J. Agr. Sci. Tech. (2018) Vol. 20: 1059-1070

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

R. Ghorbani[1], F. Moradian[1*], and P. Biparva[1]

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,

[1] Department of Basic Sciences, Sari Agricultural Sciences and Natural Resources University, P. O. Box: 578, Sari, Islamic Republic of Iran.
*Corresponding author; e-mail: f.moradian@umz.ac.ir

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).

MATERIALSAND 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 ($C_6H_8O_6$) as a stabilizing agent. In the first step, 1.6 g $FeCL_3$ 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 (1drop/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).

$$2\ FeCl_3+6\ NaBH_4+18\ H_2O \rightarrow 2\ Fe^0+6\ NaCl+6\ B(OH)_3+21\ H_2 \quad (1)$$

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 were 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 ($CaCO_3$)] broth medium (1.0% yeast extract, 2.0% D-(+)-glucose, 2.0% $CaCO_3$) 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

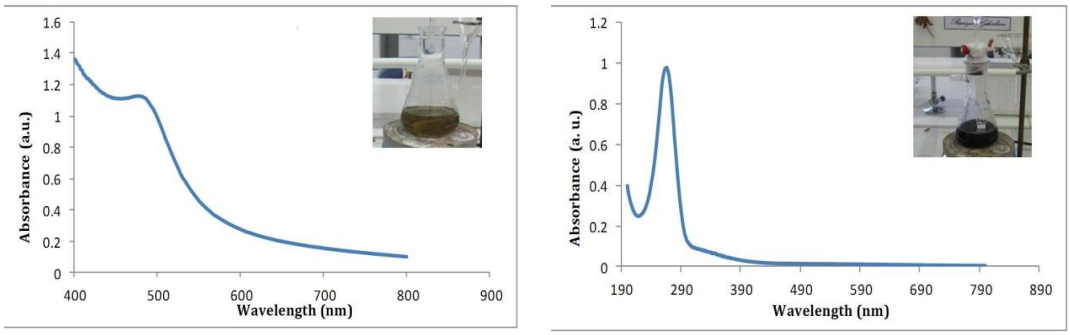
At first, various concentrations of iron nanoparticle (0, 50, 150, 300, 350, 450, and 550 µM) and copper nanoparticle (0, 60,120,180, 240, 300, and 360 µM) were mixed with 20 mL of nutrient agar medium, thus the plate containing nanoparticles was prepared. Then, 20 µL of the bacteria with 10^{-4} dilution 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 10^8 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 $(NH_4)_2SO_4$, 0.01 mM $MgSO_4$, 60.34 mM K_2HPO_4, 333.07 mM KH_2PO_4, 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 (Corrbet 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'GATTTACCAATGCCACCC3') 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



(a) (b)

Figure 1. UV-Visible spectra of zero valent copper nanoparticle (a), and UV-Visible spectra of zero valent iron nanoparticle (b).

was used as a reference for data normalization. All the samples, including no-RT and no-template controls, were analyzed in triplicate. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method, as follows:

$\Delta\Delta C_T$= ΔC_T (treated sample)-ΔC_T (untreated sample),

ΔC_T= C_T (*hrpE* gene)-C_T (*16S rRNA*),

Where, C_T is the threshold Cycle value for the amplified gene (Livak and Schmittgen, 2001).

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 Figure1. 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%

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

Xanthomonas campestris pv. pelargonii	
Concentration	CFU mL^{-1}
Fe (µM)	
0	8.4×10^8
50	6.4×10^8
Cu (µM)	
0	8.8×10^8
60	8×10^8

$$\text{CFU mL}^{-1}= \frac{[(\text{Number of colonies plate surface})\times(\text{Dilution factor}= 10^n)]\times 1}{\text{The volume of bacteria on plates used}} \quad (2)$$

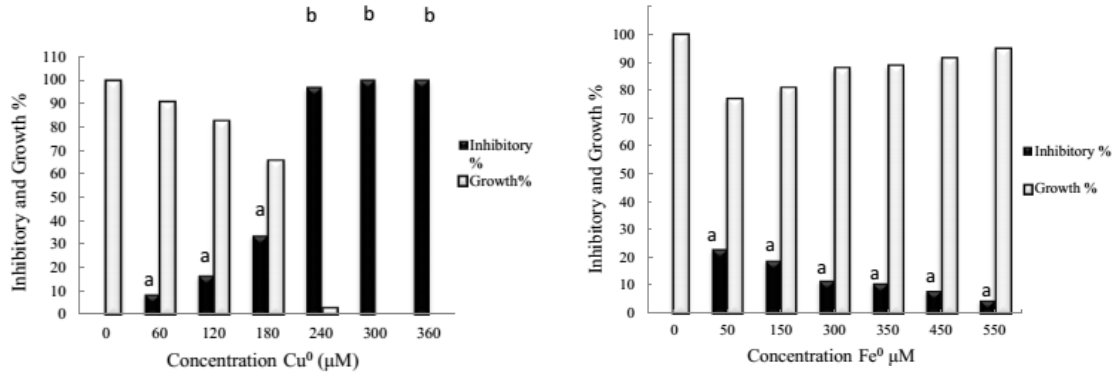
reduction indicates a low bactericidal effect; 50–70% reduction indicates an expressive bactericide; greater than 70% reductions is considered a powerful bactericidal effect (Sayilkan *et al*., 2009).The results indicated 180 µM concentration of copper nanoparticles had expressive bactericidal effect. 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).



(A) (B)

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.

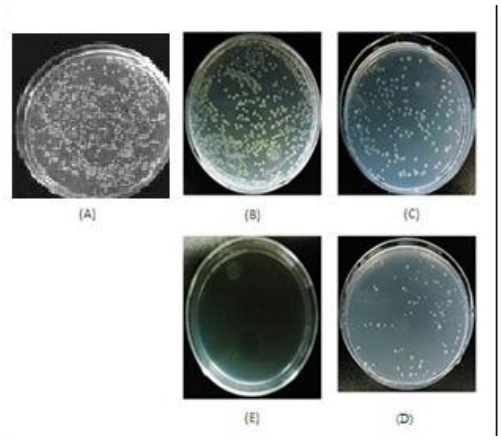


Figure 3. The number of bacterial colonies grown on plates. (A) Colonies of *Xanthomonas campestris* without nanoparticles; (B) Treatment with 120 µM; (C) 180 µM; (D) 240 µM, and (E) 360 µM copper.

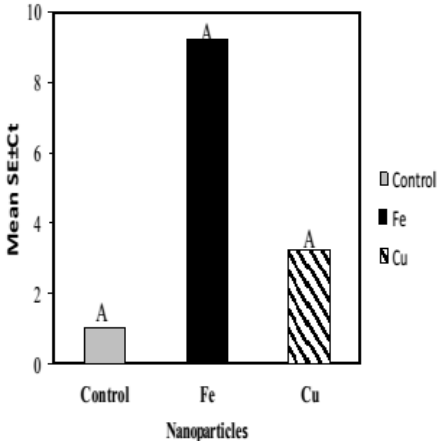


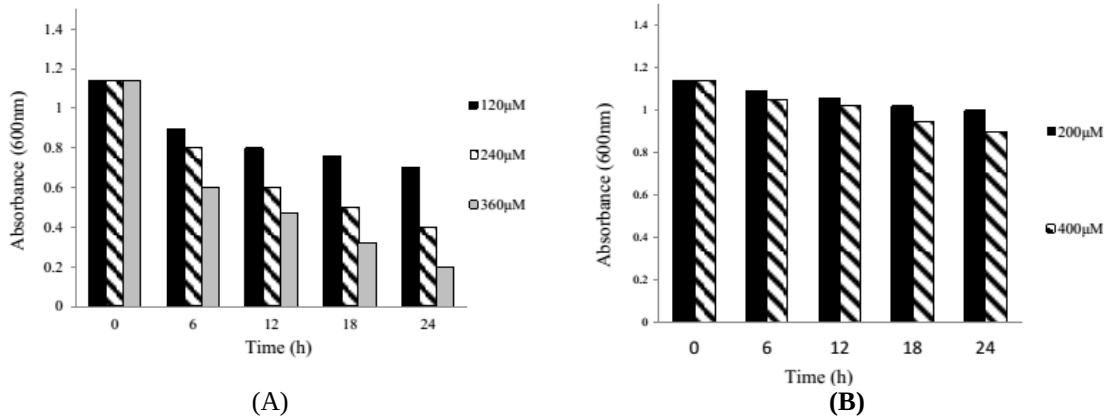
Figure 5. Relative *hrpE* gene expression levels after treatment by iron and copper nanoparticles and untreated as control.

Expression of Phatogenic 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



(A) **(B)**

Figure 4. Changes the optical density of *Xanthomonas campestris* bacteria after treatment with various concentrations of (A) copper (B) iron, nanoparticles in intervals 0, 6, 12, 18 and 24 hours. The black column shows 120 µM concentrations; the stripe column shows 240 µM, and the grey column shows 360 µM.

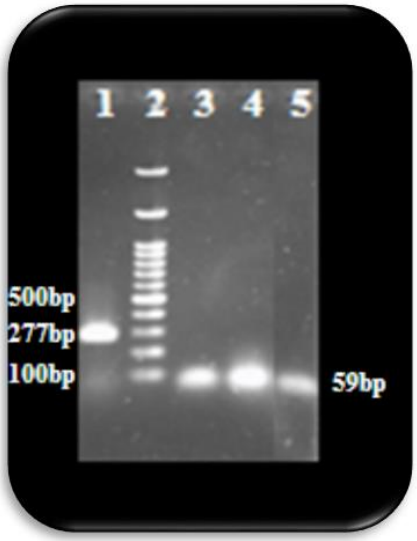


Figure 6. Electrophoresis of amplification product of *hrpE* and *16S rRNA* genes by Real-Time PCR in agarose gel 1.5%. (Lane 1) 16S rRNA; (Lane2) Molecular weight marker 100 bp (Fermentas); (Lane 3) *hrpE* gene amplification product in bacteria as Control; (Lane 4) Bacteria treated with iron, (Lane 5) Bacteria treated with copper.

this gene with Tukey and Duncan methods and revealed no statistically significant difference (P> 0.05) between three groups of the control and those treated with nanoparticles of iron (19.9±0.20) and copper (3.27±0.42). Table 2.

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.

Group	Number	$2^{-\Delta\Delta CT}$	Mean SE±Ct	Duncan/ Tukey
Control	3	1.09 1.12 0.81	1.010082	A
Fe	3	9.16 9.55 8.85	9.194057	A
Cu	3	2.84 4.10 2.86	3.268595	A

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^{o} nanoparticles against pathogenic bacteria and the results showed that the Cu and Fe^{o} 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, Borcherding *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*, *Salmonellaparatyphi* 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

antimicrobial activity (Cioffi *et al.*, 2005; Ren *et al.*, 2009). Copper reacts with the -SH groups of enzymes, consequently, this reaction leads to the inactivation of the proteins (Jeon *et al*., 2003). The main mechanism of antibacterial activity shown by nanoparticles might be oxidative stress generated via ROS (Tran *et al*., 2010; Mahdy *et al*., 2012) including superoxide radicals (O_2–), hydroxyl radicals (–OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), which can cause damage to proteins and DNA in bacteria. Copper ions inside the bacterial cells 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 (Stohs and Bagchi, 1995; Kim, *et al*., 2000; Behera *et al*., 2012). In the present study, pathogenic *hrpE* gene expression was investigated in the presence of nanoparticles, using Real-Time PCR for the first time. *HrpE* is unique to the genus of *Xanthomonas* and shows no sequence similarity to other *pilin* genes (Weber and Koebnik, 2006). Expression of *hrp/hrc* genes is tightly controlled. They are expressed at a very low level in vitro in nutrient-rich media, but can be induced in infected plant tissues (Boureau *et al*., 2002). *X. campestris* produces filamentous structures, the Hrp pili, at the cell surface under *hrp*-inducing conditions (Weber *et al*., 2005). Depending on the bacterial species, different environmental factors such as the concentration of divalent cations, osmotic pressure, pH, and salts concentrations have an important role in regulating gene expression (Huynh *et al*., 1989; Xiao *et al*., 1992; Wei *et al*., 1992; Salmeron and Staskawicz, 1993; Boureau *et al*., 2002). Our results showed an over expression of this gene in the presence of nanoparticles. It is assumed that the positive charge on the surface of nanoparticles with negative charge of bacterial cell membrane creates electrostatic attraction, and this physical contact of the nanoparticles as a signal causes production of a surface appendage, named the Hrp pilus.

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

This original research was performed in Sari Agricultural Sciences and Natural Resources University, Department of Basic Sciences. We are thankful for receiving facilities and assistance.

REFERENCES

1. Anderson, M. J. and Nameth, S. T. 1990. Development of a Polyclonal Antibody-Based Sero Diagnostic Assay for the Detection of *Xanthomonas campestris* pv. *pelargonii* in Geranium Plants. *Phytopathol.*, **80(4)**: 357-360. doi.org/10.1094/PHYTO-01-14-0014-R.
2. Babushkina, I. V., Borodulin, V. B., Korshunoy, G. V. and Puchinjan, D. M. 2010. Comparative Study of Antibacterial Action of Iron and Copper Nanoparticles on Clinical *S. aureus* Strain. *J. Med. Sci. Res.*, **6**: 11-14.
3. Barzan, E., Mehrabian, S. and Irian, S. 2014. Antimicrobial and Genotoxicity Effects of Zero-Valent Iron Nanoparticles. *Jundishapur J. Microbiol.*, **7(5):** e10054. doi:10.5812/jjm.33933.
4. Behera, S. S., Patra, J. K., Pramanik, K., Panda, N. and Thatoi, H. 2012. Characterization and Evaluation of Antibacterial Activities of Chemically Synthesized Iron Oxide Nanoparticles. *World J. Nano. Sci. Eng.*, **2(04)**: 196.

5. Benedict, A.A., Alvarez, A. M. and Pollard, L. W. 1990. Pathovar-Specific Antigens of *Xanthomonas campestris* pv. *begoniae* and *X. campestris* pv. *Pelargonii* Detected with Monoclonal Antibodies. *Appl. Environ. Microbiol.*, **56(2)**: 572-574.
6. Bhattacharya, D. and Gupta, R. K. 2005. Nanotechnology and Potential of Microorganisms. *Crit. Rev. Biotechnol.*, **25(4)**: 199 -204.
7. Boch, J. and Bonas, U. 2010. *Xanthomonas* AvrBs3 Family-Type III Effectors: Discovery and Function. *Phytopathol.*, **48(1)**: 419. doi.org/10.1094/PHYTO-01-14-0014-R.
8. Borcherding, J., Baltrusaitis, J., Chen, H., Stebounova, L., Wu, C. M., Rubasinghege, G. and Comellas, A. P. 2014. Iron Oxide Nanoparticles Induce *Pseudomonas aeruginosa* Growth, Induce Biofilm Formation, and Inhibit Antimicrobial Peptide Function. *Environ. Sci. Nano.*, **1(2)**: 123-132. DOI: 10.1039/C6EN00159A.
9. Boureau, T., Routtu, J., Roine, E., Taira, S. and Romantschuk, M. 2002. Localization of hrpA-Induced *Pseudomonas syringae pv. tomato* DC3000 in Infected Tomato Leaves. *Mol. Plant Pathol.*, **3(6)**: 451-460.doi: 10.1111/mpp.12439.
10. Brar, S. K., Verma, M., Tyagi, R. D. and Surampalli, R. Y. 2010. Engineered Nanoparticles in Waste Water and Waste Water Sludge: Evidence and Impacts. *Waste Manage.*, **30(3)**: 504-520.
11. Chaudhari, V. R., Haram, S. K., Kulshreshtha, S. K., Bellare, J. R. and Hassan, P. A. 2007. Micelle Assisted Morphological Evolution of Silver Nanoparticles. *Colloids Surf. A.*, **301(1)**: 475-480.
12. Chiang, H. M., Xia, Q., Zou, X., Wang, C., Wang, S., Miller, B. J. and Fu, P. P. 2012. Nanoscale ZnO Induces Cytotoxicity and DNA Damage in Human Cell Lines and Rat Primary Neuronal Cells. *J. Nano. Sci. Nanotechnol.*, **12(3)**: 2126-2135.doi:10.1166/jnn.2015.10348.
13. Cioffi, N., Torsi, L., Ditaranto, N., Tantillo, G., Ghibelli, L. and Sabbatini, L. 2005. Copper Nanoparticle/Polymer Composites with Antifungal and Bacteriostatic Properties. *Chem. Matr.*, **17**: 5255-5262.
14. Daughtrey, M. L. and Wick, R. L. 1995. Vascular Wilt Diseases. In: "*Geraniums IV*", (Ed.): White, J. W. The Grower's Manual, Ball Publishing, Geneva, III: 237-242.
15. Daughtrey, M. L., Wick R. L. and Peterson, J. L. 2006. *Compendium of Flowering Potted Plant Diseases*. *Am. Phytopathol. Soc. (APS)*, St Paul MN.
16. Fenselau, S., Balbo, I. and Bonas, U. 1992. Determinants of Pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* Are Related to Proteins Involved in Secretion in Bacterial Pathogens of Animals. *Mol. Plant. Microbe Interac.*, **5**: 390-390. doi: 10.1094/MPMI-03-16-0056-R.
17. Fu, P. P., Xia, Q., Hwang, H.M., Ray, P. C. and Yu, H. 2014. Mechanisms of Nanotoxicity: Generation of Reactive Oxygen Species. *J. Food Drug Anal.*, **22(1)**: 64-75. doi:10.1002/14651858.
18. Huynh, T. V., Dahlbeck, D. and Staskawicz, B. J. 1989. Bacterial Blight of Soybean: Regulation of a Pathogen Gene Determining Host Cultivar Specificity. *Sci.*, **245**: 1374-1377.
19. Jang, M. H., Lim, M. and Hwang, Y. S. 2014. Potential Environmental Implications of Nanoscale Zero-Valent Iron Nanoparticles for Environmental Remediation. Environ. *Health Toxicol.*, **29**: e2014022. Doi:10.5620/eht.e2014022.
20. Jeon, H. J., Yi, S. C. and Oh, S. G. 2003. Preparation and Antibacterial Effects of Ag–SiO2 Thin Films by Sol–Gel Method. *Biomatr.*, **24(27)**: 4921-4928. doi: 10.3389/fmats.2016.00030.
21. Khati, P. 2017. Nanoparticles as Antimicrobial Agents against Medically Important Pathogens. *Int. J. Appl. Pharm. Biol. Res.*, **2(2)**: 56-66.
22. Kim, J. H., Cho, H., Ryu, S. E. and Choi, M. U. 2000. Effects of Metal Ions on the Activity of Protein Tyrosine Phosphatase VHR: Highly Potent and Reversible Oxidative Inactivation by Cu^{2+} Ion. *Arch. Biochem. Biophys.*, **382(1)**: 72-80. doi: 10.1016/j.
23. Kumar, S. A., Abyaneh, M. K., Gosavi, S. W., Kulkarni, S. K., Pasricha, R., Ahmad, A. and Khan, M. I. 2007. Nitrate Reductase-Mediated Synthesis of Silver Nanoparticles from AgNO3. *Biotechnol. Let.*, **29(3)**: 439-445.

24. Liu, Q. M., Zhou, D. B., Yamamoto, Y., Ichino, R. and Okido, M. 2012. Preparation of Cu Nanoparticles with $NaBH_4$ by Aqueous Reduction Method. *Trans. Nonferrous Metals Soc. China*, **22(1)**: 117-123.
25. Livak, K. J. and Schmittgen,T. D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, **25(4)**: 402-408.
26. Mahapatra, O., Bhagat, M., Gopalakrishnan, C. and Arunachalam, K. D. 2008. Ultrafine Dispersed CuO Nanoparticles and Their Antibacterial Activity. *J. Exp. Nano. Sci*., **3(3)**: 185-193. doi.org/10.1080/17458080.
27. Mahdy, S. A., Raheed, Q. J. and Kalaichelvan, P. T. 2012. Antimicrobial Activity of Zero-Valent Iron Nanoparticles. *Int. J. Mod. Eng. Res.*, **2(1)**: 578-581.
28. Manulis, S., Valinsky, L., Lichter, A. and Gabriel, D. W. 1994. Sensitive and Specific Detection of *Xanthomonas campestris* pv. *pelargonii* with DNA Primers and Probes Identified by Random Amplified Polymorphic DNA Analysis. *Appl. Environ. Microbiol.*, **60(11)**: 4094-4099. doi:10.1128/AEM.02023-16.
29. McPherson, G. M., Bradbury, J. F. and Preece, T. F. 1977. *Descriptions of Pathogenic Fungi and Bacteria.* CMI No 560, 2 PP.
30. Moorman, G. W. 2016. *Bacterial Blight of Geraniums, Penn State Extension*. Penn State College of Agricultural Sciences. http://extension.psu.edu/pests/plant-diseases/all-fact-sheets/bacterial-blight-of-geraniums.
31. Morones, J. R., Elechiguerra, J. L., Camacho, A., Holt, K., Kouri, J. B., Ramírez, J. T. and Yacaman,M. J. 2005. The Bactericidal Effect of Silver nanoparticles. *J. Nanotechnol.*, **16(10)**: 2346.
32. Pal, A., Shah, S. and Devi, S. 2007. Synthesis of Au, Ag and Au–Ag Alloy Nanoparticles in Aqueous Polymer Solution. *J.* Colloids Surf. A., **302(1)**: 51-57.
33. Raffi, M., Mehrwan, S., Bhatti, T. M., Akhter, J. I., Hameed, A., Yawar, W. and Hasan,M. M. 2010. Investigations into the Antibacterial Behavior of Copper Nanoparticles against *Escherichia coli. Ann. Microbiol.*, **60(1)**: 75-80.
34. Ren, G., Cheng, E. W. C., Vargas-Reus, M. A., Reip, P. and Allakar, R. P. 2009. Characterization of Copper Oxide Nanoparticles for Antimicrobial Applications. *Int. J. Antimicrob. Age.*, **33**: 587-590.
35. Ruparelia, J. P., Chatterjee, A. K., Duttagupta, S. P. and Mukherji, S. 2008. Strain Specificity in Antimicrobial Activity of Silver and Copper Nanoparticles. *Acta Biomater.*, **4(3)**: 707-716.doi: 10.1016/j.
36. Salmeron, J. M. and Staskawicz, B. J. 1993. Molecular Characterization and hrp Dependence of the *Avirulence* Gene avrPro from *Pseudomonas syringae* pv. *tomato*. *Mol. Gen. Genet.*, **239(1-2)**: 6-16.
37. Sayilkan, F., Asilturka, M. and Kirazb, N. 2009. Photocatalytic Antibacterial Performance of sn^{4+} Doped Tio_2 Thin Films on Glass Substrate. *J. Hazard Matr.*, **162**: 1309-1316.
38. Selvarani, M. and Prema, P. 2013. Evaluation of Antibacterial Efficacy of Chemically Synthesized Copper and Zerovalent Iron Nanoparticles. *Asian J. Pharm. Clin. Res.*, **6(3)**:222-227.
39. Stohs, S. J. and Bagchi, D. 1995. Oxidative Mechanisms in the Toxicity of Metal Ions. *Free Radic. Biol. Med.*, **18(2)**: 321-336. doi: 10.1016/j.
40. Tran, N., Mir, A., Mallik, D., Sinha, A., Nayar, S. and Webster, T. J. 2010. Bactericidal Effect of Iron Oxide Nanoparticles on *Staphylococcus aureus*. *Int. J. Nanomed.*, **5**: 277-283. doi: 10.2147/IJN.S106299.
41. Vauterin, L., Hoste, B., Kersters, K. and Swings, J. 1995. Reclassification of *Xanthomonas*. *Int. J. Syst. Bacteriol.*, **45(3)**: 472-489.
42. Weber, E. and Koebnik, R. 2006. Positive Selection of the Hrp pilin HrpE of the Plant Pathogen *Xanthomonas*. *J. Bacteriol.*, **188(4)**: 1405-1410. doi:10.1128/JB.00384-16.
43. Weber, E., Ojanen-Reuhs,T., Huguet, E., Hause, G., Romantschuk, M., Korhonen, T. K. and Koebnik, R. 2005. The Type III-Dependent Hrp Pilus Is Required for Productive Interaction of *Xanthomonas campestris* pv. *vesicatoria* with Pepper Host Plants. *J. Bacteriol*., **187(7)**: 2458-2468. doi:10.1128/JB.00384-16.

44. Wei, Z. M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, H. and Beer, S. V. 1992. Harpin, Elicitor of the Hyper Sensitive Response Produced by the Plant Pathogen *Erwinia amylovora*. *Sci. (Washington)*, **257**: 85-88.
45. Xiao, Y., Lu, Y., Heu, S. and Hutcheson, S. W. 1992. Organization and Environmental Regulation of the *Pseudomonas syringae* pv. *syringae* 61 hrp Cluster. *J. Bacteriol.*, **174(6)**: 1734-1741. doi:10.1128/JB.00384-16.
46. Xie, Y., He, Y., Irwin, P.L. and Jin, T. 2011. Antibacterial Activity and Mechanism of Action of Zinc Oxide Nanoparticles Against *Compylobacter jejuni*. Appl. Environm. Microbiol., **77(7)**: 2325-2331
47. Yin, J. J., Lao, F., Fu, P. P., Wamer, W. G., Zhao, Y., Wang, P. C. and Liang, X. J. 2009. The Scavenging of Reactive Oxygen Species and the Potential for Cell Protection by Functionalized Fullerene Materials. *Biomatr.*, **30(4)**: 611-6.doi: 10.3389/fmats.2016.00030

اثرات ضد باکتریایی متفاوت نانو ذرات مس و آهن بر رشد باکتری *Xanthomonas campestris* و بیان ژن پاتوژنیک ***hrpE*** آن

ر. قربانی، ف. مرادیان، و پ. بی‌پروا

چکیده

بیماری های گیاهی باعث آسیب شدید به محصولات کشاورزی شده و باید به طور موثری مدیریت شوند .اهمیت اقتصادی بیماری‌های گیاهی و فقدان اقدامات موثر کنترل آن به تحقیقات جدید در این زمینه منجر شده است. فن‌آوری نانو یکی از روشهای جدید برای کنترل بیماری است. هدف از این مطالعه بررسی اثر ضد باکتریایی نانو ذرات مس و آهن ضد گونه زانتاموناس کمپستریس و همچنین مطالعه اثرات نانوذرات در بیان ژن بیماری‌زا hrpE بود. نانوذرات آهن و مس صفر ظرفیتی توسط روش احیا شیمیایی سنتز شدند .غلظت‌های مختلف نانوذرات آهن و مس در محیط کشت جامد باکتری مورد استفاده قرار گرفتند و حداقل غلظت مهاری (MIC)و حداقل غلظت کشندگی (MBC)با استفاده از روش‌های شمارش کلنی و چگالی نوری تعیین شدند. اثر نانوذرات بر بیان ژن بیماری زا hrpEبا استفاده از Real Time PCR بررسی شد. زانتاموناس کمپستریس در معرض نانوذره صفر ظرفیتی آهن نشان داد که با افزایش غلظت نانو آهن نرخ رشد افزایش یافته بود .اما درصد رشد باکتری زانتاموناس کمپستریس با افزایش غلظت نانو مس کاهش یافت. میزان بیان ژن بیماری‌زا hrpE در مس و آهن به ترتیب ۹ و ۳ برابر افزایش یافته بود. نانوذرات مس و آهن اثرات مختلفی بر رشد باکتری زانتاموناس نشان دادند.