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Biotechnological Aspects of Propagation of Black Poplar Hybrids "San Giorgio" and "Ghoy"

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Abstract. Energy independence of the country can be solved in several ways. One of them is the creation of energy plantations of woody plants. The area increase of such plantations is limited by the insufficient amount of high-quality planting material. Hybrid plants of the genus *Populus* are of considerable interest when creating plantations. Given that not all hybrids are successfully propagated by conventional vegetative methods, the use of biotechnological methods, namely microclonal propagation, solves the mentioned problem. This method allows obtaining a large amount of high-quality, uniform planting material. The purpose of this study was to develop the technology of microclonal propagation of black poplar hybrids *'San Giorgio'* and '*Ghoy*'. The study used generally accepted biotechnological methods in the author's modification at all stages of obtaining planting material of regenerating plants of poplar hybrids. Studies have established a positive effect when obtaining an aseptic culture of sterilising substances 0.1% $AgNO_3$, 2.5 and 1.25% NaClO, 0.05% sodium merthiolate and 0.1% $HgCl_2$. The largest number of aseptic viable explants was obtained by cultures using a 0.05% solution of sodium merthiolate after exposure for 5 and 10 minutes. When rooting microshoots on a hormone-free nutrient medium ½ MS and with the addition of 0.1-1.0 mg/l IBA, it was established that the best parameters for rooting microshoots are on a hormone-free nutrient medium ½ MS. Adaptation was carried out for 4 weeks in a greenhouse on a substrate that included components of peat:sand:perlite in a ratio of 1:1:1. The viability of regenerating plants of black poplar hybrids '*San Giorgio*' and '*Ghoy*' was more than 91%. The conducted research and the obtained results will be useful for producers of planting material and the scientific environment, which develop renewable energy sources through the creation of energy plantations of fast-growing woody plants

Keywords: explant, nutrient medium, phytohormones, substrate, planting material, *in vitro*

Introduction

Global threats associated with climate change and military aggression adversely impact the development of food and energy security in most countries of the world. This is primarily due to their dependence on oil, natural gas, and food exporting countries. In modern Ukrainian realities, the issues of energy independence and the introduction of national energy-efficient technologies are also of particular importance [1-3].

One of the solutions to Ukraine's energy security is the creation and operation of energy tree plantations. The rather slow implementation of the modern transformational approach to the creation of renewable forest resources in the Ukrainian forestry practice is explained, among other things, by the insufficient production of planting material for the creation of energy plantations. For this purpose, as the long-term experience of foresters in Italy, France, Slovakia, Hungary, and many other countries of the world shows, it is better to use planting material of hybrids of fast-growing tree species, namely the genus *Populus* [4-6]. The simultaneous use of conventional and modern methods of plant propagation allows obtaining a large amount of planting material and fully meeting the needs for it to create energy plantations. The use of a certain method of obtaining planting material is determined by the characteristics of hybrids, since they cannot always be propagated by conventional vegetative methods. In this case, as practice

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shows, it is better to use biotechnological methods, namely microclonal propagation [7-9].

Literature Review

O. Yu. Chornobrov used fragments of microshoots with one bud and leaf plates as initial explants when developing the technology of micropropagation of poplar hybrids. Effective sterilisation of shoot fragments of cultivars P. × *canadensis* ‘Robusta’ and P. × *canadensis* ‘Dorskamp’ was achieved by applying 0.1% $HgCl_2$ for 10-12 minutes. For microshoots of cultivars P. × *canadensis* ‘Tardif de Champagne’, P. × *canadensis* ‘I-45/51’ and P. × *canadensis* ‘Blanc du Poitou’, exposure to 2.5% NaClO for 8-10 min followed by transfer in 1.0% $AgNO_3$ was optimal. The formation of microshoots by direct morphogenesis in the tissues of the leaf blades of plants of the hybrids P. × *canadensis* ‘I-45/51’, P. × *canadensis* ‘Tardif de Champadne’, P. × *canadensis* ‘Robusta’, P. × canadensis ‘Dorskamp’ was recorded on the nutrient Murashige and Skoog (MS) medium [10] with 1.0 mg/l BAP (N^6 – benzylaminopurine) and 0.5 mg/l NAA (α-Naphthaleneacetic acid) under illumination of 2.0-3.0 klx. Actively growing microshoots of stem-derived plants of hybrid P. × *canadensis* ‘Tardif de Champadne’ were obtained on MS with the addition of 0.1 mg/l BAP and 1.0 mg/l IAA (β-indolyl-3-acetic acid) and 7.5 g/l of glucose [11].

Upon developing approaches to microclonal reproduction of the green-barked form of aspen (*Populus tremula* L.), S.Yu. Bilous established the need for individual selection of nutrient medium for cultivation of different types of explants at each subsequent stage of microclonal reproduction. It was determined that the best option is a nutrient medium with the following composition: 0.5 mg/l TDZ (thidiazuron) + 1 g/l activated carbon and 0.25 mg/l kinetin 1 + 1 g/l activated carbon, which ensure the realisation of not only the induction of organogenesis, but also the morphogenetic potential of the explant with the formation of rooted plants [12].

Chinese scientists have developed a step-by-step micropropagation technology of regenerating Ussuri poplar plants. Cultivation of buds was carried out on nutrient medium for woody plants (WPM) [13] with the addition of different concentrations of BAP, IBA (3-Indolebutyric acid) and TDZ. The formation of microshoots from buds ranged within 75.2-78.0%. Rooting of microshoots took place within a week on MS nutrient medium, with the content of macro- and micro-salts reduced by half. Regenerating plants were adapted for 4 weeks on a substrate comprising such components as earth:vermiculite:perlite. Subsequently, the containers with regenerating plants were moved to the landfill [8].

Lithuanian researchers developed an alternative approach to the micropropagation of the hybrid (*Populus alba* L. × *P. tremula* L.), which was based on limiting the gas exchange between the internal and external environment of the culture vessel, and not on the application of exogenous hormones. Explants, apical and internodal parts of shoots were cultivated in sealed (stoppered Paratilm) or unstoppered glass culture tubes on hormone-free nutrient medium WPM. Proliferation of microshoots on apical explants was observed in closed culture tubes, but not in unstoppered ones. The difference between the two variants increased over time in the number of microshoots and became threefold after three months of cultivation. Shoots taken from stoppered culture tubes could be distinguished by longer shoot length compared to shoots from unstoppered tubes during the next stage of cultivation under the same conditions [14]. When developing the technology of microclonal propagation, researchers consider the specific features of propagated plants and apply generally accepted biotechnological methods in the author’s modification: starting from the selection of explants from mother plants, ending with the adaptation of regenerating plants to environmental conditions.

Considering the need for continuous improvement of technological processes of microclonal reproduction while taking into account the local factors that determine the success of obtaining planting material, the purpose of this study was to improve the technology of micropropagation of black poplar hybrids *‘San Giorgio*’ and ‘*Ghoy*’, as promising species for the creation of energy plantations.

The originality of this study lies in the optimisation of the process of obtaining regenerating plants of black poplar hybrids *‘San Giorgio*’ and ‘*Ghoy*’.

Materials and Methods

Parts of one-year shoots 10-25 cm long were used to select initial explants. Donor plants of black poplar hybrids ‘*San Giorgio*’ and ‘*Ghoy*’ are located at the poplar mother plantation of the educational and research nursery of the Department of Forest Restoration and Melioration of the National University of Life and Environmental Sciences of Ukraine. Dormant and germinated buds were used as initial explants for introduction into *in vitro* culture.

During the development of stepwise sterilisation of intact plants, the following sterilising substances were used for different exposure times: 0.1% solution of silver nitrate ($AgNO_3$), 2.5 and 1.25% solution of sodium hypochlorite (NaClO), 0.05% solution of sodium merthiolate and 0 .1% mercury chloride ($HgCl_2$).

Aseptic and viable explants were cultivated on a nutrient medium according to the MS prescription with a halved content of macro- and microsalts (½ MS) and the addition of 0.05-1.0 mg/l IAA, IBA, BAP and kinetin (6-(Furfurylamino)purine).

The obtained microshoots were rooted on ½ MS nutrient medium without growth regulators and with 0.1-1.0 mg/l IBA. Cultivation conditions: illumination 2.0-3.0 klx, photoperiod 16 h, temperature 24±1 °C, humidity 70%.

Adaptation to closed soil and substrate conditions was carried out in a greenhouse using a substrate consisting of components of peat:sand:perlite in a ratio of 1:1:1. Adaptation took place for 4 weeks in a greenhouse at 24±1 °C and a humidity of 80%.

Results and Discussion

The development of the technology for growing planting material of regenerating plants started with the acquisition of a well-growing aseptic culture. At this stage, a scheme for step-by-step sterilisation of the original explants with the selection of sterilising substances, their concentration, and exposure time is developed. The main task is to obtain aseptic, viable explants for further micropropagation.

Analysing the number of aseptic and viable explants after the use of sterilising substances, it was established that sodium hypochlorite and mercuric chloride are the least effective (Table 1). When using solutions of 1.25% and 2.5% sodium hypochlorite, aseptic explants were obtained in the black poplar hybrid ‘*San Giorgio*’ from 53% to 89%, in

'*Ghoy*' – from 76% to 95%. But in the future, the number of viable explants substantially decreases, which is due to the toxicity of this solution. At the same time, a feature of the '*San Giorgio*' hybrid was its insensitivity to different concentrations of sodium hypochlorite solutions and exposure time for obtaining viable explants.

Table 1. Sterilisation efficiency of explants of black poplar hybrids '*San Giorgio*' and '*Ghoy*'

No.	Name of the sterilising agent and concentration	Exposure, min	Aseptic explants, %		Viable explants, %	
			San Giorgio	*Ghoy*	*San Giorgio*	*Ghoy*
1	0.1% mercury chloride ($HgCl_2$)	5	67±0.5	99±0.4	38±0.6	47±0.4
2	0.1 % mercury chloride ($HgCl_2$)	10	61±0.5	82±0.5	21±0.5	28±0.4
3	2.5% sodium hypochlorite (NaClO) solution	10	85±0.3	90±0.6	–	36±0.4
4	2.5% sodium hypochlorite (NaClO) solution	15	89±0.6	95±0.5	–	41±0.4
5	1.25% sodium hypochlorite (NaClO) solution	10	53±0.3	76±0.4	–	32±0.3
6	1.25% sodium hypochlorite (NaClO) solution	15	78±0.5	87±0.6	–	40±0.4
7	0.05% sodium mertiolate solution	5	92±0.4	93±0.5	83±0.5	85±0.6
8	0.05% sodium mertiolate solution	10	97±0.4	99±0.5	92±0.4	95±0.5
9	0.1% silver nitric acid solution	5	93±0.6	98±0.4	44±0.5	55±0.4
10	0.1% silver nitric acid solution	10	88±0.5	90±0.3	33±0.6	32±0.5

Due to the influence of the concentration of sterilising substances and exposure time, a positive effect on obtaining aseptic explants was found: in the hybrid '*San Giorgio*' – from 53% to 97%, and in the hybrid '*Ghoy*' – from 76% to 99%. Notably, increasing the time of exposure to the 0.1% silver nitrate solution and 0.1% mercuric chloride led to a substantial decrease in the number of aseptic and viable explants.

The study discovered the effective production of an aseptic culture using a 0.05% solution of sodium merthiolate with exposure for 5 and 10 minutes. Increasing the exposure time from 5 to 10 min positively influenced the increase of aseptic and viable explants: from 92% to 97% aseptic and 83% to 92% viable explants of the '*San Giorgio*' hybrid, from 93% to 99% aseptic and from 85% to 95% viable explants of '*Ghoy*' hybrid.

Other researchers also found a specific reaction of explants to the concentration of the sterilising substance and exposure time depending on the poplar hybrid upon obtaining an aseptic culture of poplar hybrids [11].

Based on the conducted research on obtaining an aseptic culture of poplar hybrids '*San Giorgio*' and '*Ghoy*', an effective method of sterilisation of the original explants has been developed, which is successful from the standpoint of introduction into culture *in vitro*.

After 6-7 days, aseptic viable explants were transferred to ½ MS nutrient medium with growth regulators IAA, IBA, BAP, and kinetin at a concentration of 0.05-1.0 mg/l (Table 2).

Table 2. Dependence of the formation of microshoots on the concentration of growth regulators

Modification of the nutrient medium	Name of the growth regulator	Concentration, mg/l	The number of explants that formed microshoots, %		Height of microshoots, cm	
			San Giorgio	Ghoy	San Giorgio	Ghoy
1	IAA	0.05	21±0.3	22±0.4	2.0±0.22	2.1±0.25
2		0.1	18±0.4	17±0.5	1.7±0.26	1.8±0.27
3		0.2	13±0.5	12±0.4	1.5±0.21	1.7±0.24
4	IBA	0.05	36±0.4	36±0.5	1.9±0.25	2.0±0.24
5		0.1	24±0.5	25±0.5	1.7±0.25	1.9±0.25
6		0.2	18±0.7	19±0.6	1.5±0.26	1.6±0.23
7	BAP	0.25	86±0.3	85±0.4	4.3±0.21	4.5±0.19
8		0.5	77±0.4	79±0.5	4.0±0.19	4.1±0.21
9		1.0	74±0.5	75±0.6	3.8±0.22	4.0±0.19
10	kinetin	0.25	63±0.4	65±0.4	3.7±0.20	3.9±0.22
11		0.5	60±0.3	62±0.6	3.1±0.22	3.3±0.20
12		1.0	49±0.6	48±0.7	3.0±0.19	3.2±0.21

The use of IAA and IBA in low concentrations in the nutrient medium showed a weak stimulating effect on the formation of microshoots of poplar hybrids. Due to their action, the emergence of microclones was observed in up to 36% of explants. The formed microshoots were thin and often vitrified, while their growth took place. The results upon using indolylbutyric acid turned out to be better than upon using indolylacetic acid.

When the concentration of growth regulators of the auxin group increased to 0.2 ml/l, the formation of lignified, hard, non-morphogenic callus and weak roots, which were unsuitable for further adaptation, was observed.

Proceeding from the obtained data, the addition of IAA and IBA to the nutrient medium for the multiplication of microshoots, regardless of the concentration of auxins, did not give the expected result. The use of these growth regulators for the formation of microclones of black poplar hybrids '*San Giorgio*' and '*Ghoy*' can be considered ineffective.

Compared to auxins, cytokines BAP and kinetin showed more intense shoot formation when added to the nutrient medium. Notably, the inclusion of BAP in the nutrient medium in all variants, compared to kinetin, caused intensive formation of microshoots from 75% to 85%, depending on the concentration. Therewith, the best stimulation of the emergence of microshoots (85%) occurred on the nutrient medium with 0.25 mg/l BAP. Microshoots of poplar hybrids '*San Giorgio*' and '*Ghoy*' obtained on nutrient medium ½ MS + 0.25 mg/l BAP also prevailed in height compared to all other options (Fig. 1).

Figure 1. General appearance of microshoots of black poplar hybrids '*San Giorgio*' and '*Ghoy*' on nutrient medium ½ MS + 0.25 mg/l BAP

In studies [8; 15], which covered the microclonal propagation of poplar hybrids, intensive formation of microshoots was also obtained with the content of cytokine group growth regulators in the nutrient medium. This is because cytokinins contribute to the elongation of cells and the formation of explant tissues, which further affects the formation of mesenteries and the growth of the stem. They also positively affect the induction of adventitious buds. Microshoots were rooted on a hormone-free ½ MS medium and with 0.1-1.0 mg/l IBA to the nutrient medium (Table 3).

Table 3. Specific features of rooting poplar hybrids '*San Giorgio*' and '*Ghoy*'

Modification of the nutrient medium	IBA concentration, mg/l	% of rooted microshoots	The average number of roots, pcs.
1	–	92±0.3	2.5±0.18
2	0.1	92±0.5	1.9±0.25
3	0.5	93±0.6	2.2±0.21
4	1.0	94±0.7	2.4±0.24

As the above data shows, there were 92% of rooted microshoots on a hormone-free nutrient medium ½ MS (Fig. 2). The addition of IBA slightly increased the percentage of rooted microshoots by 1% and 2% at concentrations of 0.5 and 1.0 mg/l, respectively, compared to hormone-free nutrient medium.

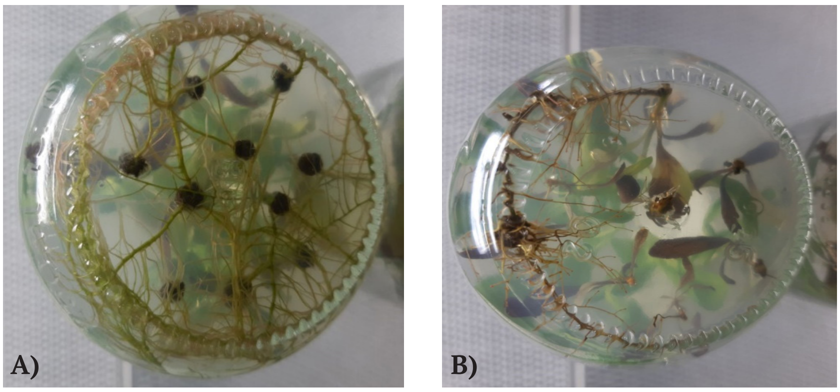

Figure 2. The root system of black poplar hybrids '*San Giorgio*' (A) and '*Ghoy*' (B) on hormone-free nutrient medium ½ MS

Cultivation of regenerating plants on hormone-free nutrient medium ½ MS for two weeks improved the habit of the plants: the stem of the rooted microshoots gradually extended, the leaf plate grew, and the petioles of the leaves lengthened (Fig. 3).

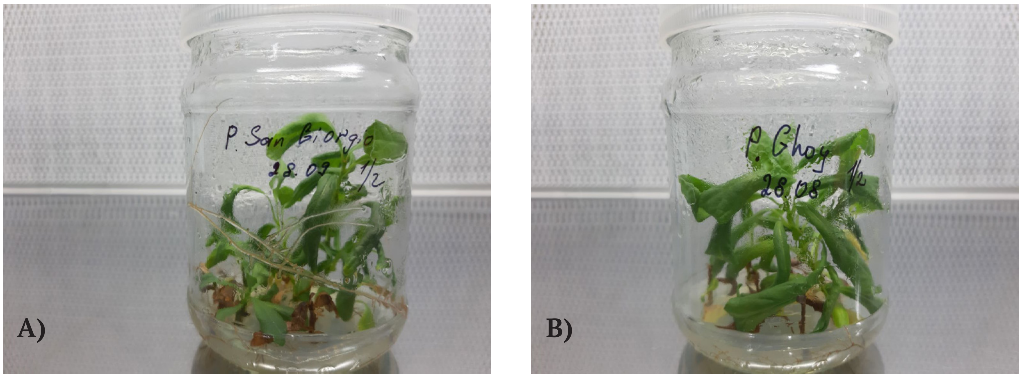

Figure 3. Regenerating plants of *Populus nigra* hybrids '*San Giorgio*' (A) and '*Ghoy*' (B) on a hormone-free nutrient medium ½ MS

During the adaptation of regenerating plants of poplar hybrids, most researchers used multicomponent substrates, which included peat, sand, perlite, vermiculite, soil, which is conditioned upon the ratio of poplars to the soil environment [16; 17].

When the regenerating plants reached a height of 4-5 cm, they were adapted to the conditions of closed soil. Adaptation was carried out on a substrate that included components of peat:sand:perlite in a ratio of 1:1:1. After 4 weeks of adaptation, the survival rate of regenerative plants was over 91%.

Conclusions

An effective method of sterilisation of initial explants of poplar hybrids '*San Giorgio*' and '*Ghoy*' was developed. The highest percentage of aseptic, viable explants was obtained using a 0.05% solution of sodium merthiolate with exposure for 10 minutes. It was found that intense shoot formation occurred with the addition of BAP and kinetin cytokines to the nutrient medium. Low concentrations of IAA and IBA showed a weak stimulating effect on the formation of microshoots of poplar hybrids. The most intense shoot formation occurred on nutrient medium ½ MS + 0.25 mg/l BAP.

Addition of 0.1-1.0 mg/l IBA to the nutrient medium slightly increased the rooting percentage of microshoots. Based on the results of the study, the technology of microclonal propagation of black poplar hybrids '*San Giorgio*' and '*Ghoy*' was developed, which includes obtaining aseptic and viable explants using a 0.05% sodium merthiolate solution with exposure for 10 min, micropropagation on nutrient medium ½ MS + 0.25 mg/l BAP rooting of microshoots on a hormone-free nutrient medium ½ MS, adaptation of regenerating plants to closed soil conditions on a substrate of peat:sand:perlite in a 1:1:1 ratio. The developed technology for obtaining regenerant plants can be used in the future for other black poplar hybrids.

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Біотехнологічні аспекти розмноження гібридів тополі чорної «San Giorgio» та «Ghoy»

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Анотація. Енергетичну незалежність країни можна вирішити декількома шляхами. Одним із них є створення енергетичних плантацій деревних рослин. Збільшення площі таких плантацій обмежується недостатньою кількістю якісного садивного матеріалу. Значний інтерес при створенні плантацій становлять рослини гібридів роду *Populus*. Враховуючи, що не всі гібриди успішно розмножуються традиційними вегетативними способами, використання біотехнологічних методів, зокрема мікроклонального розмноження, вирішує згадану проблему. Даний спосіб дає змогу отримати велику кількість якісного однорідного садивного матеріалу. Метою досліджень була розробка технології мікроклонального розмноження гібридів тополі чорної *'San Giorgio'* та '*Ghoy*'. При проведенні досліджень використовували загальноприйняті біотехнологічні методи в авторській модифікації на всіх етапах одержання садивного матеріалу рослин-регенерантів гібридів тополі. Дослідженнями встановлено позитивний вплив при отриманні асептичної культури стерилізуючих речовин 0,1 % $AgNO_3$, 2,5 та 1,25 % NaClO, 0,05 % мертіоляту натрію та 0,1 % $HgCl_2$. Найбільшу кількість асептичних життєздатних експлантів отримували культури за застосування 0,05 %-го розчину мертіоляту натрію при експозиції 5 та 10 хв. При укорінення мікропагонів на безгормональному живильному середовищі ½ MS та з додаванням 0,1-1,0 мг/л ІМК встановлено, що кращі параметри укорінення мікропагонів на безгормональному живильному середовищі ½ MS. Адаптацію упродовж 4 тижнів здійснювали в теплиці на субстраті до складу якого входили компоненти торф: пісок: перліт у співвідношенні 1:1:1. Приживлюваність рослин-регенерантів гібридів тополі чорної '*San Giorgio*' та '*Ghoy*' становила більше 91 %. Проведені дослідження та отримані результати будуть корисними для виробників садивного матеріалу та наукового середовища, які працюють у напрямі розвитку відновлювальних джерел енергії через створення енергетичних плантацій швидкорослих деревних рослин

Ключові слова: експлант, живильне середовище, фітогормони, субстрат, садивний матеріал, *in vitro*