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Biodegradable Silver Nanoparticles Gel and Its Impact on Tomato Seed Germination Rate in In Vitro Cultures

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Abstract: Nanotechnology plays an important role in many fields of science and the economy. A special example of nanostructures is silver nanoparticles (AgNPs) created following the principles of green chemistry, i.e., without the use of toxic reducing compounds. The common tomato (*Solanum lycopersicum*) is a popular vegetable whose germination and growth process are studied by using, e.g., in vitro cultures. The aim of the experiment was to evaluate the inhibitory effect of the biodegradable gels containing silver nanoparticles on the development of microbial infection and to evaluate their influence on the germination degree of Tomato (*Solanum lycopersicum*) seeds in in vitro plant cultures. Based on macroscopic and microscopic observations, all experimental samples showed the presence of Gram-positive bacilli as well as mould fungi of the genus *Rhizopus*, *Alternaria* and *Aspergillus*. The study showed that the biocomponents containing silver nanoparticles obtained by using xylose as a reducing agent limit the development of microbial infection and stimulate the germination rate of tomato seeds. They could find their application as biodegradable raw materials in the production of modern disinfecting preparations for research in in vitro cultures. This study allowed to identify new research directions, especially to evaluate the metabolic regulation of seedlings treated with biodegradable silver nanoparticles.

Keywords: AgNPs; sodium alginate; bacteria; fungi; tomato; in vitro culture



Citation: Rutkowski, M.; Krzemińska-Fiedorowicz, L.; Khachatryan, G.; Bulski, K.; Kołton, A.; Khachatryan, K. Biodegradable Silver Nanoparticles Gel and Its Impact on Tomato Seed Germination Rate in In Vitro Cultures. *Appl. Sci.* **2022**, *12*, 2722. <https://doi.org/10.3390/app12052722>

Academic Editor:
Ioannis Kartsonakis

Received: 6 February 2022

Accepted: 4 March 2022

Published: 6 March 2022

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1. Introduction

In the 21st century, nanotechnology is an interdisciplinary branch of science, describing structures sized 100 nm or less, allows characterizing innovative possibilities of using nano-scale metals with biological properties important for the industry [1–3]. Metal nanoparticles find interdisciplinary applications in various fields, e.g., silver is used in the production of food packaging, cosmetics or medical bandages [4–6]. The synthesis of silver nanoparticles can be physical, chemical or biological. The type of the used method influences the properties of the obtained nanostructures [7,8]. One of the most desirable properties exhibited by metal nanoparticles seems to be antimicrobial activity resulting from the shapes assumed in the structure of the carrier in which they are embedded. This biological activity described in the literature is based on the interaction of nanostructures with microbial cell surfaces, influencing metabolic processes in microbial cells, which in turn lead to the

destruction of internal structures, inhibition of microbiota development and microbial contamination [9–12]. A special example of a carrier stabilizing the formation of silver nanoparticles are substances of natural origin, which show innovative properties that allow the creation of plastic gels. Biopolymers as biodegradable and intranasalable substances have the advantage of low toxicity to the ecosystem because they quickly decompose into basic compounds. Among such substances, examples of sodium alginate or chitosan can be cited [13–17]. Sodium alginate is a biopolymer consisting of D-mannuronic and L-guluronic acid. In the food industry, it is used to create modern food packaging. As a substance derived from the natural environment, it is a non-toxic matrix for the creation of components containing active biological factors, e.g., silver nanoparticles [18,19]. On the other hand, chitosan is a biodegradable polymer used in the food industry and bioengineering for the process of sprinkling active substances. When using chitosan, it is possible to create components containing biologically active factors [20–22]. Tomato (*Solanum lycopersicum*) is a popular vegetable belonging to the *Solanaceae* family. Tomato fruits are an important part of the human diet due to the wide range of bioactive compounds such as carotenoids, flavonoids, antioxidants and anti-cancer compounds [23–25]. The production of tomato fruit is one of the largest undertakings in the world. In 2018, it reached 182 million tonnes. The largest tomato producing countries are the United States, China and Turkey, while Spain and Italy are the largest tomato producers on the European market [26].

The tomato is also an example of a model organism [27] and can be used in a variety of in vitro experiments [28–30]. In vitro cultivation requires prior preparation of the plant material-sterilisation. If the culture is carried out from seeds, surface sterilisation of the seeds is usually applied [31]. Many methods of surface sterilisation of seeds are known [32,33], but they are not always effective. Many authors observed culture contamination despite the use of surface sterilisation and high care during handling or with prolonged treatment with disinfectants, lower levels of germination and seedling survival were observed [32]. The cause of such phenomenon may be microorganisms located inside the seeds [34]. Plant organisms, including the tomato (*Solanum lycopersicum*), can become a reservoir for many strains of microorganisms. These microorganisms can survive inside the seed structure of dormant seeds and, as a result of the germination process, move to the newly formed seedling. Potential microorganisms that inhabit the plant are bacteria of the genus *Bacillus*, *Pseudomonas*, *Acinetobacter* and *Staphylococcus*. The large diversity of plant microbiota results from changes in the environment in which the plant grows [28,35,36]. Infection of the culture may be observed after seed rupture—germination begins. Decontamination of such a culture is very difficult. The phenomenon forces the need to find an alternative method of limiting microbial growth using innovative technology, the use of which would be consistent with the postulates of “green chemistry”, and therefore potentially safe for the proper growth of plants and the development of the natural environment.

The aim of this study was to produce biodegradable alginate-based material containing silver nanoparticles, describe their structure and properties and to test their applicability during in vitro culture of tomato using seeds. It was hypothesised that an appropriate concentration of nanoparticles would reduce pathogen growth but not seed germination.

2. Materials and Methods

2.1. Synthesis of Gels with Silver Nanoparticles

Reagents used in the synthesis of nanosilver containing gels.

Chemical reagents were used to prepare the nanocomposites, i.e., sodium alginate (Sigma-Aldrich), glycerine (99.5%, Sigma-Aldrich, Poznan, Poland)—as an excipient (plasticiser), AgNO₃ (Aldrich, Poznan, Poland, 99.99%, PubChem CID: 24470), NH₃ (Sigma-Aldrich, Poznan, Poland, PubChem CID: 18944693) and D-(+)-xylose (Sigma-Aldrich, Poznan, Poland, BioXtra, PubChem CID: 135191) and deionized water.

A 1.5% sodium alginate solution was prepared by gelatinizing 3 g of sodium alginate (Sigma-Aldrich) with 197 g of deionized water in a magnetic stirrer (Heidolph MR3002)

with a connected thermostat (70 °C). After completion of the gelatinizing process, 1.5 g of glycerine (99.5%, Sigma-Aldrich) was added as a plasticizer. 200 mL of the polysaccharide gel was stirred and 2 mL of Tollens solution was added. 1.5 mL of 4% D-(+)-xylose were added to the gel, the temperature was reduced to 55 °C and the mixture was stirred for another 10 min. The final nanosilver concentration in the gel was 150 ppm. Then, dilutions of gel solutions containing silver nanoparticles in three concentrations in the volume ratio (1:2), (1:5) and (1:10) were prepared, which allowed to obtain three concentrations of silver nanoparticles: 75 ppm, 30 ppm and 15 ppm. A sterile water solution was determined as a control.

2.2. UV–Vis Absorption Spectrophotometry

The UV-VIS absorption spectra were obtained using a Shimadzu 2101 scanning spectrophotometer (Shimadzu, Kyoto, Japan), with a range covering 200–800 nm.

2.3. FTIR-ATR Spectrophotometry of Composites

The FTIR spectra of the produced composites were analyzed within the 4000–700 cm^{-1} range, using a MATTSON 3000 FT-IR spectrophotometer (Madison, WI, USA) equipped with a 30SPEC 30 Degree Reflectance adapter (MIRacle ATR, PIKE Technologies Inc., Madison, WI, USA).

2.4. Scanning Electron Microscopy (SEM)

The shape and size of the obtained nanostructures characterized using a JOEL 7550 high-resolution SEM (Akishima, Tokyo, Japan) equipped with a Retractable Backscattered-Electron detector (RBEI) (Akishima, Tokyo, Japan).

2.5. Tomato In Vitro Cultures—Germination and Infection Test

Sterile autoclaved standard MS [37] medium (1/2 MS, 0.4% phytigel, 1% sucrose, pH 5.8) was poured on the Petri dishes, then 1 mL of prepared gels with silver nanoparticles in different concentrations (75 ppm, 30 ppm, 15 ppm) were poured on the surface of the solidified medium. It was observed for 7 days to see if any infection appeared. The gels were free of pathogens and the experiment could be continued (data not presented). In the subsequent experiments, tomato seeds (*Solanum lycopersicum*) of the cultivar Kmicic (Plantico, Stare Babice, Poland) were used. Following plates were prepared in a similar manner (sterile 1/2 MS, 0.4% phytigel, 1% sucrose, pH 5.8), and 1 mL of different concentrations (75 ppm, 30 ppm, 15 ppm) of prepared nanosilver gels were poured onto the surface of the solidified medium under sterile conditions. Control plates were treated with sterile distilled water. Twenty non-sterile tomato seeds were sown on each plate (Experiment 1). The plates were closed and kept in the dark at 20 °C. Every 24 h the degree of contamination of the tested plates as well as seed germination was checked. The number of germinated seeds in each plate was counted and expressed as a percentage of all the seeds sown on a plate (germination rate). In addition, the number of seeds at which infections occurred was counted and expressed as a percentage of all seeds in the plate (infection rate). The experiment lasted 120 h. Each treatment was performed in 3 replications.

Another experiment was conducted on similarly prepared plates (sterile 1/2 MS, 0.4% phytigel, 1% sucrose, pH 5.8) with the difference that before sowing the seeds were immersed in different solutions: sterile water, nanosilver solutions of different concentration (75 ppm, 30 ppm, 15 ppm), and subjected to surface sterilisation with an aqueous solution of sodium hypochlorite (Experiment 2). For surface sterilization of seeds with hypochlorite, 0.1% NaOCl dissolved in water was used, seeds were treated with the solution for 5 min, followed by two rinses with 70% ethanol and two rinses with sterile water. After the tomato seeds were immersed (5 min) in the appropriate solutions, they were sown into the prepared plates, 20 seeds per one. Three plates were prepared for each treatment. The plates were closed and kept in the dark at 20 °C. Every 24 h the germination rate and the infection rate were checked as described above. The experiment lasted 120 h.

2.6. Identification of Isolated Microorganisms

The isolated microorganisms were identified based on macroscopic and microscopic characterization. The macroscopic characterization of bacterial colonies included the determination of the morphological features of the colony (size, shape, elevation or profile, transparency, colour of the colony, structure and smell). The microscopic characterization of the bacterial colonies was based on the morphological evaluation of the Gram-stained preparations. The microscopic analysis provided data on: the size of the cells, their shape and arrangement in relation to each other. The macroscopic analysis of fungi included the determination of morphological features: colony size in a Petri dish, colony appearance, consistency, degree of fluffiness, colour, smell, possible pigment diffusion into the substrate and the separation of substance droplets. In the microscopic characterization of the colonies, the preparations in the Lugol's liquid were observed. Size, shape, surface appearance and arrangement of spores, size and shape of conidiophores were determined [38–41].

2.7. Statistical Analysis

Statistical analysis of the obtained results was performed with the use of Statistica version 13.3. Objects were compared using analysis of variance and Fisher's Least Significant Difference (LSD) test. The letters represent homogeneous groups determined with the assumed significance level of 0.05. Lack of letters means no statistically significant differences. Statistical analysis was performed for each term separately.

3. Results

3.1. UV–VIS Absorption Spectrophotometry

UV-VIS analysis indicated the formation of Ag nanoparticles, a typical peak in the range of 350–600 nm was obtained (Figure 1) [18,42].

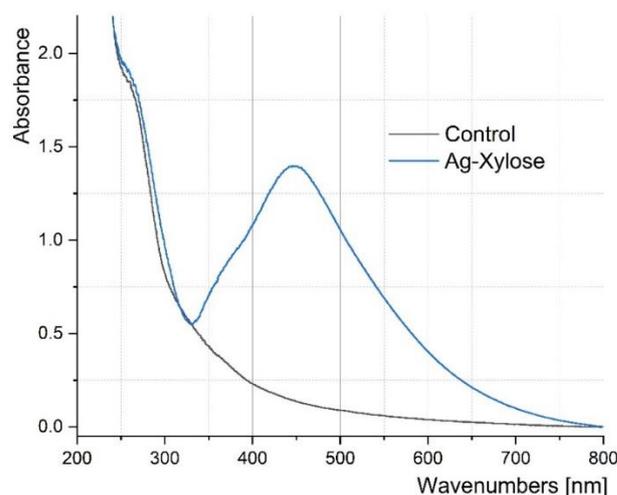


Figure 1. UV–VIS spectra of control (black line) and Ag-xylose (blue line).

3.2. Scanning Electron Microscopy (SEM)

The width of the peak band indicates that the formed nanoparticles are characterized by different sizes, which has been confirmed by images from a scanning electron microscope.

The obtained silver nanoparticles were characterized by regular and spherical shapes, their sizes varied between 5–20 nm (Figure 2).

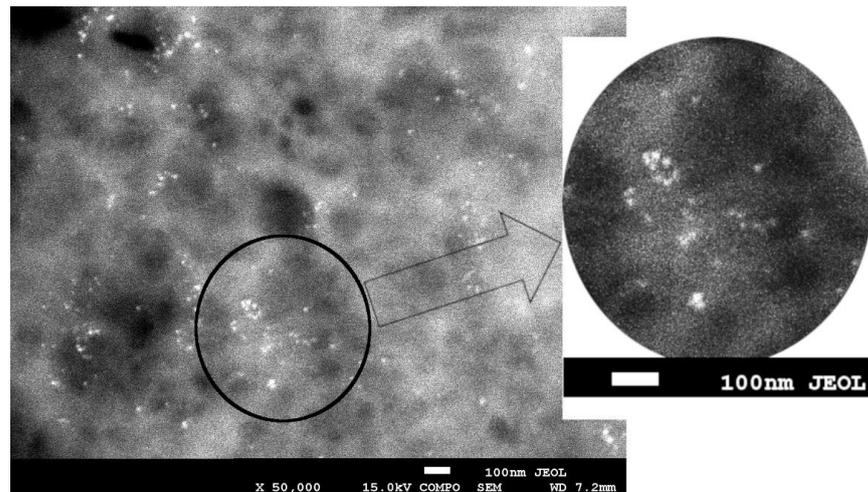


Figure 2. SEM micrographs of AgNPs taken at $\times 50,000$ magnification.

3.3. FTIR-ATR Spectrophotometry

In the presented FTIR spectra (Figure 3) we observed the characteristic spectrum of the sodium alginate with a broad band centred at approximately 3210 cm^{-1} (hydroxyl groups stretching), low intensity bands at about 2915 cm^{-1} (attributed to $-\text{CH}_2$ groups), two peaks at 1603 cm^{-1} and 1408 cm^{-1} (asymmetric and symmetric stretching modes, respectively, of carboxylate salt groups ($-\text{COONa}$), and a number of vibrations in the range of $1100\text{--}990\text{ cm}^{-1}$ (glycoside bonds in the polysaccharide (C-O-C stretching) [43]. The absence of significant changes in the shape of the obtained spectra indicates that the synthesis of nanometals did not cause structural changes in the alginate polymer.

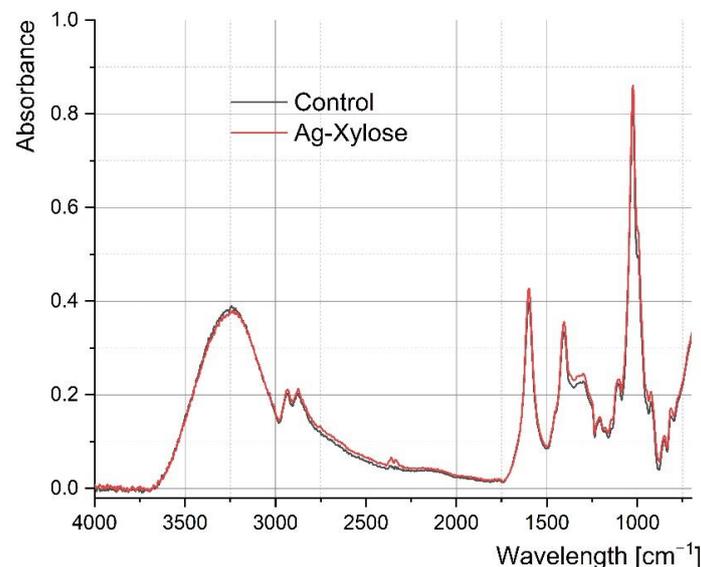


Figure 3. FTIR absorption spectra of control (black line) and Ag-Xylose (red line).

3.4. Germination and Infection Rate in Tomato In Vitro Culture

3.4.1. Experiment 1

In the first experiment, silver nanoparticle solutions of different concentrations and water as control were applied to a sterile medium in a plate. The first infections (Figure 4) were found 48 h after the start of the experiment therefore the results after 24 h are not shown in the figure. The highest infection rate was recorded in the control (sterile water applied to the surface of the medium) and the injection of silver nanoparticles reduced the infection rate at 48 h of the experiment. The number of infections increased over time.

After 120 h of the experiment, the dishes treated with water and the lowest concentration of nanoparticles were all infected. However, the application of 75 ppm nanoparticles significantly reduced the number of infections.

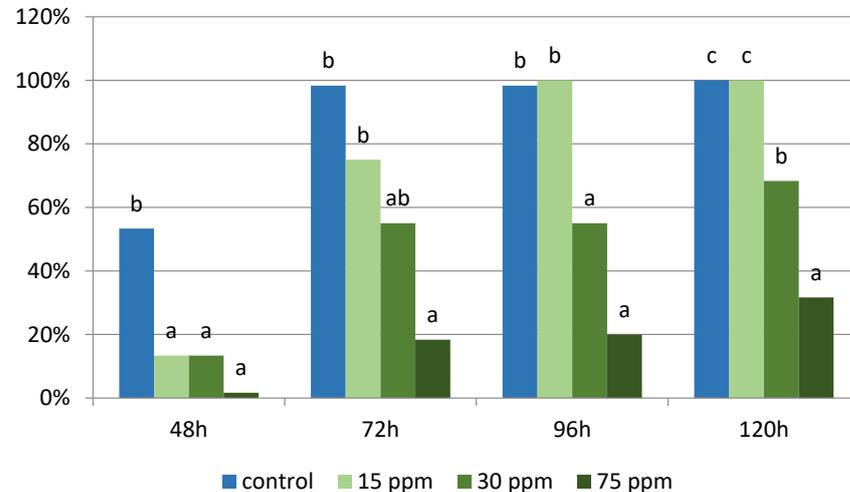


Figure 4. Rate of infections of tomato seed culture after application of different concentrations of silver nanoparticles (75 ppm, 30 ppm and 15 ppm). The control sample was treated with sterile water. Treatments were compared using analysis of variance and Fisher's LSD test for each term separately. Parameters in columns denoted with the same letters (a. b. c.) do not differ statistically at the confidence level of $p < 0.05$.

In the case of the germination rate, the first seeds started to germinate after 48 h in all experimental trials (Figure 5). However, during this time the seeds germinated similarly, regardless of the treatment applied. The differences in seed germination rate were already noted after 72 h of observation. The highest number of seeds germinated was observed after the application of 75 ppm of silver nanoparticles and the lowest in the control (72 h). At the last observation term, it was noted that the application of all concentrations of silver nanoparticles significantly improved germination, while seeds treated with water germinated the worst.

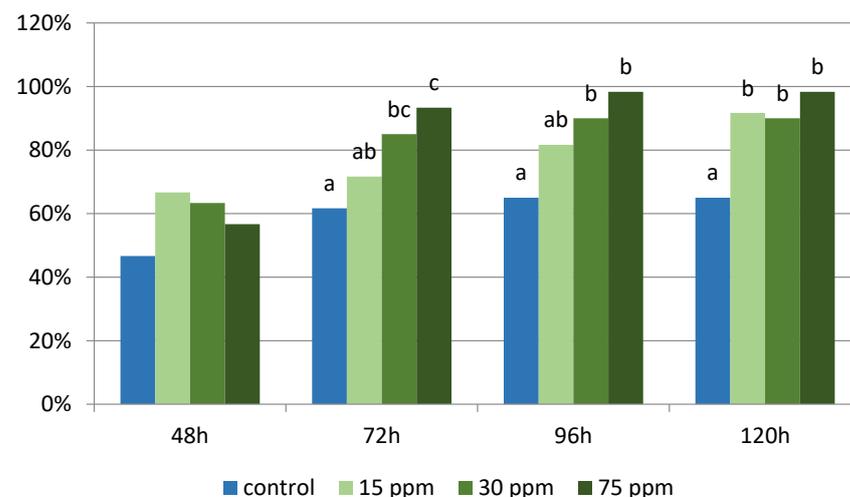


Figure 5. Germination of tomato seeds in culture after application of different concentrations of silver nanoparticles (75 ppm, 30 ppm and 15 ppm). The control sample was treated with sterile water. Treatments were compared using analysis of variance and Fisher's LSD test for each term separately. Parameters in columns denoted with the same letters (a. b. c.) do not differ statistically at the confidence level of $p < 0.05$. Lack of letters means no statistically significant differences.

3.4.2. Experiment 2

In the second experiment, seeds were soaked in the respective solutions before sowing onto the prepared medium. As in the first experiment, the first infections were recorded after 48 h of the experiment (Figure 6). During this observation, the highest number of infections was recorded in seeds pre-treated with sterile water and the lowest in those treated with hypochlorite. The application of silver nanoparticles reduced the infection rate compared to the control. Although no infections were observed in seeds dipped in hypochlorite after 48 h, infections in these seeds appeared over time. It is noteworthy that after 120 h from the start of the experiment, infection levels in seeds treated with hypochlorite and nanosilver solutions at concentrations of 75 ppm and 30 ppm were similar and significantly lower than those of the control or the lowest concentration of nanoparticles. The percentage of germination is shown in Figure 7. The first germinating seeds were observed 48 h after sowing. There was no difference in germination as a result of the applied solutions into which the seeds were immersed before sowing.

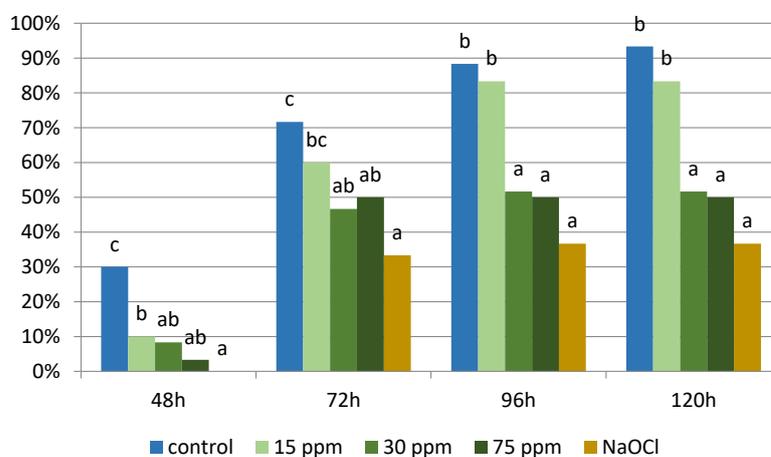


Figure 6. Rate of infections of tomato seed after immersion in solutions of different concentrations of silver nanoparticles (75 ppm, 30 ppm and 15 ppm) or sodium hypochlorite (NaOCl). The control sample was treated with sterile water. Treatments were compared using analysis of variance and Fisher’s LSD test for each term separately. Parameters in columns denoted with the same letters (a. b. c.) do not differ statistically at the confidence level of $p < 0.05$.

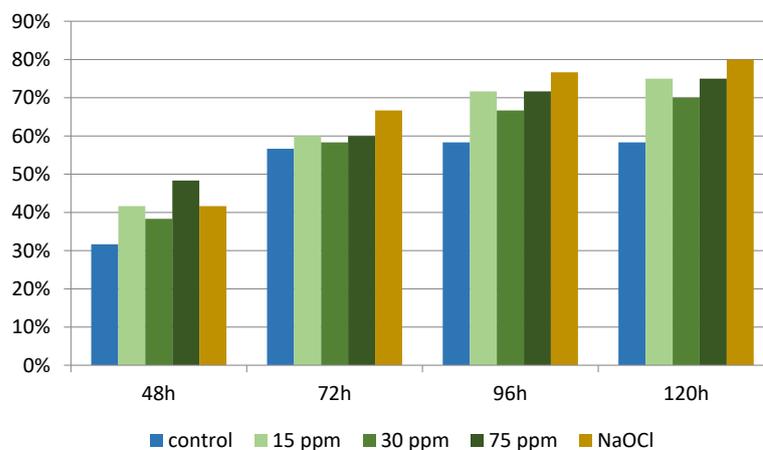


Figure 7. Germination of tomato seeds after immersion in solutions of different concentrations of silver nanoparticles (75 ppm, 30 ppm and 15 ppm) or sodium hypochlorite (NaOCl). The control sample was treated with sterile water. Treatments were compared using analysis of variance and Fisher’s LSD test for each term separately. The letters represent homogeneous groups determined with the assumed significance level of 0.05. Lack of letters means no statistically significant differences.

3.5. Isolated Microorganisms

In all experimental trials, the presence of microorganisms was found. In the case of bacteria, the Gram-positive bacilli were found. In the case of fungi, the genus of *Alternaria*, *Rhizopus* and *Aspergillus* were found.

4. Discussion

Preventing the development of undesirable microbiota seems to be one of the main topics of research on the practical application of preparations containing active agents such as silver nanoparticles, as evidenced by the works [9–11,44] carried out over several years. Due to their antimicrobial activity, components containing biologically active silver nanoparticles could be a potential alternative to disinfectants used so far in in vitro cultures. However, for this to happen, a number of analyses should be carried out to assess the degree of germination of seeds and other plant structures used in vitro. The potential lack of necrotic changes in seedlings, presented in this paper, raises some hopes for a non-toxic, time-delayed release of silver nanoparticles from the structure of sodium alginate.

MS medium is commonly used in in vitro cultures of tomato [45]. Sucrose is usually added to the medium as an easy source of carbon chains for plants [46,47], but it can also be used by microorganisms [48]. Sometimes sugar is omitted from the nutrient solution to reduce infection [34], but there is a risk of slower plant growth. The possibility of using silver nanoparticles to decontaminate *Arabidopsis* seeds and tomato leaf explants were demonstrated by Mahna and co-worker [49]. However, it was observed that with complete sterilising effect (no culture infection) long exposure to silver nanoparticle solutions (30 and 60 min) has an inhibitory effect on *Arabidopsis* seed germination and high concentrations of silver nanoparticles (250–2000 ppm) even at shorter exposure inhibit germination. Therefore, silver nanoparticles are a promising sterilizing agent but it is important to select an appropriate concentration and assess the risk of seed germination inhibition. Toxic effects of silver nanoparticles on plants in vitro cultures were also observed in *Aldrovanda vesiculosa* [50]. Therefore, work on the application of nanoparticles to in vitro cultures is still ongoing and requires, on the one hand, the search for new methods of producing silver nanoparticles and, on the other, testing their effect on specific cultures. In the presented work, a modern method of nanoparticle production using alginate was used and, most importantly, no toxic effect on germinating tomato plants was observed at any of the concentrations used. However, not every concentration used was equally effective in protecting against culture infection. In experiment 1 (non-sterile seeds were placed on culture media), the application of a 75 ppm silver nanoparticle solution gave the highest inhibitory effect on infection development. In contrast, the application of a 15 ppm solution did not reduce the development of infection. In the second experiment (seeds were treated with different solutions before placing them on the plates), the greatest reduction in infection development was obtained with a 75 and 30 ppm solution of nanosilver and a solution of sodium hypochlorite. Despite the best performance of these solutions, infections of 50% were still observed. In addition, in other studies, the appearance of microorganisms was observed after the application of hypochlorite [34]. Infections were also observed in barley embryo cultures after hypochlorite treatment [51]. The addition of silver nanoparticles to the MS medium decreased the frequency of these infections which points to the effectiveness of nanosilver in reducing infection—as in our experiments. The use of silver nanoparticles as a solution poured on the surface of the medium in the first experiment improved seed germination compared to the control. In the second experiment, seeds germinated at similar levels in all treatments. In the study by Bill et al. [52], similarly to this study, the stimulation of plant seedlings growth was demonstrated on the example of garden petunias in in vitro cultures. Dehkourdi et al. [53] showed that with an increased concentration of TiO₂ nanoparticles, the germination process of parsley seeds (*Petroselinum crispum*) was stimulated in in vitro cultures. Similarly, Shinde et al. [54] found the stimulation of germination of corn seeds (*Zea mays*) caused by the presence of nanoparticles in culture. Therefore, it can be said that nanoparticles can be potential biostimulators in the

process of plant germination and the applied in the present experiment concentrations of silver nanoparticles had no toxic effect and did not reduce tomato seed germination, and in some cases even stimulated germination.

One of the new ideas for human, animal or plant pathogens prevention is the composition of new materials with antibacterial or antifungal properties, with long-acting times and without negative environmental impact. One of the potential solutions are metallic nanoparticles (NPs), especially silver nanoparticles. Their antimicrobial effect results from the cell membrane and DNA damages or interaction with enzymes from thiol groups [55,56]. It should be emphasized that the morphology, stability, type, aggregation, and concentration of nanoparticles are the main properties that determine their toxic effect on biological systems [57]. The results of our study showed that for both non-sterile and sterile seeds in vitro cultures, the lowest value of the infection rate was observed for the concentration of 75 ppm of AgNPs. The worst effect of AgNPs was observed at a concentration of 15 ppm. Therefore, our research confirmed the influence of the concentration of metal nanoparticles on antimicrobial properties, which was described by other authors—higher concentration of NPs increases their antimicrobial properties [58–61]. However, the concentrations of AgNPs, used in our research, were too low to completely inhibit the growth of microorganisms, because in all experimental trials, the presence of microbes was found. The results of our research showed the growth of Gram-positive bacilli as well as *Alternaria*, *Rhizopus* and *Aspergillus* fungi (in all samples). All these microorganisms are common in the natural environment. Although previous biological studies reveal potential inhibition of growth of Gram-positive and Gram-negative bacteria as well as microscopic fungi as a result of interactions of NPs [62–66], the nanotoxicity towards Gram-positive cells was significantly less, possibly due to the presence of a thicker peptidoglycan layer [67]. Antifungal properties of AgNPs usually concern the study of various types of yeast, there are merely a few studies concerning the interaction of molds with AgNPs. The research by Żarkowska et al. [68] showed that the sensitivity of mold fungi to AgNPs depends on the mould species. The study also showed that, for example, concentration 2.5 mg/L of AgNPs was sufficient for the inhibition of *Aureobasidium pullulans*, but 10 times higher concentration was needed for the *Aspergillus niger* growth inhibition [69].

5. Summary

Based on the conducted research, it is concluded that biocomponents containing silver nanoparticles obtained by using xylose as a reducing agent limit the development of microbial infection and stimulate the germination degree of tomato seeds. They could find their application as biodegradable raw materials in the production of modern disinfecting preparations for the research in in vitro cultures. Work on the growth of plant seedlings treated with metal nanoparticles demonstrates the stimulating effect of nanostructures.

This experiment is a pilot study, based on which further analyses of the effect of components containing biologically active silver nanoparticles on the germination of vegetable seeds and the inhibition of microbial infection of seeds will be carried out in the future.

Author Contributions: Conceptualization, M.R., A.K. and L.K.-F.; methodology, G.K., K.K., A.K., K.B. and M.R.; software, K.K., A.K. and K.B.; formal analysis, K.K., A.K., G.K., L.K.-F. and M.R.; investigation, K.K., G.K. and A.K.; writing—original draft preparation, M.R., A.K., G.K. and K.B.; writing—review and editing, K.K., G.K. and A.K.; visualization, K.K., A.K. and K.B.; supervision, G.K. and A.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a subsidy of the Ministry of Science and Higher Education for the University of Agriculture in Krakow.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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