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Field assessment of calcium, copper and zinc ions on plant recovery and disease severity following infection of Huanglongbing (HLB) disease

Khairulmazmi Ahmad[1]*, Kamaruzaman Sijam[2], Habibuddin Hashim[3], Zamri Rosli[4] and Arifin Abdu[5]

[1]Department of Crop Science, Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Kampus Bintulu, Sarawak, Malaysia.
[2]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.
[3]Biotechnology Research Center, Malaysian Agricultural Research and Development Institute (MARDI), Serdang Selangor, Malaysia.
[4]Department of Forestry Science, Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Kampus Bintulu, Sarawak, Malaysia.
[5]Department of Forest Management, Faculty of Forestry, University Putra Malaysia, Serdang Selangor, Malaysia.

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The effect of Ca, Cu and Zn ions on plant recovery and disease severity following infection of huanglongbing disease was carried out at a commercial citrus orchards belonging to Lembaga Pertubuhan Peladang (LPP) Terengganu, situated at Durian Mentangau, Dungun, Terengganu. Application of Zn or Cu ions in combination with Ca was able to delay disease incidence and severity of hunglongbing disease. These treatments (C/Ca/Cu and C/Ca/Zn) also showed slightly reduction of AUDPC value on infected citrus trees. In general, analysis of macronutrients and micronutrients content in citrus leaves showed significantly different at the beginning of the study (0 to 6 months after treatment) but no significant different were observed at the end of the study period (6-9 months after treatment) as noted on Mg, P, Ca and Zn analysis. Results for specific leaf area showed no significant different among treatment as observed in leaf area, total leaf length and total leaf width. However, specific leaf area was increased gradually in all treatments over the period of the study. The overall results of field trial showed that infected citrus trees treated with combination of Ca and Zn ions at 600 and 10 ppm, respectively resulted in significant increase ($P \leq 0.05$) in terms of fruit production and total soluble solid (TSS) content only.

Key words: Huanglongbing, *candidatus* liberibacter asiaticus, field assessment, micronutrients and macronutrients.

INTRODUCTION

In Malaysia, huanglongbing (HLB) disease is considered a very important disease of citrus and many efforts have been made to contain the disease spread in the orchards as well as in the country. Reports from Department of Agriculture Malaysia (2005) and Khairulmazmi et al. (2008a) showed that HLB was detected throughout Peninsular Malaysia including Sabah and Sarawak. About 2458 ha or 69.7% from 3526 ha of citrus cultivation areas in Peninsular Malaysia have been detected positive visually (Azizah and Zazali, 2005). A further study confirmed the occurrence and spread of HLB disease in Peninsular Malaysia (Khairulmazmi et al., 2008b; 2009). In Terengganu as an example, HLB was distributed

*Corresponding author. E-mail: kcik@hotmail.com. Tel: + 6086 855409.

widely, increasing from 641 ha in 2001 to 1262 ha in 2004 (Azizah and Zazali, 2005). The causal agent, *Candidatus* liberibacter asiaticus was characterized genetically based on the outer membrane protein (OMP) and 16S rDNA genes (Khairulmazmi et al., 2009). Furthermore, the causal agent is believed to be indigenous species (Khairulmazmi et al., 2008b). Severe HLB infection was reported on many commercial citrus species such as *Citrus reticulata*, *C. suhuiensis* and *C. madurensis* but mild infection was reported on *C. grandis* (Khairulmazmi et al., 2008a).

In Malaysia many commercial citrus species such as *C. reticulata* and *C. suhuiensis* are among the most susceptible species to micronutrients deficiency such as Zn, Fe and Cu as well as HLB disease (Khairulmazmi et al., 2008a). Foliar sprays with micronutrient fertilizer towards susceptible species showed good promise to improve micronutrients deficiency (Ozbek, 1969). The most important breakthrough regarding this issue appears to be the finding of Dybing and Currier (1961) that the presence in spray solutions of an efficient surfactant at a concentration of about 0.5% markedly enhanced the penetration of iron and other chemical into leaves tissue. As compared with some other crops, an application of balanced fertilizer is more important for citrus trees because they showed strong reaction against of either deficiencies or excesses of nutrients. However, the reaction of host is always depending on their root systems (Ozbek, 1969).

The obvious symptoms of micronutrients deficiency and HLB symptoms in citrus orchards showed typical symptoms of chlorosis pattern such as leaf yellowing and/or leaf mottling. These symptoms appeared as result of reduced chlorophyll content in leaves. Other deficiency symptoms which are always associated with Zn and Fe deficiencies are small and narrow leaves (Bryan, 1957). Yield loss due to HLB disease alone is not easy to evaluate because sometimes only certain sectors of trees are affected and this result only in small losses. In another case, the entire citrus tree is affected and it may cause total yield loss (da Graca, 1991). A study conducted by Aubert et al. (1996) showed that 65% of citrus trees in Reunion Island were badly damaged and rendered unproductive within seven years after planting. Similar situation was reported in Thailand by Roistacher (1996). Their study showed that citrus trees generally started to decline within five to eight years after planting. In order to make profit the groves must live for a minimum of 10 years (Roistacher, 1996).

Citrus is very important industry and HLB disease is the main threat to the industry in Malaysia. To date, there is no specific treatment available to overcome HLB disease. However, one possible method is by suppressing HLB severity in the orchards using inorganic elements amendment. According to Sugimoto et al. (2007), inorganic elements amendment showed promising results to suppress the growth of *Phytophthora sojae*. Other studies have also showed that inorganic elements have suppressive effect against many *Phytophthora* species (Slade and Pegg, 1993; Toppe and Thinggaard, 1998). Previous studies showed that N, Ca, Zn, Cu and other microelements showing good potential in suppressing disease severity caused by fungi and bacteria (Naar, 2006; Long et al., 2000; Bockus and Davis, 1993). However, to date very limited information is available on the effect of micro or/and macronutrients on HLB disease. Therefore, in the present study Ca, Zn and Cu ions were applied to suppress HLB disease as well as to increase life span of infected citrus trees in order to maintain economical fruit production. The specific objective of this study was to evaluate the effects of Ca, Zn and Cu ions amendment on citrus plant recovery and disease severity following infection of HLB disease.

MATERIALS AND METHODS

Disease control trials

A field trial plot was conducted in commercial citrus orchards belonging to Lembaga Pertubuhan Peladang (LPP) Terengganu, situated at Durian Mentangau, Dungun, Terengganu, where the disease has affected citrus production for several years. The study was performed on citrus trees of susceptible species namely honey mandarin (*C. reticulata)*. The age of the trees was about eight years and planted on a mineral soil. Based on the data reported by Khairulmazmi et al. (2008a), the disease incident in this plot was about 60% consisting of three levels of disease severity namely severe, moderate and mild infections. The symptomatic leaves were tested positive against HLB disease using conventional PCR test (Khairulmazmi et al., 2008b; 2009). Citrus tree used in this experiment was selected based on uniformity in size and level of disease severity. Selected trees were tagged and labeled properly. Monthly temperature and rainfall during the trials were collected from nearby a meteorological station.

This experiment was divided into three blocks namely Block A, B and C and four treatments namely Treatment A (C), B (C/Ca), C (C/Ca/Cu) and D (C/Ca/Zn). Every block consisted of 160 trees. Planting distance between citrus trees was 5 × 5 m. In these trials, Treatment A was assigned as control treatment (C). For the control treatment, citrus trees were given only organic fertilizer at 2 kg/tree for every three months as recommended by the manufacturer (Table 1). Standard farm management practices were applied in all treatments such as light pruning whereby infected twigs or small branches were removed to reduce source of inoculums and to stimulate new shoot or branches. Pesticide was applied to control population of vector and N:P:K fertilizer (2 kg/tree) was given at every three months.

For Treatment B (C/Ca), citrus trees were given similar treatment as the control. However, Ca (600 ppm/ha) was amended to the trees every three months. Treatments were applied using fogging type sprayer. Treatment C (C/Ca/Cu), citrus trees were given similar treatment as the control. However, combination of Ca (600 ppm/ha) and Cu (10 ppm/ha) were amended to the trees every three months. Lastly, Treatment D (C/Ca/Zn) citrus trees were treated similar as control treatment. However, combination of Ca (600 ppm/ha) and Zn (10 ppm/ha) were amended to the trees every three months.

Disease assessments in commercial orchard plot trails

Four disease variables were used to evaluate the effect of Ca, Cu

Table 1. Types of treatments given on infected honey mandarin (*C. reticulata)* used in field trials conducted at commercial citrus orchards at Durian Mentangau, Dungun Terengganu.

Treatments	Time applied	Micronutrient Application*			
		Organic fertilizer (2 kg/tree)	Calcium ($CaCl_2$) 600 ppm/ha	Zinc (Zn-EDTA) 10 ppm/ha	Copper (Cu-EDTA) 10 ppm/ha
Treatment A (C)	March, Jun, September	√	-	-	-
Treatment B (C/Ca)	March, Jun, September	√	√	-	-
Treatment C (C/Ca/Cu)	March, Jun, September	√	√	-	√
Treatment D (C/Ca/Zn)	March, Jun, September	√	√	√	-

and Zn ions amendment on plant recovery and severity against HLB disease i.e. disease incidence and disease severity, specific leaf area, micronutrient and macronutrient analysis and fruit quality and quantity.

Disease incidence and disease severity

HLB symptoms on citrus trees were recorded for yellowing or mottling leaves and twigs dieback. To evaluate the disease incidence, the number of citrus trees with apparent symptoms of HLB disease over the total number of trees was assessed for each treatment. The following formula was adopted to calculate percentage of disease incidence:

$$\%\text{ disease incidence} = \frac{\text{Total infected citrus trees}}{\text{Total number of trees evaluated}} \times 100$$

However, data for disease severity was recorded separately according to their symptoms namely leaf yellowing or mottling and twigs dieback. For disease severity evaluation, the disease severity grading was defined based on the symptoms that existed on plant canopy. The grading system was as follows:

1. No symptom (no symptom observed on plant canopy) = 0

2. Mild (leaf mottling or yellowing / twigs dieback observed on 1 to 30% plant canopy) = 1

3. Moderate (leaf mottling or yellowing / twigs dieback observed on 31 to 50% plant canopy) = 2

4. Severe (leaf mottling or yellowing / twigs dieback observed on 51 to 100% plant canopy) = 3

The below formula was adopted to calculate percentage of disease severity;

$$\%\text{ disease severity} = \frac{X_1 + X_2 + \ldots + X_n}{Y \times \text{Maximum rating scale}} \times 100$$

Where: $X_{1..n}$ = score of disease severity of each citrus plant and Y = total number of plants at the same experiment.

The data for disease incidence and disease severity were collected and transformed using arc sine transformation and analyzed according to analysis of variance (ANOVA) with means comparison using least significant different test ($LSD_{0.05}$) at 5% confident level. The area under disease progress curve (AUDPC) was then assessed using the same data plotted as disease progress curve. The highest AUDPC value proved that disease incidence, disease severity and yield loss of citrus trees were high in the treatments. The AUDPC was calculated according to the formula of Campbell and Madden (1990).

Micronutrient and macronutrient analysis

The samples were washed using 0.01 M ethylene diamine tetra acetic (Na-EDTA) and afterwards rinsed with distilled water. The samples were bagged and dried immediately using oven at 60°C until constant weight was affined. The dried samples were grounded using a mechanical grinder. Standard ashing method (Faculty of Agriculture, 1981) was used to digest the samples and the constant of micro and macronutrient determined using atomic absorption spectrophotometer (AAS) (Perkin Elmer 5000, Perkin Elmer, USA).

Assessment of specific leaf area

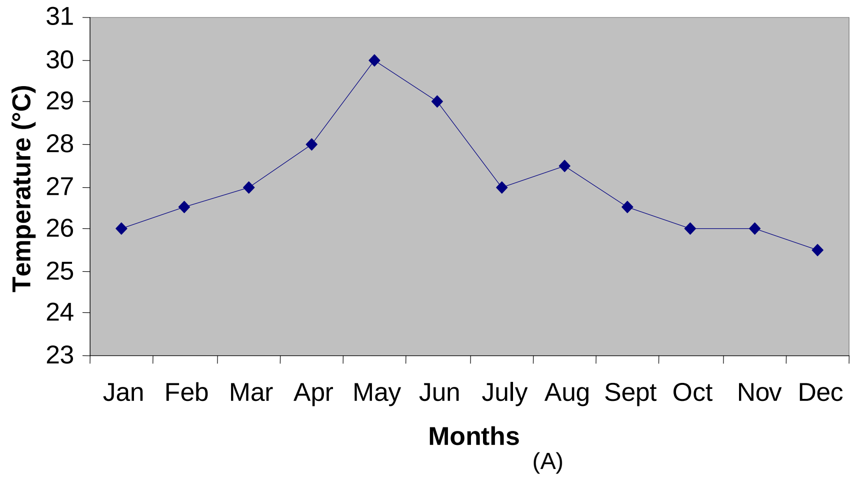
Infected citrus trees were assessed based on the methods of Ho (1984) and Corley and Bruere (1981). Data on specific leaf areas for each treatment were collected every three months.

Assessment of fruit quality and quantity

Twelve months after treatment, data on fruit quality and quantity were evaluated. Fruit quality was assessed based on fruit grades and total soluble solid (TSS). The fruits were graded based on the following scale:

i. AA grade = 200-250 g/fruit
ii. A grade = 150-199 g/fruit
iii. B grade = 100-149 g/fruit
iv. C grade = less 100 g/fruit

For fruit quantity, number of fruit per branch from each treatment was evaluated and weighed using a digital balance. The total soluble solid (TSS) content of fruits was determined using hand refractometer (N-3000E, Atago, Japan). Ten grams of fruit was homogenized using a kitchen blender with 40 ml of distilled water. The mixture was filtered through cotton wool. A drop of the filtrate was then placed on the prism glass of a refractometer to obtain the TSS reading (Ranganna, 1986).



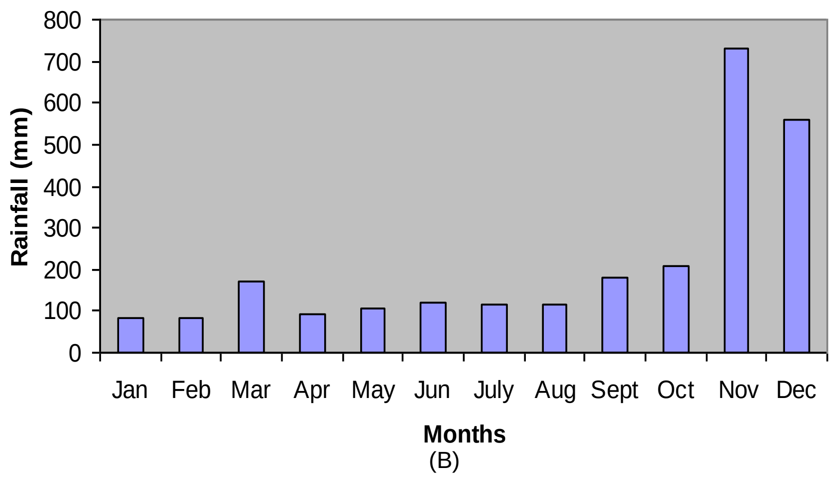


Figure 1. Ten year's mean (A) monthly temperature and (B) rainfall throughout the year in Terengganu (1998-2008).

Statistical analysis

Data were analyzed using SAS® Software. Treatment effect was tested using ANOVA and the mean comparison separated using least significant different (LSD) test at 5% probability level (Gomez and Gomez, 1984).

RESULTS

Effect of Ca, Cu and Zn Ions on disease incidence and disease severity of infected citrus trees

The mean temperature and rainfall for every month during experimentation are summarized in Figures 1A and 1B. The month with the highest temperature was May (30°C) and this was followed by Jun (29°C) and April (28°C). The period with high rainfall was November and December with volumes of 730 and 560 mm, respectively. The effect of treatment application on the disease incidence and disease severity of HLB disease was monitored during this season in the experimental plots.

The effect of the assayed treatments to the control of HLB disease of citrus in Durian Mentangau, Terengganu was summarized in Figures 2A, 2B and 2C. Different treatments showed different efficacy at different period of evaluation. Results of percentage disease incidence in Figures 2A showed generally disease incidence on citrus leaf increased over times of evaluation. Disease incidence increased rapidly starting from June until September and it kept stable afterwards. This disease took six to nine months to reach 100% disease incidence depending on treatments applied. In Treatment A (C), disease incidence progressing faster than Treatments B (C/Ca), C (C/Ca/Cu), and D (C/Ca/Zn). It reaches the peak (100% disease incidence) within six months after the first treatment was applied. On the other hand, Treatment B (C/Ca), C (C/Ca/Cu) and D (C/Ca/Zn) took nine months to reach 100% disease incidence. Treatment A (C) was also recorded as having the highest AUDPC values followed by Treatments D (C/Ca/Zn), C (C/Ca/Cu) and B (C/Ca) of 465.0, 451.5, 447.0 and 442.5,

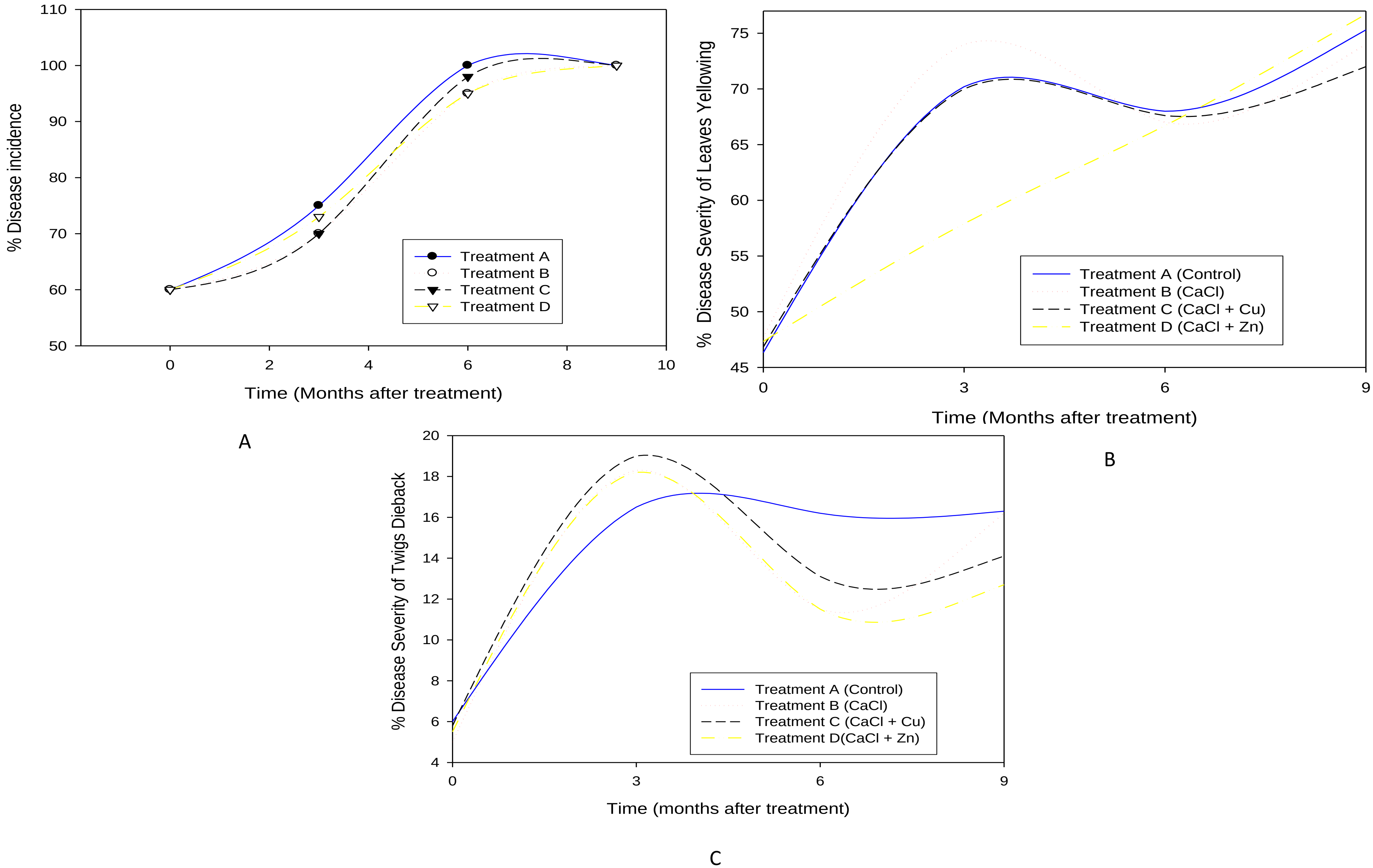


Figure 2. (A) Disease progress curve for HLB disease incidence and (B and C) disease severity on honey mandarin (*Citrus reticulata)* after treated with calcium, zinc and copper ions.

Table 2. Area under the disease progress curve (AUDPC) values for disease incidence, disease severity caused by HLB disease based on the four treatments.

Treatment	AUDPC value			
	Disease* incidence	Disease severity* of leave yellowing	Disease severity* of twigs dieback	Mean AUDPC
Treatment A (C)	465.0a	382.1a	87.8a	311.6a
Treatment B (C/Ca)	442.5a	394.0a	79.7a	305.4a
Treatment C (C/Ca/Cu)	447.0a	381.6a	81.5a	303.4a
Treatment D (C/Ca/Zn)	451.5a	344.8a	80.1a	292.1a

*Mean within block of each treatment.
Means within a column having the same letter is not significantly different at $P \leq 0.05$

respectively (Table 2). From these results it showed that treatments given were able to reduce yield loss and delay disease incident in the trial plot study.

For disease severity assessment, similar trends were observed. Results of percentage disease severity in Figures 2B and 2C showed disease severity on citrus leaves and twigs dieback increased over the time of evaluation. Based on leaf yellowing or mottling symptoms, Treatment B (C/Ca) recorded the highest percentage disease severity (74%) three months after treatment. However, disease severity was decreased for the following months (3-6 months after treatment). Based on AUDPC values, Treatment B (C/Ca) recorded the highest value followed by Treatments A (C), C (C/Ca/Cu) and D (C/Ca/Zn) with a value of 394.0, 382.1, 381.6 and 344.8, respectively. In terms of twigs dieback severity (Figure 2C), the degree of severity alleviated in the initial study, from 6 to 19% (0-3 months after treatment) as observed in Treatment C (C/Ca/Cu) but it started to decline three to six months after treatment and remained constant afterwards. Treatment A (C) showed the highest percentage disease severity of twigs dieback compared to other treatment. Based on AUDPC values for twigs dieback (Table 2), Treatment A(C) was recorded the highest value followed by Treatments C (C/Ca/Cu), D (C/Ca/Zn) and B (C/Ca) with a value of 87.8, 81.5, 80.1 and 79.7, respectively. However, based on mean value of AUDPC (Table 2) Treatment A (C) gave the highest value of 311.6 followed by treatment B (C/Ca), C (C/Ca/Cu) and D (C/Ca/Zn). Again these values showed no significant different between treatments.

Relationship of nutrient content in citrus leaves with HLB disease

One of the effects of HLB disease to the infected citrus trees was disruption of nutrient uptake by the trees. Most of the previous report showed that the infected citrus trees generally exhibited micronutrient deficiency especially Zn deficiency. In these field trials several important macronutrients such as Mg, K, P and Ca and micronutrients such as Zn and Cu were evaluated.

Comparison of macronutrients content in citrus leaves showed significantly different at beginning of study (0-6 months after treatment) as noted on Mg, P and Ca analysis in Treatments C (C/Ca/Cu), D (C/Ca/Zn) and C (C/Ca/Cu), respectively. However, it showed no significant different 6 months after treatment. Most of macronutrients evaluated such as P, K and Mg for every treatments showed positive increment nine months after treatment as compared to pre-treatment period (Figures 3A, 3B, 3C and 3D). It is especially noted on Treatment D which consisted combination of Ca and Zn ions. Upon comparing the macronutrients content in citrus leaves with the nutritional standard it was found out that concentration of Mg, K and P were at optimum level as recommended by University of California standard value for macronutrient content (Table 3). For instance, nutritional standard value for Mg was between 0.26-0.60%, however Mg content in our trials recorded before treatments were ranged from 0.44-0.78% and the value increased between 0.64-0.74% (high level) nine after months depending on the treatments. On the other hand, Ca content was recorded below the optimum level, 3.0–5.5% on Treatments A (C), B (C/Ca) and C (C/Ca/Cu) with a value of 2.53, 2.75 and 2.69, respectively. However, Treatment D (C/Ca/Zn) was at optimum level with a value of 3.59%. The concentration of Ca was increased to optimum level (3.93–5.64%) three months after treatments and then it decreased (2.39–2.79%) again to the sub-standard value nine months after treatment.

Significant different was observed on Zn analysis as noted on Treatment C (C/Ca/Cu) and D (C/Ca/Zn). Generally, Zn and Cu content for every treatment showed positive increment over times of evaluation compared with micronutrient content before treatment application (Figures 3E and 3F).

This was especially observed on Treatment D (C/Ca/Zn) whereby upon comparing micronutrient content in citrus leaves with the nutritional standard (Table 4) it was noted that concentration of Zn was below the optimum level. For instance, optimum level of nutritional standard for Zn was 25-100 ppm; in our trials Zn content

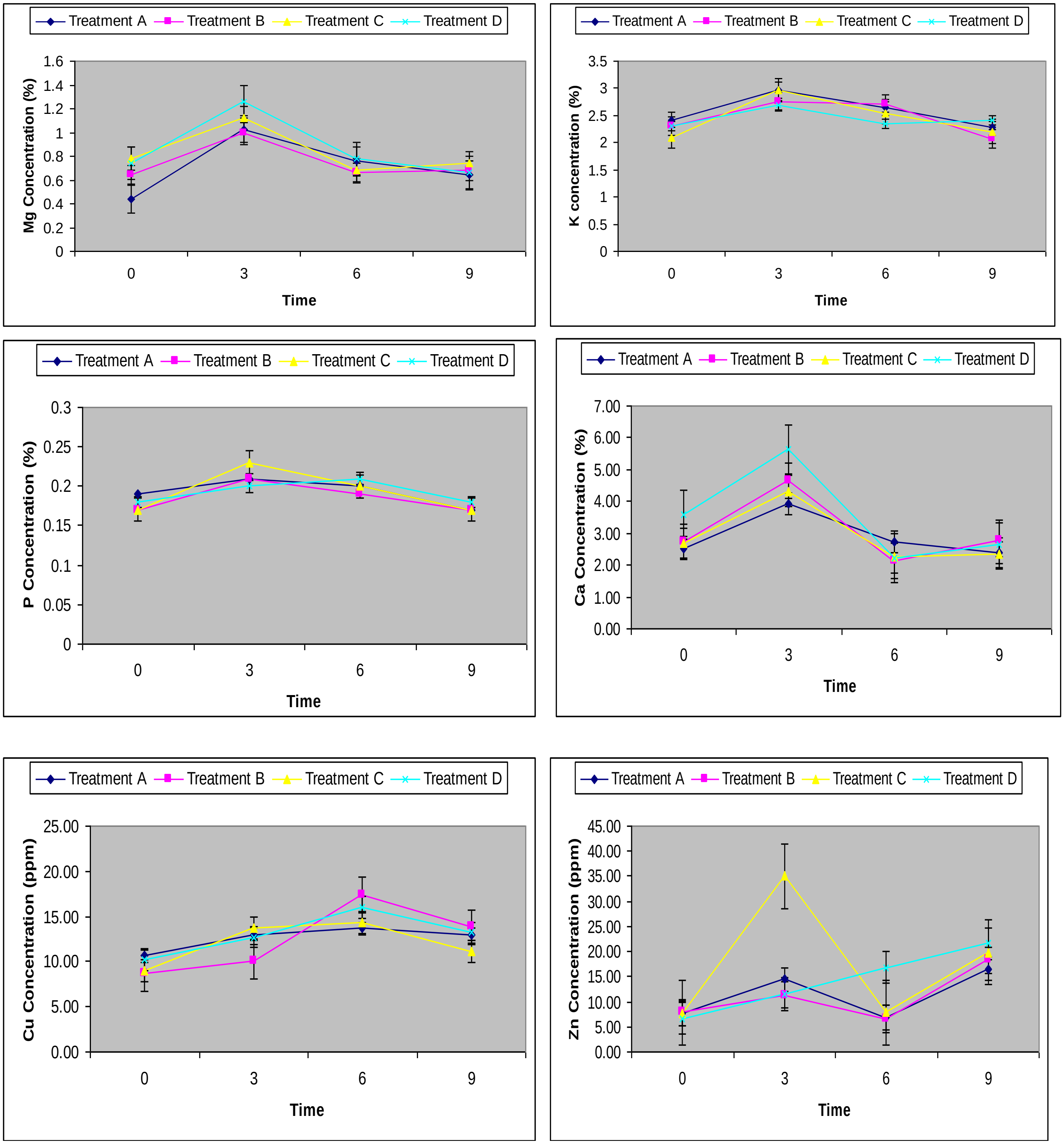


Figure 3. The effect of calcium, zinc and copper ions on the macronutrient (P, K, Mg, Ca) and micronutrient (Cu and Zn) content in citrus leaves following infection with HLB disease.

recorded before treatment application was from 6.70–7.90 ppm. This value indicated that citrus trees tested in all study plots were at deficiency level. However, the value was increased between 16.58–21.58 ppm (low level) nine months after the first treatment application depending on the treatment applied. Even though it showed improvement after treatment was given, nevertheless it still far from sufficient. In contrast, analysis of Cu content in citrus leaves in this trial indicated that the concentration was at optimum level. Cu concentration

Table 3. Nutritional standards for macronutrient in citrus leaves as recommended by University of California (University of California Cooperative Extension, 2003).

Parameter	Nitrogen (%)	Phosphorus (%)	Potassium (%)	Sodium (%)	Calcium (%)	Magnesium (%)
Deficiency	2.2	0.09	0.4	0.14	1.5	0.164
Low	2.2-2.4	0.09-0.11	0.40-0.69	0.14-0.19	1.6-2.9	0.16-0.25
Optimum	2.5-2.7	0.12-0.16	0.70-1.09	0.2-0.3	3.0-5.5	0.26-0.60
High	2.7-2.8	0.17-0.29	1.1-2.0	0.4-0.5	6.0-6.9	0.7-1.1
Excess	3.0	0.3	2.3	0.6	7.0	1.2

Table 4. Nutritional standards for micronutrient in citrus leaves as recommended by University of California (University of California Cooperative Extension, 2003).

Parameter	Zinc (ppm)	Iron (ppm)	Copper (ppm)	Boron (ppm)	Manganase ppm)	Molybdenum (%)
Deficiency	16.0	36.0	3.6	21.0	16.0	n.a
Low	16.0-24.0	36.0-59.0	3.6-4.9	21.0-30.0	16.0-24.0	n.a
Optimum	25.0-100.0	60.0-120.0	5.0-16.0	31.0-100.0	25.0-200.0	n.a
High	110-200	130-200	17.0-22.0	101-260	300-500	n.a
Excess	300	250	22.0	260	1000	n.a

n.a = not available

Table 5. The relationship between concentrations of Ca, Mg, Cu and Zn ions, dieback and leaf yellowing symptoms at nine months after treatment application (n=200).

Parameter	Ca Conc.	Mg Conc.	Cu Conc.	Zn Conc.	Time evolution	Dieback	Leaf yellowing
Calcium (Ca) Conc.	1.0	0.874**	- 0.232	0.188	- 0.373	0.468	- 0.028
Magnesium (Mg) Conc.		1.0	0.041	0.353	- 0.160	0.628**	0.189
Copper (Cu) Conc.			1.0	0.230	0.595*	0.439	- 0.232
Zinc (Zn) Conc.				1.0	0.394	0.534*	0.188
Time evaluation					1.0	0.536*	- 0.373
Dieback						1.0	0.809**
Leaf yellowing							1.0

**Significant at P≤0.01.
* Significant at P≤0.05.

was increased over time of evaluation.

Relationship of macronutrients and micronutrients content with HLB disease was summarized in Table 5. Based on the Pearson’s correlation analysis, micro-nutrient (Cu) and macronutrient (Ca) content interacted negatively with the symptoms of HLB disease in general. For instance, Ca and Cu showed negative correlation (-0.028 and -0.232) with leaf yellowing symptom. It means that increase in concentration of Ca and Cu in citrus leaves was able to suppress the severity of HLB symptoms in the field. However, the interactions were not significantly different; it could be due to the inability of the trees to uptake the fertilizers effectively. It could be also due to the trees suffered from the disease severely.

Effect of Ca, Cu and Zn Ions on citrus leaf development

Assessment of citrus leaf development was carried out. Detail data of mean leaf area, leaf length and leaf width were summarized in Table 6. The data showed that there were no significant different among treatments observed on leaf areas, total leaf lengths and total leaf widths. There were also no significant different observed between assessment times. In general, the trees showed poor response to the treatments applied by showing slight improvement in terms of leaf area at nine months after treatment application as compared to leaf areas at three months after treatment.

Table 6. The effect of Ca, Cu and Zn ions on leaf lengths, leaf widths and leaf areas of citrus leaves following infection of HLB disease.

Treatments	Mean leaf length (cm^2)			Mean leaf width (cm^2)			Mean leaf area (cm^2)		
	3 MAT	6 MAT	9 MAT	3 MAT	6 MAT	9 MAT	3 MAT	6 MAT	9 MAT
Treatment A (C)	5.82a	6.77a	6.72a	2.96a	3.02a	3.1a	17.23a	20.45a	20.83a
Treatment B (C/Ca)	6.43a	6.22a	6.97a	2.95a	2.83a	3.2a	18.97a	17.6a	22.3a
Treatment C (C/Ca/Cu)	6.71a	6.62a	7.02a	3.12a	3.09a	3.13a	20.93a	20.46a	21.97a
Treatment D (C/Ca/Zn)	6.3a	7.01a	6.86a	2.89a	3.32a	3.33a	18.21a	23.27a	22.84a

MAT = Month after treatment.
Values followed by the same letter in the same column are not significant differences at P≤0.05.

Table 7. Correlation between Ca, Cu and Zn ions, leaf length, leaf width and leaf area at nine months after treatment application (n=200).

Parameter	Ca Conc.	Cu Conc.	Zn Conc.	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)
Calcium (Ca) Conc.	1.0	- 0.232	0.188	0.077	0.43	- 0.004
Copper (Cu)Conc.		1.0	0.230	0.566*	0.536*	0.496
Zinc (Zn)Conc.			1.0	0.491	0.510*	0.502*
Leaf length (cm)				1.0	0.889**	0.895**
Leaf width (cm)					1.0	0.988**
Leaf area (cm^2)						1.0

**Significant at P≤0.01.
* Significant at P≤0.05.

Table 8. Effect of Ca, Cu and Zn ions on total soluble solids (TSS), mean fresh weight and mean number of fruits/branch of citrus fruits.

Treatments	Mean fresh weight (g)	Mean number of fruits/branch	Mean TSS (Brix%)
Treatment A (C)	60.5a	1.10a	7.5a
Treatment B (C/Ca)	43.8a	1.13a	7.1a
Treatment C (C/Ca/Cu)	57.1a	1.77b	7.4a
Treatment D (C/Ca/Zn)	58.9a	1.37ab	8.5b

Values followed by the same letter in the same column are not significant differences at P≤0.05.

Relationship of macronutrient and micronutrient content with citrus leaf development was summarized in Table 7. Based on the Pearson's correlation analysis, generally macronutrient such as Ca and micronutrients such as Zn and Cu interacted positively with citrus leaf development. It means that increase in nutrient concentration in a leaf may increase the leaf length, leaf width and leaf area. In the case of Cu content, application at 10 ppm/ha of Cu concentration showed significant difference (P≤0.05) with correlation values of 0.566 and 0.536 for leaf length and leaf width, respectively. Similar response was observed on Zn application and showing significant difference (P≤0.05) with correlation values of 0.510 and 0.502 for leaf width and leaf area, respectively. It proved that application of micronutrients fertilizer managed to improve citrus leaf development. On the other hand, application of Ca showed no significant different with leaf length, leaf width and leaf area of citrus leaves.

Effect of Ca, Cu and Zn ions on citrus fruit production

In these field trials, citrus trees treated with combination of Ca and Cu ions showed significant different in terms of fruit production as compared to the Treatment A (C). For instance, the average number of fruits produced at the end of study was 1.77 per branch in Treatment D (C/Ca/Zn) as compared to Treatment A (C) with 1.1 per branch (Table 8). Infected citrus trees fertilized with a combination of Ca and Cu ions at concentrations of 600 ppm and 10 ppm respectively produced 61% more fruits than the Treatment (A). However, other treatments such as Treatments B (C/Ca) and D (C/Ca/Zn) were not significantly different. It means that fertilizers were supplied as either Ca alone or in combination of Ca and Zn at similar rate made little improvement of citrus yield.

Assessment on fruit grades were summarized in Figures4 and 5, which showed no significant different but varied

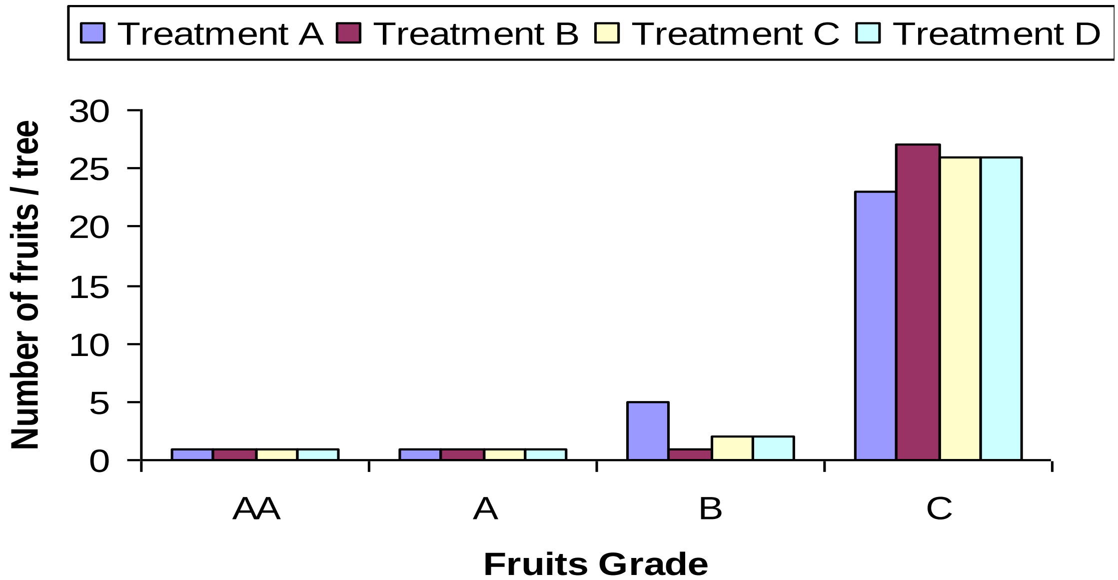


Figure 4. Effect of Ca, Cu and Zn ions on the citrus fruit grade. Values followed by the same letter in the same fruit grade are not significant differences at P≤0.05.

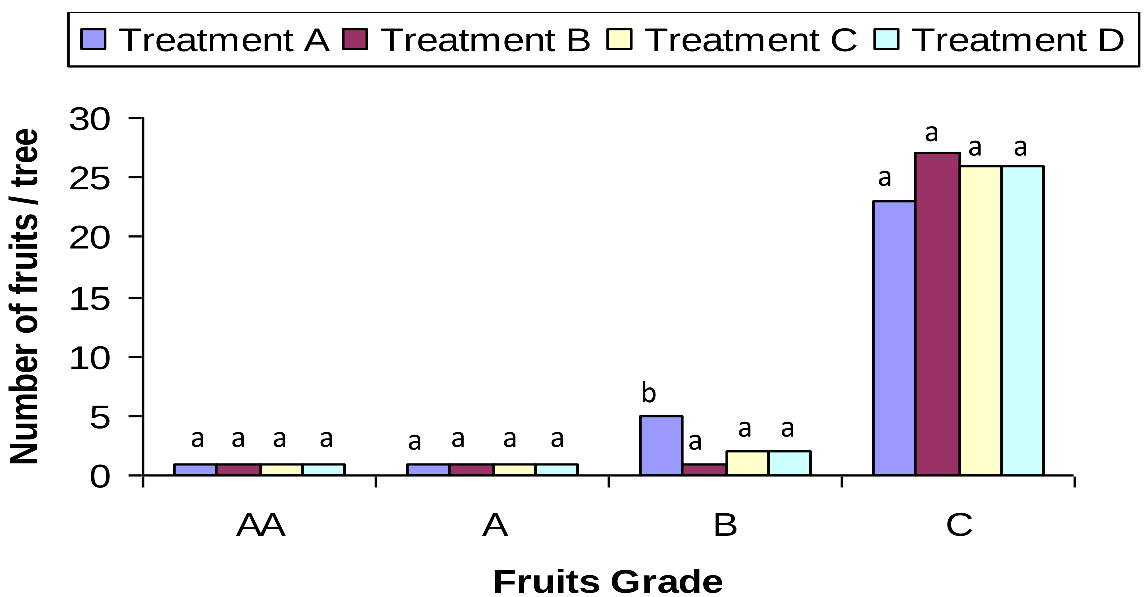


Figure 5. Effect of Ca, Cu and Zn ions on the citrus fruit grade. Values followed by the same letter in the same fruit grade are not significant differences at P≤0.05.

slightly between treatments especially noted at Treatments A (C) and B (C/Ca). Treated citrus trees mostly producing fruits under the grade C group, with the fruit weight is being less than 100 g/fruit followed by grade B, A and AA. The HLB disease disrupting the uptake of nitrogen fertilizer and other important nutrients that were required for fruits development especially during early phase of fruits development and this effect caused in poor fruit development and production.

The mean fresh weight of fruits in Treatments D (C/Ca/Zn), C (C/Ca/Cu), B (C/Ca/Zn) and A (C) were 58.85, 57.05, 43.84 and 60.50 g, respectively (Table 8). Based on this data, they also showed no significant different in terms of total fresh weight of fruits. Interestingly, for total soluble solids (TSS) content of citrus fruits showed significant differences in Treatment D (C/Ca/Zn) as compared with Treatment A (C) (Table 8). It means that combination application of Ca and Zn ions managed to improve fruit quality in terms of TSS content.

DISCUSSION

HLB disease is currently managed in Malaysia by application of insecticides to control vector population, uprooting infected trees and light pruning of infected branches to reduce inoculums source, effective quarantine and recently using disease-free planting materials. However, until now there is no successful method or treatment available in Malaysia able to control HLB bacterium in the orchards effectively. Therefore, information on host nutrients in relation to disease severity is considered important in the management of HLB disease in the orchards and it may offer alternative option to conventional agriculture practices (Sugimoto et al., 2007). Now days, removal and destruction of infected trees are the only practical method in order to prevent further disease spread within an orchard (da Graca, 1991). However, recommendation to uproot suspected HLB infected trees always face with objections from groups of growers. These groups hesitated to believe or do not have confidence that their trees with typical symptoms of HLB disease were truly infected by *Candidatus* Liberibacter asiaticus. This objection was most likely due to confusion between symptoms of HLB with those symptoms caused by Zn or Mn deficiencies and/or by other plant diseases (Garnier and Bove, 2000; Weinert et al., 2004). In certain cases, more than one pathogen may be present in the same tree. In Malaysia, another two important diseases of citrus namely citrus tristeza virus (CTV) and *Phytophthora* collar or root rot disease (Ko, 1996) were reported to infect citrus trees in the orchards. Both diseases were also causing symptoms of leaves yellowing, mottling, stunting and dieback of twigs. Moreover, nitrogen deficiency also caused leaf yellowing symptom and this further complicates the diagnosis of HLB disease.

As an alternative control measure, it was proven that Ca application managed to reduce the severity of several diseases caused by root and stem pathogens such as the fungi, *Rhizoctonia, Sclerotium, Botrytis* and *Fusarium oxysporium* (Agrios, 2005). Copper ion application to the soil was able to reduce disease severity caused by fungi, *Gaeumannomyces graminis* and *Claviceps purpurea* and also bacterial disease, *Pseudomonas chicorii* (Agrios, 2005). The effect of Ca on disease resistance seems to result from its effect on the composition of cell wall and their resistance to penetration by pathogen. In contrast, Mg and Zn ions showed positive correlation 0.628 and 0.534, respectively with dieback symptom and their interaction were significantly difference but it showed no significant difference with leaf yellowing symptom. It means that increase in concentration of Mg and Zn ions in citrus leaves would increase the severity of dieback symptom. It is not surprising because based on the previous report it was proven that addition of magnesium increased the severity of corn leaf blight caused by *Cochliobolus heterostrofus* (Agrios, 2005).

Researchers proved that micronutrients play very important role in plants for protection to bacterial and fungal diseases (Hacisalihoglu et al., 2008). Study by Yamazaki et al. (1996) was also proved that bacterial wilt resistant tomato is associated with high Ca level in the leaves. However, there is still limited information on the effect of mineral nutrients on plant diseases and their interactions. Marschner (1995) reported that nutrients decreased the severity of plant diseases by inducing resistance to the host because of their roles in cellular defense systems. Minerals such as S, B and Ca perform differently but important functions in the plant defense systems. Sulfur for example, plays an important role in producing number of chemical compounds such as glutathiones, glucosinolates, and phytoalexins. All of these plant compounds were actively involved in disease resistance in plants. Besides that B is an essential micronutrient which plays a role in improving fruit set, cell wall structures as well as antibody production in plants (Marschner, 1995). It is also involved in metabolic regulator to prevent damage from reactive oxygen species. Calcium is an important macronutrient with several key functions including structural roles in membranes and cell wall and messenger for transducing external signals (Marschner, 1995). A study by Del Reviro and Veyrat (1966) have shown that trees sprayed with urea plus micronutrients such as Zn, Mn, Cu and B produces large number of citrus fruit. In terms of disease suppression, a recent study by Sugimoto et al. (2007) showed that application of micro inorganic elements such as Mn, Zn and Cu and macro inorganic elements such as P, K, Mg and Ca manage to reduce *Phytophthora* stem rot disease of soybean by reducing the growth rate and zoospore release of the pathogen, *P. sojae* and at the same time increase the yield of soybean. On the other hand, micronutrients were also served another role in plant system. From a previous study, reported that the most important problem associated with the cultivation of mandarin orange is unfruitfulness (Del Rivero et al., 1969). Some of the success treatment has been obtained with the use of other mandarin varieties as pollenizer, girdling and high rates of nitrogen fertilization, either by foliar or soil application. The addition of micronutrients to the foliar nitrogen spray even where no visible deficiency symptoms were observed appears to reduce the problem in some cases (Del Rivero et al., 1969).

Based on our findings, the effect of Ca, Zn and Cu ions application generally showed slight protection against the bacterial disease but it is inconsistent in some cases. Based on our experience and observation in study plots at Durian Mentangau and others citrus orchards in Malaysia, citrus trees with severely infection with HLB disease generally would not give positive response to the treatments applied. On the other hand, citrus trees with mild to moderately infection levels always showed positive response. Hence, good and proper fertilization and pesticides spraying programs is very important steps especially at the beginning stage because it helps the plant to be more tolerant to the disease. Based on our findings, citrus trees treated with combination of Ca and Zn ions at 600 and 10 ppm, respectively resulted in significant increase (P≤0.05) in terms of fruit production and total soluble solid (TSS) content. This treatment also managed to delay disease incidence and reduced AUDPC value of disease incidence and disease severity. It also managed to improve mean leaf lengths, leaf widths and mean leaf areas of treated trees. The improvement observed on treated trees was probably due to the enhancement of trees' tolerance as had been reported for other plant-pathogen systems (Oostendorp et al., 2001; Agrios, 2005). Application of Ca only showed no significant protection in these trials and its application would be considered as a complementary treatment in the management of bacterial disease and combination with other micronutrients such as Zn and Cu showing a more synergistic effect to control HLB disease. However, as mentioned above there is no specific treatment successful enough to control HLB disease in the orchards. Perhaps by combination of the present treatments together with the good agriculture practices would increase and improve the efficiency of disease control of HLB disease in Malaysia by delaying disease onset and prolong lifespan of citrus trees in order to maintain fruit production

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