Influence of GO-Ag and GO-CuO composites on micropropagated shoots during adaptation to greenhouse environment

O Zakharova¹², E Kolesnikova³, E Kolesnikov², P Baranchikov¹, N Strekalova¹ and A Gusev¹²*

¹Derzhavin Tambov State University, 33 Internatsionalnaya Street, Tambov 392000, Russia
²National University of Science and Technology “MISIS”, 4 Lenisnky Avenue, Moscow 119991, Russia
³All-Russian Scientific Research Institute of Sugar Beet and Sugar named after A L Mazlumov, 86 VNIISS, Ramon district, Voronezh region 396030, Russia

*E-mail: nanosecurity@mail.ru

Abstract. This paper focuses on the effects produced by GO-Ag and GO-CuO composite materials on microclones of hybrid white poplar and aspen (Populus alba x Populus tremula), hairy birch (Betula pubescens), crack willow (Salix fragilis), red oak (Quercus rubra) and scots pine (Pinus sylvestris) during their adaptation to greenhouse environment. The study revealed a positive effect of microclones treatment with GO-CuO and GO-Ag solutions at a concentration of 3 μg/l. The number of surviving and adapted microclones increased considerably, as did such parameters as the height of the plantlets, the number of leaves and the plantlets condition on a 1 to 5 scale. Photosynthetic activity was high in the white poplar and aspen hybrid, in birch, willow and pine. At the same time, in oak, the maximal stimulation of biomorphological parameters was accompanied by the decrease in photosynthetic activity. GO-Ag solution at a concentration of 4.5 μg/l produced an inhibitory effect on all the studied species except red oak.

1. Introduction

In the current economic situation, the microclonal propagation method seems to be the most promising and effective one as it quickly produces many plants that are clones of each other.

Tissue culture technique for micro propagation includes several stages: 1) initiation and establishment of culture; 2) multiplication in the aseptic environment; 3) rooting of shoots; 4) hardening of plantlets moved to greenhouse conditions.

The establishment of plantlets in soil with their further adaptation to the field environment is the final and the most critical stage. The success of the whole micropropagation process often depends on it [1].

Adaptation of microclones to ex vitro conditions, also known as hardening, is the most cost- and labor-consuming process. The industrial microclonal propagation suffers heavy losses when plantlets are transferred to non-sterile conditions. As very often their development is arrested, the plantlets lose leaves and die. In some cases, less than 50% of the plantlets survive the transferal.

If the microclones survive the adaptation stress, then they take root in the non-sterile conditions. Successful ex vitro establishing of the plantlets allows for the industrial microclonal propagation.
The success of the plant adaptation is largely determined by the selection of media for the rooting stage. The in vitro plantlets often do not develop fully functional root hairs, cuticle and stomata; considerable losses may occur when the cloned shoots are transplanted to non-sterile environment. Thus, the soil composition and structure should be carefully selected for each specific plant species and variety [2]. Hardening of the microcloned plantlets can have a positive effect on their further growth and development. As a result, the hardened and adapted plants can be established in the field [3].

According to a well-established opinion, the industrial microclonal propagation is viable only when the stage of transferal of the plantlets to non-sterile conditions is successful.

Application of nanoparticles and nanomaterials as antimicrobial agents is one of the key directions of the nanobiotechnology development [4].

Silver and copper oxide nanoparticles are well known for their antibacterial activity [5, 6]. However, the researchers have described both positive and adverse effects of these nanomaterials on plants [7-10]. Graphene oxide also possesses antibacterial properties [11-13]. Moreover, hydrophilicity of graphene oxide makes it a useful material for stabilizing hydrophobic particles in colloidal systems [14]. It should also be noted that copper is an essential micronutrient, which is incorporated in many proteins and enzymes, therefore, playing a significant role in plant health and nutrition [15].

Based on the aforesaid, the aim of our research is to elucidate the effects of nanopreparations based on graphene oxide and metallic nanoparticles on the microclonal shoots of such woody plants as white poplar and aspen hybrid (Populus alba x Populus tremula), hairy birch (Betula pubescens), crack willow (Salix fragilis), red oak (Quercus rubra), and scots pine (Pinus sylvestris) in the period of their adaptation to greenhouse environment when the plantlets previously cultivated in the aseptic conditions are exposed to severe contamination with phytopathogenic microorganisms.

2. Methods and materials

The graphene oxide (GO) nanoparticles were fabricated by the chemical exfoliation method, i.e. by Hummers method [16], consisting in graphite oxidation with sulfuric acid and potassium permanganate. Average size of the flakes varies from 0.1 to 3 μm while their mean thickness is less than 1 nm.

The silver nanoparticles were obtained by electric explosion of conductive wire in the atmosphere of inert gases (Advanced Powder Technologies, LLC, Tomsk, Russia). Particles are predominantly spherical in shape. The arithmetic average particle size is about 100 nm. Individual particles, on average, have sizes of 70-150 nanometers and form microagglomerates.

The copper oxide nanoparticles were obtained by the chemical deposition method with subsequent reduction of the analytically pure Cu(NO₃)₂·6H₂O solution in the alkaline medium [17]. Copper oxide particles are flakes (as thick as 100 nm) united in aggregates about 5 μm in size.

The nanocomposite materials were produced by mixing the aqueous colloidal solutions of the components with subsequent ultrasound treatment, while the ratio of graphene oxide to silver/copper oxide was 1:10.

The analysis of the obtained composite materials was carried out on the high-resolution scanning electron microscope “Merlin” (Carl Zeiss, Germany) with an energy dispersive analyzer “10mm² SDD Detector - X-Act” (figure 1).

SEM and EDX analysis showed the presence of silver and copper oxide nanoparticles, concentrated on graphene oxide flakes. This indicates the successful formation of nanocomposite materials.

The study of the nanomaterials effects on microclones of hybrid white poplar and aspen (Populus alba x Populus tremula), hairy birch (Betula pubescens), crack willow (Salix fragilis), red oak (Quercus rubra), and scots pine (Pinus sylvestris) was carried out during their adaptation to greenhouse environment. Plantlets of the uniform height with 4-5 leaves and at least 2 cm-long roots were transferred to the non-sterile conditions of the greenhouse. The plantlets with a well-developed stem and root system were removed from the tubes with long forceps or with a special hook. The roots were rinsed from the remaining agar with 1 % potassium permanganate solution. Then, the microclones were planted into technological cassettes filled with moist cultivation soil and placed into a greenhouse propagating frame.
Figure 1. Results of SEM and EDX analysis of the samples of composite materials: (a) GO-Ag, (b) GO-CuO.

The soil consisted of pH-neutral peat and perlite at the ratio of 3:1. Perlite improves soil air permeability due to its considerable oxygen-retaining and wicking abilities. On the other hand, this material has low absorptive capacity, and plants cultivated on perlite or on soils with a high perlite content require frequent watering. The peat contained the following elements: nitrogen (N), phosphorus (P2O5), potassium (K2O). The pH value of the peat aqueous extract was 6.7. The soil was treated with fungicidal agent 24 hours prior the plantlets relocation.

After the transferal, the microclones were treated with aqueous dispersions containing GO-Ag and GO-CuO preparations at 3 or 4.5 µg/l concentrations. The reference growth regulator 2,4-epibrassinolide at a concentration of 667 µg/l was used for positive control.

The pots with the plantlets were placed in a greenhouse and cultivated at 22-24 °C and humidity of 85-90%.
In order to assess the root-taking and morphometric characteristics, the following parameters were measured: a number of surviving regenerated plants, %; plant height in cm; a number of leaves, pcs; a number of wilted leaves, pcs; a number of axillary shoots, pcs; a number of adapted regenerated plants, %; the microclones condition on a 1 to 5 scale.

Biochemical evaluation was carried out by measuring the photosynthesis activity which serves as the integral diagnostic indicator of plant resistance against stressors. The photosynthesis activity in the chlorophyll-containing tissues was determined by means of a fluorimetric indicator of physiological state IFSR-2 (Genty et al. method) [18].

3. Results and discussion

For adaptation of rooted microclones of the white poplar x aspen hybrid, the 3 μg/l concentrations of the two studied solutions produced better results than the same substances at 4.5 μg/l.

The best results were observed after foliage application of GO-CuO (3 μg/l). The number of surviving microclones reached 92%, thus exceeding the control by 32%. The mean plantlets height after 3 weeks of the experiment was 9.2 cm, while the mean number of leaves was 11 pcs. The microclones condition reached 5 points, while 60% of the plantlets were fully adapted to the new conditions (table 1).

Table 1. Adaptation effectiveness indicators for the white poplar x aspen hybrid microclones.

| Active substances in the solution | Number of surviving microclones, % | Plant height, cm | Number of leaves, pcs | Number of wilted leaves, pcs | Number of adapted regenerated plants, % | Number of axillary shoots, pcs | Microclones condition on a 1 to 5 scale |
|---------------------------------|-----------------------------------|-----------------|----------------------|-----------------------------|----------------------------------------|-----------------------------|---------------------------------------|
| Control                         | 60.0±5                            | 8.0±0.8         | 8±0                  | 2±0                         | 50.0±3                                 | 0                           | 4±0                                   |
| 2,4-epibrassinolide             | 70.0±5                            | 8.5±0.4         | 10±1                 | 2±1                         | 60.0±2                                 | 0                           | 5±0                                   |
| GO-CuO 3 μg/l                  | 92.0±3                            | 9.2±0.4         | 11±1                 | 0±0                         | 60.0±2                                 | 0                           | 5±0                                   |
| GO-Ag 3 μg/l                   | 72.0±3                            | 7.6±1.1         | 8±0                  | 2±0                         | 63.0±3                                 | 0                           | 5±0                                   |
| GO-CuO 4.5 μg/l                | 60.0±8                            | 8.5±0.7         | 6±0                  | 3±0                         | 35.0±4                                 | 0                           | 4±1                                   |
| GO-Ag 4.5 μg/l                 | 48.0±7                            | 7.0±0.6         | 7±1                  | 4±1                         | 36.0±4                                 | 0                           | 3±1                                   |

The lowest values were noted after the treatment with the solution containing 4.5 μg/l of GO-Ag. Only 48% of the plantlets survived; this value falls short of the control by 12%. Also, this group displayed the minimal height and the highest number of wilted leaves, while the number of adapted microclones did not exceed 36% compared with 50% of adapted plants in the control group.

For hairy birch rooted microclones adaptation, the concentration of 3 μg/l of all the nanomaterials also produced better results than the concentration of 4.5 μg/l.

The best results were observed after foliage application of GO-CuO and GO-Ag at a concentration of 3 μg/l. The number of surviving microclones reached 91% upon GO-CuO application and 90% after treatment with GO-Ag, thus exceeding the control by 23% and 24%, respectively. The mean plantlets height after 3 weeks of the experiment was 5.8 cm and 5.3 cm, with the mean number of leaves equaling 6 pcs. The microclones condition reached 5 points on a 1 to 5 scale, while 70% of the plantlets were fully adapted to the new conditions with only 1 wilted leaf (table 2).

The lowest values were noted after the treatment with the solution containing 4.5 μg/l of GO-Ag. The number of surviving plantlets fell short of the control by 15%, and the number of adapted plants decreased by 20% compared to the control. The microclones condition reached 3 points on a 1 to 5 scale.
Table 2. Adaptation effectiveness indicators for hairy birch microclones.

| Active substances in the solution | Number of surviving microclones, % | Plant height, cm | Number of leaves, pcs | Number of wilted leaves, pcs | Number of adapted regenerated plants, % | Number of axillary shoots, pcs | Microclones condition on a 1 to 5 scale |
|----------------------------------|-----------------------------------|-----------------|----------------------|----------------------------|----------------------------------------|-------------------------------|--------------------------------------|
| Control                          | 65.0±5                            | 4.0±0.2         | 5±1                  | 4±0                        | 50.0±5                                 | 0                            | 4±0                                  |
| 2,4-epibrassinolide               | 70.0±3                            | 4.5±0.5         | 6±1                  | 2±0                        | 60.0±2                                 | 0                            | 5±0                                  |
| GO-CuO 3 μg/l                    | 91.0±4                            | 5.8±0.3         | 6±0                  | 1±0                        | 70.0±2                                 | 0                            | 5±0                                  |
| GO-Ag 3 μg/l                     | 90.0±2.3                          | 5.3±0.2         | 6±1                  | 2±0                        | 65.0±3                                 | 0                            | 5±0                                  |
| GO-CuO 4.5 μg/l                  | 60.0±6                            | 4.0±0.4         | 5±1                  | 3±0                        | 35.0±4                                 | 0                            | 4±0                                  |
| GO-Ag 4.5 μg/l                   | 50.0±6                            | 4.1±0.2         | 5±0                  | 4±0                        | 30.0±4                                 | 0                            | 3±0                                  |

The same tendencies were noted in crack willow. The best results were observed after foliage application of GO-CuO solution at a concentration of 3 μg/l. The number of surviving microclones reached 100%, thus exceeding the control by 20%. The mean plantlets height after 3 weeks of the experiment was 5.8 cm with the mean number of leaves equaling 6 pcs. The microclones condition reached 5 points on a 1 to 5 scale, while 70% of the plantlets were fully adapted to the new conditions with only 1 wilted leaf.

The lowest values of the adaptation effectiveness parameters were recorded upon the treatment with GO-Ag at a 4.5 μg/l concentration (table 3).

Table 3. Adaptation effectiveness indicators for crack willow microclones.

| Active substances in the solution | Number of surviving microclones, % | Plant height, cm | Number of leaves, pcs | Number of wilted leaves, pcs | Number of adapted regenerated plants, % | Number of axillary shoots, pcs | Microclones condition on a 1 to 5 scale |
|----------------------------------|-----------------------------------|-----------------|----------------------|----------------------------|----------------------------------------|-------------------------------|--------------------------------------|
| Control                          | 80.0±3                            | 4.8±0.2         | 5±1                  | 3±0                        | 50.0±2                                 | 0                            | 4±1                                  |
| 2,4-epibrassinolide               | 85.0±2                            | 5.0±0.2         | 6±1                  | 2±0                        | 60.0±2                                 | 0                            | 5±0                                  |
| GO-CuO 3 μg/l                    | 100.0±0                           | 6.2±0.1         | 6±0                  | 1±1                        | 70.0±1                                 | 0                            | 5±0                                  |
| GO-Ag 3 μg/l                     | 100.0±0                           | 5.6±0.2         | 5±0                  | 2±0                        | 65.0±3                                 | 0                            | 5±0                                  |
| GO-CuO 4.5 μg/l                  | 71.0±2                            | 4.0±0.1         | 5±1                  | 2±0                        | 35.0±4                                 | 0                            | 4±0                                  |
| GO-Ag 4.5 μg/l                   | 70.0±3                            | 2.9±0.3         | 5±1                  | 4±0                        | 30.0±4                                 | 0                            | 3±1                                  |

The analysis of the red oak growth dynamics showed that the treatment with 3 μg/l solution of GO-Ag increased the number of surviving plantlets by more than 2 times compared with the control; the maximal values for plant height and for adapted microclones number were observed in the same group. At the same time, the maximal number of leaves (6 pcs per plant) was in the group treated with GO-CuO at 3 μg/l, similar to the number of leaves in the control group. Also, in the group treated with GO-CuO at 3 μg/l, we observed the least number of wilted leaves; here 50% of microclones survived, compared with 30% of surviving plantlets in the control. The number of adapted plantlets was three times higher than that in the control (table 4). One should note that every variant of treatment increased the number of surviving plantlets. GO-CuO and GO-Ag solutions applied to the soil at a concentration of 4.5 μg/l, reduced the plantlets height by about 1 cm; the values of all the other parameters remained close to that of the control.
Table 4. Adaptation effectiveness indicators for red oak microclones.

| Active substances in the solution | Number of surviving microclones, % | Plant height, cm | Number of leaves, pcs | Number of wilted leaves, pcs | Number of adapted regenerated plants, % | Number of axillary shoots, pcs | Microclones condition on a 1 to 5 scale |
|----------------------------------|-----------------------------------|------------------|----------------------|-----------------------------|-----------------------------------------|-------------------------------|--------------------------------------|
| Control                          | 30.0±5                            | 3.5±0.2          | 4±1                  | 2±0                         | 10.0±2                                  | 0                             | 3±0                                  |
| 2,4-epibrassinolide               | 45.0±3                            | 3.9±0.1          | 6±1                  | 2±0                         | 20.0±1                                  | 0                             | 3±0                                  |
| GO-CuO 3 μg/l                    | 50.0±2                            | 3.6±0.1          | 6±0                  | 1±1                         | 30.0±1                                  | 0                             | 4±0                                  |
| GO-Ag 3 μg/l                     | 70.0±2                            | 4.2±0.2          | 4±0                  | 2±0                         | 50.0±1                                  | 0                             | 4±0                                  |
| GO-CuO 4.5 μg/l                  | 40.0±6                            | 2.5±0.3          | 2±1                  | 2±0                         | 10.0±2                                  | 0                             | 4±1                                  |
| GO-Ag 4.5 μg/l                   | 50.0±7                            | 2.6±0.3          | 2±1                  | 3±0                         | 10.0±3                                  | 0                             | 4±0                                  |

In case of scots pine microclones, GO-Ag solution at a concentration of 3 μg/l produced the best results. The number of surviving microclones reached 70%, thus exceeding the control by 10%. The mean plantlets height reached its maximal value of 4.8 cm, as did the mean number of leaves – 12 leaves per plant. The maximal number of adapted plants, namely, 70% of the plantlets, was observed in the same group (table 5).

Table 5. Adaptation effectiveness indicators for scots pine microclones.

| Active substances in the solution | Number of surviving microclones, % | Plant height, cm | Number of leaves, pcs | Number of wilted leaves, pcs | Number of adapted regenerated plants, % | Number of axillary shoots, pcs | Microclones condition on a 1 to 5 scale |
|----------------------------------|-----------------------------------|------------------|----------------------|-----------------------------|-----------------------------------------|-------------------------------|--------------------------------------|
| Control                          | 60.0±3                            | 3.0±0.2          | 8±1                  | 0±0                         | 60.0±2                                  | 0                             | 5±1                                  |
| 2,4-epibrassinolide               | 60.0±2                            | 3.5±0.2          | 10±1                 | 0±0                         | 60.0±2                                  | 0                             | 5±0                                  |
| GO-CuO 3 μg/l                    | 60.0±4.5                          | 4.6±0.1          | 10±0                 | 0±0                         | 60.0±3                                  | 0                             | 4±0                                  |
| GO-Ag 3 μg/l                     | 70.0±2.5                          | 4.8±0.3          | 12±1                 | 0±0                         | 70.0±4                                  | 0                             | 5±0                                  |
| GO-CuO 4.5 μg/l                  | 60.0±2.0                          | 3.4±0.5          | 6±0                  | 1±0                         | 30.0±2.2                                 | 0                             | 4±1                                  |
| GO-Ag 4.5 μg/l                   | 30.0±3.0                          | 3.3±0.6          | 6±0                  | 1±0                         | 30.0±5.0                                 | 0                             | 3±1                                  |

The treatment with GO-CuO at 3 μg/l also produced good results. The lowest values were noted in the variant of GO-Ag at 4.5 μg/l, when the number of surviving and adapted plantlets decreased by two times in comparison with the control. The microclones were evaluated at 3 points on a 1 to 5 scale, this being the worst value for the overall plant condition.

Biochemical analysis was carried out for the variants with the most positive response to the treatment with nanomaterials. Photosynthetic activity measurements in the white poplar x aspen hybrid cultivated in the medium treated with GO-CuO at a concentration of 3 μg/l showed (figure 2) that they were characterized by a higher level of photosynthetic activity compared with the control. At the same time, the parameter variability within each individual plant was lower, thus prompting a suggestion that the treatment not only increases the resistance potential in plants but also improves stability of their condition.

The analysis of the effect produced by composite materials on photosynthetic activity in hairy birch plantlets showed that the highest results were produced by GO-Ag at 3 μg/l (figure 3). The obtained data, as well as the results of biomorphological research, allow one to assume that GO-Ag nanoparticles solutions at 3 μg/l can increase resistance potential in hairy birch microclones if applied during in vivo adaptation.
Figure 2. Photosynthetic activity of the white poplar x aspen hybrid microclones.

Figure 3. Photosynthetic activity of the hairy birch microclones.

Photosynthetic activity measurements in crack willow microclones showed that the treatment with GO-CuO at a concentration of 3 μg/l increased the studied parameter (figure 4); the plants from this group combined a higher level of photosynthetic activity with reduced parameter variability within each individual plant.

Figure 4. Photosynthetic activity of the crack willow microclones.
Unlike in the above described species, where increase in photosynthetic activity happened in the same treatment variants that produced the strongest stimulating impact on the biomorphological parameters, in the red oak microclones, inverse relationship was detected. The treatment with GO-Ag solution at 3 $\mu$g/l had the strongest stimulating effect on the adaptation processes while photosynthetic activity was below the activity observed in the control group (figure 5). At the same time, it should be noted that the parameter variability within each individual plant in the treated group was considerably below the level of variability observed in the control group.

![Figure 5. Photosynthetic activity of the red oak microclones.](image)

For the scots pine plantlets, the highest values of adaptation parameters were detected when treated with GO-Ag solution at 3 $\mu$g/l. When we carried out the photosynthetic activity analysis in this group, the results showed that in the treated plants, photosynthesis processes were more than twice as active as in the control (figure 6).

![Figure 6. Photosynthetic activity of the scots pine microclones.](image)

4. Conclusion
In order to summarize the results, we would like to note that the plant treatment with GO-CuO and GO-Ag solutions at a concentration of 3 $\mu$g/l had a pronounced positive effect on the plant adaptation process. The application of these solutions considerably increased the number of surviving and adapted
microclones as well as plant height, the number of leaves and overall plantlet condition on a 1 to 5 scale. We assume that antibacterial activity of the nanoparticles contributed the most to this improvement [12]. Besides that, the nanoparticles may have influenced expression of the genes responsible for the synthesis of the growth-influencing phytohormones [19].

The analysis of photosynthetic activity in the white poplar x aspen hybrid, birch, willow, and pine plantlets revealed the increase in the values of the parameters and the reduction in their variability within an individual plant, which can point at a high resistance potential and stability of the condition of these microclones. Stimulation of photosynthetic activity by copper nanoparticles was described by Nekrasova et al. in their paper [20].

At the same time, the red oak microclones displayed a “non-standard” reaction to the treatment with nanopreparations. Namely, the treatment with GO-Ag solution at 3 μg/l produced the maximal level of stimulation of biomorphological parameters, while the photosynthetic activity fell below the values observed in the control. The decrease in photosynthetic activity may be attributed to the toxic action of silver nanoparticles on the photosynthetic system of the studied oak plants [21, 22].

The solution of GO-Ag at a concentration of 4.5 μg/l had a considerable toxic impact on all the plants except red oak. Phytotoxic effects of silver and graphene oxide nanoparticles have been described in a number of papers [23-26]. The researchers have observed that the toxic effects are produced mainly by high concentrations of nanoparticles; this agrees with the results of our research. The noted resistance of the oak plants against the adverse effects of nanoparticles may be attributed to a high content of tannin and other phenolic compounds in the tissues. It is known that phenolic compounds perform protective functions preventing mechanical damage to the cell walls as well as reducing the amount of reactive oxygen intermediates [27], i.e. they effectively block the main assumed phytotoxicity mechanisms of nanoparticles [28, 29]. However, the mechanisms of the effects of GO-Ag solutions on oak plants require further study.

Thus, composite materials based on graphene oxide and silver and copper oxide nanoparticles can be considered promising components of sterilizing products, taking into account their potential phytotoxicity at high concentrations.

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