Report

on

Survey of Greening Disease and Its Transmitting Insect, Citrus Psylla

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Introduction

Huanglongbing (HLB), previously known as citrus greening is a devastating disease of citrus. It affects all citrus cultivars and causes rapid decline of trees. HLB is caused by a phloem limited fastidious bacterium, *Candidatus* Liberibacter a gram negative bacterium belonging to alpha proteabacteria (Jagoueix *et al.,* 1994). Until now, at least 3 species of *Candidatus* Liberibacter have been reported to be associated with HLB. The species *Ca* L. africanus causing African HBL and *Ca* L. asiaticus causing Asian HLB have been described previously (Jagoueix *et al.,* 1997). Recently, *Ca* L. americanus has been named for the causal bacterium of HLB in Brazil (Chung and Brlansky, 2005). The Asian HLB is the heat tolerant type, as symptom occurs at temperature up to 35°C while the African HLB induces symptom under cool condition at 20-25 °C (da Graca, 1991).

The early symptoms of HLB are a leaf yellowing that usually starts from one branch or one part of the tree. Hence, the Chinese name Huanglongbing (yellow dragon) is descriptive of this symptom (Jagoueix *et al.,* 1997). The leaf symptoms include a blochy mottle, interveinal chlorosis of leaf blade, island of greening tissue, narrow and upright of new leaves which are similar to zince deficiency symptom. Fruit are small, and abnormal in appearance (lopsided) with aborted seeds, and have a bitter taste. Many fruit fall prematurely and those remain on the tree do not color properly. Affected trees at advance stage of disease show dieback of twig ad gradually decline. In China the disease was reported to kill young tree within 1-2 year (da Graca, 1991).

Distributions of HLB are through a contaminated citrus propagation material and through transmission by psyllid vectors. Two species of psyllid are involved in spread of HLB. *Diaphorina citri* Kuwayama, the Asian citrus psylla, and *Trioza erytreae* occurring in Africa were reported to transmit Asian HLB and African HLB, respectively (da Graca, 1991).

HLB has been reported to affect citrus productions world wide including Asian, Africa, the Indian subcontinent and the Arabian Peninsula. Recently, it was discovered in Brazil and Florida in 2004 and 2005, respectively (Texeira *et al.,* 2005 and Chung and Brlansky, 2005). The disease was found for the first time in Iriomote, the Southernmost island in Japan, in 1988 (Miyakawa and Tsuno, 1989). Since then, the disease has spread northword, posing a great threat to the main production area. Earlier, HLB was reported to destroy citrus trees in Thailand in 1973 (Schwarz, 1973) and the disease is still continue to involve in the death the tree until now.

In this report, Prince of Songkla University and Tokyo University of Agriculture collaborated on the survey of HLB and its psyllid vector in Chiang Mai, the main citrus production area in the north and some provinces in the south of Thailand. This was to evaluate the condition of HLB in Thailand interm of disease management.

Materials and Methods

1. Survey of citrus greening disease and its psyllid vector

1.1 Thailand

The greening disease and the vector survey in Thailand was conducted in the north (Chiang Mai province) and the south (Krabi and Songkla provinces). Two surveys were made in citrus orchards at Pongnoi and Panda research station, Royal Project Foundation, Chiang Mai while one survey was carried out at Krabi and Songkla. In the south the survey was made in commercial orchard in Krabi and in Prince of Songkla University experimental plot, Songkla. Citrus trees were examined for psyllid vector and diseased symptoms including intervienal chlorosis and mottle leaves, yellow shoot, dieback of twig or limb and decline. Leaf samples of citrus trees were randomly or selectively collected from each orchard or plot. The samples were then assayed by PCR for the presence of greening bacterium. Total DNA was extracted from leaf sample using CTAB method described by Dellaporta *et al.* (1983). The greening bacterium DNA was selectively amplified by primers specific to 16S rRNA gene of the bacterium (Sdoodee *et al.,* 1999). PCR amplified products were determined by 1% agarose gel electrophoresis.

1.2 Japan

Field diagnosis of greening disease based on symptoms and spyllid vector examination were performed at citrus orchard in Okinawa on November 29, 2007 during the visit to Laboratorys of Tropical Horticulture and Tropical Plant Protection, Tokyo University of Agriculture, November 20 – December 1, 2007.

2. DNA sequence analysis of citrus greening bacterium 16S rRNA gene

Greening bacterium DNA (16S rRNA gene) from Honey tangor collected from PSU, Songkla was amplied by PCR and the PCR DNA was sequenced using automated sequencer, ABI prism, Applied Biosystems 3130XL genetic Analyzer, Hitachi (Fig 1 A, B, C) according to manufactor instruction. DNA sequence analysis were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). The sequence analysis was performed by research team from Laboratory of Plant Protection, Tokyo University of Agriculture and Prince of Songkla University under supervision of Prof. Keiko Natsuaki (Fig 1D).

Result and discussion

1. Survey of greening disease and its psyllid vector

1.1 Thailand

North (Chiang Mai)

Results from PCR (Fig 2) test indicated that 19/67 (28%) citrus sample collected (Fig 3) from Royal Project Foundation research station (Pongnoi and Pangda) were infected with greening bacterium. The citrus greening infected varieties found at Royal project research station including mandarin cv. King, Ponkan, Okisu, Matsuyama wase, and Sugriyama, sweet orange cv. Valencia, F11 Navel and Kumquat (Table 1 and 2). Incidence of greening disease was common in Ponkan mandarin at Pangda station (Table 2). Symptoms on the diseased trees were similar to that reported elsewhere (Chung and Brlanky, 2005; da Graca, 1991; Miyakawa and Tsuno, 1989 and Sdoodee *et al*., 1999) including yellow shoot, interveinal chlorosis of leaves and decline (Fig 4). Although 28% of collected sample was found to be infected by greening bacterium, this could be a source of inoculum for further spread by psyllid vector because the vectors were also found at the same plot (Fig 3C, D). In addition more of the decline trees were found at Pangda and Pongnoi research station (Fig 5) for the second survey on January, 2007. Moreover, incidence of greening diseases was also notice as determine by symptoms in commercial orchard at citrus groove, Fang district (Fig 6 and 7).

South (Krabi and Songkla)

Citrus greening disease was detected in Honey (Shogun) tangor at commercial orchard in Krabi province (Table 3). However, the incidence was low in the commercial orchard comparing to the disease found at Prince of Songkla university (PSU) experimental plot (Table 3). This plot was also infested by psyllid vector. At least 4 varieties of citrus grown in the same area at PSU were found to be infected with greening as assayed by PCR (Fig 8, 9) including Honey tangor, Indian lime, Pomelo, Somjuke tangerine and Trifoliata orange (Table 3). The low incidence of citrus greening disease in commercial orchard could be related to heavily spray of insecticide. Interestingly, citrus greening bacterium was detected by PCR in jasmine orange grown at Prince of Songkla university (Fig 8, 10). The jasmine orange was reported to be the preference host plant of psyllid vector (*Diaphorina citri*) (Halbert and Manjunath, 2004). The vectors was also found to propagate on the infected jasmine orange (Fig 10).

Monitoring infestation of *D. citri* in Sumjuke tangerine plot was previously conducted at PSU Klong Hoi Klung research station, Songkla. It was found that infestation of *D. citi* was related to flushing of citrus trees which was induced by rainfall (Fig 11)

1.2 Okinawa, Japan

Survey on the citrus greening based on symptom only was also conducted a commercial orchard in Okinawa during November 29, 2007 (Fig 12). The greening affected Shekwasha (*Citrus depressa* Hayata) was observed in citrus orchard at Tominato and Yako. The diseased citrus tree had declined and dieback (Fig 12A) and the new shoot showed yellow leaves (Fig 12B). The nymphs of *D. citri* were also found on the young shoot of Shewasha (Fig 12C). In addition, jasmine orange hedge

grown near by Shekwasha orchard at Yako revealed chlorosis leaves similar to greening symptom (Fig 10D).Moreover, research team of Okinawa Prefectural Agricultural Research Center has conducted experiment on natural infection of *C. depressa* by psyllid vector at Tominato (Fig 12E). Currently, greening infected Shekwasha bud woods from Okinawa were graft inoculated on healthy seedling by research team, National Institute of Fruit Tree Science, Tsukuba for further study on greening disease in Japan (Fig 13).

2. DNA sequence analysis of citrus greening bacterium 16S rRNA gene

The complete sequence of greening bacterium (*Ca* Liberibacter asiaticus) 16S rRNA gene, Thai isolate from Honey tangor was obtained as shown in below. Comparison of greening bacterium 16S rDNA sequence between Thai isolate from Honey tangor and Okinawa isolate resulted in 100% identity and similarity. The result was similar to that reported by Subandiyah *et al.* (2000). Therefore, citrus greening disease in Okinawa and in the south of Thailand could possibly caused by the same strain of greening bacterium.

16S rDNA sequence of citrus greening, Thai isolate

1	GACTTCGCAA	CCCATTGTAA	CCACCATTGT	AGCACGTGTG	TAGCCCAGCC
51	CATAAGGGCC	ATGAGGACTT	GACGTCATCC	CCACCTTCCT	CCGGCTTATC
101	ACCGGCAGTC	CCTATAAAGT	ACCCAACATC	TAGGTAAAAA	CCTAAACTTG
151	ATGGCAACTA	GAGGCAGGGG	TTGCGCTCGT	TGCGGGACTT	AACCCAACAT
201	CTCACGACAC	GAGCTGACGA	CAGCCATGCA	GCACCTGTGT	AAAGGTCTCC
251	GAAAAGAAAA	TACCATCTCT	GATATCGTCC	TATACATGTC	AAGGGCTGGT
301	AAGGTTCTGC	GCGTTGCATC	GAATTAAACC	ACATGCTCCA	CCGCTTGTGC
351	GGGCCCCCGT	CAATTCCTTT	GAGTTTTAAT	CTTGCGACCG	TACTCCCCAG
401	GCGGAGTGCT	TAATGCGTTA	GCTGCGCCAC	TGAATGGTAA	ACCACCCAAC
451	AGCTAGCACT	CATCGTTTAC	GGCGTGGACT	ACCAGGGTAT	CTAATCCTGT
501	TTGCTCCCCA	CGCTTTCGCG	CCTCAGCGTC	AGTATCAGGC	CAGTGAGCCG
551	CCTTCGCCAC	CGGTGTTCCT	CCGAATATCT	ACGAATTTCA	CCTCTACACT
601	CGGAATTCCA	CTCACCTCTC	CTAAACTCTA	GACAACCAGT	ATTAAAGGCA
651	GTTCCAAGGT	TGAGCCCTGG	GATTTCACCT	CTAACTTAAT	CGCCCGCCTA
701	CGCGCCCTTT	ACGCCCAGTT	ATTCCGAACA	ACGCTCGCCC	CCTTCGTATT
751	ACCGCGGCTG	CTGGCACGAA	GTTAGCCGGG	GCTTCTTCTC	CGAATACCGT
801	CATTATCTTC	TCCGGCGAAA	GAGCTTTACA	ACCCTAAGGC	CTTCTTCACT
851	CACGCGGCAT	GGCTGGATCA	GGGTTGCCCC	CATTGTCCAA	TATTCCCCAC
901	TGCTGCCTCC	CGTAGGAGTC	TGGGCCGTGT	CTCAGTCCCA	GTGTGGCTGA
951	TCGTCCTCTC	AGACCAGCTA	TAGATCGTAG	CCTTGGTAGG	CTCTTACCCT
1001	ACCAACTAGC	TAATCCAACG	CAGGCTCATC	TCTCTCCAAT	AAAATCTTTC
1051	CCCCAATAGG	GCGTATACGG	TATTAGCACA	CGTTTCCATG	CGTTATCCCG
1095	TAGAAAAAGG	TAGATTCCTA	CGCGTTACTC	ACCCGTCTGC	CGCTC

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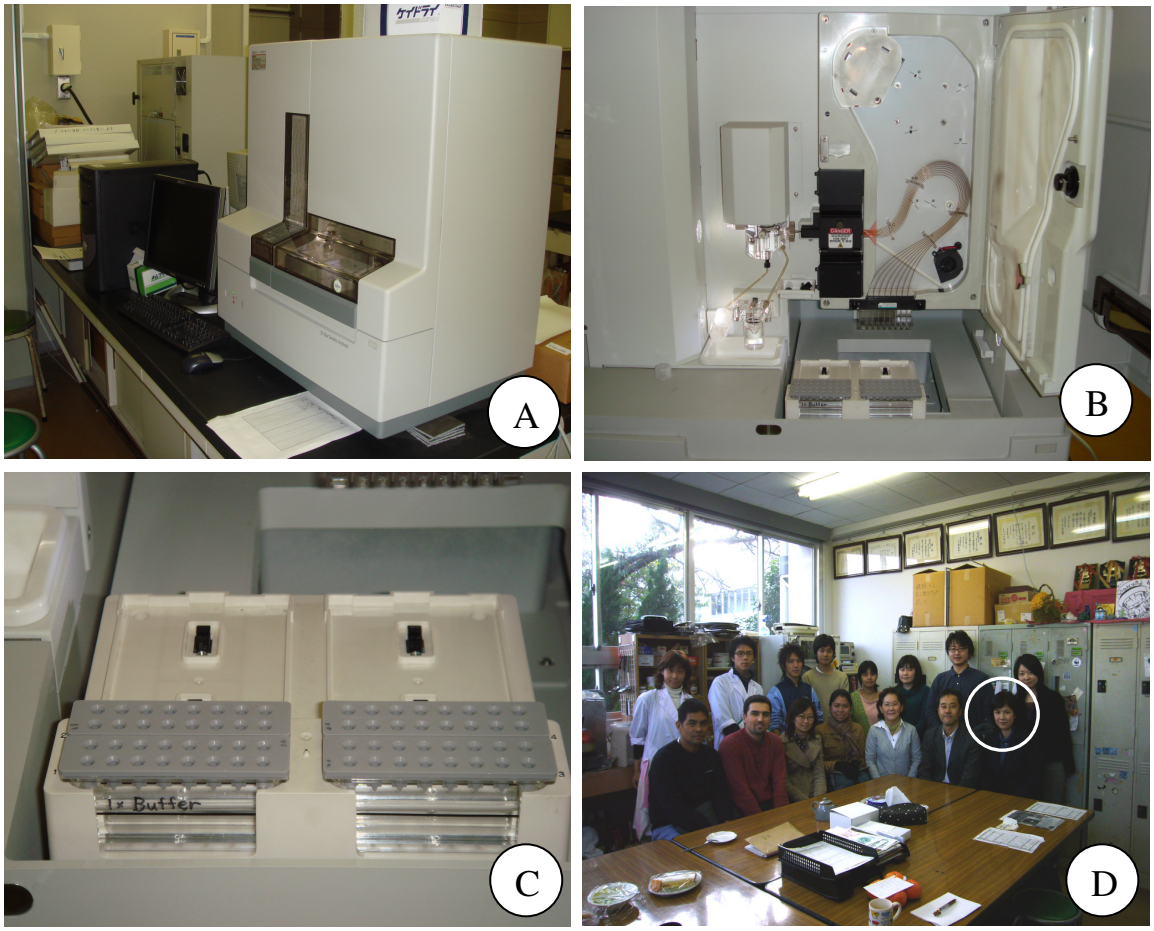

Fig 1. A, B, C Automated DNA sequencer at Tropical Plant Protection Laboratory, Tokyo University of Agriculture
D Research team at Tropical Plant Protection Lab Lead by Professor Keiko Natsuaki

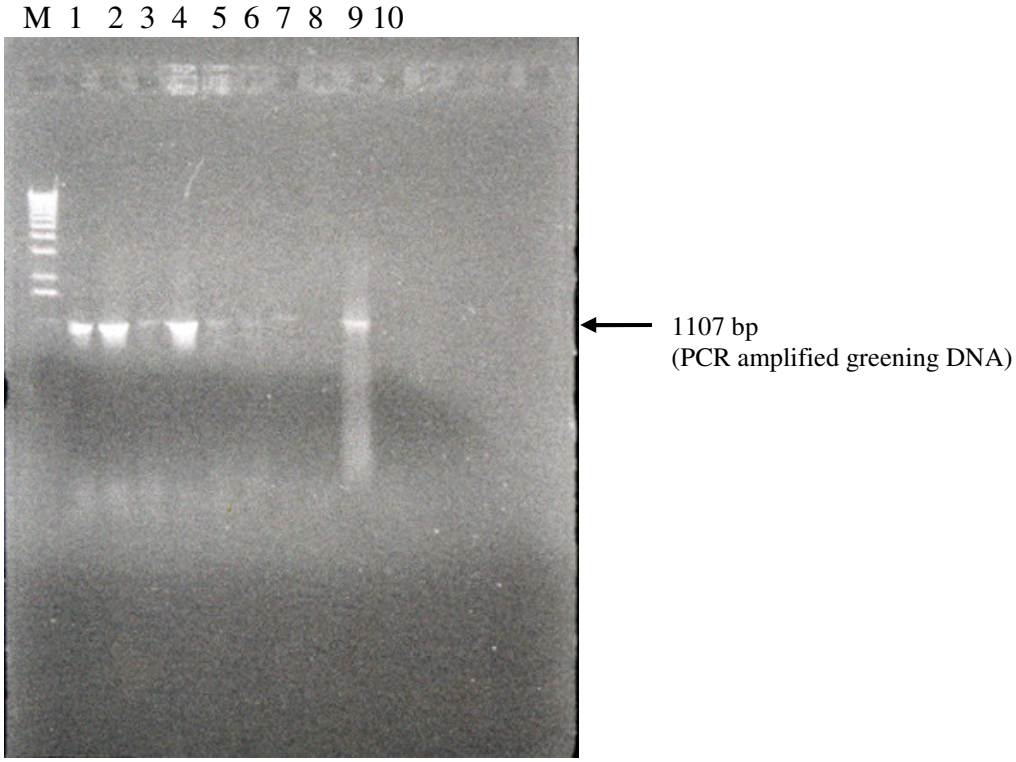

Fig. 2 Greening DNA amplified by PCR using primers specific to 16S rRNA gene from citrus sample collected from Royal Project Foundation research station, Chiang Mai.

Lane M	1 kb marker (GIBCO, BRL)
Lane 1, 2, 3	Ponkan mandarin, Panda station
Lane 4	Matsuyama wase mandarin, Pongnoi station
Lane 5, 6, 7, 8	F11 Navel orange, Pongnoi station
Lane 9	Greening DNA (positive control)
Lane 10	Deionized water (negative control)

Fig 3. Survey of citrus greening disease and its psyllid vector at Royal project foundation research station in Chiang Mai, Thailand.

A Field diagnosis based on symptoms

B Sample collection for assaying greening pathogen

C Professor Shuichi Iwaori examined for psyllid vector

D Nymph of psyllid (*Diaphorina citri*) vector on citrus young shoot

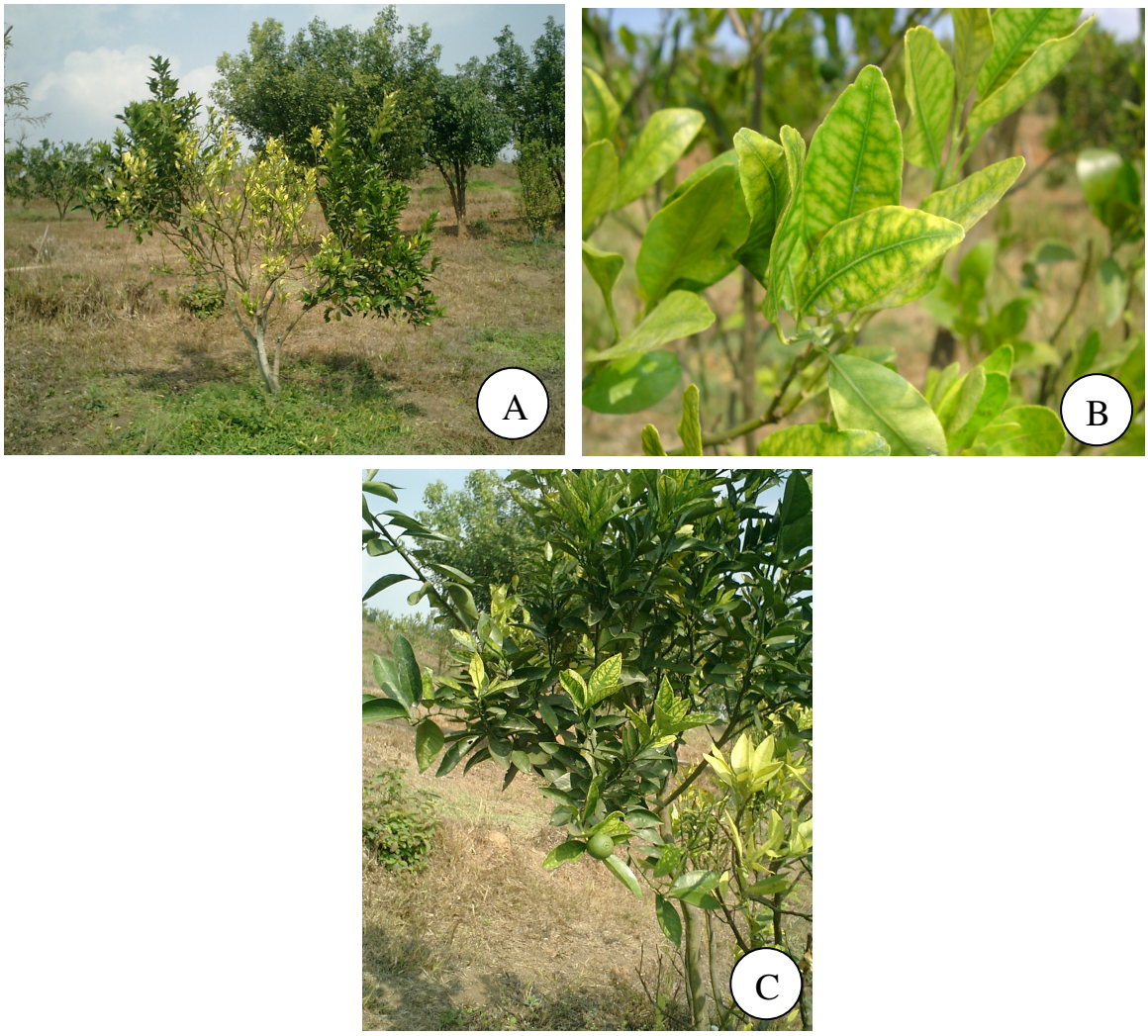

Fig 4. Greening infected citrus tested by PCR at Royal Project Foundation Research Station.

A Valencia sweet orange showing yellow shoot, Pongnio station

B Ponkan mandarin showing interveinal chlorosis symptom at Pangda station

C Interveinal chlorosis on Valencia infected leaves

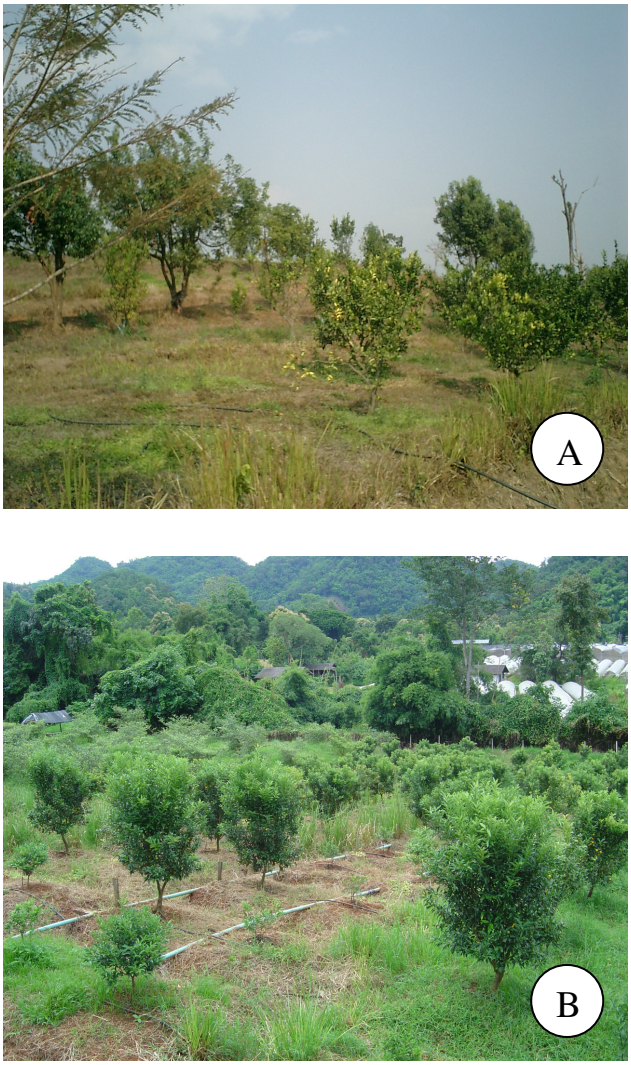

Fig 5. Greening infested orchards at Royal Project Foundation.
A Pongnoi station
B Pangda station

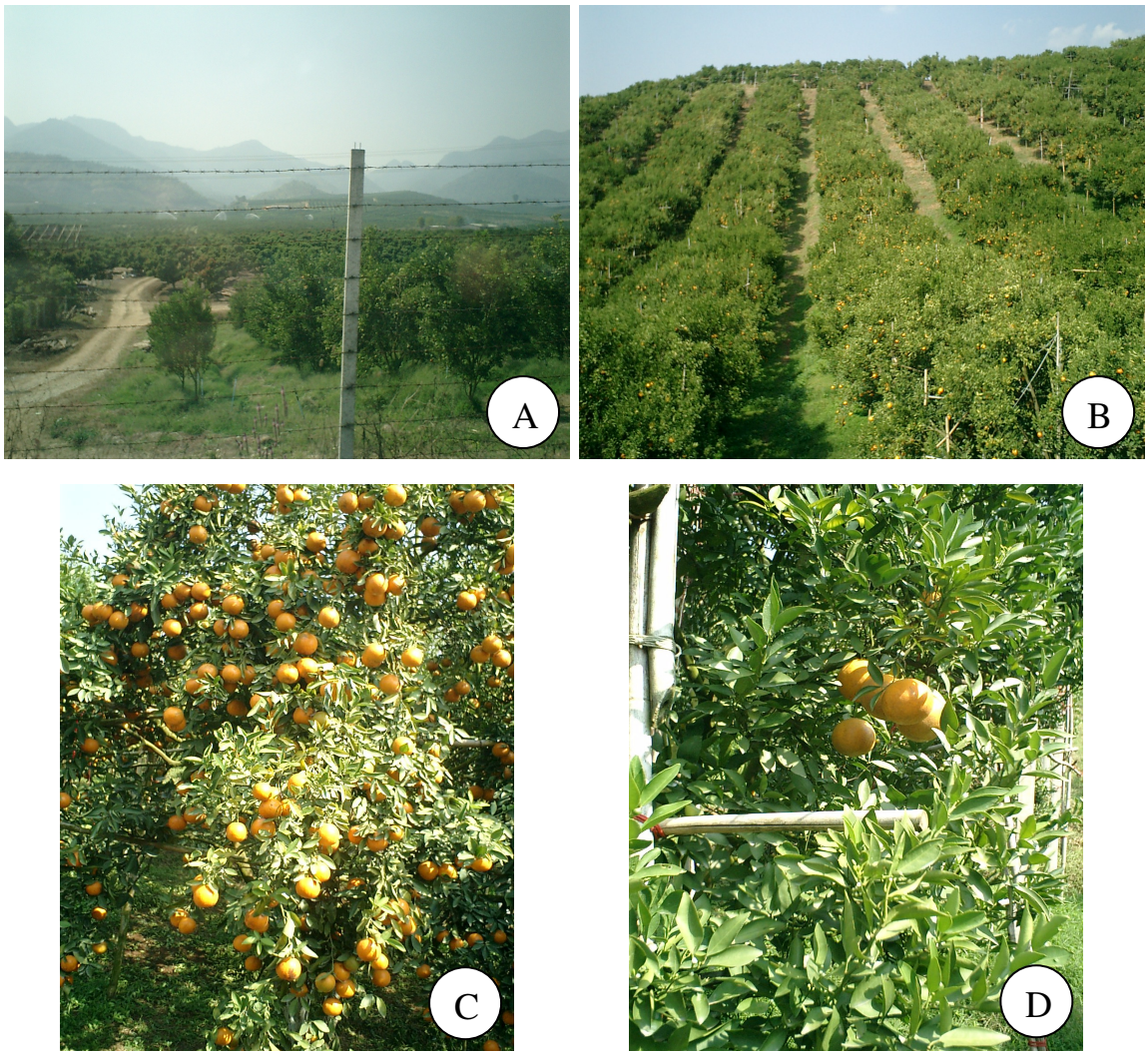

Fig 6. A Citrus groove at Fang District, Chiang Mai
B Tanatorn orchard
C Kaewan mandarin
D Honeydew (Shogun) mandarin

Fig 7. A Ocean mandarin, Tanatorn orchard, Chiang Mai
B Ocean mandarin showing typical greening symptom

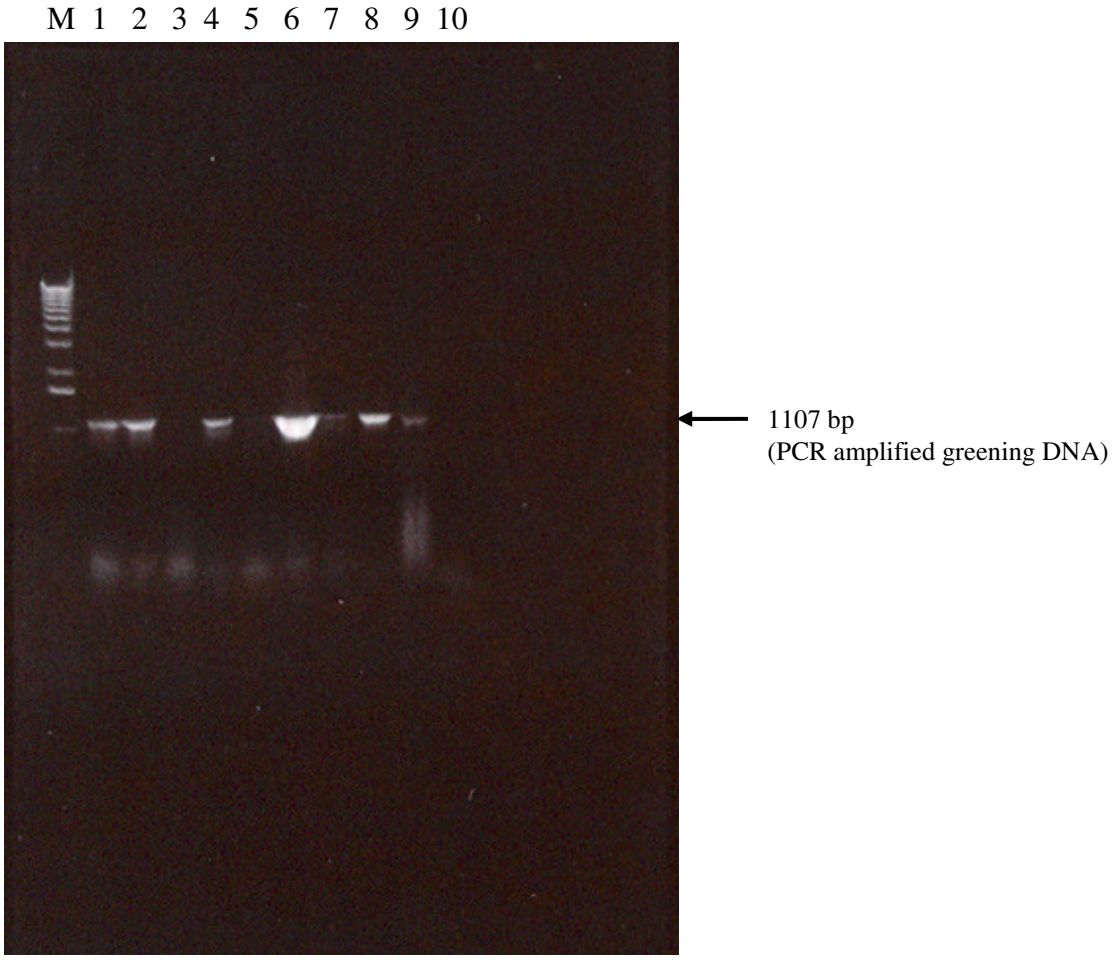

Fig 8 Greening DNA amplified by PCR using primers specific to 16S rRNA gene from citrus and jasmine orange collected from Prince of Songkla University, Hat Yai.

Lane M	1 kb marker (GIBCO, BRL)
Lane 1, 2,	Pomelo
Lane 3, 4, 5	Jasmine orange (Murraya paniculata)
Lane 6, 7, 8	Somjuke tagerine
Lane 9	Greening DNA (positive control)
Lnae 10	Deionized water (negative control)

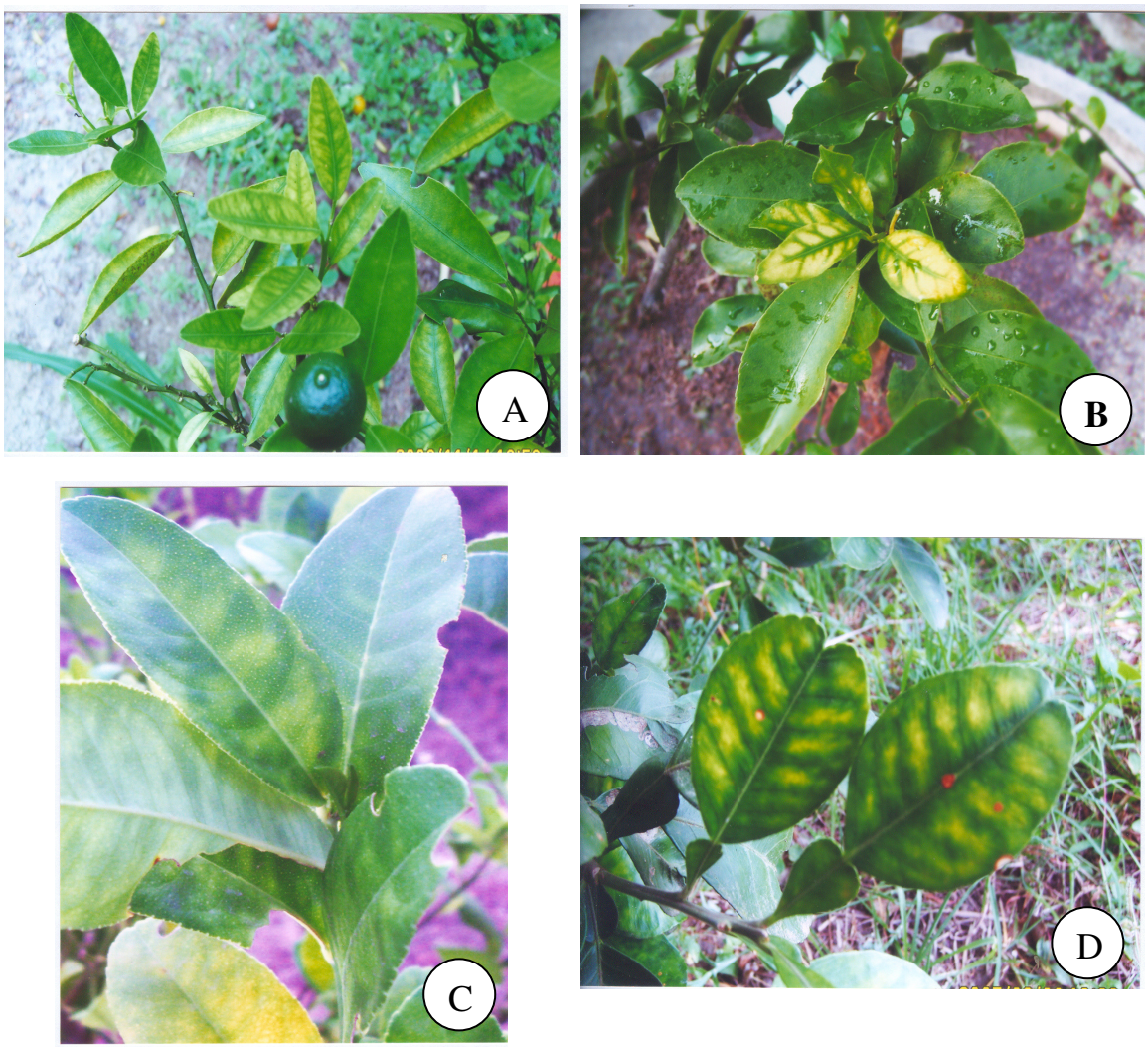

Fig 9. Citrus greening disease found in Songkhla province, South Thailand.
A Honey dew (Shogun) mandarin
B Somjuke tangerine
C Indian or Mexican lime
D Pomelo

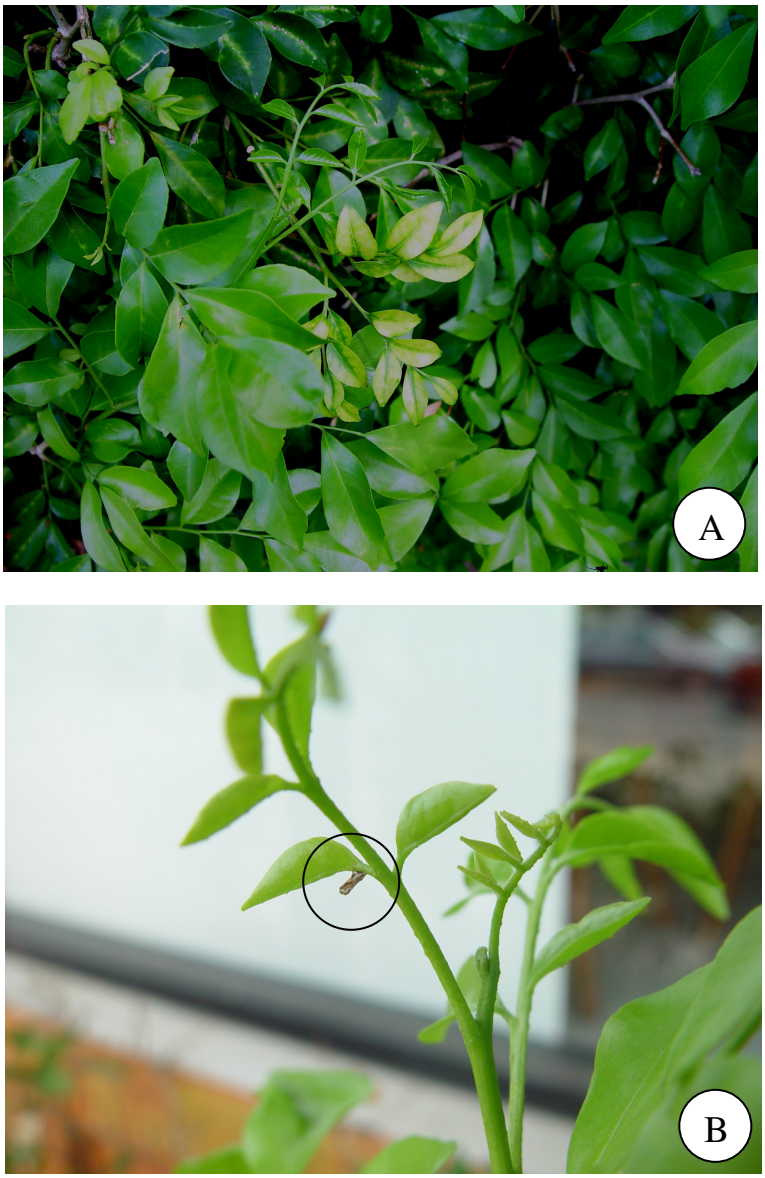

Fig 10. A Jasmine orange (*Murraya paniculata*) infected with greening bacterium as tested by PCR showing yellow leaves with green veins in Hat Yai, Thailand

B Psyllid vector (*Diaphorina citri*) found on jasmine orange

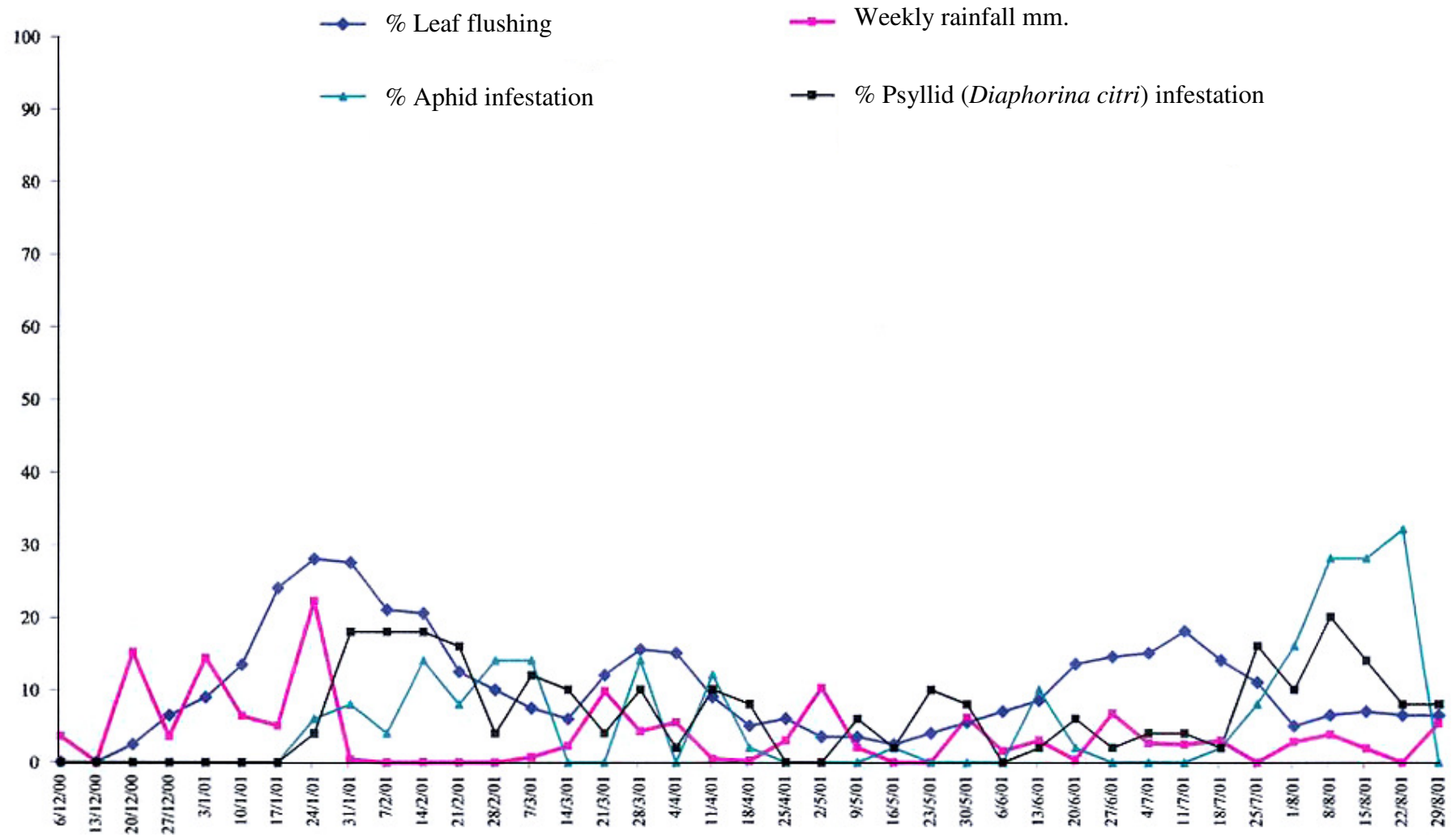

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Fig 11 Greening and tristeza insect vector monitoring of Sumjuke tangerine plot, PSU Klong Hoi Khung research station, Songkla, Thailand.

Fig 12. Citrus greening disease in Okinawa, Japan

A Dieback and decline Shekwasha (*Citrus depressa*) caused by greening

B *C. depressa* showing yellow leaves with green veins

C Nymphs of *Diaphorina citri* found on *C. depressa* young shoot

D Jasmine orange hedge near by showing greening symptom

E Greening experiment in Okinawa, *C. depressa* seedling (pot plant) have been grown near by infested orchard for natural infection by psyllid vector, Dr.Shinji Kawano (circle), Research leader, Okinawa, Prefectural Agricultural Research Center

Fig 13. Research work on citrus greening at National Institute of Fruit Tree Science, Tsukuba

A Dr. Taru Iwanami and Shekwasha (*Citrus depressa*) healthy seedlings

B *Citrus depressa* seedlings inoculated with infected bud wood from Okinawa showing yellow diffusion leaves

Table 1. Detection of greening pathogen by PCR in citrus from Pongnoi research station, Royal project foundation, Chiang Mai, North of Thailand.

Citrus varieties	PCR test[1]
Eureka Lemon	0/1
Honey (Shogun) tangor	0/1
King mandarin	2/5
Matsuyama wase mandarin	1/7
Navel orange	0/3
F 11 Navel	3/6
Okisu mandarin	2/6
Ponkan mandarin	1/6
Sugriyama mandarin	1/4
Valencia orange	1/3

[1] Using primers specific to 16S rRNA gene of greening bacterium

Table 2. Detection of greening pathogen by PCR in citrus from Pangda research station, Royal project foundation, Chiang Mai.

Citrus varieties	PCR test[1]
Honey (Shogun) tangor	0/3
Kumquat	1/9
Ponkan mandarin	7/13

[1] Using primers specific to 16S rRNA gene of greening bacterium

Table 3. Detection of greening pathogen by PCR in citrus from the south of Thailand.

Citrus varieties	PCR test[1]
<u>Krabi province</u>	
Honey (Shogun) tangor	2/7
<u>Songkla province</u>	
Honey (Shogun) tangor	2/3
Indian lime	3/3
Pomelo	2/3
Somjuke tangerine	3/3
Trifoliata orange	3/3

[1] Using primers specific to 16S rRNA gene of greening bacterium