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Identification of physical and biochemical characteristic of mandarin *(Citrus reticulata)* fruit infected by Huanglongbing (HLB)

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Abstract

This study was conducted on an infected 8 year old *Citrus reticulata* orchard in Terengganu, Malaysia. Huanglongbing (HLB) was successfully detected using conventional PCR in samples with blotchy mottling and midrib yellowing symptoms. Different treatments of antibiotic (Oxi-tetracycline), GA_3 and foliar fertilizer were tested on the HLB-infected citrus plants. The chemical treatments were applied before flowering (February 2008) and during fruit set (June 2008). Changes in fruit quantity and quality parameters of *Citrus reticulata* were studied after harvesting. Low titratable acidity (TA) was measured on T7, T6 and a high rate of TA was achieved on T8 as control. High SSC percentage was recorded in T7 followed by T2 and T8. A high peel thickness percentage, fruit weight, was recorded on T7 and T2 while low values were observed on T8 and T5. High juice percentage was recorded on T2 followed by T7 and low juice percentage was recorded on control (T8). High pulp percentage was observed on T2 followed by T7 and a low rate was measured on T8.Finally, the best treatments were T7 and T4 to increase the HLB-infected fruit quantity and quality.

Keywords: *Citrus reticulata,* Chemical treatments, HLB-infected fruit.
Abbreviation: ANOVA-Analysis of Variance; DMRT-Duncan's multiple range tests; g-grams; GA_3-gibberellic acid; HLB-Huanglongbing; mg-Milligrams; ml-millilitre; L-litre; µl-Microliter; RCBD-randomized complete block design; t/ha-tonne per hectare; TEM-transmission electron microscope; TA-titratable acidity; SSC-soluble solids contents; SAS-statistical analysis system.

Introduction

The exact location of the origin of citrus is not clearly identified. Most researchers report that citrus originated in South-East Asia. Some species of citrus originated from Malaysia. Citrus is grown in more than 100 countries in tropical, subtropical and Mediterranean climates. It is also the leading fruit crop grown in the world (Khan 2007). Huanglongbing (HLB) disease has been threatening the world's citrus industry. Previously called citrus greening disease, HLB is the most destructive disease to citriculture in Asia (Garnier et al. 1984). The most characteristic symptom of HLB disease is an asymmetrical blotchy mottling of leaves. The fruit produced by infected trees is small, green, underdeveloped, and misshapen, with aborted seeds and bitter in taste. Thus, the fruit cannot even be marketed as juice. Fruits also have higher acid and lower sugar content. The fruits do not colour properly, remaining green on the shaded side (hence the name "greening disease") (Bove 2006; Gottwald et al. 2007; Halbert and Manjunath 2004). In 1989 the occurrence of HLB disease in Malaysia was confirmed by field symptoms and transmission electron microscope (TEM) by (Lim et al. 1990), however, until now there is still little available information regarding this disease (local HLB disease) in Malaysia. In 2005, it was revealed that approximately 2,458 ha from the total of 3,526 ha of the citrus cultivation areas in Malaysia showed the presence of the HLB disease symptoms (Azizah and Zazali 2005). HLB is difficult to control, especially when the psyllid *(Diaphorina citri)* population has already established in the citrus orchard. Management of HLB in areas where the disease is endemic depends largely upon culture control. Infected trees should be removed as soon as symptoms appear (Van Vuuren and da Graça 1993). In infected citrus orchards, the common practice is that if the infected trees are 5 years old or less, the trees are completely removed. If they are 6 to 10 years old, the trees are completely removed if infection has reached 75%; otherwise only the branches are removed. If it is more than 10 years old, the affected branches of up to 40% of the tree are cut off (Buitendag and Von Broembsen 1993; Van Vuuren and da Graça 1993). No curative methods exist to control HLB in citrus. However, studies have shown that antibiotics injected into infected citrus trees provide a temporary remission of symptoms (Lim et al. 1990; Gottwald et al. 2007; Halbert and Manjunath 2004; Van Vuuren and da Graça 1993). Injection with antibiotics has been recommended as part of an integrated management programme in India (Nariani 1981). Halbert and Manjunath (2004) reported that symptoms reappear 1 to 1.5 years after injection of the antibiotics. Antibiotics such as penicillin and tylosine were effective in suppressing the development of HLB symptoms at 200µg/ml. Streptomycin and cefaloridine were also effective at 500 µg/ml (Bove 2006). Some chemicals such as foliar fertilizer were also effective in suppressing HLB symptoms in infected citrus orchards. Urea (46% N) has been applied as a foliar spray to Clementine mandarins (citrus reticulate) prior to bud break, at full bloom and at June drop. The rates tested were in the range of 0.8% to 1.6% urea with 10L of the spray applied per tree. A significant increase in flower number, fruit set and yield was obtained with the pre-bloom application. The application of urea at full bloom and at the physiological drop also increased the yield (El-Otmani et al. 2002). A similar result was also obtained when gibberellic acid (GA_3) (15 mg/l) was applied at the green bud stage (Milind 2008). In all cases, yield increase was due to an increase in the number of fruits per tree and in fruit size. Consequently, export yield was improved and fruits could be harvested earlier (Milind 2008; Ritenour and Stover 1999; Pozo et al. 2000). Other literature reports that nitrogen, calcium, zinc, copper, cadmium, nickel and other microelements showed some potential to suppress diseases caused by fungi and bacteria (Bockus and Davis 1993; Long et al. 2002; Naar 2006). Antibiotics were also used to control the greening disease pathogen. Different types of antibiotics were injected into HLB-infected citrus trees to relieve temporary symptoms (Halbert and Manjunath 2004). Nariani (1981) recommended injection of antibiotics as a part of an integrated management program in India. Leaf symptoms of HLB were reduced when Martinez et al., (1970) used tetracycline hydrochloride, whereas Cheema et al.(1986) obtained complete control of HLB using penicillin carbendazin. The best results were observed when tetracycline hydrochloride was applied through the injections in the spring season (Aubert and Bove 1980; Graca and Korsten 2004). In South Africa, tetracycline was applied up to 20 g/adult tree with high capacity compressors working at 10 kg/cm^2 on greening-infected citrus trees. Trees treated with rolitetracycline showed reduced symptoms of HLB. However, the main control measurement in South Africa is based on the use of healthy nursery trees and effective systemic insecticides against the vector *T. erytreae* via trunk treatments (Buitendag 1991). Without any control measures, HLB evolution in an affected orchard is fast (Bove 2006). In China, without controlling HLB, it only takes about 5 years for a citrus orchard to reach 100% infection (Zhao 1981). In the Reunion Island, the time needed to reach 100% infection was estimated to be 13 years. Generally, HLB-infected citrus trees start to decline five to eight years after cultivation. However, the groves must live for a minimum of 10 years in order to make a profit (Roistacher 1996). It was reported that the life span of citrus orchards with good management and control measures can be extended up to 12 years or more (Hung et al. 2001). However, there is currently no specific treatment available to overcome HLB disease in citrus in the world, including Malaysia. This and the above mentioned reasons make HLB the single most important threat to the citrus industry. This study was conducted to evaluate the effects of antibiotic (Oxi-tetracycline), GA_3 and foliar fertilizer (commercial name; Vita-Grow) on HLB-infected *Citrus reticulata* (Limau Madu) trees to achieve good agricultural practice and to determine the effect of chemical treatments on the quantity and quality of the fruits.

Materials and methods

Research area and condition

Research evaluations were carried out in Kuala Berang, Terengganu, Malaysia. The state of Terengganu overlooks the South China Sea on the east side of Malaysia. It has a strong tropical monsoon climate, with relatively uniform temperature within the 21 °C to 32 °C range. From January until April, the weather is dry and warm, with humidity in the lowlands being consistently high, between 82 and 86 percent annually. Terengganu's average rainfall is 2,032 mm to 2,540 mm per year, with the most rain falling between November and January. The orchard of *Citrus reticulata* cv. Limau Madu was cultivated in 2000 and it has been unproductive since that time because of HLB. A total of 72 trees of *Citrus reticulata* were selected for this experiment. A randomized complete block design (RCBD) with three replications was used. Three trees were selected as a treatment unit on each replication for each treatment. GA_3, antibiotic, foliar fertilizer and a combination of these chemicals were tested (Table 1). Aqueous solutions of the treatments were prepared and sprayed on whole trees according to the method described earlier by Saleem et al. (2007). Moreover, 2 g/L of antibiotic was injected in the stem at 15-25 cm above the soil (Fig.1). The treatments were applied before flowering (February, 2008) and in fruit set (June, 2008).

Table 1. List of chemical treatments to reduce the HLB disease severity and improve fruit quantity and quality

Treatments
*Antibiotic (Oxi-tetracycline) 2g/L
Gibberellic acid (GA_3) 15 mg/L
Foliar fertilizer (Vita-Grow) 5 ml / L
Antibiotic (2 g/L) + GA_3 (15 mg/L)
Antibiotic (2 g/L) + foliar fertilizer (20 ml /4 L)
GA_3 (15 mg/L) + foliar fertilizer (20 ml /4 L)
Antibiotic (2 g/L)+ GA_3 (15 mg/L)+ foliar fertilizer (20 ml /4 L)
Control (none-treated)

*Antibiotic was injected in the trunks 15 cm above the soil surface

Fig1. Injection of antibiotics; (A) making a hole on the trunk of the tree, (B) placing the tube, (C) preparing the antibiotic container, (D) an overall view of the whole injection set up.

Leaves sample collection, DNA extraction and PCR protocol

The leaves samples collected from the trees with 8 years old in this experiment which were showed HLB symptom to determine the *Candidatus* Liberibacter asiaticus bacteria via PCR methods. The leaves transferred to the plant pathology laboratory for DNA extraction and PCR identification

Table 2. Mean comparison of biochemical analyses under different treatments using the Duncan multiple rang test (DMRT)

Treatment [a]	TA [b] (%)	SSC (%)	SSC/TA (%)
T1	6.33 c	11.60 b	1.83 cd
T2	5.90 de	12.26 ab	2.08 b
T3	6.10 cde	11.60 b	1.90 cbd
T4	6.80 b	12.13 ab	1.78 d
T5	6.26 dc	11.70 b	1.87 cd
T6	5.73 e	11.53 b	2.01 cb
T7	5.33 f	12.86 a	2.41 a
T8	7.70 a	9.73 c	1.26 e

[a] Means within a column with different letters are significantly different and means followed by the same letters are not significantly different according to DMRT test at P≤ 0.05 levels. [b] The percentage data were converted to their arcsine-square root equivalents in order to normalize data sets prior to analysis. T1: Antibiotic. T2: GA_3. T3: foliar fertilizer. T4: Antibiotic + GA_3. T5: Antibiotic + foliar fertilizer. T6: GA_3 + foliar fertilizer. T7: Antibiotic + GA_3 + foliar fertilizer. T8: Control.

purposes. Total DNA was extracted from the leaves tissue using hexadecyltrimethyl ammonium bromide (CTAB) method (Hung et al. 1999). The DNA was isolated from approximately +100 mg of frozen leaves or fresh leaves samples. The conventional PCR was carried out to detect the HLB disease in this study. The 16S ribosomal DNA (16S rDNA) fragment of *Candidatus* Liberibacter asiaticus was identified using a specific primer pairs (OI1 as forward primer: '5-GCG CGT ATG CAA TAC GAG CGG CA-3' and OI2c as reverse primer: '5-GCC TCG CGA CTT CGC AAC CCA T-3'). The PCR was performed on 25 µl reaction mixture followed the method which was described by Hung et al., 1999.

Physical and biochemical changes of the fruits

Changes in the fruit quantity and quality parameters including yield, fruit weight, peel, pulp, peel/pulp and juice percentage, soluble solids contents (SSC), titratable acidity (TA) and SSC/TA of *Citrus reticulata* were studied after harvesting the fruits. The fruit samples were collected and brought to the pathology laboratory for data gathering and analysis.

Fruit sampling and physical analysis

Fruit yield was recorded as ton per hectare. Samples of 20 representative fruits were collected randomly from all sides of each tree and the average fruit weight (g) was calculated. The fruits were washed with tap water, dried under shade then cut into two halves. Peel thickness (mm) was measured using a vernier caliper. After peeling, the juice was extracted in a beaker to obtain the average fruit juice weight (ml)/fruit, in addition, the peel and pulp were weighed (g) separately and the quantities of were all expressed on a percentage basis.

Biochemical analysis of fruit

The fruit juice quality analysis, including soluble solids content (SSC), titratable acidity (TA), and SSC/TA ratio, were performed following the standard procedures mentioned by Saleem et al., (2007) at the Laboratory of Horticultural Sciences, University of Putra, Malaysia. The soluble solids content of *Citrus reticulata* juice were determined with a hand refractometer (Model N1, Atago) at room temperature. One or two drops of sap from pulp presses were placed on the prism glass of the refractometer. The reading of SSC (%) was recorded with the refractometer that was pointed directly at a light source. For titrable acidity, 10 gram samples were taken from the pulp of each fruit. The pulp was blended with 40mL of distilled water using a blender (Mix-798S, National, Malaysia) and filtered through a No.2 filter paper. The TA was determined according to the titration method by Ranganna (1977).

Statistical analysis

Data were collected and analysed by using Analysis of Variance (ANOVA), and significant differences between treatments were compared by the Duncan's multiple range tests (DMRT) method with statistical analysis system (SAS Version 9) to separate the treatment means at the ≤ 0.05% level of probability. Data from counting were arcsine-transformed prior to analysis.

Results

HLB detection

16S rDNA fragments with molecular weight of 1160 bp were successfully amplified from the HLB-infected *Candidatus* Liberibacter asiaticus, which was showed blotchy mottled and midrib yellowing symptoms (Fig.2). The samples for PCR detection were taken from the midrib of the leaves which was showed the HLB-symptom. One pair primers: OI1 (forward primer) and OI2c (reverse primer) for amplification of 16S rDNA was used. In blotchy mottling symptom HLB detected where the bond of PCR amplification was not clear than midrib yellowing symptom. Using conventional PCR HLB was successfully detected in the fertilizer deficiency symptom, bond of PCR amplification wan not clear as detected in blotchy mottling symptom (Fig.2).

Effects of chemical treatments on physical characteristics

Comparisons of the minimum and maximum values for the physical characteristics of *Citrus reticulata* are also recorded and presented in Table 3. Peel thickness was significantly increased among the treatments. High peel thickness percentages were recorded for T7 (2.20) and T2 (2.08) while low values were observed for T8 (1.16) and T5 (1.75). However, T2 (2.08), T3 (1.85), T4 (1.90) and T6 (1.90) were statistically similar. The fruit weight was significantly increased among the treatments compared to the control. High fruit weight was recorded for T7 (135.00) followed by T2 (129.33) and T4 (121.33), while low fruit weight was measured for T8 (96.33) followed by T1 (103.33). No significant differences were observed for T2, T3 and T4. A significant difference of juice percentage was measured among the treatments. High juice percentage was recorded for T2 (47.96) followed by T7 (47.88) and T3 (47.66) compared to other treatments. Low juice percentage was recorded for the control (T8= 40.33%) followed by T5 (44.66), T1 (45.33), T4 (47.16) and T6 (47.28). Pulp and peel percentage were also significantly increased by the treatments. High pulp percentage was observed for T2 (47.96) followed by T7 (47.87) and T4 (47.16) compared to the control (T8; 40.33). Among the different treatments, the lowest pulp percentage was recorded for T5 (44.66). Pulp percentage was significantly similar for T1, T2, T3, T4, T6 and T7. Significant differences were observed among the treatments compared to the control. Among the treatments, the highest peel percentage was measured for T7 (16.36) while the lowest peel percentage was recorded for T1 (13.31). Pulp/peel ratio and yield were also measured. The highest ratio was observed for T8 (4.06) followed by T4 (2.25) and T5 (2.68) while the lowest ratio was observed for T7 (2.11) compared to the other treatments. Yield was measured based on tonnes/hectare (Table 3). There were significant differences among the treatments. A low yield was recorded for the control (3.75 t/ha) while a high yield was observed for T7 (12.90 t/ha) followed by T2 (12.43) and T4 (12.40 t/ha).

Effects of chemical treatments on biochemical characteristics of Citrus reticulata fruits

The comparison of the mean values of some of the biochemical characteristics of *Citrus reticulata* are presented in Table 2. Significant differences were observed among the biochemical parameters such as TA, SSC and SSC/TA ratio. All the experimental treatments significantly decreased TA compared with the control. A low TA was measured for T7 (5.33), T6 (5.73) and T2 (5.90) compared to the control (7.70). A high rate of TA was achieved for T8 as control (7.70), T4 (6.80) followed by T1 (6.33), T5 (6.26) and T3 (6.10) (Table 2). Soluble solid content (SSC) was also recorded using a refractometer at room temperature. It was significantly affected by different treatments compared with the control (T8). SSC was increased in all treatments. A high SSC percentage was recorded for T7 (12.86) followed by T2 (12.26), T4 (12.13), T5 (11.70), T3 (11.60), T1 (11.60) and T6 (11.53). SSC were statistically similar among the T1, T2, T3, T4, T5 and T6 (Table 2). SSC/TA ratio was also measured among the different treatments. Significant differences were observed between the treatments. A High SSC/TA ratio was observed for T7 (2.41) and T2 (2.08) followed by T6 (2.01), T3 (1.90), T5 (1.87), T1 (1.83) and T4 (1.78) compared to the control (T8; 1.26) (Table 2).

Discussion

Molecular detection methods have been difficult to develop since the greening organism has not yet been cultured (Halbert and Manjunath 2004). Polymerase chain reaction (PCR) for HLB detection was developed in 1996 based on the amplification of 16S rDNA fragments (Jagoueix et al. 1996). HLB was successfully detected from the *C. reticulata* orchard with 8 years old. HLB was detected very easily in the sample with midrib yellowing compared with the sample with blotchy mottling symptom. Conventional PCR test showed present of HLB on plants with severe symptom of blotchy mottling because they have the highest titers of Liberibacters (Teixeira et al. 2008). Hajivand et al. (2009b)

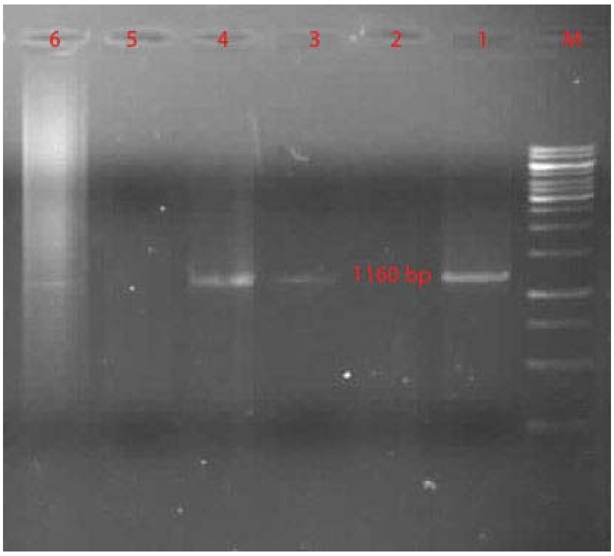

Fig 2. 16S rDNA fragments with molecular weight of 1160 bp were successfully amplified from the infected samples. M: Marker (1000 bp Invitrogen); line 1: positive sample; line 2: water; line 3: blotchy mottling symptoms; line 4: midrib yellowing symptoms; 5: negative control; line 6: fertilizer deficiency symptom.

detected HLB in peninsular Malaysia using OI1 and OI2c primer in eighteen citrus spices. He also detected HLB bacteria in the root system of citrus reticulate seedlings used the same primers (Hajivand et al. 2009a). The HLB-infected trees exhibited fruit drop. In some cases, a greenish discoloration developed on fruits at the peduncle rather than the styler end, as in the normal case (Halbert and Manjunath 2004; Bove 2006). The infected-fruits are small, lopsided, hard and have a bitter taste (Bove 2006, 2007). However, chemical treatments such as antibiotics, GA_3 and foliar fertilizer affected the quantity and quality of the fruit in this study. Typically, a direct application of antibiotics was not effective in improving fruit quantity and quality but fruits with low acidity were produced when infected plants were given antibiotic + GA_3 + foliar fertilizer (T7) and GA_3 + foliar fertilizer (T4). It has been reported that the application of GA_3 has improved the fruit set in Clementine mandarins but that it has little influence on Satsuma mandarins (Manuel et al. 1992). Seed abortion is common in HLB-infected citrus trees (Meyer et al. 2007; Bove 2006; Batool et al. 2007). The application of GA_3 reduces the incidence of both rind puffiness in mandarins and splitting in varieties with a thin rind. No research has been done concerning the effects of direct or a combination application of GA_3 on fruit quantity and quality in HLB-infected citrus trees. However, GA_3 is an important plant growth regulator for maintaining the citrus fruit rind in better condition and for controlling skin disorder in fruits (George 2008; Machado and Soccol 2004; Manuel et al. 1992). Low peel thickness percentage was obtained when T1 (antibiotic), T4 (antibiotic + GA_3) and T7 (antibiotic + GA_3 + foliar fertilizer) were used. Agusti et al., (2002) concluded that GA_3 prevents the increase of the peel weight, increases its resistance to puncturing and reduces its rate of chlorophyll losses. This relationship was found between the GA_3 effect on chlorophyll retention in the rind and the prevention of puffiness, together with its effects on increasing the compactness of the albedo, which supports the conclusion that puffiness is determined by the viability of the

Table 3. Mean comparison of physical analysis under different treatments using the Duncan multiple rang test

Treatment [a]	Peel thickness (%)	Fruit weight (g)	Juice %	Pulp Weight %	Peel Weight %	Pulp/peel Weight %	Yield (t/ha)
T1	1.80 c	103.33 ed	45.33 ab	45.33 ab	13.31 c	2.85 b	11.73 dc
T2	2.08 ab	129.33 ab	47.96 a	47.96 a	14.09 bc	2.63 bc	12.43 ab
T3	1.85 bc	115.33 bcd	47.66 a	47.16 ab	13.04 cd	2.85 b	11.33 d
T4	1.90 bc	121.33 abc	47.16 ab	47.66 a	15.60 ab	2.25 cd	12.40 ab
T5	1.75 c	105.66 ed	44.66 b	44.66 b	14.79 abc	2.68 cb	11.70 dc
T6	1.90 bc	113.33 cd	47.28 ab	47. 28 ba	13.65 bc	2.70 cb	12.00 bc
T7	2.20 a	135.00 a	47.88 a	47.88 a	16.36 a	2.11 d	12.90 a
T8	1.16 d	96.33 e	40.33 c	40.33 c	11.33 d	4.06 a	3.75 e

[a] Means within a column with different letters are significantly different and means followed by the same letters are not significantly different according to DMRT test at P≤ 0.05 levels. [b] The percentage data were converted to their arcsine-square root equivalents in order to normalize data sets prior to analysis. T1: Antibiotic. T2: GA_3. T3: foliar fertilizer. T4: Antibiotic + GA_3. T5: Antibiotic + foliar fertilizer. T6: GA_3 + foliar fertilizer. T7: Antibiotic + GA_3 + foliar fertilizer. T8: Control.

flavedo cell layers. Moreover, fruit with a high percentage of pulp, juice and fruit weight was obtained when T7 (antibiotic + GA_3 + foliar fertilizer) was applied to the infected citrus trees. The fruit yield was also increased by GA_3 and other chemical treatments such as foliar fertilizer on the HLB-infected citrus trees. GA_3 has been applied routinely to citrus trees such as navel orange and mandarin to improve the peel quality and prevent fruit drop. The pre-harvest application of gibberellic acid, in general, is used to maintain fruit peel quality on the tree and in storage, and reduces postharvest decay in several citrus fruits such as mandarins (Davies and Albrigo 1994). In the Nagpur mandarin, GA_3 (10-20 mg/L) treatment at break improves fruit firmness and reduces fruit weight loss during storage (Ladaniya 2004). The application of GA_3 (10 mg/L) did not significantly affect the abscissic acid content (Tao et al. 2002). The GA_3 pre-harvest applications are reported to increase juice percentage from 2-5% in Hamlin, Pineapple and Valencia. The peel was thinner and firmer in the treated fruits (Davies and Stover 2003). Ritenour and Stover (1999) also reported that GA_3 treatment significantly reduced TSS (10.50 ºBrix, compared to 11.10 º Brix in the control) and the TSS/TA ratio (9.62, compared to 11.52 in the control) at harvest.

Conclusion

The major milestones for this study were an increase in the fruit quantity and quality in an infected orchard. Finally, the best treatments were T7 and T4 to improve the fruit quality and quantity in HLB-infected *Citrus reticulata* fruits. The commercial benefit of this study can benefit the citrus industry in Malaysia, as well as throughout the world by increasing the life span of citrus trees.

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References

Agusti M, Martinez F, Mesejo C (2002) A review of citrus fruit quality. Physiological Basis and Techniques of Improvement. Agrociencia VIN2:1-16

Aubert B, Bove JM Effect of penicillin or tetracycline injections of citrus trees affected by greening disease in Reunion Island. In: Calavan. EC, Garnsey. SM, Timmer. LW (eds) Proc. of the 8th Conf. of the Intl. Organ. of Citrus Virologists., Riverside, CA, 1980. IOCV, Riverside, CA., pp 103-108

Azizah MJ, Zazali G (2005) Status kemerebakan dan impak Penyakit Greening Limau terhadap industry limau di Malaysia. Jabatan Pertanian Malaysia. Bengkel Kebangsaan Pengurusan Penyakit Greening Limau.

Batool A, Iftikhar Y, Mughal SM, Khan MM, Jaskani MJ, Abbas M, Khan IA (2007) Citrus greening disease; A major cause of citrus decline in the world; A Review. Horticultural Science 4 (34):159–166

Bockus WW, Davis MA (1993) Effect of nitrogen fertilizers on severity of tan spot of winter wheat. Plant Disease 77:508-310

Bove JM (2006) Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. Journal of Plant Pathology 88 (1):7-37

Bove JM (2007) Etiology of three recent diseases of citrus in São Paulo State: Sudden death, variegated chlorosis and huanglongbing. IUBMB Life 4 (2007):346-354

Buitendag CH (1991) The current status and the control of greening disease of citrus in the Republic of South Africa. Citrus Journal 1:35-40

Buitendag CH, Von Broembsen LA (1993) Living with citrus greening in South Africa. Paper presented at the Proc. of the 12th Conf. IOCV, Riverside, University of California, Riverside, November 23-27, 1992

Cheema SS, Kapur SP, Sharma OP (1986) Chemo-therapeutic controls of greening disease of citrus through bud dip treatment. Indian Journal of Virology (2):104-107

Davies F, Stover E (2003) New uses for old plant growth regulators. Citrus Industry, November, 21.

Davies FS, Albrigo LG (1994) Citrus. Redwood Books Trowbridge, Wiltshire, Great Britain

El-Otmani M, Taibi FZ, Lmoufid B, Ait-Oubahou A, Lovatt CJ (2002) Improved use of foliar on clementine mandarin to manipulate cropping in a sustainable production system. International Society for Horticultural Science:167-175

Garnier M, Danel N, Bové JM (1984) Aetiology of citrus greening disease. Annales de Institut Pasteur Microbiologie (Inst Pasteur) 135 (1):169-179. doi:10.1016/S0769-2609(84)80075-7

George EF (2008) Plant growth regulators III: Gibberellins, ethylene, abscisic acid, their analogues and inhibitors; miscellaneous compounds. In: George EF, Hall MA, Klerk G-JD (eds) Plant Propagation by Tissue Culture. pp 283-333. doi:10.1007/978-1-4020-5005-3_7

Gottwald TR, da Graça JV, Bassanezi RB (2007) Citrus Huanglongbing the pathogen and its impact. Online Plant Health Progress. doi:10.1094/PHP-2007-0906-01-RV

Graca JV, Korsten L (2004) Citrus Huanglongbing: Review, present status and future strategies. In: Diseases of Fruits and Vegetables, vol 1. pp 229-245. doi:10.1007/1-4020-2606-4_4

Hajivand S, Thohirah LA, Kamaruzaman S, Siti NAA (2009a) Determination of the presence of Huanglongbing in seeds and movement of the pathogen in *Citrus reticulata*. American Journal of Applied Sciences 6 (6):1180-1185

Hajivand S, Thohirah LA, Kamaruzaman S, Siti NAA (2009b) Differential reaction of citrus species in Malaysia to Huanglongbing (HLB) disease using grafting method. American Journal of Agricultural and Biological Sciences 4 (1):32-38

Halbert SE, Manjunath KL (2004) Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. The Florida Entomologist 87 (3):330-353

Hung TH, Wu ML, Hong JS (1999) Development of rapid method for the diagnosis of citrus greening disease using polymerase chain reaction. Journal of Phytopathol 147 (1999):599-604. doi:10.1046/j.1439-0434.1999.00435.x

Hung TH, Wu ML, Su HJ (2001) Identification of the Chinese box orange (*Severinia Buxifolia*) as an alternative host of the bacterium causing citrus Huanglongbing. European Journal of Plant Pathology 107 (2):183-189

Jagoueix S, Bové JM, Garnier M (1996) PCR detection of the two *Candidatus* Liberobacter species associated with greening disease of citrus. Molecular and Cellular Probes 10 (1):43-50

Khan IA (2007) Citrus genetics, breeding and biotechnology. pp.1-370. 1st edn. CABI Publishing, Cambridge

Reduction in post-harvest losses of fruit and vegetabale. Final report of NATP Project, Indian Council of Agricultural Research , New Delhi (2004).

Lim WH, Shamsudin BO, Ko W (1990) Citrus greening disease in Malaysia. In: Aubert B, Tontyaporn S, Buangsuwon D (eds) Rehabilitation of citrus industry in the Asia Pacific region, International Conference on Citriculture, Chiang Mai, Thailand, 4-10 February 1990. UNDP-FAO. Rome, Rome, pp 100-105

Long DH, Lee FN, Tebeest DO (2002) Effect of nitrogen fertilization on disease progress of rice blast on susceptible and resistant cultivars. Plant Disease 48:403-409

Machado CMM, Soccol CR (2004) Kinetics of gibberella fujikuroi growth and gibberellic acid Production by solid-state fermentation in a packed-bed column bioreactor. Biotechnology Progress 20 (5):1449-1453. doi:10.1021/bp049819x

Manuel T, Lorenzo Z, Eduardo P-M (1992) Gibberellins and parthenocarpic ability in developing ovaries of seedless mandarins. Plant physiology 99:1575-15781

Martinez AL, Nora DM, Armedilla AL (1970) Suppression of symptoms of citrus greening disease in the Philippines with tetracycline antibiotics. Plant Disease Reporter 54 (1970):1007-1009

Meyer JM, Hoy MA, Singh R (2007) Low incidence of *Candidatus* Liberibacter asiaticus in *Diaphorina citri* (Hemiptera: Psyllidae) populations between Nov 2005 and Jan 2006: Relevance to Management of Citrus Greening Disease in Florida. Florida Entomologist 90 (2):394-397

Milind SL (2008) Citrus Fruit: Biology, Technology and Evaluation. Elsevier USA

Naar Z (2006) Effect of cadmium, nickel and zinc on the antagonistic activity of *Trichoderma sp*. against *Pythium irregulare* Buisman. Acta Phytopathologia Entomologica Hungarica 41 (3-4):193-202

Nariani TK (1981) Integrated approach to control citrus greening disease in India. International Society of Citriculture 1:471-472

Pozo L, Kender WJ, Burns JK, Hartmond U, Grant A (2000) Effects of gibberellic acid on ripening and rind puffing in 'Sunburst' mandarin. Paper presented at the Selected Proceedings of the Florida State Horticultural Society, Florida,

Ranganna S (1977) Manual of analysis of fruit and vegetables products. McGraw-Hill Publishing Company, New Delhi.P: 634.

Ritenour MA, Stover E (1999) Effects of gibberellic acid on the harvest and storage quality of Florida citrus fruit. The Proceedings of the Florida State Horticultural Society 112:122-125

Roistacher CN (1996) The economics of living with citrus diseases: Huanglongbing (greening) in Thailand. Paper presented at the 13th Conference of the International Organization of Citrus Virologists (IOCV), Riverside,

Saleem BA, Malik AU, Farooq M (2007) Effect of exogenous growth regulators on fruit drop and fruit quality in *citrus* cv. Blood Red. Pakistan Journal of Agricultural Sciences 44 (2):289-294

Tao J, Zahang SL, Chen KS, Zhao ZZ, Chen JW (2002) Effects of GA_3 treatment on changes of pigments in peel of citrus fruit. Acta Horticulture Sinica 19:566-568

Teixeira DC, Saillard C, Couture C, Martins EC, Wulff NA, Eveillard JS, Yamamoto PT, Ayres AJ, Bove JM (2008) Distribution and quantification of *Candidatus* Liberibacter americanus, agent of Huanglongbing disease of citrus in São Paulo State, Brasil, in leaves of an affected sweet orange tree as determined by PCR. Molecular and Cellular Probes 22 (3):139-150

Van Vuuren SP, da Graça JV (1993) Variable transmission of African greening to sweet orange. Paper presented at the 12th Conference of the International Organization of Citrus Virologists, Riverside,

Zhao XY (1981) Citrus yellow shoot (Huanglungbin) in China. A review, International Society of Citriculture 1:466-469