Effects of graphene oxide on white poplar X ASPEN (Populus alba x Populus tremula) hybrid microsprouts at various growth stages

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Abstract. Graphene-like materials are promising in biotechnology as antimicrobial agents and plant growth stimulants. We investigated the effects of graphene oxide nanoparticles in the composition of the MS0 medium on microclone survival of the poplar and aspen hybrid. In the early stages of cultivation, graphene oxide improved microclone survival by 6–20%. However, then, along with some stimulation, negative effects were observed, manifested in a decrease in the development of the root system and a decrease in the thickness of the leaf lamina and the diameter of the stem. Our results indicate the promise of using graphene oxide in the early stages of the development of microclones of white poplar and aspen hybrids.

1. Introduction

Plant cell cultures are widely used both in the general and applied areas of plant biology such as cytology, embryogenesis, morphogenesis, nutrition, pathology and germplasm conservation, genetic manipulation, large scale microcloning and production of phytopathogen-free plants. Success of in vitro manipulations with plant cultures depends on the methods for surface disinfection, on the selected growth medium and growth-regulating chemicals as well as on donor plant genotype and physiological status and on cell cultures maintenance conditions. Culture media usually consist of macro- and microelements, amino acids, organic additives, vitamins, nitrogen sources, plant growth regulators and solidifiers and their compositions have a pronounced effect on morphogenetic potential of explants. Optimization of plant mineral nutrition promotes explants growth and morphogenesis. It also induces cell proliferation, organogenesis, somatic embryogenesis, improves stems quality and content of bioactive substances in cell and organ cultures [1]. Both chemical composition and physical properties of growth media should conform to the demands of each stage of microsprout development. At the first stage various antioxidants, such as cysteine, glutathione or ascorbic acid are added to the media in order...
to inhibit damage from hydrolyzing enzymes thus preventing death of explants. Use of carbon materials fosters offshoots growth due to absorption toxic substances produced by explants. Khodakovskaya et al. demonstrate that multiwalled carbon nanotubes have the ability to enhance the growth of tobacco cell culture (55–64% increase over control) in a wide range of concentrations (5–500 μg/mL). Activated carbon (AC) stimulates cell growth (16% increase) only at low concentrations (5 μg/mL) while dramatically inhibits the cellular growth at higher concentrations (100–500 μg/mL) [2].

Authors of some studies report metallic nanoparticles (NPs) have positive effects when used during microcloning of plants. For instance, in the process of tobacco explants cultivation on modified medium containing TiO2 NPs, high antibacterial activity of the studied NPs was observed [3, 4]. The best results for potato cells cultivation were obtained in the Murashige and Skoog medium (MC0) containing 1% (wt./wt.) of TiO2 NPs [5], while no adverse effects on further growth and development of the culture cells was observed. Addition of 60 mg/L of TiO2 NPs to the MC0 medium eliminated bacterial contamination in barley callus cultures [6].

Microbial contamination is a grave issue for plant cell cultures cultivation as it can lead to total destruction of all the experimental specimens. Both the explants and the laboratory environment act as sources of contamination. According to the accepted procedure for creating in vitro cultures various organs collected from plants undergo surface sterilization. Disinfection of explants is the most important procedure preceding the actual process of in vitro cultivation because in the growth media microorganisms develop quicker than explants and the former can seriously affect the culture initiation. Nevertheless, antimicrobial treatments are often ineffective. Various chemical agents can be used for obtaining sterilized explants, for example, bromine water, sodium hypochlorite, mercuric chloride, silver nitrate, antibiotics and fungicides, but the concentrations of the chemicals and exposure time may affect the quality of explants. Besides, some of the sterilizing agents show phytotoxic action [7, 8]. In the study [9] graphene was found to penetrate tomato seed husks that might facilitate water uptake, resulting in faster germination and higher germination rates. The authors of [10] investigated the effects of unfunctionalized graphene oxide and amine-functionalized graphene oxide on wheat (Triticum aestivum) after 9 days of hydroponic culture. They found that the incubation with both nanomaterials did not affect the final seed germination rate. The plant growth was enhanced under amine-functionalized graphene oxide exposure, and the root and stem lengths were increased. Several studies are focused on the phytotoxic effects of graphene based materials. Authors of [11] reports graphene significantly inhibited plant growth and biomass of cabbage, tomato, and red spinach. Graphene cytotoxicity has been reported in the experiment on Arabidopsis thaliana cell culture [12], as well as amplification of the phytotoxicity of arsenic in wheat by graphene oxide [13].

However, the influence of graphene-like materials on microclonal propagation of tree cultures lacks thorough study. Thus, the aim of this work was to reveal the effects of graphene oxide in the cultural medium on the development indicators of white poplar and aspen (Populus alba L. х Populus tremula L.) hybrid microclonal sprouts.

2. Materials and methods
Graphene oxide (GO) NPs were prepared by the chemical exfoliation method (i.e. the Hummers method) [14], where graphite is oxidized with sulfuric acid and potassium permanganate. Concentrated H2SO4 (69 ml) was added to the mixture of graphite flakes (3 g, 1 wt. equivalent) with NaNO3 (3 g, 0.5 wt equivalent), the obtained mixture was cooled down to 0°C. Then KMnO4 (9 g, 3 wt equivalent) was added slowly, in order to keep the temperature of the reaction mixture below 20°C. After that the reaction mixture was heated up to 35°C and stirred for 30 min, at the same time 138 ml of water was added slowly to it. The reaction temperature was kept at 98°C by heating the mixture for 15 min on an electric heater. After this the heating was switched off and the reaction mixture was cooled down in a water-bath for 10 min. Then 420 ml of water and 3 ml of 30% H2O2 were added. The resulting mixture was run through a sieve (U.S. Sieve Size (WS Tyler, 300_m)) and then filtered through polyester fiber (Carpenter, Co). The obtained product was centrifuged (for 4 h at 4000 rpm), the supernatant fluid was decanted. The remaining solid material was rinsed successively with 200 ml of water, 200 ml of
30% HCl and 200 ml of ethanol (2×); after each rinsing cycle the mixture was run through a sieve (U.S. Sieve Size Standard), filtered through polyester fiber and centrifuged (for 4 h at 4000 rpm) while the supernatant fluid was decanted. The resulting material was coagulated with 200 ml of ether and the obtained suspension was filtered through a PTFE membrane with the pore size of 0.45 μm.

The obtained material was studied by atomic-force microscopy (AIST-NT instrument (AIST-NT, Russia) in semi-contact tapping mode with NTMDT AFM tips), Raman scattering spectroscopy (Thermo DXR Raman microscope (Thermo Scientific, USA) with 532 nm laser at 1mW power through 100× objective), optical (Biolam M-1 microscope, LOMO, Russia) and scanning electron microscopy (Vega3 microscope, Tescan, Czech Republic).

In order to prepare the samples for studying by Raman scattering spectroscopy, optical microscopy and scanning electron microscopy the colloidal solution of NPs was applied onto a silicon wafer and dried at 90 °C for 1 h.

For the survival rate study stem cuttings from the white poplar and aspen hybrid containing apical and axillary buds were cultured in vitro. Softwood shoots were taken in June from field grown plants. The 20-cm-long cuttings were stored at a low positive temperature for the period from 3 to 5 days. The cut surfaces were renewed and water was changed every 3 days. Then the shoots were washed in running water, cut into 3-5 cm segments and thoroughly washed in warm water with a surface-active agent. After that the cuttings were washed for 35 min in solution consisting of 200 µL of 2% sodium hypochlorite and 200 ml of distilled water with subsequent rinsing for 10 min in distilled water being thus prepared for sterilization. Sodium hypochlorite solutions were used as main sterilizing agents.

After sterilization the explants were planted into agar-solidified micro- and macro-salts Murashige and Skood medium (MS0) [15, 16]. The parts damaged by the sterilizing agent were removed before the cuttings were placed into the agarized growing medium.

The test tubes containing explants were placed on the light box shelves and cultivated at +24°C and 70% relative air humidity with 16 h photoperiod (5000 lx).

The newly developed microshoots were removed from the initial explants and were planter into the growing medium containing micro- and macro-elements and enriched with hormones and NPs promoting further growth and propagation.

The white poplar and aspen hybrid explants and microclones were cultivated on MS growth medium with addition of 0.75, 1.5, 3, 6 or 15 μg of GO per liter of the medium. In each variant, 10 seedlings were cultivated. The experiment was carried out in triplicate.

Throughout the cultivation process the dynamics of the morphometric parameters of the test tube plants was analyzed every second day, the following parameters were measured: the plant height, number of green leaves, number of yellow leaves, number of roots, condition on a 1 to 5 scale.

When the developed regenerants reached the height of 2 to 3 cm they were removed into culture tubes with the rooting medium, ½ woody plant medium (WPM) was used for this purpose [17]. GO NPs were added to the growth medium at the concentration of 1.5 μg of GO per liter of the medium in order to stimulate rhizogenesis.

Biochemical evaluation was carried out by measuring the photosynthesis activity which is the integral diagnostic indicator of the general plant resistance against stressors and by studying the oxidoreductases class enzymes, catalase in particular. It is established that the degree of catalase activity can be considered a practicable indicator for evaluating the activity of protection systems in organisms [18].

The photosynthesis activity in the chlorophyll-containing tissues was estimated by means of a fluorimetric indicator of physiological state IFSR-2 according to the method suggested in [19].

The catalase activity assay was carried out by the permanganometric method - H₂O₂ titration in sulphuric acid. The enzyme activity was expressed as standard units (act. units or 1 mg of H₂O₂ per min) while taking into account that 1 ml of 0.1 μ KMnO₄ corresponds to 1.7 mg of hydrogen peroxide.

The histological and cytological studies of leaf lamina tissue measuring such criteria as stomatal density (i.e. the number of stomata per mm²) and stomatal pore size were carried out by means of a Video Test Morphology 4.0 hardware-software complex.
In order to study the effects of NPs on the white poplar and aspen hybrid microclones the plantlets of the uniform height with 4-5 leaves and at least 2 cm-long roots cultivated in the protected environment were used. The plantlets with well developed stem and root system were removed from the tubes with long forceps and with a special hook. The remaining agar was rinsed from the roots with 1 % potassium permanganate solution, the sprouts were planted into technological cassettes filled with moist cultivation soil and then placed into the greenhouse propagating frame.

The cultivation soil consisted of pH-neutral peat (Lama Torf, Russia) and perlite at the ratio of 3:1. The peat contained the following elements: nitrogen (N), phosphorus (P₂O₅), potassium (K₂O). The plants were kept in the controlled atmosphere at 22-24 °C and humidity of 85-90 %.

The planting cassettes willed with the soil were treated with solution of the reference growth regulator 2,4-epibrassinolide at 667 μg per liter and with GO solution at 3 μg per liter. Both solutions were prepared in distilled water.

3. Results and discussion

Figure 1 presents the results of atomic-force microscopy of the samples. The average size of the flakes varies from 0.1 to 3 μm (figure 1a, 1c) while their mean thickness is less than 1 nm (figure 1b, 1d), which indicates single layer GO.

Figure 1. The results of atomic-force microscopy of GO samples. Individual flakes marked by arrows.
In the Raman spectrum (figure 2a) one can observe the main lines characteristic for GO D (at 1338 cm\(^{-1}\)) and G (at 1590 cm\(^{-1}\)). Figure 2b represents a micrograph of the sample in the same spot where the Raman spectrum was taken. One can see folds and creases on the surface of individual flakes.

![Raman Spectrum](image1)

![Micrograph](image2)

**Figure 2.** The results of Raman scattering spectroscopy (a) and optical micrograph of the GO film on a silicon wafer (b). Folds and creases on the surface of individual flakes marked by arrows.

Figure 3 represents a scanning electron micrograph of the GO film on a silicon wafer after air-drying. Folds and creases on the surface of individual flakes can be clearly seen.

![Scanning Electron Micrograph](image3)

**Figure 3.** Scanning electron micrograph of the GO film on a silicon wafer after air-drying at 90 °C. Folds and creases on the surface of individual flakes marked by arrows.

After 1.5 months of cultivation a number of well-developed and free of any morphological anomalies microclones of the white poplar x aspen hybrid were obtained from the apical and axillary meristems (figure 4).
During the cultivation process a positive effect of the NPs solutions on the growth processes in the tissue culture of the white poplar x aspen hybrid meristems was observed. The results presented in table 1 show the positive effect of GO in the cultivation medium on the analyzed parameters. The number of uninfected microclones was 100% at a concentration of GO 1.5 - 15 μg / L (figure 5), against a background of 80% in the control. However, it is worth noting that a dose of 15 μg / L inhibited the further development of seedlings, according to other indicators. The number of surviving passed microclones exceeded the control by 6-20% at 0.75 - 6 μg / L. The maximum stem length and the largest number of additional shoots were noted in groups 1.5 and 3 μg / L, root occurrence was observed here.

Table 1. Biomorphological parameters of the white poplar x aspen hybrid microclones cultivated on soils containing various concentrations of GO NPs.

| NPs in cultivation medium, μg/L | Number of sterile microclones, pcs/% | Number of surviving microclones, pcs/% | Stem height, cm | Number of leaves, pcs | Number of additional shoots, pcs | Root occurrence | Microclones condition, on a 1 to 5 scale |
|-------------------------------|-------------------------------------|---------------------------------------|----------------|----------------------|-------------------------------|----------------|--------------------------------------|
| Control                       | 80.0                                | 80.0                                  | 3.5            | 2                    | 0                             | 0              | 5                                    |
| GO 0.75                       | 86.0                                | 86.0                                  | 3.5            | 2                    | 0                             | 0              | 5                                    |
| GO 1.5                        | 100.0                               | 100.0                                 | 3.8            | 5                    | 1                             | +              | 5                                    |
| GO 3                          | 100.0                               | 100.0                                 | 4.0            | 3                    | 2                             | +              | 5                                    |
| GO 6                          | 100.0                               | 90.0                                  | 3.8            | 2                    | 2                             | 0              | 3                                    |
| GO 15                         | 100.0                               | 70.0                                  | 3.4            | 2                    | 0                             | 0              | 2                                    |

Cuttings were taken from the normally developed microclones and transplanted into growing media enriched with hormonal components.

After 1-month cultivation on the growing media containing the following hormonal components: 0.2 mg/L of 6-benzylaminopurine (BAP) + 0.1 mg/L of indoleacetic acid (IA) + 0.3 mg/L of gibberellic acid (GA) we observed height reduction of the studied plants, while on the media containing both hormones and GO the number of additional shoots increased to up to 4 shoots per plant (table 2). The maximal stem height of 3.8 cm was registered in the plants cultivated on the hormone-free medium containing GO, in the control group the stem height was 3.5 cm. Introduction of GO NPs into the growing media also increased the number of surviving microclones up to 100%. One should note that roots were developed only by the microplants cultivated on GO-containing medium. The maximal number of leaves was observed in the same case - 5 leaves per plant.

Further studies of the influence of GO NPs at a concentration of 1.5 μg/L on the white poplar x aspen hybrid microclones at the rooting stage prior to transplanting into the soil showed adverse effect of GO NPs on the rooting process as only 30% of the microclones took root, which is by 20% less than in the control (table 3).
Table 2. Biomorphological parameters of the white poplar x aspen hybrid microclones cultivated for 1 month on media containing hormonal components and GO NPs at a concentration of 1.5 μg/L.

| Version                              | Number of surviving clones, % | Stem height, cm | Number of leaves, pcs | Number of additional shoots, pcs | Root occurrence | Microclones condition, on a 1 to 5 scale |
|--------------------------------------|-------------------------------|----------------|-----------------------|---------------------------------|----------------|------------------------------------------|
| Control                              | 80.0                          | 3.5            | 4                     | 0                              | 0              | 5                                        |
| 0.2BAP+0.1IA+0.3GA                   | 80.0                          | 1.5            | 4                     | 3                              | 0              | 5                                        |
| GO1.5                                | 100.0                         | 3.8            | 5                     | 1                              | +              | 5                                        |
| GO1.5+0.2BAP+0.1IA+0.3GA             | 100.0                         | 2.4            | 4                     | 4                              | 0              | 5                                        |

Table 3. Morphometric parameters of the white poplar x aspen hybrid microclones cultivated at the rooting stage under the influence of GO NPs.

| Version      | Number of surviving microclones, % | Stem height, cm | Number of leaves, pcs | Number of additional shoots, pcs | Number of microclones with roots, % | Number of roots, pcs | Microclones condition, on a 1 to 5 scale |
|--------------|-------------------------------------|-----------------|-----------------------|---------------------------------|-------------------------------------|----------------------|------------------------------------------|
| Control      | 100.0                               | 5.0             | 7                     | 0                               | 50.0                                | 1.0                  | 4                                        |
| GO 1.5       | 100.0                               | 5.1             | 6                     | 0                               | 30.0                                | 1.0                  | 4                                        |

Figure 6. A leaf of the white poplar x aspen hybrid microclone: (a) - control, (c) - in the growth media with GO; a stoma of the white poplar x aspen hybrid microclone: (b) - control, (d) - in the growth media with GO.
The histological analysis showed that on the GO-containing media the plants developed higher stomatal density per mm² and the stomata had larger openings (figures 6, 7a, 7b), these factors may result in improved heat tolerance of these plants.

![Figure 7](image)

**Figure 7.** Biomorphological parameters of: (a), (b) stomata; (c) leaf and stem.

The leaf lamina thickness and stem diameter in the white poplar x aspen hybrid treated with GO were smaller than those in the control (figure 7c). This fact may be attributed to the nanomaterial phytotoxic effects which may manifest themselves in suppression of some morphometric parameters [10, 13].

Biochemical analysis of the sprouts showed slight increase in photosynthetic activity under the GO influence (figure 8) while the parameter dispersion within an individual plant was lower compared to the control (0.01580 vs 0.00323 relative units) prompting suggestions that the treatment not only increases the resistance potential in plants but also promotes stability of their state.

Figures 6-8 demonstrate positive effect of GO on the photosynthetic and antioxidant systems as well as on stomata number and function was noted, while at the same time some decrease in leaf lamina thickness and stem diameter was observed.

The study of the white poplar x aspen hybrid microclones during their adaptation to the protected environment showed that soil treatment with the GO solution had no observable effect on the sprouts during the adaptation period. At the same time, treatment with the reference growth regulating agent increased the number of surviving microclones by 10% while the number of leaves and additional shoots also increased (table 4). The noted small increase in the proportion of adapted plants in the GO group is within the statistical error.

Thus, during the cultivation we have observed positive effects produced by GO solutions on the growth processes of the white poplar x aspen hybrid meristems in the tissue culture. In every case the number of surviving microclones exceeded that in the control by 6-20%. The number of sterile microclones in the media containing more than 0.75 μg/L of GO reached 100%, thus exceeding the same parameter in the control group by 20%. Integrally, the maximal effect was observed at the GO NPs concentration of 3 μg/L.

Further cultivation of the microsprouts on the growth media enriched with hormones showed stem height reduction, while on the media containing both hormones and GO NPs the number of additional shoots increased up to 4 shoots per plant. The maximal stem height of 3.8 cm was registered in the plants cultivated on the hormone-free medium containing GO compared to 3.5 cm stems in the control group. Introduction of GO NPs into the growing media also increased the number of surviving microclones up
to 100%. One should note that only the microplants cultivated on GO-containing medium developed roots. The maximal number of leaves was observed in the same case - 5 leaves per plant.

![Figure 8](image)

**Figure 8.** Biochemical parameters of the sprouts: (a) photosynthetic activity, (b) catalase activity.

**Table 4.** Indicators of in vivo adaptation effectiveness of the white poplar x aspen hybrid microclones.

| Version          | Number of surviving microclones, % | Regenerated plant height, cm | Number of leaves, pcs | Number of wilted leaves, pcs | Number of adapted plants, % | Number of additional shoots, pcs | Microclone s condition, on a 1 to 5 scale |
|------------------|------------------------------------|-------------------------------|-----------------------|------------------------------|-----------------------------|----------------------------------|----------------------------------------|
| Control          | 60.0                               | 8.0                          | 8                     | 2                            | 50.0                        | 0                                | 4                                       |
| 2,4-epibrassinolide | 70.0                             | 8.5                          | 10                    | 2                            | 60.0                        | 0                                | 5                                       |
| GO               | 60.0                               | 8.4                          | 7                     | 4                            | 51.0                        | 0                                | 4                                       |

Further studies of the influence of GO NPs showed adverse effect of GO NPs on the rooting process as only 30% of the microclones took root, which is by 20% less than in the control. The effect of soil treatment with the GO solution during adaptation of the plants to the protected environment was negligible.

The histological and biochemical assays showed that treatment with GO not only increased the resistance potential in plants but also promoted stability of their state.

On the one hand, the obtained results are connected with antibacterial and fungicidal properties of GO described in a number of research papers [20, 21]. The material improves microsprouts survival rate by suppressing pathogenic microorganisms. On the other hand, some researchers mention phytotoxicity of graphene-like materials [10, 13, 22]. We observed similar effects at further stages of plant development. Besides, some of the described effects can be attributed to high sorption capacity of GO [23]. In particular, phytohormones and other active molecules can be aggregated on its surface and thus their biological activity can be extended.
Conclusion

Thus, we studied the effects of GO nanoparticles in the composition of the MS0 medium on microclonal seedlings of the poplar and aspen hybrid. In the early stages of cultivation, GO improved microclone survival by 6–20%. However, then, along with some stimulation, negative effects were observed, manifested in a decrease in the development of the root system and a decrease in the thickness of the leaf lamina and the diameter of the stem.

Our results prove that GO is a promising nanomaterial for application at the early stages of cultivation of white poplar and aspen hybrid microclones.

Acknowledgments

This study was supported by the Ministry of Education and Science of the Russian Federation, project no. RFMEFI57417X0159.

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