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БАЯНДАМАЛАРЫ

ДОКЛАДЫ

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РЕСПУБЛИКИ КАЗАХСТАН

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R. A. Turganova[1,2], E. D. Djangalina[1,2], E. A. Shadenova[1], A. I. Kapytina[1], G. K. Kamshybayeva[1,2]

[1]Institute of General Genetics and Cytology, Almaty, Kazakhstan;
[2]Al-Farabi Kazakh National University, Almaty, Kazakhstan.
E-mail: ranaexotic97@mail.ru

FEATURES OF THE INTRODUCTION INTO IN VITRO CULTURE AND MICROPROPAGATION OF PAULOWNIA TOMENTOSA

Abstract. *Paulownia sp.* are tall and fast-growing perennial plants that grow faster than all woody plants in the world. In many countries, *Paulownia sp.* are used as a raw material in bioenergy, furniture industry, landscape gardening and technologies for phytoremediation. In this study for the first time in Kazakhstan, conditions of *Paulownia tomentosa (P. tomentosa) in vitro* cultivation and propagation have been optimized, also the factors influencing the morphogenetic activity of primary explants have been studied. Along with the adaptation potential of *Paulownia tomentosa* microclones to the *ex situ* conditions, laboratory standing order for microclonal reproduction have been evaluated.

For sterilization of *P. tomentosa* explants are recommended to use 50% domestos and 0.1% merthiolate. Hormone-free WPM medium was considered as the most optimal for the *in vitro* propagation. Infrared light is highly recommended for *P. tomentosa* microclones adaptation, due to its ability to stimulate the formation plants aboveground biomass and root system.

For Kazakhstan, the research of this type of tree crops is a relevant, new and promising direction. The development of microclonal propagation of *Paulownia tomentosa* will accelerate the process of introduction of Paulownia in our Republic.

Keywords: *Paulownia tomentosa*, sterilization, micropropagation, nutrient medium, clones, ex situ adaptation, infrared light.

Introduction. Paulownia (Adam's tree) is a perennial (height up to 15-20 meters) and fast-growing deciduous plant with large leaves (20-50 cm) and attractive scented inflorescences, the plant belongs to the *Scrophulariaceae* family. *Paulownia sp.* growth rate surpasses the woody plants all over the world. The annual growth rate is more than 1.5 m [1, 2].

Such countries as China, Japan, Brazil, Europe, and the United States distribute Paulownia as a valuable garden-park crop. In addition, Paulownia is used as a raw material in bioenergy, as an effective way to mitigate the climate changes, as a good glucose feed in livestock racing and even as a phytoagent in contaminated soil phytoremediation process [3–5].

Current economic, social, and ecological conditions are crucially important for the rare or endangered plants preservation and biodiversity problems, initiated by the habitats loss and anthropogenic activities. Nowadays, the plant micropropagation technique based on the cell and tissue culture methods has been evolved in a wide commercial application [6].

Major biotechnology companies apply the method not only for mass production of planting material but in the breeding process, obtaining new hybrids and cultivars over a much shorter period. The clonal micropropagation allows getting healthy, virus-free planting material, whereas, the multiplication factor rate reaches 10^3-10^7 plants per year within a short time. It is several thousand times more compared to the traditional method of vegetative propagation [7, 8] . Moreover, plantation production is relevant within the context in view of increasing demands for commercial wood, and population of genetically aligned trees can be accessed through clonal micropropagation. Thus, accurate prediction of the plantations development dynamics can become real [9, 10].

The high demand for planting material in the domestic and international markets for landscape gardening and bioenergy led to the building effective ways of micropropagation of *Paulownia sp.* for the rapid and mass distribution. Over the past several decades, numerous laboratories researched the *Paulownia sp*. aiming to expand the technology for microclonal reproduction, study the organogenesis processes, and evolve the protocols for genetic transformation [11–14].

Furthermore, for Kazakhstan, the study of the Paulownia species is a relevant and promising direction. Currently, in the Southern regions of Kazakhstan researches on the *Paulownia tomentosa* (*P. tomentosa*) introduction has begun. In 2019, about 37,000 *P. tomentosa* seedlings were successfully planted in the Saryagash district of the South Kazakhstan region. Nowadays, pilot projects are being constructed to adapt *P. tomentosa* to the climatic conditions of the Mangistau region, by the request of the International Center for Green Technologies and Investment Projects.

Considering the economic importance, as well as the current and potential future of the using *Paulownia sp.,* the development of biotechnological approaches to the crop propagation are based on the application of micropropagation technology, which will help to accelerate the Paulownia introduction in our Republic.

Research goal. Studying the features and optimization conditions of microclonal reproduction of *P. tomentosa* in order to obtain high-quality planting material.

Materials and methods. *Explants sterilization*. Stem segments 2-3 cm long with one axillary bud were used as explants for introduction into *in vitro* culture. At the first step of sterilization, the segments were kept in a soapy solution (7 min), then washed three times with distilled water (2 min). Further work was carried out in a laminar box by treating explants with different sterilizing agents such as Whiteness, Merthiolate, and Domestos in various concentrations. Sterilization options are shown in Table 1. After sterilization, the explants were washed three times in the sterile distilled water (5 min), placed on a sterile filter paper, and planted on a nutrient media.

Micropropagation. For the cultivating of the *P. tomentosa* explants, were used Variety of the nutrient media with different combinations and concentrations of phytohormones (benzylaminopurine [BAP], indolyl-3-butyric acid [IBA], indole-3-acetic acid [IAA], 2,4-dichlorophenoxyacetic acid [2,4-D], and gibberellic acid [GA]) were used for the *P. tomentosa* explants cultivation. However, to induce the morphogenesis, explants were cultivated in the WPM medium without growth regulators for 28 days. The obtained shoots were separated from the primary maternal explant and independently cultivated again to the freshly prepared medium during the 4 passages.

The shoots were developed from buds within 2-4 weeks and separated from the explant after 4 weeks of cultivation.

Among the shoots, the largest ones were selected, with a well-developed leaf blade with a length of 1.5 – 2.5 cm. Shoots were divided into single-node segments which were subcultured in fresh WPM medium without growth regulators for 10-12 days for the root formation.

The selected explants were cultured under 16-hours photoperiod, fluorescent illumination, and at the temperature of 22-24°C. The explants viability, growth and development were assessed weekly. The experiments were carried out in 3-4 replications. One replication contained 60 explants.

Microshoots adaptation to ex situ conditions. Adjustment of microshoots to *ex situ* conditions was carried out by placing rooted 10-14 days shoots in containers with universal soil, where peat was a dominant component. Rooted shoots were cultivated under 24-26°C and high humidity conditions. Planting was carried out by placing one plant per container (250 g). Before planting into the soil regenerated plants were 2-3 cm in height with intensely colorful leaves and developed root system. Therefore, to successful adaptation in open ground conditions, regenerated plants with at least 1 cm root length were selected.

Regenerated root clones with a small amount of nutrient medium were planted in the containers filled by moist soil mixture, then closed on top via plastic caps. White fluorescent lamps WL-40 (white light) and fluorescent lamps FL-40-4 (predominance of radiation in the red spectral region) were used to investigate the illumination conditions impact to the adaptation of regenerated plants. The white lamps illumination intensity was 6000 lux, infrared - 3000 lux. To produce a greenhouse effect, some of the plants were grown 7 days under infrared illumination, and then after visual checking their viability plants were transferred to illumination with the former light. The rest of the plants were grown only under white

light. Monitoring of the plant adaptation to open ground conditions was carried out weekly. The survival rate was assessed according to the following parameters: plant height, leaves quantity, leaf length and width. The experiment was carried out in three replications. In a number of works had been shown that lighting conditions were of great importance for the adaptation of tree and shrub species [15].

Watering of the regenerants was carried out as the soil dries up. Spraying of vegetative mass was carried out in the morning and in the evening. Depending on the survival rate of the clones, the plastic caps were removed after 7-14 days.

Results and discussion. *Object sterilization.* The sterilizing agents selection for introduction of the explants under *in vitro* conditions is one of the most difficult steps in micropropagation. At this stage, it is necessary to select both the concentration of sterilizing substances and the exposure time in the sterilizing solution.

Merthiolate at the concentrations of 0.1%, 1%, 15%, 20%; Whiteness and Domestos were used as sterilizing agents. Domestos was used in two concentrations: undiluted solution (100%) and diluted 1:2 (50%), Whiteness - only undiluted. The exposure time for all sterilizing agents was 5-15 minutes. There was found that the explants greatest survival rate (100%) was observed under 0.1% merthiolate for 5 and 15 minutes, and 50% -100% domestos for 5 minutes. At the same time, the most well-developed shoots were formed after explants sterilization with 0.1% merthiolate for 5 minutes. However, increasing the merthiolate concentration up to 1% sharply reduced the number of surviving explants to 20%. An increase in the exposure time up to 10 and 15 minutes with both 50% and 100% domestos reduced the survival rate of explants by 80%, 20% and 86%, 40%, respectively (table 1).

Thus, the investigation of *P. tomentosa* sterilization showed that the most suitable sterilizing agents for introduction of *P. tomentosa* into *in vitro* culture are 0.1% merthiolate, 50% and 100% domestos. This study is an important stage to research the possibility of introduction into *in vitro* the culture and technology development for micropropagation of *P. tomentosa*.

Table 1 - The explants survival rates under different sterilizing agents, concentrations and time exposure

Sterilizing agent	Concentration, %	Time exposition, min.	Total number of explants, pcs.	Number of infected explants, pcs.	Number of sterile regenerated explants, pcs.	Survival rates of explants, %
Merthiolate	0,1	5	60	0	60	100
		10	60	10	50	83
		15	60	0	60	100
	1	5	60	0	12	20
		10	60	0	15	25
		15	60	0	0	-
	15	5	60	15	0	-
		10	60	5	0	-
		15	60	6	0	-
	20	5	60	5	0	-
		10	60	6	0	-
		15	60	6	0	-
Domestos	50	5	60	0	60	100
		10	60	5	48	80
		15	60	1	13	20
	100	5	60	0	60	100
		10	60	4	52	86
		15	60	0	24	40
Whiteness	100	5	60	9	20	33
		10	60	7	10	16
		15	60	30	-	-

Optimization of micropropagation conditions for P. tomentosa. Woody Plant Medium (WPM) and Murashige Skoog (MS) were used for the explants cultivation. Seven different media with different phytohormones combinations and concentrations were studied aiming the optimization of the hormonal composition: benzylaminopurine (BAP), indolylbutyric acid (IBA), indoleacetic acid (IAA), a-naphthylacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and gibberellic acid (GA).

Also, in the experiment various explants isolated from mother tree were used. It was determined that the leaves segments had a high ability to callusogenesis. The appearance of a dense green callus was noted on all tested media variants containing phytohormones, on the 18th day of cultivation.

For inducing organogenesis, dense callus was passaged on a medium for regeneration with the addition of 0.1 mg L^{-1} BAP, 0.2 mg L^{-1} NAA, and 0.1 mg L^{-1} GA. However, the callus cultivation during one month did not give the positive results, the callus grew without visible signs of differentiation, also necrosis was observed.

More successful *in vitro* induction of morphogenesis was the using of nodal shoot segments with axillary buds. Shoots regrowth and the additional buds' establishment in the explants were observed after 3 weeks of cultivation on inducing medium with 0.1 mg L^{-1} BAP and 0.4 mg L^{-1} IAA.

The axillary buds' morphogenetic activity was researched cultivating them on WPM and MS medium without growth regulators for 28 days. There was found that active bud growth and shoot emergence were observed only on WPM medium. Nevertheless, growth processes of explants cultivated on MS medium were less intense. Additionally, after 2 weeks of the cultivation on the MS medium, necrotic processes were noted in the buds' lower part, while after 2-3 months of the cultivation on the MS medium mass death of explants was detected. Only isolated buds formed 1–2 cm long shoots, which soon turned yellow and necrotic (figure 1). The obtained shoots were separated from the primary maternal explant and independently cultivated again on a freshly prepared medium during 4 passages.

The data shows that within the *P. tomentosa* introduction into *in vitro* culture, due to the mineral composition, WPM medium is a most optimal and promotes the direct plant regeneration, thus, WPM is the most favorable for microclonal reproduction.

Subsequently, shoot development took place in the newly formed buds (within 2-4 weeks). After 4 weeks of cultivation, the grown shoots were separated from the main explant. Among the shoots, there were selected by the most complete species traits (green leaves, high growth) those ones which reached 1.5-2.5 cm in height and had a well-developed leaf blade.

It is important to point out that there was observed a formation of a powerful root system of *P. tomentosa* under *in vitro* conditions. Already on the 3rd day of cultivation, there was seen the formation of 0.5-0.6 cm long roots. During the following four weeks, the length of the roots reached about 3 cm.

Furthermore, to get high-quality healthy planting material, the shoots were divided into single-node segments and subcultured on a fresh WPM medium without growth regulators for 10-15 days for root formation.

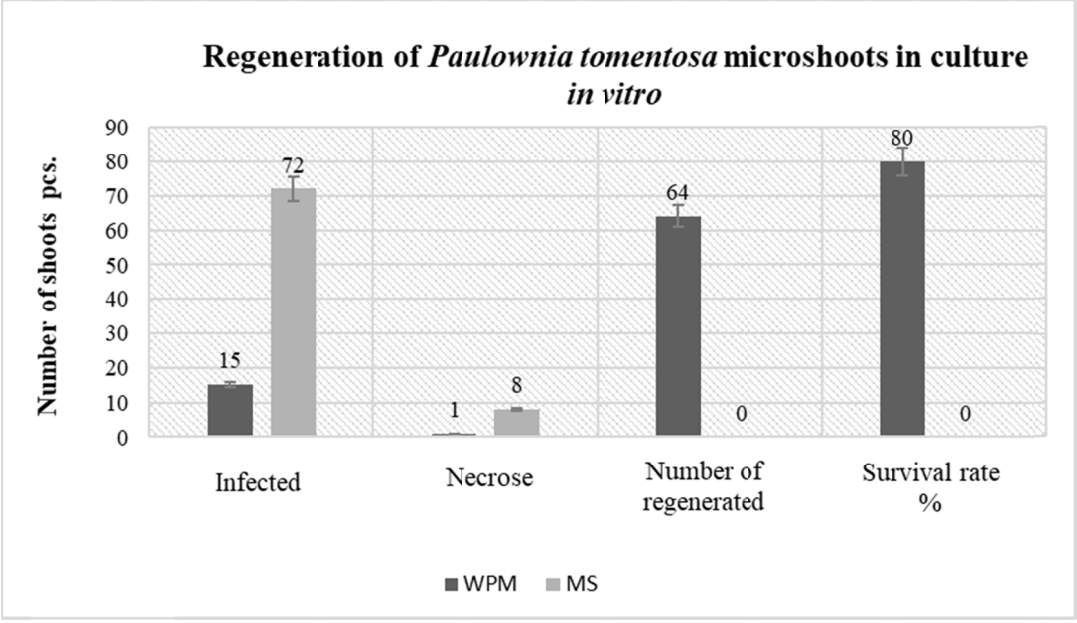

Figure 1 - Influence of the nutrient media composition
on the *P. tomentosa* microshoots regeneration in *in vitro* culture

Adaptation of P. tomentosa microclones to open ground conditions. A majority of *P. tomentosa* microplants grown under LF40-4 lamps with a predominance of radiation in the red part of the spectrum developed better (figure 2). Resulting from the observation of the growth dynamics of regenerated plants in open ground, plants grown first 7 days under infrared illumination easily adapted to non-sterile conditions and better acclimatized. It was confirmed by more intensive growth.

In addition, infrared light had a positive effect on the growth of the *P. tomentosa* aboveground biomass. Whereas, the quantity of formed leaves was slightly lower under the white light, leaves greatly varied in length and width (figure 3). The leaves length and width of plants grown under infrared light were on average twice larger (4.3 cm and 4.9 cm, respectively) (table 2).

The root length measuring after 4 weeks of *P. tomentosa* cultivation in open ground conditions demonstrated the root length increasing by 4 times. The root length before planting in open ground ranged between 3.5 cm and 3.8 cm, while after 4 weeks – between 12.5 cm and 12.8 cm.

a) b)

Figure 2 - Influence of light conditions on the *P. tomentosa* microclones growth in open ground conditions during 4 weeks
a) Infrared light; b) White light

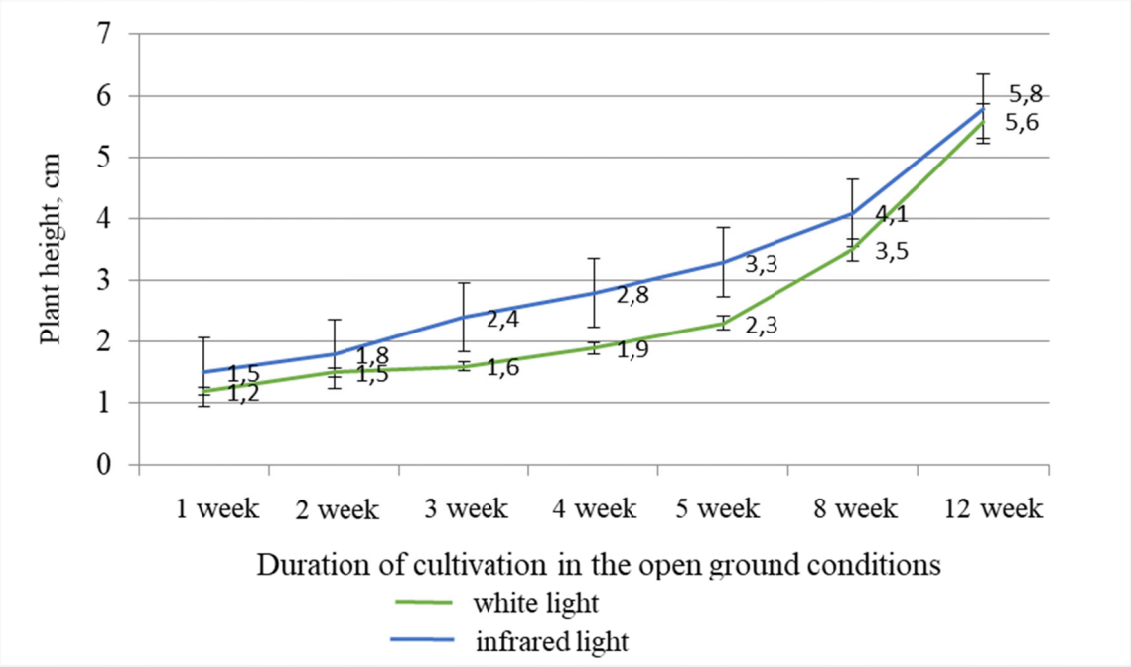

Figure 3 - Growth dynamic of *P. tomentosa* grown under different light conditions

Therefore, the *P. tomentosa* microclones adaptation to open ground conditions showed a high coefficient of plant survival. The number of surviving plants under white light was no more than 50%, and under infrared light - 92%.

Table 2 - Influence of illumination on the growth of *P. tomentosa* aboveground biomass in *ex situ* conditions

Planting time	Leaves quantity, pcs		Leaf length, cm		Leaf width, cm	
	White light	Infrared light	White light	Infrared light	White light	Infrared light
1 month	5,1 ± 1,4	6,5 ± 1,3	2,1 ± 1,1	4,3 ± 1,3	2,5 ± 1,0	4,9 ± 1,3
2 month	5,0 ± 2,2	6,2 ± 1,5	3,5 ± 1,5	5,0 ± 1,6	3,1 ± 1,5	4,9 ± 1,6

Accordingly, the conducted studies allowed us to create a protocol for *P. tomentosa* micropropagation (figure 4). Using the protocol, we can get 66 healthy, viable *P. tomentosa* microclones within a month (from 1 shoot – approximately 11-15 pcs). These microclones are adapted to the open ground conditions and can be used, for instance, in various landscaping programs.

Figure 4 - Protocol for *P. tomentosa* micropropagation

Conclusion. Throughout the study, the features of *P. tomentosa* microclonal reproduction and the most optimal conditions for its introduction and reproduction in *in vitro* culture were established. Thus, the most effective sterilizing agents for disinfection are 50% of domestos and 0.1% of merthiolate. Also, the *P. tomentosa* explants showed a high regenerative capacity in a hormone-free WPM medium. When acclimatizing *P. tomentosa* microclones to open ground conditions, the use of infrared light to stimulate the formation of the plants aboveground biomass and the root system is highly recommended. The survival rate of microclones under infrared light was 92%.

Р. А. Турганова[1,2], Э. Д. Джангалина[1,2] ,
Э. А. Шаденова[1], А. И. Капытина[1], Г. Қ. Қамшыбаева[1,2]

[1]Жалпы генетика және цитология институты, Алматы қаласы, Қазақстан
[2] Әл-Фараби атындағы қазақ ұлттық университеті, Алматы қаласы, Қазақстан

IN VITRO ДАҚЫЛЫН ЕНГІЗУ ЖӘНЕ *PAULOWNIA TOMENTOSA* ӨСІМДІГІНІҢ МИКРОКЛОНАЛДЫ КӨБЕЮІНІҢ ЗЕРТТЕУ ЕРЕКШЕЛІКТЕРІ

Аннотация. *Paulownia sp* - биік және тез өсетін көпжылдық өсімдіктер, олар әлемдегі кез келген ағаш өсімдіктеріне қарағанда тез өседі. Осыған байланысты әлемнің көптеген елдерінде *Paulownia sp* биоэнергетикада, жиһаз өндірісінде, ландшафтық көгалдандыруда және ластанған топырақты фиторемедиациялау технологиясында шикізат ретінде қолданылады.

Жұмыс *Paulownia tomentosa*-ны *in vitro* дақылдарына енгізу жағдайларын оңтайландыруға, алғашқы эксплантардың морфогенетикалық белсенділігіне әсер ететін факторларды зерттеуге және микроклондардың ашық жер жағдайларына бейімделуіне арналған. *Paulownia tomentosa* –ны экстракорпоральды дақылына

енгізу мен, көбейтудің оңтайлы шағдайлары белгіленді. Стерилизация үшін ең тиімді зарарсыздандырғыштар 5-10 минуттық экспозициясында 50% доместос және 0,1% мертиолат болып табылды. Жүргізілген зерттеулердің нәтижесінде қысқа мерзімде сау, сапалы отырғызу материалын алуға мүмкіндік беретін *Paulownia tomentosa*-ның микроклоналды көбеюіне арналған зертханалық ережелер жасалды.

Қазақстан үшін, ағаш дақылдарының осы түрін зерттеу өзекті, жаңа және перспективті бағыт болып табылады. Микроклональды көбею технологиясын қолдану негізінде, *Paulownia tomentosa* өсірунің биотехнологиялық тәсілдерін әзірлеу, біздің Республикада енгізу процесін жеделдетеді.

Түйін сөздер: *Paulownia tomentosa,* зарарсыздандыру, микроклональды көбею, қоректі орта, клондар, ex situ бейімделу, инфрақызыл жарық.

Р. А. Турганова [1,2], Э. Д. Джангалина[1,2], Э. А. Шаденова[1], А. И. Капытина[1], Г. К. Камшыбаева[1,2]

[1]Институт общей генетики и цитологии, Алматы, Казахстан
[2]Казахский национальный университет им. аль-Фараби, Алматы, Казахстан

ОСОБЕННОСТИ ВВЕДЕНИЯ В КУЛЬТУРУ *IN VITRO* И МИКРОКЛОНАЛЬНОГО РАЗМНОЖЕНИЯ *PAULOWNIA TOMENTOSA*

Аннотация. Технология микроклонального размножения растений активно развивается в настоящее время и находит широкое коммерческое применение для получения качественного посадочного материала и ускоренного размножения ценных видов и сортов древесных растений. *Paulownia sp* – многолетние высокорослые и быстрорастущие растения, темпы роста которых опережают все существующие в мире древесные культуры. В связи с этим во многих странах мирах *Paulownia sp* используется как сырье в биоэнергетике, мебельной промышленности, в ландшафтном озеленении и технологиях фиторемедиации загрязненных почв. Высокий спрос на посадочный материал на внутреннем и международном рынках вызвал необходимость разработки эффективных протоколов микроразмножения для быстрого и массового распространения *Paulownia sp*.

Работа посвящена оптимизации условий введения в культуру *in vitro Paulownia tomentosa*, исследованию факторов, влияющих на морфогенетическую активность первичных эксплантов, изучению условий адаптации микроклонов к условиям открытого грунта. Установлены наиболее оптимальные условия введения и размножения в культуре *in vitro* данного вида. Для стерилизации наиболее эффективными стерилизующими агентами являются 50% доместос и 0,1% мертиолят в экспозиции 5-10 минут. Для введения в культуру *in vitro Paulownia tomentosa* наиболее оптимальной по минеральному составу является среда WPM, которая способствует прямой регенерации растений. Наибольшая частота регенерации отмечена при культивировании в качестве эксплантов узловых сегментов побега с пазушными почками на безгормональной среде WPM. Для адаптации микроклонов *Paulownia tomentosa* к условиям открытого грунта рекомендуется использовать инфракрасный свет, который стимулирует формирование надземной части, корневой системы растений и высокую выживаемость микроклонов (до 92%). В результате проведенных исследований разработан лабораторный регламент микроклонального размножения *Paulownia tomentosa,* который позволяет за короткий срок получать большое количество здорового, качественного посадочного материала.

Для Казахстана изучение данного вида древесных культур является актуальным, новым и перспективным направлением. Разработка биотехнологических подходов для размножения *Paulownia tomentosa* на основе использования технологии микроклонального размножения будет способствовать ускорению процесса интродукции павловнии в нашей Республике.

Ключевые слова: *Paulownia tomentosa*, стерилизация, микроклональное размножение, питательная среда, клоны, адаптация ex situ, инфракрасный свет.

Information about authors:

Turganova R.A., Institute of General Genetics and Cytology, Ave. al-Farabi 93, Almaty, Kazakhstan, Senior laboratory assistant at the laboratory genetics and reproduction of forest cultures, Master's student al-Farabi Kazakh National University; ranaexotic97@mail.ru; https://orcid.org/0000-0001-7538-7340;

Djangalina E.D., Institute of General Genetics and Cytology, Ave. al-Farabi 93, Almaty, Kazakhstan. Leading Researcher at the laboratory genetics and reproduction of forest cultures, Candidate of Biological sciences, Senior lecturer of the Department of molecular biology and genetics al-Farabi Kazakh National University; djangalina@rambler.ru; https://orcid.org/0000-0002-1884-0732;

Shadenova E.A., Institute of General Genetics and Cytology, Ave. al-Farabi 93, Almaty, Kazakhstan, Head of laboratory of genetics and reproduction of forest cultures, Candidate of Agricultural Sciences; shadel08@mail.ru; https://orcid.org/0000-0002-7844-4264;

Kapytina A.I., Institute of General Genetics and cytology, Ave. al-Farabi 93, Almaty, Kazakhstan, Senior laboratory assistant at the laboratory genetics and reproduction of forest cultures, master of biotechnology; anastasiya.kapytina@mail.ru; https://orcid.org/0000-0001-5029-1107;

Kamshybayeva G.K., Institute of General Genetics and Cytology, Ave. al-Farabi 71, Almaty, Kazakhstan, Senior laboratory assistant at the laboratory genetics and reproduction of forest cultures, PhD student, al-Farabi Kazakh National University; gkamshebaeva@gmail.com; https://orcid.org/0000-0001-7015-8143;

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