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The Application of Copper and Silver Nanoparticles in the Protection of *Fagus sylvatica* Wood against Decomposition by *Fomes fomentarius*

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Abstract: Wood technology is constantly looking for environmentally friendly technological solutions. It seems that nanotechnology can provide green and environmentally friendly alternatives for wood protection. In this study, the antifungal activity of silver and copper nanoparticles against the white rot fungus *Fomes fomentarius* was investigated under in vitro conditions and with respect to the protection of *Fagus sylvatica* wood. In both experiments, 5, 25 and 50 ppm of nanoparticles were used. The results of the in vitro test showed selective antifungal activity of the nanoparticles. All concentrations of copper nanoparticles stimulated mycelial growth. The stimulation was inversely proportional to the concentration. Silver nanoparticles inhibited fungal colony growth at the highest concentration (50 ppm) and did not affect growth at concentrations of 5 and 25 ppm. Silver nanoparticles increased the rot resistance of beech wood, but only at the highest concentration. Decay caused by *F. fomentarius* was stimulated by copper nanoparticles at the highest concentration. Probably, the fungus used copper as a micronutrient for better growth and increased the activity of extracellular enzymes. Our results from in vitro tests are consistent with those obtained on beech wood specimens, showing that the concentrations of the two nanoparticles used were too low to protect the beech wood from decomposition by xylophagous fungus.

Keywords: decay resistance; environmentally friendly technology; in vitro and decay tests; nanoparticles concentration; wood decomposing fungus; wood preservative

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1. Introduction

European beech [*Fagus sylvatica* L.] is an important forest-forming species in both Europe and Poland [1]. It accounts for 6.1% of Polish forest area and 6.8% of volume [2]. Beech is also planted in parks, cities and street tree alleys [3]. The wood of beech is widely used in Europe because of its hardness, wear-resistance, strength, excellent bending capability and relatively low price. Beech wood is also widely used in the production of lumber, veneer, flooring, furniture, musical instruments, plywood and turned objects. On the other hand, beech wood is considered as non-durable or perishable with poor microorganism resistance [4], therefore, the question arises as to whether the use of copper and silver nanoparticles can be treated as preservatives for beech wood against rot fungi attack?

Fomes fomentarius (L.) Fr. is one of the most common white rot fungi of beech [1,3]. It causes decay in living standing trees and can live saprotrophically for many years after

the tree has died [5]. It has a worldwide distribution [6], and plays an important role in the utilization of lignocellulosic materials through bioremediation, biorefining, bioincising, bioengineering, biofuels, and biopulping [7]. It is considered as a pretreatment agent for biopulping, and reduces industrial pollution. As *F. fomentarius* can depolymerize lignin, nanotechnology application seems to have a great potential in wood protection. Nowadays, nanotechnology is finding wider and wider application in various fields of the economy, including wood preservation [8,9]. Small dimensions of nanoparticles (in the range of 1–100 nm) and relatively large surface areas ensure that the physicochemical and biological properties of nanoparticles differ significantly from those of bulk materials of the same type [10]. The dimensions of pores in the pit membrane of the bordered pits in the wall of wood cells are in the angiosperm range of 5–420 nm, it should be emphasized, however, that these values are usually less than 100 nm [11], so most of nanoparticles could potentially penetrate easily into the cell walls of wood and be more difficult to leach compared to conventional formulations [12]. The degree of leachability and efficacy of preservatives depends, inter alia, on their micro-distribution within the cell wall which has been studied for more than half a century [13–18]. For example, the efficacy of copper-based preservatives' distribution into the cell wall depends on the species of wood [19], the morpho-anatomical characteristic of the wood [20], and is strongly related to the distribution of lignin [21]. Matsunaga et al. [18] and Archer [22] found that the micro-distribution of particulate copper was different to that of soluble copper because of particulate accumulation in the lumen of wood cells and pit chambers. Semi-quantitative analysis performed by Matsunaga et al. [20] revealed that the amount of copper increased in the following order: secondary wall in the tracheids < middle lamellae (the most lignified part of cell wall) < membrane of half-bordered pits < tori in tracheid pits < deposits in longitudinal parenchyma cells. In turn, Freeman and McIntyre [23] stated that the fixation of micronized copper occurs mainly through deposition in pit chambers and on tertiary cell wall layers, and not through chemical reaction. Perhaps the unique properties of nanometals, e.g., nanozinc, will provide a more even distribution within the wood cell wall and may show reduced leachability due to increased reactivity with the woody substrate.

So far, several experiments have been conducted to investigate the sensitivity of wood rot fungi to nanoparticles and the application of different types of nanoparticles as wood preservatives (e.g., [24,25]).

However, although many studies have been carried out, they have not provided a clear answer regarding the effectiveness of NPs. Moreover, they are also difficult to compare because they involve different nanoparticles (type, size, shape, concentration), fungi, and wood species. Some studies show the effectiveness of metalnanoparticles and metal oxid nanoparticles in limiting decomposition of different wood species by various decay fungi. AgNPs showed high antifungal activity at low concentrations against *Trametes versicolor* and protected the wood of *Populus* spp. [26] and the wood of three tropical species (*Acacia mangium*, *Cedrela odorata*, and *Vochysia guatemalensis*) [27]. AgNPs also increased the resistance of *Pinus sylvestris* wood to *Coniophora puteana* [28]. Nanoparticles of silver, copper, and zinc oxide at high concentrations have been shown to be effective in protecting Scots pine, European beech, and Paulownia wood against *T. versicolor* [29,30]. In addition, titanium dioxide nanoparticles prevent the colonization of *Hypocrea lixii* and *Mucor circinelloides* on eight different wood species [31].

Several researchers have shown that nanoparticles were completely ineffective against wood rot fungi in some cases. Moya et al. [32] tested AgNPs at low concentrations to protect nine tropical wood species against *T. versicolor* and *Lenzites acuta*. The resistance of the wood species was higher against the white rot fungus *T. versicolor* and lower against the brown rot fungus *L. acuta*. AgNPs were ineffective in protecting *C. odorata* and *Vochysia ferruginea* against *T. versicolor* and *Tectona grandis* against *L. acuta*. These differences in vulnerability to decay are likely due to mechanism used by white rot and brown rot fungi. This fungal classification is based on the ability to degrade lignin (white rot only) along with cellulose and hemicellulose, which is caused by the different enzymatic activity of these fungi [33].

White rot fungi use oxidative and hydrolytic enzymes that gradually degrade cellulose while lignin is completely mineralized by enzyme systems including lignin peroxidase, manganese peroxidase, versatile peroxidase and laccase [34]. The details about brown rot mechanisms are still under discussion, but it is generally agreed that brown rot fungi use a two-step oxidative-enzymatic mechanism [35].

In the study by Kartal et al. [12], copper, zinc and boron nanoparticles were ineffective in protecting southern yellow pine wood against *Antrodia* sp., but inhibited wood degradation caused by *T. versicolor*. The third fungus tested in this study, *Gloeophyllum trabeum*, was insensitive to zinc and boron nanoparticles. Zinc oxide nanoparticles inhibited Scots pine wood decay caused by *Serpula lacrymans*, while they were ineffective against *Poria placenta* [36]. Similar inconclusive results were obtained in other studies [37–39].

Our previous in vitro research has shown the selective antifungal activity of AgNPs and CuNPs depending on the type of nanoparticles and fungal species. A brown rot fungus—*Sparassis crispa*—was not sensitive to either type of nanoparticle at any of the concentrations tested. The growth of *Meripilus giganteus*, a white rot fungus, was significantly reduced by all concentrations of both types of nanoparticles. In turn, two other species, *Fistulina hepatica* (brown rot) and *Grifola frondosa* (white pocket rot), were found to be sensitive to the AgNPs but not to the CuNPs [24].

The use of fungicides to preserve old trees (which often have the status of natural monuments) by controlling the spread of fungal rots is still poorly understood. To date, the study conducted with copper-carbon nanoparticles demonstrated their effectiveness in protecting trees from the blue stain fungus *Ophiostoma minus* as well as the white rot fungus *T. versicolor*. This work found no evidence of phytotoxicity of these copper–carbon nanoparticles [40].

Modern forestry and wood technology in the European Union and Poland are constantly searching for environmentally friendly technological solutions. Products with low toxicity to humans and the environment are a challenge for science. It seems that nanotechnology can provide green and environmentally friendly alternatives in plant and wood protection [41]. Such considerations were behind the work described in this paper. Nanoparticles are found to be less toxic to people and animals than synthetic fungicides and their multiple modes of action constitute a key feature contributing much to obstruct the emergence of resistance in fungi [42,43], and the aim of the study was to determine the effect of CuNPs and AgNPs on the growth of the white rot fungus *F. fomentarius* in vitro and to evaluate the efficacy of these two nanoparticles in protecting beech wood against this fungus. We assumed that the tested nanoparticles could find application both in the protection of old trees and natural monuments and as wood preservatives, especially after natural disasters and in the need to protect large quantities of wood in forests and wood yards.

2. Materials and Methods

2.1. Biological Materials

European beech (*Fagus sylvatica* L.) wood comes from a 55-year-old stand growing in the Rogów forest district (51.916289, 19.912083) in central Poland. According to the standard EN 113, wood test specimens with dimensions 15 × 25 × 50 mm^3 (tangential × radial × longitudinal) were taken from the middle part of the radius, from the butt part of the trunk (0.5–1.5 m). The specimens were free from defects. They were conditioned at 20 °C and 65% relative humidity for two weeks. After conditioning, the specimens were weighed. The mean air-dry density of beech wood was 0.678 g/cm^3.

A culture of *Fomes fomentarius* mycelium was used. The strain came from the culture collection of the Department of Forest Protection of the Warsaw University of Life Sciences. It was isolated from a dead silver birch (*Betula pendula*).

2.2. Nanoparticles

The two types of metal nanoparticles selected were AgNPs and CuNPs—well known for centuries for their antimicrobial properties. Moreover, copper-based biocides have provided protection against fungal decay and termite damage to wood since the 1930s [23,44,45]. Additionally, among the metallic nanoparticles, Ag nanoparticles have been the most studied as antifungal agents, followed by Cu nanoparticles. These nanoparticles have shown promising activity against different species of phytopathogenic fungi.

Samples of commercially available solutions of AgNPs and CuNPs were purchased from Nano-Koloid sp. z o. o., Karolino, Poland, a licensee of Nano Technologies Group, Inc. (NY, USA), manufactured under European patent EP2081672 A2. According to the manufacturer, they are produced in a physical process, consist of about 100 atoms (about 5 nm) and are suspended in demineralized water. The concentration of nanoparticles in the commercially available product is 50 ppm.

2.3. In Vitro Assay

The experiment was performed in Petri dishes with a diameter of 9 cm. *F. fomentarius* was cultured on malt extract agar (MEA) [46]. Nanoparticles were added to the medium at concentrations of 5, 25, or 50 ppm. Control dishes contained only MEA. Each experimental combination (type and concentration of nanoparticles) was subject to 10 replicates (i.e., 10 Petri dishes). The inoculum consisted of 2-week-old fungal cultures in the form of discs with a diameter of 3 mm. The fungi were incubated in the dark in thermostats set at a temperature of 22 °C. Measurements of mycelial diameter were made after 3, 5, 6, and 7 days. The timing of the measurement was related to the growth rate of the mycelium, as the measurement was taken at the time when the growing piece of mycelium reached the edge of the Petri dish. The "cross" reading of two measurements was made at the bottom of each dish with an accuracy of 1 mm.

Based on the results obtained (after 7 days), an inhibition or stimulation index was calculated according to the following formula:

$$I/SI = (DT - DC)/DC \times 100\%, \quad (1)$$

where I/SI—is the inhibition/stimulation index (%), DT is the diameter of the fungal colony on the test plate, and DC is the diameter of the fungal colony on the control plate.

This formula for the index is such that a negative value indicates inhibition of fungal growth, while a positive value indicates stimulation.

2.4. Decay Test

Wood specimens were sterilized twice in an autoclave (second sterilization after 24 h) at 121 °C. On the first day, the material was sterilized for 20 min, and on the second day for 10 min. After sterilization, the wood specimens were placed in a laminar chamber and allowed to cool for 2 to 3 h. They were then soaked for one hour in chilled sterile distilled water (control specimens) or in an AgNPS or CuNPs solution at three concentrations: 5 ppm, 25 ppm, or 50 ppm. A total of 256 wood specimens were tested (32 wood specimens per variant × 2 nanoparticle types × 3 concentrations, AgNPs control, CuNPs control). The treated and untreated specimens were placed in Kolle flasks with MEA medium on a two-week-old mycelium of *F. fomentarius*. Kolle flasks were incubated in a culture chamber (Q-Cell 700, Poll Lab, Wilkowice, Poland) for 60 or 120 days at 22 °C and 70 ± 5% relative humidity.

After 60 days, half of the Kolle flasks (16 specimens) from each variant were randomly selected. The remaining wood specimens were collected after 120 days of the experiment. The specimens were cleaned to remove fungal mycelium, oven dried to a constant weight, and reweighed. Then, the percentage weight loss was calculated.

2.5. SEM Analysis

To evaluate the morphology of control wood and nanoparticles-treated wood, small specimens were cut and then examined using a scanning electron microscope (FEI Quanta 200; Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Statistical Analysis

The antifungal activities of of AgNPs and CuNPs against *F. fomentarius* on the medium MEA, mycelial diameter and weight loss of beech specimens were analyzed using two-way ANOVA with nanoparticle concentration and time as factors. For comparison of mean values was performed using Tukey HSD test. The values of the characteristics were in accordance with the assumptions of ANOVA, based on the homogeneity of variances—Levene test, and the normal distribution of the data was checked with the Shapiro–Wilk test. Statistical analyses were performed using R version 4.2.1 (The R Foundation for Statistical Computing, Vienna, Austria). The stat package was used for ANOVA, and the emmeans package in R was used for mean estimation and the Tukey HSD test. The accepted significance level was $p \leq 0.05$.

3. Results

3.1. In Vitro Assay

The radial growth rate of the mycelium of *F. fomentarius* on the medium containing CuNPs at a concentration of 50 ppm did not differ from the control variant during the experimental period (7 days). However, at concentrations of 5 and 25 ppm, mycelial growth was significantly higher from the fifth day of the experiment compared to the control. After 7 days of the experiment, radial mycelial growth on the medium containing AgNPs at concentrations of 5 and 25 ppm was not statistically different from mycelial growth in the control variant without AgNPs. However, the 50 ppm concentration significantly inhibited mycelial growth compared to the control (Figure 1a,b, Table 1).

Table 1. Results of two-way analysis of variance for colony diameters of *Fomes fomentarius* and weight loss of beech wood as a result of the action CuNPs and AgNPs in CuNPs and AgNPs.

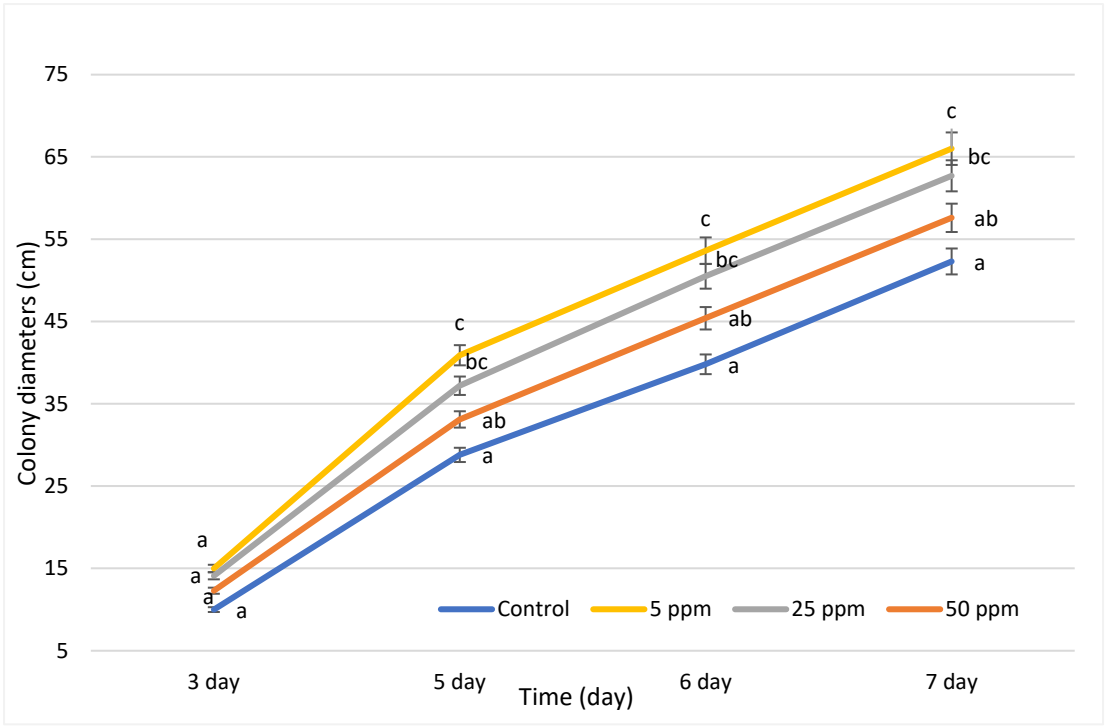
Effect	CuNPs		AgNPs	
	F	***p* Value**	**F**	***p* Value**
Colony dimeters				
Nanoparticle concentration	39.36	<0.0001	17.16	<0.0001
Time	663.33	<0.0001	263.41	<0.0001
Interaction between nanoparticle concentration and time	1.40	0.1935	1.74	0.0851
Weight loss				
Nanoparticle concentration	59.08	<0.0001	109.65	<0.0001
Time	11.97	0.0022	18.65	<0.0001
Interaction between nanoparticle concentration and time	0.46	0.7126	2.01	0.1159

The stimulation/inhibition index showed stimulation of mycelial growth of *F. fomentarius* growing on medium containing CuNPs. The extent of stimulation was inversely proportional to the concentration used. In the case of AgNPs, the stimulation/inhibition index was positive (stimulation) at a concentration of 5 and 25 ppm and negative (inhibition) at a concentration of 50 ppm (Figure 2).

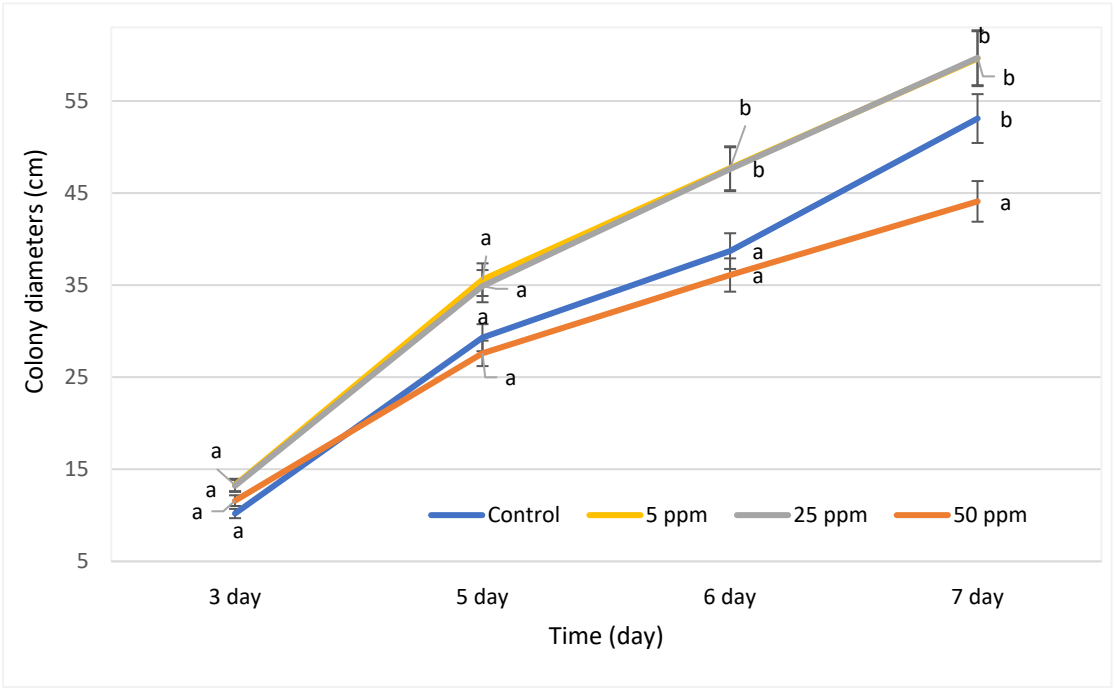
3.2. Decay Test

The mean weight loss of beech wood specimens was different after treatment with AgNPs and CuNPs. The development of decay was stimulated by CuNPs at concentrations of 25 ppm and 50 ppm after 60 days and at concentration of 50 ppm after 120 days. In both cases, the differences in weight loss were not large and amounted to 3–4%, but were

statistically significant. Specimens treated with AgNPs at the highest concentration—50 ppm—had significantly lower mean mass losses than untreated specimens and those treated with concentrations of 5 ppm and 25 ppm at both 60 and 120 days (Figure 3, Table 1).



(**a**)



(**b**)

Figure 1. (**a**) Antifungal activities of different concentrations of CuNPs and (**b**) AgNPs against *F. fomentarius* on MEA medium. Different letters indicate significant differences determined by Tukey HSD test ($p \leq 0.05$). Error bars indicate standard errors.

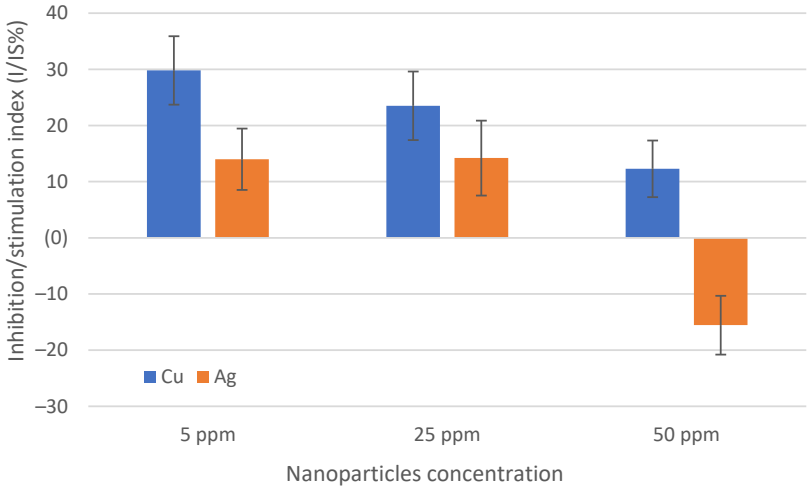
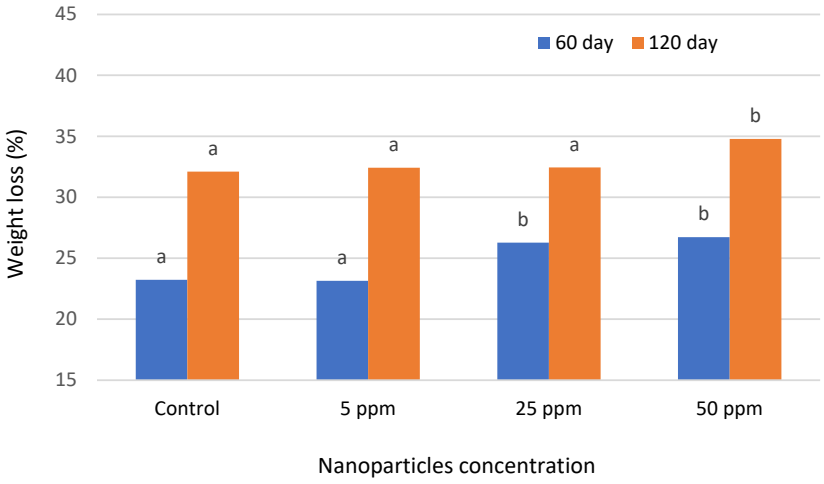
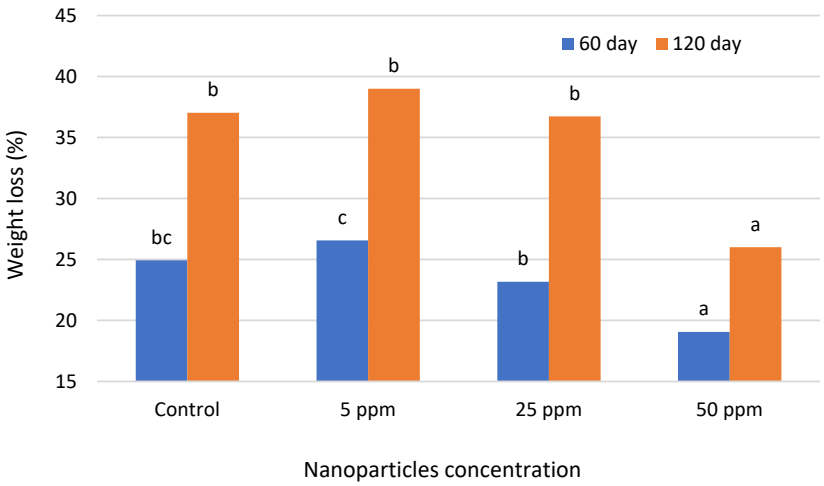


Figure 2. Inhibition/stimulation index (I/IS %) of *F. fomentarius* mycelia growth on MEA medium containing CuNPs or AgNPs during 7 days. Error bars indicate standard errors.



(**a**)



(**b**)

Figure 3. (**a**) Weight loss (%) of beech wood samples treated with CuNPs and (**b**) AgNPs after 60 and 120 days. Different letters indicate significant differences determined by Tukey HSD test ($p \leq 0.05$).

3.3. SEM Analysis

The untreated wood specimens exhibited the typical characteristics of beech wood, i.e., a semi-ring porous type composed of vessels, fibrous and parenchyma cells. We recognized a solitary distribution of vessels and in radial groups. The perforated plates were both simple and compound. The rays varied in size, and the broad rays were conspicuous by eye. No decay was observed in the untreated wood specimens.

The treated wood specimens exhibited varying degrees of decomposition of the layered walls of the wood cells. The decomposition caused by the activity of lignocellulose-degrading enzymes of *F. fomentarius* was observed both in the specimens treated with AgNPs and CuNPs. The hyphae mainly penetrated the lumen of the vessels but were also observed on the surface of the whole wood specimens (Figure 4a–c). The vessels were often collapsed, and some had completely degraded walls, resulting in irregularly shaped holes visible in the cross-sections (Figure 4a). Although the degree of decay was visible in the images from SEM, the color of the nanoparticles-treated wood was similar to that of the control wood.

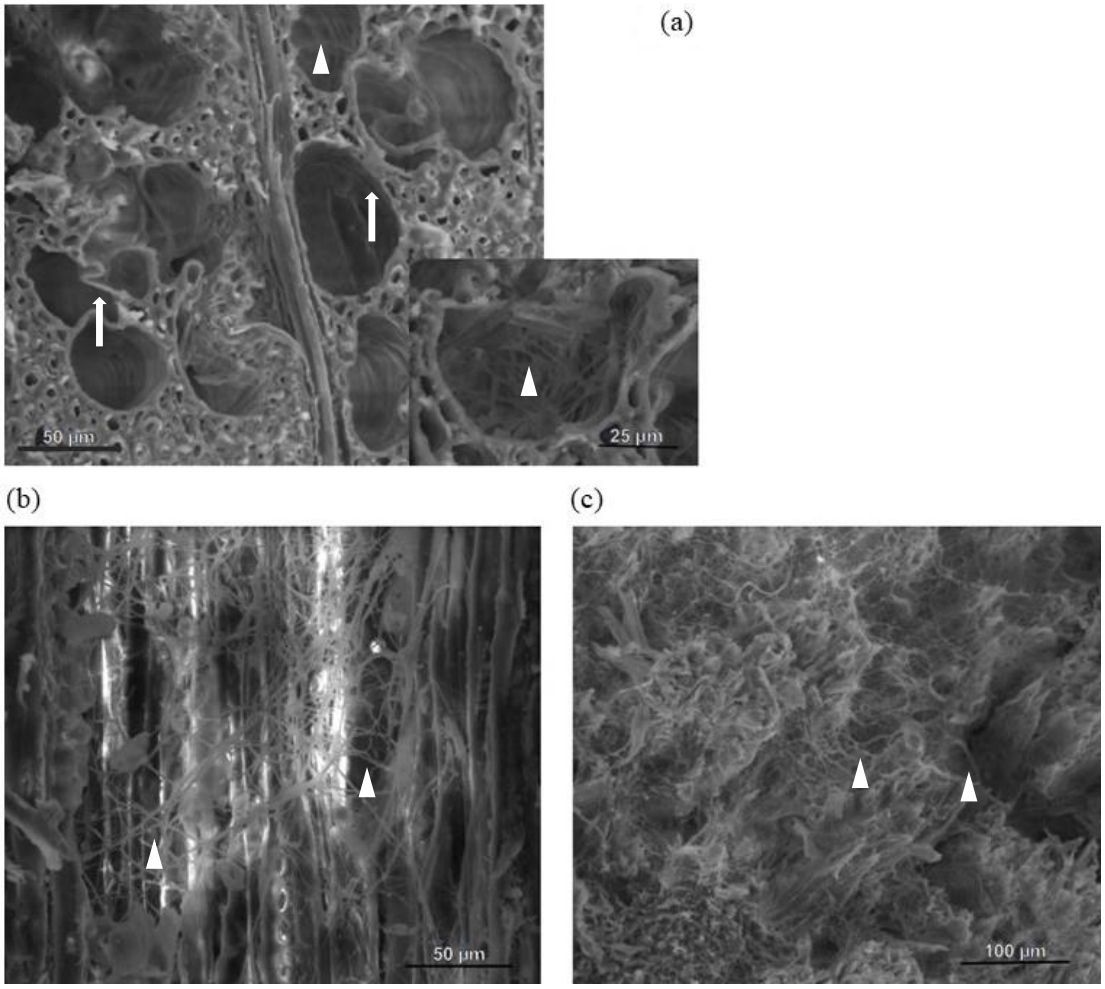


Figure 4. (**a**,**b**) SEM images of wood treated with CuNPs and (**c**) AgNPs after 120 days of the experiment. The concentration of both nanoparticles was 50 ppm. (**a**) Cross-sectional area showing advanced decomposition of vessel walls and visible hyphae in vessel lumen. (**b**) The tangential area of the wood specimen with visible hyphae on its surface. (**c**) Cross-sectional area of the wood specimen. The mycelium completely covered the surface of the examined wood specimen, arrows—strongly thinned cell wall of vessels due to strong degradation of its chemical compounds, arrowheads—colonization of hyphae in cell lumina of vessels (**a**) and on the surface of the wood specimens (**b**,**c**).

4. Discussion

The effects of AgNPs and CuNPs on fungi in vitro vary significantly depending on the size [47], the type and concentration of NPs, the fungal species [24,48], and the media composition [49]. In our study, we observed radial growth stimulation of *F. fomentarius* by CuNPs at the two highest concentrations 25 and 50 ppm, lack of effect with CuNPs at 5 ppm and AgNPs at 5 and 25 ppm, and growth inhibition by AgNPs at the concentration of 50 ppm. Although many studies indicate high antifungal activity of NPs even at low concentrations [47,50], our study cannot confirm this. However, they are consistent with the results of Aleksandrowicz-Trzcińska et al. [24]. Baldrian [51] claimed that there is a correlation between copper tolerance of decay fungi in wood and in agar. In our study, the results of the in vitro test for both NPs correlate well with the mass losses determined by the decay test.

The results suggest that the rotting of beech wood caused by *F. fomentarius* was not only not inhibited by CuNPs but stimulated by the application of nanoparticles at higher concentrations. Our results are difficult to compare with those of other researchers because they involve different species of wood and fungi, different types of nanoparticles, their size and concentration. The experiment most similar to ours was performed by Bak and Németh [39]. They investigated CuNPs in rot prevention of beech wood caused by the white rot fungus *Coriolus versicolor*, but the nanoparticles were smaller (2–4 nm) than in our experiment and were applied at different concentrations of 0.5%–2%. The results obtained by Bak and Németh [39] suggest that CuNPs did not provide effective protection, and the results are in agreement with ours.

Copper is toxic to most fungi even at very low concentrations [51], which is why copper-based biocides have been used since the 1930s to protect wood from decay fungi [25]. At the same time, copper is also a metal necessary for fungal growth [23]. White rot fungi secrete three major classes of ligninolytic enzymes: lignin peroxidases, manganese-dependent peroxidases, and laccases [52]. Some studies show that copper in millimolar amounts stimulates the formation of these enzymes by these fungi [53–55], but copper at higher concentrations is extremely toxic to microbial cells [56]. Most likely, the copper from the CuNPs of *F. fomentarius* was used to increase the growth rate and enzyme production, thus increasing the degree of wood rot [30] but we still believe that CuNPs are suitable to protect beech wood from fungi, but higher concentrations than those used in our experiment should be tested. The result we obtained, i.e., the lack of protection of wood from decomposition by CuNPs, could also be the result of different tolerance to heavy metals, including copper, among fungal species, but also within strains of the same species [51].

Our study demonstrated the antifungal activity of AgNPs. The average weight losses of wood samples were significantly lower, but only after treatment with nanoparticles at the highest concentration of 50 ppm. It is also important to note that the application of nanoparticles at concentrations of 5 and 25 ppm did not provide protection against *F. fomentarius*, and even after the application of AgNPs at a concentration of 50 ppm, wood protection was not satisfactory. After 60 days, the average weight loss was 19% and after 120 days 26%.

Results similar to ours, indicating partial protection of wood treated with AgNPs, were obtained by Can [28]. In contrast, high antifungal activity of AgNPs with variable sizes from 10 to 30 nm was demonstrated by Casado-Sanz et al. [26] at very low concentrations from 5 to 20 ppm. A similar high protective effect was obtained by Akhtari and Arefkhani [29] with AgNPs of size 10–80 nm, but at a high concentration of 400 ppm. We believe that the AgNP concentrations used were too low, as with CuNPs, because silver has been known for its antimicrobial properties for centuries. Silver ions inhibit the activity of extracellular enzymes of both white rot and brown rot fungi [30,51]. Metallic silver oxidizes under humid conditions, leading to the formation of silver ions. The oxidation process is a slow reaction, but in the case of NPs, their small size provides a larger surface area and thus a larger area available for oxidation than bulk materials [27,32,39].

Our results from SEM analysis confirmed the low protection of beech wood by CuNPs and AgNPs against *F. fomentarius*. The lignin-rich middle lamella and all wall layers composed of cellulose, hemicellulose, and lignin were utilized by the fungus, resulting in deterioration of the physical properties of the wood samples studied.

The aim of our research was to test the feasibility of using NPs as wood preservatives for the protection of old, monumental trees and wood in forests and on timber depots. For this purpose, we used the application method in which the samples were soaked in NPs solutions. The size of the NPs used was 5 nm, so they could easily enter the interior of the cells [57]. However, regardless of the size of the NPs, this method is less effective than the vacuum-pressure impregnation method [58], which has been used in many studies with NPs (e.g., Pařil et al. [30]). On the other hand, similar results from the in vitro and decay tests indicate that regardless of how the NPs were applied, their concentrations were too low to inhibit radial mycelial growth and decomposition of the wood samples.

The results of our study and those of other authors show that it is possible to achieve resistance to decay fungi in wood by using AgNP and CuNP. However, based on these results, it is difficult to make a definite statement about what factors must be fulfilled to achieve a high level of wood protection. NPs of different sizes and shapes, applied at different concentrations, were tested for the protection of different wood species against different types of fungi. The methods of applying the NPs also varied. Therefore, further research should be conducted.

5. Conclusions

The in vitro assay showed stimulation of mycelial growth of *F. fomentarius* by CuNPs. The extent of stimulation was inversely proportional to the concentration used. AgNPs inhibited radial growth of the mycelium, but only at a concentration of 50 ppm. Lower concentrations (5 and 25 ppm) were unaffected. The in vitro tests for both NPs correlate well with the mass losses determined by the decay test. An increase in the resistance of beech wood to decay caused by *F. fomentarius* was obtained only when AgNPs were applied at the highest concentration (50 ppm). In the application method where the wood was soaked in a NP solution, the concentrations of both NPs were too low to efficiently protect the beech wood from decomposition by the xylophagous fungus.

Author Contributions: J.P. and M.A.-T. conceptualized and designed the study. J.P., M.T., A.A. and E.Z. carried out the experiments. J.P., M.S., M.A.-T. and M.T. organized the database and performed the analysis. M.A.-T. wrote the manuscript with contributions from J.P., M.T. and T.O. All authors have read and agreed to the published version of the manuscript.

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

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