na-hypochlorite in bottle. (B) Clean budwoods and detached buds and scions for grafting. (C) Excising a bud from budwood. (D) Cutting a scion to be used for grafting. (E) Excising an opening site for grafting. (F, G & H) Procedure of fixing a bud or scion on graft site of rootstock with stretched band of parafilm.

(Contd)

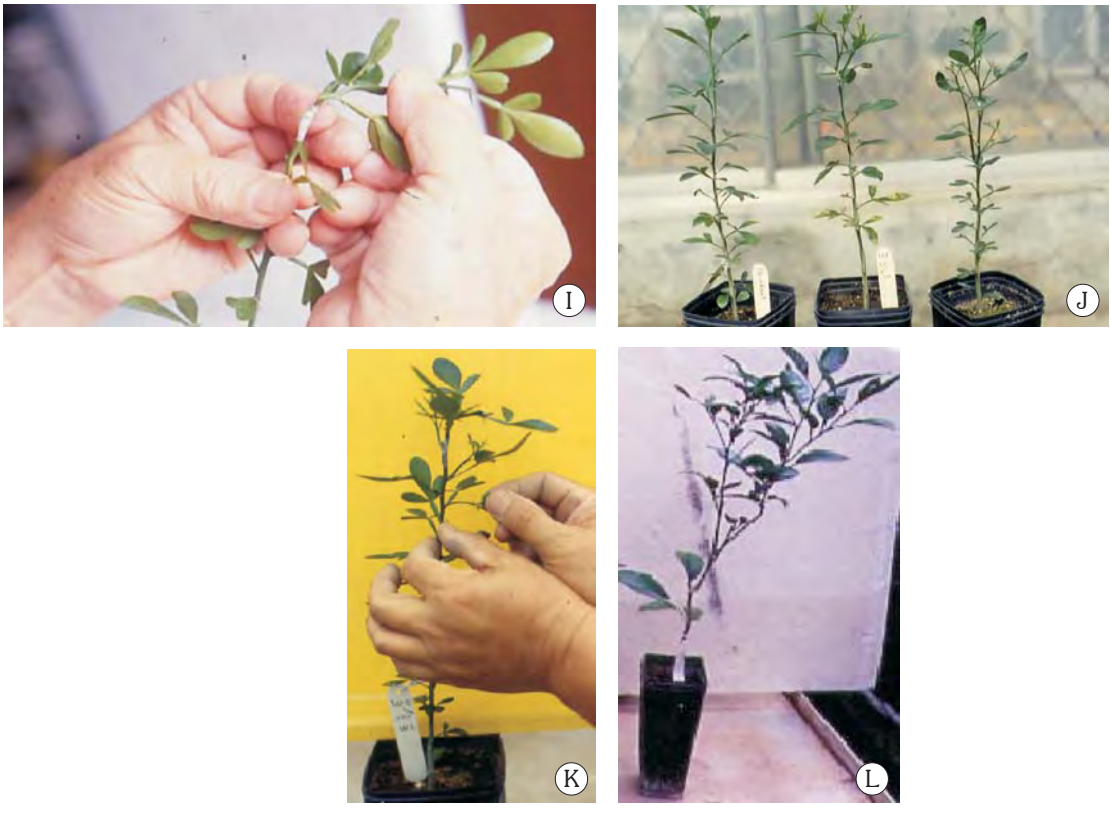

Fig. 31 (Contd). (I) Bending the upper part of rootstock seedling above the graft toward the opposite side. (J) New shoots come out from budded citrange seedlings. (K) Removing axiliary sprouts. (L) A well-grown budded nursery plant in container ready for transplanting to the field.

5. CULTIVATION AND HEALTH MANAGEMENT OF CITRUS SAPLINGS

5.1. Planting of pathogen-free seedlings in an orchard

PF citrus seedlings are best planted in a new area free of diseased citrus trees. However, they can also be planted in the existing citrus-cultivating areas after the diseased trees and alternative host plants are completely removed. The planting layout of PF seedlings comprises a quincunx system i.e. four seedlings at each corner of a square with the fifth one in the center. Plant spacing should be adjusted in relation to the species and cultivars of citrus, rootstock, and environmental factors. In general, the recommended plant spacing for sweet orange and mandarin (500 plants/hectare) is 5 meters between rows and 4 meters between plants; and 6 m × 6 m for pummelo and grapefruit (Fig. 32 A - C). A planting hole of about 100 cm diameter and 60 cm depth is dug on the planting bed (ridge) (Fig. 32 B and D). The topsoil and subsoil is hollowed out from the planting hole and placed separately to avoid mixing them with each other. An adequate amount of organic manure (10~20 kg) and dolomite or lime stone powder (1~2 kg), or super-phosphate lime (0.5~1 kg) are mixed with the dug soils (Fig. 33 A). The topsoil is returned to the bottom of the planting hole, and the subsoil is placed on the topsoil in the hole (Fig. 33 B - C). The PF seedling is planted in the center of the planting hole on elevated ridge (15 cm) (Fig. 33 D). Some growers cover the planting rows with rice straw after watering (Fig. 32 B).

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

The PF citrus trees can grow luxuriantly and begin fruiting as early as two years after transplanting by following appropriate health management and cultural practices such as watering, fertilization, and pruning (Fig. 34). Further information on the recommended horticultural practices has been detailed by Timmer and Duncan (1999) and Chang and Bay-Petersen (2003).

Health management of pathogen-free citrus seedlings in orchards needs to be properly performed using following strategies:

1. Prompt elimination of HLB-diseased citrus trees and alternative host plants to prevent reinfestation of healthy trees;

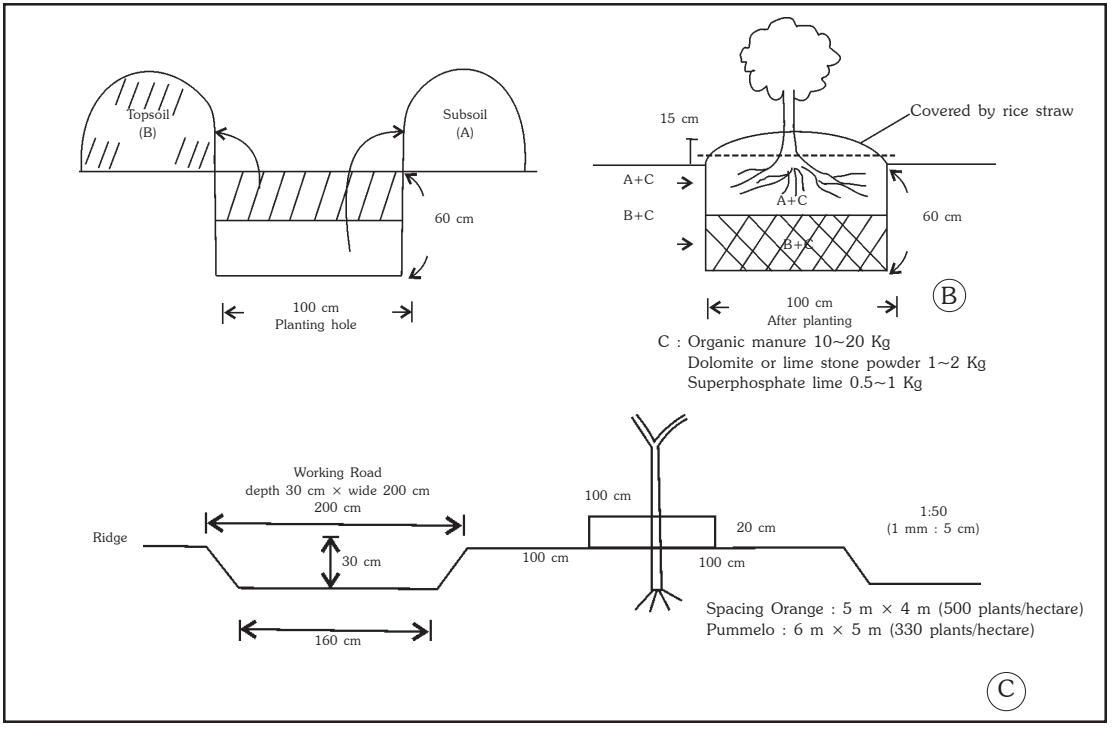

Fig. 32. (A) A view of planting field including ridges and canals for irrigation and drainage. (B) A profile view of a planting hole with topsoil and subsoil placed separately (left) and showing planting procedure (right). (C) Diagram showing measurements of planting ridge, working road, inter-row space, and planting space. (D) Digging a planting hole 100 cm wide × 60 cm deep.

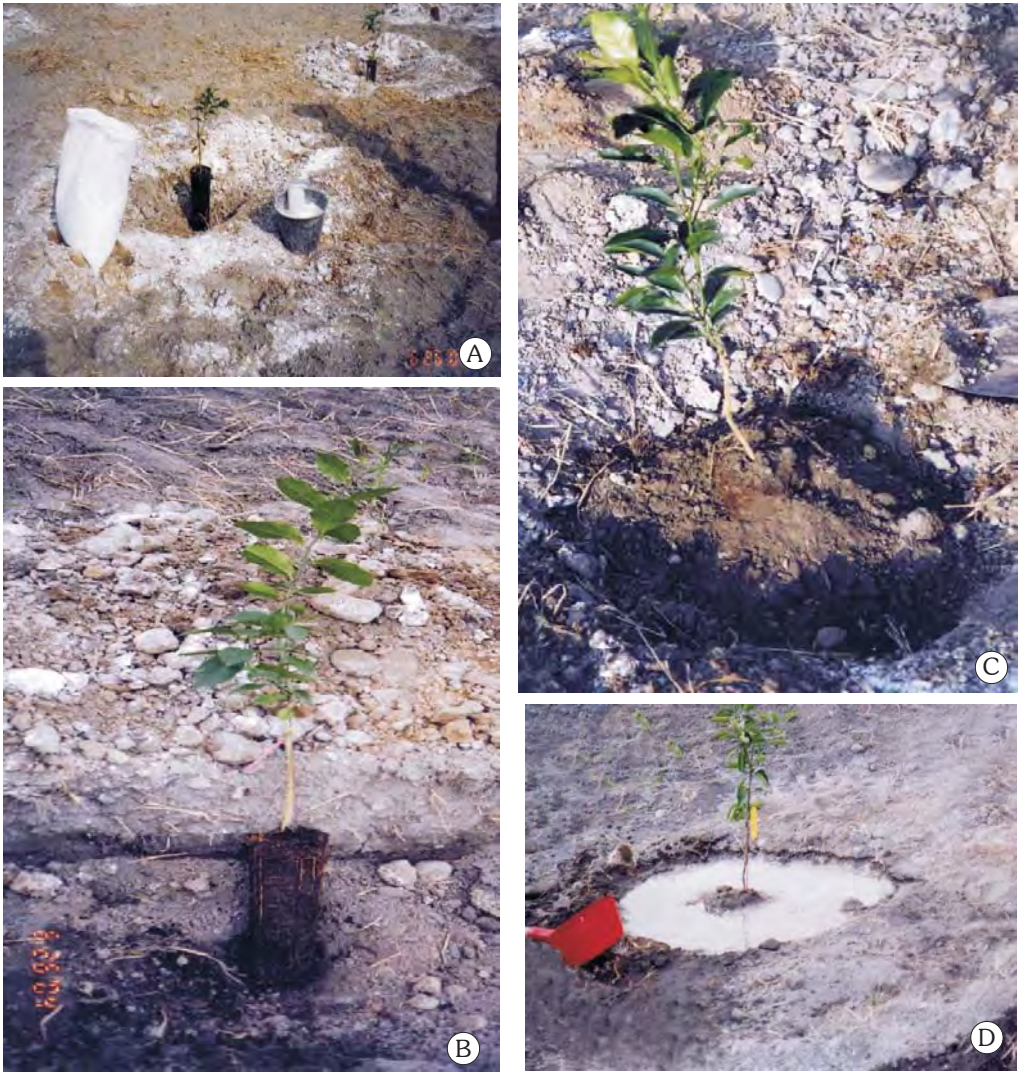

Fig. 33. Planting of PF citrus seedlings. (A) The dug out topsoil and subsoil placed around a planting hole to be mixed with manure and dolomite or lime stone powder. (B) Planting the seedling after taking off the plastic container. (C) Covering with enriched soil. (D) Watering transplanted seedling.

2. Protection of pathogen-free trees from vector transmission by effectively spraying insecticides such as Dimethod and Lannate at critical sprouting time, and biological control of the vector using natural enemies including *Tamarixia radiata* (Fig. 35);
3. Guarding the orchards by physical barriers such as wind breaker or distance barrier;
4. Pre-immunization of healthy foundation stock by protective mild strains of CTV against severer strains;
5. Chemotherapy of HLB-infected citrus trees: Tetracycline (Achromycin) infusion has been tested by some citrus growers using the antibiotic transfusion method of Su and Chang (1976). Recently, the efficacy of the antibiotic therapy has been greatly improved by use of an air-pressured injector (Fig. 36). Three applications (2 autumn, 1 spring) of 1,000 ppm Achromycin (2~4 L/tree) by air-pressured injector of 80 lbs have provided the best curing efficacy to diseased trees (Fig. 37). No HLB symptoms have reappeared in the injected trees, which are producing normal fruits. Pruning upper die-back branches improves the therapeutic efficacy. The antibiotic transfusion 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 works best for the HLB-affected large trees in which the diseased branches constitute not over half of the canopy and these are in the early stage of disease development.

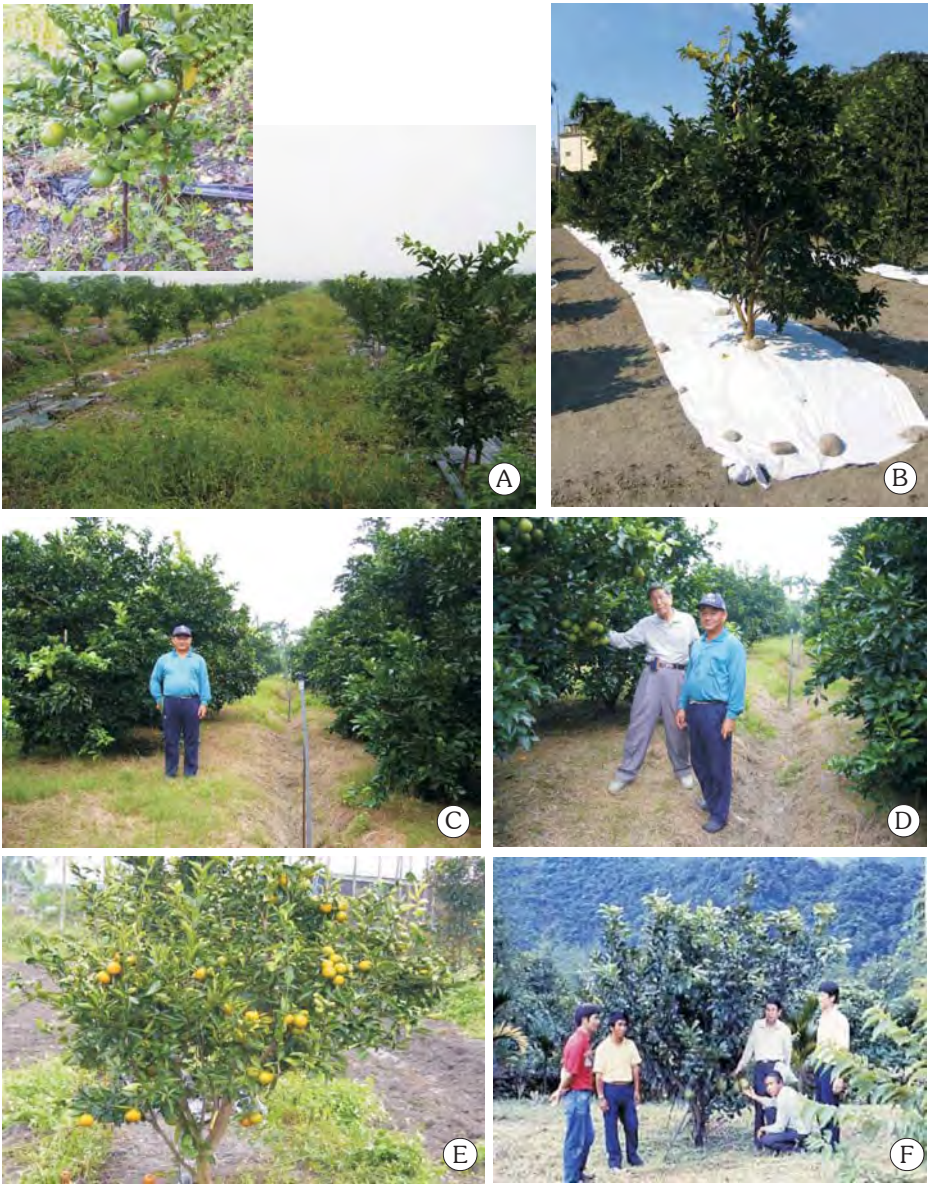

Fig. 34. Rehabilitation of citrus orchards planted with PF seedlings. (A) A large-scale orchard planted with PF sweet orange of Honjian cultivar one year after transplanting, and a 1-year old tree bearing fruits (upper left corner). (B) Vigorous growth of 2-year old sweet orange trees grown on water-proof polythene sheet (Tyrek) mulching ridge for improving sugar content of fruit. (C) Luxuriant growth of 3-year old sweet orange trees in orchard planted with PF seedlings. (D) Normal bearing of fruits of high yield on the 3-year old trees also showing the author and a grower. (E) A 2-year old PF tangor tree of Murcott bearing a large number of good quality fruits. (F) A 2-year old PF pummelo tree (Wentan) showing fruiting.

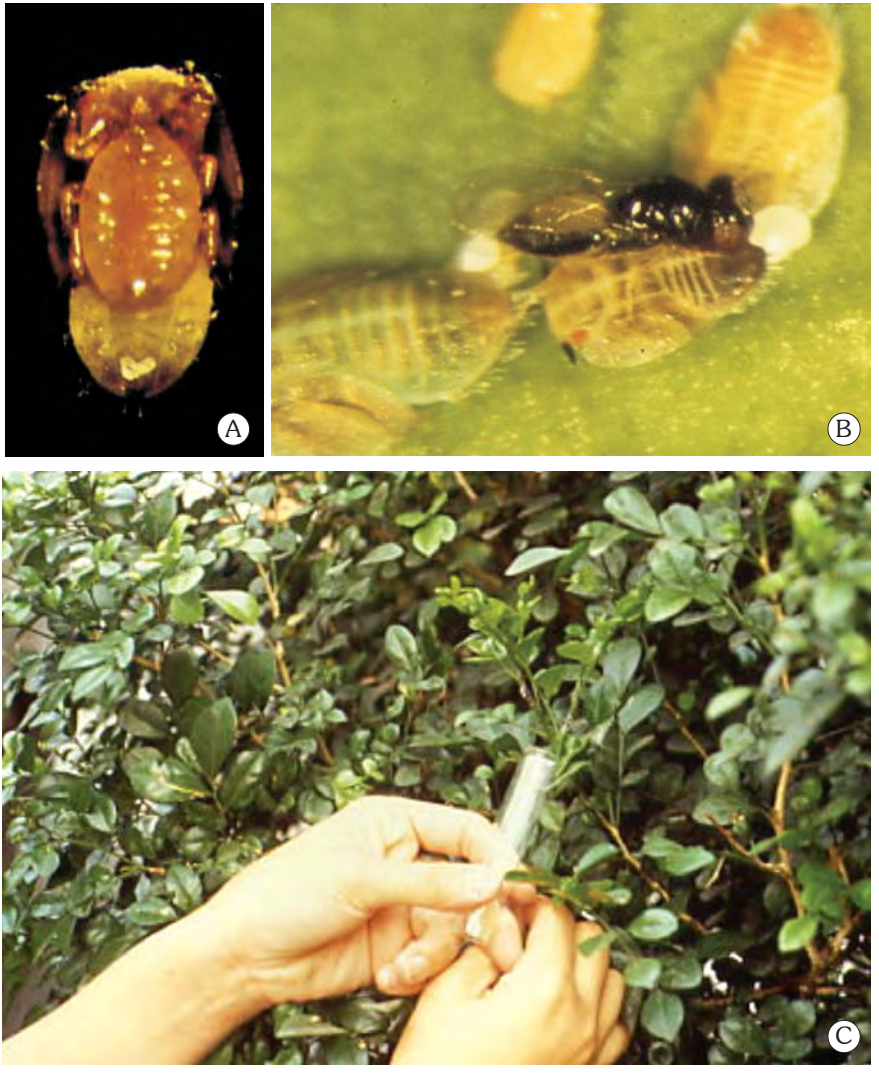

Fig. 35. Biocontrol of vector psylla/psyllids by a Eulophid wasp (Tamarixia radiata).
(A) Ectoparasitism of a wasp nymph on a psyllid. (B) A wasp adult laying egg on psyllid nymph. (C) Field releasing of cultured Eulophid wasps on jasmine orange shrub.

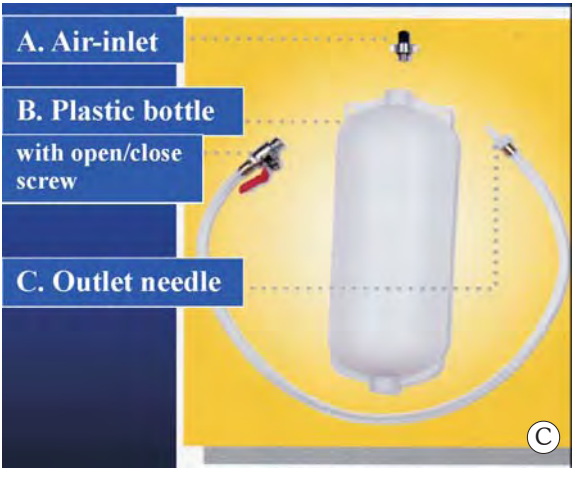

Fig. 36. Chemotherapy of HLB-diseased citrus tree by antibiotic injection. (A) Injection of 2~4 L of 1,000 ppm Achromycin with air-pressured injector near the basal portion of trunk. (B) A cordless battery-driven drill. (C) Diagram of air-pressured plastic injector.

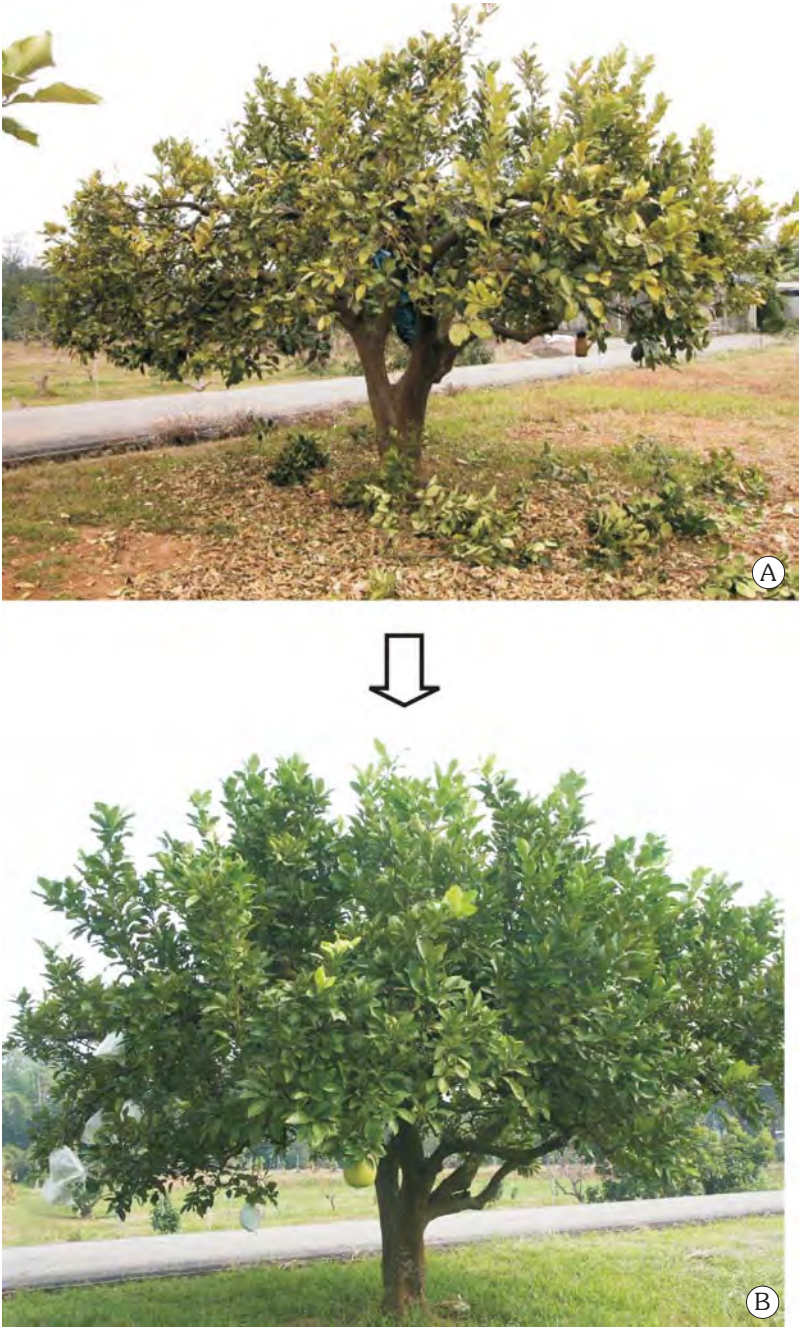

Fig. 37. Recovery of HLB-affected pummelo tree injected with Acromycin. (A) HLB-affected pummelo tree with yellow mottling symptoms before injection. (B) Acromycin treated tree showing normal growth.

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Asia-Pacific Consortium on Agricultural Biotechnology

The Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB), was established in 2003 under the umbrella of the Asia-Pacific Association of Agricultural Research Institutions (APAARI)— an initiative of Food and Agriculture Organization that has been promoting appropriate use of emerging agri-technologies and tools in the region.

APCoAB's mission is "To harness the benefits of agricultural biotechnology for human and animal welfare through the application of latest scientific technologies while safeguarding the environment for the advancement of society in the Asia-Pacific Region".

APCoAB's main thrust is:

- To serve as a neutral forum for the key partners engaged in research, development, commercialization and education/learning of agricultural biotechnology as well as environmental safety in the Asia-Pacific region.
- To facilitate and promote the process of greater public awareness and understanding relating to important issues of IPR's *sui generis* systems, biosafety, risk assessment, harmonization of regulatory procedures, and benefit sharing in order to address various concerns relating to adoption of agricultural biotechnology.
- To facilitate human resource development for meaningful application of agricultural biotechnologies to enhance sustainable agricultural productivity as well as product quality, for the welfare of both farmers and consumers.

ASIA-PACIFIC ASSOCIATION OF AGRICULTURAL RESEARCH INSTITUTIONS

The Asia-Pacific Association of Agricultural Research Institutions (APAARI), established in 1990 at the initiative of FAO, is an apolitical, neutral, non-profit forum of Agricultural Research Institutions, National Agricultural Research Systems (NARS) in the Asia-Pacific region, in the pursuit of common objectives.

The 'Mission' of APAARI is to promote the development of national agricultural research systems in the Asia-Pacific region through facilitation of intra-regional and inter-institutional, and international co-operation/ partnership.

The overall objectives of APAARI are to foster agricultural research for development in the Asia-Pacific region so as to help address the concerns of hunger, poverty, environmental degradation and sustainability of agricultural production. More specifically, the objectives are as follows:

a. Promote the exchange of scientific and technical know-how and information in agriculture;

b. Encourage the establishment of appropriate co-operative research and training programs in accordance with identified regional, bilateral or national needs and priorities;

c. Assist in prioritizing NARS/Regional needs, strengthening of research organizational and management capabilities of member institutions including information and communication technology;

d. Strengthen cross-linkages among national, regional and international research centers and organizations, including universities, through involvement in jointly planned research and training programs; and

e. Promote collaborative research among member institutions including need based support to regional research networks.