Microbial Pathogenesis 115 (2018) 358–362

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Bactericidal effect of silver nanoparticles against propagation of *Clavibacter michiganensis* infection in *Lycopersicon esculentum* Mill

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ARTICLE INFO

Keywords:
Silver nanoparticles
Lycopersicon esculentum Mill
Clavibacter michiganensis

ABSTRACT

This study explored the use of silver nanoparticle as a bactericidal against the propagation of *Clavibacter michiganensis* onto tomatoes (*Lycopersicon esculentum* Mill). In Mexico, tomato production covers about 73% of the total vegetable production but it is affected by outbreak of bacteria canker caused by *Clavibacter michiganensis subspecies michiganensis* (Cmm). Silver ions possess inhibitor properties, bactericides and high specter antimicrobials. In this study, 6 groups of culture were prepared using 6 different petri dishes where silver nanoparticles of varying concentrations (120, 84, 48, 24, 12 and 0 μg) were added. Furthermore, each group was observed for 20 min, 1, 2, 12 and 24 h. The optimum concentration is 84 μg, which shows an average of 2 Cmm colonies after 20 min. Further increase to 120 μg shows no significant change. However, the average colonies was observed for 48 μg after 1, 2, 12, and 24 h. The obtained results indicate that silver nanoparticles are a promising inhibitor, bactericide and high a specter antimicrobial for treatment or prevention of Cmm.

1. Introduction

Drug-resistant bacteria are evolving pathogens with strong resistance profiles and their spread is difficult to contain, making a negative impact on plants and animals health and diseases [1–5]. *Clavibacter michiganensis* subsp. *michiganensis* (Smith), which causes bacterial canker; *Pseudomonas syringae pv.* tomato (Okabe) which causes bacterial speck; *Pseudomonas corrugata* which causes bacterial pith Necrosis; *Xanthomonas vesicatoria* which causes bacterial spot; and *Ralstonia solanacearum*, which causes bacterial wilt pose a serious and constant threat to glasshouse- and field-grown tomatoes (*Lycopersicon esculentum* Mill) [6]. This makes the bacterial diseases economically important worldwide. Bacterial canker caused by *Clavibacter michiganensis* subsp. *Michiganensis* Cmm. could lead to chlorosis, wilting, vascular infections and possibly death of the plant. It is difficult to control this due to lack of conventional resistant in tomato cultivars [7]. Moreover, antibiotics are not active against some pathogens, and they are forbidden in several countries because they are questionable for a number of reasons [8,9].

Presently, the main measures employed to mitigate these diseases is the by using Copper treatments [10] and suitable agronomic practices like fertilization, irrigation and seed certification. However, it is difficult to alleviate their damage because variable colonization schemes and environmental factors play a vital role in the spread of phytobacteria on tomato crops, having no effective precautionary measures [11]. Recently a number of natural substances are suggested but further studies are required to establish and optimize their efficacy [3–5].

The use of nanotechnology helps in exploring the biological properties of previously established bactericidals by controlling their size to boost the performance. For decades, Silver has been known for its bactericidal effect, but the development of antibiotics declined its medical applications. Presently, World Health Organization listed silver sulfadiazine as a vital anti-infective topical medicine [12,13]. The use of silver nanoparticle could be promising since bulk silver is effective.

Several authors have confirmed the bactericidal effect of silver nanoparticles against Gram positive and Gram negative bacteria,

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https://doi.org/10.1016/j.micpath.2017.12.075
Received 12 December 2017; Received in revised form 26 December 2017; Accepted 30 December 2017
Available online 03 January 2018
0882-4010/

Table 1
Experimental design to test Nano silver effect in *Clavibacter*.

Nano-Ag dilution from 12 mg/ml		1			2			3			4			5			6		
		$1/1 \times 10^{-2}$			$1/1 \times 10^{-3}$			$1/1 \times 10^{-4}$			$1/1 \times 10^{-5}$			$1/1 \times 10^{-6}$			Control negative		
		120 µg/ml			12 µg/ml			1.2 µg/ml			120 ng/ml			12 ng/ml			0 ng/ml		
	Time	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3	R1	R2	R3
A	20 min	1			2			3			4			5			6		
B	1 h	7			8			9			10			11			12		
C	2 h	13			14			15			16			17			18		
D	12 h	19			20			21			22			23			24		
E	24 d	25			26			27			28			29			30		

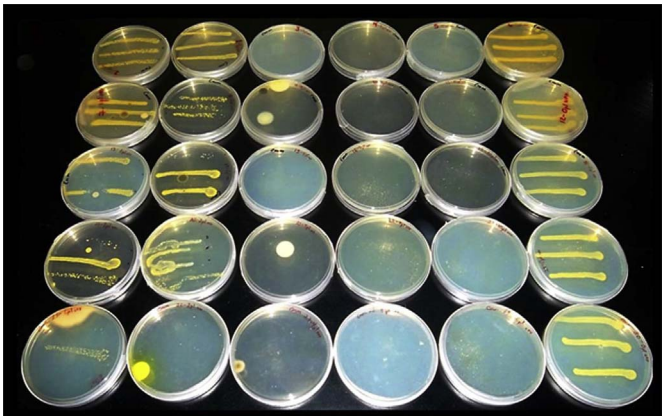

Fig. 1. Petri dishes supplemented with *Cmm* and then incubated with silver nanoparticle (6 groups, representing 6 different concentrations of silver nanoparticles (120, 84, 48, 24, 12 and 0 µg) with 5 crop dishes each, representing incubation periods (20 min, 1, 2, 12 and 24 h)).

however, a distinct elucidation of the bactericidal mechanism is needed [13–15]. Lara et al. [12] investigated the effect of silver nanoparticles suspension against different clinically important drug-resistant pathogens (multidrug-resistant *Pseudomonas aeruginosa*, ampicillin resistant *Escherichia coli* O157:H7 and erythromycin resistant *Streptococcus pyogenes*) using luciferase-based assay. They reported that both drug-susceptible and drug-resistant bacteria (Gram negative and Gram positive) are vulnerable to silver nanoparticle treatment, and the antibacterial activity follows bactericidal mechanism rather than bacteriostatic. Immediately after the contact between the nanoparticles and the bacteria was established, the bacterial growth rate was inhibited. The report of Verma and Mehata [16] confirms the effectiveness if silver nanoparticle on *Escherichia coli*. Previously, other researchers have used silver nanoparticles against Gram positive bacteria like *Staphylococcus aureus* [17–19]. Moreover, the antiviral ability of silver nanoparticles against type hepatitis B virus and human immunodeficiency virus (HIV-1) - [20,21] has been proven. Therefore, this study explored the use of silver nanoparticle as a bactericidal against the propagation of Cmm. The effect of dosage on the antimicrobial activity was also investigated.

2. Material and methods

Three g/L of Difco Luria Broth Base Miller (241420) was added to 0.4 g of Na_2HPO_4-H_2O (*disodium phosphate*) and 0.50 mg of KH_2PO_4 (monophasic potassium phosphate) for a total medium of 200 ml for the culture of *Cmm* and sterilized at 28 °C in a pressure cooker to simulate autoclave function.

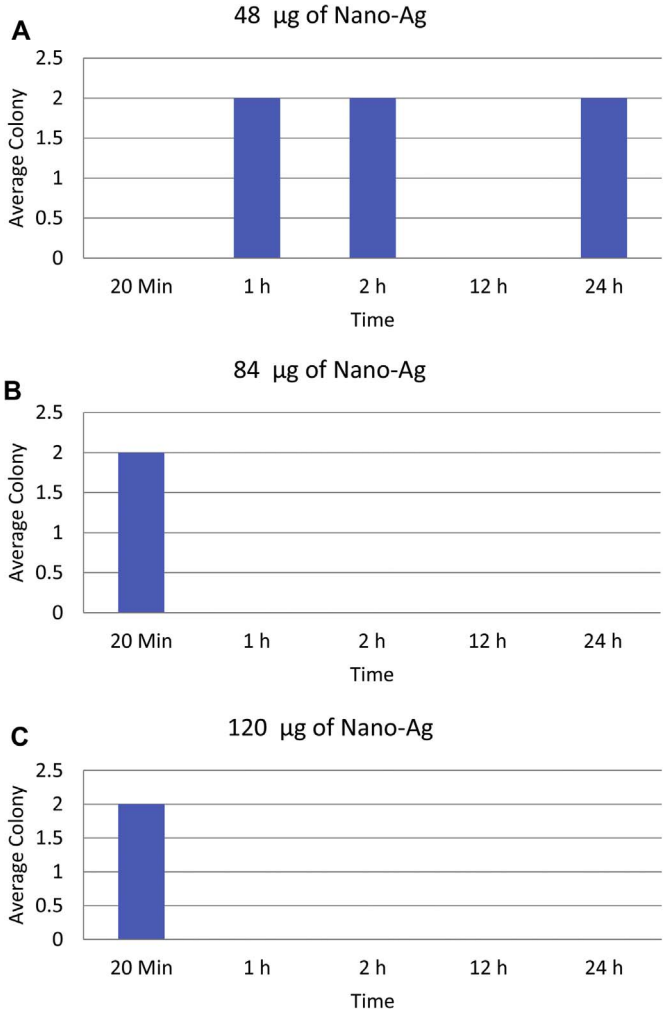

Fig. 2. Average colony against time.

2.1. Inoculation of culture medium for Cmm

The inoculation of *Cmm* was performed using two lighters in a medium totally closed to prevent wind currents, and with the disinfection and cleaning of the place with ethylic alcohol desnaturated (Ethyl Alcohol Desnatured, A4075-4). From the jar with media of 200 ml, 20 ml was extracted into a 50 ml Falcon tube of, which was used as a control. The rest of media (180 ml) was added less of 5 ml of Cmm and was immediately incubated at 25 °C in an incubator (ECHO-Therm in 40 Chilling Incubator) for two days.

Then its continue with the extraction of 2 ml of control and 2 ml of

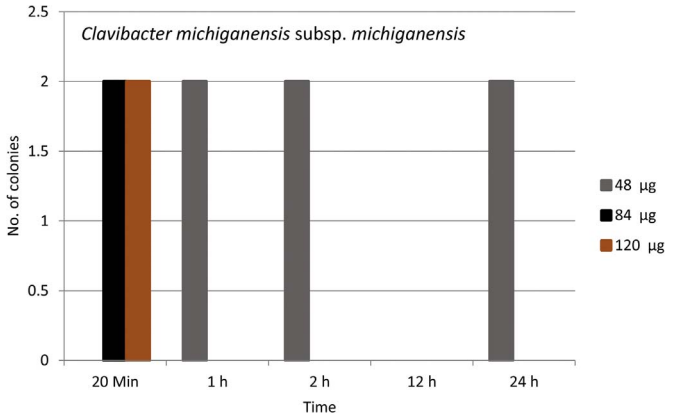

Fig. 3. Results obtained in the best three concentrations of silver nanoparticles (120, 84 and 48 µg).

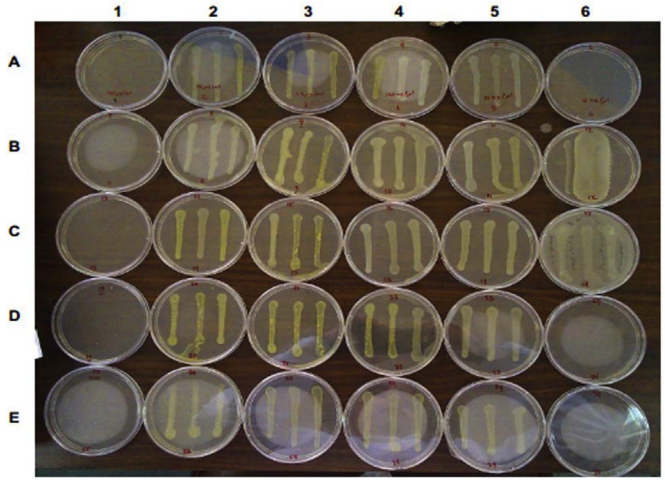

Fig. 4. Crops of the three best concentrations found in this study of silver nanoparticles (120, 84 and 48 µg), with a maximum two copies each, but with better effectiveness in time for the concentrations used.

Cmm cultivated previously, each one collocated in it respective bucket *(*Zuzi*)* and were cover immediately with parafilm paper to avoid contamination. Each bucket was analyzed into a spectrophotometer (Elyptica, Ely-2000*)* to determine the number of bacteria present after 2 days of inoculation. The parameters for the spectrophotometer are wave longitude of 600 nm, maximum value and minimum of 100 and 1, respectively and it was adjusted at a temperature of 33 °C. After three consecutive days, the bacteria number was obtained using spectrophotometer considering the parameters previously mentioned with an optic density of 5×10^8, The observations are 67 million, 78 million and 78, 500, 000 million bacteria of Cmm.

2.2. Agar media

1000 ml of media NYB modified (*NBYm)* and NBY-agar, 15.5 g/L of Difco Luria Broth Miller Base (241420), 2 g of Na_2HPO_4-H_2O (*disodium phosphate*) and 250 mg of KH_2PO_4 (monophasic potassium phosphate) and 15 g of agar were prepared. The solution was emptied into 30 petri dishes (VWR-Polyester*)* under a laminar flow.

2.3. Experimental model

A total of 30 petri dishes were used, including 6 groups with 5 dishes each, where the groups were assigned different concentrations of silver nanoparticle: 120, 84, 48, 24, 12 and 0 µg. Furthermore, each concentration was subjected to 20 min, 1, 2, 12 and 24 h incubation (see Table 1).

2.4. Gen sequence

Once isolated and the crops are morphologically identified, they were sent to the University of California at San Diego, for the determination of their sequence to know the identity of the crops. It was observed that the crop only has two or three punctual mutations, which arecorroborated in the chromatogram, but more than 99% identical to the sequences of *C. michiganensis* reported in the Genbank. The following is the sequence of the gen obtained from the crop of *C. michiganensis*:

GAGCTTGCTCTGGCGGATCAGTGGCGAACG GGTGAGTAACACGT GAGTAACCTGCCCCCG ACTCTGGGATAACTGCTAGAAATGGTAGCT AATACCGGATATGACGATTGGCCGCATGGT CTGGTCGTGGAAAGAAT TTCGGTTGGGGAT GGACTCGCGGCCTATCAGGTTGTTGGTGAG GTAA TGGCTCACCAAGCCTACGACGGGTAG CCGGCCTGAGAGGGTGACCGG CCACACTGG GACTGAGACACGGCCCAGACTCCTACGGGA GGCAGCA GTGGGGAATATTGCACAATGGGC GAAAGCCTGATGCAGCAACGCCGC GTGAGG GATGACGGCCTTCGGGTTGTAAACCTCTTT TAGTAGGGAAG AAGCGAAAGTGACGGTACC TGCAGAAAAAGCACCGGCTAACTACGT GCC AGCAGCCGCGGTAATACGTAGGGTGCAAGC GTTGTCCGGAATTA TTGGGCGTAAAGAGCT CGTAGGCGGTTTGTCGCGTCTGCTGTGAAA TCCCGAGGCTCAACCTCGGGTCTGCAGTGG GTACGGGCAGACTAGAG TGCGGTAGGGGAG ATTGGAATTCCTGGTGTAGCGGTGGAATGC GCA GATATCAGGAGGAACACCGATGGCGAA GGCAGATCTCTGGGCCGTAA CTGACGCTGA GGAGCGAAAGCATGGGGAGCGAACAGGATT AGATAC CCTGGTAGTCCATGCCGTAAACGT TGGGAACTAGATGTGGGGACCAT TCCACGG TCTCCGTGTCGCAGCTAACGCATTAAGTTC CCCGCCTGGG GAGTACGGCCGCAAGGCTAA AACTCAAAGGAATTGACGGGGGCCCGC ACA AGCGGCGGAGCATGCGGATTAATTCGATGC AACGCGAAGAACC TTACCAAGGCTTGACAT ATACCGGAAACATGCAGAAATGTGTGCCCC GCAAGGTCGGTATACAGGTGGTGCATGGTT GTCGTCAGCTCGTGTCG TGAGATGTTGGGT TAAGTCCCGCAACGAGCGCAACCCTCGTTC TATG TTGCCAGCACGTAATGGTGGGAACTC ATAGGAGACTGCCGGGGTCAA CTCGGAGGA AGGTGGGGATGACGTCAAATCATCATGCCC CTTATGTC TTGGGCTTCACGCATGCTACAA TGGCCGGTACAAAGGGCTGCGATAC CGTAA TGTGGAGCGAATCCCAAAAAGCCGGTCTCA GTTCGGATTGAG GTCTGCAACTC.

Description: Clean sequence of *C. michiganensis* gen reported in Genbank.

3. Results and discussion

In the first phase of the results, it was observed that the 30 petri dishes in 6 groups with 5 crop dishes with the times and micrograms are mentioned before in the methodology (Table 1), (Fig. 1). The results obtained for different times at the different concentrations applied are shown in Figs. 2 and 3. In the second phase of the results, 30 petri dishes, in 6 groups with 5 cultured dishes with the times and micrograms were also used (Figs. 1 and 4).

All this by the culture of 6 groups into 6 petri dishes, where the concentrations of silver nanoparticles on 120, 84, 48, 24, 12 and 0 µg were added, and each concentration was subjected to different incubation time of 20 min, 1, 2, 12 and 24 h. The obtained results can be consider like an option to treat or prevent the damages caused by Cmm.

The production of tomatoes (*Lycopersicon esculentum,* Mill.) in Mexico accounts for 73% of the production of vegetables, but it was affected by the appearance of bacteria canker caused by Cmm. The symptomatology of this infection is the marginal withering of the leaflets and can appears at any age of the plant, generating circular necrotic spots, which looks round like a halo of white color. The use of silver ions has been reported to exhibit inhibitor properties, bactericides and high specter antimicrobials.

In this study, the average ratio of the smallest bactericidal dosage to the lowest inhibitory concentration showed that silver nanoparticles

exhibit a bactericidal rather than bacteriostatic effect on bacteria canker. Typically, bactericidal agents are clinically preferred because killing of bacterial is expected to produce a quicker technique to eliminate the infection, enhance clinical results, and minimize the possible emergence of resistance and the spread of infection. If pathogens are eliminated rather than inhibited, the resistance mutations that could occur due to antibiotic pressure are eradicated [22].

The observed differences in bactericidal activity found among the different compared groups (drug-resistant vs. susceptible, Gram positive vs. negative) are insignificant, which shows that silver nanoparticles are broad-spectrum antibacterial agents. These observations corroborates with previous reports by other researchers. They proved that silver nanoparticles exert a similar effect on Gram positive and Gram negative strains pathogenic bacteria [23,24]. Shrivastava et al. [25] suggested that Gram-negative bacteria are less vulnerable to silver nanoparticles due to the presence of positive charges on silver nanoparticles, which relate to the Gram negative lipopolysaccharide with a greater affinity versus the Gram positive cellular wall. This is assumed to have a fewer positively charged active sites [25].

On the other hand, in our results *Clivibacter michiganensis* infection in *Lycopersicon esculentum* Mill was less vulnerable (though insignificant) to silver nanoparticles. Therefore, the presence of lipopolysaccharide may not be responsible for a higher receptivity of the bacterial cells to the bactericidal activity of silver nanoparticles. Rather, it is possible that lipopolysaccharide trapped and blocked the positive charges of silver nanoparticles, making the Gram-negative bacteria less susceptible. Definitely, silver nanoparticles anchored on the cell membrane surface penetrate the bacteria, restricts its function, and release the silver ions. Lok et al. [26], Sondi and Salopek-Sondi [27] reported that silver nanoparticles try to locate the bacterial membrane, thereby dissipating the proton motive force. Thus, for silver nanoparticles to exhibit antibacterial effect, the cell membrane must be targeted.

Prominent of the factors responsible for bacteria infectivity is their ability to rapidly reproduce, an attribute that is capable of preventing a viable infection. As presented by time-kill assays, silver nanoparticles were effective towards preventing bacterial growth in a time and dosage dependent manner.

Our results indicate that silver nanoparticles exhibit a similar mode of action to that of silver ions, by forming a complex with electron donor groups, which contain nitrogen, oxygen or sulfur atoms that are normally present as thiols or phosphates [28] on amino acids and nucleic acids. Similar to silver nanoparticles, silver ions also exhibit a broad range of mechanisms, which include denaturing of the 30s ribosome subunit, subduing the proteins and preventing respiratory enzymes, enzymes expression essential for ATP production [29]. This helps to induce reactive oxygen species production [29,30], disrupting and destabilizing the outer membrane [26], and dimerizing and binding DNA and RNA [31].

Silver ion-resistant strains are reported to undergo modification in the response to antibiotics, like the acquisition of resistance to sulfonamides, ampicillin, tetracycline, chloramphenicol, streptomycin and mercuric chloride [32].

Chopra [32] reported that the instability of phenotype is capable of reflecting reversions of the chromosomal mutations engendering silver resistance, particularly if they enforce fitness costs, or probably demonstrate loss of plasmids encoding resistance [32]. In addition to their instantaneous antibacterial effect against various bacteria (that are drug-resistant) and bactericidal effect, silver nanoparticles exhibit certain characteristics offered by the silver in general. This is because noble metal induces low bacterial resistance [33] and exhibits minimal side effects and low toxicity when consumed since ~2–4% is retained in the tissues after ingestion. A prominent health effect is argyria, an irremediable skin pigmentation that is mainly aesthetic concern [34].

The bactericidal effect of silver nanoparticles against bacteria that are multidrug-resistant could be employed together with polymer technology and advances in impregnation method to broaden the range of utilization of silver nanoparticles in disinfection of medical equipment and supplies, preservation of food, and surface decontamination of items like kitchenware and toys [29].

4. Conclusions

This study investigates the bactericidal effect of silver nanoparticles dosage against propagation of *Clivibacter michiganensis* infection in *Lycopersicon esculentum* Mill. The best concentrations is 84 μg at a minimum incubation time of 20 min, however the concentration of 48 μg only obtained two colonies with time instability. Further, increase in the concentration to 120 μg yield no significant effect. Therefore, silver nanoparticle is a promising inhibitor, bactericide and high specter antimicrobial for treatment or prevention of Cmm. The results suggest that silver nanoparticle is an effective biocide against *Clavibacter michiganensis* as drug-resistant bacteria, which makes it a potential candidate for use in pharmaceutical products and medical devices that may help to prevent the transmission of drug-resistant pathogens.

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