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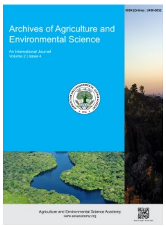

ORIGINAL RESEARCH ARTICLE

CrossMark

Detection of citrus greening disease and field efficacy of anti-pathogen chemicals against the disease in mandarin (*Citrus reticulata* Blanco.) in Gulmi, Nepal

Abhinav Poudel , Kamal Kafle * and Susan Subedi

Faculty of Agriculture, Agriculture and Forestry University, Rampur, Chitwan, NEPAL
*Corresponding author's E-mail: lotuskafle56@gmail.com

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ABSTRACT

Citrus cultivation in Nepal faces a persistent decline due to the widespread prevalence of Huanglongbing (HLB), also known as Citrus Greening Disease, across citrus-growing regions. This has resulted in significant economic losses for farmers, prompting them to actively seek preventive and remedial measures. In a study conducted at Resunga Municipality and Dhurkot Rural Municipality, Gulmi, aimed at addressing this decline, 51 orchards were assessed for HLB using the starch iodine test. Concurrently, the efficacy of anti-pathogen chemicals in managing citrus greening disease in mandarin (*Citrus reticulata* Blanco) was evaluated. An experiment was designed, incorporating eight treatments in a Randomized Complete Block Design (RCBD) with three replications. Each replication included eight treatments designated as follows: T1: Neem oil 5ml/l + Lentinan 2ml/l, T2: Neem Oil 5ml/l + *B. amyloliquefacians* 5ml/l, T3: Neem oil 5ml/l + *Pseudomonas* 5ml/l, T4: Imidacloprid 0.02% + Copper Oxychloride 0.025%, T5: Imidacloprid 0.02% + Streptocycline 250ppm, T6: *B. thuringiensis* 2ml/l + *Pseudomonas* 5ml/l, T7: *B. thuringiensis* 2ml/l + *B. amyloliquefacians* 5ml/l, T8: control. Results revealed that among the orchards tested, 18 were positive for HLB, representing 35.2% of the sample size. Notably, plots treated with Neem oil + *B. amyloliquefacians* and *B. thuringiensis* + *B. amyloliquefacians* exhibited the most significant reduction in disease severity compared to the control. Based on these findings, foliar application of Neem oil at a concentration of 5ml/l along with *B. amyloliquefacians* at 5ml/l, and *B. thuringiensis* at 2ml/l along with *B. amyloliquefacians* at 5ml/l at monthly intervals showed promising results in reducing the severity of citrus greening.

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INTRODUCTION

Citrus plants, which are members of the Rutaceae family, encompass numerous species and are characterized by their longevity as perennial shrubs and trees. They hold significant importance as fruit crops globally, being cultivated extensively in tropical, subtropical, and regions with borderline subtropical/temperate climates. These plants can be propagated through various methods such as seedling, rootstock, layering, and grafting, and they thrive within a temperature range of 14°C to 40°C. However, their optimal growth temperature is typically around 30°C (Whiteside *et al*., 1993). Citrus stands as one of the most vital high-value crops cultivated in arid to semi-arid agroecological zones, facing significant challenges that affect both its productivity and quality. Among these challenges, insect pests pose a notable threat (Chandrasekaran *et al*., 2021). As of 2019, global citrus production totalled 143,755.6 thousand metric tons (FAO, 2020). In Nepal, citrus holds a prominent position as a major fruit crop, covering 28.29% of the total fruit-growing area. The mid-hill region, spanning from 26°45' to 29°40' North latitude and 80°15' to 88°12' East longitude, is considered ideal for mandarin cultivation in Nepal. Despite its considerable potential, mandarin production in Nepal remains low, attributed to various factors including insect pests, diseases, management issues, and a shortage of healthy seedlings. Of these challenges, citrus greening disease stands out as a significant obstacle faced by farmers, contributing to a sharp decline in mandarin production across Nepal. All citrus cultivars in Nepal are susceptible to greening, resulting in decreased yields typically observed 5-8 years post-plantation. Globally, citrus greening disease is recognized as a major factor hampering productivity, posing a grave threat to the citrus industry. Huanglongbing, the causative agent of citrus greening disease, is attributed to phloem-restricted bacteria, including *Candidatus liberibacter asiaticus*, *C. liberibacter africanus*, and *C. liberibacter americanus*. Its transmission occurs primarily through the Citrus psylla insects, such as Diaphorina citri and Trioza erytreae (Paudyal, 2016).

In Nepal, the HLB disease was first reported in Pokhara in 1968 (Regmi & Lama, 1988). Although biological indexing and PCR tests for HLB are reliable, they are considered highly expensive, time-consuming, and labour-demanding, so they are unsuitable for indexing large samples (Li *et al*., 2019). To solve this problem, a rapid and straightforward field diagnostic iodine test was developed to study the samples. Anatomical studies conducted in the 1960s found a massive accumulation of starch in leaf samples of HLB-infected citrus trees: Starch accumulation in HLB-infected leaves is up to six times more than in healthy leaves (Etxeberria *et al*., 2008). This technique has been adapted as one of the most accessible diagnostic tools for HLB as the starch readily reacts with iodine, resulting in a very dark grey-to-black stain. This diagnosis method has been reported to have more than 90% agreement with PCR analysis (Irey *et al*., 2006). Chamberlin and Irey compared starch-based field tests for HLB to the results from real-time PCR to test field samples from 1759 suspected symptomatic trees. They found that 85% of the samples were positive by RT-PCR versus 78% favourable for the starch test. Therefore, they recommended that the starch test be considered a valuable tool for HLB diagnosis in the field but not a substitute for PCR-based testing (Irey *et al*., 2006).

Mandarin fruit have been recognized as an important food and integrated as a part of our daily diet, playing key roles in supplying energy and nutrients and in health promotion (Liu *et al*., 2012). So, the demand of mandarin goes on increasing but the production doesn't meet up the demand. The lower production and inferior quality of mandarin is due to the citrus greening in Nepal. To increase the production within a country various action has been taken by government of Nepal, NGOs/INGOs, co-operatives and other bodies. But, for the effective and long term planning to eliminate the citrus greening in Nepal, the prevalence and severity study of citrus greening is utmost needed (Bové, 2006; Regmi & Yadav, 2007; Regmi *et al*., 2010). However, very little information regarding the prevalence and severity of citrus greening in Nepal are available. Realizing the significance of the study, the present experiment has been undertaken to assess the citrus greening test on mandarin by scratch method and the efficacy of anti-pathogen chemicals against the disease in Mandarin (*Citrus reticulata* Blanco) in Gulmi district, Nepal.

MATERIALS AND METHODS

The study was conducted from February 2022 to July 2022. Samples were collected from major mandarin growers within the research site, and an iodine-based starch test was performed to identify the presence of HLB (Huanglongbing), a citrus disease. An experimental setup was implemented at the PMAMP's field to assess the effectiveness of anti-pathogen chemicals against HLB in Mandarin (*Citrus reticulata* Blanco). The detailed activities undertaken during the research period are outlined below:

Selection of site

The research was conducted in Resunga Municipality and Dhurkot Rural Municipality, located in the Gulmi district which is shown in Figure 1. The iodine-based starch test was carried out in the primary citrus-growing areas of these municipalities. These areas were selected based on their status as major mandarin-producing zones, designated as citrus zones by PMAMP.

Sample size and sampling technique

Resunga Municipality and Dhurkot Rural Municipality were purposively chosen for the study. Mandarin orchards were selected using a simple random sampling method, and the sample size was determined using Raosoft software.

Sample collection

Samples were collected from trees exhibiting strong symptoms of HLB, while those with mechanical or physical injuries or symptoms clearly related to other issues were avoided. Two to four symptomatic plants were sampled from each orchard, with three leaves from healthy and undamaged branches being collected. The trees were located on both southern and northern-facing slopes and were managed under common farmer horticultural practices.

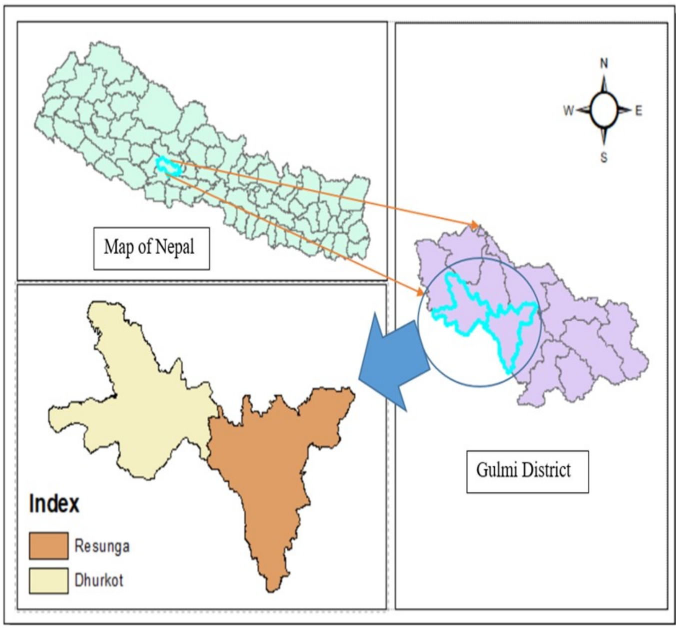

Figure 1. *Map of Nepal showing study area (Resunga and Dhurkot of Gulmi district).*

Procedure of iodine-based starch test by scratch method

The collected leaves underwent testing using the scratch method with a 1.2% iodine solution, similar to commercially produced laboratory iodine solutions like Tincture of Iodine. Water-proof abrasive paper with a mesh size of 120 was cut into small rectangles, each measuring one and a half inches. The upper surface of an infected leaf was scratched at least 20 times using a small piece of abrasive paper. The tissue scrapings were gently washed off from the sandpaper into water by rocking the bag by hand. The paper was then removed from the liquid suspension, and a drop of iodine solution was added, followed by a waiting period of 2 to 3 minutes.

Citrus greening scale

The study measured data such as types of HLB symptoms, percentage of disease incidence, and severity. The percentage of disease incidence was calculated based on the number of observed symptomatic plants out of the total number of assessed plants, using the formula outlined by Khairulmazmi *et al.* (2008).

$$\% \text{ disease incidence} = \frac{\text{Total infected citrus trees}}{\text{Total no of trees evaluated}} \text{ x } 100\%$$

The percentage of disease severity was defined based on the symptoms that existed. The grading system is as follows.

i) No symptoms (no symptoms observed on the plant canopy) = 0
ii) Mild (from 1 to 30% of the canopy) = 1
iii) Moderate (from 31% to 50% of the canopy) = 2
iv) Severe (More than 50% of the canopy) = 3

The below formula was adopted to calculate the percentage of the disease severity (Khairulmazmi *et al.*, 2008).

$$\% \text{ Disease severity} = \frac{X1 + X2 + X3 + \cdots . + Xn}{Y * \text{Maximum rating scale}}$$

Whereby;
X = sum score of disease severity of each citrus plant
Y = total number of plants in the same experiment

Anti-pathogen chemical trial

Two citrus groves infected with HLB were selected to conduct 2 same but independent experiments. Each grove was used for pesticide application in each replication. The experiment took place under open field conditions, utilizing local cultivars commonly grown by farmers in the mid-hills of Nepal. Various concentrations of insecticides and pesticides were applied to assess their impact on the severity scale of Huanglongbing disease in mandarin orchards. The experimental and treatment designs adopted in Mandarin Orchard are shown in Tables 1 and 2.

Table 1. Experimental design adopted in mandarin orchard.

S. No.	Particulars	Trial details
1	No of experiments	2
	In each experiment	Details
1	Experimental design	Randomized complete block design (RCBD)
2	Replication (Blocks)	3
3	Treatments	8
4	Total number of plots	24
5	Number of plants per plot	2
6	Variety used	Local

Preparation of treatment for spray

The treatments were prepared as follows:

Imidacloprid 0.02%: One milliliter of Imidacloprid was dissolved in 5 liters of water to create a 200-ppm solution.

Streptocycline 250 ppm: One milliliter of Streptocycline was dissolved in 4 liters of water to form a 250-ppm solution.

Copper Oxychloride 0.025%: One gram of copper oxychloride was dissolved in 4 liters of water to produce a 250-ppm solution.

Neem oil 5ml/l: Five milliliters of Neem oil was dissolved in 1 liter of water.

Lentinan 2ml/l: Two milliliters of Lentinan were dissolved in 1 liter of water.

Pseudomonas 5ml/l: Five milliliters of Pseudomonas were dissolved in 1 liter of water.

Bacillus amyloliquefacians 2ml/l: Two milliliters of Bacillus amyloliquefacians was dissolved in 1 liter of water.

Bacillus thuringiensis 2ml/l: Two milliliters of Bacillus thuringiensis was dissolved in 1 liter of water.

Pesticide application was conducted in the evening to minimize evaporation loss, and adhesives were included in the pesticide solution to enhance adherence to treated surfaces, thereby reducing wash-off due to rain or irrigation.

Table 2. Treatment details.

Treatments	Chemicals and their dosage
T_1	Neem oil 5ml/l + Lentinan 2ml/l
T_2	Neem Oil 5ml/l + *Bacillus amyloliquefacians* 5ml/l
T_3	Neem oil 5ml/l + *Pseudomonas* 5ml/l
T_4	Imidacloprid 0.02% + Copper Oxychloride 0.025%
T_5	Imidacloprid 0.02% + Streptocycline 250ppm
T_6	*Bacillus thuringiensis* 2ml/l + *Pseudomonas* 5ml/l
T_7	*Bacillus thuringiensis* 2ml/l + *Bacillus amyloliquefacians* 5ml/l
T_8	Control

Data collection and statistical analysis

Observations were recorded from tagged plants at various intervals as required. The citrus greening scale of individual plots was assessed before treatment application, with subsequent data collected monthly for a total of five times. Collected data were systematically organized and entered into MS Excel. Statistical analysis was performed using R Studio software. Means were compared using Duncan's Multiple Range Test (DMRT) at a 5% significance level (Gomez & Gomez, 1984) to evaluate the effectiveness of treatments based on the aforementioned parameters.

Table 3. Observation of citrus greening disease test in different location.

S. No.	Location	Observation
1	28° 4'35.34"N 83°9'47.23"E	Positive
2	28° 4'36.51"N 83° 9'48.62"E	Negative
3	28° 4'36.23"N 83° 9'45.98"E	Positive
4	28° 4'37.62"N 83° 9'47.02"E	Negative
5	28° 4'37.67"N 83° 9'50.96"E	Negative
6	28° 4'47.00"N 83° 9'44.00"E	Positive
7	28° 4'26.21"N 83° 9'44.84"E	Negative
8	28° 4'9.00"N 83° 9'22.00"E	Positive
9	28° 4'10.36"N 83° 9'26.93"E	Negative
10	28° 4'47.11"N 83° 9'37.71"E	Negative
11	28° 4'48.90"N 83° 9'42.98"E	Negative
12	28° 4'18.25"N 83° 9'11.67"E	Positive
13	28° 4'12.08"N 83°10'19.22"E	Negative
14	28° 4'7.13"N 83°10'16.44"E	Positive
15	28° 4'7.26"N 83°10'14.27"E	Negative
16	28° 4'5.33"N 83°10'14.61"E	Negative
17	28° 3'58.43"N 83°10'8.91"E	Positive
18	28° 5'25.00"N 83° 8'36.00"E	Negative
19	28° 5'0.98"N 83° 9'55.56"E	Negative
20	28° 5'3.46"N 83° 9'55.46"E	Positive
21	28° 4'29.68"N 83° 9'40.07"E	Positive
22	28° 5'7.07"N 83° 8'42.00"E	Positive
23	28° 5'3.48"N 83° 8'34.83"E	Negative
24	28° 5'2.73"N 83° 8'34.78"E	Negative
25	28° 5'17.89"N 83° 8'19.44"E	Negative
26	28° 5'16.88"N 83° 8'23.75"E	Positive
27	28° 5'14.79"N 83° 8'26.81"E	Negative
28	28° 5'17.26"N 83° 8'16.77"E	Negative
29	28° 5'24.69"N 83° 8'17.91"E	Positive
30	28° 5'0.65"N 83° 8'42.17"E	Negative
31	28° 4'28.52"N 83° 8'51.20"E	Negative
32	28° 4'25.73"N 83° 8'51.19"E	Negative
33	28° 4'9.98"N 83° 8'50.18"E	Negative
34	28° 4'15.90"N 83° 8'52.62"E	Positive
35	28° 4'14.56"N83° 8'58.71"E	Negative
36	28° 5'47.36"N 83°15'27.93"E	Positive
37	28° 5'55.39"N 83°15'31.59"E	Negative
38	28° 5'55.53"N 83°15'27.81"E	Negative
39	28° 6'1.34"N 83°15'25.46"E	Negative
40	28° 6'0.95"N 83°15'30.23"E	Positive
41	28° 5'49.48"N 83°15'23.54"E	Negative
42	28° 5'39.41"N 83°15'23.19"E	Negative
43	28° 5'36.00"N 83°15'46.63"E	Negative
44	28° 5'19.10"N 83°15'23.63"E	Positive
45	28° 5'20.33"N 83°15'14.74"E	Negative
46	28° 5'27.08"N 83°15'16.83"E	Positive
47	28° 4'6.28"N 83°15'15.04"E	Negative
48	28° 4'43.69"N 83°14'11.95"E	Negative
49	28° 5'40.84"N 83°14'54.93"E	Negative
50	28° 5'46.52"N 83°14'54.93"E	Negative
51	28° 4'22.69"N 83°14'15.30"E	Positive

RESULTS AND DISCUSSION

Occurrence and detection of citrus greening disease

Out of 51 mandarin orchards surveyed, 18 orchard samples tested positive for HLB which is 35%of the total sampled as shown in Table 3. This finding is on par with the finding of (Knorr & Shah, 1971), (Schwarz, 1970) which found that more than 40-70% trees are infected with CGD. Conversely, the remaining 33 orchard samples yielded negative reactions when subjected to a 1.2% iodine solution formulation, akin to commercially produced laboratory iodine solutions such as Tincture of Iodine. This study highlights a significant prevalence of citrus greening, with the test indicating a rate as high as 35.29%.

Efficacy of different anti-pathogen chemicals against citrus greening in mandarin

Two distinct setups were established within the affected area to assess the effectiveness of various anti-pathogen chemicals. The severity of citrus greening disease on mandarin was observed to be impacted by the application of these treatments as shown in Tables 4 and 5. Upon comparing all treatments, plots treated with combinations such as (Neem oil + *B. amyloliquefaciens*) and (*B. thuringiensis* + *B. amyloliquefaciens*) exhibited the lowest disease severity scale (0.67). This was closely followed by plots treated with (Imidacloprid + Streptocycline) and (*B. thuringiensis* + *Pseudomonas*). Conversely, the control plots displayed the most significant disease severity scale.

Figures 2 and 3 shows the percentage reduction of severity of treated plots over control plots. The plot treated with (Neem oil + *B. amyloliquefaciens*) followed by plot treated with (*B. thuringiensis* + *B. amyloliquefaciens*) were the highest percentage reduction over the control plot. The severity scale of citrus greening was notably reduced through the application of combinations such as (Neem oil + *B. amyloliquefaciens*) and (*B. thuringiensis* + *B. amyloliquefaciens*), followed by (Imidacloprid + Streptocycline) and (*B. thuringiensis* + *Pseudomonas*). According to Weathersbee & McKenzie (2005), Neem bio-pesticides were observed to repel and increase the mortality of the psylla population. Additionally, pesticidal proteins derived from bacteria like Bacillus thuringiensis showed efficacy against the Asian Citrus Psyllid and Diaphorina citri (Fernandez-Luna *et al.*, 2019). *Bacillus thuringiensis* primarily acts by lysing the midgut epithelial cells, penetrating the target membrane and forming pores (Bravo *et al.*, 2007). The main compound in Neem oil, Azadirachtin, exhibits insecticidal activity and acts as a feeding inhibitor. It delays the development and growth of insects, reducing fecundity and fertility, altering behavior, and inducing anomalies in eggs, larvae, and adults of insects and mites. Given that citrus psylla serves as the primary vector of citrus greening, the effects of Neem oil may have controlled these insects and subsequently mitigated greening in citrus (Zanuncio *et al.*, 2016). However, our research results contradict those of Wang *et al.* (2017), who reported a higher suppression of *C. liberibacter* asiaticus by streptocycline compared to other biopesticides.

Table 4. Efficacy of different anti-pathogen chemicals against citrus greening in Mandarin (First Experiment).

Treatment	Disease severity scale				
	Severity scale before spray	Severity scale after 1 month of 1st spray	Severity scale after 1 month of 2nd spray	Severity scale after 1 month of 3rd spray	Severity scale after 1 month of 4th spray
T1 (Neem oil 5ml/l + Lentinan 2ml/l)	2	1.83	1.67	1.33[b]	1.33[b]
T2 (Neem oil 5ml/l + *B. amyloliquefaciens* 5ml/l)	1.83	1.67	1.3	1[b]	0.67[c]
T3 (Neem oil 5ml/l + *Pseudomonas* 5ml/l)	2.17	2	1.83	1.5[b]	1.33[b]
T4 (Imidacloprid 0.02% + Copper Oxychloride 0.025%)	1.67	1.5	1.5	1.33[b]	1.33[b]
T5 (Imidacloprid 0.02% + Streptocycline 250 ppm)	1.83	1.67	1.5	1.17[b]	1.17[bc]
T6 (*B. thuringiensis* 2ml/l + *Pseudomonas* 5ml/l)	1.83	1.83	1.67	1.17[b]	1.17[bc]
T7 (*B. thuringiensis* 2 ml/l + *B. amyloliquefacians* 5ml/l)	1.83	1.67	1.33	1[b]	0.67[c]
T8 (Control)	2	2	2	2.17[a]	2.17[a]
LSD (0.05)	NS	NS	NS	0.63*	0.52***
SE_m(+/-)	-	-	-		
CV, %	-	-	-	26.17	24.31
Grand Mean	-	-	-	1.375	1.229

NOTE: The common letter(s) within the column indicates the non-significant difference based on the Duncan Multiple Range Test (DMRT) at a 0.05 significance level. NS- Not significant, '*' – p<0.05 '**' – p<0.01 '***' – p<0.001.

Table 5. Efficacy of different anti-pathogen chemicals against citrus greening in Mandarin (Second Experiment).

Treatment	Disease Severity Scale				
	Severity scalebefore spray	Severity scale after 1 month of 1st spray	Severity scale after 1 month of 2nd spray	Severity scale after 1 month of 3rd spray	Severity scale after 1 month of 4th spray
T1 (Neem oil 5ml/l +Lentinan 2ml/l)	1.83	1.67	1.67	1.33[bc]	1.33[bc]
T2 (Neem oil 5ml/l + *B. amyloliquefaciens* 5ml/l)	2	1.83	1.67	1[c]	0.67[d]
T3 (Neem oil 5ml/l + *Pseudomonas* 5ml/l)	2.17	2	1.83	1.67[b]	1.5[b]
T4 (Imidacloprid 0.02% + Copper Oxychloride 0.025%)	1.83	1.67	1.5	1.33[bc]	1.33[bc]
T5 (Imidacloprid 0.02% + Streptocycline 250 ppm)	1.83	1.5	1.5	1.17[c]	1[cd]
T6(*B. thuringiensis* 2ml/l + *Pseudomonas* 5ml/l)	2	1.67	1.33	1.17[c]	1[cd]
T7(*B. thuringiensis* 2 ml/l + *B. amyloliquefacians* 5ml/l)	1.83	1.5	1.33	1[c]	0.67[d]
T8 (Control)	2.17	2.17	2.17	2.17[a]	2.17[a]
LSD (0.05)	NS	NS	NS	0.45**	0.42***
SE_m(+/-)	-	-	-		
CV, %	-	-	-	18.89	19.93
Grand Mean	-	-	-	1.354	1.208

NOTE: The common letter(s) within the column indicates the non-significant difference based on the Duncan Multiple Range Test (DMRT) at a 0.05 significance level. NS- Not significant, '*' – p<0.05 '**' – p<0.01 '***' – p<0.001.

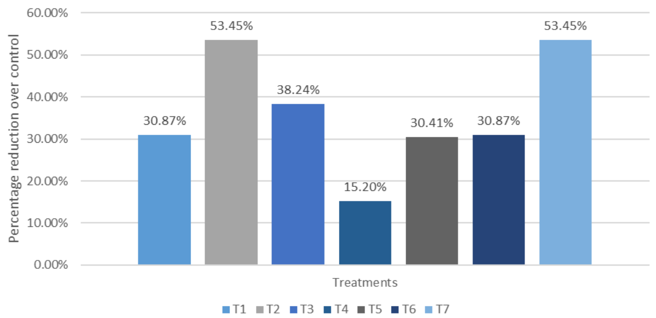

Figure 2. *Percentage reduction of severity index by different combinations of treatments overcontrol in first experiment.*

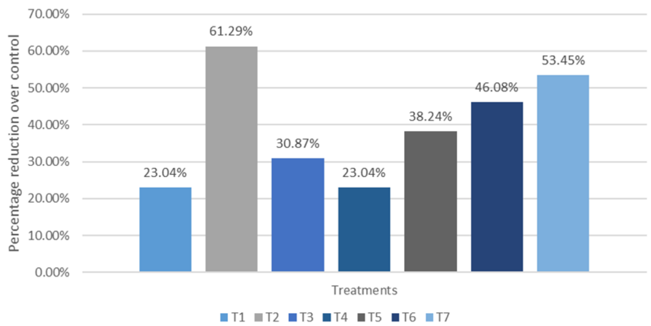

Figure 3. *Percentage reduction of severity index by different combinations of treatments over control in second experiment.*

Conclusion

After comparing all treatments, the plots treated with (Neem oil + *B. amyloliquefaciens*) and (Bacillus thuringiensis + Bacillus amyloliquefaciens) exhibited the least disease severity scale, underscoring the effectiveness of these anti-pathogen chemicals against citrus greening disease.

DECLARATIONS

Author contribution statement

Conceptualization: AP. and KK.; Methodology: AP.; Software and validation: AP., SS., and KK.; Formal analysis and investigation: KK.; Resources: AP; Data curation: AP.; Writing—original draft preparation: AP, KK, and SS.; Writing—review and editing: KK, SS.; Visualization: SS.; Supervision: KK and SS.; Project administration: AP.; Funding acquisition: AP. All authors have read and agreed to the published version of the manuscript.

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REFERENCES

Bové, J. M. (2006). Huanglongbing: A destructive, newly emerging, century-old disease of citrus. *Journal of Plant Pathology*, *88*(1), 7–37. https://doi.org/10.4454/jpp.v88i1.828

Bravo, A., Gill, S. S., & Soberón, M. (2007). Mode of action of Bacillus thuringiensis Cry and Cyt toxins and their potential for insect control. *Insecticidal Toxins and Their Potential for Insect and Pest Control*, *49*(4), 423–435. https://doi.org/10.1016/j.toxicon.2006.11.022

Chandrasekaran, M., Pirithiraj, U., & Soundararajan, R. P. (2021). Asian Citrus Psyllid: A Threat to Citriculture. *Biota Research Today*, *3*(1), 074–076.

Etxeberria, E., Gonzalez, P., Dawson, W., & Spann, T. (2008). An Iodine-Based Starch Test to Assist in Selecting Leaves for HLB Testing 1. *EDIS*, *2008*. https://doi.org/10.32473/edis-hs375-2007

FAO. (2020). *World Food and Agriculture—Statistical Yearbook 2020*. FAO. https://doi.org/10.4060/cb1329en

Fernandez-Luna, M. T., Kumar, P., Hall, D. G., Mitchell, A. D., Blackburn, M. B., & Bonning, B. C. (2019). Toxicity of Bacillus thuringiensis-derived pesticidal proteins Cry1Ab and Cry1Ba against Asian citrus psyllid, *Diaphorina citri* (Hemiptera). *Toxins*, *11*(3), 173.

Gomez, K. A., & Gomez, A. A. (1984). *Statistical procedures for agricultural research*. John wiley & sons.

Irey, M., T. Gast, & T.R. Gottwald. (2006). Comparison of visual assessment and polymerase chain reaction assay testing to estimate the incidence of the huanglongbing pathogen in commercial Florida citrus. *Annual meeting of the Florida State Horticultural Society*, *119*, 89–93.

Khairulmazmi, A., Kamaruzaman, S., Habibuddin, H., Jugah, K., & Rastan, S. O. S. (2008). Cloning and sequencing of Candidatus Liberibacter asiaticus isolated from citrus trees in Malaysia. *International Journal of Agriculture And Biology*, *10*(4), 417–421.

Knorr, L. C., & S. M. Shah. 1971. World Citrus problem V. Nepal. *FAO Plant Protection Bulletin*, *19*(4), 73-79.

Li, J., Li, L., Pang, Z., Kolbasov, V. G., Ehsani, R., Carter, E. W., & Wang, N. (2019). Developing Citrus Huanglongbing (HLB) Management Strategies Based on the Severity of Symptoms in HLB-Endemic Citrus-Producing Regions. *Phytopathology*, *109*(4), 582–592. https://doi.org/10.1094/PHYTO-08-18-0287-R

Liu, Y., Heying, E., & Tanumihardjo, S. A. (2012). History, global distribution, and nutritional importance of citrus fruits. *Comprehensive reviews in Food Science and Food Safety*, *11*(6), 530-545.

Paudyal, K. P. (2016). Technological Advances in Huanglongbing (HLB) or Citrus Greening Disease Management. *Journal of Nepal Agricultural Research Council*, 41–50. https://doi.org/10.3126/jnarc.v1i0.15735

Regmi, C., & Lama, T. K. (1988). Greening Incidence and Greening Vector Population Dynamics in Pokhara. *International Organization of Citrus Virologists Conference Proceedings (1957-2010)*, *10*(10). https://doi.org/10.5070/C54g19n0bh

Regmi, C., & Yadav, B. P. (2007, January). Present status of Huanglongbing in some western districts of Nepal. In *Proceeding of the Fourth National Horticulture Seminar on Horticulture for Food Security, Employment Generation and Economic Opportunities, Kirtipur, Kathmandu, Nepal*.

Regmi, C., Devkota, R. P., Paudyal, K. P., Shrestha, S., Ayres, A. J., Murcia, N., & Durán-Vila, N. (2010). Shifting from seedling mandarin trees to grafted trees and controlling huanglongbing and viroids: a biotechnological revolution in Nepal. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 17, No. 17).

Schwarz, R. E. 1970. seasonal graft transmissibility and quantification of gentisoyl marker of Citrus greening by single primer DNA amplification fingerprinting. please Horticulture. 3: 128s

Wang, N., Pierson, E. A., Setubal, J. C., Xu, J., Levy, J. G., Zhang, Y., Li, J., Rangel, L. T., & Martins, J., Jr. (2017). The *Candidatus Liberibacter*—Host interface: Insights into pathogenesis mechanisms and disease control. *Annual Review of Phytopathology*, *55*, 451–482.

Weathersbee, A. A., III, & McKenzie, C. L. (2005). Effect of a neem biopesticide on repellency, mortality, oviposition, and development of Diaphorina citri (Homoptera: Psyllidae). *Florida Entomologist*, *88*(4), 401–407.

Zanuncio, J. C., Mourão, S. A., Martínez, L. C., Wilcken, C. F., Ramalho, F. S., Plata-Rueda, A., Soares, M. A., & Serrão, J. E. (2016). Toxic effects of the neem oil (*Azadirachta indica*) formulation on the stink bug predator, *Podisus nigrispinus* (Heteroptera: Pentatomidae). *Scientific Reports*, *6*(1), 30261.

Whiteside, J. O., Garnsey, S. M., & Timmer, L. W. (1993). Compendium of Citrus Diseases. American Phyto pathological Society. APS Press, pp 80.