Stimulating and toxic effects of graphene oxide on *Betula pubescens* microclones

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**Abstract.** We have studied the impact of graphene oxide (GO) at 1.5 – 15 µg/L on *Betula pubescens* microclones. Discovered, that the nanomaterial protects the plants against phytopathogens at the stage of culture establishment and promotes a 25% increase in the shoots survival rate. Further growth stimulation was observed in the groups treated with 1.5 and 3 µg/L, where the shoots length increased by 1.8 and 1 cm, and the number of leaves increased by 2 and 1.5 times respectively. 15 µg/L reduced the height of shoots by 20%. At the multiplication stage addition of GO enhanced the plantlets survival rate by 20% and had a stimulating effect on the birch regenerants growth, as well as on the number of leaves. The data on the plantlets survival rate and their morphometric analysis results correspond well with the biochemical assessment, namely in the GO group both photosynthetic and catalase activity exceeded those of the control group by 2 and 1.5 times, respectively. At the same time, histological analysis exhibits reduced number of stomata in the GO-treated group. At the rooting stage addition of 1.5 µg/L GO to the growth medium increased the number of plantlets with roots by 10% compared to control.

1. **Introduction**

A large body of research articles about graphene applications in various areas have been published since 2007, due to its unique properties. Nanomaterials from the graphene family have been a subject of intense development and are used in numerous fields, including electronics [1], conductivity [2], energy generation [3], batteries [4], sensors [5] and more [6, 7]. Besides, graphene-like materials are very promising for biomedical applications [8, 9], such as bioelectronics [10], antibacterial agents manufacturing [11, 12], tissue engineering [13, 14], drug and gene delivery [15 - 17], biosensing [18, 19], cancer therapy [20] and others [21 - 23]. A number of papers prove that graphene-based materials possess high potential in plant cultivation. For example, positive influence of GO on the root length, the area of leaves, the number of leaves, and the formation of flower buds in *Arabidopsis thaliana* L.
has been established [24]. Other authors showed that graphene penetrates seed husks. The penetration might break the husks to facilitate water uptake, resulting in faster germination [25]. It was found that a low dose of GO significantly promoted the germination of spinach and chive in soil. Oxygen-containing functional groups of GO collected water, and the hydrophobic sp2 domains transported water to the seeds to accelerate the germination of plants [26].

At the same time, some research works report opposite results. Begum P. et al. showed that cabbage, tomato, and red spinach exposed to varying concentrations (500 to 2000 mg/L) of graphene displayed a considerable decrease in the growth rate and biomass development [27]. Chen et al. used transmission electron microscopy (TEM) to indicate that exposure to GO at a concentration above 1000 μg/mL resulted in a decrease in root length, shoot length and relative biomass, along with obvious damage to plant tissue structures [28].

Thus, the question of the real impact of graphene-based materials on plants has not been resolved yet. Besides, the influence of graphene and its derivatives on the plant tissue cultures has never been carried out. The results of such work might be of considerable value for biotechnology.

In our work we examine the influence of GO, fabricated by the chemical exfoliation of graphite, upon downy birch (Betula pubescens Ehrh.) culture during microclonal propagation.

2. Methods and materials
GO nanoparticles were prepared by the chemical exfoliation method (i.e. the Hummers method) [29], 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 300 μm (U.S. Sieve Size (WS Tyler, Mentor, Ohio, USA)) and then filtered through polyester fiber (Carpenter, Co, Richmond, Virginia, USA). 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; 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 polytetrafluoroethylene (PTFE) membrane with the pore size of 0.45 μm.

The obtained material was studied by Raman scattering spectroscopy (Thermo DXR Raman microscope (Thermo Scientific, USA) with 532 nm laser at 1mW power through 100× objective), scanning electron microscopy (Vega3 microscope, Tescan, Czech Republic) and atomic-force microscopy (SmartSPM, AIST-NT, Russian Federation) in semi-contact tapping mode with atomic force microscopy tips (NT-MDT Spectrum Instruments, Zelenograd, Russian Federation).

For the in vitro culture initiation plant samples with apical and axillary buds were used. Before sterilization the cuttings were washed with surface-active substance and thoroughly rinsed in distilled water. Then the samples were cut into 3-5 cm segments and sterilized for 35 min in the solution of 200 μL 5% sodium hypochlorite in 200 mL distilled water with subsequent rinse in distilled water. The major sterilization was carried out for 15 min in a laminar flow cabinet, in the solution consisting of 15 mL 5% sodium hypochlorite and 200 mL distilled water, succeeded by rinsing in sterile distilled water. Under aseptic conditions the sterilized shoots were cut into 1.5 - 2 cm explants, each with a single axillary bud, and transferred into Murashige and Skoog nutrient medium (MS0) [30, 31]. The test tubes with the explants were placed in the growth chamber at +24 °C under light/dark 16/8 photoperiod, 5000 lx light intensity and 70% relative air humidity. The emerging shoots were sliced.
off and subcultured onto new media with hormones and the nanomaterial added at concentrations of 0.75, 1.5, 3, 6 and 15 μg/L of the growth medium.

During the cultivation period the following morphometric parameters were assessed: plant height, number of leaves, number of roots, general condition evaluation (according to 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).

During the subsequent multiplication stage the 1.5 - 2 cm microshoots, with 1 or 2 internodes each, were transplanted into the growth media containing 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 1.5 μg/L nanoparticles has also been analyzed. Besides, at the multiplication stage we carried out histological examination of the shoots using a VideoTesT-Morphology 4.0 hardware and software complex (Argus-BIO, St. Petersburg, Russia). For biochemical analysis of the shoots we measured photosynthesis activity and catalase activity via the spectrophotometric method.

At the final stage of the research the ability of GO to promote rhizogenesis was studied. The well-formed regenerants (2 -3 cm high) were isolated and transferred to the rooting ½ woody plant medium (WPM) [32]. The nanomaterial was added at 1.5 μg per 1 L of growth medium, in order to promote rhizogenesis.

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

3. Results and discussion

3.1. GO samples analysis

Raman spectroscopy showed (figure 1) two prominent GO bands - the D-band at 1338 cm⁻¹ and G-band at 1590 cm⁻¹ [33-35].

![GO Raman spectrum](image)

**Figure 1.** GO Raman spectrum.

SEM examination displayed planar structure of the sample, free from crystalline phase particles (figure 2).
Atomic force microscopy analysis showed that the lateral dimensions of the flakes were in the range from 0.1 to 3 μm, while their mean thickness was less than 1 nm (figure 3).

Thus, the analysis of the fabricated GO sample showed that the material had the characteristic planar morphology with the lateral dimensions of individual flakes in the range from 0.1 to 3 μm, and their mean thickness less than 1 nm.

3.2. GO impact on the explants at the stage of culture establishment

After 1.5 months of incubation a positive influence of the GO solutions upon the downy birch meristem growth in the culture was observed. The number of surviving and sterile microclones, i.e. the ones with no signs of infection, was 100% at the nanomaterial concentrations above 0.75 μg/L, exceeding the control by 25% (figure 4(a))
The height of the shoots and number of leaves almost doubled at GO concentrations of 1.5 and 3 µg/L. The shoots length increased by 1.8 and 1 cm (figure 4 (b)), while the number of leaves increased by 2 and 1.5 times (figure 4 (c)), respectively. At the same time, GO 15 µg/L in the growth medium had a pronounced suppressing effect on the shoots development, their length decreased by 0.4 cm. In all the other cases the parameters did not deviate from the control. One should note that in the 1.5 µg/L group the microclones developed roots, while there were no roots in the control. In the same group the microplants were in the perfect condition according to the 5-point scale (table 1).

### Table 1. Microclones condition according to a 5-point scale.

|          | Condition |
|----------|-----------|
| Control  | 4         |
| GO 0.75 µg/L | 4         |
| GO 1.5 µg/L  | 3         |
| GO 3.0 µg/L  | 5         |
| GO 6.0 µg/L  | 3         |
| GO 15.0 µg/L | 2         |

Thus, the research established that GO at 1.5 – 15 µg/L has a positive impact on the microclones protection against phytopathogens at the stage of culture establishment, while simultaneously increasing the shoots viability by 25%. Nevertheless, the further growth was promoted only in the 1.5 and 3 µg/L variants, where the shoots length increased by 1.8 and 1 cm, and the number of leaves increased by 2 and 1.5 times respectively. 15 µg/L suppressed the plant development reducing the height of shoots by 20%. The microclones in the 1.5 µg/L group developed roots and displayed the
best general condition according to a 5-point scale, that is why this group was selected for further multiplication.

3.3 GO impact on the explants at the multiplication stage
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.

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

| Growth medium composition | Number of surviving clones, % | Height of shoots, cm | Number of leaves, pcs | Number of additional shoots, pcs | Root occurrence |
|---------------------------|-------------------------------|----------------------|-----------------------|-------------------------------|-----------------|
| Control                   | 80.0±4.6                      | 2.3±0.2              | 3                     | 0                             | 0               |
| 0.2BAP+0.1IA+0.3GA        | 78.0±3.9                      | 1.5±0.3              | 4                     | 3                             | 0               |
| GO1.5                     | 100.0                         | 3.8±0.4              | 4                     | 0                             | +               |
| GO1.5+0.2BAP+0.1IA+0.3GA  | 100.0                         | 2.4±0.5              | 4                     | 3                             | 0               |

As one can see from table 2, GO introduced into the growth medium, both separately and in combination with phytohormones, increased the plantlets survival rate by 20%. The highest shoots were also observed in the GO group - 3.8 cm against 2.3 cm in the control (table 2, figure 5).

The minimum result of 1.5 cm was detected in the variant with phytohormones and zero GO, that is hormones introduction inhibited the shoots growth. Combination of hormones with GO produced the results similar to those in the control group. Apparently, the negative influence of hormones was cancelled out by GO effect. Nevertheless, the plantlets treated with hormones, though shorter in length, developed axillary shoots, while the control and GO groups showed only single shoots. The number of leaves (4 leaves per plantlet) was similar in all the variants except control, where the plantlets developed only 3 leaves each. At this stage root development was observed in the hormone-free group treated with 1.5 µg/L GO.

![Figure 5](image1.png) ![Figure 5](image2.png)

**Figure 5.** Microclones external appearance: a) control; b) medium with GO.

3.4 Histological and biochemical examination of the microclones at the multiplication stage
Histological examination of leaf laminae from downy birch regenerants displayed a significant increase in the stomatal opening area and in degree of opening against a notable decrease in the stomatal area and density in the GO group (table 3, figure 6).
Table 3. Histological parameters of the stomatal apparatus condition in downy birch leaves.

| Version | Stomatal opening area, µm² | Stomatal area, µm² | Stomatal density, pcs/mm² | Degree of stomatal opening, S stom./S open |
|---------|---------------------------|-------------------|--------------------------|-----------------------------------------|
| Control | 86.04±5.3                 | 1208.32±113.4     | 2.99±0.8                 | 0.071                                   |
| GO      | 150.99±9.8                | 915.61±95.1       | 2.39±0.6                 | 0.16                                    |

The data obtained from assessment of the explants morphological parameters suggest activation of compensatory mechanisms, when decrease in the number of stomata per unit area is balanced out by increase in stomatal opening, and the overall transpiration efficiency is improved.

The biochemical parameters analysis showed that photosynthetic activity almost doubled while catalase activity increased by 1.4 times (table 4).

Table 4. Microclones biochemical parameters

| Variant | Photosynthesis Fv/Fm (rel. units) | Catalase activity |
|---------|-----------------------------------|-------------------|
| Control | 0.345 ± 0.023                     | 2.79 ± 0.57       |
| GO      | 0.623 ± 0.013                     | 3.83 ± 0.43       |

The analysis of GO impact on microclones at the multiplication stage revealed that GO in the growth medium improves the microclones survival rate by 20%, promotes growth of regenerants and leaf formation regardless of hormonal composition of the medium. At the same time, only the plantlets cultivated in the presence of GO without hormones developed roots, while only the group receiving phytohormones without GO developed axillary shoots. The data on the regenerants survival rate and their morphometric analysis results correspond well with the biochemical assessment, namely in the GO group both photosynthetic and catalase activity exceeded those of the control group by 2 and 1.5 times, respectively. It is a well-established fact that reduction in photosynthetic activity alters morphometric parameters, for example, it leads to a decrease in leaf lamina area. Catalase is an enzyme particularly active in young viable plant tissues and organs. Decrease in viability significantly reduces the enzyme activity [36]. Histological analysis exhibits reduced number of stomata in the GO variant and the consequent compensatory increase in the functional activity of the remaining stomata. Thus, GO at the multiplication stage produced differently directed effects on the downy birch microclones.

Rooting stage. At the final stage of the research we studied the ability of GO to enhance rhizogenesis. We have discovered that addition of 1.5 µg/L GO to the growth medium increases the number of plantlets with roots by 10% (table 5).
## Table 5. GO effects at the rooting stage.

| Variant | Number of surviving microclones, % | Height of shoots, cm | Number of microclones with roots, % | Number of roots, pcs | Microclones condition according to a 5-point scale |
|---------|-----------------------------------|----------------------|-------------------------------------|----------------------|--------------------------------------------------|
| 0       | 100.0                             | 4.0 ± 0.6            | 35.0 ± 3                           | 1.0                  | 4                                               |
| GO      | 100.0                             | 4.3 ± 0.4            | 45.0 ± 2                           | 1.0                  | 4                                               |

Some slight growth activation has also been observed in the group treated with GO.

### Conclusion

The research revealed positive effect of 1.5 – 15 µg/L GO on the microclones protection against phytopathogens at the stage of culture establishment and showed 25% increase in the shoots survival rate. Nevertheless, further growth stimulation was observed only in the groups treated with 1.5 and 3 µg/L GO, where the shoot length increased by 1.8 and 1 cm, and the number of leaves increased by 2 and 1.5 times respectively. 15 µg/L suppressed plant development reducing the height of shoots by 20%. The microclones in the 1.5 µg/L group developed roots and displayed the best general condition according to a 5-point scale. The noted effect might be attributed to a high adsorption property of GO. It is known that activated charcoal is often used in tissue culture to improve cell growth and development. The promotor effects of activated charcoal on morphogenesis may be mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and the resulting decrease in the toxic metabolites, phenolic exudation and brown exudate accumulation [37, 38].

At the multiplication stage addition of the nanomaterial to the growth media enhanced the plantlets survival rate by 20% and had a stimulating effect on the birch regenerants growth, as well as on the number of leaves, regardless of the phytohormones content in the medium. At the same time, only the plantlets cultivated in the presence of GO without hormones developed roots, while only the group receiving phytohormones without GO developed axillary shoots. Xie et al. showed that GO and IAA have a synergistic effect on the Brassica napus root system in case of cotreatment, while treatment with 25 mg/L GO alone inhibited root development [39].

The data on the plantlets survival rate and their morphometric analysis results correspond well with the biochemical assessment, namely in the GO group both photosynthetic and catalase activity exceeded those of the control group by 2 and 1.5 times, respectively. While histological analysis exhibited reduced number of stomata in the GO-treated group.

At the rooting stage addition of 1.5 µg/L GO to the growth medium increased the number of plantlets with roots by 10% compared to control.

Probably, the effects of graphene oxide on plants, like other nanomaterials, is based on the formation of reactive oxygen species [40], which cause the activation of antioxidant enzymes, controlled by phytohormones [41]. It was found that nanomaterials affect the level of expression of genes responsible for the synthesis of phytohormones [42]. Thus, the effects noted by us can be associated with the effect of GO on the complex system of plant regulation at the biochemical and gene levels.

Thus, in most cases GO addition to the growth media had a positive effect on the birch microclones development, with the exclusion of high concentrations. Nevertheless, the mechanisms of GO action upon plants require further study. The results of this work may be used in the biotechnology of plant microclonal propagation.
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