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Article

Silver armor against bacteria: A battle of antimicrobial effectiveness

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SUMMARY

With an estimated one billion dollars loss in the US each year, pathogenic bacteria cause devastating losses in fruit tree orchards, vineyards, and food crops. Treatments rely on five major antibiotics, out of these the most common treatments used world-wide to suppress plant pathogenic bacteria include: streptomycin, oxytetracycline, kasugamycin, oxolinic acid and gentamicin. However, continuous applications of antibiotics to plants exert selective pressure on plant-associated bacteria, leading to the development of antibiotic resistance. This has given rise to the development of treatments using bactericidal nano-metals, such as Copper, Zinc, Magnesium and Silver Therefore, this study evaluates if a low-voltage Do-It-Yourself (DIY) method will produce silver solutions with antibacterial activity. We hypothesize that DIY colloidal silver at 3 ppm will inhibit the growth of Gram-negative crop pathogens (e.g., Serratia marcescens) and Gram-positive pathogens (e.g., Bacillus cereus) more effectively than water, but less than the commercial AgNP at 1,000 ppm. Treatment effects were compared to a positive control of 1,000 ppm silver nanoparticles via the Kirby-Baur method. This is a commercially available silver solution known to kill bacteria species. AgNP treatments were: 1,000 ppm AgNP (positive control); 5 ppm AgNP, 3 ppm AgNP; and negative blank water control. The efficacy was compared across three Gram-negative and two Gram-positive bacteria species. The positive control solution, 1,000 ppm AgNP, was the most effective bactericide across all bacteria species, interestingly the 5 ppm treatment produced zones of clearance half the size of the positive control for two of the Gram-negative species. Activity at 5 ppm suggests that simple DIY systems can produce effective low-cost, bactericidal nano-silver solutions. Further improvements of DIY methods may provide low-cost treatment solutions against some bacteria species.

INTRODUCTION

Increasing antibiotic resistance in healthcare settings is making Gram-negative bacterial species more virulent (1). For example, *Serratia marcescens* (*S. marcescens*) a Gram-negative pathogen that causes black rot in citrus and other agricultural diseases, has developed antibiotic resistance (2, 3). Similarly, approximately 75% of *Branhamella catarrhalis* (*B. catarrhalis*) strains produce β-lactamase that cleaves the β-lactam ring of β-lactam antibiotics thereby blocking entry of antibiotics into the bacteria cell inactivating drug treatments, become resistant to penicillin and its derivatives (4).

Gram-negative species have a second outer membrane which functions as a permeability barrier that limits the entry of many current antibiotics making them more resistant to antibiotic treatments (5). However, AgNPs are less effective on Gram-positive bacteria due to increased peptidoglycan in the cell wall, while Gram-negative bacteria's lipopolysaccharide layer attracts the positively charged AGNPs (6). In contrast to common antibiotic treatments, silver nanoparticles (AgNPs) enter bacterial cells through porin proteins, accumulate due to limited export mechanisms, and subsequently damage cell membranes (6–8). Toxic silver concentrations often hinder protein synthesis via ribosomal decay (8). Manufacturers use these properties in commercial colloidal silver products, such as health supplements and cleaning products, and to coat the surfaces of many household appliances and materials (9).

Silver nanoparticles (AgNPs) can impair DNA and RNA function by causing oxidative stress and damage, which occur due to toxicity at high concentrations (>1280 ppm) (6, 10). Researchers have successfully used AgNPs to treat Gram-negative bacterial infections (10). For example, AgNPs successfully suppressed the Gram-negative bacterium *Candidatus Liberibacter asiaticus*, an alphaproteobacterium that causes Huanglongbing disease in citrus trees (11). The efficacy of bactericidal effects depends on AgNPs' size, shape, and concentration (8, 9, 12). Studies have reported that smaller AgNPs (< 50 nm diameters) have greater activity, with both positive and negative effects in plant treatments (14, 15).

Researchers who develop nanosilver treatments and products continue to demonstrate significant suppression of many bacterial pathogens (9, 16–18). In this study, we evaluated a Do-It-Yourself (DIY) colloidal silver solution as a cost-effective antibacterial treatment. We developed a method for running a current through common silver jewelry to isolate AgNPs and then compared the antibacterial effects of this accessible AgNP treatment to expensive commercial products. We hypothesized that a low-voltage DIY-produced colloidal silver solution would have antibacterial activity.

AgNP antimicrobial activity involves four steps: (i) adhesion of AgNPs to the cell wall or membrane and its disruption; (ii) intracellular penetration and damage; (iii) oxidative stress; and (iv) modulation of signal transduction pathways (7, 8). Bacteria are more susceptible to the antibacterial effects of smaller-sized AgNPs (5 nm to 20 nm) (19–23), which are effective at low concentrations (5 ppm to 50 ppm) (24–26).

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When researchers use a voltage from 20 V to 30 V to create colloidal silver, they produce smaller-sized particles when the anodizing time was less than 4 hours (27). Therefore, low-voltage DIY systems can produce average AgNPs sizes between 43 nm–85 nm by limiting anodizing production time to between 2 and 4 hours at 20 V (27).

This study used the Kirby-Bauer test to compare the effects of AgNP treatments on two Gram-positive species (*Bacillus cereus* and *Micrococcus luteus*) and three Gram-negative species (*Branhamella catarrhalis, Rhodospirillum rubrum,* and *Serratia marcescens*). The Kirby-Bauer assay evaluates a treatment's bactericidal effects by visualizing zones of clearance (ZOC). Our results supported the hypothesis that a low-voltage DIY-system could produce AgNP solutions with antibacterial activity. Specifically, the 5 ppm treatment produced ZOC about half as great as the positive control AgNP 1,000 ppm treatment on two of the bacteria species tested.

RESULTS

The commercially available AgNP solution had the silver nanoparticle size confirmed using transmission electron microscopy (**Figure 1**). The antimicrobial activity against Gram-positive and Gram-negative species was tested using the Kirby-Bauer test, comparing the effects of the positive control with the DIY silver nanoparticle (AgNP) treatments. Within each bacterial species the zone-of-clearance (ZOC) of the positive control, the 1,000 ppm treatment, was significantly greater than all other treatments, followed by the 5ppm treatment (**Figure 2** and **Figure 3**).

The Kirby-Bauer test showed bacteria inhibition induced by DIY AgNP on Gram-positive species *Bacillus cereus*, (*B. cereus*) with the control 1,000 ppm (ZOC = 18.20 mm). The 5 ppm treatment (ZOC = 2.50 mm) was significantly greater than both the 3 ppm treatment (p = 0.19331, ZOC = 0.0 mm) and distilled water (p = 0.9, ZOC= 0.0 mm) (ANOVA: $F(3,80) = 53.3$, $p < 0.01$, post-hoc comparison of means using Tukey test $p \leq 0.05$). Similarly, for *Micrococcus luteus* (*M. luteus*), the 1,000 ppm (ZOC = 18.50 mm), with the ZOC for 5 ppm (ZOC = 2.50 mm) and the 3 ppm (ZOC = 2.20 mm) being statistically different from distilled water ($p > 0.1$, ZOC = 0.19 mm) (ANOVA: $F(3,80) = 14.9$, $p < 0.05$, post-hoc comparison of means using Tukey test ($p \leq 0.05$)

The DIY produced AgNP showed antimicrobial activity on four of the five bacteria species. For *Branhamella catarrhalis*, the ZOC was significantly greater for the 1,000 ppm (ZOC = 27.20 mm). The 5 ppm treatment (ZOC = 14.88 mm) was significantly greater compared to the 3 ppm treatment (ZOC = 3.13 mm), and to distilled water (ZOC = 0.0 mm) (ANOVA: $F(3,80) = 53.3$, $p < 0.05$, post-hoc comparison of means using Tukey test ($p \leq 0.05$) *Similarly for Rhodospirillum rubrum* (*R. rubrum)*, the ZOC was significantly greater for 1,000 ppm treatment (ZOC = 7.5 mm) and the 5 ppm treatment (ZOC = 5.10) mm) ($p < 0.05$) to all other treatments (3 ppm treatment, p = 0.3) and distilled water (ZOC = 0.00, p = 0.5) (ANOVA: $F(3,80) = 1.6$, $p < 0.05$, post-hoc comparison of means using Tukey test ($p \leq 0.05$)). In contrast for *S. marcescens*, the 1,000 ppm treatment (ZOC=16.37 mm) was statistically significantly different (ANOVA: $F(3,80) = 235.1$, $p<0.001$), from all other treatments which were zero. Except for *S. marcescens*, all other bacteria in the 5 ppm treatment produced significantly greater ZOC compared to the 3 ppm treatment.

DISCUSSION

For real-world applications, AgNP solutions are effective because their small diameter produces antibacterial activity. Our study results indicate that the positive control AgNP, which had small (10 nm), spherical, citrate-stabilized nanoparticles, was most effective at producing a greater zone of clearance (ZOC) and suppressing bacteria for 72 hours after treatment in all tested bacteria. In our study, AgNP solutions at concentrations greater than 5 ppm had significant antibacterial effects against Gram-negative species (*B. catarrhalis* and *S. marcescens*). This finding is significant because Gram-negative bacteria are often more resistant to antibacterial products and antibiotics in healthcare settings (5). Researchers have also shown that AgNP treatments on citrus trees are effective against the Gram-negative bacterium *Candidatus Liberibacter asiaticus*, which severely threatens global citrus production (11, 28). To better understand the properties of AgNP treatments, we need to use other microbial techniques, such as a minimum inhibitory concentration assay, or study the mechanism of changes in bacteria concentration. Nanoparticles smaller than 100 nm have both a high surface area to volume ratio and a high dispersion rate (29, 30). These properties make nanosilver colloids suitable for use as an antibacterial agent in bandages, clothing, materials, and antiseptic sprays (31, 32–34).

Differences reported in nanosilver activity, including in commercial products, arise from variations in the concentration of small-diameter AgNP (< 50 nm) or differences in agglomeration, which affects efficacy by altering the size, shape, or stability of the AgNP (6, 7, 8, 15, 17, 18, 20). Furthermore, unknown proprietary blends or compositions—such as surfactants, stabilizers, and adjuvants—in each formulation may contribute to the observed differences in bacteriostatic or bactericidal effects (6, 35, 36). While concerns over the use of silver in various products affecting environmental microbial communities are increasing, studies have shown that silver

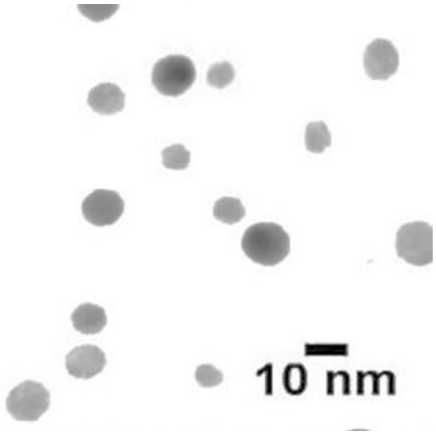

Figure 1: Transmission Electron Micrograph showing average 10 nm diameter size of the silver nanospheres in the positive control solution (1,000 ppm). Five microliters of undiluted stock AgNP 1,000 ppm solution were deposited on a formvar coated grid, 200 mesh for two minutes. The grid was dried overnight at 50°C in a drying oven. The sample was visualized using the STEM Model S-4800, Hitachi, conditions for imaging were set to 25 kV on TEM/SEM view (USDA, ARS, Electron Microscopy Laboratory, Ft. Pierce, FL).

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ions rapidly form less toxic, insoluble salts in natural environments (37, 38). Like many trace metals, silver ions become immobilized in the soil when they encounter organic matter, clays, and metal oxides, as they form complex molecules (38, 39).

The outer lipopolysaccharide membrane, which is unique to Gram-negative bacteria, serves as an effective defense mechanism against many antibiotics. However, the lipopolysaccharide layer is negatively charged which attracts the positive charged AgNPs' to the outer membrane, where it causes damage. The increased permeability makes Gram-negative bacteria susceptible to nanometal treatments (4, 8, 40). Previous reports showed that AgNP smaller than 100 nm have improved bactericidal activity (34). A primary challenge in producing effective colloidal AgNP solutions is the tendency for high agglomeration, which forms particle assemblies of various shapes and sizes and results in larger-diameter nanoparticles with larger surface areas (29, 30, 32, 33, 34, 41, 42). To address the problem of agglomeration and its effect on particle size, commercial solutions use stabilizing agents (21). For instance, researchers commonly accept that smaller nanoparticles are more active and that bacteria are more susceptible to the antibacterial effects of smaller (10–20 nm) AgNP (19–22) (**Figure 1**). Our study supports previous research that demonstrate the size-dependent activity of AgNP on microbes (6, 29, 32, 34).

A limited review comparing retail colloidal silver products to DIY solutions revealed that the homemade colloidal AgNP solution at a concentration of 5 ppm is approximately 50% lower in cost than commercial alternatives of the same concentration and quantity ($45--70 USD). However, researchers did not evaluate another important factor: the longevity or persistence of the DIY AgNP treatment after application. The activity and persistence will significantly impact cost-benefit analyses. If DIY-produced AgNP solutions are to become a cost-effective treatment, they will need to address the problem of unwanted agglomeration during production. Commercial companies have solved this issue by developing stabilizing agents to prevent agglomeration. Common stabilizing agents that prevent nanoparticle agglomeration include polymers like polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP), as well as surfactants such as sodium dodecyl sulfate (SDS) and polyoxyethylene (20) sorbitan monolaurate (Tween 20) (41). These molecules introduce enough repulsive forces to counteract the AgNPs' thermodynamic attraction (43). One widely used solution involves using capping agents, such as citrate, during nanoparticle synthesis (30). Citrate-capped AgNP are the most widely used form in research because citrate is readily available, low cost, considered safe, and has a strong affinity for silver (44, 45).

In conclusion, our study's results demonstrate the bactericidal activity of AgNP solutions produced using a low-voltage DIY system. Many companies are already commercializing colloidal silver solutions to target bacterial pathogens especially in healthcare settings, antimicrobial silver is expanding its therapeutic applications to reduce bacterial resistance (9, 46, 47 48). Researchers have recently begun to investigate silver applications for crops. For new citrus pathogens, researchers can develop silver solutions as an effective disease management treatment (37). An increasing number of research reports support developing AgNP for silver antibacterial treatments, with over 1084 registered products

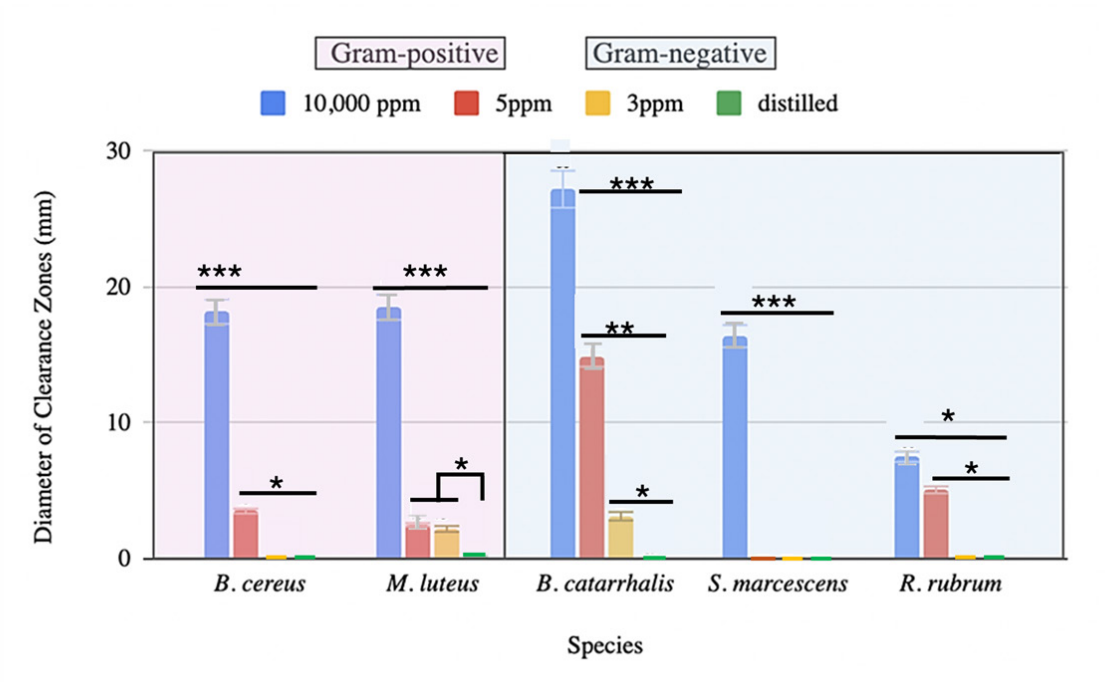

Figure 2: Zones of clearance (ZOC) within treatments for all five bacteria species at 24 hours after treated. Background colors (Pink) Gram-positive bacteria (Left) and (Blue) Gram-negative bacteria (Right). Blue bars are positive control 1,000 ppm AgNP, significant from all other treatments. The red bars are the 5 ppm AgNP treatment. The yellow bar indicates 3 ppm AgNP and the green bar indicates distilled water control (no effect). ANOVA One-way T-test and Tukey's HSD Test used to determine statistical difference between treatment means within each bacteria species. Each bar displays the mean and standard errors. *** ($p \leq 0.001$); ** ($p \leq 0.01$); * ($p \leq 0.05$).

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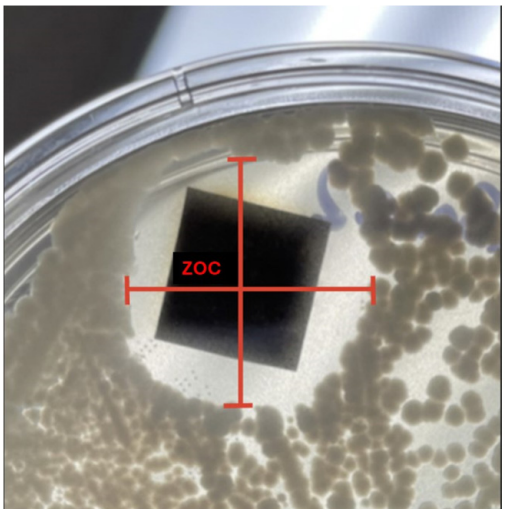

Figure 3: Example of measurement of the Zones of Clearance, ZOC, 24 hours after treatment on *Micrococcus luteus*. Shown is positive control Treatment-1, 1,000 ppm AgNp. Each paper square diameter was measured and used to calculate the difference in ZOC beyond the treated paper square.

that target microbial pathogens of humans and agricultural livestock and crops (48). Future studies should determine the antimicrobial properties of AgNP treatments for more bacteria species to clarify their application in healthcare and agricultural settings. Furthermore, exploring how to improve the production efficiency of smaller, more stable AgNP that exhibit less agglomeration will significantly advance the adoption of AgNP in agriculture. The development of low-cost production systems (homemade or DIY) combined with emerging plant-based capping have potential to become an essential part of global sustainable agriculture.

METHODS

Bacteria Cultures

Five bacteria species were purchased as plate cultures from the BSL-1 kit from Carolina Biological Supply (Item #154615). Gram-negative cultures were the following: *Branhamella catarrhalis*, *Serratia marcescens* and *Rhodosprillum rubrum*. Gram-positive cultures were the following: *Bacillus cereus* and *Micrococcus luteus*. Nutrient agar plates were provided in the kit. The experiment had five biological replicates; each culture was grown from a single colony to account for natural variability. Individual colonies were picked using sterile toothpicks and each individual colony was mixed in 0.5mL nutrient media (provided in kit: Nutrient agar is a general-purpose solid medium supporting growth of a wide range of non-fastidious organisms. It typically contains (mass/volume): 0.5% peptone – this provides organic nitrogen. 0.3% beef extract/yeast extract – the water-soluble content of these contributes vitamins, carbohydrates, nitrogen, and salts). These tube cultures were grown overnight at 35˚C in an incubator. The individual tubes of each culture were vortexed for 10 seconds prior to use; a sterile plastic loop was dipped once into each colony which was streaked onto plates to produce the bacterial lawns to be tested. The sterilized, blot paper squares treated with AgNP, 4 per plate, were added and the plates were sealed, dated, and kept at room temperature (approximately 22 -25˚C).

Kirby-Baur Method

Using 60 nutrient agar petri dishes in four separate trials, the Kirby-Bauer method was used to determine bacterial suppression across treatments. Each plate was streaked with one of the five bacterial species (*B. cereus, B. catarrhalis, M. luteus, R. rubrum, S marcescens*). Squares (2x2) of sterilized paper (Profile, Louisville, Kentucky, 177˚C) were saturated with each treatment of nanosilver solution. Each petri dish was then divided into four quadrants, and a treatment square was placed in each. The four treatments: 1,000 ppm (positive control), Treatment-2, 5ppm, Treatment-3, 3ppm, and Treatment-4, distilled water (negative control). Each of the four treatments were placed on each plate.
After 24, 48, and 72 hours, the ZOC around each treatment square were measured and averaged in millimeters, through the diameter of each side looking through the plastic lids of plates.

ZOC Testing

Petri dishes were treated with bacterial species. The plates were then treated with all four silver treatments via the Kirby-Baur Method. The Petri dishes were incubated at 33 ˚C for 24 hours. The Zone of Clearance, ZOC, around each treatment square was recorded in millimeters. Plates were incubated for another 24 hours at room temperatures, approx. 22-25 ˚C. The ZOC were quantified using two measurements across perpendicular diameters.

Treatments The experiment had five biological replicates, each grown from a separate bacterial culture to account for natural variability. Bacteria were cultured overnight at 35 ˚C in an incubator. The overnight cultures in each trial started from a single colony and were streaked on nutrient agar plates.

Transmission Electron Microscopy (TEM)

Five microliters of undiluted stock AgNP 1,000 ppm solution were deposited on formvar coated grids, 200 mesh, for 1 min (Lacey F/C 200 mesh Au, Cat No. 01882G, Ted Pella, Inc., (49). Grids were then sequentially rinsed 3 times by dipping 30 times into 20 mL deionized, syringed-filtered water (0.2 µm, Part No. 431219, Corning®, NY 14831). Excess water was removed using lab wipes. Grids were placed in an open glass petri dish and dried overnight at 50°C in a drying oven (Fisher Econotemp™, lab oven model 15G). For imaging, grids were mounted in specimen holders specific to TEM. Conditions for imaging were set to 25 kV on TEM/SEM view using a STEM Model S-4800 (Hitachi High Technologies America, Inc, Pleasanton, CA, USA), contrast and focus were adjusted occasionally according to quality of images (**Figure 1**).

Colloidal Silver

The U.S. Department of Agriculture provided the stock silver solution. (10 nm diameter Silver Nanospheres, 1,000 ppm. Aqueous, 2 mM Sodium Citrate. (nanoComposix, Cat. No.: SKU: AGCN10-50M). For the DIY-Colloidal silver, AgNP were produced starting with a silver jewelry chain (98% Silver, 9 Inches, Spain) and two U.S. quarters (1963) (50). The four treatments were: 1,000 ppm AgNP (positive control) ; 5ppm AgNp, 3ppm AgNp; and negative control distilled water. In brief required materials included: alligator clips, eight 9-volt batteries, glass jars, and distilled water (Pure Life™, Zephyrhills, Florida). Silver diameter and concentrations follow

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previous studies that have shown antibacterial effects with small diameter AgNP (8 - 50 nm diameters) at concentrations between 4 ppm - 20 ppm (6, 39; 24; 25; 26, 48).

Statistical Analysis

The data were summarized as the mean ± standard error for all data sets. The data were then subjected to a One-way analysis-of-variance (ANOVA) using Analyse-it® Statistical analysis add-in for Microsoft Excel, significance, Excel (version 7.2.10 68) and/or ANOVA using Social Science Statistics (51). Means separation post-hoc test Tukey's (HSD) with differences considered statistically significant at the 5% level ($p \leq 0.05$).

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