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Differential Reaction of Citrus Species in Malaysia to Huanglongbing (HLB) Disease using Grafting Method

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Abstract: Problem statement: Huanglongbing (HLB) is a phloem limited disease in citrus caused by a fastidious bacterium called '*Candidatus* Liberibacter' found in Africa, Asia and United States of America (USA). HLB can severely reduce vigor and yield or kill all citrus trees within 5 years. There is a need to screen and identify suitable rootstock for propagation of clean plan materials for citrus in the tropics. This study was conducted to detect the presence of HLB on 18 selected citrus species and to categorize the level of infection and susceptibility of citrus species to HLB. **Approach:** Eighteen citrus species were assessed for susceptibility to HLB by graft transmission from source infection (*Citrus reticulata*). **Results** HLB was detected in 15 species 6 months after grafting using PCR test. **Conclusion:** The species could be categorized in five groups: Severe group (72-58% severity) which includes *C. reticulata*, *C. sinensis*, *C. reshni* cv. *cleopatra*, moderate group (50- 41% severity) includes *Fortunella* sp. cv. Kasturi Chinai, *C. macrophylla*, *C. microcarpa*, mild group (25-17% severity) which included *C. medica*, *C. aurantifolia*, *Citrus* sp. (natural biotype), *C. jambhiri*. The tolerant group which did not show any HLB symptoms but tested positive by PCR test includes *C. aurantium* and *C. aurantifolia*. The resistant groups which include *C. grandis* cv. Limau Bali, *C. hysterix* and *Citrus* sp. cv. Limau Tembikai showed no symptoms and were tested negative for HLB.

Key words: Huanglongbing, citrus greening disease, disease severity, citrus rootstock

INTRODUCTION

Citrus is believed to have originated from the region within Northeast India, South China, Indonesia and Peninsular Malaysia. It is an extremely important crop on a world basis and the total world production of citrus was estimated at over 73 million metric tons[1]. In Malaysia, citrus is grown in commercial orchards, backyard orchards and small holdings in various parts of the country. For conservation purposes citrus collections was established, which have notable genetic diversity, particularly of the pummelo and some of the related genera and appear to be fairly well maintained. Some are also observed in areas such as the Taman Negara National Park in Pahang and the Danum Valley in Sabah[2].

Huanglongbing (HLB), commonly known as citrus greening, is one of the most serious diseases that affect citrus fruit. HLB has destroyed an estimated 60 million trees in Africa and Asia[3-5] and occurs in more than 40 countries including Malaysia[1]. In Malaysia HLB was first detected in Cameron Highland in 1990. Survey done by Azizah and Zazali[6] revealed that approximately 70% of the cultivated area with citrus in Malaysia were affected by HLB disease[6]. In separate studies, it was shown that HLB was successfully experimentally transmitted from the infected citrus to periwinkle (*Catharanthus roseus*) and a non-*rutaceous* host by means of dodder (*Cuscuta campestris*)[7]. HLB is caused by an uncultured phloem limited bacterium that was first characterized in 1994 with the 16S rDNA sequence and classified to be a new genus in the α-Proteobacteria subdivision[7,8].

Polymerase Chain Reaction (PCR) for HLB detection was developed in 1996 based on the amplification of 16SrDNA fragments[9]. The pathogen

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of HLB has many isolates in various hosts so far. It cannot be diagnosed easily by conventional procedures such as electron microscopic examination of ultra-thin sections, bioassay on indicator plants and Enzyme-Linked Immunosorbent Assay (ELISA) with polyclonal or monoclonal antibodies. However molecular tolls such as PCR are a very effective, simple and sensitive tool for HLB detection[7,8]. Therefore this study was conducted to detect the presence of HLB on 18 selected citrus species for rootstock through grafting and to categorize the level of infection and susceptibility of 18 citrus species to HLB in Malaysia.

MATERIALS AND METHODS

Planting materials: Seeds were obtained from the Malaysian Agricultural Research and Development Institute (MARDI) and the Department of Agriculture in Terengganu, Malaysia. Seeds of 18 citrus rootstocks were sown in seed trays using a soil mixture of soil, peat and sand (2:3:1). The seedlings were transplanted 15-20 days after germination into a 16 cm diameter pot. The seedlings were ready for grafting when they were 40-50 cm tall. All experimental plants were grown in an insect proof screen house.

Source inoculums and graft transmission success: Source of HLB inoculums was collected from *Citrus reticulate* cv. Limau Madu which was obtained from University Putra Malaysia (UPM) and confirmed by PCR test. Infected scions were grafted on 18 citrus species (Table 1). Side grafting method[10] was chosen to ensure high rate of HLB transmission via vector transmission using *Diaphorina citri* which has vector preference. Terminal shoots of 10 cm long were randomly collected from trees with typical symptoms.

Table1: Percentage of grafting success of 18 selected citrus species

Sr. No	Scientific name	Local name	Grafting success (%)
1	*C. jambhiri*	Rough lemon	80
2	*C. grandis*	Limau Bali	78
3	*C. aurantium*	Limau Samur	75
4	*C. reticulate*	Limau madu	75
5	*C. medica*	Limau susu	70
6	*C. microcarpa*	Limau kasturi	69
7	*C. sinensis*	Limau lankat	68
8	*C. aurantifolia*	Limau nipis	65
9	*Citrus* sp.	Limau naga	65
10	*C. reshni*	Cleopatra mandarin	65
11	*C. aurantifolia*	Limau (Mexican lime)	62
12	*Citrus* sp.	*(natural biotype)	60
13	*C. hysterix*	Limau purut	60
14	*Fortunella* sp.	Limau kasturi chini	58
15	*Citrus* sp.	*(natural biotype)	55
16	*Citrus* sp.	*(natural biotype)	55
17	*C. macrophylla*	Machrophylla	50
18	*Citrus* sp.	Limau tembikai	35

*: Unknown citrus species in Malaysia

Twigs were then kept in the transparent plastic bags and placed in a cool box to maintain their freshness.

Vegetative growth assessment of inoculated citrus seedlings: The seedlings were washed thoroughly rinsed in tap water, followed by distilled water and then dried at 65°C for 4-5 days to measure the total dry weight in inoculated and non inoculated plants. Plant height and stem diameter of non inoculated and inoculated citrus species were measured. Data were collected, analyzed and means were separated using Duncan's Multiple Range Test (DMRT).

Disease severity: Disease severity was determined according to alternative rating scale proposed by Bowen[11] and Kranzm[12] on infected plants[11,12]. Base on the leaf symptoms, the scale includes: 0 = no symptom, 1 = Mild (blotchy mottling symptoms observed from 1-30% on seedlings canopy), 2 = Moderate (yellowing symptoms observed from 31-50% on seedlings canopy), 3 = Severe (blotchy mottling, midrib yellowing and twigs dieback symptoms observed of more than 50% on seedling canopy).The disease severity was measured using the formula below:

$$\text{Disease severity} = \frac{\sum(a \times b)}{N.Z} \times 100\%$$

Where:

$\Sigma(a \times b)$ = Sum of the symptomatic plant and their corresponding score scale
N = Total number of sampled plant
Z = Highest score scale

DNA extraction of citrus tissues: Leaf samples were collected for evaluation from seedlings which were inoculated by grafting method. DNA extraction from citrus tissues was prepared following the method described by Hung *et al*.[13]. DNA was extracted from HLB-infected tissue using Cetyl Trimethyl Ammonium Bromide (CTAB). The pellets were washed with 70% ethanol, dried and resuspended in 100 µL TE buffer.

Polymerase chain reaction (PCR) conditions, primers and gel electrophoresis: PCR was performed using 25 µL of reaction mixture containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM of dATP, dTTP, dCTP and dGTP, 50 ng forward primer, 50 ng reverse primer, 0.75 units of Taq DNA polymerase and 200 ng genomic DNA. The thermal cycle condition was: 1 cycle at 95°C for 2 min 35 cycles at 95°C for 40 sec, 60°C for 1 min and 72°C 1 min then followed

by a 72°C extension for 10 min. Specific primers pair, composed of the forward primer of OI1 (5'-GCG CGT ATG CAA TAC GAG CGG CA-3') and reverse primer of OI2c (5'-GCC TCG CGA CTT CGC AAC CCA T-3'), was used to amplify the 16S ribosomal DNA fragment. Amplification of DNA were determined by electrophoresis on 1.2% agarose gel for about 30-45 min and visualized by ethidium bromide staining[13].

RESULTS

Graft transmission success: All 18 citrus species were grafted using infected scions of *Citrus reticulata*. The performance of citrus plants varied in terms of grafting success. Result in Table1, shown the highest grafting success was observed on *C. jambhiri*, *C. grandis, C. aurantium and C. reticulate* and *C. medica* (from 70-80%). The scion used for grafting on these rootstocks grew and was normal growing development. Low grafting success was observed on three citrus which are the natural biotypes (*Citrus* sp.), *C. macrophylla* and *Citrus* sp. cv. Limau tembicai (from 35-85%).

Effects of HLB on vegetative growth: Mean comparison of total dry weight, plant height and stem diameter of none inoculated, inoculated and reduction rate of citrus species were measured six months after inoculation. There were significant difference (p<0:05) between total dry weights of non inoculated citrus species. The highest total dry weight was observed on *C. jambhiri*, *C. aurantium*, *C. macrophila* than on *Citrus* sp. cv. Limau Tembiki, *Citrus* sp. (natural biotype), *C. aurantifolia* cv. Mexican Lime and *Citrus* sp. cv. Limau Naga. Plant heights of non-infected citrus seedlings were significantly different on *C. jambhiri*, *C. aurantium*, *C. macrophila* and citrus sp. cv. Coleopatra than on *Citrus* sp.cv. Limau Tembiki, *Citrus* sp. (natural biotype) and *C. aurantifolia* cv. Mexican Lime. Stem diameter of citrus species was also significantly different at probability statistic level of 5%. The highest stem diameters were observed on *C. jambhiri*, *C. aurantium* and *C. aurantifolia* than other species such as *Citrus* sp. (natural biotype) and *Citrus* sp. cv. Limau Tembiki (Table 2).

In inoculated citrus species, mean comparisons of total dry weight, plant height and stem diameter were significantly different (at p<0:05). Total dry weight, plant height and stem diameter of inoculated citrus species were significantly higher on *C. hysterix, C. grandis*, *C. aurantium* and *C. aurantifolia* than on *C. reticulate* cv. Limau Madu, *C. sinensis* cv. Limau Lankat, *C. microcarpa* cv. Limau Kasturi *and Citrus* sp. (natural biotype) (Table 2). Mean comparisons revealed the highest total dry weight, plant height and stem diameter in non-infected than infected citrus species.

Reduction rate percentages were measured to compare the inoculated and non inoculated citrus species. Reduction rate of total dry weight, plant height and stem diameters on seedlings which showed severe symptoms of HLB were higher than the species which showed less HLB symptom, tolerant or resistance species too. Highest reduction rate of total dry weight, citrus plant height and stem diameter were observed on *C. reticulata*, *C. sinensis*, *C. macrophylla* and *C. microcarpa* than on *C. grandis*, *C. hysterix*, *Citrus* sp. cv. Limau tembiki, *C. aurantifolia* and *C. aurantium*.

Table 2: Comparison between mean of total dry weight, plant height, stem diameters and reduction rate on inoculated and non inoculated of citrus species six months after inoculation

Citrus species		Inoculated			Non inoculated			Reduction rate (%)		
Scientific name	Local name	Total dry weight (g)	Plant Height (cm)	Stem diameter (mm)	Total dry weight (g)	Plant Height	Stem diameter (cm)	Total dry weight (mm)	Plant height	Stem diameter
C. grandis	Limau bali	71.40a	72.8a	19.3a	76.00def	74.0cd	19.5c	6.05	3.00	5.00
C. hysterix	Limau purut	67.50ab	68.2abcd	17.6bcd	71.17gh	69.5ef	19cd	4.17	2.00	7.36
C. medica	Limau susu	65.42abc	68.4abc	16.7bcdef	77.00bcde	76.0bcd	18de	14.28	11.60	19.14
C. aurantium	Limau samur	68.40ab	69.4ab	19.0a	79.73ab	78.5ab	23.5a	15.03	11.16	7.22
C. aurantifolia	Limau nipis	65.20abc	69.0ab	14.6efgh	78.10bcd	77.0abc	17.5e	16.51	10.40	16.60
C. macrophila	Macrophila	62.00bcd	64.2bcdef	16.0cdefg	79.13abc	78.0ab	20.0c	18.38	15.00	12.00
C. aurantifolia	Mexican lime	56.80de	59.0fg	18.6ab	69.20hi	66.5fg	21.0b	21.64	17.70	20.00
Citrus sp.	Limau tembikai	59.06cde	61.2cdefg	15.3defgh	61.00j	66.0g	16.5f	3.20	2.18	7.30
C. reshni	Coleopatra mandarin	58.70cde	60.0fg	14.0fgh	78.07bcd	77.3d	18.0de	17.90	11.30	11.40
C. jambhiri	Rough lemon	66.70ab	68.0abcde	19.3ab	81.73a	80.0a	24.0a	24.80	20.05	22.22
Fortunella sp.	Kasturi chini	55.10de	59.5fg	15.5defg	75.00ef	74.0cd	19.5c	26.53	19.60	32.44
Citrus sp.	Limau naga	56.40de	61.0defg	17.2bcde	69.17hi	69.0efg	19.5c	18.46	11.60	12.00
Citrus sp.	Limau 3	56.60de	60.6fg	16.6bcdef	66.50i	67.0efg	19.5c	14.88	10.00	15.00
Citrus sp.	limau2	58.90cde	61.4cdefg	14.0fgh	66.50i	66.0g	16.0f	11.50	7.00	12.50
Citrus sp.	limau1	58.20de	60.8efg	15.3defgh	68.50hi	67.0efg	17.5e	15.00	11.00	12.60
C. microcarpa	Limau kasturi	54.60e	59.6fg	15.2defgh	75.73def	73.5d	19.0cd	27.90	18.90	18.40
C. sinensis	Limau lankat	48.32f	56.0gh	13.2gh	76.50cde	76.0bcd	19.0cd	36.80	26.31	31.00
C. reticulata	Limau madu	43.70f	50.4h	12.5h	73.50fg	70.0e	18.0de	40.50	28.00	30.00

Means within column followed by the same letters are not significant at p = 0.05

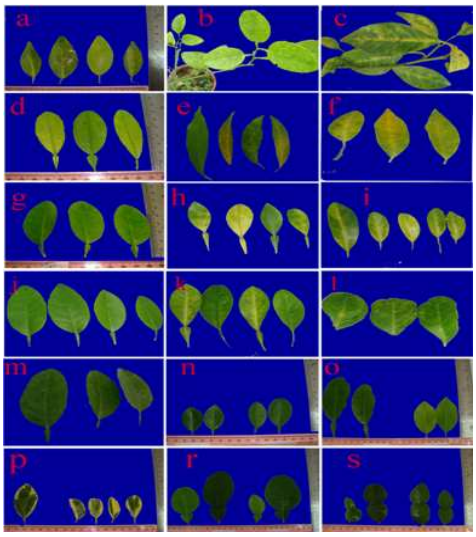


Fig. 1: Symptoms of HLB observed on different citrus species six months after graft inoculation; (a): *C. reticulate*; (b): *C. reshni* cv. Cleopatra; (c): *C. sinensis*, (d): *C. macrophylla*; (e): *C. aurantifolia* cv. Limau Nipis; (f): *C. microcarpa* cv. Limau Kasturi, (g): *Fortunella* sp.; (h): *Citrus* sp.; (i): *Citrus* sp., (j): *Citrus* sp.; (k): *C. jambhiri*; (l): *C. medica*; (m): *Citrus* sp.; (n): *C. aurantifolia*; (o): *C. aurantium*; (p): *C. grandis*; (r): *C. hysterix*; (s): *Citrus* sp. cv. Limau tembikai

Symptom expression of HLB: Symptoms expression is shown in Fig. 1. It was observed that *C. grandis* cv. Limau Bali, *C. hysterix* cv. Limau purut and *Citrus* sp. cv. Limau tembikai showed no symptom of HLB after 6 months of inoculation and leaves remained green. Also no symptom of HLB occurred on *C. aurantifolia* cv. Mexican Lime and *C. aurantium* six months after graft inoculation. *C. sinensis* cv. Limau Lankat, *C. reticulata* cv. Limau Madu and *C. reshni* cv. Cleopatra showed severe symptoms of HLB at the sixth month after inoculation. *C. reticulata* showed midrib yellowing, yellowing and mild twig dieback six months after graft inoculation. *C. sinensis* and *C. reshni* cv. Cleopatra showed blotchy mottling and yellowing on leaf. On *C. macrophylla* fertilizer deficiency symptom was observed, but *C. aurantifolia* cv. Limau nipis showed mild blotchy mottling and midrib yellowing. *C. microcarpa* cv. Limau kasturi and *Fortunella* sp. were able to show blotchy mottling and mild yellowing on main veins. Mild midrib yellowing were observed on tow *Citrus* sp. (citrus natural biotype), *C. jambhiri* and *C. medica*, but on other citrus natural biotype and *Citrus* sp. cv. Limau Naga, mild blotchy mottling were observed.

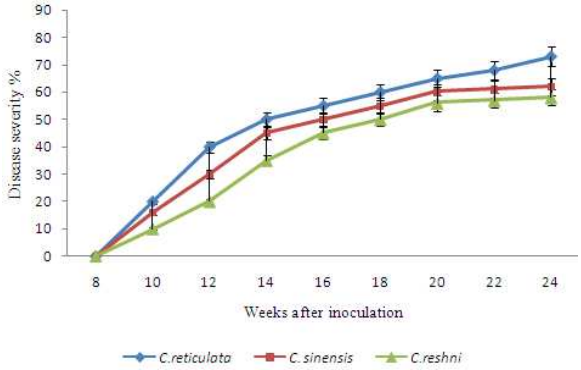


Fig. 2: Progress of HLB disease severity on severe group of citrus species

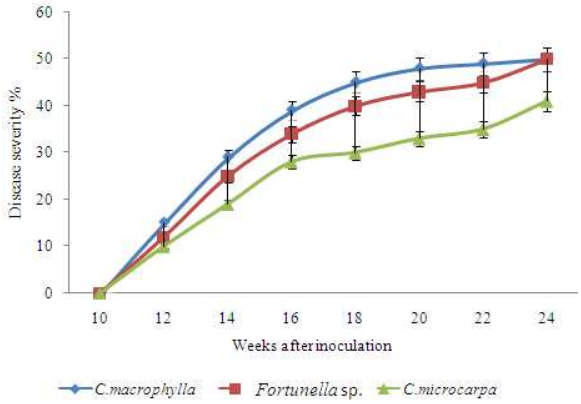


Fig. 3: Progress of HLB disease severity on moderate group of citrus species

Disease severity of HLB on citrus species: Disease severity was evaluated on individual inoculated seedlings. There were significant differences of disease severity observed among the 18 citrus species. *C. reticulata*, *C. sinensis and C. reshni* cv. Cleopatra showed high level of severity with value of 94.45-50% respectively. The symptom of HLB started to show 8 weeks after inoculation. This species showed severe symptom of HLB after 24 weeks (6 months) of inoculation (Fig. 2). *Fortunella* sp., *C. mycrophylla* and *C. microcarpa* showed moderate symptom of HLB with the value of 41-50% respectively.

This symptom started on week 10 after inoculation and showed severe symptom six months after inoculation (Fig. 3). *C. medica*, *C. aurantifolia*, *Citrus* sp. *C. jambhiri* and three citrus biotype *(Citrus* sp.) showed mild symptom of HLB with the value of 17-25% starting 14 weeks after inoculation and showed the highest symptom also 6 months after inoculation (Fig. 4). *C. aurantifolia*, *C. aurantium*, *C. grandis* and *C. hysterix* did not show symptom of the HLB 6 months after inoculation.

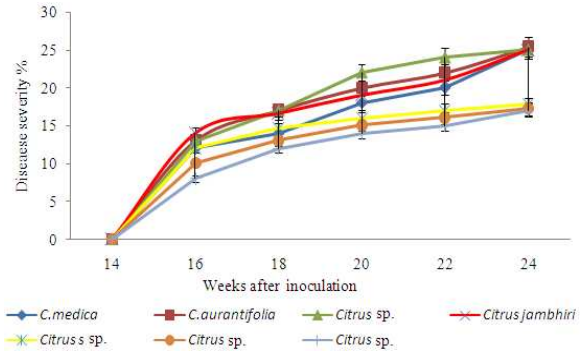
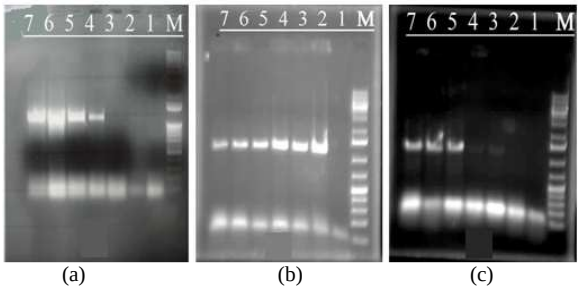


Fig. 4: Progress of HLB disease severity on mild group of citrus species



(a) (b) (c)

Fig. 5: 116s rDNA fragments with molecular weight of 1160 bp were successfully amplified from infected; (a): M. Marker (1): Water; (2): *Citrus* sp. cv. Limau Tembikai, (3): *C. grandis* cv. Limau Bali; (4): *C. medica*; (5): *Citrus* sp. cv. Limau Naga; (6): *Citrus* sp. (natural biotype); (7): *Citrus* sp. (natural biotype); (b): M. Marker (1): Water; (2): *C. reticulate*; (3): *C.sinensis*; (4): *C. microcarpa* cv. Limau Kasturi; (5): *C. aurantifolia* cv. Limau Nipis; (6): *C. reshni* cv. Cleopatra; (7): *C. macrophylla*; (c): M. Marker (1): Water; (2): *C. hysterix* cv. Limau Purut; (3): *C. aurantifolia* cv. Mexican Lime (4): *C. aurantium*; (5): *Citrus* sp. (natural biotype); (6): *C. jambhiri*; (7): *Fortunella* sp.

Detection of HLB pathogen on the species using PCR test: The amplified PCR product is 1160bp which is the targeted 16S rDNA gene sequence region of the HLB pathogen amplified by the OI1 and OI2c primer sets. According to the result HLB was detected on fifteen citrus species (Table 3 and Fig. 5).According to the PCR test HLB was not present on 3 of the species including *C. grandis*, *C. hysterix* and *Citrus* sp.cv. Limau Tembiki (Fig. 5) and also the seedling did not show symptoms of HLB 6 months after graft inoculation. Results of PCR test show HLB was present on *C. reticulata* and *C. sinensis* (Fig. 5) and showed

Table 3: Percentage of positive PCR test of citrus species

Citrus species	Local name	Positive PCR test (%)
C. reticulata	Limau Madu	100.00
C. sinensis	Limau Lankat	83.30
C. reshni	Cleopatra	66.67
C. microcarpa	Limau kasturi	66.67
Citrus sp.	Limau Naga	66.67
C. macrophylla	Machrophylla	66.67
Fortunella sp.	Limau kasturi chini	66.67
C. aurantifolia	Limau Nipis	50.00
C. medica	Limau susu	50.00
C. jambhiri	Rough lemon	50.00
Citrus sp.	natural biotype	33.34
Citrus sp.	natural biotype	33.34
Citrus sp.	natural biotype	33.34
C. aurantium	Limau Samur	16.00
C. aurantifolia	Mexican lime	16.00

height infection with the value of 83.3-100% respectively among the citrus species. Results in Table 2 also showed that HLB was present on *C. microcarpa* cv. Limau Kasturi, *C. reshni* cv. Cleopatra, *Citrus* sp. cv. Limau Naga, *C. macrophylla* and *Fortunella* sp. (Fig. 5) and infection rate were 66.67%.

HLB was also detected on *C.aurantifolia* cv. Limau Nipis, *C. medica* and *C. jambhiri*. The results of PCR test (Table 3) has shown 50% rate of infection. HLB was present on 3 *Citrus* sp (natural biotype) with 33.3% rate of infection. *C. aurantifolia* and *C.aurantium* showed lowest infection (16% positive PCR test of each species), but on symptom expression these species did not show any symptom of HLB. However HLB was present on 15 citrus species which were tested in the experiment and HLB was not present on 3 species including *C. grandis*, *C. hysterix* and *Citrus* sp. cv. Limau Tembikai. PCR is certainly a very effective, simple and sensitive tool for HLB detection. However, *Candidatus* Liberibacter is very low in concentration and unevenly distributed in its natural hosts.

DISCUSSION

Transmission of citrus greening occurs primarily via infective citrus psyllids, grafting and it is transmissible experimentally through dodder. However psyllid species feed and survive on citrus and citrus relatives. The side grafting method was chosen to transmit the HLB on species on high rate of inoculation. This study demonstrated high infection of HLB using grafting method. The scion used for grafting on these rootstocks grew and was normal growing development. *C. reticulata* and Limau Tembikai were less compatible with infected scion and the growth was disrupted. In this case scion survived and grew 3-5 cm after 6 months and the success rate of grafting was 35%. When the

scion and rootstock are from the same species grafting was be high successful. A successful graft union depends on good contact between the cambium of the rootstock with the cambium of the scion. The bacteria can be transmitted in orchards or nurseries by grafting and experimentally by several species of dodder (*Cuscuta* spp.)[19]. Reduction rate of total dry weight, plant height and stem diameters on seedlings which showed severe symptoms of HLB were higher than the species which showed less HLB symptom, tolerant or resistance species too. Because the seedlings infected with HLB are usually stunted and it will be unproductive 4-5 years after planting. The infected seedlings to HLB was showing low rate of growing. Ahmad evaluated the effects of HLB infection on growth performance on *C.reticulata* based on the percentage reduction rate of total biomass and plant height. It was observed that high reduction rate of total biomass and plant height with the value of 56.2% and 39.4% respectively on infected honey mandarin[2]. Citrus species was showing different symptom of the HLB. Seedlings which become infected by HLB usually developed one or more yellow shoots. Leaves become thicker, with enlarged and corky veins and green areas are lacking. In later stages, zinc-like deficiency symptoms developed, followed by leaf drop and twig dieback[14]. Ahmad was not able to detect HLB on *C. grandis* in green house after six months of infection[2]. These results also agree with the results which were reported by Manicom and Vuuren[16], where they reported HLB symptoms on *C. reticulata* and *C.sinensis* (sweet orange) are more severe but lemon and grapefruits are tolerant. They also reported that *C. aurantifolia* and *C. grandis* are more tolerant to HLB. Some species and cultivars of citrus are somewhat tolerant to HLB. Most of the sweet orange trees became infected with the HLB pathogen and subsequently declined, while grapefruit was more tolerant[15]. In general, sweet oranges, mandarins and tangelos are most susceptible, grapefruit and lemon are more resistant and limes, *Poncirus trifoliate* and citranges are the most tolerant[17]. Ahmad in Malaysia also reported that no HLB symptoms were observed on pummelo but *C. reticulata* cv. Honey Mandarin, *C. madurensis* cv. Calamondin and *C. aurantium* show severity with value of 75%, 65% and 50% respectively, but he transferred HLB pathogen to that species using *Diaphorina citri* vector[3]. Infected orange (*C. sinensis*), mandarin *(C. reticulata*) and tangelo (*C. reticulata* x *C. paradise*) produce the most severe symptoms and infected trees die within 3-5 years[18]. PCR test on this study also demonstrated that HLB was absent on *C.grandis*, *C.hysterix*, *Citrus* sp. cv. limau Tembiki. These species were shown a normal growing during six months after inoculation. PCR is certainly a very effective, simple and sensitive tool for HLB detection. However, *Candidatus* Liberibacter is very low in concentration and unevenly distributed in its natural hosts. The PCR based assay detected almost all Asian HLB strain collected from different countries in Asia such as Malaysia[13]. Based on this study, it can be concluded that HLB can be identified on fifteen citrus species six months after graft infection. The species that were infected include: *C. reticulata*, *C. sinensis*, *C. reshni* cv. Cleopatra, *C.microcarpa*, *C. medica*, *Citrus* sp. (Natural Biotype), *Citrus* sp. (natural biotype), *Citrus* sp. cv. Limau Naga,*Citrus* sp. (natural biotype), *C. jambhiri*, *Fortunella* sp., *C. aurantifolia* and *C. aurantium*. But symptom expression and severity of HLB were different between species. However, *C.grandis* cv. Limau Bali, *C. hysterix* cv. Limau Purut *Citrus* sp. cv. Limau Tembiki showed negative reaction of HLB by PCR test and on these species no symptom showed six months after graft inoculation. According this study *C. aurantifolia* and *C. aurantium* was without symptom and showed 16% positive of HLB. However according to this study citrus species could be categorized as severe, moderate, mild, tolerant and resistant. Severe citrus species on HLB include *C.reticulata*, *C. sinensis*,*C. reshni* cv. Cleopatra. Moderate species include *Fortunella* sp., *C.macrophylla* and *C. microcarpa* cv. Kasturi. Mild species include *C. medica*, *C. aurantifolia* cv. Limau Nipis, *Citrus* sp. (natural biotype) and *C. jambhiri*. Tolerant species include *C. aurantium* and *C.aurantifolia*. Finally, resistant species include *C. grandis*, *C. hysterix* and *Citrus* sp. cv. Limau Tembikai. Ahmad used graft and insect vector to transmit HLB to evaluate citrus species against HLB. He reported that jasmine orange and pummelo were resistant to infection and no HLB symptom was observed even 6 months after inoculation. HLB isolate was also not detected in leaf tissue by PCR test[2].

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