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# Features of Triploid Aspen Clonal Micropropagation Using Modern Growth-Stimulating Preparations

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***Abstract.*** The creation of fast-growing triploid aspen plantations in the area of wood processing enterprises has high importance in terms of increasing demand for deciduous wood associated with the development of board production and the prospects for the introduction of innovative technologies for deep processing of wood. The article presents the results of studies of triploid aspen clones of Kostroma origin at the stages of "introduction to *in vitro* culture", "proper micropropagation", and "rooting of microshoots" with the use of growth-regulating substances. At the "introduction culture to *in vitro*" stage, the most effective sterilizing agents were silver nitrate with the concentration of 0.2 %, Lysoformin 3000, 5 % applied for 15 min and sulema, 0.2 % used for 10 min. At the "proper micropropagation" stage, the total length of triploid aspen shoots did not differ significantly depending on the composition of the nutrient media studied but was slightly exceeded the options with Woody Plant Medium. An increase by 1.2–2.6 times was observed in the total length of triploid aspen microshoots *in vitro*, when the concentration of 6-benzylaminopurine in the nutrient medium was increased from 0.5 to 1.0 mg/L. The maximum value of the total length (5.6 cm) of tryploid aspen microshoots *in vitro* was observed on Woody Plant Medium at the 6-benzylaminopurine concentration of 1.0 mg/L and the presence of Epin-Extra preparation of 0.5 mg/L. At the "rooting of microshoots" stage the number, average, and total length of triploid aspen roots *in vitro* had no statistically significant differences depending on the nutrient medium composition. Increasing the concentrations of indole-3-butyric acid and indole-3-acetic acid from 1.0 to

2.0 mg/L in the nutrient medium increased the total length of regenerated plant roots of triploid aspen *in vitro* by 2.3–2.4 times. The maximum total length (5.1 cm) of triploid aspen roots *in vitro* was observed on Woody Plant Medium with indole-3-butyric acid at a concentration of 1.0 mg/L. Clonal micropropagation appears to be a perspective for accelerated production of elite planting material of triploid aspen for the purposes of laying out timber plantations.
***Keywords:*** triploid aspen, clone, forest plantations, clonal micropropagation, *in vitro*, organogenesis, rhizogenesis, growth-regulating substances

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Научная статья

# Особенности клонального микроразмножения триплоидной осины с применением ростостимулирующих препаратов

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***Аннотация.*** В условиях возрастающего спроса на древесину лиственных пород в связи с развитием плитного производства и перспективами внедрения инновационных технологий глубокой переработки древесины создание быстрорастущих плантаций триплоидной осины вблизи деревоперерабатывающих предприятий является актуальным. Приведены результаты исследований клонов триплоидной осины костромского происхождения на этапах введения в культуру *in vitro*, собственно микроразмножения и укоренения микропобегов с применением росторегулирующих веществ. На этапе вве-

дения в культуру *in vitro* наиболее эффективными стерилизующими агентами оказались нитрат серебра концентрацией 0,2 % и «Лизоформин 3000», 5 %, применявшиеся в течение 15 мин, а также сулема, 0,2 %, использовавшаяся в течение 10 мин. На этапе собственно микроразмножения суммарная длина побегов триплоидной осины не имела существенных различий в зависимости от состава исследуемых питательных сред, однако была незначительно больше в вариантах с питательной средой WPM. При повышении в питательной среде концентрации цитокинина 6-БАП от 0,5 до 1,0 мг/л наблюдалось увеличение суммарной длины микропобегов триплоидной осины *in vitro* в 1,2–2,6 раза. Максимальная суммарная длина микропобегов (5,6 см) триплоидной осины *in vitro* отмечена на питательной среде WPM при концентрации цитокинина 6-БАП 1,0 мг/л и наличии препарата «Эпин-Экстра» в концентрации 0,5 мг/л. На этапе укоренения микропобегов количество, средняя и суммарная длина корней триплоидной осины *in vitro* не имели статистически значимых различий в зависимости от состава питательной среды. Повышение в питательной среде концентраций ИМК и ИУК от 1,0 до 2,0 мг/л способствовало увеличению суммарной длины корней растений-регенерантов триплоидной осины *in vitro* в 2,3–2,4 раза. Максимальная суммарная длина корней (5,1 см) триплоидной осины *in vitro* отмечена на питательной среде WPM с ауксином ИМК в концентрации 1,0 мг/л. Использование клонального микроразмножения перспективно для ускоренного получения элитного посадочного материала триплоидной осины в целях закладки лесосырьевых плантаций.

***Ключевые слова:*** триплоидная осина, клон, лесные плантации, клональное микроразмножение, *in vitro*, органогенез, ризогенез, росторегулирующие вещества

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## *Introduction*

In recent decades, global forestry has seen a steady trend of transition from traditional forest practice towards plantation cultivation of wood with a short rotation cycle and the use of modern biotechnology [9]. About 1 million hectares of plantation crops are created annually in the world for producing pulpwood, sawlogs, and fuel wood. Forest plantations provide up to 17 % of the world wood's consumption [19]. The creation of target forest plantations and the transition to the arrangement of sustainable forest management will allow wood deep processing enterprises to solve the problem of bringing raw materials closer to production, reduce the cost of creating forest infrastructure, and ensure their further development. Large-scale development of forest plantations for cultivation of fast-growing wood species with target wood is necessary for the practical combination of forestry intensification and forest management. It will eliminate the lack of small-sized wood raw materials for the development of pulp, board and biofuel industries [12]. Plantation forest growing enables to accelerate by 1.5–3 times the production of target wood compared to the traditional method [4]. The practice of the developed countries (Germany, Finland, Canada, Italy, etc.) shows the wide possibilities for rapid industrial cultivation of fast-growing plantation stands for processing industry and fuel-energy purposes.

In Russia, aspen (*Populus tremula* L.), one of the fastest-growing and fastest-ripening wood species, is among the promising species for plantation cultivation that

are a source of raw materials and biofuels. Its wood is used in the pulp and paper industry, the construction, the production of wood panels and many other areas [13]. Modern technologies of deep wood processing open new directions for usage of aspen wood: wood biofuel production, environmentally friendly pressed and composite materials, nanocellulose, raw materials for the pharmaceutical, the food, and the fragrance industries, etc. However, the widespread use of aspen is hindered by the massive damage to trees by stem rot caused by the false aspen tinder fungus (*Phellinus tremulae* (Bond.) Bond. et. Boriss.). According to Academician A.S. Yablokov, 70–90 % of aspen stands are infected with stem rot [24]. The average infestation of aspen allotments in the Kostroma region at the age of 41–50 years is 30 % with an 80 % occurrence of affected allotments; and 54 % with an 85 %, respectively, at the age of 51–60 years [22].

The Kostroma region is unique in the productivity of triploid forms of aspen (*Populus tremula* gigas), first selected in Russia in the Sharya forest district by Academician A.S. Yablokov, who proved their ploidy. Later, works on aspen breeding for productivity and resistance to rot diseases with the selection and reproduction of promising forms for target cultivation in several regions of Central Russia were carried out for 40 years [7, 23, 24]. Triploid aspen is called Giant aspen for its fast growth and high productivity. Triploid clone No. 27 in the genetic reserve in the Sharya district of the Kostroma region had a stock of 340 $m^3$/ha by the age of 25 years, which was 2 times higher than the stock of ordinary aspen [6]. At the same age, test crops planted from root shoots of clone No. 27 in the Ivanteevka Forest Tree Nursery (Moscow region) had a stock of more than 300 $m^3$/ha and were suitable for pulpwood harvesting, whereas the common diploid aspen (clone No. 29) gave a stock of 100 $m^3$/ha in similar conditions [25]. At the age of 52 years, the stock of aspen giant clone No. 27 reached 500 $m^3$/ha, exceeding the stock of diploid clone No. 29 by more than 30 % [2]. At the same time, triploid aspen retains resistance to stem rot.

The importance of preservation and reproduction of a valuable aspen gene pool is caused by the unique silvicultural qualities of both triploid and fast-growing diploid aspen clones selected in the Sharya forest district in terms of fast growth, resistance to rots, and high quality wood. Plantation cultivation of elite aspen clones can be realized on the base of the genetic reserve. This acquires great importance due to the increasing demand for hardwood in connection with the development of board production and the prospects of introducing innovative technologies for deep mechanical, chemical and energy processing of wood. The creation of fast-growing aspen plantations is especially relevant in the area of modern forest sector enterprises, which use timber from soft-wooded broadleaved species.

Currently, plantations of triploid aspen are laid out in the following regions of Russia: the Leningrad region, the Voronezh region, the Moscow region, the Republic of Mari El, and the Republic of Tatarstan. The Saint Petersburg Forestry Research Institute (SPBNIILH) is the most experienced in plantation cultivation of highly productive aspen. In several forestries in the Leningrad region, aspen plantations were planted using experimental planting material grown by the clonal micropropagation method [20, 26]. Felling age of aspen plantations is 30 years; productivity is up to 400 $m^3$/ha [5]. The Research Institute of Forest Genetics, Breeding, and Biotechnology (VNIILGISbioTech) carried out experiments on

plantation cultivation of hardwood for 30 years that resulted in aspen yield with an average trunk volume of 1.1–1.8 $m^3$. The average wood stock was 720 $m^3$/ha; the basic wood density was 395 kg/$m^3$ [19]. There is an opinion that wood stock of about 100 $m^3$/ha is achieved in triploid aspen plantations within 15 years [12]. The same authors suggest that in North-West Russia triploid aspen plantations at the felling age of 20–30 years in optimal forest site conditions will produce a target wood stock of 150–300 $m^3$/ha. The experience of Sweden and Finland has shown that using the plantation method of triploid aspen cultivation it is possible to produce healthy wood for pulpwood production as early as 12–14 years after planting [13].

It is necessary to provide accelerated production of elite planting material on the industrial basis in order to lay out timber plantations of triploid aspen as a producer of raw material and biofuel. For the purposes of plantation cultivation, it is advisable to use modern methods of biotechnology, such as clonal micropropagation, which allows to produce a large amount of high-quality, healthy planting material, including species poorly propagated by the traditional methods, in a short time throughout a year [21]. So far, studies on the introduction of triploid forms of aspen to *in vitro* culture have been carried out by a number of Russian scientists from different regions of the country [1, 8, 10, 14, 17, 28]. Since 2010, studies have been carried out at the Central European Forest Experimental Station of the All-Russian Research Institute for Silviculture and Mechanization of Forestry (ARRISFM) [3, 16, 27]. There is a need to improve the technology of triploid aspen clonal micropropagation with the use of modern sterilizing agents and growth-stimulating preparations.

The research is aimed at studying the features of clonal micropropagation of triploid aspen clones of Kostroma origin at the stages of "introduction culture to *in vitro*", "proper micropropagation", and "rooting of microshoots" with the use of modern sterilizing and growth-regulating substances.

## *Research objects and methods*

The research was carried out at the Laboratory of Clonal Micropropagation of the Central European Forest Experimental Station of ARRISFM in 2019–2022 according to the generally accepted methods [11, 21]. We used explants obtained from the apical meristems of plants of triploid aspen clone No. 27 selected from the genetic reserve in the Sharya district of the Kostroma region as the study objects.

The following solutions were used as basic sterilizing agents at the stage of "introduction culture to *in vitro*": sulema, 0.1 %; detergent "Domestos", 1:3; hydrogen peroxide, 30 %, chlorinated lime, 1:1; silver nitrate, 0.2 %; preparations of eco-sterilizer chlorine free, 5 %, and Lysoformin 3000, 5 %. Sterilization time: 5, 10, 15, and 20 min. The viability of explants was recorded as the ratio of the number of survivors to the total number of those introduced to the culture. The plants were cultivated in the hormone-free (control) nutrient medium according to Murashige and Soog (MS) [18] and Woody Plant Medium (WPM) [15], including the options with a 2-fold dilution of the mineral base, in a light room at 23–25 °C, 75–80 % humidity and a 16/8 h photoperiod.

At the stage of "proper micropropagation" 6-benzylaminopurine (6-BAP) at concentrations of 0.5 and 1.0 mg/L and Epin-Extra preparation at a concentration

of 0.5 mg/L were used as cytokinin growth regulator in the nutrient medium. At the stage of "rooting of microshoots" Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA) were used as auxins at concentrations of 0.5 and 1.0 mg/L. The number, average and total length of microshoots (on the 30th day) and roots (on the 45th day) per a regenerated plant were recorded. Experiments were carried out in 10-fold biological and 2-fold analytical replications. Fifteen test-tube plants were recorded in each option.

Experimental data were statistically processed using AGROS v.2.11 software and Microsoft Office Excel 2016. A two-way analysis of variance was applied, where factor A – nutrient medium; factor B – concentrations of growth-regulating substance. Reliability of differences between the average data from the experimental options was estimated using the least significant difference for the 5 % level of significance ($LSD_{05}$).

*Research results and discussion*

The studies revealed that at the stage of "introduction culture to *in vitro*" of triploid aspen explants the most effective basic sterilizers were silver nitrate (0.2 %) and Lysoformin 3000 (5 %) with sterilization time of 15 min, as well as in case of using sulema for 10 min. In these options, the viability of explants reached 80–82 %. With the increase in sterilization time for sulema treatment to 15 and 20 min, explant viability decreased sharply to 42 and 18 %, respectively, which appears to be related to the mercuric chloride phytotoxicity. At the sterilization time of 5 min, the percentage of viable explants, when treated with the studied sterilizing agents, was low and did not exceed 2–20 %; the remaining explants died of infection (table 1).

Table 1

**The viability of triploid aspen (clone No. 27) explants depending on sterilizing agents and sterilization time, %**

| Sterilizing agent | Sterilization time, min | | | |
|---|---|---|---|---|
| | 5 | 10 | 15 | 20 |
| Hydrogen peroxide, 30 % | 18 | 28 | 76 | 24 |
| Sulema, 0.2 % | 2 | 80 | 42 | 18 |
| Chlorinated lime, 1:1 | 10 | 46 | 50 | 20 |
| Silver nitrate, 0.2 % | 14 | 42 | 80 | 42 |
| Eco-sterilizer chlorine free, 5 % | 20 | 24 | 42 | 50 |
| Lysoformin 3000, 5 % | 12 | 40 | 82 | 32 |

At the stage of "proper micropropagation", there were no significant differences in the number of shoots among triploid aspen regenerated plants depending on the nutrient medium composition; 1.8–2.3 pcs on average. The number of shoots of regenerated plants increased on average by 1.3–1.9 times with the 6-BAP concentration increase from 0.5 to 1.0 mg/L. The highest number of triploid aspen microshoots was observed at the 6-BAP concentration of 1.0 mg/L, and when Epin-Extra preparation was added at the concentration of 0.5 ml/L. It was 3.0 pcs on average, while on WPM it reached the maximum of 3.8 pcs (table 2).

Table 2

**The number of triploid aspen (clone No. 27) microshoots *in vitro* depending on the nutrient medium composition and the concentration of growth-regulating substances, pcs**

| Nutrient medium | Concentration of 6-BAP, mg/L | | | | Average |
|---|---|---|---|---|---|
| | Without preparation | | With addition of Epin-Extra, 0.5 ml/L | | |
| | 0.5 | 1.0 | 0.5 | 1.0 | |
| Control | 0.6 | 1.2 | 0.9 | 1.8 | 1.1 |
| MS | 1.1 | 1.9 | 1.4 | 2.9 | 1.8 |
| MS 1/2 | 1.5 | 1.9 | 1.5 | 3.0 | 2.0 |
| WPM | 1.6 | 2.0 | 1.9 | 3.8 | 2.3 |
| WPM 1/2 | 1.4 | 1.5 | 1.8 | 3.4 | 2.0 |
| Average | 1.2 | 1.7 | 1.5 | 3.0 | – |

Note: $LSD_{05}$ factor A = 1.32, factor B = 1.21, general = 1.80.

The average length of triploid aspen shoots had no statistically significant differences depending on the nutrient medium composition and ranged from 1.8 to 2.3 cm. The average length of triploid aspen shoots slightly increased by 1.3 times, with the 6-BAP concentration increase from 0.5 to 1.0 mg/L, and addition of Epin-Extra preparation, while in options without the preparation it was 1.7 cm at similar concentrations (table 3).

Table 3

**The average length of triploid aspen (clone No. 27) microshoots *in vitro* depending on the nutrient medium composition and the concentration of growth-regulating substances, cm**

| Nutrient medium | Concentration of 6-BAP, mg/L | | | | Average |
|---|---|---|---|---|---|
| | Without preparation | | With addition of Epin-Extra, 0.5 ml/L | | |
| | 0.5 | 1.0 | 0.5 | 1.0 | |
| Control | 1.0 | 1.2 | 1.5 | 1.9 | 1.4 |
| MS | 1.5 | 1.4 | 1.8 | 2.5 | 1.8 |
| MS 1/2 | 1.7 | 1.6 | 1.9 | 2.6 | 1.9 |
| WPM | 2.0 | 1.8 | 2.2 | 3.0 | 2.3 |
| WPM 1/2 | 1.7 | 1.9 | 2.4 | 2.8 | 2.2 |
| Average | 1.6 | 1.6 | 2.0 | 2.6 | – |

Note: $LSD_{05}$ factor A = 1.87, factor B = 1.59, general = 1.92.

The total length of triploid aspen shoots was the longest (on average 5.6 cm) in the options with WPM, and only 3.5–4.8 cm in other options; although the differences were not statistically significant. The 6-BAP concentration increase in the nutrient medium from 0.5 to 1.0 mg/L and the addition of Epin-Extra preparation to the nutrient medium resulted in a considerable increase in the total length of triploid aspen shoots on average by 2.3 times, and in options without the preparation – by 1.8 times. The total length of triploid aspen shoots reached the maximum value (11.4 cm) on WPM at the 6-BAP concentration of 1.0 mg/L and presence of Epin-Extra preparation of 0.5 mg/L (table 4).

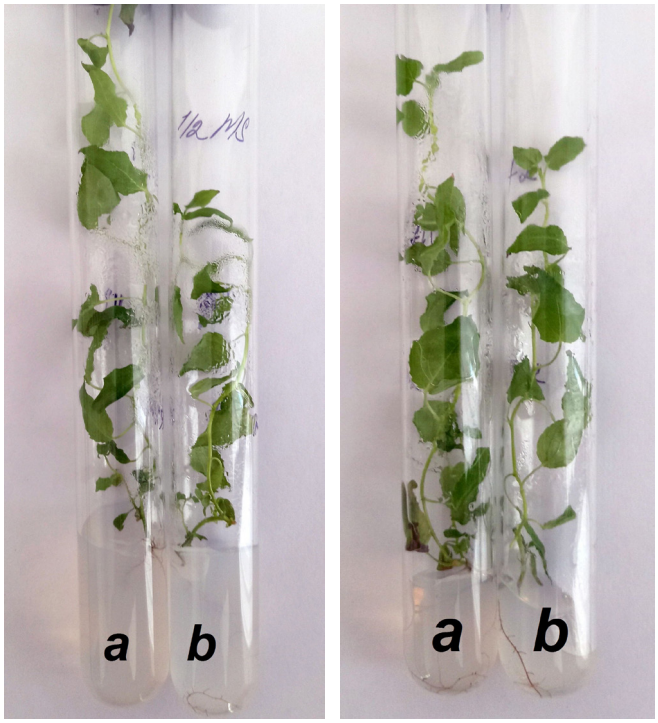
Table 4

**The total length of triploid aspen (clone No. 27) microshoots *in vitro* depending on the nutrient medium composition and the concentration of growth-regulating substances, cm**

| Nutrient medium | Concentration of 6-BAP, mg/L | | | | |
|---|---|---|---|---|---|
| | Without preparation | | With addition of Epin-Extra, 0.5 ml/L | | Average |
| | 0.5 | 1.0 | 0.5 | 1.0 | |
| Control | 0.7 | 1.5 | 1.4 | 3.4 | 4.5 |
| MS | 1.6 | 2.7 | 2.5 | 7.3 | 3.5 |
| MS 1/2 | 2.6 | 3.0 | 2.8 | 7.8 | 4.1 |
| WPM | 3.2 | 3.6 | 4.2 | 11.4 | 5.6 |
| WPM 1/2 | 2.4 | 2.9 | 4.3 | 9.5 | 4.8 |
| Average | 1.9 | 2.7 | 3.0 | 7.9 | – |

Note: $LSD_{05}$ factor A = 1.67, factor B = 2.01, general = 1.96.

At the stage of "rooting of microshoots *in vitro*" (fig.) no significant differences were revealed in the number of roots of triploid aspen regenerated plants depending on the nutrient medium composition; it was 1.8–2.5 pcs.




*1* *2*

Triploid aspen regenerated plants *in vitro* on MS (*1*) and WPM (*2*): *a* – complete medium; *b* – 2-fold mineral dilution medium

Number of roots per triploid aspen plant increased on average by 1.5 times with the IBA concentration increase from 1.0 to 2.0 mg/L in the nutrient medium, and by 1.8 times in the options with IAA (table 5).

Table 5

**The number of triploid aspen (clone No. 27) roots *in vitro* depending on the nutrient medium composition and the concentration of auxins, pcs**

| Nutrient medium | Concentration of auxin, mg/L | | | | |
|---|---|---|---|---|---|
| | IBA | | IAA | | Average |
| | 0.5 | 1.0 | 0.5 | 1.0 | |
| Control | 1.0 | 2.1 | 1.1 | 1.6 | 1.5 |
| MS | 1.9 | 3.3 | 1.5 | 2.2 | 2.2 |
| MS 1/2 | 2.3 | 3.2 | 1.3 | 2.5 | 2.3 |
| WPM | 2.7 | 3.4 | 1.3 | 2.4 | 2.5 |
| WPM 1/2 | 1.3 | 3.0 | 1.0 | 2.0 | 1.8 |
| Average | 1.8 | 3.0 | 1.2 | 2.1 | – |

Note: $LSD_{05}$ factor A = 1.70, factor B = 1.54, general = 1.63.

The average length of triploid aspen roots had no statistically significant differences depending on the nutrient medium composition and was 1.0–1.3 cm. Concentrations of IBA and IAA also had no effect on the average length of triploid aspen roots (table 6).

Table 6

**The average length of triploid aspen (clone No. 27) roots *in vitro* depending on the nutrient medium composition and the concentration of auxins, cm**

| Nutrient medium | Concentration of auxin, mg/L | | | | |
|---|---|---|---|---|---|
| | IBA | | IAA | | Average |
| | 0.5 | 1.0 | 0.5 | 1.0 | |
| Control | 0.5 | 0.9 | 0.6 | 0.8 | 0.7 |
| MS | 0.9 | 1.2 | 1.0 | 1.0 | 1.0 |
| MS 1/2 | 0.8 | 1.3 | 1.2 | 1.3 | 1.2 |
| WPM | 1.0 | 1.5 | 1.0 | 1.5 | 1.3 |
| WPM 1/2 | 1.0 | 1.5 | 1.0 | 1.5 | 1.3 |
| Average | 0.8 | 1.3 | 1.0 | 1.2 | – |

Note: $LSD_{05}$ factor A = 0.68 , factor B = 0.76, general = 0.90.

The total length of triploid aspen roots had no statistically significant differences depending on the nutrient medium composition and ranged on average from 2.4 to 3.2 cm. The total length of triploid aspen roots *in vitro* increased by 2.4 times with the increase in IBA concentration from 0.5 to 1.0 mg/L, and by 2.3 times in case of IAA. Herewith, the maximum total length of triploid aspen roots (5.1 cm) was observed in the option with WPM and the IBA concentration of 1.0 mg/L (table 7).

Table 7

**The total length of triploid aspen (clone No. 27) roots *in vitro* depending on the composition nutrient medium and the concentration of auxins, cm**

| Nutrient medium | Concentration of auxin, mg/L | | | | |
|---|---|---|---|---|---|
| | IBA | | IAA | | Average |
| | 0.5 | 1.0 | 0.5 | 1.0 | |
| Control | 0.5 | 1.9 | 0.7 | 1.3 | 1.1 |
| MS | 1.7 | 4.0 | 1.5 | 2.2 | 2.4 |
| MS 1/2 | 1.8 | 4.2 | 1.3 | 3.3 | 2.7 |
| WPM | 2.7 | 5.1 | 1.3 | 3.6 | 3.2 |
| WPM 1/2 | 1.3 | 4.5 | 1.0 | 3.0 | 2.5 |
| Average | 1.6 | 3.9 | 1.2 | 2.7 | – |

Note: $LSD_{05}$ factor A = 1.53, factor B = 1.42, general = 1.60.

The results of previous experiments on replanting of adapted plants of triploid aspen with closed root system obtained by the *in vitro* method (without addition of growth-stimulating preparations) to the open ground [4] suggest the prospects of using the clonal micropropropagation method with the addition of modern growth-stimulating preparations for accelerated production of planting material of triploid aspen forms for further plantation laying out.

## *Conclusions*

1. Silver nitrate, 0.2 %, and Lysoformin 3000, 5 %, with sterilization time of 15 min, as well as sulema, 0.2 % with, sterilization time of 10 min, proved to be the most effective sterilizing agents at the stage of "introduction to *in vitro* culture" during clonal micropropropagation of triploid aspen.

2. The total length of triploid aspen shoots showed no significant differences depending on the composition of the nutrient media studied, while being slightly longer in the options with WPM.

3. Increasing the 6-BAP concentration in the nutrient medium from 0.5 to 1.0 mg/L promoted a significant increase in the total length of triploid aspen microshoots *in vitro* when Epin-Extra preparation at the concentration of 0.5 mg/L was added to the nutrient medium.

4. The total length of triploid aspen microshoots *in vitro* reached its maximum value on WPM at the 6-BAP concentration of 1.0 mg/l and in the presence of Epin-Extra preparation of 0.5 mg/L.

5. The number, average, and total length of triploid aspen roots *in vitro* had no statistically significant differences depending on the composition of the nutrient medium studied.

6. The total length of regenerated plants' roots increased significantly during clonal micropropagation of triploid aspen *in vitro,* with the IBA and IAA concentrations increasing from 1.0 to 2.0 mg/L in the nutrient medium.

7. The maximum total length of triploid aspen roots *in vitro* was observed in the option with WPM and the IBA concentration of 1.0 mg/L.

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