CuO nanoparticles effects on poplar×aspen hybrid clones at various stages of microclonal propagation

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Abstract. We have studied the effects of CuO nanoparticles with the mean lateral dimension 300×500 nm and thickness about 50 nm on hybrid poplar×aspen clones at various stages of micropropagation. It has been established that CuO nanoparticles display a strong sterilizing activity and enhance rhizogenesis in microplants. At the culture establishment stage exposure to 1.5 - 3 µg/L CuO increased the number of leaves and roots, and stimulated the root system development. At 15 µg/L it had an inhibiting effect on the studied parameters. At the multiplication stage we observed a considerable decrease in the height of shoots, and thickness of leaf lamina and stem diameter. At the same time a significant increase in the regenerants survival rate was observed (+20%), together with improved rhizogenesis. During the rooting stage, a 30% increase in the number of microclones with roots was detected, as well as a twofold increase in the number of roots on each plant. Besides, the nanomaterial had a stimulating effect on the shoots growth, increasing their height by 25%. Thus, it has been established that CuO nanoparticles produce differently directed effects on the growth and development of poplar×aspen hybrid microclones, depending on the micropropagation stage.

1. Introduction

Plant tissue culture technology is a prominent method in plant biology, it has become of major industrial importance in the area of large-scale plant propagation and production of phytopathogen-free plants. The success of all the in vitro manipulations with plant microclones depends not only on genotype and physical status of the donor plants, explant types and incubation conditions, but also on the sterilization methods of the explant surface, on the employed growth media and on plant growth regulators. A large number of recent research papers prove that nanotechnologies are gaining a rightful place in agriculture, in particular, they are successfully applied in yield enhancement and crop protection against diseases. For example, in works [1, 2] numerous developments of nanopesticides are considered, with the most common components being polymers, inorganic nanoparticles (e.g., silicon dioxide, titanium dioxide)
and nanoemulsions. It is pointed out that the mechanisms of action of nanopesticides on phytopathogens are poorly understood, as well as the effects of nanopesticides on the environment. Biosynthesis of AgNPs by Serratia sp. BHU-S4 (bsAgNPs) exhibited strong antifungal activity against Bipolaris sorokiniana, the spot blotch pathogen of wheat. Interestingly, 2, 4 and 10 μg/ml concentrations of bsAgNPs accounted for complete inhibition of conidial germination, whereas in the absence of bsAgNPs, conidial germination was 100% [3]. The authors [4-6] emphasize the importance of using biofriendly methods for the synthesis of nanoagrochemicals and their comprehensive study for environmental safety. The issues of the effectiveness and safety of nanoparticles, which are promising in agricultural technologies, are also discussed in articles [7-9].

Nanomaterials can be used not only for direct plant growth enhancement, but also they can improve plant development by eliminating contamination of explants with phytopathogens. Microbial contamination is an acute problem in cell culture, as it can totally destroy the experimental samples. The antimicrobial activity of nanoparticles has been proven by studies [10]. A wide range of nanoparticles, including silver, aluminum oxide, copper oxide, iron oxide, gold, zinc oxide and titanium dioxide, have been reported to possess antimicrobial properties against various microorganisms [11]. Studies show that treatment with nanoparticles leads not only to elimination of microbial contaminants from explants, but also plays a positive role in callus induction, organogenesis, somatic embryogenesis, etc. [12]. Adding nanosilver (4 mg/L) to the growth media was fully effective to control olive explants internal contaminations and no harmful effects were observed on explants and their growth [13]. In [14] the authors report that pathogen-free cultures were obtained after potato and tobacco axillary buds were sterilized and cultivated on the Murashige and Skoog nutrient medium with added titanium dioxide. Numerous researches have displayed positive effects of nanoparticles on callus induction and on shoots regeneration and growth. For example, the study of Tecomella undulata revealed an increase in the mean number of fresh shoots per explants, the percentage of explants producing shoots, and plant survival when the explants were incubated in MS media supplemented with a combination of 10 mg/L silver nanoparticles, 2.5 mg/L benzylaminopurine (BAP) and 0.1 mg/L indole acetic acid (IAA) [15]. The highest percentage of shoot formation (89.6%) was obtained when Stevia rebaudiana shoot nodes were incubated in the presence of 1 mg/L of ZnO nanoparticles in the medium [16].

In our research we reveal CuO nanoparticles (CuO NPs) effects on hybrid poplar×aspen (Populus alba L. × Populus tremula L.) regenerants at various stages of microclonal propagation. Previously, other authors have shown positive influence of copper-containing nanoparticles on plants in in vitro experiments. For example, Verbena bipinnatifida seedlings treated with 5 μg/L CuSO₄ NPs added to MS medium showed 52% increase in the shoot length, 21% increase in the root length and 39% increase in the fresh weight, compared to control [17]. In an experiment on Mentha longifolia copper (0.5 mg/l) and cobalt (0.8 mg/l) nanoparticles were added to modified MS0 nutrient medium and have led to increase of microplant height and growth index by 45-48.4 %, the quantity of internodes by 29.4-33.9 % and quantity of shoots by 55.6-66.2 %, while reproduction coefficient reached 30-40 % [18].

2. Materials and methods

In this work we used CuO NPs fabricated via the chemical precipitation method [19]. Copper oxide nanoparticles were synthesized by chemical precipitation from a solution Cu(NO₃)₂•6H₂O (A. R. purity) according to the reaction (1):

\[ \text{Cu(NO}_3\text{)}_2 + 2\text{NaOH} = \text{Cu(OH)}_2 \downarrow + 2\text{NaNO}_3, \]

with subsequent transformation of copper hydroxide into copper oxide in aqueous basic solution. The nanoparticles were obtained using reagents from Sigma-Aldrich (USA). The obtained nanoparticles were characterized by scanning electron microscopy (TESCAN VEGA3 scanning electron microscope, the Czech Republic) and by dynamic light scattering using a Zetasizer Nano device (Malvern Instruments, UK).
To prepare the colloidal solution, 0.3g nanoparticles were dispersed in 100 mL distilled water (pH=7.1±0.2) and treated in an ultrasound bath Ultrasonic Cleaner CD-4800 (Codyson,China) for 2 min (70 W, 44 Hz, volume – 1.4 L). Thus, a 3 g/L colloidal solution of nanoparticles was obtained.

In this work we followed the conventional methods for in vitro cultivation of isolated plant organs [20]. For the in vitro culture initiation plant cuttings with apical and axillary buds were used. The cuttings were harvested from outdoor-growing plants. The samples were sterilized in sodium hypochlorite solution. After sterilization the explants were transferred into Murashige and Skoog nutrient medium (MS0) [21, 22]. To modify the nutrient medium with nanoparticles, instead of water, we used colloidal aqueous solutions of CuO NPs at concentrations of 0.75, 1.5, 3, 6, and 15 μg/L, obtained by diluting the initial solution (3 g/L). Nanoparticle-free growth medium was used as control. The test tubes with the explants were placed in the growth chamber at +24 °C under LD 16:8 photoperiod, 5000 lx light intensity and 70% relative air humidity. The following morphometric parameters were assessed: height of shoots, number of leaves, number of roots, general condition evaluation (5-point scale).

During the subsequent multiplication stage the well-developed microclones were grafted and transplanted into the growth media containing the following hormonal agents: 0.2 mg/L benzylaminopurine (BAP), 0.1 mg/L indoleacetic acid (IAA) and 0.3 mg/L gibberellic acid (GA). The synergistic effect of the phytohormones and nanoparticles at 1.5 μg/L has also been analyzed. Materials and reagents from Sigma-Aldrich (USA) were used for the study.

At this stage we carried out histological and cytological examinations by means of VideoTesT-Morphology 4.0 hardware / software complex (Argus-BIO, Russia). For biochemical analysis of the shoots we measured induction of variable fluorescence of chlorophyll-a in assimilatory tissues (Fv/Fm), which makes it possible to assess the activity of the photosystem-2 chlorophyll-containing tissues and can serve as a diagnostic indicator of the state of the photosynthetic apparatus of plants. The intensity of photosynthesis of chlorophyll-containing tissues was recorded using an IFSR-2 fluorimeter (Russia) according to the Genty method [23]. Catalase activity was determined by the standard permanganometric method - the titer of H2O2 with potassium permanganate in the presence of sulfuric acid. The enzyme activity was expressed in standard units (mg H2O2 per minute), taking into account that 1 ml of 0.1 M KMnO4 corresponds to 1.7 mg of hydrogen peroxide [24]. At the rooting stage well-formed woody plant regenerants (2 - 3 cm high) were isolated and transferred to culture bottles with the rooting ½ woody plant medium (WPM) [25]. The nanomaterial was added at 1.5 μg per 1 L of growth medium, in order to promote rhizogenesis.

For statistical data processing Microsoft Excel 2010 (Descriptive Statistics software package) with one-way analysis of variance (ANOVA) at a 5% predetermined level of significance was used.

3. Results and discussion

3.1. Nanoparticles characterisation

SEM examination of the CuO NPs (figure 1) showed that the obtained particles had a plate-like shape with mean dimensions 300×500 nm and thickness of about 50 nm.

From the image one can see that the particles were aggregated into larger agglomerates. Dynamic light scattering study revealed that the mean size of the particles and aggregates in the solution was within the 170 – 340 nm range (figure 2).
3.2. Culture establishment

Assessment of CuO NPs effects at the culture establishment stage displayed their high efficiency as a sterilizing agent in the variants where the concentrations of nanoparticles in the growth medium was above 0.75 μg/L (figure 3a).

CuO NPs added to the growth medium at 0.75–3 μg/L enhanced the number of surviving microclones. At 1.5 μg/L we observed a 100% survival rate, against 80% in the control. 0.75 μg/L nanoparticles increased the parameter by 5%, while in 3 μg/L CuO NPs exposed plants the survival rate
was 10% higher than that in the control group. At the same time, higher CuO NPs concentrations had a toxic effect, 6 μg/L suppressed the parameter by 5% and exposure to 15 μg/L resulted in a 10% decrease in the microclones survival rate.

Analysis of the shoots height revealed no significant effects from the concentrations below 15 μg/L, while the maximum dose of CuO NPs inhibited the microclone growth by 15% (figure 3b). The clones exposed to 1.5 μg/L CuO NPs developed 6 leaves, this was the maximum number that exceeded the control values by a factor of 3. The nanoparticles concentration increase to 3 μg/L reduced the number of leaves to 3 per microclone. 0.75 and 6 μg/L produced no effect on the measured parameter, while 15 μg/L evidently suppressed it, in this group the mean number was 1 leaf per microclone, that is half the number of leaves developed by control (figure 3c).

At 1.5 μg/L and 3 μg/L CuO NPs the microclones developed 2 axillary shoots each, in the 6 μg/L group the microclones developed 1 axillary shoot, while in control, as well as in the 0.75 and 15 μg/L groups, the regenerants developed no axillary shoots at all (table 1).

Table 1. CuO NPs effects on biomorphological parameters of the regenerants.

| Variant          | Number of axillary shoots | Presence of roots | Microclones condition according to a 5-point scale |
|------------------|---------------------------|-------------------|---------------------------------------------------|
| Control          | 0                         | 0                 | 5                                                 |
| CuO NPs 0.75 μg/L| 0                         | 0                 | 5                                                 |
| CuO NPs 1.5 μg/L | 2                         | +                 | 5                                                 |
| CuO NPs 3.0 μg/L | 2                         | +                 | 5                                                 |
| CuO NPs 6.0 μg/L | 1                         | 0                 | 3                                                 |
| CuO NPs 15.0 μg/L| 0                         | 0                 | 2                                                 |

As one can see from table 1, CuO NPs at 1.5 and 3 μg/L stimulated root formation. The overall condition of the regenerants exposed to 6 and 15 μg/L deteriorated and was evaluated at 3 and 2 points, respectively, indicating the suppressing activity of copper nanoparticles at these concentrations.

The obtained results show that at the culture establishment stage CuO NPs acted as an efficient sterilizing agent enhancing the survival rate of multiclones at concentrations below 6 μg/L. No significant effect on the regenerants height has been observed, except in the 15 μg/L CuO NPs exposed plants, where 15% growth suppression has been registered. At the same time, exposure to 1.5 μg/L CuO NPs led to a threefold increase in the number of leaves, the maximum number of axillary shoots was also observed in this group. Rhizogenesis was stimulated by 1.5 and 3 μg/L CuO NPs, only the clones from these groups developed roots. Exposure to 6 and 15 μg/L CuO NPs worsened the microclones condition according to a 5-point scale, making it 3 and 2 points respectively. Thus, the conducted research determined 1.5 μg/L as the optimal concentration for the microclones development, the further study was conducted on the plants from this group. CuO NPs at 6 and 15 μg/L displayed the strongest toxicity at the culture establishment stage.

3.3. Multiplication

At the multiplication stage cuttings were taken from the microclones and were transplanted into the media containing phytohormones and nanoparticles at 1.5 μg/L, as this concentration displayed the best results during the previous stage. The results of the study are presented in table 2.

As one can see from table 2, CuO NPs introduced into the growth medium, both separately and in combination with phytohormones, increased the plantlets survival rate by 20%. Similar to the first stage, nanoparticles had no influence on the shoot growth, while hormones had a repressing effect. In the 0.2 BAP+0.1mg/L IAA+0.3 mg/L GA variant the microclones height was reduced by a factor of 2.3, and when the nanomaterial was added to phytohormones the microclones height was reduced by a factor of 1.5. Hormones had an inhibiting effect on the leaf development too. While the regenerants from control and from the group exposed solely to CuO NPs had 6 leaves each, the plants exposed to hormones, and to the hormones + CuO NPs combination had 4 and 5 leaves respectively. The highest number of axillary shoots (3) was in the group treated only with hormones, in the CuO NPs -exposed
groups the microclones grew, on the average, 2 axillary shoots, while in the control group the microplants developed no axillary shoots at all. At this stage root development was observed in the hormone-free group exposed to CuO NPs.

Table 2. Biomorphological parameters of the regenerants at the multiplication stage.

| Growth medium composition | Number of surviving microclones, % | Height of shoots, cm | Number of leaves | Number of axillary shoots | Presence of roots |
|---------------------------|-----------------------------------|----------------------|-----------------|-------------------------|--------------------|
| Control                   | 80.0±4.1                          | 3.5±0.4              | 6               | 0                       | 0                  |
| 0.2 BAP+0.1mg/L IAA+0.3 mg/L GA | 80.0±2.9                          | 1.5±0.3              | 4               | 3                       | 0                  |
| CuO NPs 1.5 µg/L          | 100.0                             | 3.5±0.4              | 6               | 2                       | +                  |
| CuO NPs 1.5 µg/L + 0.2 BAP+0.1mg/L IAA+0.3 mg/L GA | 100.0                             | 2.3±0.5              | 5               | 2                       | 0                  |

At the multiplication stage a histological examination of the microshoots was carried out. The analysis of nanoparticles effects upon the stomatal morphometric parameters revealed a small increase in the area of stomatal pores and in the degree of stomatal opening in the 1.5 µg/L CuO NPs variant, this can be explained by the transpiration process adjustment to the decrease in stomatal area and number of stomata per unit area in this group (table 3, figure 4). In the variant with the nanoparticles and hormones combined, the area of stomatal pores decreased by more than 30% compared to control and degree of stomatal opening decreased by almost 10%. Besides, in this variant the stomatal area was 30% below the control values. This reduction in all the parameters was partially compensated by an increased number of stomata per sq.mm. One should also note that exposure to CuO NPs had an adverse effect on such histological parameters as leaf lamina thickness and stem diameter that were much lower than in control (table 3). In the CuO 1.5 µg/L group these parameters were below the control by 12 and 36%, while in the group exposed to CuO NPs 1.5 µg/L + 0.2 BAP+0.1mg/L IAA+0.3 mg/L GA they were below the control by 5 and 20%, respectively.

Table 3. Histological parameters of the hybrid poplar×aspen clones.

| Growth medium composition | Stomatal pore area, µm² | Stomatal area, µm² | Stomatal density, pcs/mm² | Degree of stomatal pore opening, Sstom./Sopen | Leaf lamina thickness, µm | Stem diameter, µm |
|---------------------------|------------------------|--------------------|---------------------------|-----------------------------------------------|--------------------------|------------------|
| Control                   | 74.88±5.4              | 563.41±25.4        | 5.18±0.4                  | 0.14±0.02                                     | 100.06±8.3               | 1206.38±42       |
| CuO NPs 1.5 µg/L          | 80.63±3.2              | 522.29±21.3        | 4.19±0.7                  | 0.15±0.03                                     | 88.17±6.2                | 782.38±28.1      |
| CuO NPs 1.5 µg/L + 0.2 µg/L BAP + 0.1mg/L IAA + 0.3 mg/L GA | 51.19±4.6              | 388.57±15.8        | 6.46±0.7                  | 0.13±0.02                                     | 94.96±6.1                | 976.83±35.3      |
Figure 4. Stoma of a hybrid poplar×aspen microclone: a) control, b) CuO NPs 1.5 µg/L, c) CuO NPs 1.5 µg/L + 0.2 BAP+0.1mg/L IAA+0.3 mg/L GA.

The analysis of photosynthetic and catalase activity revealed that CuO NPs exposure had no significant impact upon the studied parameter, regardless of the phytohormones content in the medium (table 4).

Table 4. Microclones biochemical parameters.

| Variant | Photosynthesis Fv/Fm (rel. units) | Catalase activity, mg H$_2$O$_2$/min |
|---------|----------------------------------|------------------------------------|
| Control | 0.57±0.016                       | 4.56±0.57                          |
| CuO NPs 1.5 µg/L | 0.57±0.004 | 3.32±0.23                          |
| CuO NPs 1.5 µg/L + 0.2 BAP+0.1mg/L IAA+0.3 mg/L GA | 0.63±0.003 | 4.37±0.44                          |

The results of examinations show that CuO NPs had negative effect on the regenerants development at the multiplication stage, exposure to the nanomaterial led to significant reduction in the plant height, leaf lamina thickness and stem diameter. These adverse effects can be attributed to the toxic activity of copper as a heavy metal, or to specific phytotoxicity of CuO NPs [26]. Nevertheless, one should note a significant increase in the microclones survival rate (+20%) in the groups exposed to nanoparticles. This effect is probably explained by antimicrobial properties of CuO. Exposure to nanoparticles without hormones promoted rhizogenesis, as this was the single variant where we observed root development.

3.4. Rooting stage

At the final stage of our experiment we assessed the ability of CuO NPs to stimulate rhizogenesis. A 30% increase in the number of microclones with roots was detected, as well as a twofold increase in the number of roots on each plant. Besides, the nanomaterial had a stimulating effect on the shoots growth, increasing their height by 25% (table 5).

Table 5. CuO NPs effects at the rooting stage.

| Variant | Number of surviving microclones, % | Height of shoots, cm | Number of microclones with roots, % | Number of roots | Microclones condition according to a 5-point scale |
|---------|-----------------------------------|----------------------|-------------------------------------|----------------|-----------------------------------------------|
| Control | 100                               | 5.0±0.8              | 50±3.6                              | 1              | 4                                            |
| CuO NPs 1.5 µg/L | 100                             | 6.2±0.6              | 80±2.8                              | 2              | 5                                            |

As one can see from table 5, according to a 5-point scale, the condition of microclones exposed to CuO NPs 1.5 µg/L was evaluated as excellent, while the control plants condition gained only 4 points.
The results of our study of CuO NPs effects at various stages of poplar×aspen hybrid micropropagation indicate that it is a promising sterilizing agent. Antimicrobial activity of CuO NPs has been reported in a number of research papers [27-31], in particular, against such plant-dangerous microorganisms as fungi of the genus *Penicillum* and *Alternaria solani*. Probably, we observed a similar effect in our study.

At the culture establishment stage 1.5 - 3 µg/L CuO NPs increased the number of leaves and roots, and stimulated the root system development, thus enhancing the micropropagation process at this stage. At the same time, the nanomaterial at 15 µg/L had an inhibiting effect on the studied parameters. At the multiplication stage the effect was, on the whole, negative, as we observed a considerable decrease in the height of shoots, and thickness of leaf lamina and stem diameter. Nevertheless, here, as at the previous stage, a significant increase in the regenerants survival rate was observed (+20%), together with improved rhizogenesis. Positive effects of the studied nanomaterial upon the root development have also been revealed at the rooting stage where a 30% increase in the number of microclones with roots was detected, as well as a twofold increase in the number of roots on each plant. Besides, the nanomaterial had a stimulating effect on the shoots growth, increasing their height by 25%. CuO NPs produced dose-dependent increases in root diameter for lettuce (+52%) and carrot (+26%) seedlings, as reported in [32]. A twofold increase in the root and stem length compared to the control group have been observed at 0.01 g/L of copper oxide nanoparticles in the growth medium during in vitro experiments [33]. Exposure to 20 mg/L CuO NPs promoted root formation in Stevia rebaudiana [34], the number of rooting explants increased by nearly 20%.

### 4. Conclusion

The copper oxide nanoparticles produce differently directed effects on the growth and development of poplar×aspen hybrid microclones, depending on the micropropagation stage. CuO NPs have a strong sterilizing activity and enhance rhizogenesis in microplants. At the culture establishment stage exposure to 1.5-3 µg/L CuO NPs increased the number of leaves and roots, and stimulated the root system development. At the same time, the nanomaterial at 15 µg/L had an inhibiting effect on the studied parameters. At the multiplication stage the effect was, on the whole, negative, as we observed a considerable decrease in the height of shoots, and thickness of leaf lamina and stem diameter. At the same time, here, as at the previous stage, a significant increase in the regenerants survival rate was observed, together with improved rhizogenesis. Positive effects of the studied nanomaterial upon the root development have also been revealed at the rooting stage where a increase in the number of microclones with roots was detected, as well as a twofold increase in the number of roots on each plant. Besides, the nanomaterial had a stimulating effect on the shoots growth, increasing their height.

Thus, our study has shown the efficiency of using CuO NPs at the stage of introducing tissues of a hybrid of poplar and aspen, as well as birch into an in vitro culture, as a sterilizing and stimulating agent, especially as an activator of rhizogenesis. At the same time, concentration-dependent toxic effects were noted, manifested in a decrease in the viability of seedlings. Further assessment of biological effects at later stages of ontogenesis, as well as identification of the mechanisms of the effect of nanomaterials on plants, require additional research. This research results must be taken into account in planning biotechnological applications of copper oxide nanoparticles.

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