ANTIBACTERIAL EFFECT OF SELECTED NANOPARTICLES AS REVEALED BY DOUBLING TIME OF TREATED XANTHOMONAS CAMPESTRIS PV. CAMPESTRIS CULTURES

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Abstract: Besides many possibilities of applications of nanoparticles in the field of medicine, diagnostics, molecular biology, bioorganic chemistry or remediation of environment, there is also a potential of employment of nanoparticles as a tool for elimination and control of bacteria invading plant tissue. In this experiment an antibacterial activity of selected nanoparticles based on silver, gold and bimetallic silver/copper was tested on bacteria Xanthomonas campestris pv. campestris (Xcc) (strain 1279a). The strongest inhibitory effect represented by doubling time of treated cultures was measured in the presence of the smallest silver nanoparticles (9 nm) at the highest concentration (5 ppm).

Key Words: Nanoparticles, Xanthomonas campestris, doubling time, antibacterial effect

INTRODUCTION

Borm et al. (2006) consider nanoparticles (NPs) as particles with size from 1 to 100 nm. Properties of NPs depend on the used material, size, shape and their surface area. Recently, the use of NPs in industry and in manufacture of commercial products recorded large increase. Understanding of interaction mechanisms between NPs and biological systems on the molecular level is still insufficient (Barren et al. 2009). Simultaneously, new ways of NPs applications are found but many of that are still in the phase of testing (Ngomsik et al. 2005, Uheida el al. 2006). One of the most important properties of metal nanoparticles is their antibacterial activity. Nanoparticles slow down growth of bacteria or eliminate bacteria by disrupting the function of the cell wall (Ahmed et al. 2016). The antibacterial properties of silver NPs are widely employed in healthcare industry and they can also be used for the control of bacterial and fungal plant diseases (Yo et al. 2009). Similar effects have nanoparticles based on copper (Cu NPs) or titanium dioxide (Giannousi et al. 2013, Paret et al. 2013). On the other hand, certain concentrations of zinc oxide NPs proved an inhibition of seed germination and root elongation (Hrdinová 2011). Study focused on the application of gold nanoparticles on aquatic plant Ceratophyllum demersum showed an inhibitory effect as well (Ostroumov et al. 2014).

Xanthomonas campestris is a gram-negative quarantine bacterium belonging to strain of proteobacteria. This bacterium has been genetically divided into more than 140 pathovars and each of them has a different range of hosts. Xanthomonas campestris pv. campestris (Xcc) usually infects system of species from Brassicaceae family and exhibits characteristic symptoms as black rot caused by blackening of vascular bundles and V-shaped necrosis proceeding from the edges to central vessel (Park et al. 2004). Xcc belongs to seed-borne pathogens and it enters plants through hydathodes, stomata, roots or injuries. Xcc infects a wide scale of Brassicaceae family species including cabbage, cauliflower, broccoli, radish, or Arabidopsis. Therefore, this bacterium is an economically important pathogen (Williams 1980). The epidemics of Xcc are repeated worldwide and regularly while areas with high temperature and humidity are the most affected. Thus, significant losses of agricultural products are
caused by Xcc epidemics (Qian et al. 2005). Despite increasing knowledge, there are no effective approaches for elimination of Xcc from seeds and crops.

MATERIAL AND METHODS

The nanoparticles were purchased from NPIC s.c. (G. Celichowski J. Grobelny, Poland). Silver nanoparticles were tested in 9, 19, 35 and 61 nm sizes, gold NPs in 9, 19 and 35 nm sizes and silver-copper NPs in sizes of 29 and 69 nm. Particular emphasis in the choice of supplier was placed on ability to get nanoparticles in solutions without any biological toxicity for biologically active systems such as plants or bacteria. Dissolving medium was used as a control in this experiment.

Measurement of inhibitory effect of NPs against Xcc

For testing, the strain 1279a of *Xanthomonas campestris pv. campestris* was chosen. The strain was obtained from the collection of The University of Warwick HRI (UK). Antibacterial activity of NPs was tested by spectrometric measurement of growing activity under influence of different types of nanoparticles. For this measurement, the spectrophotometer SpectraMax PLUS384 from Molecular Devices (Sunnyvale, California) and 96-well plates were used.

Firstly, Xcc were cultivated overnight in liquid LB medium (20 ml of medium, 30 °C, 160 rpm). Obtained substance was used to inoculate experimental cultures. Cultivation of bacteria with added nanoparticles was performed in 96-well plates. The final volume of cultures was 200 µl. Cultures were inoculated into a new LB medium (initial OD$_{600}$ nm=0.08). The final concentration of NPs (5; 3.75; 2.5; 1.25; and 0.5 ppm) was added into the cultures. As a control, a reference solution (NPIN s.c.) was used. Each type and each concentration of NPs was prepared in triplicate. Cultures were incubated at 30  °C with continual mixing (100 rpm) for 6 hours. During the cultivation, the optical density (600 nm of wavelength) of cultures was measured. Sterile LB medium was used as a blank. Interval of measurement was determined on every 10th minute. Values of obtained ODs were used for calculation of bacteria doubling time (T min.). Software SoftMax Pro v4.6 (Molecular Devices, Sunnyvale, California), Statistica and Microsoft Excel were employed for data acquisition and evaluation.

RESULTS AND DISCUSSION

Au NPs

The highest increase of Xcc doubling time (31 minutes) with gold nanoparticles was measured at the size of 19 nm with a concentration of 5 ppm (see Figure 1). The doubling time was also increased at the 35 nm Au NPs but the effect was not as strong as at 19 nm Au NPs (less than 15 minutes at the concentration of 5 ppm). In most of the concentrations of 9 nm Au NPs the doubling times were below the control. The explanation of this phenomenon requires additional experiments but is out of our scope to search for growth inhibition.

Ag/Cu NPs

Description of Ag/Cu-based NPs antibacterial activity is possible to find in literature (Singh et al. 2014, Yasir et al. 2016). The aim of including this type of NPs was to verify the possibility of synergistic effect of silver-copper composited nanoparticles. The doubling time was affected at the variant with size of 29 nm in a concentration of 5 ppm (16 minutes). Variants with lower concentrations as well as 69 nm sizes didn’t have any significant effect on the doubling time (see Figure 2).

Ag NPs

Four kinds of Ag NPs with diameter of 9, 19, 35 and 61 nm were tested. Variant of 35 nm diameter showed statistically significant effect with concentrations of 1.25 ppm and higher. Other sizes described within Fig 3 (19 nm, 61 nm) showed significant effect from the concentration of 2.5 ppm and higher. An influence of concentration is noticeable, when in the case of variants with the highest concentration (5 ppm) the difference of doubling time compared to control was from 18 to 21 minutes (see Figure 3).
Figure 1 Doubling time of Xcc 1279a in the presence of 9, 19, and 35 nm Au NPs

* Statistically significant differences compared to control are highlighted as hatched columns

Figure 2 Doubling time of Xcc 1279a in the presence of 29 and 69 nm Ag/Cu NPs

* Statistically significant differences compared to control are highlighted as hatched columns

Figure 3 Doubling time of Xcc 1279a in the presence of 19, 35 and 61 nm Ag NPs

* Statistically significant differences compared to control are highlighted as hatched columns
A unique position in the context of this study was showed by the variant of 9 nm diameter Ag NPs. Doubling time of 5 ppm concentration was increased by 1016 minutes compared to the control. This is many times more than other tested variants. Significantly above the average of the experiment, it was also the variant with the concentration of 3.75 ppm (doubling time increased by 151 minutes) (see Figure 4).

**Figure 4 Doubling time of Xcc 1279a in the presence of 9 nm Ag NPs**

*Statistically significant differences compared to control are highlighted as hatched columns*

There are many studies focused on the antibacterial activity of nanoparticles. Nanoparticles have an influence on the growth and development of bacteria and development of bacterial biofilm. Shristavata et al. (2007) reported 60% slowdown of the gram-negative bacterium *Escherichia coli* grown in LB medium in the presence of silver nanoparticles at a concentration of 5 mg/ml. Concentration of 10 mg/ml showed 90% growth retardation and concentration of 25 mg/ml showed complete inhibition. On the other hand, gram-negative bacteria *Staphylococcus aureus* showed no slowdown at a concentration of 25 mg/ml of Ag NPs and concentration of 100 mg/ml showed only partial slowdown. Thus there are indications that the antibacterial effect of individual nanoparticles is specific for each species and before targeted application it is necessary to verify this effect on the specific bacterium. Based on our preliminary study, use of Ag NPs against Xcc seems to be promising.

Gold nanoparticles used in this study didn’t prove so high inhibitory effect as for silver NPs. But interestingly, Mu et al. (2016) published a study which showed significantly higher antimicrobial properties of chitosan-streptomycin conjugates bound to the gold nanoparticles. These substances had higher ability to eliminate biofilms of gram-positive and gram-negative bacteria and proved to be more effective than chitosan conjugates, or streptomycin alone. However, limited availability of this type of nanoparticles didn’t let us to verify these effects against Xcc, we received information only about the unmodified Au NPs.

Ag/Cu NPs showed the smallest effect on the doubling time of Xcc cultures from all three types of NPs used in this experiment. On the other hand, Giannousi et al. (2013) published study describing the positive antibacterial effect of copper-based nanoparticles. *Bacillus cereus*, *Bacillus subtilis*, *E. coli*, *X. campestris* and *S. aureus* were exposed to Cu and Cu$_2$O NPs. The half-maximal inhibitory concentration (IC$_{50}$) and 100% inhibitory concentration (IC$_{100}$) were determined and were various depending on the size, type and concentration of nanoparticles. However, the results of our study didn’t confirm potential synergistic effect of these composite nanoparticles. Effect of Ag/Cu NPs was smaller than Ag NPs.

**CONCLUSION**

The results of this experiment showed as the most effective silver-based nanoparticles. Ag NPs 9 nm size variant at a concentration of 5 ppm showed an increase of Xcc doubling time by more than 570% compared to control. Same size of these nanoparticles at concentration of 3.75 ppm also increased
the doubling time of Xcc by more than 84%. The best result for the gold nanoparticles was shown in the case of 19 nm size at the concentration of 5 ppm (doubling time extended by 19%). Bimetallic Ag/Cu NPs showed the slightest antibacterial effect of all tested nanoparticles. Although the effects of nanoparticles on biological systems are intensively studied, the exact mechanism of their action is not completely understood. There is no possibility to exactly determine which property of the nanoparticles is the most important in its antimicrobial effect. Acquired results represent solid basis for further experiments.

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REFERENCES


