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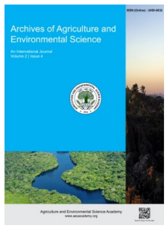

ORIGINAL RESEARCH ARTICLE

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Chemical inducers, nutrient management, guava intercropping and insecticides can reduce Huanglongbing incidence and severity in Sweet orange

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ABSTRACT

Huanglongbing (HLB) or citrus greening is the most economically devastating disease of citrus in the world. HLB is a vector-borne disease and transmitted by Asian Citrus psyllid (ACP). HLB is now a serious threat to the cultivation and expansion of Sweet orange and Mandarin in Bangladesh. As no suitable cure is available against the disease, inducing plant immunity by chemical inducers or nutrient management and intercropping could be an effective way to combat this challenge. In this study, two inducers *viz.*, Bion (Acibenzolar S-methyl) and Bactroban (Bismerthizol), nutrients formulations SICOGREEN® (soil application) and foliar spray, intercropping with guava, spraying guava leaf extract (10%), foliar spray with insect growth regulators (IGR) such as Heron (Lufenuron), insecticides such as Neonicotinoids/ Imidachloropid + Thiomethoxam and foliar spray of *Beauveria bassiana* (Commercial formulation) showed comparatively better performance as compared to untreated control considering both HLB incidence and severity in both locations (Haluaghat and Bhaluka) of Sweet orange orchards. All these treatments reduced HLB incidence by 57.5 to 89.44% and HLB severity by 54.16 to 80.35% in Sweet orange considering both Haluaghat and Bhaluka orchards. The results revealed that Bion (Acibenzolar S-methyl), nutrients formulations SICOGREEN® (soil and foliar application), intercropping with guava, spraying guava leaf extract, foliar spray of insecticides can be integrated to reduce HLB incidence and severity in Sweet orange. Some of these treatments have also some positive effects on plant growth and yield parameters of Sweet orange as compared to control. These results comprehensively suggest that chemical inducers and nutrient management seem a better alternative to control HLB aimed to increase tree lifespan and productivity.

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INTRODUCTION

Citrus huanglongbing (HLB), also known as citrus greening, is the most destructive disease of citrus and has been rapidly spreading worldwide, resulting in significant economic losses (Bové, 2006; FAO, 2012; Khan and Razi, 2018). HLB has been known in East Asia for over a century and is currently widespread in most citrus areas of Asia, Africa, and the Americas (Gottwald *et al.*, 2007). HLB has been reported previously in Sweet orange and Mandarin in Bangladesh (Tipu *et al.*, 2017, 2020). Estimates showed that HLB caused a loss of about 100,000 citrus acres since 2007 in Florida and had cost Florida's economy approximately $3.6 billion in lost revenues since 2006 which also reduced its production by 74% (Gottwald, 2010;

Wang and Trivedi, 2013; Putnam and Hudson, 2018). However, there is no such estimation of losses available in Bangladesh due to HLB.

Citrus HLB is caused by a phloem-limited fastidious α-proteobacterium belonging to the 'Candidatus' genus Liberibacter (Jagoueix *et al.*, 1994; Pelz-Stelinski *et al.*, 2010). There are three species of 'Ca. Liberibacter' have been identified to cause HLB: 'Ca. L. asiaticus' (Las), 'Ca. L. africanus', and 'Ca. L. americanus' (Gottwald, 2010; Bové, 2012; Li *et al.*, 2017). These bacteria are unculturable *in vitro* culture medium. Asian Citrus psyllid (*Diaphorina citri*) in Asia and the Americas (Bové, 2006; Halbert, 2005; Teixeira *et al.*, 2005) and African citrus psyllid (*Trioza erytreae*) in Africa (Bové, 2006) are the two known vectors of HLB pathogen. 'Ca. L. asiaticus' (Las) and Asian Citrus psyllid are the most prevalent and important throughout HLB-affected citrus-growing areas worldwide (Bové, 2006). Las propagates in the phloem of the host plants, resulting in die-back, small leaves, yellow shoots, blotchy mottles on leaves, corky veins, malformed and discolored fruit, aborted seed, premature fruit drop, root loss, and eventually tree death (Bové, 2006; Gottwald *et al.*, 2007; Wang and Trivedi, 2013). The life span for the profitable productivity of infected citrus trees is dramatically shortened as the disease severity increases and the yield is significantly reduced (Gottwald *et al.*, 2007). The understanding of the bacterial pathogen's virulence mechanism is limited due to the difficulty in culturing Las and its uneven distribution in the citrus hosts (Paudyal, 2016; Tipu *et al.*, 2020). So far, most molecular insights of the HLB biology and Las pathogenicity are derived from the genome sequences of Las and other related Liberibacters (Duan *et al.*, 2009; Lin *et al.*, 2011; Leonard *et al.*, 2012; Wulff *et al.*, 2014).

An integrated control program has been recommended for HLB in commercial orchards by the United Nations Development Program, Food and Agriculture Organization (FAO, 2012) Southeastern Asian citrus rehabilitation project (Aubert, 1990). The program highlights controlling psyllid vectors with insecticides, reducing inoculum through the removal of HLB-symptomatic trees, propagating and using pathogen-free budwood and nursery trees. In Florida, foliar nutrition programs coupled with vector control are often used to slow down HLB's spread and reduce the devastating effects of the disease (Gottwald, 2010). These control practices have shown a limited impact on preventing the further spread of HLB. Recently, various treatment strategies including applications of penicillin and streptomycin (Zhang *et al.*, 2011), enhanced nutrient program (Gottwald *et al.*, 2012), thermotherapy (Hoffman *et al.*, 2013), soil-conditioners (Xu *et al.*, 2013), and small molecules targeting Las virulence traits including osmotic stress tolerance (Pagliai *et al.*, 2014), have been examined for HLB disease management and some showed promising progress. However, no effective approach has been established to control HLB and stop it from spreading to new citrus-production areas.

Induced resistance, either locally or systemically, may confer long-lasting protection against a broad spectrum of plant diseases (Durrant and Dong, 2004; Walters *et al.*, 2013). The plant defense mechanisms can be activated by pathogens (Durrant and Dong, 2004), beneficial microorganisms (Weller *et al.*, 2012; Zamioudis and Pieterse, 2012; Tang *et al.*, 2018), or by chemical inducers (Walters *et al.*, 2013; Hu *et al.*, 2018). Tremendous effort has been put into the development of agents that can mimic natural inducers of resistance. These include acibenzolar-S-methyl (ASM), benzothiadiazole (BTH), 2,6-dichloroisonicotinic acid (INA), β-aminobutyric acid (BABA), oligosaccharide from plant and fungal cell walls, and probenazole. These agents could induce plant resistance effectively against a wide range of pathogens including bacteria, fungi, viruses, nematodes, and parasitic weeds (Beckers and Conrath, 2007), even though effects varied with concentrations pathosystems (Vallad and Goodman, 2004; Walters *et al.*, 2005). For example, soil applications of systemic acquired resistance (SAR) elicitors induced systemic resistance against canker under greenhouse conditions and showed season-long control of canker epidemics on young citrus trees (Francis *et al.*, 2009). In addition, BABA induced citrus resistance against psyllids in the greenhouse (Tiwari *et al.*, 2013), suggesting BABA's potential for the management of HLB. In certain nutrient/SAR programs, salicylic acid (SA) and/or its analogs were applied as foliar amendments to act against the HLB pathogen by activating the SAR pathway and the effects on disease expression of HLB-infected trees and fruit yield remain to be demonstrated (Stansly *et al.*, 2014). Overall, no conclusive study has been conducted regarding how to control HLB by inducing plant defense. HLB symptoms could be reduced by foliar applications of micronutrients, especially Mn, Zn, B, and Mg, (Pustika *et al.*, 2008; Shen *et al.*, 2013; Stansly *et al.*, 2014). Some other studies have shown the benefits of enhanced nutritional programs for HLB management (Pustika *et al.*, 2008; Shen *et al.*, 2013; Stansly *et al.*, 2014). HLB causes declines in the canopy and root system (Graham *et al.*, 2013) and since leaves typically persist up to 2 years in citrus, it would be expected that alleviation of disease symptoms would require a 2-year window to rebuild the root system and canopy before yield would recover. It appears that longer-term studies with foliar applications of Mn, Zn, B, and Mg are required with the twin goals of first aiding the recovery of the root systems and the canopy followed by recovery of yield. Some growers in the Florida citrus industry are using phosphites as the anionic complement to the cationic micronutrients in the salts used to apply essential nutrients to the foliage, such as $Mn_3(PO_3)_2$ and $Zn_3(PO_3)_2$. Early studies of phosphites use in agriculture involved evaluating their nutritional (Rickard, 2000) and other horticultural benefits, including increased citrus flowering, fruit set, yield, and higher fruit quality (Rickard, 2000). Elicitors are compounds that stimulate any type of defense in the plant and favor the synthesis of secondary metabolites under both abiotic and biotic stress conditions and can be applied through foliar sprays. Within these compounds, carbohydrates, lipids, (glyco)peptides, (glyco)proteins, vitamins, and phytohormones can be found (Wallace *et al.*, 1954; Angelova *et al.*, 2006; Boubakri *et al.*, 2016; Thakur and Sohal, 2013; Tsagkarakis *et al.*, 2012). Among the compounds studied to activate defense mechanisms in plants against the attack of biotic agents (macro- and microorganisms) are SA, phytohormones, chitosan, and thiamine (vitamin B1) (Ahn *et al*., 2005; El-Hadrami *et al*., 2010; Thakur and Sohal, 2013).

In the present study, the effects of chemical inducer, elicitor, nutrient element, insecticide and entomopathogenic fungus on HLB progression under field conditions were investigated to determine the feasibility of the integrated use of these control approach in controlling citrus HLB.

MATERIALS AND METHODS

Experimental locations

Field experiments were conducted in two in situ Sweet orange orchards located in Bhaluka and Haluaghat, Mymensingh from 2017 to 2020. The age of Sweet orange plants in Haluaghat was 2 years and the age of Bhaluka Sweet orange plants is three years at the time of experiment setting. Standard citrus fertilization, minimum weed and pest control measures were taken for all the plants in both Orchards.

Design and treatments

Randomized Complete Block Design (RCBD) was followed with three replications. Each replication contains three plants. The following treatments were used: T_0 = Control (Untreated citrus plants), T_1 = Chitosan (an elicitor), T_2 = Bion (Acibenzolar S-methyl, a chemical inducer), T_3 = Bactroban (Bismerthizol, a chemical inducer), T_4= Balanced nutrition with micronutrients formulations SICOGREEN® (soil application), T_5 = Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray), T_6 = Intercrop with guava, T_7 = Spray guava leaf extract (10%), T_8= Foliar spray with insect growth regulators (IGR) such as Heron (Lufenuron), T_9 = Foliar spray with insecticides such as Neonicotinoids/Imidachloropid + Thiomethoxam and T_{10} = Foliar spray with *Beauveria bassiana* (Commercial formulation).

Application of the treatments

The following treatments were applied in situ orchards two times before the rainy season and two times after the rainy season. Chitosan was dissolved in 1% acetic solution and was sprayed with a concentration of 1000 ppm on the surface of the citrus plants. The bio-activator, bion [acibenzolar-S-methyl (ASM)] was applied as foliar spray @ 200 mL (500 ppm) per tree at an interval of 90 days. The chemical activator, Bismerthizol was applied as both foliar spray and soil drench @ 200 mL per tree at a concentration of 200 ppm at an interval of 60 days. The micronutrient was applied as both foliar spray and soil drench at 200 mL per tree (@ 0.2%) at an interval of 60 days. One guava seedling was planted in the middle of four citrus plants to assess the survival efficacy of citrus psyllid bug, the insect vector carrying the Las. Heron 5 EC (Lufenuron), (Haychem Bangladesh Ltd.) was applied @ 200 mL (75 ppm) per tree at an interval of 60 days six times in a year. Two insecticides *viz*., Confider (Neonicotinoids) and Actara (Thiomethoxam) were sprayed @ 100 mL (0.5%) per tree at one-month intervals during the rainy season (July to October) because the availability of the Asian citrus psyllid is high at that time.

Data collection

Data were collected on the following parameters-citrus greening incidence, citrus greening severity, plant height (cm), number of branches and number of fruits

Monitoring disease progress

To address whether the treatments have any effect on the disease progress, the citrus trees were evaluated based on disease symptoms and presence of the Las at 6 and 12 months in a year. For disease symptoms, the trees were visually examined for the presence of typical citrus greening symptoms, such as asymmetric mottling and thickening of veins in mature leaves. Further, the presence of Las was determined based on the polymerase chain reaction (PCR) test using specific primers for the detection of Las (Tipu *et al*., 2017).

Assessment of HLB infection

Leaf sampling: Leaf samples for the detection of *Candidatus Liberibacter asiaticus* (Las) were collected as visual ratings. For each target tree, samples containing 3-4 green twigs of 6 to 8 inches long with approximately twenty leaves, preferably with the petiole still attached and with well recognizable symptoms, were collected. Samples were kept in double bags in cool conditions, preferably in plastic containers containing silica gel. The leaf samples without symptoms were collected as the symptomless carrier of Las.

PCR based confirmation or detection of Las

Extraction of genomic DNA: Collected leaf samples were washed with sterile distilled water and 70% ethanol and dried on blotting paper to remove excess water. Leaf midribs were ripped off and chopped with sterilized scissors. Approximately 60mg of leaf tissues were processed by freezing with liquid nitrogen in a microcentrifuge tube and crushed into a fine powder using a micro pestle. The Las genomic DNA was extracted using the Wizard Genomic DNA purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Briefly, approximately 60mg of midrib tissues were processed by freezing with liquid nitrogen in a microcentrifuge tube and crushed into a fine powder using a micro pestle. For digestion, 600 µl of Nuclei Lysis Solution was added. It was vortexed for 1-3 seconds to wet the tissue and incubated after 15 minutes in a water bath. Then 3 µl of RNase Solution was added to the cell lysate. The sample was mix by inverting the tube 2-5 times. The samples have then incubated the mixture are 37°C for 15 minutes in a water bath. The example was allowed to cool to room temperature for 5 minutes before proceeding to the next step. 200 µl of protein Precipitation Solution was added. It was then vortex vigorously at high speed for 20 seconds. The mixture was centrifuged for 3 minutes at 15,000×g.

The precipitated proteins were formed a tight pellet. The supernatant containing the DNA (leaving the protein pellet behind) was removed carefully. It was then transferred to a clean microcentrifuge tube containing 600 µl of room temperature isopropanol. The solution was mixed gently by inversion until thread-like strands of DNA from a visible mass. Then it was centrifuged at 15,000×g for 1 minute at room temperature. The supernatant was decanted carefully. 600 µl of room temperature 70% ethanol was added. The tube was inverted gently several times to wash the DNA. It was then centrifuged at 15,000×g for 1 minute at room temperature. The ethanol was aspirated carefully. The tube was then inverted onto clean absorbent paper, and the pellet air-dried for 15 minutes. Five µl of DNA Rehydration Solution was added. The rehydrated DNA was incubated at 4°C for overnight. Finally, the DNA was stored at -20°C.

PCR confirmation of Las: Las was identified by PCR using primers Las606 (5´-GGAGAGGTG AGTGGA ATTCCGA-3´), and LSS (5´-ACCCAACATCTAGGTAAAAACC-3´) as described Fujikawa and Iwanami (2012) previously. PCR reactions were performed using GoTaq® Green Master Mix (Promega, Madison, USA) in 25 µl reaction mixture containing GoTaq® Green Master Mix 12.5 µl, forward and reverse primer 1 µM for each, template DNA DNA 100 ng and 9.5 µl of ddH2O. The PCR conditions were 9 min of pre-denaturation at 96°C, followed by 35 cycles of 30 s of denaturation at 96°C, 30 s of annealing at 55°C, 1 min of extension at 72°C, and then a single final extension of 7 min at 72°C.

Visualization of PCR products: The PCR products were visualized in 1% agarose gel containing 0.2 µg ethidium bromide per 100 ml gel from the stock solution. After electrophoresis, the gel was placed under a UV transilluminator (GelView Master, Dynamics, UK) for visualization of DNA bands. The UV light of the apparatus was switched on. The image of the desired bands on the gel was viewed on the monitor and saved on the computer disc (CD-R) for taking photographs.

Statistical analysis

RCBD design was followed for field experiments and Mstat-C statistical program was used for data analyses. DMRT was used to compare the treatment means.

RESULTS AND DISCUSSION

Effect of different treatments on the citrus greening incidence (% citrus greening infected plant) and severity

Citrus greening incidence: Experiments were set up *in situ* Sweet orange Orchards in Haluaghat and Bhaluka, Mymensingh. The greening suspected trees were confirmed by PCR using Las specific primers. The results showed that all suspected trees yielded a fragment size 500bp which confirmed trees were infected with Las. In Haluaghat orchard, the highest (33%) citrus greening incidence was recorded in T_0(Control) while the lowest (3.7%) citrus greening T_3 = Bactroban (Bismerthizol, a chemical inducer) followed by T_2 [Bion (Acibenzolar S-methyl, a chemical inducer)] and T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)] T_6 [Intercropping with guava] (Table 1 and Figure 1A). Moderate level of citrus greening incidence ranged by 11.11 to 18.51 were observed when plants were treated with T_1[Chitosan (an elicitor)], T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application)], T_5[Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray)], T_7 (30%) [spray guava leaf extract (10%)], T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)] and T_9 [Foliar spray with insecticides such as Neonicotinoids/ Imidachloropid + Thiomethoxam] and T_{10}= Foliar spray with *Beauveria bassiana* (Commercial formulation).

On the other hand in Bhaluka orchard, the highest (24.62%) citrus greening incidence was recorded in T_0(Control) while the lowest (2.22%) greening incidence was recorded in no infections were observed in T_3 [Bactroban (Bismerthizol, a chemical inducer)], T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application)], T_6 [Intercrop with guava, T_7 = Spray guava leaf extract (10%)], T_7 [Spray guava leaf extract (10%)], T_9 [Foliar spray with insecticides such as Neonicotinoids/Imidachloropid + Thiomethoxam] and T_{10}[Foliar spray with *Beauveria bassiana* (Commercial formulation)] (Table 1 and Figure 1A). Intermediate level of citrus greening incidence (3.7 to 8.88%) was recorded T_1[Chitosan (an elicitors)], T_2 [Bion (Acibenzolar S-methyl, a chemical inducer)], T_5 [Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray)] and T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)].

Citrus greening severity: In the case of citrus greening severity, in Haluaghat orchard the maximum (1.22) citrus greening severity was recorded in T_0(Control) while the lowest (0.22) greening severity were recorded in T_2 [Bion (Acibenzolar S-methyl] followed by T_6 [Intercrop with guava], a chemical inducer)] and T_3 [Bactroban (Bismerthizol, a chemical inducer)]. The moderate levels of greening severity were recorded in followed by while the lowest citrus greening severity was recorded in followed by T_6 [Intercrop with guava] (Table 1 and Figure 1B) Moderate level of citrus greening severity were recorded in T_1 [Chitosan (an elicitor), T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application)], T_5 [Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray)], T_7 [Spray guava leaf extract (10%)] and T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)], T_9 [Foliar spray with insecticides such as Neonicotinoids/ Imidachloropid + Thiomethoxam] and T_{10} [Foliar spray with *Beauveria bassiana* (Commercial formulation)].

In the case of citrus greening severity, in Bhaluka orchard the maximum (0.66) greening severity was recorded in T_0(Control) while the lowest (0.11) greening severity was recorded in T_3 [Bactroban (Bismerthizol, a chemical inducer)], T_4 [Balanced

Table 1. Effect of different treatments on the citrus greening incidence (% citrus greening infected plant) and severity.

Treatment	Citrus greening incidence (% plant infection)		Citrus greening severity	
	Haluaghat	Bhaluka	Haluaghat	Bhaluka
T_0	33.33 a (7.05)	24.62 a (3.52)	1.22	0.56 a
T_1	18.52 ab (4.31)	4.99 b (2.20)	0.58	0.44 ab
T_2	7.41 bc (2.05)	8.88 b (1.97)	0.22	0.22 ab
T_3	3.70 c (1.60)	2.22 b (0.70)	0.22	0.11 b
T_4	11.11 abc (2.95)	2.22 b (0.70)	0.44	0.11 b
T_5	22.22 abc (4.11)	4.44 b (1.36)	0.44	0.22 ab
T_6	11.11 abc (2.40)	2.22 b (0.70)	0.33	0.11 b
T_7	18.52 ab (4.31)	2.22 b (0.70)	0.47	0.11 ab
T_8	7.63 abc (2.62)	3.70 b (1.40)	0.56	0.22 ab
T_9	14.81 abc (3.85)	2.22 b (0.70)	0.67	0.11 b
T_{10}	11.11 abc (3.40)	2.22 b (0.70)	0.44	0.11 b
Level of significance	*		NS	*
CV (%)	40.98	97.15	69.44	18.8

Data are the averages of three replications and each replication consists three plants. Values in the parentheses are the transformed values with arcsine transformation. Values with same letters in each column are statistically similar. Citrus greening severity was assessed using a 3-point scale where 0 representing no apparent HLB symptoms, 1 suspect citrus greening symptom, and 2 likely citrus greening symptoms. T_0 = Control (Untreated citrus plants), T_1 = Chitosan (an elicitors), T_2 = Bion (Acibenzolar S-methyl, a chemical inducer), T_3 = Bactroban (Bismerthizol, a chemical inducer), T_4= Balanced nutrition with micronutrients formulations SICOGREEN® (soil application), T_5 = Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray), T_6 = Intercropping with guava , T_7 = Spray guava leaf extract (10%), T_8= Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron), T_9= Foliar spray with insecticides such as Neonicotinoids/Imidachloropid + Thiomethoxam and T_{10} = Foliar spray with *Beauveria bassiana* (Commercial formulation).

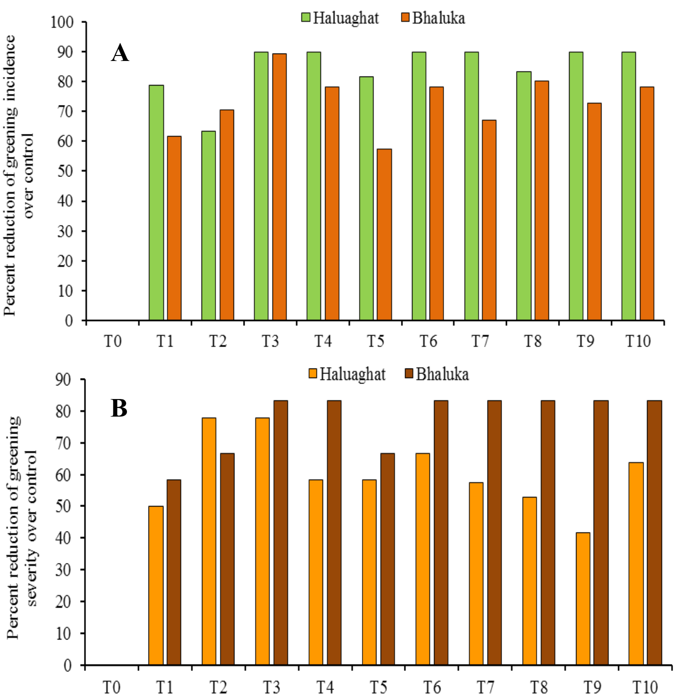

Figure 1. *Effect of different treatments on the percent reduction of citrus greening incidence (A) and severity (B) in Sweet orange in both Haluaghat and Bhaluka orchards. T_0 = Control (Untreated citrus plants), T_1 = Chitosan (an elicitors), T_2 = Bion (Acibenzolar S-methyl, a chemical inducer), T_3 = Bactroban (Bismerthizol, a chemical inducer), T_4= Balanced nutrition with micronutrients formulations SICOGREEN® (soil application), T_5 = Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray), T_6 = Intercropping with guava , T_7 = Spray guava leaf extract (10%), T_8= Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron), T_9 = Foliar spray with insecticides such as Neonicotinoids/ Imidachloropid + Thiomethoxam and T_{10}= Foliar spray with Beauveria bassiana (Commercial formulation).*

nutrition with micronutrients formulations SICOGREEN® (soil application)], T_6 [Intercrop with guava], T_7 [Spray guava leaf extract (10%)], T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)], T_9[Foliar spray with insecticides such as Neonicotinoids/Imidachloropid + Thiomethoxam] and T_{10} [Foliar spray with *Beauveria bassiana* (Commercial formulation)] (Table 1 and Figure 1B). Moderate level (0.22 to 0.33) of citrus greening severity were recorded in T_1 [Chitosan (an elicitor), T_2 [Bion(Acibenzolar S-methyl] T_5 [Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray)].

Considering both citrus greening incidence and severity in both Haluaghat and Bhaluka orchards, T_3 [Bactroban (Bismerthizol, a chemical inducer)], T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application)], T_6 [Intercrop with guava], T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)] and T_{10} [Foliar spray with *Beauveria bassiana* (Commercial formulation)] showed comparatively better performance as compared to control and other treatments (Figures 1A and 1B).

PCR based confirmation of HLB infection: Symptomatic trees were confirmed by PCR using Las specific primers Las606 and LSS. The results revealed that all suspected trees were positive by PCR. An amplicon size 500bp confirmed the presence of Las in samples collected from suspected trees of the respective treatment (Figure 2).

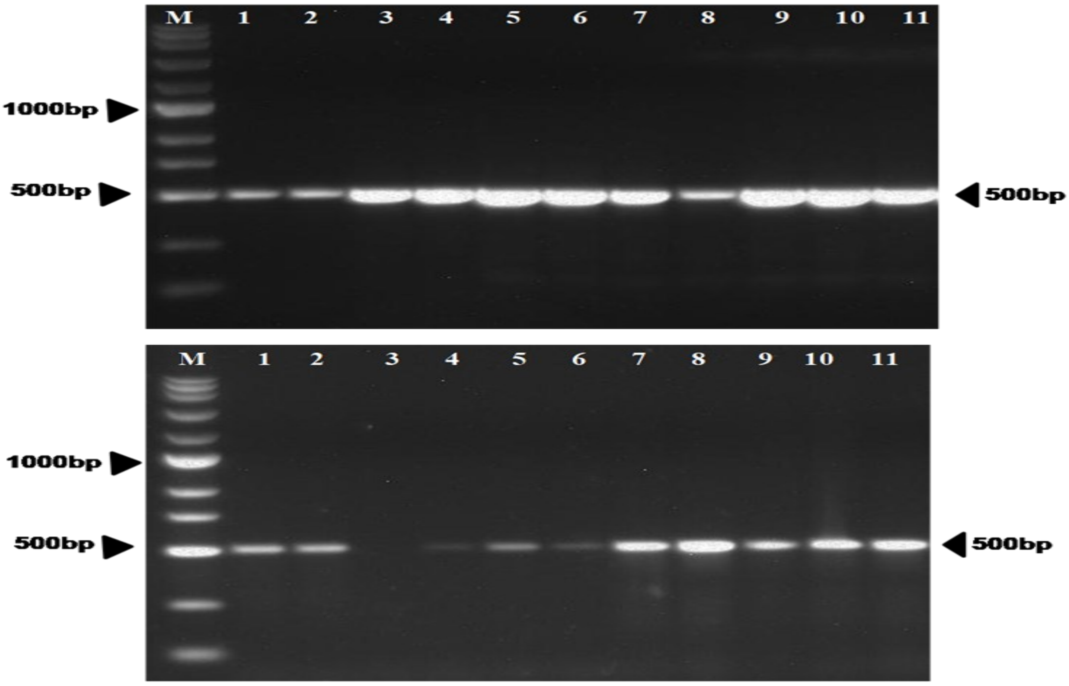

Figure 2. *PCR confirmation of citrus greening suspected trees using Las specific primers Las606 and LSS in Haluaghat (A) and Bhaluka (B). DNA collected from leaf samples obtained from trees under 1: T_0 [Control (Untreated citrus plants)], 2: T_1 [Chitosan (an elicitors)], 3: T_2 [Bion (Acibenzolar S-methyl, a chemical inducer)], 4: T_3 [Bactroban (Bismerthizol, a chemical inducer)], 5: T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application)], 6: T_5 [Balancednutrition with micronutrients formulations SICOGREEN®(foliar spray)], 7: T_6 [Intercropping with guava], 8: T_7 [Spray guava leaf extract (10%)], 9: T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)], 10: T_9[Foliar spray with insecticides such as Neonicotinoids/Imidachloropid + Thiomethoxam and 11: T_{10} [Foliar spray with Beauveria bassiana (Commercial formulation)].*

Effect of different treatments on the growth and yield of Sweet orange (Malta) plants citrus

Plant height: Data on plant height, number of branches, number of fruits per plant were recorded. The results showed that no significant differences were observed among the treatments in relation to plant height at Haluaghat Orchard. However, the maximum (294 cm) plant height was observed in T_5 (Balanced nutrition with micronutrients formulations SICOGREEN® as a foliar spray) followed by T_6 (Intercropping with guava) that resulted 292 cm plant height while the minimum (256.67cm) plant height was recorded in control treatments (T_0). All other treatments *viz.*, T_1 [Chitosan (an elicitor)], T_2 [Bion (Acibenzolar S-methyl, a chemical inducer)], T_3 [Bactroban (Bismerthizol, a chemical inducer)], T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application] T_5 [Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray), T_7 [Spray guava leaf extract (10%)], T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)], T_9 [Foliar spray with insecticides such as Neonicotinoids/ Imidachloropid + Thiomethoxam] and T_{10}[Foliar spray with *Beauveria bassiana* (Commercial formulation)] that resulted 257.22 to 283.89 cm (Table 2).

Significant differences were observed among the treatments in relation to plant height in Bhaluka Orchard. In this orchard, statistically similar plant height was recorded in T_3 [Bactroban (Bismerthizol, a chemical inducer)] (253.33cm) and T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application)] (257.78cm). The maximum (300cm) plant height was recorded in T_6 (Intercropping with guava) which was statistically similar to T_0 (Untreated citrus plants), T_1 [Chitosan (an elicitor)], T_2 [Bion (Acibenzolar S-methyl, a chemical inducer)], T_5 [Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray)], T_7 [Spray guava leaf extract (10%)], T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)], T_9 [Foliar spray with insecticides such as Neonicotinoids/ Imidachloropid + Thiomethoxam] and T_{10} [Foliar spray with *Beauveria bassiana* (Commercial formulation)] that resulted in a plant height ranged from 273.33 to 294.44cm (Table 2).

Number of branches per plant: A significant effect was observed among the treatments with regard to the number of branches per plant in Haluaghat Orchard. The minimum (10.44) number of branches was counted in T_1 [Chitosan (an elicitor)] which was statically similar to T_0 [Control (Untreated citrus plants)] and T2 [Bion (Acibenzolar S-methyl, a chemical inducer)] with number branches 17.11 and 18.33, respectively. However, the maximum (30.67) number of branches per plant was counted in T_9[Foliar spray with insecticides such as Neonicotinoids/Imidachloropid + Thiomethoxam] which was statistically similar to T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application)], T_5 [Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray)], T_6 [Intercropping with guava], T_7 [Spray guava leaf extract (10%)] and T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)] with a number of branches ranged by 23.56 to 26.67. A statistically similar number of branches were observed in T_3 [Bactroban (Bismerthizol, a chemical inducer] and T_{10}[Foliar spray with *Beauveria bassiana* (Commercial formulation)] (Table 2).

Table 2. Effect of different treatments on the growth and yield of Sweet orange (Malta) plants Citrus.

Treatment	Plant height (cm)		No. of branches/plant		No. of fruits per plant	
	Haluaghat	Bhaluka	Haluaghat	Bhaluka	Haluaghat	Bhaluka
T_0	256.67	286.67 a	17.11 de	29.11	26.44	8.56 ab
T_1	266.67	294.44 a	10.44 e	25.44	12.39	6.22 ab
T_2	278.89	291.11 a	18.33 cde	24.11	19.56	11.22 ab
T_3	280.00	253.33 b	21.00 bcd	29.11	15.56	14.89 ab
T_4	283.89	277.78 ab	23.67 abcd	29.44	28.61	7.67 ab
T_5	294.44	292.22 a	28.44 ab	29.78	19.11	20.78 a
T_6	292.22	300.00 a	25.22 abcd	31.78	16.89	5.22 ab
T_7	280.00	291.67 a	23.56 abcd	30.67	13.00	10.22 ab
T_8	282.78	284.44 ab	26.56 abc	28.56	22.67	6.78 ab
T_9	282.22	281.11 ab	30.67 a	31.44	24.89	10.67 ab
T_{10}	257.22	273.33 ab	19.67 cd	25.78	24.22	4.00 b
Level of significance	NS	*	*	NS	NS	*
CV (%)	8.79	5.92	20.40	17.57	47.68	8.47

Data are the averages of three replications and each replication consists three plants. Values with same letters in each column are statistically similar. T_0 = Control (Untreated citrus plants), T_1 = Chitosan (an elicitors), T_2 = Bion (Acibenzolar S-methyl, a chemical inducer), T_3 = Bactroban (Bismerthizol, a chemical inducer), T_4= Balanced nutrition with micronutrients formulations SICOGREEN® (soil application), T_5 = Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray), T_6 = Intercropping with guava , T_7 = Spray guava leaf extract (10%), T_8= Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron), T_9= Foliar spray with insecticides such as Neonicotinoids/ Imidachloropid + Thiomethoxam and T_{10}= Foliar spray with *Beauveria bassiana* (Commercial formulation).

No Significant effect was observed among the treatments with regard to the number of branches per plant in Bhaluka Orchard. The minimum (24.11) number of branches was counted in T2 [Bion (Acibenzolar S-methyl, a chemical inducer)] T_1 [Chitosan (an elicitor)] T_{10} [Foliar spray with *Beauveria bassiana* (Commercial formulation)] with the number of branches per plant 25.44 and 25.78, respectively. The maximum (31.78) number of branches were recorded in T_6 [Intercropping with guava] followed by T_9[Foliar spray with insecticides such as Neonicotinoids/Imidachloropid + Thiomethoxam], T_7 [Spray guava leaf extract (10%)], T_5 [Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray)], T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application)], T_3 [Bactroban (Bismerthizol, a chemical inducer)] and T_1 [Chitosan (an elicitor)] with a number of branches ranged by 29.11 to 31.44 (Table 2).

Number of fruits per plant: A significant effect was observed among the treatments with regard to number of fruits per plant in Haluaghat Orchard. The maximum (20.78) number of fruits per plant was counted in T_5 [Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray)] which was statistically similar to T_2 [Bion (Acibenzolar S-methyl, a chemical inducer), T_3 [Bactroban (Bismerthizol, a chemical inducer)], T_7 [Spray guava leaf extract (10%)] and T_9[Foliar spray with insecticides such as Neonicotinoids/Imidachloropid + Thiomethoxam] with a number of fruits ranged by and T_{10}= Foliar spray with *Beauveria bassiana* (Commercial formulation). The minimum (4.0) number of fruits per plant was counted in T_{10} [Foliar spray with *Beauveria bassiana* (Commercial formulation)]. A statistically similar number of fruits per plant was counted in T_1 [Chitosan (an elicitor)], T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application)], T_6 [Intercropping with guava] and T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)] with a number of fruits per plant ranged by 5.22 to 7.67 (Table 2).

In the Haluaghat orchard, no significant effect was observed among the treatments with regard to the number of fruits per plant. The minimum (28.61) number of fruits per plant was counted in T_4 [Balanced nutrition with micronutrients formulations SICOGREEN® (soil application)] followed by T_0 [Control (Untreated citrus plants)]. An almost similar number of fruits per plant were recorded in T_9[Foliar spray with insecticides such as Neonicotinoids/Imidachloropid + Thiomethoxam], T_{10}[Foliar spray with *Beauveria bassiana* (Commercial formulation)] and T_8 [Foliar spray with insect growth regulator (IGR) such as Heron (Lufenuron)]. T_2 [Bion (Acibenzolar S-methyl, a chemical inducer)] and T_5 [Balanced nutrition with micronutrients formulations SICOGREEN®(foliar spray)] resulted in similar number of fruits per plant. Similarly, T_3 [Bactroban (Bismerthizol, a chemical inducer)] and T_6 [Intercropping with guava] showed a similar number of fruits per plant (Table 2).

It is well documented that a wide range of biotic and abiotic agents are able to induce resistance to pathogen infection in various plants (Durrant and Dong, 2004; Van-Loon *et al.*, 2006; Walters *et al.*, 2013). In this study, we observed that Bion (Acibenzolar S-methyl, a chemical inducer), T_3 (Bactroban (Bismerthizol, a chemical inducer), T_4 (Balanced nutrition with micronutrients formulations SICOGREEN® (soil application), T_5 (Balanced nutrition with micronutrients formulations SICOGREEN® (foliar spray) and intercropped with guava] showed comparatively better performance as compared to control and other treatments considering both citrus greening incidence and severity in both Haluaghat and Bhaluka Sweet orange orchards. We observed that Bion (Acibenzolar S-methyl) reduced HLB incidence and severity by 70.55 and 72.22%, respectively considering both Haluaghat and Bhaluka orchards.

These results are in accordance with a recent report which showed that application of several plant defense inducers, such as ß-aminobutyric acid (BABA), 2,1,3-benzothiadiazole (BTH), and 2,6-dichloroisonicotinic acid (INA), singly or in combination suppressed Las growth *in planta* and the progress of citrus greening symptoms (Li *et al.*, 2016). Another inducer, Bactroban (Bismerthizol) reduced the citrus greening incidence by 88 and 90% in Haluaghat and Bhaluka, respectively. The citrus greening severity has been reduced by 77 and 83%, respectively by the application of Bactroban. Bismerthiazol has been widely used to control *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* infections in China (Liang *et al.*, 2015a, 2015b). Bismerthiazol has also been demonstrated to control effectively citrus canker by both inhibiting the growth of *X. citri* ssp. *citri* and triggering the plant's host defense response through the expression of several pathogenesis-related genes and the non-expresser of PR genes in 'Duncan' grapefruit, especially at early treatment times (Yu *et al.*, 2016). These possibilities in citrus against HLB need to be investigated.

SICOGREEN (a micronutrient plus hormonal formulation) reduced citrus greening incidence by 66 and 90%, in Haluaghat and Bhaluka orchards, respectively when applied as soil drenching. However, this micronutrient formulation reduced citrus severity by 58 and 83% in Haluaghat and Bhaluka orchards, respectively when applied as soil drenching. On the other hand, SICOGREEN when applied as foliar spray reduced citrus greening severity by 90 and 83% in Haluaghat and Bhaluka orchard, respectively. Mineral nutrients are important for the growth and development of plants and microorganisms, and are important factors in plant-disease interactions. How each nutrient affects a plant's response to disease is unique to each plant-disease complex, and in general, nutrient-pathogen interactions are not well understood. For example, calcium deficiency can lead to membrane leakage of sugars, amino acids, and other low-molecular weight compounds that then become available for pathogen use. On the other hand, many nutrient metals at elevated concentrations have broad antibacterial properties so pathogens that directly or indirectly reduce these plant nutrients may have an advantage. Relatively little is known about the changes in plant nutrition associated with citrus greening despite its leaf symptoms often being characterized as "nutrient deficiency-like." Recent analyses comparing symptomatic (blotchy mottle) and asymptomatic leaves from citrus greening infected trees and leaves from healthy trees have shown that citrus greening increased K while Mg, Ca, and B decreased. The micronutrients Zn and Mn, whose deficiency symptoms are commonly seen on citrus greening-infected trees, were not actually deficient in citrus greening-infected samples when the dry mass of the samples was corrected for the large amounts of starch accumulation caused by citrus greening. It remains to be seen whether remedial foliar applications of these or other nutrients can reduce the effects of citrus greening. However, in the present study, reduced levels of citrus greening incidence and severity by SICOGREEN primarily hinted effect of macro and micro-nutrient in reducing citrus greening incidence and severity. Foliar micro-nutrient deficiencies are a noted symptom of citrus greening affected citrus trees (Spann and Schumann, 2009). Therefore, foliar applications of micronutrients have been used by an increasing number of citrus growers in Florida to help mitigate citrus greening-induced deficiencies and counter the debilitating effects of the disease. Asymptomatic leaves from citrus greening-infected trees showed a significant decrease in K which is linked to plant pathogen susceptibility of those tissues. The decreases in Ca, Mg, and B are from restrictions of nutrient uptake, transport, or metabolism induced by citrus greening infection (Span and Schumann, 2009). Foliar nutrition for reducing citrus greening severity is promising in Florida, USA (Spann *et al.*, 2011). A growing trend in Florida is the manipulation of nutrition and irrigation regimes to reduce the effects of citrus greening on tree health, fruit production and fruit quality and nutrients, the trees may show less severe disease symptoms, including milder effects on fruit production and yield. The use of intensive fertigation practices that promote water and nutrient uptake within a limited root zone would be appropriate to minimize nutrient leaching and accelerate tree development particularly during the time of spring flush (Kadyampakeni *et al.*, 2014). Research that demonstrated that citrus greening symptoms could be reduced by foliar applications of micronutrients, especially Mn, Zn, B, and Mg, and other physiologically active compounds, such as salicylic acid and phosphite (Pustika *et al.*, 2008; Shen *et al.*, 2013; Stansly *et al.*, 2014). Combined soil and/or foliar application of the above nutrients to stimulate FRLD and improve root lifespan on citrus greening affected Sweet oranges with emphasis on root-zone soil pH (Atta *et al.*, 2020). Moreover, two insecticides such as IGR [Heron (Lufenuron)] and Neonicotinoids performed better as compared to control and some other treatments. The IGR, Lufenuron reduced HLB incidence and severity by 80.22 and 68.05%, respectively considering both Haluaghat and Bhaluka Sweet Orange orchards. IGRs are known to be highly effective in killing immatures, especially nymphs, of several sucking insect pests, including ACP. The majority of the data on the efficacy of IGRs against ACP comes from laboratory studies (Boina *et al.*, 2010; Tiwari *et al.*, 2012) while few studies evaluated their efficacy in the field (Qureshi and Stanslay, 2007; Abbaszadeh *et al.*, 2011; Rao and Shivankar, 2011). Three IGRs, pyriproxyfen (juvenile hormone mimic), buprofezin and diflubenzuron (both are chitin synthesis inhibitors), showed promising ovicidal and nymphicidal activities against ACP, as well as adverse effects on reproduction (both fecundity and egg viability) and morphology of adults emerging from treated older nymphs (Boina *et al.*, 2010; Tiwari *et al.*, 2012). Under field conditions, the protection offered by IGRs, (diflubenzuron, flufenoxuron, lufenuron, novaluron and pyriproxyfen) ranged from 3 days (diflubenzuron) to 4–6 weeks (lufenuron and flufenoxuron) (Qureshi and Stanslay, 2007; Abbaszadeh *et al.*, 2011; Rao and Shivankar, 2011 and Farmanullah and Gul, 2005). Given the potential of IGRs to reduce adult fecundity and control immatures, IGRs are an important and promising rotational tool in insecticide resistance management (IRM) programs for ACP.

The Neonicotinoids/Imidachloropid + Thiomethoxam reduced HLB incidence and severity by 72.77 and 62.5%, respectively considering both Haluaghat and Bhaluka Sweet Orange orchards. Field studies conducted on young King mandarin trees in Vietnam and mature (five-year-old) Valencia orange trees in the United States, however, found that foliar application of neonicotinoids (clothianidin, imidacloprid and thiamethoxam) and an anthranilic diamide (cyantraniliprole) gave the longest-lasting protection (8–9 weeks), and among the three neonicotinoids, imidacloprid provided maximum control of adults (50–90%) (Ichinose *et al*., 2012; Tiwari and Stelinski, 2013). In general, broad-spectrum insecticides, such as OPs, carbamates and SPs, exhibited more rapid killing of both adults and nymphs of ACP than systemic neonicotinoids, but neonicotinoids showed longer-lasting residual activity (Yasuda *et al*., 2006; Hayashikawa *et al*., 2006). As a result, broad-spectrum insecticides need more frequent applications than neonicotinoids. Neonicotinoids, being systemic in nature and with long-lasting residual activity, can protect the adults from disease transmission (30 min–7 h) (Capoor *et al*., 1974; Xu *et al*., 1988; Roistacher, 1991) could be disrupted with antifeedants. In support of this idea, pymetrozine applied at the labeled rate significantly reduced the feeding activities of adults on treated plants or directly treated adults, as measured by electrical penetration graphs (EPGs) (Biona *et al*., 2013). This effect resulted in reduced disease transmission by ACP adults feeding on treated plants compared with controls (Biona *et al*., 2013).

Intercropping with guava in Sweet orange orchards reduced citrus greening incidence by 66 and 90% and citrus greening severity by 66 and 83% in Haluaghat and Bhaluka orchards, respectively. Hall *et al.* (2008) reported the effect of guava on adult ACP. They found that adult ACP released into cages containing the only citrus generally moved faster to citrus than when either guava or cotton was present. They also observed a greater number of adults were consistently observed on citrus over time in cages with only citrus as compared to in cages with citrus in the presence of guava or cotton. They explained that this might be due to differences in the total plant surface area in cages with citrus alone compared to citrus caged with another plant. Mortality rates of adults were increased in cages containing both citrus and guava in one of two studies. While significant reductions in infestations of adults on young grapefruit sometimes occurred in cages containing both citrus and guava in the greenhouse, the reductions were not enough to verify the Vietnamese guava effect. These results are also supported by Beattie *et al.* (2006) and Gottwald *et al.* (2010). They reported that infestations of ACP and, consequently, incidences of citrus greening disease in citrus are greatly reduced when citrus is interplanted with guava, *Psidium guajava* L. (plant family Myrtaceae). The authors speculated that guava volatiles or phytotoxins might be responsible for reducing infestations of the psyllid on citrus. Putative guava volatiles may interfere with the psyllid's ability to locate and infest citrus grown next to guava, or they might repel psyllids away from citrus. Putative guava toxins might negatively affect the biology of the psyllid, interfering with psyllid reproduction in citrus. The reports from Vietnam prompted greenhouse investigations in Florida.

Conclusion

It can be concluded Bion (Acibenzolar S-methyl), nutrients formulations SICOGREEN® (soil and foliar application), intercropping with guava, spraying guava leaf extract, foliar spray of insecticides can be integrated to reduce HLB incidence and severity in Sweet orange. Some of these treatments have also some positive effects on plant growth and yield parameters of Sweet orange as compared to control. These findings collectively suggest that chemical inducers and nutrient management can pose a better alternative to control HLB sustainably to increase tree lifespan and productivity.

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