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Article

Silver Nano Chito Oligomer Hybrid Solution for the Treatment of Citrus Greening Disease (CGD) and Biostimulants in Citrus Horticulture

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Abstract: Citrus greening disease (CGD), or Huanglongbing (HLB), is principal in Citrus farming because of its severe damage, decreased yield, poor quality of fruit, and frequent disappearance before harvest. Present research blended silver nanoparticles (AgNPs) colloid with chitooligomers and monomers (COAMs) to create the composites referred to as a "hybrid solution". The hybrid solution has been synthesized for antimicrobial effects and plant growth stimulation. The hybrid solution was applied to cure the CGD, which was then tested using the results of the iodine test kit. Study results revealed that the mechanism for antibacterial properties is efficient and leads to cell death by silver ions (positive charge) attached to the cell wall of pathogens (negative charge). COAMs increase AgNPs' ability to adhere to negatively charged bacterial cytoplasmic membranes and amplify their electrostatic interaction. There are synergistic antibacterial effects of AgNPs–COAMs. The findings reveal a notable increase in the shoot length of leaves which may be the reason for the growth stimulation function of the hybrid solution due to the properties of COAMs as a biostimulant. The hybrid solution-treated trees yielded around 104.50 ± 4.56 kg of fruits by the trunk or foliar application. Citrus fruits did not show the presence of any silver residues, as displayed by the results of the accumulation test. Compared to conventional ampicillin, the hybrid treatment of CGD was significantly more cost-effective, increased productivity, and had minor accumulation. The acquired results may also be applied to further plant disease treatment.

Keywords: citrus greening disease; Huanglongbin; chitosan oligomers; monomer; hybrid solution; silver nanoparticles (AgNPs)

1. Introduction

Citrus is a genus of flowering trees and shrubs in the Rue family. Rutaceae includes essential crops such as oranges, lemons, grapefruit, and limes, ranking among Thailand's principal export crops. Roughly 20,487 hectares are under cultivation; 95.3% were accountable in the north. Citrus worth 1138.26 million Baht were exported in 2021, totaling 47,318.94 tons [1]. Citrus greening disease (CGD), or Huanglongbing (HLB), is critical in Citrus farming because of its severe damage, decreased yield, poor quality, and frequent disappearance before harvest. Asymmetrical, blotchy mottling patterns on leaves are crucial disease symptoms. *Candidatus Liberibacter* species are primarily responsible for the disease and cannot be cultivated on a culture medium [2]. One of the primary vectors for transmitting the disease by feeding on Citrus leaves is the Asian Citrus psyllid. It can carry a bacterium for many days and transfer it to an uninfected plant when feeding on it. The phloem tissues of the roots and leaves are severely obstructed because of bacterial migration inside these two parts of the plant. As a result, obstruction to the circulation of nutrients and sugars in the interior tissues leads to leaf loss, varying fruit size, which can impair the flavor and texture of fruits, premature fruit drop, and eventually, the death of the tree [2–6]. The excessive accumulation of starch in the remaining parenchyma cells of the aerial parts and photosynthetic cells is one of the most apparent CGD symptoms in Citrus trees that the iodine test can identify. One can differentiate whether a Citrus tree is infected with the CGD by observing the color change in the leaves and determining if the leaves turn blue or purple (positive test). The leaves are considered to be healthy if there is no change in the color pattern [7].

Since the disease commenced, numerous techniques have been applied to treat CGD, including eradicating the insect vector, administering insecticides, and using broad-spectrum antibiotics to treat the symptoms in infected trees. The use of antibiotics in field crops is undoubtedly constrained by the emergence of microbial resistance, which also poses a significant risk to human health due to its indirect effects. They are seriously concerned about the Citrus orchard's ecology, where antibiotic residues have begun to accumulate in the water, soil, and Citrus plants. These might impact the food chain and result in bacterial resistance that can be transmitted to humans. The disease cannot currently be treated with chemicals or any other types of controls. Therefore, it is evident that a long-term cure for CGD infection in plants is closely required [8].

Chitooligomers, also known as chitooligosaccharides (COS) or chitosan oligomers, are produced by the degradation of chitosan. Their degrees of polymerization (DPs) are less than 20, and their average molecular weight is less than 3900 Da. [9]. It also has excellent solubility and outstanding qualities, including biodegradability, biocompatibility, adsorptive capacities, and nontoxicity like chitin and chitosan [10]. Applying chitosan and its derivatives to plants can inhibit pathogens, particularly fungi. Previous research proved that chitosan and its products can boost the immune system and promote plant growth [11–13].

Numerous studies of silver nanoparticles (AgNPs) demonstrate potential antiphytopathogenic effects and plant disease management, including fungi, bacteria, and viruses [14]. The application of AgNPs is becoming much more attractive in various hybrid products and is being reconsidered for their outstanding characteristics [15–17]. Previous studies report on the nanocomposite production of silver nanoparticles with chitosan and its derivative displayed nanocomposites with improved features that might be used as an antibacterial agent. Silver was nano-mediated, with chitosan as a reducing and stabilizing agent [18]. Mei et al. (2020) observed and reported the synergistic antibacterial effect of nanocomposite of COS and AgNPs [19]. It is possible to enhance the surface charge of AgNPs by conjugating it with COS, intensifying their electrostatic interactions and improving their capacity to cling to negatively charged bacterial cytoplasmic membranes. COS can also make it easier for bacteria to absorb AgNPs by functioning as an antibacterial agent in multivalent binding [20,21]. There is a lack of comprehensive studies specifically focused on the effectiveness of silver nanochito oligomer hybrid solutions for CGD treatment and as biostimulants in citrus horticulture. The mechanisms underlying the efficacy of these hybrid solutions in combating CGD and acting as biostimulants are not well understood. Integrating silver nanoparticles and chitosan oligomers offers a novel approach for CGD treatment and plant growth stimulation. The synergistic effects of the hybrid solution may enhance its efficacy against CGD pathogens and promote Citrus plant growth.

This approach provides a sustainable, eco-friendly solution with the potential for targeted delivery, simultaneously addressing disease management and plant growth promotion. The main objective of this study is to replace antibiotics with the efficient and safe treatment of CGD by using hybrid silver nanoparticles and chito-oligomers to treat the CDG in Citrus and to evaluate whether their use is cost-effective and secure. Further research is needed to optimize the formulation parameters of the hybrid solution for maximum effectiveness.

2. Materials and Methods

2.1. Hybrid Solution Preparation

AgNPs were designed using pure mineral (99.99%) procured from the Bangkok Assay Office Co., Ltd., Bangkok, Thailand. AgNPs were prepared by (Navatech Co., Ltd., Chiang Mai, Thailand) extracting natural silver metal followed by implementing the previously described methods [22]. The solid silver was dissolved using nitric acid and then reduced using sodium hypophosphite as the reducing agent. Polyvinyl pyrolydene (PVP) is a stabilizer. The process was controlled at a temperature below 80 °C. The final product is a nanosilver with 12 nm of average diameter. Chito-oligomers and monomers (COAMs) were prepared via chitosan degradation by using cold plasma and ultrasonic methods. Mix AgNPs with COAMs by heating and stirring for 1–3 h. Ultrasonic and nonthermal atmospheric pressure plasma (cold plasma) technologies can produce chitosan oligomer synergistically. Firstly, chitosan polymer was depolymerized by using high-intensity ultrasonic waves with operating frequencies of 20 kHz to 100 kHz Digital Ultrasonic Cleaner (Vevor, Jiangsu, China). Then the adhesion enhancer was added at room temperature and stirred continuously with a magnetic stirrer (Velp Scientifica, Usmate Velate MB, Italy) for 12 h [23]. Tween-80 (Micro-Master, Thane, India) was mainly used as an emulsifier to prepare the chito-oligomer and silver solution compatibility. The stability of the chito-oligomer and silver emulsion have been tested as years have passed. The result of the solution mixture investigated the physical properties as apparent, acidity, and particle size.

2.2. Citrus Greening Disease Testing (CDG)

The experimental design for CGD testing was investigated using the in-directed method. Diagnosis criteria for showing symptoms of CGD consider the splotchy mottling of the entire leaf, as shown in Figure 1. The leaf sample with and without symptoms was brought up to the laboratory. An iodine test kit (Navatech Co., Ltd., Chiang Mai, Thailand) was employed to determine whether the greening disease infected Citrus trees.

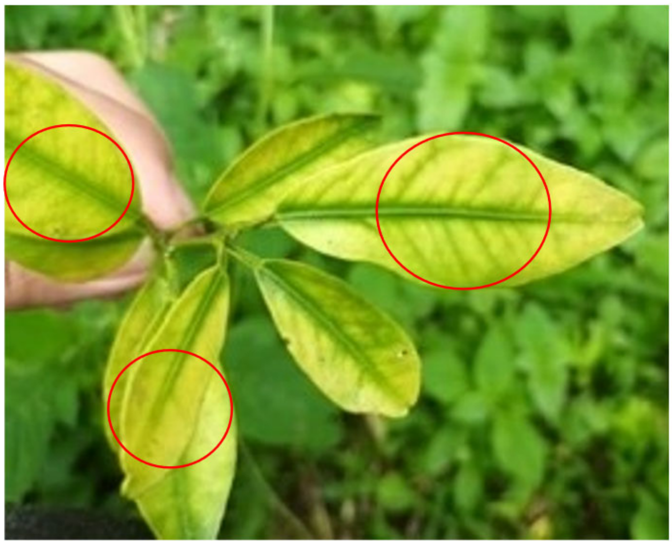

Figure 1. Citrus leaves with CGD show entire leaves' splotchy mottling (red circle).

The testing procedure was initiated by crushing the Citrus leaves (3 g) with distilled water (3 mL) in the mortar. The crushed solution was filtered and put in a transparent sampling sachet. Then, the iodine litmus paper was dipped into the sachet and allowed to sit for 1 min, and the color change was observed. Later, a dropper was used to drop iodine solution into the sachet, qualifying it for 1 min, and the color change was monitored. Finally, the observed color of the solution in a sachet was observed to see if it turned blue or purple, meaning a positive result or infected tree detection. In contrast, no color change means no infection or negative impact, as shown in Figure 2.

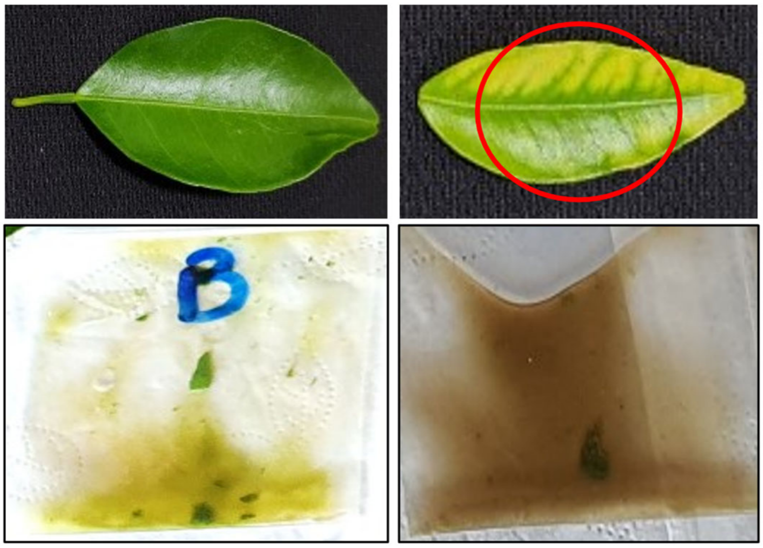

Figure 2. The color of the iodine testing solution of CGD (diseased leaf with red circle) compared to asymptomatic (Control) (B).

2.3. Field Crops Test

A Citrus plantation in the Fang district, Chiang Mai province, Thailand, was selected for the present experiment. A total of 50 Citrus trees were selected with CGD, confirmed using iodine test kits. The Citrus trees were between 2.00 and 2.50 m tall, with plantation and row spacing of 4.5 m. The selected experimental trees were around ten years old, and the Citrus tree canopy was 2 m wide. Three treatments, such as ampicillin, AgNPs, and AgNPs-COAMs (hybrid solution), were applied in five different circumstances. Each experiment was repeated three times every month for each plant. Gibberellic acid is a plant hormone which was additionally added to groups 3 and 5. The applied methods are shown in Table 1. Following the experimental plans, testing substances at the specified concentration were injected into the trunks of the chosen Citrus trees (trunk injection). Every group received a total of 800 mL of the solution.

Table 1. Group of kinds of treatment.

Group	Treatment
1	Ampicillin
2	AgNPs
3	AgNPs + Gibberellic acid
4	The hybrid solution
5	The hybrid solution + Gibberellic acid

2.4. Treatment Determines

The treatment and designed experimental were applied and employed. The liquid and litmus iodine test kits were used to confirm the CGD. The physical changes in Citrus leaves were observed as the criteria for disease improvement, as shown in Table 2. The Citrus trees were counted for shoot length, number of leaves, leaf area, and greenness of leaves. Each parameter was repeated three times to eliminate human error.

Table 2. Physical Citrus leaves criteria and determination.

Physical Leaves Criteria	Determination
Shoot length	Centimeter (cm)
Number of leaves	Number of leaves
Leaf area	Centimeter square (cm^2)
Greenness of leaves	Greenness SPAD index

2.5. Growth and Yield Analysis

The synergetic effects of the hybrid solution were tested on the Citrus trees. A hybrid solution (AgNPs-COAMs) was employed to control and treat the CGD. In this experiment, mixing the hybrid solution with water at a ratio of 1:500 was also used to treat the disease via foliar application. The hybrid solution was added to the water in a ratio of 1:500 and sprayed on the Citrus trees to study foliar application and shoot/fruit enhancement [24].

2.6. Statistical Analysis

A completely randomized (CRD) experiment was designed for statistical analysis. The statistical F-test was employed to verify the impact of physical changes at 99% confidence, $p < 0.01$.

3. Results

3.1. Physical Properties and Particle Size of Hybrid Solution

The solution mixture of silver nano and chito-oligomer was fully developed with the "OSIL" trade name market by Navatech Co., Ltd., Thailand. The visual observations of the hybrid solution are a brown–gold liquid, with standard viscosity, and a pH ranging from 5 to 7. The average particle size of the hybrid solution was 282.48 + 3.56 nm, as shown in Table 3. The particle size was measured by (Horiba Nanosizer ES 300, Kyoto, Japan) NSTDA (National Science and Technology Development Agency) Characterization and Testing Service Center (NCTC), Thailand.

Table 3. The particle size of the hybrid solution.

Measurement	Z Average (d.nm)	PdI (Mn)
1	278.8	0.314
2	284.3	0.203
3	284.8	0.186
4	283.6	0.234
5	277.3	0.339
6	286.1	0.200

3.2. Citrus Greening Disease Assessment

The Citrus leaves were examined before and after the hybrid solution treatment, as shown in Figure 3. The iodine test kit results displayed Citrus leaves showing symptoms of greening disease in Figure 3a–c. The iodine test kit confirmed with blue/purple color, and the iodine test kit (litmus paper test) reconfirms the blue/purple color of CDG. No symptoms of greening disease were observed in the control Figure 3c–e. The iodine and iodine test kits (litmus paper test) were asymptomatic.

3.3. Field Test Results

The research data was collected during January–March 2022 and March–May 2022. Iodine test kits were used to evaluate all treatments, and all groups were tested for negative results. Citrus trees treated with AgNPs + COAMs hybrid solution have shoot lengths that are significantly ($p < 0.01$) more prolonged than those treated with ampicillin throughout two periods. Still, no differences exist in the number of leaves, leaf area, or greenness index across treatments, as observed in Tables 4 and 5. The characteristic of leaves of all treatments is also shown in Figure 4.

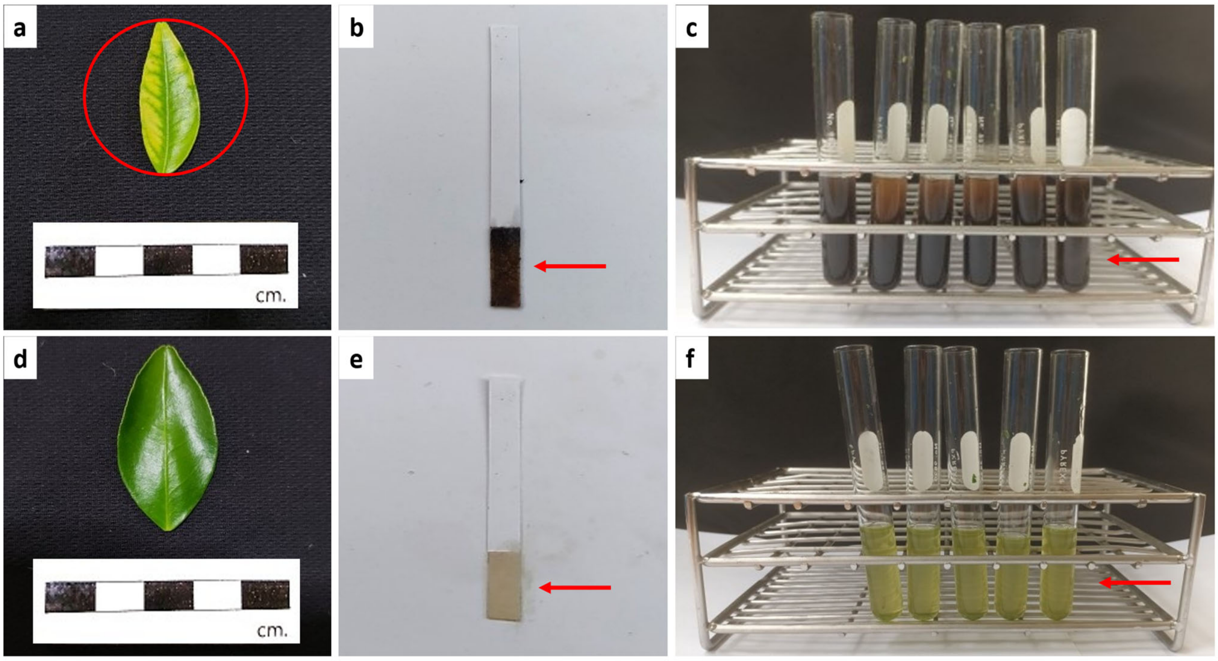

Figure 3. (**a**) Citrus leaf showing symptoms of greening disease highlighted with red circle, (**b**) the blue/purple color of iodine test kit with red arrow mark (litmus paper test), (**c**) the blue/purple color of iodine test, (**d**) no symptoms of greening disease green leaf, (**e**) no color changing of litmus paper test with red arrow mark, (**f**) and no color changing of iodine test.

Table 4. Result of treatments for shoot length and the number of leaves.

Treatment	January–March 2022		March–May 2022	
	Shoot Length (cm)	The Number of Leaves (no.)	Shoot Length (cm)	The Number of Leaves (no.)
1. Ampicillin	9.62 ± 0.13	7.09 ± 0.15	9.78 ± 0.35	42.7 ± 0.14
2. AgNPs	10.20 ± 0.21	7.23 ± 0.22	10.22 ± 0.28	7.57 ± 0.13
3. AgNPs + Gibberellic acid	16.85 ± 0.51	8.65 ± 0.21	17.37 ± 0.17	8.99 ± 0.11
4. The hybrid solution	11.58 ± 0.43	7.53 ± 0.26	12.43 ± 0.11	8.29 ± 0.17
5. The hybrid solution + Gibberellic acid	17.10 ± 0.25	8.45 ± 0.14	17.10 ± 0.23	8.78 ± 0.21
F-test	**	ns	**	ns
c.v. %	5.19 ± 0.01	1.11 ± 0.02	17.56 ± 0.04	21.82 ± 0.03

ns = not significantly different, ** = significant difference at $p < 0.01$.

Table 5. Result of treatments for leaf area, greenness index, and iodine test kit.

Treatment	January–March 2022			March–May 2022		
	Leaf Area (cm^2)	Greenness Index (SPAD)	Iodine Test Kit (−/+)	Leaf Area (cm^2)	Greenness Index (SPAD)	Iodine Test Kit (−/+)
1. Ampicillin	10.30 ± 0.34	59.92 ± 0.32	negative	10.42 ± 0.11	60.42 ± 0.13	negative
2. AgNPs	9.87 ± 0.21	54.59 ± 0.14	negative	9.94 ± 0.18	54.99 ± 0.11	negative
3. AgNPs + Gibberellic acid	8.87 ± 0.12	54.97 ± 0.16	negative	9.27 ± 0.24	55.54 ± 0.28	negative
4. The hybrid solution	10.51 ± 0.36	54.26 ± 0.27	negative	10.75 ± 0.19	54.86 ± 0.21	negative
5. The hybrid solution + Gibberellic acid	6.87 ± 0.87	59.28 ± 0.17	negative	6.93 ± 0.28	59.91 ± 0.11	negative
F-test	ns	ns	-	ns	ns	-
c.v. %	6.41 ± 0.12	20.55 ± 0.21	-	25.07 ± 0.13	8.06 ± 0.11	-

ns = not significantly different.

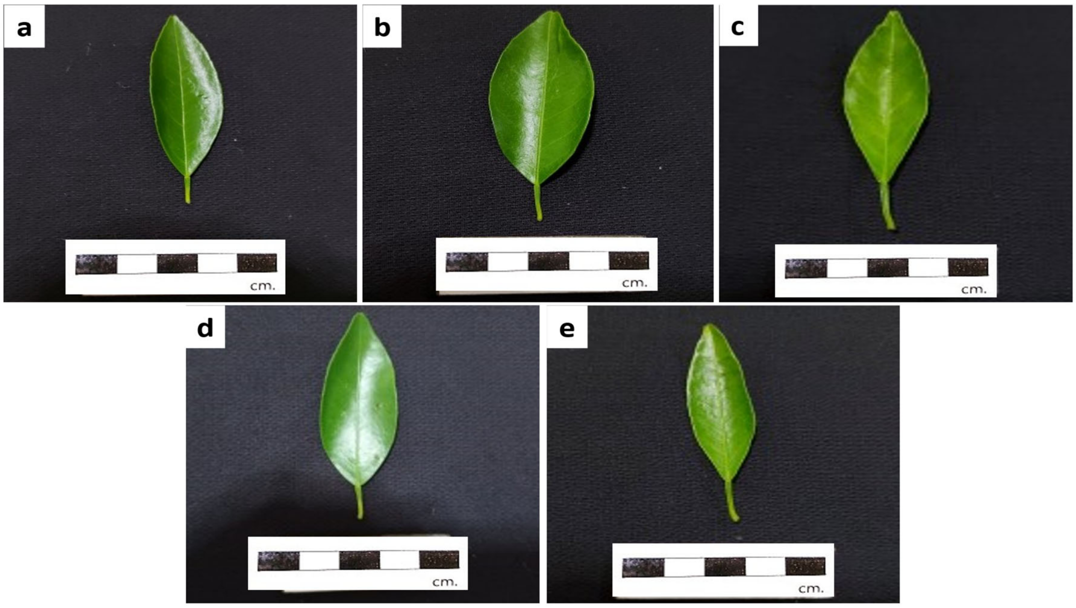

Figure 4. Characteristics of Citrus leaves after receiving the following treatments of (**a**) Ampicillin, (**b**) AgNPs (**c**) AgNPs + Gibberellic acid, (**d**) The hybrid solution, (**e**) The hybrid solution + Gibberellic acid.

3.4. Residual Analysis (LOD)

The ripened Citrus fruits were randomized, collected, and sent to the Institute of Product Quality and Standardization, Maejo University, Thailand, to analyze for residues of silver nanoparticles in the fruits using the ICP-OES technique. ICPMS is commonly used to quantify particles in environmental samples efficiently and precisely [25]. The report shows no detection of any silver particles in the Citrus fruits, as shown in Table 6.

Table 6. ICP-OES detection analysis of the Citrus fruits.

Test Item	Results	Unit	Method
Silver	Not found [1]	mg/kg	AOAC (2019), 999.10 detected by ICP-OES

Note: Limit of Detection (LOD) 0.01 mg/L.

3.5. Yield of Citrus Fruits

According to the present findings, the foliar application of a hybrid solution results in negative iodine test kits compared to the baseline (before treatment). The hybrid solution supports the growth of Citrus trees and enhances their fruiting ability. The plants began to exhibit more shooting shoot tips (terminal buds), young leaves, and flowers after ten days of treatment, and by twenty-five days, the plants had more shooting leaves and flowers. As shown in Figure 5, it was observed that the foliar application of hybrid solution enhances the plant height and shoot formation.

The results also show that the physical features of Citrus trees have changed. Figure 5 demonstrates how the same tree displays improvement in shooting leaves and flowering compared to prior treatment, and Figure 6 indicates that they are also exceedingly fruitful after the treatment. As per visual observations in Figure 5 the Citrus trees start shooting more leaves and flowering after treatment (Figure 5: 1–3) compared to before treatment (Figure 5: 4).

Figure 5. Shooting leaves and flowering after treatment (1–3) compared to before treatment (4).

Figure 6. Fruitfulness (red circle) of the Citrus tree after hybrid solution treatment.

Based on the percentage of incomplete Citrus fruits and the total yield in kilograms, it was determined from the Citrus harvest data in this experiment that the ampicillin treatment group's percentage of preliminary fruit was 25%. The fruit yield results are displayed in Table 7. The hybrid solution's percentage of incomplete fruits was 5% in both the trunk injection and foliar application groups. Citrus fruits' new yield (kg) was relatively improved with ampicillin treatment. The ampicillin yields 71.25 kg of Citrus, while the hybrid-solution-treated trees produce around 104.50 $\pm$ 4.56 kg of fruit. No significant difference was observed when the trunk or foliar application was injected with the hybrid solution. The results revealed that a hybrid solution enhances fruit yield and tree development.

Table 7. The yield of Citrus trees.

Criteria of Comparison	Ampicillin	AgNPs-COAMs	AgNPs-COAMs
Application method	Trunk injection	Trunk injection	Foliar application
Yield weight per Citrus tree (kg)	95	110	115
% Of incomplete fruit	25%	5%	5%
Net of Yield (kg)	71.25	104.50	109.25

4. Discussion

AgNP formulation is an alternative therapy for diseased trees in crop fields [8]. Hybrid solutions of AgNPs-COAMs were amalgamated using the cold plasma technique. A previous study suggested a unique approach for reducing supported Ag nanoparticles (AgNPs) using atmospheric pressure dielectric barrier discharge (DBD) cold plasma, devised without the need for any environmentally or physiologically dangerous reducing chemicals [26]. Cold plasma acoustic waves can cause cavitation, forming, growing, oscillating, and imploding bubbles inside the liquid solution. During the implosion of the bubbles, the locally high pressure and temperature can separate chitosan polymer into monomers or fragments. Secondly, these monomers or fragments in a liquid solution are exposed to cold plasma for subsequent polymerization. Cold plasma is a partially ionized gas comprising ions, electrons, radicals, etc. In this cold plasma, only the electrons hold relatively high temperatures (~1–10 eV), while the temperature of other plasma species is close to ambient temperature. The electrons, radicals, and ionized monomers in a cold plasma state are responsible for binding to larger molecules. In addition, the degree of polymerization tends to decrease as plasma pressure increases. For this reason, the atmospheric pressure (high pressure) plasma also advantageously promotes chitosan oligomerization [27].

The negative iodine test kit result confirms AgNPs-COAMs hybrid solution's ability to treat CGD. This may be because AgNPs in the hybrid solution demonstrate potential antiphytopathogenic effects and plant disease management, including fungi, bacteria, and viruses, via numerous mechanisms, including the positive charge of silver ions attached to the opposing charge cell wall of pathogens, which causes cell death [28]. The AgNPs solution displayed considerable antiadherence activity, metabolic passiveness, impaired membrane integrity, and time-dependent death of *R. solanacearum* at varying doses. Furthermore, when grown in AgNPs, the micromorphological features of both fungal hyphae and bacterial cells were altered. Previous study results indicate that such artificially generated AgNPs with sufficient antipathogenic potential could be a safe and efficient alternative to chemical control strategies used to manage phytopathogens with the potential for causing losses in agricultural production in various agroecological niches [29]. AgNPs can potentially be employed as antifungal agents and to control phytopathogens [30,31].

Nanoparticles made from organic and natural materials that are not harmful to the surroundings and promote the sustainable cultivation of crops have a promising future as a creative and effective plant biostimulant [32]. The preceding study reported that using AgNP increased leaf and bulb biomass, leaf greenness index, and bloom abundance. Furthermore, the plants treated with AgNPs began anthesis more promptly and had extended flower longevity, implying a longer decorative period, which is a top priority in floriculture [33]. Consequently, biofabricated AgNPs can be used as a stimulant for development in plant tissue cultures to improve micropropagation techniques. Furthermore, AgNPs enable the practical application of nanotechnology in plant sciences by developing regulated media components for in vitro growth [34]. According to an investigation, the AgNPs examined increased initial growth and biomass production in tomato plants and enhanced nutritional status, whereas germination did not change significantly within experimental circumstances. Although applying AgNPs reduced plant height, it increased root growth, root number, and the production of fresh and dry biomass of shoots and fresh biomass of fruits [35].

5. Conclusions

The hybrid solution of COAMs and AgNPs has been successfully synthesized as an antiphytopathogen product. Its molecule is 282.48 nm or 0.28 microns, allowing it to readily pass through the plant leaf's stomata size of 19.1–71.5 microns and enabling it to be used as a foliar application. The negative iodine test kit result confirms their ability to treat CGD. The synergistic antibacterial effects of AgNPs-COAMs were observed. It could be enhanced by conjugating COAMs to AgNPs, and one can increase the surface charge of those particles. This increases their ability to adhere to negatively charged bacterial cytoplasmic membranes and amplifies their electrostatic interactions. COAMs can enhance the bacterial absorption of AgNPs by acting as an antibacterial agent in multivalent binding. The outcome of the foliar application of the hybrid solution also reveals a notable increase in shoot length. The AgNPs-COAMs hybrid solution could produce growth stimulation due to the properties of COAMs. This hybrid approach might replace antibiotics for the treatment of CGD since antibiotics in field crops are limited by the emergence of microbial resistance, which also poses a severe risk to human health related to its indirect effects. This study's results suggest that the AgNPs-COAMs hybrid solution can be applied to other plant diseases that require further field research.

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Abbreviations

Citrus Greening Disease (CGD), Huanglongbing (HLB), Silver Nanoparticles (AgNPs), Chitooligomers and Monomers (COAMs), Completely Randomized Design (CRD).

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