Biochemical and histological evaluation of the characteristics of potential environmental sustainability of hardwood microclones (poplar, birch) grown in experimental nutrient media

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Abstract. Histological characteristics of lamina tissues (numerical values, area of stomata and width of stomatal pore), indicators of photosynthetic and enzymatic activity of the leaves of poplar (Populus alba L. × Populus tremula L.) and birch (Betula pubescens Ehrh.) microclones have been studied as criteria levels of stability degrees of plant tissue resistance to drought and fungal diseases. The stimulation of microclone potential resistance to the effects of various stressors has been revealed when adding a solution of TiO₂ and ZnO nanoparticles into a cultivation substrate.

1. Introduction
Forests are one of the most productive land ecosystems. They play a key role in the existence of humanity on our planet. Forests stabilize and regulate climate, reduce the intensity of dangerous weather phenomena, prevent soil erosion, provide shelter for about 80% of terrestrial animals, plants and insects species, and still provide food and shelter to nearly a quarter of the population living on our planet.

Forests cover about 30 percent of the land surface. However, forest area is steadily decreasing, as a result of human activity. Reproduction of forest resources using innovative technologies is one of the most urgent tasks of forest science practice [1, 2].

A promising innovative direction in forest restoration is the use of biotechnology methods based on the latest achievements in molecular biology, biochemistry, gene and cell engineering. At present, methods of plant micropropagation are being actively developed, but their use is complicated by a number of problems related, in particular, to the low adaptive capacity of microplants at the cloning stage and their susceptibility to various abiotic and biotic stressors [3, 4].

The use of synthetic nanoparticles with high antiseptic properties at different stages of cloning, as well as the ability to activate growth processes, allow solving these problems [5-7]. A review of scientific publications on the biocidal properties of ZnO and TiO₂ nanoparticles showed the promise of using these nanomaterials as antimicrobial agents, including biotechnological
targets [5, 6, 8, 9]. It was previously proved that zinc oxide and titanium dioxide nanoparticles have photocatalytic activity, high penetration, and are relatively safe for multicellular organisms [9, 10].

Evaluating the response of microclones to stressors and analyzing the potential resistance of plants to pathogens are the important points in the development of the most efficient nanoparticle complexes for introduction into the cultivation substrate during microclonal reproduction [11, 12].

Quality, viability and stability to pathogens of the resulting explants can be determined both directly and indirectly. It is known that histological characteristics of the leaf blade are direct indicators of plant resistance to fungal diseases. Enzymatic and photosynthetic activities are mediated indicators of plant organism resistance to the effects of stressors [4, 8, 2].

The purpose of this work was a histological and biochemical assessment of the characteristics of the potential ecological stability of microclones Populus (Populus alba L. × Populus tremula L.) and Betula (Betula pubescens Ehrh.), grown in vitro on experimental nanomodified nutrient media.

2. Materials and methods

Objects under study: poplar (Populus alba L. × Populus tremula L) and birch (Betula pubescens Ehrh.) microclones, grown in experimental culture media.

The main assessment methods were: histological test using VIDEO-TEST morphology-4 complex (Carl Zeiss AG) with a freezing microtome, catalase activity was determined by the classical permanganometric method; integral evaluation of physiological state of micro plants was carried out on the basis of the “Functional Diagnostic System for Fruit Plant Species” [13] with the use of IFSR-2 device (fluorometric indicator of physiological status) defining the variable fluorescence of chlorophyll in the cells (Fv/Fm), which reflects photosynthetic activity of the cells and expressed in relative units (RU) [13,14].

Dispersion of the Fv / Fm indicator within the same plant was estimated and calculated by the method of variance analysis of the primary Fv / Fm indicators [13, 14]. At each microplant in the variant, at least 100 measurement points were taken into account, followed by averaging the obtained variance values in the variant. The dispersion of the Fv / Fm indicator within the same plant acts as an independent value, which characterizes the stability of the functional state of the plant [13, 14].

As a composite material for the nutrient medium, zinc oxide and titanium dioxide nanosupplements were used. The average particle size of zinc oxide in the nutrient medium was 250 - 550 nm; particles of titanium dioxide - 300 - 400 nm [5, 8, 10].

Experience options: 1–500 μl TiO₂ (10 g / l); 2-500 μl of ZnO (10 g / l); 3 to 200 mg / l ceftriaxone; control - standard cultivation medium according to the prescription Murashige-Skoog (MS) (table 1).

| Table 1. Variants of culture media used in experiments* |
|-----------------------------------------------|
| **Populus (Populus alba L. × Populus tremula L.)** | 1P - MS + 10 g/l TiO₂ |
| | 2P - MS + 10 g/l ZnO |
| | 3P - MS + 200 mg/l ceftriaxone |
| | CP – control (MS) |
| **Betula (Betula pubescens Ehrh.)** | 1B - MS + 10 g/l TiO₂ |
| | 2B - MS + 10 g/l ZnO |
| | 3B - MS + 200 mg/l ceftriaxone |
| | CB – control (MS) |

*The number of explants per variant n = 20
3. Results and discussion

The effect of nano-additives for culture mediums on morphometric, histological and physiological characteristics of microclones in poplar (white poplar-aspen hybrid) and white birch micropropagation have been studied in this paper in order to assess their potential resistance to abiotic and biotic stressors.

The following characteristics of leaf tissue have been studied: stomato density (number of stomata per 1 mm²), stomata area and stomatal pore width. It is known that they can be criterion signs of stability degree of plant tissue to drought and fungal diseases. The smaller the stomata and the stomatal pore, the higher the resistance to fungal diseases. High drought tolerance is also indicated by a high density of small stomata or rarely located large stomata with narrow stomatal pores. Large stomata with a wide stomatal pore are characterized by a high level of gas exchange, which indicates higher heat resistance of the tissue [5, 15, 11, 9, 10].

Histological study of the leaf blades of microplants depending on the cultivation substrate has showed that leaf blades of Populus microclones grown in the cultivating substrate according to the Murasige-Skoog formulation with the addition of 500 µl of TiO₂ nanoparticles solution (10g/l) have: small stomata area (756.37 µm² on average), rather narrow stomatal pore (17.63 µm on average) and an average stomata density (5.258 ± 0.23 RU) (figure 1; table 2).

![Figure 1](image)

**Figure 1.** The size and shape of stomata of Populus microclones leaves depending on the variant of culture medium.
The addition of zinc oxide and antibiotics into the cultivation substrate has also had a positive effect on the potential resistance of cultivated plants to pathogens. However, the effect was slightly lower - the stomata area was 1.5 times less in zinc oxide option than in the control one, and the width of the stomatal pore decreased only slightly (20.3 microns compared with 25.8 µm in the control one). However, average 1.7 times increase in stomatal density indicates a potential increase in the drought tolerance of these clones.

Similar studies were performed by R. Brayner, R. Ferrari-Ilion et al. [6], D. Zhou, A. Keller et al. [10], T. Matsunaga, R. Tomoda et al. [16]. Based on the results of these studies, it was found that the nanoparticles of zinc oxide (ZnO) and titanium dioxide (TiO$_2$) in the nanoform are effective antibacterial and antifungal agents. It was noted that nanoscale zinc oxide can have a different morphology, as a result of which it can exhibit high antibacterial activity against a wide range of bacteria and fungi. In the works of L.K. Adams, D.Yu. Lyon, P.J. Alvarez [5], J. Zhang [17] and L. Zhang, Y. Jiang et al. [9] showed that titanium dioxide (TiO$_2$) in the nanoform acts as a photocatalyst, that is, it is an agent generating reactive oxygen species and an effective means of combating many bacteria and viruses from a number of taxonomic groups.

Slightly different response to the use of nano-modified media was found in *Betula* microclones: introduction of titanium dioxide nanoparticles to the cultivation substrate has a less significant positive effect on the explants than the introduction of zinc oxide (figure 2; table 2).

![Figure 2. The size and shape of stomata of Betula microclones leaves depending on the variant of culture medium.](image-url)
In this variant, histological characteristics of leaf microplant plates (stomatal area, width of stomatal pore and stomatal density) indicated the highest level of potential resistance of cultivated clones to both pathogens and stressors, such as overmoistening and drought. The above mentioned characteristics (in the variants with the addition of titanium dioxide and antibiotic to the cultivation substrate) are better than characteristics in the control variant (without addition of nanoparticles), but they are inferior to zinc oxide alteration (table 2).

Table 2. Histological characteristics of leaf blades of *Populus* and *Betula* microclones, depending on the variant of culture medium nano-modification.

|                   | Control option (MS) | MS + 10 g/l TiO₂ | MS + 10 g/l ZnO | MS + 200 mg/l ceftriaxone |
|-------------------|---------------------|------------------|-----------------|---------------------------|
| *Populus*         |                     |                  |                 |                           |
| Size of the stomata, µm² | 806.86±37.91        | 756.37±40.33    | 516.41±34.21    | 831.99±46.12              |
| Width of stomatal pores, µm | 25.85±3.99         | 17.63±2.97      | 20.31±4.11      | 25.01±3.78                |
| Density of stomata, RU | 4.19±0.33           | 5.26±0.23       | 6.82±0.28       | 4.62±0.31                 |
| *Betula*          |                     |                  |                 |                           |
| Size of the stomata, µm² | 1272.63±47.91       | 1430.69±41.19   | 1084.4±58.42    | 761.96±66.43              |
| Width of stomatal pores, µm | 27.88±5.19         | 20.17±3.11      | 16.45±4.31      | 25.18±4.19                |
| Density of stomata, RU | 2.27±0.26           | 4.75±0.31       | 6.58±0.28       | 3.59±0.25                 |

It has been previously found that the integral diagnostic indicator of plant stress resistance is the measurement of induction of chlorophyll-a variable fluorescence in assimilation tissues (Fv/Fm) [14, 16, 13]. This characteristic makes it possible to evaluate the activity of the photosystem-2 of chlorophyll-containing tissues and reflects the state of the photosynthetic plant apparatus. The correctness of using methods for determining the intensity of photosynthetic apparatus to identify the degree of stress in the physiological state of a plant is confirmed, in particular, by the fact that photosynthesis is one of the most vulnerable processes for oxidative damage. The value of the dispersion level of Fv/Fm indicator within one plant serves as an additional marker of the plant state. It indicates the degree of stability of metabolic processes in it. High dispersion values indicate an unstable functional state of the plant organism. Oxidoreductase enzymes, and, in particular, catalase are very important in protective system of any organism. The level of the enzyme activity can be considered as an indicator that actually reflects the state of the protective systems [6, 12].
Analysis of photosynthetic and enzymatic activity of the samples has showed that *Populus* and *Betula* microplants have the greatest potential resistance to stressors in the variants with the use of nano-modified media. Microclones have the highest photosynthetic activity (from 0.621 to 0.650 RU) with the lowest dispersion of the indicator within one plant (0.000284 - 0.001236 RU). It indicates not only a sufficiently high resistance potential of these plants but also the stability of their condition (figure 3).

**Figure 3.** Photosynthetic activity of *Betula* and *Populus* microclone leaves, depending on the variant of culture medium.

**Figure 4.** Activity of catalase enzyme in *Betula* and *Populus* microclone leaves, depending on the variant of culture medium.
A picture of catalase enzyme activity is the same. The indicators range from 5.4 to 6.75 mg H$_2$O$_2$/min in the best variants, while they vary from 2.8 mg H$_2$O$_2$/min up to 4.2 mg H$_2$O$_2$/min in other variants (figure 4).

Indicators of photosynthetic and catalase enzyme activity in the leaves of *Populus* and *Betula* microclones, grown on a cultivation substrate with the addition of antibiotics are higher than those of control microplants, but they are inferior to plants, grown on a cultivation substrate with the addition of nanoparticles (figures 3, 4).

4. Conclusions
According to the results of histological, physiological and biochemical studies of leaf blades of *Populus* (*Populus alba* L. × *Populus tremula* L.) and *Betula* (*Betula pubescens* Ehrh.) microclones it has been established that *Populus* microplants grown on a cultivation substrate with the addition of titanium dioxide nanoparticles have the greatest potential resistance to the effects of stressors. The cultivation substrate with addition of zinc oxide nanoparticles is more efficient for *Betula* microclones. According to leaf histological characteristics of the above-mentioned plants (width of stomatal pore, the area of stomata and their density), their high potential resistance to pathogens can be predicted and high rates of photosynthetic and enzyme activities of leaf catalase indicate potential resistance to abiotic stressors.

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