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# Eliminating the Pathogen *Xanthomonas hortorum* pv. *carotae* from Carrot Seeds Using Different Types of Nanoparticles

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**Abstract:** There exists a wide range of plant pathogens that are commonly referred to as seed-borne pathogens due to their dominant mode of spread. Treating seeds to eliminate such pathogens is therefore very important in contemporary seed production. In the present study, eight types of nanoparticles were evaluated for their effectiveness against *Xanthomonas hortorum* pv. *carotae*, a seed-borne pathogen that affects plants of the Apiaceae family. Initially, parameters considering the inhibitory and bactericidal activity of individual nanoparticles were evaluated under in vitro conditions. In this way, three nanoparticles based on copper, silver, and silver/selenium composite were identified as being the most effective. Subsequently, their ability to eliminate *Xanthomonas hortorum* pv. *carotae* from artificially infected carrot seeds was tested. This was achieved through the qPCR quantification of the pathogen in 14-day-old plantlets developed from seeds inoculated with Xhc. Based on the obtained results, copper-based nanoparticles were the most effective, resulting in an approximately 10-fold decrease in the occurrence of Xhc in plantlets compared to the untreated control. Taking into account the fact that *X. hortorum* pathovars also attack other important horticultural crops, the presented results may have a much wider scope than just carrot seeds.

**Keywords:** *Xanthomonas hortorum*; carrot; seed treatment; nanoparticles; pathogen elimination



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## 1. Introduction

There are a number of plant pathogens that threaten crop production. In terms of economic loss, it is estimated that 10 bacterial [1] and fungal pathogens [2] cause the greatest economic losses worldwide. According to Mansfield et al. [1], among the 10 most economically harmful bacteria, three species are from the genus *Xanthomonas*: *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas campestris* pathovars, and *Xanthomonas axonopodis* pathovars.

Pathogens of the genus *Xanthomonas* also include *Xanthomonas hortorum* pv. *carotae* (Xhc), which is capable of successfully colonizing the leaves of plants in the Apiaceae family [3]. Hosts from farm crops include common carrot (*Daucus carota* L.), garden parsley (*Petroselinum crispum* (Mill.) Fuss, 1886), root celery (*Apium graveolens* L.), lovage (*Levisticum officinale* Hill, 1756), and dill (*Anethum graveolens* L.) [4].

In the these plant species, Xhc causes a disease known as bacterial blight of carrot, characterized by round, water-soaked lesions on the abaxial surface of leaves, which reduces their photosynthetically active area [4]. In the final infection stages, leaves usually harden and dry. However, the leaves may not be the only parts that are damaged by the infection. Xhc can also damage petioles, peduncles, stems, flowers, and leaflets. A comprehensive review of the taxonomy, genomics, and symptoms is presented by Dia et al. [3].

From an epidemiological standpoint, Xhc represents a bacterium capable of transmission through seeds [5]. Therefore, it not only poses challenges for vegetable producers, but also presents serious complications for seed producers of economically important plants of the Apiaceae family [5,6]. Thanks to the global mass distribution of plant products, including seeds, the ease of pathogen spread has become a highly relevant contemporary issue [7].

Regarding the possible approaches for eliminating seed-borne pathogens, there are more or less traditional techniques, ranging from the use of fungicides/bactericides to physical methods such as heat or UV treatment, and include the utilization of natural substances or treatment by biological control agents [8]. From a historical perspective, one of the oldest preventive measures for seed decontamination is hot water treatment. The first deliberate hot water treatment of seeds can be traced back to the late 19th century [9]. This method is still used today, with great significance as a preventive measure, particularly for seed-borne pathogens such as *Alternaria* spp. [9,10] and *Xanthomonas campestris* pv. *Campestris* [10]. The frequently mentioned negative aspects of hot water treatment include its questionable effectiveness in the complete elimination of the pathogen [11] and the reduced germination in seeds treated in this manner, as demonstrated for *Xanthomonas campestris* pv. *vitians* by Carrise et al. [12]. There are also approaches that use chemical substances to eliminate seed-borne pathogens, such as 1% sodium hypochlorite, as reported by Carrise et al. [13], and 5% hydrogen peroxide, as reported by Perneczny et al. [14]. A comprehensive review of currently available individual techniques is presented by Moumni et al. [8].

However, some newer approaches are emerging and could theoretically serve as more efficient or environmentally friendly alternatives for seed treatment. One of them is the use of nanoparticles, due to their antimicrobial properties [13]. Their mechanism of action can differ depending on their chemical composition, but the principle of their antimicrobial properties is usually described as: (i) disruption of the peptidoglycan layer of the bacterial cell wall [14], (ii) toxicity by the release of metal ions in cytoplasm that lead to imbalanced nutrient uptake [15], (iii) leakage of intracellular components, ion imbalance, and eventual cell lysis [16], (iv) ROS induction and antioxidant production [17], and (v) interaction with bacterial DNA, leading to strand breaks and consequent disruption of vital cellular functions [18]. Comprehensive overviews of the observed mechanisms of the action of nanoparticles against phytopathogens are presented by Adisa et al. [19] and Ali et al. [20]. Regarding the use of nanoparticles to protect against pathogens within the *Xanthomonas hortorum* group, there are reports of applying Ag-dsDNA-GO nanoparticles for protection against copper-tolerant strains of *X. hortorum* pv. *gardenia* [21]. Additionally, simonkolleite nanoparticles were used for seed treatment to provide protection against tomato bacterial spot caused by *Xanthomonas hortorum* pv. *gardneri* [22]. The effects of titanium dioxide-based nanoparticles were also tested for their potential protective properties against *Xanthomonas hortorum* pv. *pelargonii* [23] after foliar application.

The prevailing method of employing nanoparticles to manage phytopathogens involves applying the treatment primarily by spraying leaves or entire plants [24]. On the other hand, only a limited number of articles cover the application of nanoparticles to suppress seed-borne pathogens [11,25–28]. This is perhaps a little surprising, considering that treating seeds with nanomaterials may be more environmentally friendly and safer than spraying whole plants in a field [29]. Thus, treating seeds with nanomaterials to protect them against phytopathogens remains a challenge, especially in the situation where a synergistic effect in terms of improved growth parameters can be observed after seed treatment [24]. However, it remains necessary to fill important gaps in the knowledge before recommending broader application of seed treatment with nanoparticles in practice.

The aim of the present study was to contribute to this field of research by testing eight nanoparticles to identify critical points in this kind of application. Our attention was focused mainly on factors such as the concentration and effective composition of nanoparticles and practical limitations. As an experimental model, we selected a combination of Xhc,

a seed-borne pathogen, and carrot seeds artificially inoculated by this pathogen. Based on the obtained results, the most suitable variants were identified, and it was possible to formulate some recommendations. These recommendations will enable more effective application of nanoparticles to eliminate problematic seed-borne pathogens.

## 2. Materials and Methods

### *2.1. Xhc Strain and Growth Conditions*

Xhc strain NCPPB 4410, obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB, London, UK), was maintained on Luria agar (LA; HiMedia, Mumbai, India) at 28 °C for 24 h and stored in cryotubes at −80 °C for long-term storage. For all experiments, the Xhc strain was cultured in Luria broth (LB; HiMedia, Mumbai, India) at 28 °C for 24 h at 150 rpm on an orbital shaker (Biosan, Riga, Latvia) and the bacterial suspension was adjusted to a concentration of $1 \times 10^8$ cfu $mL^{-1}$, based on optical density at 600 nm ($OD_{600}$) using a spectrophotometer (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). The bacterial suspension was serially diluted 10-fold to a concentration of approximately $1 \times 10^6$ cfu $mL^{-1}$ using LB for the in vitro experiment and sterile phosphate-buffered saline (PBS) for the in planta experiment.

### *2.2. In Vitro Experiment: Evaluation of Nanomaterials for Antibacterial Activity under In Vitro Conditions*

Eight nanomaterials with different properties (Table 1) were tested against Xhc strain NCPPB 4410 in in vitro assays. All tested nanomaterials were provided by the Department of Chemistry and Biochemistry, Mendel University in Brno, Czech Republic, and their properties were determined using standard procedures, as presented in the references listed in Table 1.

**Table 1.** Nanomaterials tested against Xhc strain NCPPB 4410 in in vitro assays and dilutions used for determination of minimum bactericidal concentration.

| Nanomaterial | Size | Shape | Concentration of Stock Solution (mg $L^{-1}$) | Dilution Factor of Stock Solution |
|---|---|---|---|---|
| AgNPs_29 [11] | 2 nm | Spherical | 100 Ag | 10×–100× ** |
| AgNPs_30 [11] | 22 nm | Spherical | 100 Ag | 2×–8× *<br>10×–100× |
| AgSeNPs_8 [30] | 60 nm | Cluster plates | 3703 Ag<br>748 Se | 10×–100×<br>200×–1000× *** |
| CuNPs_50 [30] | 10 nm | Spherical | 545 Cu | 2×–8× |
| CuNPs_53 [30] | 100 nm | Spherical | 3974 Cu | 10×–100× |
| rGO-Cu_25 [31] | Flakes, 1–10 nm thickness | Plate | 1694 Cu | 2×–8× |
| rGO [32] | Flakes, 1–10 nm thickness | Plate | 3100 rGO | 2×–8× |
| SeNPs_40 [30] | 150 nm | Spherical | 517 Se | 2×–8× |

* Stock solutions of nanomaterials were diluted by 2×, 4×, and 8× with sterile Luria broth. ** Stock solutions of nanomaterials were diluted by 10×, 20×, 30×, 40×, 50×, 60×, 70×, 80×, 90×, and 100× with sterile Luria broth medium. *** Stock solutions of nanomaterials were diluted by 200×, 400×, 500×, 600×, 700×, 800×, 900×, and 1000× with sterile Luria broth medium.

To evaluate the antibacterial effect of nanomaterials, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined, according to da Silva et al. [33]. The MIC was determined using the microdilution method on 96-well microplates. Each well had a total volume of 100 µL and contained a suspension of Xhc strain NCPPB 4410 (approximately $1 \times 10^6$ cfu $mL^{-1}$) and nanomaterials at the concentrations shown in Table 1 diluted with sterile LB. Bacterial suspension without nanomaterials served as a positive control. The microplates were incubated on the shaker (Biosan, Riga, Latvia) for 24 h and $OD_{600}$ was measured using the spectrophotometer (SPECTROstar

Nano, BMG Labtech, Ortenberg, Germany). MBC was determined by subculturing 5 µL of sample from each well on LA without the antimicrobial agent and incubation at 28 °C for 24 h. MBC was considered as the lowest concentration that prevented detectable bacterial growth. All assays were carried out in triplicate.

A time-kill assay was performed to determine the time required for the effective elimination of Xhc from carrot seeds, according to NCCLS [34]. Briefly, 5 mL of nanomaterial solution at the concentration determined as MBC (10 mg $L^{-1}$ of Ag for AgNPs_29, 397 mg $L^{-1}$ of Cu for CuNPs_53, and 53 mg $L^{-1}$ of Ag and 11 mg $L^{-1}$ of Se for AgSeNPs_8) were mixed at a 1:1 ratio with bacterial suspension in LB at a concentration of approximately $1 \times 10^6$ cfu $mL^{-1}$. The same volume of bacterial suspension without nanomaterials served as a growth control. Samples were incubated at 28 °C and 150 rpm on the orbital shaker (Biosan, Riga, Latvia), and 100 µL of tested suspensions was sampled at 14 points: 0, 10, 20, 30, 40, 50, and 60 min and 2, 3, 4, 5, 6, 12, and 24 h of incubation. Samples were serially diluted 10-fold using sterile phosphate-buffered saline (PBS) and cultivated on LA at 28 °C for 24 h. After incubation, the colonies visible on the medium were counted.

### *2.3. In Planta Experiment*

#### 2.3.1. Artificial Inoculation of Carrot Seeds with Xhc Strain

Prior to artificial inoculation with the Xhc strain, the cv. Galaxy carrot seeds were disinfected with hypochlorite solution at a concentration of 2.5% for 2 min, rinsed with sterile distilled water 5 times, and air-dried on filter paper in a laminar flowbox for 30 min. Disinfected seeds were inoculated with Xhc strain NCPPB 4410 suspended in sterile PBS at a concentration of approximately $1 \times 10^6$ cfu $mL^{-1}$ using a vacuum, according to Roberts et al. [35]. Seeds inoculated with sterile PBS were used for negative control.

#### 2.3.2. Treatment and Germination of Seeds with Nanomaterials

Based on the in vitro experiment, 3 of the most effective nanomaterials were selected: AgNPs_29, AgSeNPs_8, and CuNPs_53. To evaluate the effectiveness of the selected nanomaterials in eliminating Xhc from seeds, the carrot seeds previously inoculated with Xhc were treated as follows: 200 seeds per treatment were soaked in 10 mL of the nanomaterial solution in 25 mL sterile Erlenmeyer flasks at room temperature on an orbital shaker (Biosan, Riga, Latvia) set to 150 rpm. The nanomaterials were diluted with sterile PBS at concentrations determined as MBC values during the in vitro experiment (see Table 1). To verify whether the concentration of nanoparticles correlated with their effectiveness, concentrations higher than the MBC were also tested (see Table 2). The treatment duration was selected based on the time-kill assay results: 24 h for AgNPs_29, 2 h for AgSeNPs_8, and 4 h for CuNPs_53. For the PBS-treated control, an equivalent volume of sterile PBS was used instead of nanomaterials, while the non-treated control consisted of seeds with no treatment applied after inoculation. After the treatment, the solutions were removed and seeds were transferred to sterile filter paper and air-dried in a laminar flowbox for 30 min. Treated and control seeds (50 seeds per treatment in 4 repetitions) were sown on moist sterile filter paper in transparent plastic boxes and germinated at 22 °C for 14 days, with a 16 h light/8 h dark photoperiod. Subsequently, 30 seedlings without testa from each experimental variant and repetition were randomly selected and homogenized with 2 mL sterile PBS using extraction bags (Bioreba, Reinach, Switzerland). DNA was extracted from a 500 µL homogenized sample using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol, resulting in a final volume of 50 µL. To evaluate the possible phytotoxic effect of nanoparticles, carrot seeds were treated with the highest tested concentration of nanomaterials (25 mg $L^{-1}$ of Ag for AgNPs_29, 993 mg $L^{-1}$ of Cu for CuNPs_53, 74 mg $L^{-1}$ of Ag and 15 mg $L^{-1}$ of Se for AgSeNPs_8) and germinated under the same conditions as described above. After 14 days, the number of germinated seeds was counted, and the seedlings were photographed and subsequently checked for possible deficiencies in growth and abnormalities in root and leaf anatomy.

**Table 2.** Nanomaterials tested against Xhc strain NCPPB 4410 in in planta experiment and their concentrations with regard to established minimum bactericidal concentration.

| Nanomaterial | Df | Concentration (mg $L^{-1}$) | | | |
|---|---|---|---|---|---|
| AgNPs_29 | 10× | **10** | Ag | | |
| | 8× | 13 | | | |
| | 4× | 25 | | | |
| CuNPs_53 | 10× | **397** | Cu | | |
| | 8× | 497 | | | |
| | 4× | 993 | | | |
| AgSeNPs_8 | 70× | **53** | Ag | **11** | Se |
| | 60× | 62 | | 13 | |
| | 50× | 74 | | 15 | |

Df: dilution factor used to dissolve stock solution of nanomaterial. Stock solutions were diluted with sterile phosphate-buffered saline. Concentrations of nanomaterials determined as minimum bactericidal concentration are highlighted in bold.

### *2.4. Detection of Xhc in Carrot Seedlings*

The detection of Xhc infection in carrot germinated plants was performed using real-time PCR assays based on SYBR Green dye, targeting the hypersensitive response and pathogenicity-associated phosphatase (*hpaP*) gene. The assay employed T3S_fwd (5′-CAATTGCCCTCATCTACGCA-3′) and hpaP_rev2 (5′-CTTCATGCAACTGCGACGAC-3′) primers [36]. All reactions were performed in triplicate using Luna qPCR Master Mix (NEB, Ipswich, MA, USA) following the manufacturer's instructions, and the thermal profile was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 45 s, and 70 °C for 10 s. Amplification was immediately followed by a melting analysis with a temperature ramp from 80 to 95 °C in 0.5 °C increments. All analyses were performed on the qTOWER$^3$ instrument (Analytic Jena, Jena, Germany) using qPCRsoft v.4.0 software (Analytic Jena, Jena, Germany). Based on the values obtained during the construction of the standard curve, the detection limit of the assay was determined to be $10^1$ cfu $mL^{-1}$. Xhc quantification used a cut-off $C_t$ value of 32 and a standard curve with the following characteristics: $R^2$ = 0.99753, slope −3.21, and efficiency 105%. The melting temperature of the targeted amplified product ranged from 89.6 to 90.5 °C.

### *2.5. Statistical Analysis*

Differences in the concentration of Xhc cells detected in germinated plants and in the germination rate of seeds treated with the most effective NPs were identified using variance analysis (Kruskal–Wallis ANOVA) and multiple comparison analysis. A significance level of $\alpha$ = 0.05 was used in the data analysis. All statistical analyses were carried out using the STATISTICA statistical software package (version 12, StatSoft Inc., Tulsa, OK, USA).

## 3. Results

### *3.1. Antibacterial Activity of Tested Nanomaterials In Vitro*

#### 3.1.1. MIC and MBC Determination

The antibacterial effect of eight nanomaterials against Xhc NCPPB 4410 was first evaluated in vitro by determining the MIC and MBC values (Table 3). The lowest MIC (3 mg $L^{-1}$) was observed for two silver NPs of different sizes: AgNPs_29 (2 nm) and AgNPs_30 (20 nm), with stock solutions diluted 40×. AgNPs_29 also had the lowest MBC (10 mg $L^{-1}$), while AgNPs_30 had no bactericidal effect even at the highest tested concentration (stock solution diluted 2×, 50 mg $L^{-1}$). AgSeNPs_8 also showed high activity against Xhc strain, with an MIC at 19 mg $L^{-1}$ of Ag and 4 mg $L^{-1}$ of Se (stock solution diluted 200×). Bactericidal activity of AgSeNPs_8 was observed with the use of 70× diluted stock solution, with 53 mg $L^{-1}$ of Ag and 11 mg $L^{-1}$ of Se. Between the two copper NPs, which differed in size, only the larger CuNPs_53 (100 nm) exhibited inhibitory and bactericidal effects. The MIC for CuNPs_53 was 199 mg $L^{-1}$ and the MBC was 397 mg $L^{-1}$

(stock solution diluted 20× and 10×, respectively). The MIC was slightly lower for rGO-Cu_25 than CuNPs_53 (169 mg $L^{-1}$); however, bactericidal activity was observed only at a concentration of 847 mg $L^{-1}$, approximately two times higher than that of CuNPs_53. The second nanomaterial based on rGO, but without CuNPs decorated on the surface, exhibited no antibacterial effect even at the highest tested concentrations, where the stock solution was mixed with bacterial suspension at a 1:1 ratio (1550 mg $L^{-1}$ rGO). Based on these results, three of the most effective nanomaterials were selected for subsequent in planta experiments: AgNPs_29, AgSeNPs_8, and CuNPs_53.

**Table 3.** Antibacterial efficacy of tested nanomaterials against Xhc strain NCPPB 4410 in vitro based on measured minimum inhibition concentration and minimum bactericidal concentration.

| Nanomaterial | MIC (mg $L^{-1}$) | | | | MBC (mg $L^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | Df | Ag | Cu | Se | Df | Ag | Cu | Se |
| AgNPs_29 | 40× | 3 | - | - | 10× | 10 | - | - |
| AgNPs_30 | 40× | 3 | - | - | NE | - | - | - |
| AgSeNPs_8 | 200× | 19 | - | 4 | 70× | 53 | - | 11 |
| CuNPs_50 | NE | - | - | - | NE | - | - | - |
| CuNPs_53 | 20× | - | 199 | - | 10× | - | 397 | - |
| rGO-Cu_25 | 10× | - | 169 | - | 2× | - | 847 | - |
| rGO | NE | - | - | - | NE | - | - | - |
| SeNPs_40 | NE | - | - | - | NE | - | - | - |

Df: dilution factor used to dissolve stock solution of nanomaterial. Stock solutions were diluted with sterile Luria broth medium. NE: not effective at highest tested concentration (stock solution diluted 2×).

#### 3.1.2. Treatment Duration Determined by Time-Kill Assay

To assess the minimal time necessary for eliminating Xhc cells in vitro by the three selected nanomaterials at a concentration equal to MBC, a time-kill assay was performed. The kinetics of killing Xhc strain NCPPB 4410 (Figure 1) shows that AgSeNPs_8 (53 mg $L^{-1}$ Ag and 11 mg $L^{-1}$ Se) eliminated bacterial cells after 2 h of treatment, while for CuNPs_53 (397 mg $L^{-1}$ Cu) it took twice as long (4 h). The longest time to exhibit a bactericidal effect was observed for the nanomaterial with the lowest MBC, AgNPs_29 at concentration 10 mg $L^{-1}$, which required 24 h of incubation. Based on the killing kinetics, the treatment time for each nanomaterial was assessed, and the data were utilized in the in planta experiment.

### *3.2. Effectiviteness of AgNPs_29, CuNPs_53, and AgSeNPs_8 in Elimination of Xhc in Planta*

The effectiveness of the NPs in eliminating Xhc infection from germinated plants was tested by real-time PCR (Figure 2). For all the tested treatments on non-inoculated seeds, the absence of Xhc cells was confirmed. In the case of seeds inoculated with Xhc suspension, the results obtained in the in vitro assessment of antibacterial activity were not confirmed in planta. Specifically, complete elimination of Xhc infection from seeds was not achieved at any of the NP concentrations. The highest treatment effect was observed for CuNPs_53 with a 4× diluted stock solution (Cu concentration corresponding to 993 mg $L^{-1}$), which showed a statistically significant decrease in the concentration of Xhc cells in germinated plants to $3.12 \pm 1.63 \times 10^5$ cfu $mL^{-1}$. This value is statistically lower than the amount of Xhc registered in non-treated and PBS-treated controls. In the case of AgNPs_29 and AgSe_NPs_8, Xhc cells were detected in germinated plants at a rate that was not statistically different from the control, even when the highest concentrations of these NPs were used. On the other hand, there was an evident tendency of Xhc decreasing with increasing concentration of nanoparticles used for seed treatment. Worth noting is the reduction of

Xhc in the PBS-treated control, which was, however, statistically inconclusive compared to the non-treated control.

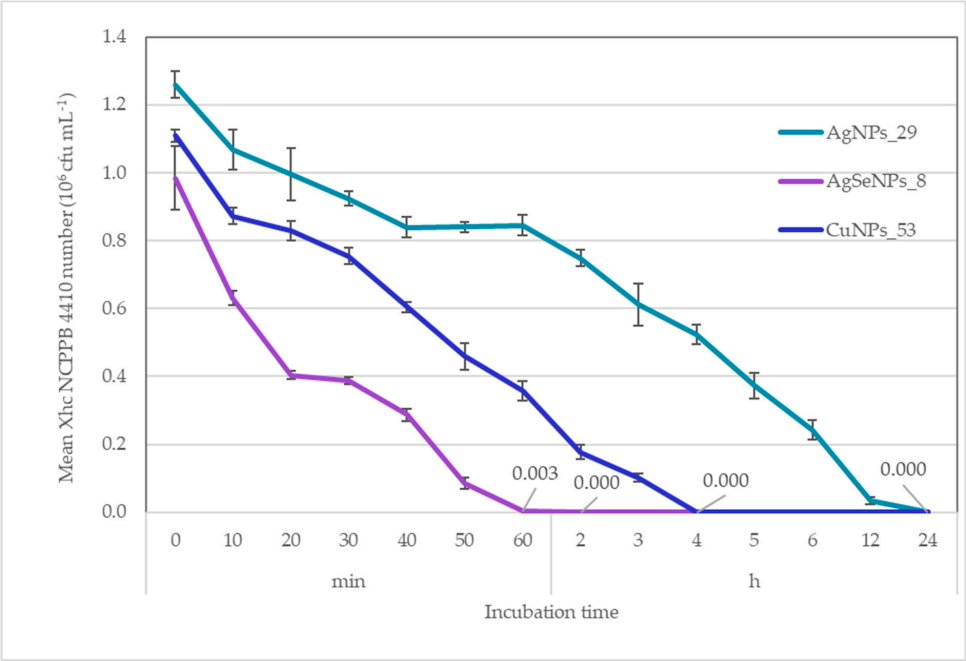


**Figure 1.** Kinetics of killing Xhc strain NCPPB 4410 at 14 time points after treatment with nanomaterials at minimum bactericidal concentration: AgNPs_29 (10 mg $L^{-1}$ Ag), CuNPs_53 (397 mg $L^{-1}$ Cu), and AgSeNPs_8 (53 mg $L^{-1}$ Ag and 11 mg $L^{-1}$ Se).

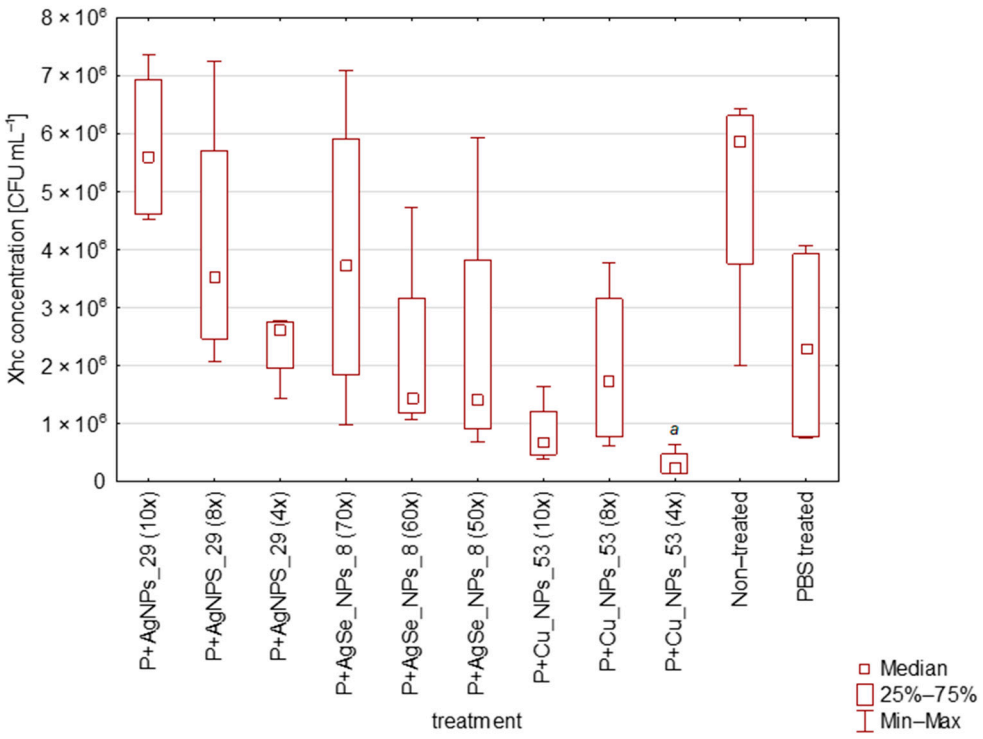


**Figure 2.** Median concentrations of Xhc cells detected in germinated plants from seeds inoculated with Xhc suspension. Median values ± SD are presented. Lowercase a indicates values statistically different from control (non-treated) variant (Kruskal–Wallis test: H (10, $N$ = 44) = 23.37093, $p$ = 0.0095).

The possible negative or positive impact of treatment with individual nanoparticles on the germination of carrot seeds was also evaluated. In comparison to non-treated and PBS-treated seeds, none of the three nanomaterials exhibited a significant positive impact on seed germination percentage (Figure 3). The highest seed germination rate was observed in seeds treated with 10× diluted CuNPs_53 (Cu concentration corresponding to 397 mg $mL^{-1}$), reaching 92.50 ± 2.75%. Surprisingly, seeds treated with 8× diluted CuNPs_53 showed the lowest germination rate (66.50 ± 3.75%). The germination rates for the remaining nanomaterials were similar to those of the non-treated seeds (85.50 ± 0.75%) and PBS-treated seeds (84.00 ± 1.00%). The significant difference in seed germination was assessed only for CuNPs_53, revealing that the 10× diluted stock solution resulted in higher seed germination compared to the 8× diluted solution. No morphological changes of germinated plants were registered.

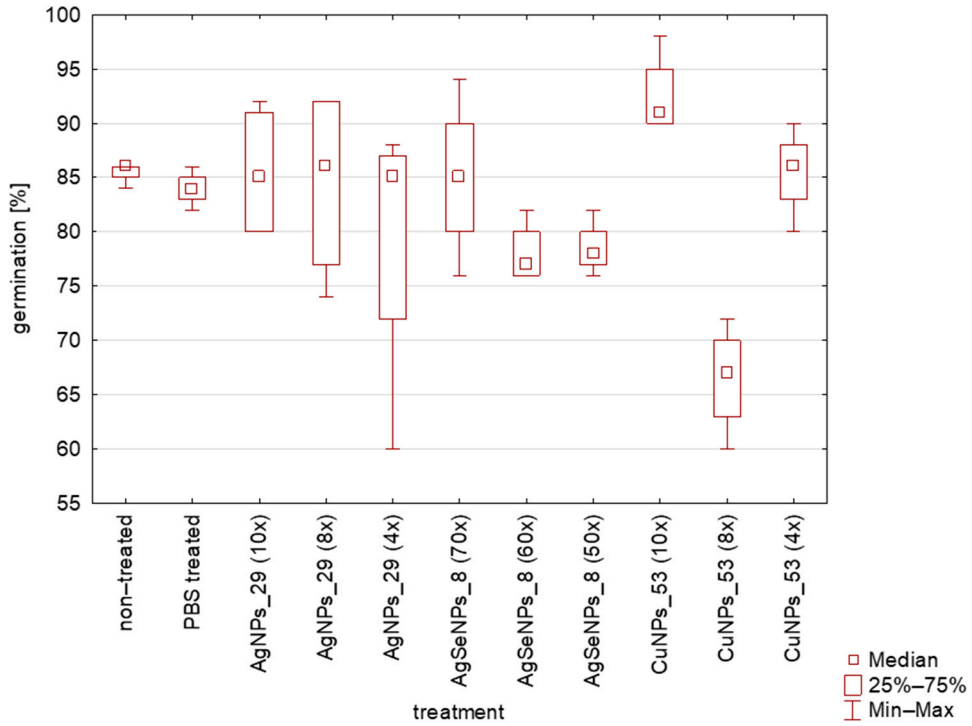


**Figure 3.** Germination of carrot seeds (%) treated with nanomaterials in concentrations used for in planta experiment (Kruskal–Wallis test: H (10, *N* = 44) = 26.01212, *p* = 0.0037).

## 4. Discussion

The aim of this study was to assess the possibility of eliminating Xhc, a harmful bacterial pathogen, using an innovative approach based on the application of nanoparticles. Considering that the main source of inoculum for Xhc is typically seeds [3], our work focused on evaluating the effectiveness of treating infected seeds as the key entities responsible for the spread of Xhc. Notably, the overall availability of approaches aimed at eliminating Xhc from seeds is very limited; most studies have tended to focus on foliar applications. For example, the use of copper-based bactericides [37] or isolates of *P. syringae* pv. *syringae* has been reported to reduce the colonization of carrot leaves by Xhc [38].

In this study, eight nanoparticles were first tested under in vitro conditions, and silver (Ag), copper (Cu), and Ag/Se composite nanoparticles were identified as the most effective. The identification of just these types of nanoparticles as the most effective is in line with the available literature. Silver nanoparticles are among the historically oldest types of nanoparticles, with their antibacterial effect being one of the first reported properties [39]. In terms of plant protection, using them might seem slightly less logical than, for example, copper nanoparticles, since copper-based products have been used for plant protection for a long time. However, examples can also be found for silver. For instance, Elemawi et al. [40]

reduced the incidence of the pathogen *Fusarium oxysporum* by using biosynthesized Ag-based nanoparticles for treatment of selected crop seeds. Jo et al. [41] significantly reduced the presence of *Gibberella fujikuroi*, a serious seed-borne fungal pathogen in rice, by treating rice seeds with silver nanoparticles. Pečenka et al. [11] successfully used silver nanoparticles to eliminate *Xanthomonas campestris* pv. *campestris* (Xcc) from artificially inoculated seeds, demonstrating significantly higher efficiency than conventional hot water treatment. An overview of the possibilities of using silver nanoparticles in plant disease management is provided in [42].

Based on in vitro tests, copper-based nanoparticles can be recommended being as suitable for protection against plant pathogenic fungi *Fusarium solani*, *Neofusicoccum* sp., and *Fusarium oxysporum* [43]. Copper-based nanoparticles have also been directly applied to tomato plants for protection against *Phytophthora infestans*. In that study, it was demonstrated that a significantly smaller dose of copper-based nanoparticles was sufficient compared to commercially available copper-based protective products [44]. Copper-based nanoparticles produced through green synthesis were successfully used to eliminate the soil-borne pathogen *Ralstonia solanacearum* during tobacco plant cultivation [45]. A more comprehensive overview of the sources of copper-based nanoparticles is presented in a review by Banik et al. [46].

The last of the three nanoparticles that was proven to have significant in vitro efficacy against Xhc was silver/selenium composite nanoparticles. Due to their more complex structure and preparation, Ag/Se composite nanoparticles are rarely used in plant protection, and, previously, they were primarily applied in the field of human medicine. An example is a publication demonstrating the effectiveness of silver-selenium nanoparticles for the eradication of dental pathogens [47]. Mittal et al. [48] compared silver and silver-selenium nanoparticles in terms of their anticancer and antimicrobial effects. A more in-depth overview of the antibacterial, fungicidal, and antiviral properties of selenium nanoparticles is provided by Serov et al. [49].

After the in vitro tests, we performed a time-kill assay, which provided important information for subsequent treatment of carrot seed. The observed differences in the efficacy of nanoparticles were surprisingly large, with the AgNPs showing a significantly slower onset than the others. A number of factors could explain these observed differences in antibacterial effect, and these are critically compared in a study by Fan et al. [50], specifically with regard to the combination of AgNPs and CuNPs.

Contrary to the results of in vitro tests, the antibacterial effectiveness of nanoparticles in seeds artificially infected by Xhc did not yield such clearly favorable results. Despite the use of higher concentrations than in the in vitro tests, there was a slight decrease in the Xhc load in treated variants compared to the control group, but this difference was usually not statistically significant. There may be several reasons why such differences between in vitro tests and treatment of inoculated seeds were observed. One is the complex structure of carrot seeds, including their roughness, fibrous spines, and longitudinal projections [51]. These structures may cause pathogen cells to remain wedged, and thus protected from the effects of the applied nanoparticles. Incomplete elimination of Xhc from seeds may subsequently result in surviving Xhc cells contaminating new seedlings, where they can further multiply. Similar trends regarding differences observed between in vitro and in vivo results have also been reported in other studies [52,53].

Even though the results obtained in the seed treatment were not fully conclusive, it is possible to note positive trends in terms of reduced Xhc concentrations in seedlings with increasing nanoparticle concentration. This suggests that the desired effect could be achieved if the concentrations used for seed treatment were increased further. Trends confirming higher efficacy with increasing nanoparticle concentration have also been reported [54–56]. On the other hand, it must be considered that increasing the nanoparticle concentration has limitations with regard to a possible negative effect on seed germination. Our results show no negative effect on seed germination in the case of AgNPs. This somewhat disagrees with [57], in which decreased germination in carrot seeds was registered after treatment

with silver-based nanoparticles. We also observed a slight decrease in germination with Ag/SeNPs at higher concentrations and, conversely, a statistically significant increase in germination with the lowest concentrations of CuNPs. There are also several examples where Cu treatment increased seed germination, but in species other than carrot [58–60]. The results of our study are generally in accordance with the information in review articles summarizing the effect of nanoparticle treatment on seed germination [61,62], which emphasize the influence of the treated plant species and nanoparticle concentration on the final effect.

The only treatment that demonstrated a statistically significant reduction of Xhc in the tested seedlings was copper nanoparticles (CuNPs) at the highest concentration, resulting in approximately 10 times fewer occurrences of Xhc in plantlets. This reduction may also have practical significance, because Xhc infestation is usually associated with large populations of the pathogen on foliage (>$10^6$ $g^{-1}$ leaf tissue) [63,64], and reducing Xhc to one-tenth by CuNP treatment could effectively mitigate economic losses in the production process. This treatment may theoretically be applicable to other pathovars of *Xanthomonas hortorum*. Additionally, considering that *X. hortorum* pathovars also attack other important horticultural crops such as lettuce, tomato, artichoke, and pelargonium, the application potential of nanoparticles may be significantly broader than solely carrot seeds.

## 5. Conclusions

In the present study, three out of eight prepared nanoparticles with the highest in vitro efficacy were selected and tested on artificially infected seeds. Copper nanoparticles (CuNPs) emerged as the most effective. The results further confirm that a responsible assessment of the suitability of nanoparticles for plant protection should not be based solely on in vitro tests of nanoparticle–pathogen interactions. It is essential to test nanoparticles under conditions as close as possible to practical applications, which typically involves monitoring the interactions in the complete triangle of plant material, pathogen, and nanoparticles. Additionally, the results suggest the need to use higher concentrations than those shown to be sufficient in in vitro tests to achieve the desired effect for in planta treatment.

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