Assessment of Different Antibacterial Effects of Fe and Cu Nanoparticles on *Xanthomonas campestris* Growth and Expression of Its Pathogenic Gene *hrpE*

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**ABSTRACT**

Plant diseases cause severe damage to agricultural production and need to be effectively managed. The economic importance of the plant diseases and lack of effective control measures have led to many research in this field. Nanotechnology is one of the new techniques for disease control. The purpose of this study was to evaluate the antibacterial effects of copper and iron NanoParticles (NPs) against a *Xanthomonas campestris* strain, as well as the study of these nanoparticles’ effects on expression of the pathogenic gene *hrpE*. The Zero-Valent Iron (ZVI) and copper nanoparticles were synthesized by chemical reduction method. Different concentration of nanoparticles of Fe and Cu were used in bacteria plate culture and the Minimum Inhibitory Concentrations (MIC) as well as Minimum Bactericidal Concentration (MBC) were determined using colony count and optical density methods. The effect of nanoparticles on pathogenic gene expression *hrpE* was studied using Real-Time PCR. *Xanthomonas campestris* strain exposed to zero-valent iron nanoparticles showed that the growth rate was increased with increase in the concentration of nano-iron. But, the growth percentage of bacteria *Xanthomonas campestris* was reduced with increase in the concentration of nano-copper. The expression levels of pathogenic gene expression *hrpE* were increased 9 and 3 fold for copper and iron, respectively. Copper and iron nanoparticles showed different effects on *Xanthomonas* growth.

**Keywords**: Antibacterial activity, Minimum bactericidal concentrations, Minimum inhibitory concentrations, Nano-copper, Nano-iron.

**INTRODUCTION**

Economic importance of plants diseases and lack of effective control measures stimulated recent studies for controlling *Xanthomonas campestris* (Bhattacharya and Gupta, 2005). Managing and controlling plant diseases efficiently is important for crop growers, environmentalists, legislators, policy makers, and implementers (Kumar et al., 2007). Recently, nanotechnology is being used for disease control (Bhattacharya and Gupta, 2005). Nanomaterials have unique chemical and biological properties which make them useful in various fields, including agriculture and medicine (Yin et al., 2009; Brar et al., 2010; Chiang et al., 2012; Fu et al., 2014). Antibacterial effects of nanomaterials has been identified and is dependent on their small size and high surface to volume ratio, which allows them to interact closely with microbial membranes (Morones et al., 2005; Ruparelia et al., 2008). Bacterial blight of geranium is the single most important disease of Pelargonium species and is caused by a bacterium named *Xanthomonas campestris* pv. *pelargonii* (McPherson et al., 1977) synonym *X.hortorum* pv. *Pelargonii* (Vauterin et al., 1995). *Xanthomonas* species can cause bacterial spots and blights of leaves, stems,
and fruits on a wide variety of plant species (Boch and Bonas, 2010). The bacterium can cause disease in all cultivated pelargonium varieties (Moorman, 2016). The bacterium usually travels from the leaf spots to the vascular system and causes a systemic wilt (Daughtrey et al., 2006). This can kill individual leaves and, in severe cases, it may kill the entire plant (Moorman, 2016). Since there is no effective chemical control, nanomaterials can be used for controlling the disease (Manulis et al., 1994).

Rapid controlling methods have economic importance for bacterial blight disease of geranium plants (Anderson and Nameth, 1990; Benedict et al., 1990). In the present study, copper and zero-valent iron nanoparticles were synthesized and their antibacterial effect against X. campestris was studied. The plant-pathogenic bacterium X. campestris possesses a Type III Secretion (TTS) system which is encoded by 23-kb hrp (hypersensitive response and pathogenicity) gene cluster (Fenselau et al., 1992). At the cell surface, the TTS system is associated with an extracellular filamentous structure, the Hrp pilus, which acts for the transmission of bacterial proteins into the plant cell cytosol. HrpE pilin is the major and unique pilus component in xanthomonads (Weber and Koebnik, 2006).

Therefore, in the present research, the effect of nanoparticles on the pathogenic gene expression of hrpE in X. campestris was studied using Real-Time Polymerase Chain Reaction (PCR).

**MATERIALS AND METHODS**

**Synthesis of Nanoparticles**

**Zero-Valent Iron**

All solutions were prepared in deionized water. Zero-valent iron nanoparticles were synthesized by chemical reduction method. In this method, the particles were prepared in aqueous phase by chemical reduction of ferrous sulfate solution (0.1M) using sodium borohydride in the presence of ascorbic acid (C₆H₈O₆) as a stabilizing agent. In the first step, 1.6 g FeCl₃ was mixed in 100 mL 30% ethanol in a glass vacuum Erlenmeyer flask and was placed on stirrer. Then, NaOH and sodium borohydride were added to iron drop by drop (1 drop/2 seconds). The solution slowly turned to black color. This solution mixture was stirred for 30 minutes at room temperature. When the color dark black was observed, it was removed from stirrer and the mixture was kept in refrigerator. The ferrous ion was reduced to zero-valent iron according to the following reaction, Equation (1).

\[ 2 \text{FeCl}_3 + 6 \text{NaBH}_4 + 18 \text{H}_2 \text{O} \rightarrow 2 \text{Fe}^0 + 6 \text{NaCl} + 6 \text{B(OH)}_3 + 21 \text{H}_2 \] (1)

**Zero-Valent Copper**

All solutions of reacting materials were prepared in distilled water. The particles were prepared in aqueous phase by chemical reduction of cupric salt solution using sodium borohydride in the presence of sodium citrate as a capping agent. The Cu nanoparticles were synthesized by chemical reduction process using copper (II) sulfate pentahydrate as precursor salt. In a typical set, 0.3 g of copper (II) sulfate pentahydrate was dissolved in 100 mL of deionized water. In a separate container, 0.1 g sodium borohydride was dissolved in 50 mL of deionized water. Then, 0.1 mM sodium borohydride was slowly added drop by drop to a solution of copper. Until the solution was yellow, 5 mL of 1% disodium citrate solution was added to the reaction mixture. Finally, after 5 minutes, the mixture on stirrer was removed and kept in refrigerator.

After synthesis of nanoparticles in an aqueous medium, UV-visible spectra of nanoparticles were taken (V-760 UV-Visible spectrophotometer JASCO) in 500 and 270 nm for copper and iron zero-valent nanoparticles, respectively.

**Bacterial Strains and Growth Conditions**
X. campestris pv. pelargonii PTCC1473 bacteria used in the present study was supplied by Iranian Research Organization for Science and Technology. X. campestris pv. pelargonii is a gram negative, rod-shaped, obligate aerobic bacterium that produces yellow colored colonies when isolated on nutrient agar. Xanthomonas strains were cultured on YGC [Y (Yeast extract), G (Glucose), C (CaCO₃)] broth medium (1.0% yeast extract, 2.0% D-(+)-glucose, 2.0% CaCO₃) at 27°C for two days. In order to determine the best CFU, serial dilution of microbial culture (10⁻¹ to 10⁻¹²) was prepared, then, 20 μL of each dilution was plated onto YGC plate and incubated at 27ºC for 24 hours. Afterwards, the colonies were counted on the plate. Bacterial colonies with 10⁻⁴ dilution were selected for experiments.

**Antibacterial Assay**

At first, various concentrations of iron nanoparticle (0, 50, 150, 300, 350, 450, and 550 μM) and copper nanoparticle (0, 60, 120, 180, 240, and 360 μM) were mixed with 20 mL of nutrient agar medium, thus the plate containing nanoparticles was prepared. Then, 20 μL of the bacteria with 10⁻⁴ dilution was poured on the plates. The numbers of colonies on the plates were counted after incubation for 48 hours at 27°C. Also, a nanoparticle-free plate was cultured under the same condition as the control. All the experiments were carried out in triplicate (Xie et al., 2011).

**Assay for Bactericidal Effect**

In order to confirm and evaluate bactericidal or bacteriostatic effects of nanoparticles, turbidity test was carried out. Twenty μL of 10⁻⁴ dilution of X. campestris was added to 10 mL nutrient broth and incubated for 24 hours at 27°C. After that, the bacteria were treated with different concentrations of iron (200, 400 μM) and copper nanoparticles (120, 240, and 360 μM). Bacterial concentrations were determined by measuring Optical Density (OD) at 600 nm (0.1 OD600 corresponding to 10⁸ cells per mL) in intervals 0, 6, 12, 18, and 24 hours. A test tube without nanoparticle was used as the control. All the experiments were carried out in triplicate.

**RNA Preparation and RT-qPCR Analysis**

X. campestris were cultured in XCM2 medium (Hrp- inducing minimal media) including: 20 mM succinic acid, 0.15 g L⁻¹ casamino acids, 7.57 mM (NH₄)₂SO₄, 0.01 mM MgSO₄, 60.34 mM K₂HPO₄, 333.07 mM KH₂PO₄, pH 6.6 and incubated at 27°C and 100 rpm for 48 hours. After the growth of bacteria, 200 μL of nanoparticle copper (240 μM) and iron (100 μM) were added and again incubated for 8 hours. After treatment, the bacterial cells were harvested by centrifugation at 4,000×g for 10 minutes at 4°C. RNA isolation was carried out using RNX Plus kit (CinnaGen Company) according to the manufacturer’s instructions. Then, DNase I treatment and Reverse Transcription (RT) reaction were performed. The synthesis of cDNA was performed using M-MLV Reverse transcriptase enzyme Thermo Scientific Company. Quantification of cDNA was performed on a Rotor-gene 6000 Real-Time PCR system (Corbett Research Australian modelRG2072D). For Real Time PCR, to amplify 59-base-pair fragment of hrpE gene: forward primer hrpE (5’ACTGCCACTCAGACCTCG3’) and reverse primer (5’GATTTCACCACGTCCTCG3’) were designed using Oligo, version 3.4; National Biosciences Inc software. Each 15 µL PCR reaction contained 7.5 µL SYBR® Green PCR Master Mix dye QIAGEN,10 pmol of each primer, 100 ng of cDNA template, 4.5 µL DI water. The amplification program was 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The 16S rRNA gene.
Figure 1. UV-Visible spectra of zero valent copper nanoparticle (a), and UV-Visible spectra of zero valent iron nanoparticle (b).

Table 1. Calculation of CFU (Colon Forming Unit) in the control samples and treated with iron and copper nanoparticles.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>CFU mL⁻¹</th>
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<tbody>
<tr>
<td>Fe (µM)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8.4x10⁸</td>
</tr>
<tr>
<td>50</td>
<td>6.4x10⁸</td>
</tr>
<tr>
<td>Cu (µM)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8.8x10⁸</td>
</tr>
<tr>
<td>60</td>
<td>8x10⁸</td>
</tr>
</tbody>
</table>

Statistical Analysis

All experiments for antibacterial activity were carried out in triplicate and data was analyzed in SPSS statistical software. Normality test was conducted to evaluate normality of the data. All data were normally distributed. The average gene expression hrpE in the control group as well as treated with nanoparticles of iron and copper were compared using Duncan and Tukey tests in SAS 9/13 software that was available in the Real-Time PCR (Rotor-gene 6000 series software 1.7).

RESULTS

Characterization of Iron and Copper NPs

Vis spectra of Cu and Fe NPs synthesized in an aqueous medium are shown in Figure 1. A yellow color of Cu NPs had absorbance in 500 nm and black color of Fe NPs was shown absorbance in 280 nm.

Antibacterial Assay

The Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentration (MBC) were determined using effect of different concentrations of nanoparticles on bacteria growth by colony count method. The numbers of colonies on the agar plates were counted after incubation for 48 hours at 27°C and CFU was calculated by Equation (2) (Table 1).

According to standard criterion of the number of bacteria, less than 20% reduction indicates no bactericidal effect; 20–50%
reduction indicates a low bactericidal effect; 50–70% reduction indicates an expressive bactericide; greater than 70% reductions is considered a powerful bactericidal effect (Sayilkan et al., 2009). The results indicated 180 μM concentration of copper nanoparticles had expressive bactericidal effect. But, in concentrations of 240, 300, and 360 μM had a powerful bactericidal effect, respectively. MIC and MBC of copper nanoparticles for X. campestris were 60 and 300 μM, respectively (Figure 2-A). In order to achieve the best inhibitory concentration of the nanoparticles, comparison of the averages of different concentrations were performed by Duncan test. The results showed statistically significant difference between different concentrations of the copper nanoparticles (P value= 0).

The results of colony count of X. campestris after treatment with iron nanoparticles showed that with the increase in iron concentration, the percentage of inhibition of bacterial growth was reduced (Figure 2-B), so that the highest percentage of inhibition of bacterial growth was 25% in 50 μM of iron concentration. Comparison of the averages of different concentrations were performed by Duncan test and showed no statistically significant difference between different concentrations of the iron nanoparticles (P> 0.05). MIC of ZVI nanoparticles for X. campestris was 550 μM. The number of colonies grown on the plate after treatment with copper nanoparticles is shown in Figure 3.

**Bacteriocidal Assay**

To evaluate bactericidal or bacteriostatic effect of iron and copper nanoparticles against X. campestris, optical density of bacteria growth was measured in liquid medium in the presence of the nanoparticles. The results showed that with increase in concentrations of copper nanoparticles (120, 240, and 360 μM), the Optical Density declined considerably (OD= 0.7, 0.4, and 0.2, respectively) (Figure 4-A). According to Duncan’s test, a statistically significant difference was observed between different concentrations of the copper nanoparticles (P= 0.01).

The results of the measurement of optical density after treatment of iron nanoparticles showed that the number of bacteria showed no significant decrease with increase in iron concentration (Figure 4-B). Also, the results of Duncan test showed no statistically significant difference between the concentrations of iron nanoparticles (P= 0.1).

![Figure 2](image_url)

**Figure 2.** Graph of inhibition and growth percentage of Xanthomonas campestris exposed to different concentrations of (A) copper (B) iron. Nanoparticles. The letters “a” and “b” represent significant differences by Duncan test in the treatments.
Expression of Phytogenic hrpE Gene

To evaluate the effect of iron and copper nanoparticles on hrpE gene expression, quantification studies were done by Real Time PCR. Bacteria grown after late-log-phase were exposed to 100 µM of iron and 240 µM of copper nanoparticles for 8 hours, then, hrpE gene expression was quantified by RT-qPCR and data were analyzed using the Comparative Critical Threshold (ΔΔCT) method. Error bars indicate standard deviations for three replicates. The expression levels of hrpE gene were found to be up regulated 3 and 9 fold, for iron and copper treatments, respectively, compared with housekeeping gene 16S rRNA as the control (Figure 5).

The amplification product of hrpE and 16S rRNA genes by Real-Time PCR in agarose gel 1.5% is shown in Figure 6.

Comparison of the average expression of
DISCUSSION

X. campestris is one of the most important pathogens in geranium species that causes spots and blights of leaves (Boch and Bonas, 2010). Under conditions unfavorable for disease development, the bacterium can survive on plant surfaces (epiphytically) or as latent infections within plants. The movement of infected, asymptomatic plants between greenhouses is the major means of dispersal of the pathogen (Daughtrey and Wick, 1995). All commercial cultivars of geranium are susceptible to X. campestris pv. pelargonii (Manulis et al., 1994). Therefore, it is very important for disease control in geranium to use new methods like nanotechnology (Bhattacharya and Gupta, 2005). In many studies, antimicrobial properties of nanoparticles have been reported. Some of metal based nanoparticles can be a better substitute to pesticides. The advantage of using nanoparticles over pesticides is that they consume less amount and have targeted action; so, nanoparticles have more susceptibility (Khati, 2017). Metal nanoparticles like Fe, Zn, Mg, Ti, Ce, Ag, Au, and Cu are considered because of the wide range of hosts and targeted actions (Cioffi et al., 2005; Ren et al., 2009). Metal based nanoparticles have cytotoxicity effect that is the result of the electrostatic

Table 2. *hrpE* gene expression were quantified byRT-qPCR and data were analyzed using the Comparative Critical Threshold (ΔΔCT) method.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>$2^{\Delta \Delta Ct}$</th>
<th>Mean SE$\pm$Ct</th>
<th>Duncan/ Tukey</th>
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<td></td>
<td></td>
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<td>9.194057</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>3</td>
<td>2.84</td>
<td>3.268595</td>
<td>A</td>
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<td></td>
<td></td>
<td>4.10</td>
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<td></td>
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<td>2.86</td>
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interaction with cell membrane depending on the charge on cell membrane (Khati, 2017).

In this study, we used zero-valent nanoparticles. The advantages of zero-valent nanoparticles are high stability and less adverse effects on the environment (Jang et al., 2014). Nanoparticles are ionized after the half-life period, with no adverse effects on animals and microorganisms in the environment. There are many techniques available for the preparation of metallic nanoparticles. Compared to other methods, the aqueous reduction method is widely used because of its advantages such as simple operation, high yield and quality, limited equipment requirements and easy to control (Chaudhari et al., 2007; Pal et al., 2007; Liu et al., 2012) and may be suitable for the formulation of new types of bactericidal materials. Selvarani and Prema (2013) evaluated antibacterial activity of chemically synthesized Cu and Fe nanoparticles against pathogenic bacteria and the results showed that the Cu and Fe nanoparticles had the antibacterial action against both gram positive and negative bacteria.

Our results of treating X. campestris with zero-valent iron nanoparticles showed that with increasing nanoparticles concentration, the percentage of growth inhibition of bacteria decreased. Also, measurement of optical density showed no significant decrease in bacterial growth with increasing iron concentration during time. Barzan et al. (2014) showed the opposite results from the study of inhibitory effect of zero-valent iron nanoparticles on plant pathogenic bacteria X. campestris (Barzan et al., 2014). Although, Borcherdung et al. (2014) demonstrated that iron oxide nanoparticles increase bacterial growth with a correlation between growth and particle size and surface area. Thus, iron nanoparticles can act as an exogenous iron source for bacterial growth. Sometimes, the nanoparticles in low concentrations show a stimulatory activity in the microorganisms while the same nanoparticles in higher concentration are toxic. Babushkina et al. (2010) showed that iron nanoparticles in concentration of 0.001 to 0.01 mg mL\(^{-1}\) pose stimulating effect on the bacterial growth, but in concentrations of 0.1 to 1 mg mL\(^{-1}\) had inhibitory effect on S. aureus (Babushkina et al., 2010). One of the assumptions is that the surface of nanoparticles of iron ions with phosphate ions found in bacterial cell proteins and biomolecules form insoluble complex and thus iron nanoparticles activity are reduced. Another assumption is that the iron ions can act as a cofactor for enzymes responsible for the growth and division of bacteria and thus growth rates are increased. But, the results of treatment with copper nanoparticles showed that with increasing Cu nanoparticles’ concentration, the percentage of inhibition of bacterial growth increased. Also, the results of absorption measurements showed that with increase in concentrations of copper nanoparticles, optical density decreased considerably. Based on these results, copper nanoparticles had an expressive antibacterial effect for the gram negative X. campestris pv. pelargonii. Mahapatra et al. (2008) tested antibacterial activity of copper oxide nanoparticles with a particle size ranging from 80 to 160 nm against Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella paratyphi and Shigella strain. The copper oxide nanoparticles showed antibacterial activity against the bacteria. It can be assumed that nanoparticles form complexes with vital enzymes as inhibitor inside cells and inhibit cellular function, resulting in cell death (Mahapatra et al., 2008). Raffi et al. (2010) reported that copper had the potential to disrupt cell function in multiple ways, since several mechanisms acting simultaneously may reduce the ability of microorganisms to develop resistance against copper. They also demonstrated that copper nanoparticles had a great antimicrobial activity against B. subtilis. This might be due to greater affinity of copper towards many amines and carboxyl groups on cell surface of B. subtilis. This suggests that release of ions into the local environment is required for optimal
Antibacterial Effects of Fe and Cu

This page contains text about the antibacterial effects of iron (Fe) and copper (Cu). It discusses how copper reacts with -SH groups of enzymes, leading to inactivation of the proteins. Copper ions inside bacterial cells can bind to deoxyribonucleic acid molecules, causing cross-linking and damage to proteins and DNA. The main mechanism of antibacterial activity shown by nanoparticles is oxidative stress generated via ROS, including superoxide radicals (O$_2^-$), hydroxyl radicals (-OH), hydrogen peroxide (H$_2$O$_2$), and singlet oxygen (1O$_2$), which can cause damage to proteins and DNA in bacteria. Copper ions may bind to deoxyribonucleic acid molecules and become involved in cross-linking within and between the nucleic acid strands, resulting in the disorganized helical structure.

**CONCLUSIONS**

Nontoxic nano materials of copper and iron were prepared in a simple and cost-effective manner. Results of the study showed that several factors influence the sensitivity or resistance of bacteria to nanoparticles. Unlike the iron nanoparticles, copper nanoparticles exhibited remarkable antibacterial activity and demonstrated a lethal effect against X. campestris, even at low concentrations. Therefore, this nanoparticle will be usable as an antibacterial agent.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


ارثار ضد باکتریایی منفیات نانودرات مس و آهن بر رشد باکتری Xanthomonas campestris آل hrpE و بیان زن پاتوژنیک آن

چکیده

بیماری های گیاهی باعث آسیب شدید به محصولات کشاورزی شده و باعث بروز می‌شود که مرگ و میر اقتصادی بیماری‌گری گیاهی و فقدان اقدامات موثر کنترل آن به تحقق پردازید. زمینه منجر به هدف اصلی این مطالعه نسبت به ضایعات باکتریایی نانودرات مس و آهن در محیط کشت، گونه Xanthomonas campestris کمپستریس و همچنین مطالعه اثرات نانوذرات در بیان hrpE با استفاده از روش‌های شمارش کلی و چگالی نوری تعیین شدند. بیانات نانوذرات مس و آهن زنجیرهی زا و مغز به ترتیب 9 و 3 برای افزایش یافته بود. نانوذرات مس و آهن اثرات مختلفی بر رشد باکتری Xanthomonas campestris داشتند.


