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Green synthesized silver nanoparticles for an effective control on soft rot disease pathogen *Pectobacterium carotovorum* and growth stimulation in pepper

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Abstract

Ayisigi, M., Cokislerel, A., Kucukcobanoglu, Y., Yalcin, T. & Aktas, L. Y. (2020). Green synthesized silver nanoparticles for an effective control on soft rot disease pathogen *Pectobacterium carotovorum* and growth stimulation in pepper. *Bulg. J. Agric. Sci., 26 (3)*, 574–584

This study aimed to evaluate the potential of green synthesized silver nanoparticles (AgNPs) to control soft rot disease caused by *Pectobacterium carotovorum* (Pc) in pepper (*Capsicum annuum* L.). Laurel (*Laurus nobilis* L.) leaf extract was used to synthesize silver nanoparticles. The green synthesized silver nanoparticles were characterized by ultraviolet-visible (UV-Vis) spectroscopy, fourier transform infrared (FT-IR) spectroscopy, zeta sizer-potential, inductively coupled plasma-mass spectrometer (ICP-MS), scanning electron microscopy (SEM-EDS). Antimicrobial activity of green synthesized AgNPs against Pc was assessed; minimum inhibition concentration (MIC) and minimum bactericidal concentrations (MBC) were recorded at 0.0625 and 0.125 mg mL^{-1}, respectively. According to *in vivo* tests AgNPs had a protective role against the soft rot disease by limiting disease severity up to 15% on pepper seedlings. Additionally, growth measurements hinted that green synthesized AgNPs have phyto-stimulative effect on growth of pepper seedlings. In conclusion, green synthesized AgNPs possess an effective and non-toxic solution to control soft rot disease factor *Pectobacterium carotovorum* in pepper cultivation.

Keywords: silver nanoparticle; green synthesis; pepper, *Pectobacterium carotovorum;* growth stimulation; plant protection; soft rot

Introduction

Pectobacterium carotovorum (Pc), formerly known as *Erwinia carotovora* is a facultative anaerobic, non-sporulating, gram negative, rod-shaped bacterium. The species is a dangerous plant pathogen that causes bacterial soft rot disease. It has a diverse host range including many important crop species such as most vegetables and ornamental plants like (Davidsson et al., 2013; Tsitsigiannis et al., 2008). The bacterium can cause disease in any plant tissue (Toth et al., 1999). The pectolytic enzymes of bacteria hydrolyse pectin rich middle lamella between plant cells which results in their separation from each other and cause damage to the plant. The disease can be spread by water, insects or tools like sickle (Davidsson et al., 2013; Tsitsigiannis et al., 2008). Although several cultural practices and biological control methods are recommended to prevent this factor (Aysan et al., 2003), these approaches are still far from limiting the pathogen's ability to infect crops.

Pepper is an important crop which is cultivated in 568.299 ha areas all over the world and its yield is 690.467 tonnes (FAOSTAT, 2017). The pepper production is seriously limited by *Pectobacterium carotovorum,* even the plants were not affected before harvest the pathogen still can cause damage after harvesting (Stommel et al., 1996).Common method to control of the disease depends primarily the use

of synthetic bactericides. However, bactericide resistance of bacteria, residue problem of the synthetic bactericides on fruits and environmental concerns led to search alternative methods of the disease control, such as using nanomaterials (Sotelo-Boyás et al., 2015).

Nanoparticles are characterized by higher surface/volume ratio, electron capturing capacity, conductivity, optic absorption and photoluminescence compared to their bulk form (López-Serrano et al., 2014). All these characteristics rendered them suitable for many different applications such as medicine, cosmetics and agriculture. In agriculture, nanoparticles are used to develop more efficient and eco-friendly agrochemicals (nano-formulations), or devices that can detect biotic or abiotic stresses before they can have any effect on production (nano-sensors) (Clemente et al., 2014). When compared to other metals, silver has the highest conductivity, and it has been used by mankind for about 7000 years (Chernousova & Epple, 2013), moreover, its antimicrobial potential and healing properties were known since 400 B.C. Silver is the most common nanomaterial and its antimicrobial properties are well-documented (Nowack et al., 2011).

The escalation in the use of AgNP brought the demand for environmentally-safe, practical methods for the synthesis of nanoparticles (Chinnappan et al., 2018) than conventional synthesis methods. Biological synthesis of nanoparticles from different biomaterials such as algae, fungi, yeast, bacteria or plant extracts, can be referred to as green synthesis (Chowdhury et al., 2016). Among these biomaterials, the viability and low cost of plants have rendered them more advantageous for AgNP production (Zayed et al., 2012). The green synthesis of metallic nanoparticles is mediated by biomolecules that are present in the plant biomass such as amino acids, secondary metabolites and proteins (Vijayaraghavan & Ashokkumar, 2017). Phenolic compounds, which are widely distributed in plants, are a group of secondary metabolites that possess antimicrobial and catalytic properties (Maddox et al., 2010). *Laurus nobilis* L. (laurel) is ever-green Mediterranean bush which is used as a medicinal and aromatic plant having higher secondary metabolites including phenols (Ishtiaque et al., 2015).

There are several reports about the effects green synthesized AgNPs from different extracts against plant pathogens. In one of the studies, it was stated that quercus mediated synthesized AgNPs had inhibited the growth of pathogens like *Erwinia amylovora*, *Pectobacterium carotovorum*, *Ralstonia solanacearum* and *Xanthomonas citri* (Chahardooli et al., 2014). Green synthesized AgNPs from *Piper nigrum* leaf extract showed inhibitory effects against *Erwinia cacticida* and *Citrobacter freundii* (Paulkumar et al., 2014). Recently, antibacterial effect of AgNPs synthesized from supernatant of a bacterium *Pseudomonas rhodesiae* against soft rot pathogen *Dickeya dadantii* was found (Hossain et al., 2019).

Several potential advantages of AgNPs in crop protection were reviewed by Pandey et al. (2019) and indicated its effect for minimizing inoculums build up, inhibiting pathogen growth, reducing disease severity, minimizing post-harvest losses, providing disease protection, being effective in low doses, resistant inducers and yield enhancers.

There are very limited number of reports on green or chemically synthesized AgNPs effect against pathogen *Pectobacterium carotovorum,* and these researches tested AgNP only *in vitro* conditions (Dzimitrowicz et al., 2018; Spagnoletti et al., 2019).To our best knowledge, this work is the first report for AgNP usage on plants to inhibit *P. carotovorum* infection under *in vivo* conditions. The current study aimed to evaluate the potential of *L. nobilis* leaf extract mediated green synthesized AgNPs to inhibit soft rot disease development on pepper and NPs effect on plant growth.

Materials and Methods

Synthesis and characterization of silver nanoparticles (AgNPs)

Green synthesis of silver nanoparticles was attained by *Laurus nobilis* L. (laurel) leaves. 10 g of thawed leaves were extracted with water (100 ml) at 60°C with continuous stirring. The synthesis of AgNP was performed at 90°C for two hours with the mixture of 1 mM silver nitrate ($AgNO_3$) solution and the plant extract (9:1). The reaction was stopped by centrifuge and the pellets were washed three times with distilled water.

Green synthesized silver nanoparticles (AgNPs) were characterized by various physicochemical techniques. The formation of silver nanoparticles was confirmed by UV-Visible spectroscopy (Thermo Scientific, UK) based on the presence of localized surface plasm on resonance peak. The morphology and size of the synthesized nanoparticles were determined by scanning electron microscopy (SEM) (Thermo Scientific, Apreo S-USA). Dried powdered samples of green synthesized AgNP were observed under high vacuum and 7.5 kV for Energy dispersive X-Ray spectroscopy (EDS) study. The surface potential and size of the nanoparticles were determined by zeta sizer-potential analysis (Malvern, UK). Fourier transform infrared spectroscopy (FT-IR) (Shimadzu, Japan) is a method used for organic material identification, measures the infrared radiation emission of the nanoparticle against the wavelength. By using this method, the components of silver nanoparticles which resulted from the reduction of silver nitrate, coated with laurel extract were characterized by Fourier Transform Infrared Spectroscopy (FT-IR). The silver content

of the nanoparticle was determined by Inductively Coupled Plasma – Mass Spectrometer (ICP-MS) analysis. Methylene Blue test was performed to see the catalytic degradation capacity of the nanoparticles by method described by Edison & Sethuraman, (2012). Due to absorption of thiazine ring at 664 nm Methylene Blue has a λ max value in absorption spectrum. Maximum absorption values (λ max) were compared to determine the formation of catalytic AgNPs.

***In vitro* tests**

Antimicrobial activity tests: Minimum inhibition concentration (MIC) for AgNP was determined by broth micro-dilution technique according to Clinical and Laboratory Standards Institute (CLSI) & M. P. Weinstein (2012). The lyophilized *Pectobacterium carotovorum* was provided by Ege University, Faculty of Science, Department of Basic and Industrial Microbiology. Bacterial strain was grown to exponential phase in Mueller-Hinton broth, overnight at 37°C. After which cell density of strain suspension was adjusted to 0.5 McFarland standards (1.5×10^8 CFU/ml). Serial dilutions of AgNP with concentrations ranging from 0.0039 mg mL^{-1} to 0.5 mg mL^{-1} were prepared. Different concentrations of Gentamycin (Sigma, UK) (0.0098-0.5 mg mL^{-1}) were used as a standard antibacterial reagent. The plates were incubated at 27°C for 24 h. MIC was defined as the lowest concentration of AgNP required to inhibit *P. carotovorum* growth after 24 h. Each test was performed in three replicates. Minimum bactericidal concentrations (MBC) of silver nanoparticles were evaluated by sub-culturing about 5–10 µL of wells on Mueller-Hinton agar plate for microorganisms.

***In planta* experimental design**

Pepper (*Capsicum annuum* L. cv. Yalova) was purchased from POLTAR agriculture company (Izmir-Turkey). Pepper seeds were germinated and grown under greenhouse conditions 28 ±2°C, 12/12 h (dark/light) photoperiod with 400 µE $m^{-2}s^{-1}$light intensity for a month in pots filled with perlite, sand and peat (1:1:1) soil mixture. The experiments were performed on one-month old pepper seedlings.

Pepper seeds were germinated and grown under greenhouse conditions and were watered two days intervals in field capacity. The bacteria and nanoparticles were applied by foliar spraying. Two days after the bacterial infection, leaves were treated with different concentrations of AgNP (0.0625 mg mL^{-1}; Pc+AgNP1, 0.125 mg mL^{-1}; Pc+AgNP2 and 0.25 mg mL^{-1}; Pc+AgNP3) or antibiotics (0.004 mg mL^{-1} Gentamycin). Moreover, the synergistic or antagonistic effect between nanoparticles and Gentamycin was also tested by adding another group (AgNP+Antibiotic). Adverse effects of AgNP on seedlings were assessed by applying the highest concentration of AgNP to non-infected pepper plants. Seedlings treated only with water served as control group. The disease severity observation and photosynthetic efficiency analysis were performed before harvest. After harvesting, leaf samples were kept in -20°C for biochemical analysis.

Growth analysis: After harvest, root-shoot length and fresh weight of the seedlings were measured. The dry weight measurements were performed by keeping plants at 65±1°C for 48 hours in oven.

Photosynthetic efficiency analysis: The maximal photosynthetic efficiency of photosystem II (*Fv/Fm*) of one-month-old intact seedlings was measured by photosynthetic efficiency analyser (Hansatech, UK). Before measurements, leaves were adapted to darkness for 30 min.

Photosynthetic pigment content: Acetone (80%) extract of the leaves (0.1 g) were used to determine the chlorophyll content based on the AOAC (2012) method and chlorophyll a, b, a/b, total chlorophyll and carotenoid contents were calculated.

Hydrogen peroxide (H_2O_2) content: Hydrogen peroxide content of the treated plants were determined by Sergiev et al. (2000) method. Leaves (0.1 g) was homogenized with 3 mL 0.1% trichloroacetic acid (TCA) and centrifuged at 4500 g for 30 min. Supernatants were added to 1 mM potassium iodure and 10 mM phosphate buffer at pH 7. The mixtures were used to determine H_2O_2 content with spectrophotometric measurements. A standard curve was prepared using different concentrations of hydrogen peroxide and a linear regression equation was calculated.

Monitoring AgNPs on leaves: Plant leaves were cut into small pieces and dehydrated in methanol series. Dehydrated samples were fixed with glutaraldehyde-cacodylate buffer according to method described by Neinhuis & Edelmann (1996). After fixation process, samples were critical point dried with carbon dioxide and were fixed on the stubs over carbon bands then monitored by the SEM.

Crop protection studies

Disease severity were calculated according to the method of Boyraz et al., (2006). Disease severity (S) was estimated by the equation according to the disease grade (0, no symptoms; 1, very small necrotic tissue; 2, small and a few necrotic tissues; 3, 1/3 of the leaf was necrotic tissue; 4, 2/3 of the leaf was necrotic tissue; 5, the leaf was completely covered with necrotic tissue).

Statistical analysis

Randomized complete block design was used for experimental design with three replicates. The data are presented as mean ± standard error of the mean and the error bars were

calculated by means of Statistical Package for Social Sciences (SPSS for Windows 16.0) software. To test differences among the means at the $P < 0.05$ level of significance, LSD Tests by SPSS were followed.

Results

Characterization of AgNPs

The synthesis of AgNPs using leaf extract of *Laurus nobilis* was monitored by UV–Vis spectroscopy to determine the surface plasm on resonance (SPR) of the formed AgNPs. The transparent colour of silver nitrate solution changed into brownish yellow after the addition of the leaf extract after fifteen minutes. The solution revealed a deep brown colour in 2 hours indicating that the synthesis was completed (Figure 1A). Silver nanoparticles exhibit specific surface plasm on resonance at wave lengths between 400–450 nm (Jyoti et al., 2016). UV band (λmax) of laurel leaf extract reduced and capped AgNPs was recorded between 400–450 nm indicating that AgNPs were formed (Ahmad et al., 2017). The highest peak was observed at 435 nm (Figure 1B).

The scanning electron microscope (SEM) uses a focused beam of electrons with high energy to generate different signals at the surface of solid specimens. The signals created by electron-sample interactions reveal information such as crystalline structure, external morphology (texture), chemical composition and orientation of materials in the sample (Argast & Tennis, 2004). SEM-EDS was used to determine the shape and configuration and the silver content of the synthesized AgNP. SEM analysis showed that nanoparticle size is 50 nm average and they have spherical shape (Figure 1C). The silver content was found around 94% by using EDS analysis for the green synthesized AgNP (Figure 1D).

The particle average size distribution and stability of the green synthesized AgNPs were evaluated by using zeta-potential and size analyser. The zeta potential is stability and dispersion parameter of metal nanoparticles and it also indicates the overall charge of the sample retained in solution

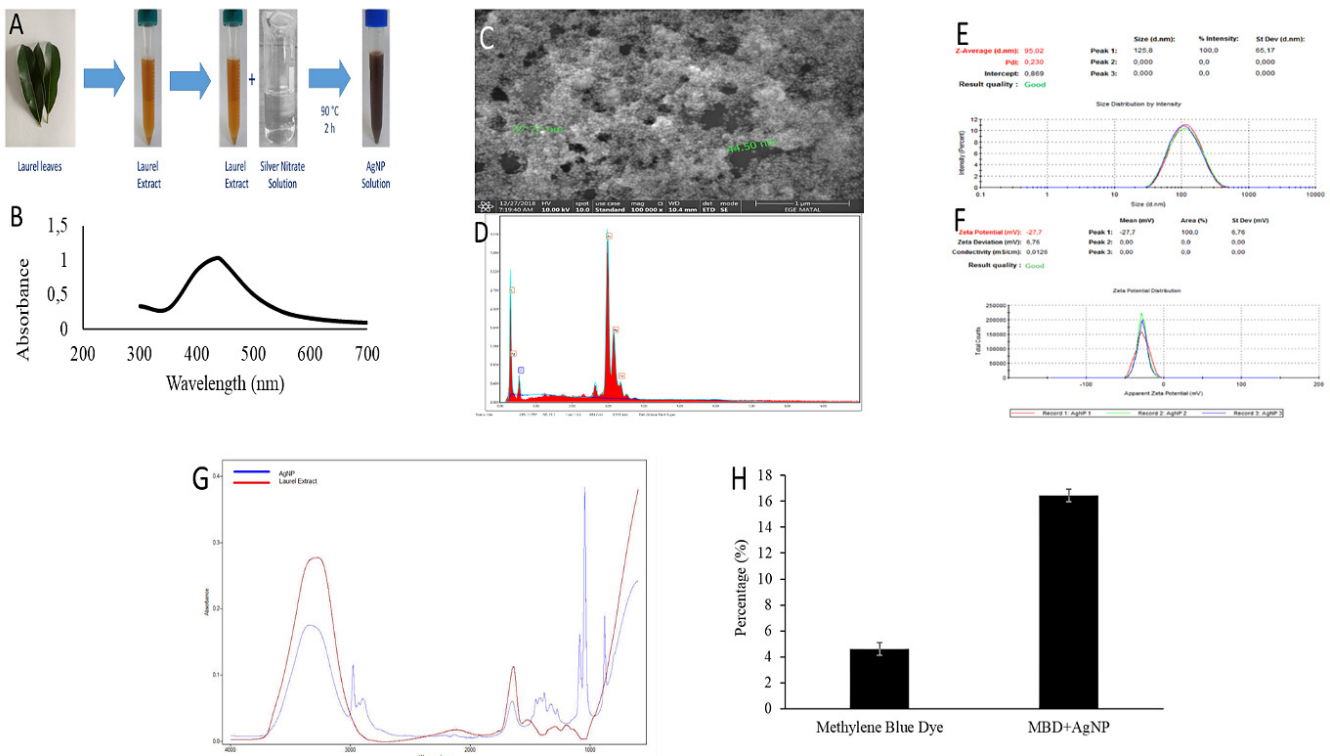

Fig. 1. (*A*) Schematic presentation of synthesis procedure of green synthesized silver nanoparticles and the conversion of silver nitrate to AgNPs with the assist of laurel extract after 2 h of incubation at 90°C; (*B*) UV–Vis spectra and specific SPR peak of AgNPs; (*C*) SEM image of green synthesized AgNPs presents the morphology and size of the nanoparticles; (*D*) EDS results and Ag percent in the sample; (*E*) Zeta size and (*F*) zeta potential of the green synthesized AgNPs; (*G*) FT-IR spectra of the green synthesized AgNPs; (*H*) Methylene blue dye decolourization activity of 1 mg mL^{-1} AgNPs

(Ahmad et al., 2017). The results showed that the particle size was equal to 95 nm and well dispersed (Figure 1E). The zeta potential was –27.7 mV for the green synthesized AgNP which indicated that the silver nanoparticles were capped with negatively charged molecules of plant (Figure 1F).

The spectra obtained by FT-IR provided the information about the present organic compounds on the nanoparticles, which may be accountable for the reduction of silver ions to AgNPs and for capping of the nanoparticles. FT-IR spectra of laurel extract and phyto-synthesized silver nanoparticles are given in Figure 1G. The extract of *Laurus nobilis* revealed three different peaks at wave number 3300, 2160 and 650 cm^{-1} that exhibited some degree shift in the corresponding silver nanoparticles. These bands may be attributed to C=C=C,–OH and C-Br stretching from polyphenolic compounds (Ahmad et al., 2017). The observed peaks in green synthesized AgNP shows C-Br stretching at 600 comes from laurel extract while C=C bending at 665 and 790 cm^{-1} that indicates alkene compounds. Also, a peak at 3271 cm^{-1} shows the shift compared to the laurel extract.

AgNPs show high catalytic activity and the increased decolourization of dyes indicates the presence of AgNPs. Decolourization percentage was calculated based on the absorbance difference. The results showed that the catalytic degradation of the dye by AgNP was responsible for the higher decolourization level which also supports the hypothesis of AgNP formation (Figure 1H).

ICP-MS elemental analysis was performed to determine the concentration of silver in AgNP. In 1 g dry nanoparticle sample, 89.08 mg silver content was detected. The silver content in nanoparticle for the highest concentration (0.5 mg mL^{-1}) was calculated as 0.0445 mg.

In vitro antimicrobial activity tests

Antimicrobial activity of green synthesized AgNPs against *P. Carotovorum* was assessed upon turbidity in wells (Figure 2A). There was no growth in the first 3 wells containing 0.5, 0.25 and 0.125 mg mL^{-1} AgNP. Hence, the MIC and MBC for *P. carotovorum* were recorded as 0.0625 and 0.125 mg mL^{-1}, respectively (Figure 2B). The antibiotic's (Gentamycin) MIC was found as 0.008 mg mL^{-1} while the MBC concentration was 0.004 mg mL^{-1}. The obtained results later were used for the *in vivo* experiments.

In planta experiments

Growth analysis: AgNP application did not cause any deleterious effect in shoot-root length and dry weights of pepper seedlings when compared to control, even it slightly enhanced shoot growth of the pepper seedlings (Figure 3A, B, C). AgNP, Pc + AgNP2, Pc + AgNP3 treatments and *P. carotovorum* infection induced root length compared to the control by 38, 27, 38, 32%, respectively (Figure 3A). Antibiotics, Pc and AgNP + Antibiotics treatments led to significant decrease in shoot length by 15, 12 and 26%, respectively while AgNP treatment caused an increase in (14%) the seedlings (Figure 3B). After *P. carotovorum* infection, lower concentrations of AgNP (Pc + AgNP1 and Pc + AgNP2) led to increase in the dry mass of the pepper plants by 25 and 38% while AgNP + Antibiotics treatment caused a significant decrease by 31% (Figure 3C).

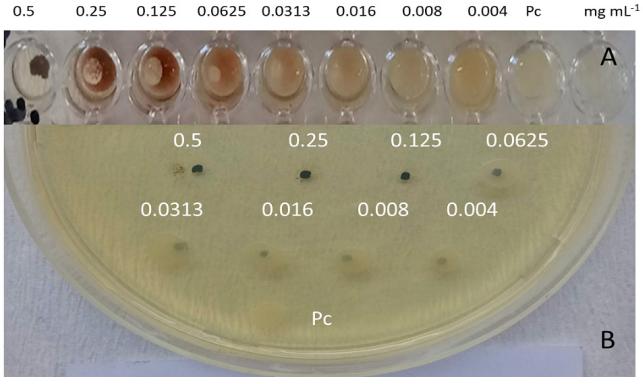

Fig. 2. Antimicrobial activity tests (*A*) MIC; (*B*) MBC test for AgNP (Pc: Only *P. carotovorum*)

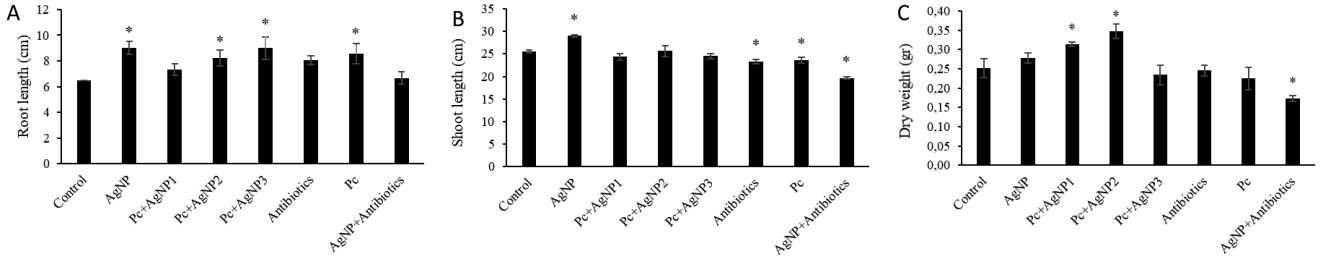

Fig. 3. (*A*) Root length, (*B*) shoot length and (*C*) dry weight measurements of pepper plants with different treatments after harvest. Bars indicate standard errors for each group.

*Statistically different at $P < 0.05$ according to LSD test

Photosynthetic efficiency analysis (Fv/Fm): *Fv/Fm* means the use of maximum efficiency at which light absorbed by PSII for reduction of Q_A. The exposure of plants to abiotic and biotic stresses in the light cause decreases in *Fv/Fm*. Among all treatments, only *P. carotovorum* infection caused 5% decrease in the *Fv/Fm* ratio (Figure 4A).

Photosynthetic pigment content: The chlorophyll a/b ratio was not significantly influenced by AgNPs and other treatments (Figure 4B). The production of chlorophyll a is generally inhibited when plants are stressed. Consequently, to compensate for the light absorption plants under stress usually increase the production of chlorophyll b due to the wider absorption spectrum of chlorophyll b which results with a decrease in chlorophyll a/b ratio (Cao et al., 2017). In this study Chl a/b ratio indicates that treatments did not cause stress on the plants. However, the seedlings treated by bacteria alone showed higher carotenoid content in comparison with other groups (Figure 4C).

Hydrogen peroxide (H_2O_2) content: The level of H_2O_2 is an important parameter to determine plant reaction to biotic and abiotic stresses. In addition, H_2O_2 is part of oxidative metabolism and is involved in various metabolism and signalling cascades such as induction of systemic acquired resistance (Kuźniak & Urbanek, 2000). The results revealed that H_2O_2 did not change in the treated seedlings except the ones treated with AgNP and antibiotics together which enhanced H_2O_2 content by 21% in seedlings (Figure 5).

AgNP monitoring on leaves: The images of the plant tissue showed the location of AgNPs on the leaf surface (Figure 6). Additionally, images of the leaf samples demonstrated the AgNPs had uptake through stomata.

Disease severity: Pepper seedlings were observed for one month (Figure 7). According to the presence of disease symptoms on leaves, the disease severity was calculated.

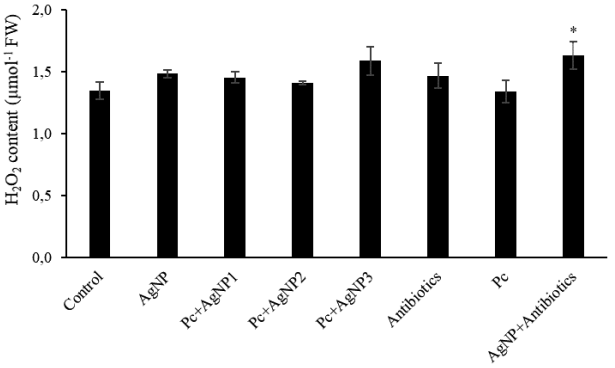

Fig. 5. Hydrogen peroxide content of pepper plants after different treatments. Bars indicate standard errors for each group.
*Statistically different at $P < 0.05$ according to LSD test

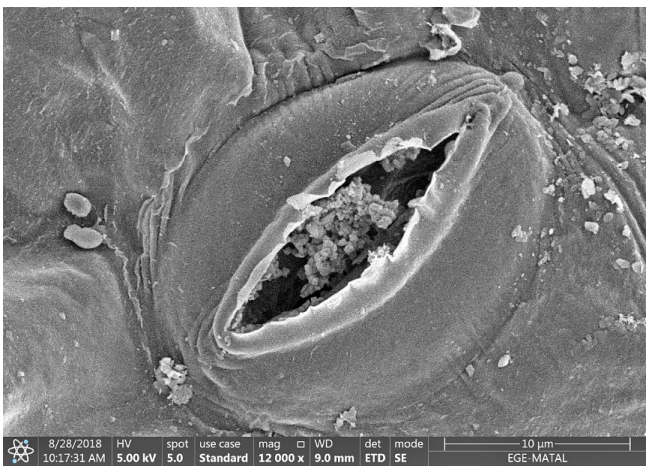

Fig. 6. SEM images of AgNP on pepper plant leaf that was sprayed after the infection which displays a nanoparticle cluster inside of the stomata

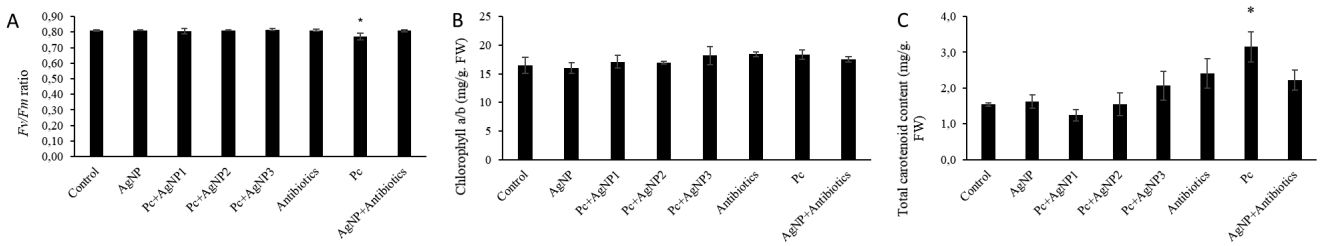

Fig. 4. (*A*) Photosynthetic efficiency (*Fv/Fm*) values of pepper plants with different treatments before harvest. Photosynthetic pigments (*B*) chlorophyll a/b (*C*) total carotenoid contents of pepper plants with different treatments after harvest. Bars indicate standard errors for each group.
*Statistically different at $P < 0.05$ according to LSD test

Table 1. Disease severity of soft rot disease on pepper seedlings

Treatments	Number of Total Leaves	Disease Severity, %
Control	218	0
AgNP	226	0
Pc + AgNP1	205	22.5
Pc + AgNP2	195	20.45
Pc + AgNP3	201	15
Antibiotics	182	0
Pc	158	56.25
AgNP+Antibiotics	170	27.41

These results showed that disease severity decreased inversely with increasing AgNP concentration. Although antibiotics treatment eliminated symptoms of the disease, yet it caused a decrease in leaf number and development. The application of AgNP + Antibiotics caused a decrease in disease severity (27.41%), however, AgNP treatments alone were found more effective (AgNP1 22.5; AgNP2 20.46 and AgNP3 15%) than AgNP + Antibiotics combined effect on pepper seedlings (Table 1).

Discussion

Characterization of silver nanoparticles (AgNPs)

The surface plasmon resonance peak located between 400 and 500 nm is characteristic for AgNPs formation (Arshadi et al., 2018) and might be result of spherical shaped nanoparticles (Zaheer, 2012) which collaborated with SEM images we obtained. SEM analysis also showed that nanoparticles are spherical in shape and have a mean size around 50 nm.

Particle size distribution shows that green synthesized AgNPs are poly-dispersed and have an average diameter ~95 nm. The measurements from the zeta sizer includes not just the particle itself, but the ions and layers of the solvent in the solution which also shows dispersion/aggregation behaviour of the particles (Costa et al., 2018; Eaton et al., 2017). Nanoparticles dispersion/aggregation behaviour is proved to be difficult to determine with dried solutions from microscopic data like SEM which results in with a difference between SEM and zeta size results for nanoparticle size. According to zeta potential results silver nanoparticles might be covered with negatively charged biomolecules which are responsible for the negative value. The negatively charged silver nanoparticles and their electrostatic interaction with

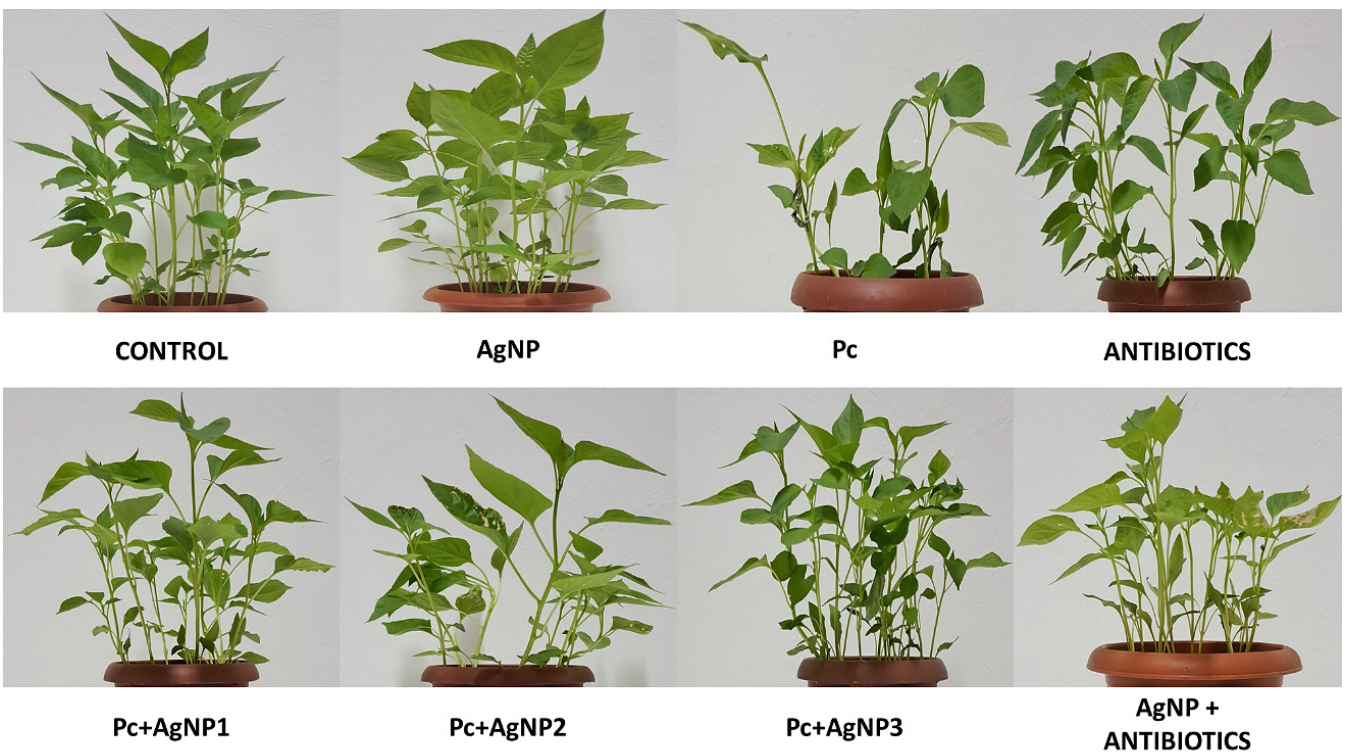

Fig. 7. The general look of different treatments on pepper plants

each other can play a part in preventing the possible aggregation, and provide long-term stability (Chowdhury et al., 2016). Polyphenolic content of the laurel leaf extract might contribute the negative potential value of AgNPs. In previous studies it was also mentioned that high negative potential value provides long term stability, good colloidal nature and high dispersity to AgNPs (Mukherjee et al., 2014).

The plant molecules which are involved in reduction of metal elements to metal nanoparticles were analysed by the FT-IR study. Plants produce free radical scavenging molecules and other metabolites that are rich in antioxidant activity (phenolics, vitamins, reducing sugar, terpenoids etc.) (Salama, 2012). The shifts in the absorbance show the changes with bonds. The decrease in the peaks between 3500–3000 shows that separated OH bonds which refer to the hydroxyl groups in phenols and alcohols. The peaks between 1250–1000 reveals the new C-O bonds were formed that might be the result of covered AgNPs. The results showed that nanoparticles are coated with phytocontent (Edison & Sethuraman, 2012). The negative potential value might be the result of the polyphenolic content of the extract based on FT-IR results.

Methylene Blue is a thiazine dye, also known as methylthioninium chloride which is a medication at the same time (Meissner et al., 2006). AgNPs and their composites are well-known for their greater catalytic activity in reduction and removal of dyes. Their role as a redox catalyst is often termed as electron relay effect (Edison & Sethuraman, 2012). The enhancement of Methylene Blue degradation rate in the presence of AgNP is a result of the electron relay effect. This result reveals that laurel leaf extract mediated synthesis of AgNPs acted as electron transfer mediators between the solution and Methylene Blue which resulted in the degradation of the dye.

ICP-MS results presented that there was very little amount (0.02225 mg silver in 1 mL of 0.25 mg mL^{-1} AgNP solution) of silver in the green synthesized AgNPs which were found as effective against *P. carotovorum*. In the previous studies it has been stated that silver NPs are biocompatible (non-toxic for human cells),due to their synthesis not involving hazardous chemicals, with high toxic effect on bacteria compared to the chemically synthesized nanoparticles (Abdelghany et al., 2018). In our study it can be seen that even with the very low concentration of silver which makes it less toxic but still effective against the bacteria.

***In vitro* antimicrobial activity tests**

There are several mechanisms for the biocidal effect of silver nanoparticles against microorganisms. These include interfering with microbial generation of reactive oxygen species, DNA replication, and contact killing (Sondi & Salopek-Sondi, 2004). The main reason for these mechanisms is the release of silver cations (Ag^{+}) that can attach to the bacterial cell wall by electrostatic forces. In addition to the effect of Ag^{+} ions, the phytochemicals coat on AgNPs might have helped in the interaction with the bacterial cell wall. The studies showed that AgNPs first attach the cell wall and change the membrane properties which effects the membrane permeability (Marambio-Jones & Hoek, 2010). Moreover, damaged membrane permeability disrupts the transport through the plasma membrane and eventually leads to cell death (Balachandran et al., 2013). It was also mentioned that the presence of phyto-constituents such as flavonoids, triterpenoids, and phenolic compounds in this leaf extract may be the cause of the enhanced antimicrobial activity (Barlanka & Gopal, 2013). *P. carotovorum* membrane consists of lipopolysaccharides (LPS) in the outer membrane which serves as an effective permeability barrier. The interaction of AgNPs with the LPS layer compromises its integrity and affects other membrane proteins hence disrupts membrane permeability (or degradation of membrane structure) (Sondi & Salopek-Sondi, 2004). The studies with the plant pathogens showed that AgNPs antibacterial activity depends on the particle size, concentration and incubation time. In one of the studies, 0.02 mg mL^{-1}Ag-based nanocomposites (Ag@ dsDNA@GO) were tested against *X. perfonans*, which is the cause of tomatoes diseases that leads to reduction in production by 10–50%, and the antibacterial activity ofAg (5 nm) @dsDNA@GO composites was achieved at0.016 mg mL^{-1} (Ocsoy et al., 2013). In another study, Hossain et al. (2019), determined that *D. dadantii* cells underwent cell death after treatment with 50 μg mL^{-1} AgNPs (20–100 nm). In this study, the determined effective concentration (MIC 0.0625 and MBC 0.125 mg mL^{-1}) has shown similarity with the results that were obtained from previous studies. The difference in the effective concentrations might be the result of the different size and treatment method of the AgNPs.

***In vivo* experiments**

After the increase of AgNP application areas, there were studies that emphasized the adverse effects of AgNPs on plant growth and development and its phytotoxic role (Tripathi et al., 2017). The literature points out the effect of AgNPs depends on several factors, including its properties (shape, size), concentration, synthesis method and conditions, as well as on the plant species applied to (Gupta et al., 2018). In May 2008, the regulation of nano-silver used in pesticide products went under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). AgNP when used as foliar spray can stop moulds, rot, fungi and other plant diseases (Sharon et al., 2010). In our study, exposure to green synthesized Ag-

NPs increased the root-shoot length, dry weights and leaf numbers. Parallel to our results, Gupta et al. (2018) reported the growth stimulatory effect of the green synthesized AgNP on rice seedlings. Moreover, it was mentioned that silver may be a growth stimulator for plants (Sharon et al., 2010).

The negative effect of the bacterial infection on shoot length was clear in the Antibiotics, bacteria, and AgNP + Antibiotics treated plants. Levard et al. (2012) showed that sulphidation has a strong effect on the properties of the AgNPs, such as surface charge, adsorbed mass of coating, aggregation state and release of Ag^+ ion. These properties will affect AgNPs fate, transport, and toxicity in the environment. Gentamycin, which is an aminoglycoside complex produced by fermentation of *Micromonospora echinospora* or *M. purpurea,* is also used as the sulfate salt. The decrease in the shoot length and dry mass resulted from the sulphidation of AgNPs in the presence of Gentamycin.

The probability of generating reactive radicals decrease when PS-II has high number of open or oxidized electron acceptors (Roach & Krieger-Liszkay, 2014). Thus, the observations revealed photo-oxidative damage in the seedlings infected with bacteria was low but there were not any adverse effects of AgNP treatments.

The photosynthetic pigments can be used as indicator of stress as well as of a plant's photosynthetic capacity (Qian et al., 2013). AgNPs synthesized by laurel leaf extract did not cause any adverse effect on photosynthetic mechanism and pigment contents which indicated the non-toxic characteristic of the nanoparticles.

Both heavy metals as well as metallic nanoparticles can cause oxidative stress and increased ROS generation in plants (Cvjetko et al., 2017). In the current study, however, the exposure of pepper plants to AgNPs after the infection with bacteria did not result in increment in ROS. Yet the treatment of AgNP and antibiotics together increased the hydrogen peroxide level due to the sulphidation of AgNP it also could be because of H_2O_2 being a signal molecule for different pathways in the plant (Levard et al., 2013; Ślesak et al., 2007).

Based on the disease severity calculations we may suggest that green synthesized AgNP has antimicrobial effect on the bacteria even after the infection of the plant. The concentration dependent decrease of disease severity was prominent and antibiotic treatment eliminated the effects of the infection. However, antibiotic treatment caused a decrease in the number of leaves. Such effect was not observed when the plants were treated with green synthesized AgNP. On the contrary, AgNPs caused an increase in the number of leaves and growth which was correlated with the results from dry mass measurements.

Conclusion

Pathogen related plant diseases like soft rot disease cause destructive effects on crop production. Using nanoparticles for plant protection has been offers an alternative against pathogens over last decades. This study showed that green synthesized silver nanoparticles inhibited soft rot disease development without causing any toxic effect on the growth of pepper plants. AgNPs even exhibited growth promoting impact on pepper seedling. Green synthesized silver nanoparticles may be used as environmentally friendly and effective tools for plant protection against soft rot disease caused by *Pectobacterium carotovorum*.

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