Silver nanoparticles for enhancing the efficiency of micropropagation of gray poplar (Populus × canescens Aiton. Sm.)

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Abstract. The main purpose of the work was to determine the prospects for the use of silver nanoparticles in woody plants tissue culture and to identify possible limitations. The paper presents the results of experiments on the use of silver nanoparticles (AgNPs) to increase the efficiency of in vitro tissue culture of woody plant cultures using the example of gray poplar (Populus × canescens Aiton. Sm.) at various stages of microclonal reproduction. It has been shown that sequential treatment with 7.5 g/L NaOCl for 30 min and 0.3 g/L AgNPs for 5 min allows obtaining 100% phytopathogen-free explants, increasing their viability. Modification of the nutrient medium with AgNPs at concentrations of 1.5...3 µg/L during multiplication and rooting reduces the phytopathogen infestation, and also stimulates the formation of the root system and accelerates the growth of the vegetative part of the shoots. In addition, the combination of photosynthetic and enzymatic activity suggests a rather high potential for stress resistance of experimental microclones in comparison with control ones.

1. Introduction
Currently, the natural habitat of woody plants is significantly reduced. The main reasons are overexploitation of wood, fires, poor reproduction of some species from seeds, contamination of planting material with phytopathogens [1] and physiologically caused slow growth of trees [2]. Therefore, the technique of in vitro micropropagation is one of the most promising options for solving the problem of obtaining planting material for forest restoration [3]. Micropropagation is the use of in vitro techniques to rapidly produce, asexually, plants that are genetically identical to the original. This method has great prospects both in the field of conservation of rare and endangered plant species, and in the creation of high quality microshoots for intensive reforestation [4].
The success of reproduction is influenced by several factors at once, among which the most important are the genotype, physiological status of donor plants, type of explants, surface disinfection methods, nutrient medium, plant growth regulators and growing conditions (light intensity, photoperiod, humidity, temperature, etc.) [5]. Since fungal and bacterial infections are barriers limiting the aseptic growth and development of woody plants [6], one of the most important factors is high-quality disinfection of the original plants, as well as preventing the contamination of the resulting microclones with phytopathogens [4, 7]. There is a fairly wide range of methods and techniques for sterilizing explants, each of which has its own advantages and disadvantages [1, 8-11]. Thus, the range of effectiveness of antibiotics is often quite narrow and extends only to bacteria, they often have a phytotoxic effect [12, 13], and with regular use, the formation of antibiotic resistance is possible [14, 15]. And the massive use of effective HgCl₂ is unacceptable due to its high toxicity and negative effect on human and animals [16]. Another potential sterilizing agent can be nanoparticles, some of which have a pronounced antibacterial and fungicidal effect [17-19].

With the active development of nanotechnology and the involvement of nanostructures in almost all spheres of life, agriculture and forestry have not been spared either [20-22]. To date, some experience has already been accumulated in the experimental use of nanoparticles to improve seed germination, stimulate plant growth and productivity, provide genetic modification of plants and protect herbaceous plants from phytopathogens [23-26]. Among all the variety of artificially synthesized nanoparticles, silver nanoparticles (AgNPs) are the most widely used as a sterilizing agent [18, 19, 24, 26-28]. In view of this, there is an assumption that the use of AgNPs, which exhibit antimicrobial activity, have high thermal stability and low volatility, may be the optimal solution in the methods of micropropagation of woody plants.

There are several works confirming the good potential in the fight against bacterial and fungal infections of wood explants. For example, in [23] the antifungal and antibacterial activity of AgNPs was evaluated upon direct processing of explants of the almond – peach hybrid G × N15 and upon addition to the nutrient medium. The results showed the high efficiency of using AgNPs at concentrations of 100 and 150 mg/L, the addition of which to the nutrient medium made it possible to reduce the contamination of explants to almost zero. However, AgNPs at concentrations greater than 150 mg/L had an unfavorable effect on kidney viability and seedling regeneration G × N15, and this effect was more pronounced when the explants were directly treated with AgNPs solution than when it was added to the medium. S. El-Kosary et al. investigated the effect of AgNPs at the concentrations of 0.125…0.5 mg/L on the level of bacterial contamination of explants of the date palm Phoenix Dactylifera L (varieties Sewi and Medjool) at different stages of micropropagation. A decrease in the level of contamination with an increase in the used concentration of AgNPs has been shown. The most effective was found to be 0.5 mg/L, which allows increasing the values of infected plants at the stage of explant formation to 97.23% compared to 80.56% in the control group, and at the multiplication stage to obtain 100% of explants free from phytopathogens. In addition, the authors noted a positive effect of AgNPs on the growth and shoot formation of explants [27]. The antibacterial effectiveness of AgNPs in the fight against diseases of coniferous plants was studied on the example of a tissue culture of Araucaria excelsa R. Br. The effects of nanosilver at all stages of micro-reproduction were studied in two experiments. In the first of them, 100, 200 and 400 mg/L AgNPs were used during standard sterilization for 60 and 180 min; in the second, nanosilver in concentrations 25, 50, 100, 200, 400, 800 and 1600 mg/L was added to the medium before autoclaving. The addition of AgNP to the culture medium inhibited the development of infection, and also reduced the percentage of bacterial infection. 400 mg/L of AgNP in nutrient media for a month reduced bacterial contamination by 62.5% compared to the control. At the same time, a high concentration (800 mg/L) of nanosilver led to necrosis of explant tissues [26]. Adding 5 mg/L AgNPs to the culture medium at the initiation stage allowed to reduce the infection of explants of downy birch Betula pubescens Ehrh by 15–25%, as well as to increase their morphogenic potential [29]. The authors of [30] propose AgNPs obtained by the "green" synthesis method as a substitute for antibiotics during sterilization of Capparis decidua explants. It has been shown that soaking explants for 20 min in a solution containing 100 mg/L AgNPs provides 100% infection-free explants with a survival rate of
95.8%. At the same time, an increase in exposure to 60 min leads to a decrease in the survival rate to 62.5%. In turn, AgNPs in high concentrations, which is shown when using 300 and 500 mg/L, although they provide a high antibacterial effect, reduce the survival rates of explants down to zero. When 50, 100, 150, and 200 mg/L AgNPs were added to the culture medium, the optimal concentration among the tested was 150 mg/L, the use of which made it possible to obtain 90.2% of bacteria-free explants and 94.4% of fungal infections with an overall 80.5% survival rate explants. The rest of the concentrations did not provide a high survival rate of the explants.

Therefore, the purpose of our work was to estimate the effect of AgNP in various concentrations on the efficiency of micropropagation of woody plants using the example of gray poplar at the initiation, sterilization, proliferation, rooting and acclimatization stages.

2. Materials and methods

2.1. AgNPs

In this work, we used AgNPs obtained by electric explosion of a conductor in an argon atmosphere ("Advanced Powder Technologies", Tomsk, Russia). Colloidal solutions were obtained under aseptic conditions using sterile distilled water. The suspensions were subjected to ultrasonic stirring using a Vibra-Cell VC 750 disperser (Sonics & Materials, USA) for 15 min at a power of 300 W and a frequency of 23.740 kHz. The analysis of the particle size and their stability in freshlyprepared colloids and in the composition of culture media for microcloning without plants was carried out using a Zetasizer Nano analyzer (Malvern Instruments, UK). Electron microscopic examination of AgNPs was carried out on a Merlin high-resolution scanning electron microscope (Carl Zeiss, Germany). Experiments were conducted using the equipment of the Derzhavin State University Center for Collective Usage (Tambov, Russia).

2.2. Nutrient medium

In the experiments at the stages of obtaining explants and their multiplication, the Murashige-Skoog culture medium (MS) [31] was used. It most commonly used for growing tissue culture or whole plants. At the stage of rooting the ½ Woody Plant Medium (WPM) nutrient medium was used [32]. At the stage of plant acclimatization in greenhouses, a substrate consisting of peat with neutral pH and 3:1 perlite was used. The pH of the aqueous extract from the peat substrate was 6.7.

2.3. Obtaining explants of gray poplar (Populus × canescens Aiton. Sm.) plants

To obtain explants we used visually healthy cuttings of gray poplar (Populus × canescens) plants growing in open ground. The work was guided by generally accepted methods of in vitro cultivation of isolated plant organs [33]. Shoots were cut under aseptic conditions into 1.5-2 cm segments with one axillary bud (culture explants) and introduced into a culture vessel on nutrient media. After being introduced into the nutrient medium, the explants were cultivated at a photoperiod of 16 h with an illumination intensity of 5 kLx, at a temperature of 25°C during the day and 19-20°C at night. Seedlings were transplanted to a new medium weekly under sterile conditions to avoid depletion of the media.

2.4. Initiation stage

At this stage, the most widely used sodium hypochlorite solution - NaOCl (at a concentration of 7.5 g/L), a colloidal solution of AgNPs (0.3 and 0.45 g/L) were used as antiseptic agents. The exposure period was 30 min. Two more groups were subjected to sequential treatment with NaOCl (30 min) + AgNPs (5 min) (7.5 g/L NaOCl + 0.3 g/L AgNPs; 5 g/L NaOCl + 0.3 g/L AgNPs). The following indicators were taken into account: sterility,%; the number of surviving microclones,%; the height of the shoots, cm; the condition of the shoots on a 5-point scale.

2.4.1. Number of sterile explants. After 14 and 28 days of cultivation, the number of sterile explants (without fungal and bacterial infection) was counted on nutrient media after sterilization.
2.4.2. **The number of surviving explants and microclones.** After 14 and 28 days of cultivation, the number of explants that survived after sterilization and had less than 30% tissue lesions were counted.

2.4.3. **Shoot height.** The height of the shoots regenerated from the introduced explants of woody plants was measured after 14 and 28 days. The dimensions were taken with a ruler from the nutrient medium to the highest point of the shoot in cm.

2.4.4. **Shoots condition on a 5-point scale.** The condition of the shoots was assessed by the appearance of the shoots regenerating from explants introduced into the tissue culture.

   Criteria for evaluation:
   - 5 points – ideal condition of shoots, green color, absence of necrotic foci, shoots tend to regenerate;
   - 4 points – good condition of shoots, green color, necrotic foci occupy no more than 10% of green mass, shoots tend to regenerate;
   - 3 points – satisfactory condition of shoots, necrotic foci occupy no more than 30% of green mass, shoots retain a tendency to regeneration;
   - 2 points – poor condition of shoots, necrotic foci occupy more than 30% of green mass, shoots do not retain a tendency to regenerate;
   - 1 point – very poor condition of the shoots, necrotic foci occupy more than 60% of the green mass, shoots do not retain a tendency to regenerate.

2.5. **Proliferation stage**

At the proliferation stage, 8 groups were selected from the microclones of the control group of the previous stage. 0.75, 1.5, 3, 6 and 15 μg/L of AgNP were introduced into the MS culture medium. To assess the stimulation of the formation of new shoots, two additional groups were formed: 1) with the addition of phytohormones stimulating shoot formation (PhH) (0.2 mg/L of 6-benzylaminopurine + 0.1 mg/L of indoleacetic acid + 0.3 mg/L of gibberelic acid), stimulating the formation of shoots and 2) PhH, additionally containing 1.5 μg/L of AgNP. Phytohormones and nanoparticles were not used in the control group. After 28 days, such indicators as sterility, %, the number of surviving microclones, %; the height of shoots, cm; the number of leaves and additional shoots, pcs; the presence of roots; the state of shoots on a 5-point scale were evaluated.

Biochemical studies were also carried out on the 28th day after the start of the multiplication stage. Measurement of the level of catalase activity was determined by the permanganatometric method according to Bertrand and Oparin - the titer of H₂O₂ with potassium permanganate in the presence of sulfuric acid.

The intensity of photosynthesis of chlorophyll-containing tissues was recorded using an IFSR-2 instrument (fluorometric indicator of physiological state) [34].

Histological studies of the tissue of the leaf blade - stomatal density, stomatal fissure size were carried out according to generally accepted methods using the VideoTesT-Morphology 4.0 hardware-software complex.

2.6. **Rooting stage**

At this stage, 6 groups were formed. AgNPs were added to the ½ WPM culture medium at concentrations of 0.75, 1.5, 3, 6, and 15 μg/L. Plants cultivated on pure ½ WPM without the addition of nanoparticles served as control. The number of rooted microclones (%) was recorded on days 7, 14, 21, and 28 of the experiment. Indicators of the state of microclones, including the number of surviving microclones, %; the height of the shoots, cm; the number of leaves and additional shoots, pcs; the number of roots, pcs; the condition of the shoots on a 5-point scale was assessed 28 days after the start of the rooting stage.

2.7. **Acclimatization stage**

For transplantation into non-sterile conditions, leveled gray poplar plants were used, having a powerful stem, 5-6 leaves and a well-developed root system with a root length of at least 2 cm. Before planting,
the roots were washed from the remains of the nutrient medium with a 1% solution of potassium permanganate. The plants were carefully removed from flasks or test tubes with tweezers with long ends and planted in technological cassettes, filled with pre-moistened soil substrate in greenhouses.

At acclimatization stage, 4 groups were formed. The number of plants in each group was 100 pcs. Since at the rooting stage, the best results were demonstrated in the groups of 1.5 and 3 μg/L AgNPs, and the use of high concentrations led to inhibition of the general state of microclones, it was decided at this stage to use only the two indicated concentrations. The control was distilled water. Fludioxonil 0.01 g/L was used as a positive control. The survival rate of microclones, %, substrate contamination, %, and biomorphological parameters of shoots were evaluated as described above.

All experiments were carried out in three repetitions. Each experimental group contained 30 microclones. Statistical data processing was performed using the Descriptive Statistics software package Microsoft Excel 2010. To assess the reliability of the differences, a one-way analysis of variance (ANOVA) with a significance level of 5% was used.

3. Results and discussion

3.1. Characterization of AgNPs

After the preparation of colloidal solutions according to the method described in 2.1, the NP size analysis was carried out, according to which the average particle size in suspensions was 30…70 nm (figure 1). The AgNPs used in this study were spherical, as evidenced by the SEM micrograph (figure 1, inset). When analyzing the particle size, we analyzed the colloidal solution and media with the maximum content of nanoparticles (15 μg/L), because it is obvious that in the presence of Brownian motion, large aggregation will be observed in solutions with a high content of nanoparticles.

![Figure 1. Dispersion composition of 15 μg/L AgNPs in water. On the insert (left) – SEM-image of AgNPs.](image)

The study of the stability of AgNPs in experimental samples of nutrient media was carried out by analyzing the dispersed composition of the media and measuring the zeta potential (figure 2). Analysis of the disperse composition of AgNPs in a freshly prepared medium showed that the particle sizes are in the range of 70 - 250 nm (figure 2a). After a three-month holding of the dispersed composition, the sizes increased to 80-350 nm (figure 2b), which indicates a tendency for the development of particle aggregation processes.

The minimum indicators of the zeta potential were recorded at the content of 0.75 and 6 μg/L of nanoparticles, however, holding the media for three months led to an increase in the values from -35 and -39 mV to -31 and -24 mV, respectively. A three-month exposure of NPs in a nutrient medium at all concentrations, except for 1.5 μg/L, led to an increase in the studied indicator (figure 2c). An increase in the particle size and a decrease in the ζ-potential indicates the instability of the AgNP suspension in
the culture medium and the presence of a tendency towards aggregation. The results obtained agree with the conclusions of other authors [35, 36].

Figure 2. Particle size distribution in freshly prepared (a) and three months aged (b) 15 μg/L AgNPs suspensions in MS, and zeta potential in freshly prepared and three months aged suspensions depending on AgNPs concentration in MS (c). *The difference is significant (p<0.05) in comparison with the values of freshly prepared media containing AgNPs.

3.2. Initiation stage

The results of a comparison of the effect of different sterilizing agents on the purity of the gray poplar explants 14 and 28 days after treatment showed that when using NaOCl at a concentration of 7.5 g/L, 93 and 75% of the explants remained sterile, respectively (figure 3a).

At the same time, only 65 and 33% were viable (figure 3b). This is probably due to the exposure of young plant tissues to chlorine, which causes necrosis. Treatment with colloidal solutions of AgNPs was significantly inferior in efficiency to a solution of 7.5 g/L NaOCl. Sterilization with colloidal solutions of 0.3 g/L AgNPs made it possible to obtain on the 14th and 28th day of the experiment only 50% of sterile explants, however, however, there was a tendency in the group to increase the number of viable explants, in comparison with the group of 7.5 g/L NaOCl.
The solution of 0.45 g/L AgNPs proved to be somewhat more effective, the use of which contributed to an increase in the number of sterile explants by 5%, while on the 14th day of the experiment the number of viables was reduced by 6.9% in comparison with the group of 0.3 g/L AgNPs (figure 3).

The most effective sterilization method was the sequential treatment with NaOCl for 30 min and AgNPs for 5 min, which made it possible to obtain 100% phytopathogen-free explants, significantly increasing their viability in comparison with the groups receiving NaOCl and AgNPs separately. Moreover, the addition of AgNPs made it possible to reduce the effective concentration of NaOCl, while increasing the percentage of viable explants on the 28th day of the experiment (figure 3).

During the experiment, a positive dynamics of the growth of primary shoots of gray poplar was observed in all experimental groups. The general condition of the primary shoots after 14 and 28 days of explant cultivation in groups treated with 7.5 g/L NaOCl and 0.3...0.45 g/L AgNPs was assessed as good. The best condition (5 points) was observed in explants treated with NaOCl + AgNPs. Shoot height in all groups on the 14th day did not differ significantly. However, by the 28th day, the maximum height of growing shoots in the group 7.5 g/L NaOCl + 0.3 g/L AgNPs was 62.5% higher than that of the group 7.5 g/L NaOCl and by 30% in group 0, 3 g/L AgNPs (table 1).

Thus, the optimal conditions for sterilization of the introduced explants, which had an antiseptic and growth-stimulating effect, for gray poplar were the use of 5 g/L NaOCl + 0.3 g/L AgNPs. This made it possible to obtain up to 100% of sterile explants with viability and regenerative capacity in 70% or more cases.

### Table 1. Biomorphological parameters of the primary shoots depending on the sterilization method.

| Sterilization method | Shoots height, cm | Clones condition on the 1 to 5 scale |
|----------------------|-------------------|--------------------------------------|
|                      | 14th day          | 28th day                             |
| 7.5 g/L NaOCl        | 0.5±0.03          | 0.8±0.05                             |
| 0.45 g/L AgNPs       | 0.6±0.02          | 0.9±0.04                             |
| 0.3 g/L AgNPs        | 0.7±0.02          | 1.0±0.04                             |
| 7.5 g/L NaOCl+0.3 g/L AgNPs | 0.6±0.03          | 1.3±0.02*                            |
| 5 g/L NaOCl+0.3 g/L AgNPs | 0.7±0.04          | 1.5±0.04*                            |

*The difference is significant compared to the values for 7.5 g/L NaOCl (p < 0.05).

Thus, the optimal conditions for sterilization of the introduced explants, which had an antiseptic and growth-stimulating effect, for gray poplar were the use of 5 g/L NaOCl + 0.3 g/L AgNPs. This made it possible to obtain up to 100% of sterile explants with viability and regenerative capacity in 70% or more cases.

### 3.3. Proliferation Stage

In 28 days after the establishment the culture from the axillary meristems of the gray poplar, it was possible to obtain morphogenic microclones without visible anomalies and to proceed to the stage of multiplication. 28 days after the start of the multiplication stage, the addition of AgNPs at a concentration of 1.5-15 μg/L made it possible to maintain sterility at the level of 100%, in contrast to the control groups, in which the sterility of micro shoots was at the level of 80%. A positive effect of AgNPs at concentrations of 1.5 and 3 μg/L on the growth processes of gray poplar meristems in tissue culture was noted. The number of survivors during the passage of microclones was 20% higher compared to the control (MS without AgNPs added). At the same time, an increase in the concentration of AgNPs to 15 μg/L led to the development of a toxic reaction in plants and a significant decrease in their viability (figure 4).

Analysis of the results of biomorphological studies confirms that AgNPs at concentrations of 1.5 and 3 μg/L have a stimulating effect on the growth and development of microclones, including stimulating the formation of the root system and accelerating the growth of the vegetative part of the shoot. The addition of PhH also stimulates the development of microclones, which is confirmed by an increase in the additional shoots in comparison with the control group, but does not affect the development of the
root system. AgNPs at concentrations of 6 and 15 μg/L cause inhibition of plant development, which is expressed in a deterioration in the general condition of microclones in comparison with the control group and groups receiving PhH and AgNPs at lower concentrations. It is noteworthy that in the group receiving 1.5AgNPs + PhH, a slight slowdown in shoot growth was observed with an increase in the stimulating effect on the generation of additional shoots in comparison with the control group and the group receiving PhH without nanoparticles. No root formation on media with PhH was observed, which is associated with the influence of cytokinin (table 2).

![Figure 4. Sterility and viability of explants on the 28th day after the start of the proliferation stage. *The difference is significant in comparison with the values of control group (p < 0.05).](image)

| Group               | Shoot height, cm | Number of leaves, pcs | Number of additional shoots, pcs | Presence of roots | Condition of microclones on a 5-point scale |
|---------------------|------------------|-----------------------|---------------------------------|------------------|---------------------------------------------|
| Control             | 3.5±0.05         | 2±0.05                | 0                               | –                | 5                                          |
| PhH                 | 1.5±0.04*        | 4±0.04                | 3                               | –                | 5                                          |
| AgNPs 0.75 μg/L     | 3.7±0.07         | 3±0.06                | 0                               | –                | 5                                          |
| AgNPs 1.5 μg/L      | 4.0±0.03*        | 6±0.06                | 2                               | +                | 5                                          |
| AgNPs 1.5 μg/L+PhH  | 2.5±0.05         | 4±0.03                | 4                               | –                | 5                                          |
| AgNPs 3 μg/L        | 4.0±0.05*        | 3±0.05                | 2                               | +                | 5                                          |
| AgNPs 6 μg/L        | 3.5±0.06         | 2±0.05                | 2                               | –                | 3                                          |
| AgNPs 15 μg/L       | 3.0±0.03         | 1±0.04                | 0                               | –                | 2                                          |

*The difference is significant in comparison with the control group.

In view of the fact that the best morphometric parameters were recorded in the AgNPs 1.5 μg/L group, additional biochemical and histological studies of this group were carried out in comparison with the control group. According to the results of studying the histological characteristics of the leaf blade of gray poplar, it can be seen that the plants of the experimental group have a similar leaf blade thickness and stem diameter (90.1 ± 3.46 and 1137.7 ± 11.42 μm, respectively), in comparison with control plants (100.06 ± 4.14 and 1206.4 ± 9.86 μm). At the same time, in the experimental leaves, a significantly large area of the stomatal fissure is observed with a similar stomatal density per 1 mm² (table 3) and the degree of their opening (figure 5a,b). Based on this, it can be concluded that the plants of the experimental group have a high potential for general plant resistance and high heat resistance. These
results are in good agreement with the data of other authors. For example, in the work [37] describes the role of stomata in the response of plants to heat and cold stress, while a large area of the stomatal fissure promotes more efficient adaptation of plants to stressful environmental conditions.

**Table 3.** Histological studies of the leaf blade of gray poplar microclones.

| Indicator                  | Control                  | AgNPs 1.5 μg/L            |
|----------------------------|--------------------------|---------------------------|
| Stomatal pore area, μm²    | 74.88±10.32              | 116.36±9.46 *             |
| Stoma area, μm²            | 553.41±9.86              | 622.15±8.22 *             |
| Stoma density, pcs / mm²   | 5.18±0.86                | 5.38±0.83                 |
| Stomatal gap opening degree, μm | 0.14±0.83               | 0.19±0.83                 |

*The difference is significant in comparison with the control group.

![Figure 5](image-url)

**Figure 5.** Stomata of gray poplar microclones in control (a) and 1.5 μg/L AgNPs (b).

Analysis of the photosynthetic activity of gray poplar samples showed that the values of the studied parameter in the control and experimental groups did not significantly differ (0.576 ± 0.016 and 0.522 ± 0.002 arb.units, respectively), which indicates an equal degree of resistance to stress and the stability of their state.

AgNPs entering plant tissues can cause disruption of the antioxidant system [38, 39]; therefore, control of the level of catalase activity as the main protective enzyme against the toxic effect of reactive oxygen species is an important stage in assessing the state of a plant. The level of activity of the enzyme catalase in the experimental group was reduced and amounted to 3.21 mg H₂O₂ / min. compared with the control group, in which this indicator was 4.565 mg H₂O₂ / min. This decrease can be caused by damage to the enzyme structure or disruption of its biosynthetic pathway in response to the presence of AgNPs.

Analysis of the results of biomorphological studies at the proliferation stage confirms that AgNPs at concentrations of 1.5 and 3 μg/L have a stimulating effect on the growth and development of microclones, including stimulating the formation of the root system and accelerating the growth of the vegetative part of the shoot. Despite the difficulty of comparing the results of different studies due to the variety of sizes, shapes, and concentrations of NPs, on the whole, our results are consistent with the previously obtained results showing the stimulating effect of NPs on plant growth and development in small doses [38, 40]. The studies carried out on morphometric, biochemical, and histological parameters show that gray poplar microclones grown with AgNPs at a concentration of no more than 1.5 μg/L have high potential resistance to stressors, regarding to the increase of stomatal pore area and stoma area in experimental group.
3.4. Rooting stage

At this stage, the survival rate of microclones in all groups was 95 ± 0.9%. Root formation was observed by the end of the second week of the experiment. Studies of the parameters of rhizogenesis of microclones indicated a stimulating effect of a solution of AgNPs at a concentration of 1.5 and 3 μg/L on the process of root formation, while the number of rooted microclones was 75 and 72%, respectively, which is more than 20% more than in the control. However, with an increase in the concentration of AgNPs, a tendency towards a decrease in the rate of rhizogenesis was observed, which indicates a suppressive effect on the development of microclones at the stage of rooting (figure 6).

**Figure 6.** Dynamics of root formation of gray poplar microclones on nutrient media of ½ WPM with AgNPs solutions.

Biomorphological parameters of microclones were recorded on the 28th day after transferring the regenerants to the ½ WPM nutrient medium. The results show that AgNPs at concentrations of 1.5 and 3 μg/L stimulate plant growth and development, which is reflected in the number of roots and the rate of their formation, as well as in the general state of microclones (table 4).

**Table 4.** Biomorphological parameters of cultivated plant samples of gray poplar at the stage of rooting under the influence of AgNPs.

| Group          | Shoot height, cm | Number of leaves, pcs | Number of additional shoots, pcs | Number of roots, pcs | Condition of microclones on a 5-point scale |
|---------------|------------------|-----------------------|----------------------------------|----------------------|-------------------------------------------|
| Control       | 5.0±0.08         | 7±0.05                | 0                                | 1                    | 4                                         |
| AgNPs 0.75 μg/L | 5.2±0.07         | 7±0.09                | 0                                | 1                    | 5                                         |
| AgNPs 1.5 μg/L | 6.2±0.04*        | 7±0.06                | 0                                | 2                    | 5                                         |
| AgNPs 3 μg/L  | 5.8±0.07         | 6±0.09                | 0                                | 2                    | 5                                         |
| AgNPs 6 μg/L  | 5.5±0.06         | 6±0.05                | 0                                | 1                    | 3                                         |
| AgNPs 15 μg/L | 5.1±0.04*        | 5±0.04*               | 0                                | 1                    | 2                                         |

*The difference is significant in comparison with the control group.

Thus, the AgNPs at concentrations of 1.5 and 3 μg/L in nutrient media at the rooting stage increased the number of microclones with the presence of roots. By stimulating the development of the root system, plant nutrition is also improved, which is clearly demonstrated in terms of the height of the shoots. In addition, the stimulation of rhizogenesis during micropropagation will significantly increase the percentage of survival of microclones at the stage of acclimatization in greenhouse.
3.5. Acclimatization stage
In the control group, microclones free from infection in the substrate were observed after four weeks in only 30.0% of cases with a survival rate of 50% (figure 7a, 7b). Fludioxonil treatment reduced the number of infections by up to 50% and increased the survival rate by up to 70%. The best results were observed when using AgNPs at a concentration of 3 μg/L, at which it was possible to obtain 100% of pathogen-free microclones and increase their survival rate up to 95% by preventing rotting of the root system under the influence of pathogenic microflora.

![Figure 7. Sterility and viability of explants on the 14th and 28th day after planting indoors.](image)

*The difference is significant in comparison with the control group. **The difference is significant in comparison with the fludioxonil group (p < 0.05).

Shoot height indices of experimental groups significantly differ from control ones and are characterized by higher indices (table 5).
Table 5. Biomorphological indicators of cultivated plant samples of gray poplar at the stage of acclimatization in greenhouse under the influence of AgNPs.

| Indicator                              | Control       | Fludioxonil | AgNPs 1.5 µg/L | AgNPs 3 µg/L |
|----------------------------------------|---------------|-------------|----------------|--------------|
| Shoot height, cm                       | 8±0.3         | 8.2±0.2*    | 8.5±0.4*       | 8.6±0.7*     |
| Number of green leaves, pcs            | 8±0.5         | 8±0.5       | 10±0.5         | 10±0.4       |
| The number of withered leaves, pcs     | 2             | 2           | 2              | 2            |
| The number of additional shoots, pcs   | 0             | 0           | 0              | 0            |
| Condition of microclones on a 5-point scale | 4 | 5 | 5 | 5 |

*The difference is significant in comparison with the control group

At the stage of plant acclimatization in greenhouses, the AgNPs at a concentration of 3 µg/L doubled the sterility indices and increased the survival rate of microclones by 45%. In addition, an increase in the biomorphological parameters of cultivated samples of gray poplar plants was observed.

4. Conclusion

Thus, there is no question that nanobiotechnology in forestry has great potential and is able to improve the quality of woody plants during reforestation and afforestation. In addition, it is imperative to develop new methods of controlling plant diseases. The totality of the described results allows us to conclude that AgNPs are highly effective and expedient for micropropagation of woody plants. However, more researches to control bioavailability, biodegradation, and long-term effects of AgNPs on woody plants are needed.

Acknowledgments

This work was supported by the Ministry of Science and Higher Education of the Russian Federation in the frame work of agreement # 075-15-2021-709.

References

[1] Hazubska-Przybył T 2019 Propagation of juniper species by plant tissue culture: A mini-review. Forests 10 1028 https://doi.org/10.3390/f10111028
[2] Baravardi H, Ranjbar G A, Kamali S and Abadi F 2014 Investigation of the effects of growth regulators on Callus induction in Juniperus excels L. Bull. Env. Pharmacol. Life Sci. 4 73
[3] Thorpe T A, Harry I S and Kumar P P 1991 Application of micropropagation to forestry Micropropagation: Technology and Application eds Debergh P C and Zimmerman R H (Dordrecht: Springer) pp 311-336
[4] Evans D E, Coleman J O and Kears A 2020 Plant Cell Culture (London: Taylor & Francis) p 208
[5] Sivanesan I and Park SW 2015 Optimizing factors affecting adventitious shoot regeneration, in vitro flowering and fruiting of Withania somnifera (L.) Dunal. Ind. Crop. Prod. 76 323 https://doi.org/10.1016/j.indcrop.2015.05.014
[6] Sarmast MK, Salehi H and Khosh-Khui M 2011 Nano silver treatment is effective in reducing bacterial contaminations of Araucaria excelsa R. Br. var. glauca explants. Acta biol. Hung. 62 477 https://doi.org/10.1556/ABiol.62.2011.4.12
[7] Cassells A C 1991 Problems in tissue culture: culture contamination Micropropagation: Technology and Application eds Debergh P C and Zimmerman R H (Dordrecht: Springer) pp 31-44
[8] Castro M R, Belo A F, Afonso A and Zavattieri M A 2011 Micropropagation of Juniperus navicularis, an endemic and rare species from Portugal SW coast. Plant Growth Regul. 65 223 https://doi.org/10.1007/s10725-011-9590-1
[9] Mihaljević I, Dugalić K, Tomaš V, Viljevac Vuletić M, Pranjić A, Cmelik Z, Puškar B and Jurkovic Z 2013 In vitro sterilization procedures for micropropagation of ‘oblacinska’ sour cherry. J. Agr. Sci. 58 117 https://doi.org/10.2298/JAS1302117M

[10] Kashani SA, Asrar M and Leghari SK 2018 In vitro callus induction and shoot formation of Juniperus excelsa of Ziarat, Balochistan, Pakistan. FUUAST Journal of Biology 8 203

[11] Sivanesan I, Muthu M, Gopal J, Tasneem S, Kim D-H and Oh J-W 2021 A fumigation-based surface sterilization approach for plant tissue culture. Int. J. Env. Res. Pub. He. 18 2282 https://doi.org/10.3390/ijerph18052282

[12] Nadha H K, Salwan R, Kasana R C, Anand M and Sood A 2012 Identification and elimination of bacterial contamination during in vitro propagation of Guadua angustifolia Kunth. Pharmacogn Mag 8 93 https://doi.org/10.4103/0973-1296.96547

[13] Teixeira da Silva J A, Nhtu T, Tanaka M and Fukai S 2003 The effect of antibiotics on the in vitro growth response of chrysanthemum and tobacco stem transverse thin cell layers (tTCLs). Sci. Hortic.-Amsterdam 97 397 https://doi.org/10.1016/S0304-4238(02)00219-4

[14] Falkiner F R 1997 Antibiotics in plant tissue culture and micropropagation — what are we aiming at? Pathogen and Microbial Contamination Management in Micropropagation ed Cassells A C (Dordrecht: Springer) pp 155-160

[15] Ray S S and Ali N 2018 Biotic contamination and possible ways of sterilization: a review with reference to bamboo micropropagation. Braz. Arch. Biol. Techn. 60 https://doi.org/10.1590/1678-4324-2016160485

[16] Yuan D 2017 Acute toxicity of mercury chloride (HgCl$_2$) and cadmium chloride (CdCl$_2$) on the behavior of freshwater fish, Percocypris Pingi. International Journal of Aquaculture and Fishery Sciences 3 066 https://doi.org/10.17352/2455-8400.000031

[17] El-Sayed I M, Salama W H, Salim R G and Taha L S 2021 Relevance of nanoparticles on micropropagation, antioxidant activity and molecular characterization of Sequoia sempervirens L. plant. Jordan Journal of Biological Sciences 14 373

[18] Gupta N, Upadhyaya C P, Singh A, Abd-Elsalam KA and Prasad R 2018 Applications of silver nanoparticles in plant protection Nanobiotechnology Applications in Plant Protection eds Abd-Elsalam KA and Prasad R (Cham: Springer) pp 247-265

[19] Kailasa S K, Park T J, Rohit J V and Koduru J R 2019 Antimicrobial activity of silver nanoparticles in Pharmacotherapy ed Grumezescu A M (Cambridge: Elsevier Inc.) chapter 14, pp 461-484

[20] Kole C, Kumar D S and Khodakovskaya M V 2016 Plant Nanotechnology: Principles and Practices (Switzerland: Springer) p 383

[21] Aslani F, Bagheri S, Muhd Julkapli N, Juraimi A, Hashemi F and Baghdadi A 2014 Effects of engineered nanomaterials on plants growth: An overview. The Scientific World Journal 2014 641759 https://doi.org/10.1159/1675-4324-2016160485

[22] Serrano L, Feregrino-Perez A, Guevara-Gonzalez R, Mendoza S and Escalante K 2020 Nanoparticles in agroindustry: Applications, toxicity, challenges, and trends. Nanomaterials 10 1 https://doi.org/10.3390/nano10091654

[23] Arab M M, Yadollahi A, Hosseini-Mazinani M and Bagheri S 2014 Effects of antimicrobial activity of silver nanoparticles on in vitro establishment of G×N15 (hybrid of almond×peach) rootstock. Journal of Genetic Engineering and Biotechnology 12 103 https://doi.org/10.1016/j.jgeb.2014.10.002.

[24] Kim DH, Gopal J and Sivanesan I 2017 Nanomaterials in plant tissue culture: the disclosed and undisclosed. RSC Adv. 7 36492 https://doi.org/10.1039/C7RA07025J

[25] Nair R 2016 Effects of nanoparticles on plant growth and development Plant Nanotechnology: Principles and Practices eds Kole C, et al. (Cham: Springer) pp 95-118

[26] Sarmast M K and Salehi H 2016 Silver nanoparticles: An influential element in plant nanobiotechnology. Mol. Biotechnol. 58 441 https://doi.org/10.1007/s12033-016-9943-0
[27] El-Kosary S, Allatif A A, Stino R, Hassan M and Kinawy A A 2020 Effect of silver nanoparticles on micropropagation of date palm (Phoenix Dactylifera L., Cv. Sewi And Medjool). *Plant Arch.* 2 9701

[28] Kim S W, Jung J H, Lamsal K, Kim Y S, Min J S and Lee Y S 2012 Antifungal effects of silver nanoparticles (AgNPs) against various plant pathogenic fungi. *Mycobiology* 40 53 https://doi.org/10.5941/MYCO.2012.40.1.053

[29] Evlakov P M, Fedorova O A, Grodetskaya T A, Zakharova O V, Gusev A A, Krutyakov YA and Baranov O Y 2020 Influence of copper oxide and silver nanoparticles on microclonal sprouts of downy birch (*Betula pubescens* Ehrh.). *Nanotechnologies in Russia* 15 476 https://doi.org/10.1134/S1995078020040035

[30] Ahlawat J, Sehrawat A R, Choudhary R and Yadav S K 2020 Biologically synthesized silver nanoparticles eclipse fungal and bacterial contamination in micropropagation of Capparis decidua (FORSK.) Edgew: A substitute to toxic substances *Indian J. Exp. Biol.* 58 336

[31] Murashige T and Skoog F 1962 A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plantarum* 15 473 https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

[32] Lloyd G and McCown B 1980 Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture *Combined Proceedings, International Plant Propagators’ Society* 30 421

[33] Mohan Jain S and Häggman H 2007 *Protocols for Micropropagation of Woody Trees and Fruits*. (Netherlands: Springer) p 559 https://doi.org/10.1007/978-1-4020-6352-7.

[34] Genty B, Briantais J-M and Baker N R 1989 The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *BBA-Gen. Subjects* 990 87 https://doi.org/10.1016/S0304-4165(89)80016-9

[35] Li X, Ke M, Zhang M, Peijnenburg W J G M, Fan X, Xu J, Zhang Z, Lu T, Fu Z and Qian H 2018 The interactive effects of diclofop-methyl and silver nanoparticles on Arabidopsis thaliana: Growth, photosynthesis and antioxidant system. *Environ. Pollut.* 232 212 https://doi.org/10.1016/j.envpol.2017.09.034

[36] Ke M, Qu Q, Peijnenburg WJGM, Li X, Zhang M, Zhang Z, Lu T, Pan X and Qian H 2018 Phytotoxic effects of silver nanoparticles and silver ions to Arabidopsis thaliana as revealed by analysis of molecular responses and of metabolic pathways. *Sci. Total Environ.* 644 1070 https://doi.org/10.1016/j.scitotenv.2018.07.061

[37] Agurla S, Gahir S, Munemasa S, Murata Y and Raghavendra AS 2018 Mechanism of stomatal closure in plants exposed to drought and cold stress. *Survival Strategies in Extreme Cold and Desiccation: Adaptation Mechanisms and Their Applications* eds Iwaya-Inoue M, Sakurai M and Uemura M (Singapore: Springer) pp 215-232

[38] Bello-Bello J J, Chavez-Santoscoy R A, Lecona-Guzmán C A, Bogdanchikova N, Salinas-Ruíz J, Gómez-Merino F C and Pestryakov A. 2017 Hormetic response by silver nanoparticles on in vitro multiplication of sugarcane (Saccharum spp. Cv. Mex 69-290) using a temporary immersion system. *Dose Response* 15 https://doi.org/10.1177/1559325817744945

[39] Zakharova O, Vasyukova I, Strekalova N and Gusev A. 2019 Effects of silver nanoparticles on morphometric parameters of hairy birch (*Betula pubescens*) at various stages of micro cloning. *IOP C Ser. Earth Env.* 392 012024 https://doi.org/10.1088/1755-1315/392/1/012024

[40] Castro-González CG, Sánchez-Segura L, Gómez-Merino F C and Bello-Bello J J 2019 Exposure of stevia (*Stevia rebaudiana* B.) to silver nanoparticles in vitro: transport and accumulation. *Sci. rep.-UK* 9 10372 https://doi.org/10.1038/s41598-019-46828-y