Effects of silver nanoparticles on morphometric parameters of hairy birch (*Betula pubescens*) at various stages of micro cloning

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**Abstract.** The paper presents the study results of silver nanoparticles (AgNPs) effectiveness for woody species explants sterilization as exemplified by hairy birch (*Betula pubescens*) at various stages of micro-clone propagation. It was shown that simultaneous application of 0.03% AgNPs+15% NaOCl at the stage of explant sterilization resulted in consistently sterile (90%) cell cultures throughout the 28-days cultivation period. When the growth medium was modified with AgNPs at concentrations of 1.5 - 3 μg/l at the multiplication and rooting stages it dramatically reduced phytopathogenic contamination of the explants and regenerants thus increasing the number of surviving plantlets up to 100%. Besides, the analysis results carried out for the photosynthetic and enzyme activity display high stress-resistance potential of the studied plants compared with the control. The obtained results allow one to conclude that AgNPs have high effectiveness and application potential when used in microclonal propagation of woody plants.

1. **Introduction**

Artificial forest regeneration has a number of advantages over the natural one, the key advantage is the high rate of restoring woody plants through cloning which is of great importance for their further use by the forestry industry [1]. At the same time, plantings grown from seeds after the traditional method often have low vitality rate because of contamination of the parent plants and seed material with various phytopathogens. This factor reduces on the rate of artificial forest regeneration [2]. Micro-clonal propagation is an effective method for rapid recovery of the felled or post-fire forest areas with high-quality and patogene-free plantlets of such woody species as oak, birch, poplar, aspen, pine, etc.

At the same time, there are some obstacles hampering successful micro-clone propagation and reducing the economic efficiency of forest restoration [3, 4], such as: the meristem tissues used for propagation are not always free from disease (viruses) and require additional chemical or thermal sterilization; high doses of phytohormones (e.g. cytokinins) may produce adverse effects on the plant development, that is why the search for the new types of growth stimulants is such an important issue; the method applicability is badly affected by the large number of plants dying for various reasons, including diseases, during the period of adaptation to the field conditions.

Recent research works prove high effectiveness of various metallic nanoparticles (NPs), e.g. silver, copper, zink particles against a wide range of microorganisms and in some cases their activity against...
viruses has been proven [5, 6]. All this makes these nanomaterials a good alternative to the existing antibiotics especially taking into account the ability of microorganisms to develop antibiotic resistivity. NPs have an advantage over thermal treatment as they require shorter treatment times, they are less harmful for the plants and they can provide prolonged activity as colloidal forms of NPs can be introduced into solid or liquid culture media. Besides, some researchers have reported that NPs may stimulate in vitro plant cell cultures growth and development. Inhibitory effects of AgNPs to plant senescence phytohormone ethylene was described [6]. It was demonstrated the positive role of Ag, TiO₂, and ZnO NPs in callus induction, organogenesis, somatic embryogenesis, somaclonal variation, genetic transformation and secondary metabolite production [7]. However, for woody plants such as birch, that form the appearance of terrestrial ecosystems, such studies have not yet been conducted.

In the present paper we present the results of our study of the AgNPs influence on the organogenesis parameters of hairy birch explants and regenerants. The aim of the study was to find out how nanosilver affects phytopathogenic contamination and survival of microclonal seedlings at the stages of multiplication and rooting.

2. Materials and methods

2.1. Silver nanoparticles

The silver nanoparticles used in this research were manufactured by the electric explosion of conductive wire in the atmosphere of inert gases method (Advanced Powder Technologies, LLC, Tomsk, Russia).

To produce the colloidal solutions the 0.3 g samples of NPs were weighed on an analytical balance ViBRA HT (Shinko Denshi, Japan, accuracy ± 0.0001) and poured into a flask containing 100 ml of distilled water. The suspensions were stirred with a glass rod for 30 sec, then a sterilized ultrasonic disperser waveguide was submerged 20 mm deep into the flask and the solution was treated for 15 min at 300 W and 23.740 kHz. A Zetasizer Nano device (Malvern Instruments, UK) was user for analyzing the particle sizes in the solution. The electron microscopic study of the AgNPs was carried out on a high resolution transmission electron microscopy JEM-2100 JEOL, Japan.

2.2 The growth media for microclone propagation

The Murashige and Skoog (MS) growth medium was used in this work [8], this medium is commonly used for cultivating cell cultures and whole plants.

2.3 Production of hairy birch explants and regenerants

In this work we followed the conventional methods for in vitro cultivation of isolated plant organs [9]. The cuttings were harvested from outdoor-growing hairy birch (Bétula pubéscens) plants. Freshly cut explants with apical and axillary buds were used for establishing axenic culture in vitro.

Prior to sterilization the plant cuttings were thoroughly washed with liquid soap and rinsed in distilled water. The washed explants were stored for 3-5 days in a cool place. The explants removed from the storage were rinsed in running water, cut into pieces from 3 to 5 cm long and thoroughly washed in warm water with detergents. The plant material was washed for 35 min in the solution consisting of 200 μl of 20% NaOCl and 200 ml of distilled water with further rinsing in distilled water for at least 10 min. After that the explants were sterilized. The sterilized explants were cut into 1.5 - 2 cm pieces and introduced into the culture tubes containing the culture medium. All the operations were carried out in the axenic environment.

For the first 14 days after introduction into culture the explants were cultivated at the 16-h photoperiod with the 5 kLx illumination intensity, the temperature was 25 °C at daytime and 19-20 °C at nighttime.

The axillary buds produced 1 or 2 shoots after 2 - 4 weeks of cultivation, the survival rate varied and the buds retained the regenerative ability. The depleted media were replaced, every 7 days the shoots were transferred to the fresh media in the axenic environment.
2.4 Influence of explant sterilization on the survival rate and morphometric parameters of clones.

Sodium hypochlorite (NaOCl) solution, which is one of the most widely used antimicrobial agents, AgNPs colloidal solution and the NaOCl+AgNPs combination were used in this work. The explants were subjected to antiseptic agents for 35 min.

For the in vitro study of the hairy birch microshoots the following parameters were determined: sterility (%), number of surviving microclones (%), shoot height (cm), number of adventitious shoots (pcs), presence of roots, microclones condition on the 1 to 5 scale.

After 14 and 28 days of cultivation on growth media was calculated: the number of post-sterilization explants free from bacterial and fungal contamination; the number of explants surviving the initial sterilization and having less than 30 % of necrotizing tissue; the height of shoots regenerated from the cultivated explants.

The microshoots condition was assessed on a 5-point scale:
- 5 – perfect microshoots condition, no necrotic foci, show a tendency to regenerate;
- 4 – good microshoots condition, the necrotic foci take up less than 10 % of the green mass, show a tendency to regenerate;
- 3 – satisfactory microshoots condition, the necrotic foci take up less than 30 % of the green mass, retain a tendency to regenerate;
- 2 – poor microshoots condition, the necrotic foci take up more than 30 % of the green mass, retain no tendency to regenerate;
- 1 – very poor microshoots condition, the necrotic foci take up more than 60 % of the green mass, the microshoots have no tendency to regenerate.

2.5 Influence of AgNPs on the morphometric characteristics clones during the multiplication stage.

In order to assess the effects produced by AgNPs and phytohormones (PhH) on the morphometric characteristics of the hairy birch microclones we formed several groups of explants sterilized with 15% solution of NaOCl. The shoots were planted in test tubes with the growth media (MS) containing 15, 6, 3, 1.5 and 0.75 μg/l of AgNPs. The group receiving hormonal components was cultivated on the medium containing 0.2 mg/l benzylaminopurine + 0.1 mg/l indoleacetic acid + 0.3 mg/l gibberellic acid (0.2 mg/l BAP + 0.1 mg/l IAA + 0.3 mg/l GA). An additional group was formed, maintained on the medium containing 1.5 μg/l AgNPs + 0.2 mg/l BAP + 0.1 mg/l IAA + 0.3 mg/l GA. The control was cultivated on unmodified MS medium.

After 42 days the following parameters were assessed: number of contamination-free microclones (%), number of surviving microclones (%), shoot height (cm), number of additional shoots (pcs), presence of roots, microclones condition on the 1 to 5 scale.

The biochemical parameters were also evaluated after 42 days of experiment. The catalase activity level was determined using the pergamometric method (Bertrand - Oparin), namely, potassium permanganate titration of H₂O₂ in the presence of sulphuric acid. The enzyme activity is expressed in the standard units (act. units or mg H₂O₂ per min) where 1 ml of 0.1 M KMnO₄ is equal to 1.7 mg of hydrogen peroxide.

The rate of photosynthesis in chlorophyll-containing tissues was determined using an IFCR-2 device (a fluorometric indicator of the physiological state) after the method described in [10].

The histological parameters of the leaf tissue, such as stomatal density (number of stomata per 1 mm²) and stomatal pore dimensions (stomatal length × stomatal width rate) were calculated after the standard practice by means of a computerized image analysis system Video Test T-Morphology 4.0.

2.6 Study of the influence of AgNPs content in the growth media on the morphometric characteristics of the hairy birch in vitro microclones during the rooting stage.

When the well-developed regenerants of the studied woody species reached the 2 - 3 cm height they were removed and transplanted into culture tubes on the rooting medium (½ WPM). In order to stimulate risogenesis 1.5 μg/l AgNPs was added to the medium. The control group consisted of regenerants cultivated on unmodified medium. Upon 7 days of transplantation the assay of the following
morphometric parameters of growth and development was carried out: number of surviving microclones, shoot height, number of leaves, number of adventitious shoots number of microclones with roots, number of roots, microclones condition on the 1 to 5 scale. The first paragraph after a section or subsection should not be indented; subsequent paragraphs should be indented by 5 mm.

3. Results and discussion

3.1. Silver NPs characterization
According to the dimensional analysis of the NPs in the colloidal solutions prepared according to the method described in 2.1, the nanoparticle size is in the 30-60 nm range (figure 1).

![Figure 1. Size distribution of AgNPs in aqueous medium. On the insert (right) – TEM-image AgNPs.](image)

3.2 In vitro study of AgNPs-based sterilizing agent effect on sterility
Figure 2a compares the results of antiseptic treatment of hairy birch explants with the traditionally used NaOCl solution, AgNPs and their combination in the process of microclonal propagation.

![Figure 2. Sterilization efficiency after 14 and 28 days: (a) sterility values of the hairy birch explants; (b) survival rate of the hairy birch explants.](image)

The weakest antibacterial effect was registered in the groups treated with AgNPs. In the groups treated with 20 % and 15 % NaOCl solutions on the 14th day from the treatment the measured characteristics were at about 90%, while on the 28th day upon the treatment the same characteristic
decreased by 1.5-2 times and came up with the values for AgNPs treatment. The NaOCl + AgNPs complex produced the best results (about 90%) even when assessed 4 weeks from the treatment.

The dependence of the explants survival rate on the antiseptic treatment type was also addressed in this study (figure 2b). According to the obtained results, after 14 days from the treatment the studied characteristic never exceeded 50% in the groups treated separately with NaOCl and AgNPs solutions. After 4 weeks from the treatment we observed further decrease in this parameter. While the combined treatment with NaOCl + AgNPs at the rates represented in the figure 2 produced the maximal positive effect. The survival rate at the 14th day measured at about 85-87%, this value decreased by the 28th day to about 71-75%, these values far exceed those obtained from the groups treated with the two substances separately.

The results of morphometric characteristics analysis performed on the post-sterilization explants and represented in the table 1 show that 0.03 % AgNPs solution used at the sterilization stage can have a stimulating effect on the plants growth and development. One should note that the highest effect resulting in increased shoot height and improved condition of the surviving shoots was observed after the combined treatment with NaOCl + AgNPs.

| Sterilisation method       | Shoots height, cm | Clones condition on the 1 to 5 scale |
|----------------------------|-------------------|-------------------------------------|
|                            | 14th day | 28th day | 14th day | 28th day |
| 20% NaOCl                  | 0.2±0.05 | 0.3±0.03 | 3        | 3        |
| 15% NaOCl                  | 0.4±0.03 | 0.6±0.05 | 4        | 3        |
| 0.03% AgNPs                | 0.5±0.02 | 0.7±0.04 | 4        | 4        |
| 0.045% AgNPs               | 0.6±0.02 | 0.8±0.05 | 4        | 4        |
| 15% NaOCl + 0.03% AgNPs    | 0.5±0.03 | 0.7±0.02 | 4        | 4        |
| 10% NaOCl + 0.03% AgNPs    | 0.7±0.04 | 1.2±0.04 | 4        | 4        |

Thus, the combined NaOCl + AgNPs solutions dramatically improved the culture sterility, increased the shoots survival rate by decreasing the amount of phytopathogens and improved the morphometric parameters values thus allowing us to conclude that the studied combination not only acts as an effective sterilizing agent, but also displays a pronounced stimulating effect on the plant growth and development.

3.3 Study of AgNPs-based sterilizing agent effects on morphometric parameters of microclones at the multiplication stage

In accordance with the used method, the morphometric parameters of the microclones were measured on the 42nd day from the explants introduction into the growth medium. The results of the study of the effects produced by the conditions of hairy birch explants and microclones cultivation on the MS growth medium containing AgNPs and PhH are presented below.

In figure 3 one can see that application of 1.5 and 3 μg/l of AgNPs has a high antiseptic effect and produces up to 100% of phytopathogen-free microclones. Improvement in microclones vitality in the same groups may be connected with the reduced phytopathogenic load and a slight stimulating effect of low-dose AgNPs. Nevertheless, increase in AgNPs concentrations is accompanied by dose-dependent inhibition of the microclones vitality, though the antiseptic effect is high. This may be connected with toxic effects of AgNPs at concentrations over 3 μg/l. Combined application of PhH and 0.75 μg/l AgNPs to the growth medium has no effect on the microclones vitality rate.
Figure 3. Sterility and vitality of microclones cultivated on AgNPs and PhH-modified media.

The morphometric characteristics analysis proves the stimulating effect of 1.5 and 3 μg/l concentrations of AgNPs on the microclones growth and development, including such parameters as root development and shoot growth rate (table 2). PhH application also has a stimulating effect on the microclones development, for example, increasing the number of leaves and adventitious shoots compared with the control group, though PhH has now effect on the root development. AgNPs at the 6 and 15 μg/l concentrations inhibit plant growth and development which is manifested by deterioration of the microclones general state when compared with the control group and the groups treated with PhH and lower concentrations of AgNPs. It is particularly remarkable that the groups treated with the AgNPs and PhH combination displayed no growth-stimulating effect, though this combination has a positive effect on the additional shoots regeneration.

Table 2. Morphometric parameters of the hairy birch shoots microclones depending on the AgNPs and PhH concentration (μg/l) in the MS media.

| Parameter                          | Control | AgNPs 0.75 | AgNPs 1.5 | AgNPs 3 | AgNPs 6 | AgNPs 15 | PhH | PhH+AgNPs 1.5 |
|-----------------------------------|---------|------------|-----------|---------|---------|---------|-----|--------------|
| Shoots height, cm                 | 2±0.05  | 2.5±0.04   | 5±0.07    | 3±0.04  | 1.8±0.05| 1.5±0.05| 1.5±0.05| 2.5±0.03    |
| Number of leaves, pcs             | 2±0.05  | 2±0.04     | 4±0.06    | 3±0.06  | 2±0.03  | 1±0.05  | 4±0.05 | 4±0.04      |
| Number of adventitious shoots, pcs| 0       | 0          | 1         | 1       | 0       | 0       | 3    | 3            |
| Presence of roots                 | -       | -          | +         | +       | -       | -       | -    | -            |
| Microclones condition on the 1 to 5 scale | 4 | 4 | 5 | 5 | 2 | 2 | 5 | 4 |

Taking into account the fact that the group treated with 1.5 μg/l AgNPs was characterized by the best morphometric parameters, we have carried out additional biochemical and histological studies of the plants from this group and compared the data with the control. The results of the lamina histological studies showed that in the treated hairy birch microclones the stomatal pores have narrower openings (figure 4 a,4 b) while the stomatal density per mm² is higher than in the untreated microclones (table 3).
**Figure 4.** A hairy birch microclone stoma: a – Control, b – AgNPs 1.5 μg/l.

**Table 3.** Parametric characteristics of histological studies of the hairy birch microclones lamina.

| Indicator                  | Control        | AgNPs 1.5 μg/l |
|----------------------------|----------------|----------------|
| Stomatal pore area, μm²    | 86.04±2.32     | 208.48±3.46    |
| Stoma area, μm²            | 1208.32±9.86   | 1232.93±12.22  |
| Stomatal density, pcs/mm²  | 2.99±0.76      | 5.38±1.13      |

From this on can assume that the general resistance potential is higher in the plants from the studied group and they have a higher heat tolerance.

The photosynthetic activity analysis showed that the plants from the group treated with 1.5 μg/l AgNPs have a higher potential resistance to the stressors as they a characterized by higher values of the studied parameters (0.593±0.002 rel.units) compared with the control (0.345±0.023 rel.units). It should be noted that the low variance in the 1.5 μg/l AgNPs group indicates a high level of stability in the plants condition.

Similar dependencies were revealed while studying catalase activity. In the 1.5 μg/l AgNPs group the values exceed those of the control group by 2.4 times (5.11±0.02 и 2.79±0.03 mg H₂O₂/min respectively). This combination of better photosynthetic and enzyme activity indicates a higher stress-resistance potential of the plants from the 1.5 μg/l AgNPs group.

### 3.4 Effects of AgNPs on morphometric parameters of hairy birch microclones at the rooting stage

After the developed regenerants were isolated and transplanted into the rooting media in culture tubes the effect of AgNPs on the microclones risogenesis was studied. According to the obtained results, represented in the table 4, use of AgNPs has a stimulating effect on root-formation and on the vegetative parts development.

**Table 4.** Morphometric parameters of the hairy birch microclones cultivated on ½ WPM media containing AgNPs.

| Indicator                                      | Control        | AgNPs 1.5 μg/l |
|------------------------------------------------|----------------|----------------|
| Microclones vitality, %                       | 100            | 100            |
| Shoots height, cm                             | 4.0±0.07       | 5.4±0.08       |
| Number of leaves, pcs                         | 6±0.1          | 6±0.09         |
| Number of adventitious shoots, pcs            | 0              | 0              |
| Number of microclones with roots, %           | 35±0.8         | 70±1.1         |
| Number of roots, pcs                          | 1              | 1              |
| Microclones condition on the 1 to 5 scale     | 4              | 5              |
Thus, AgNPs application to the growth media at the rooting stage doubles the number of microclones with roots. Stimulation of the root system improves the plant nutrition which can be judged from the height of the shoots in the tested group. Additionally, one should note that stimulated root-development may increase microclones survival index when transferred into the outdoor conditions.

Among the wide range of artificially synthesized nanoparticles, AgNPs are the most commonly used ones for sterilizing applications. A number of research papers prove their effectiveness for plant cell cultivation procedures. In [11] AgNPs solutions were used as a sterilizing components for valerian explants. The treatment was carried out at the sterilization stage, AgNPs were used at the concentrations of 25, 50 and 100 mg/l. It has been shown that treatment with the 100 % solution had the strongest effect and resulted in 89 % of contamination-free explants. The effectiveness of AgNPs for woody species explants sterilization was exemplified by Araucaria heterophylla. The treatment consisted of soaking the explants in AgNPs solutions and adding the solution to MS medium. From the results one can see that surface sterilization with subsequent 180-min treatment with 200 mg/l AgNPs solution decreases bacterial contamination of the explants by 50.2 %. The authors noted that this type of treatment had no adverse effects on the plant cells [4]. In our study preliminary direct treatment of the hairy birch explants with 0.03% AgNPs colloidal solution produced no effect. At the same time, combined application of 0.03% AgNPs+15% NaOCl resulted in sustainable sterility (about 90 %) of the cell cultures for the period of 28 days which is twice as long as the result obtained for the control. The morphometric parameters analysis reveals no toxic impact on the studied plants.

Modification of the MS growth medium with AgNPs at the concentrations of 5, 25, 50, 75 and 100 mg/l during potato (Solanum tuberosum L.) cultivation reduced the amount of microorganisms thus improving the cell cultures growth and development parameters [12]. Similar results were obtained for strawberry when cultivated on the AgNPs-modified MS medium [13]. In the paper [14] the authors evaluated the antifungal and antibacterial activities of AgNPs directly applied to G×N15 (peach × almond hybrid) explants and added to the MS growth medium. The results have shown that treatment with 100 and 150 ppm AgNPs solutions decrease the internal and external explants contamination compared with the control. Adding the AgNPs to the growth medium produced better antifungal and antibacterial effect than direct treatment of the explants. However, AgNPs in concentrations above 150 ppm displayed adverse effects inhibiting the G×N15 bud vitality and shoots regenerating ability, and this harmful impact was more pronounced after the direct treatment than in the case of application to the medium. The results of our research are in accordance with the previous data. We have revealed that application of 1.5-3 μg/l AgNPs to the growth medium eliminates phytopathogens thus increasing the explants survival rate up to 100 %. Besides, the photosynthetic and enzyme activities analysis showed higher stress-resistance potenial of the treated plants compared with the control. Still, similar to the results published by other authors [14], increase in AgNPs nanoparticles concentration in the medium above 3 μg/l reduces vitality of explants and regenerants, has an adverse effect on the microclones general condition and negatively affects the main morphometric characteristics.

4. Conclusion

Our experiments revealed that high antibacterial effectiveness of AgNPs is accompanied by some stimulating effect on the microclonal growth and development both at the multiplication and rooting stages. The morphometric assay proved that 1.5 and 3 μg/l AgNPs stimulate root formation and promote the vegetative part growth at the multiplication stage. The results of the lamina histological studies showed that in the treated hairy birch microclones the stomatal pores have narrower openings while the stomatal density per mm2 is higher than in the control. From this on can assume that the general resistance potential is higher in the plants from the studied group and they have a higher heat tolerance. The photosynthetic activity analysis showed that the results for the plants from the treated group treated are 1.7 times higher than in the control. Similar dependencies were revealed while studying catalase activity. In the 1.5 μg/l AgNPs group the values exceed those of the control group by 2.4 times. These effects may be connected with the reduced phytopathogenic load and alteration in the antioxidant status [15].
Application of AgNPs to the growth media at the rooting stage doubles the number of microclones with roots. Stimulation of the root system improves the plant nutrition which can be judged from the height of the shoots in the tested group. Additionally, one should note that stimulated root-development may increase microclones survival index when transferred into the outdoor conditions.

The body of the described results allows one to assume that AgNPs are highly effective and their application for microcloning is practicable and advantageous. At the same time, further research is necessary in order to study bioaccumulation and biodegradation of AgNPs in various parts of the plants.

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