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## Optimisation of the Protocol for Sterilising Explants of Some Deciduous Woody Plants in Culture *in vitro*

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**Abstract.** One of the methods of obtaining planting material for deciduous woody plants, in particular, common ash (*Fraxinus excelsior* L.), large-leaved linden (*Tilia platyphyllos* Scop.), and the silver birch (*Betula pendula* Roth) is micropropagation. Explant asepsis – prerequisite for microclonal plant reproduction. For the most part, chemical sterilisation with liquid substances is used for this purpose. The decontamination regime is influenced by a number of factors, in particular, the genotype of the plant. The purpose of the study is to optimise the explant sterilisation protocol of *F. excelsior*, *T. platyphyllos*, and *B. pendula* for micropropagation. For the study, 20-30 cm parts of shoots isolated from a 12-year-old *T. platyphyllos*, 10-year-old *B. pendula*, and a 15-year-old *F. excelsior* were used in February-March 2021. Plant material was cultured according to the generally accepted method on an MS nutrient medium (Murashige & Skoog, 1962). MS Excel software tools were used to process the research data, the average, and its main error were calculated. Univariate analysis of variance (ANOVA) was performed to analyse the effect of explant sterilisation mode on asepsis. The expediency of keeping plant material for a day in fungicidal preparations, in particular, in 0.1% “Samshit” solution, is shown – *F. excelsior* and 0.3% Fundazol solution – *T. platyphyllos* and *B. pendula*. The protocol of sterilisation of experimental plants (efficiency over 50%) is optimised by using a step method using 70% ethyl alcohol, 1.0% and 2.0% AgNO<sub>3</sub>, and 2.5% and 5.0% NaClO. It was identified that the effect of the sterilisation regime of experimental plants on asepsis is statistically substantial at the level of 5%. A MS nutrient medium with the addition of 0.25 mg·l<sup>-1</sup> kinetin and 2.0 g·l<sup>-1</sup> activated carbon was used to initiate explants. Further research is aimed at developing a protocol for the direct regeneration of plant microshoots of *F. excelsior*, *T. platyphyllos*, and *B. pendula* by the action of the components of the nutrient medium *in vitro*

**Keywords:** plant tissue culture *in vitro*, *Fraxinus excelsior* L., *Tilia platyphyllos* Scop., *Betula pendula* Roth, asepsis, explants, regeneration, nutrient medium, micropropagation

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## Introduction

Obtaining high-quality planting material for deciduous woody plants, in particular, common ash (*Fraxinus excelsior* L.), large-leaved linden (*Tilia platyphyllos* Scop.), and the silver birch (*Betula pendula* Roth), is an urgent task for the forestry and gardening industry of Ukraine. One of the methods of obtaining plants while preserving valuable hereditary properties is micropropagation. In contrast to conventional reproduction methods, this method allows obtaining healthy genetically homogeneous plants throughout the year from a minimum amount of donor material and substantially increases the reproduction rate (Butenko, 1964; Lutova, 2003; Smith, 2012). One of the most important conditions for micropropagation is the preparation of plant material for introduction into the conditions *in vitro*, in particular, the selection of an effective sterilisation protocol (Kalinin et al., 1980; Kataeva & Butenko, 1983). The result of micropropagation depends on sterilisation as the first stage. Therewith, it is necessary to select the appropriate sterilising substances, their concentration and exposure to holding, which effectively neutralise the biota, do not damage plant tissues, and allow obtaining high viability in the conditions of *in vitro*.

Currently, chemical sterilisation with liquid substances, less often gaseous substances, and physical reagents (ultrasound, ultraviolet radiation) are used to achieve asepsis of explants (Butenko, 1964; Kalinin et al., 1980; Kataeva & Butenko, 1983). Analysis of studies shows that the most common is chemical sterilisation with substances containing active chlorine (sodium/calcium hypochlorite, chloramine, bleach lime), hydrargium (Sulema, ethylmercurchloride, thimerosal), oxygen (hydrogen peroxide), silver (silver nitrate), alcohols (Shabanova & Mashkina, 2015; Lebedev & Shestibratov, 2016; Podhaietsky et al., 2016; Shakhov et al., 2018, 2019). Antibiotics are used to neutralise the endogenous biota of objects, but they are characterised by a selective spectrum of action (Butenko, 1964). Biocides are also used, in particular, PPM (Plant Preservative Mixture™) (Podhaietsky et al., 2016). In particular, the authors of Lebedev & Shestibratov (2016) sterilised the parts of the escape from 2-3 buds isolated from 60-year-old plus *F. excelsior* trees, in 0.1% mercury chloride for 6 minutes, followed by three-time washing with sterile distilled water. Plant material was cultured on

an MS nutrient medium (Murashige & Skoog, 1962) with the addition of BAP (6-benzylaminopurine) and IAA ( $\beta$ -indolyl-3-acetic acid). Two-stage sterilisation was used to increase the percentage of sterilisation efficiency (Skálová et al., 2012). Authors Payamnour et al. (2014) optimised the shoot sterilisation protocol of *T. platyphyllos* using soapy water to neutralise surface contamination and pre-sterilisation, which consisted of holding in a solution of  $600 \text{ mg} \cdot \text{l}^{-1}$  ascorbic acid,  $4.0 \text{ g} \cdot \text{l}^{-1}$  fungicide “Captan”, 5.0% commercial solution of NaOCl (5.0% Cl) (20 min). The plant material was then washed under running water. A solution of  $600 \text{ mg} \cdot \text{l}^{-1}$  was used for sterilisation of ascorbic acid and 10% NaOCl (5 min). For sterilisation of plant seeds of *Betula spp.* 1.0% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (48 h) at  $T=+4^\circ\text{C}$ , followed by exposure at 30.0%  $\text{H}_2\text{O}_2$  (15 min) and washing in sterile distilled water (Gaidamashvili et al., 2015). Authors Shabanova & Mashkina (2015) conducted sterilisation of hybrid explants *Populus* L. considering the isolation season. In particular, shoots isolated in winter were pretreated under non-sterile conditions in 2.0% “Domestos” (10 min), then in a mixture of solutions of 0.025% merthiolate (10 min) and 7.0% “Bilyzna” (10 min); the exposure of explants isolated in summer was 7 minutes. Effective for sinus buds of *Populus maximowiczii* Henry was holding in 70% ethyl alcohol (1 min), followed by transfer to NaClO (2% active chlorine) (10-18 min) (Kärkönen et al., 2011). Other researchers (Delgadillo-Díaz et al., 2013) developed an effective mode for sterilising explants of *Quercus spp.* with the use of 70% ethyl alcohol and 5.25% sodium hypochlorite. A 0.1% solution of merthiolate or mercury bichloride (Shakhov et al., 2019) / 12,0 %  $\text{H}_2\text{O}_2$  / NaClO with 95.2% active chlorine (Shakhov et al., 2018) was used for cherry explants isolated from the apical and lateral buds of annual shoots in March. Therewith, a number of authors note that the mode of explant sterilisation depends on a number of factors, in particular, the donor genotype, physiological state, age and type of explant, and the isolation season (Butenko, 1964; Kärkönen et al., 2011; Payamnour et al., 2014; Podhaietskyi et al., 2016; Shakhov et al., 2018, 2019).

*The purpose of the study is to optimise the explant sterilisation protocol of F. excelsior, T. platyphyllos and B. pendula for micropropagation. The study provided for solving the following tasks: 1) determine*

the effective mode of decontamination of explants in non-sterile conditions; 2) select sterilising substances and determine their optimal concentration, and exposure in sterile conditions; 3) select an effective nutrient medium at the *in vitro* initiation stage.

### Materials and Methods

For the study, 20–30 cm parts of shoots isolated from a 12-year-old *T. platyphyllos*, 10-year-old *B. pendula*, and a 15-year-old *F. excelsior* in February–March 2021 were used. Commercial fungicides were used to reduce the percentage of contamination of plant material under non-sterile conditions: 0.3% Fundazol and 0.1% Samshit (100 g<sup>\*1</sup>-1 cresoximethyl + 200 g<sup>\*1</sup>-1 diphenconazole). Shoots were treated by spraying/holding for a day / were not treated with fungicides (control). Sterilisation of plant material (4–8 cm fragments) consisted in holding (15–20 min) in a soapy solution (with the addition of a few drops of Tween-80 detergent), stirring constantly, washing in running water (15–20 min), rinsing in distilled water, immersion in 70% ethyl alcohol, sterilisation in solutions, and subsequent three-time washing in sterile distilled water (5–6 minutes in each serving). 2.5% and 5.0% NaClO, 1.0% and 2.0% AgNO<sub>3</sub> were used as sterilising agents. At the stage of introduction to culture *in vitro* an MS nutrient medium was used. The nutrient media was supplemented with 100 mg<sup>\*1</sup>-1 mesoinositol, 0.25 mg<sup>\*1</sup>-1 kinetin, 30 g<sup>\*1</sup>-1 sucrose, 2.0 g<sup>\*1</sup>-1 activated carbon, and 7.0–7.3 g<sup>\*1</sup>-1 microbiological agar. The acidity index of the medium (pH) was adjusted to the level of 5.7–5.9.

The plant material was cultivated according to the generally accepted method in a light room at a temperature of 24±1°C and illumination of 2.0–

3.0 klx with 16-hour photoperiod and relative humidity of 70–75%. Biotechnological methods were used, in particular, micropropagation using the culture of plant shoot fragments *in vitro* (Butenko, 1964; Kalinin et al., 1980; Kataeva & Butenko, 1983; Smith, 2012). The repeatability of experiments is fourfold. MS Excel software tools were used to process the research data, the average, and its main error were calculated. Univariate analysis of variance (ANOVA) was performed to analyse the effect of explant sterilisation mode on asepsis. The following abbreviations were used in the results: *F* – calculated value of the Fischer criterion; *F*<sub>crit.</sub> – critical value of the Fischer criterion. The study was conducted in the research laboratory of plant biotechnology of the National University of Life and Environmental Sciences of Ukraine “Boyars’ka LDS” in 2021.

### Results and Discussion

Explant asepsis – prerequisite for microclonal plant reproduction. One of the most common sterilising agents for the production of aseptic woody plant cultures is NaClO (Kärkönen et al., 2011; Delgado-Díaz et al., 2013; Payamnour et al., 2014; Shabanova & Mashkina, 2015). Therewith, the difficulty of obtaining a substantial proportion of aseptic material of woody plants is due to morpho-physiological features, so by analogy with other researchers, (Skálová et al., 2012; Payamnour et al., 2014; Gaidamashvili et al., 2015) two-stage sterilisation was used. Results of the authors’ research (Butenko, 1964; Payamnour et al., 2014) indicate the feasibility of using fungicides for pre-sterilisation. Action of fungicides and sterilising substances (ethyl alcohol, NaClO, AgNO<sub>3</sub>) for explants are shown in Table 1.

**Table 1.** Efficiency of sterilisation of fragments of shoots of experimental plants in the conditions of *in vitro*

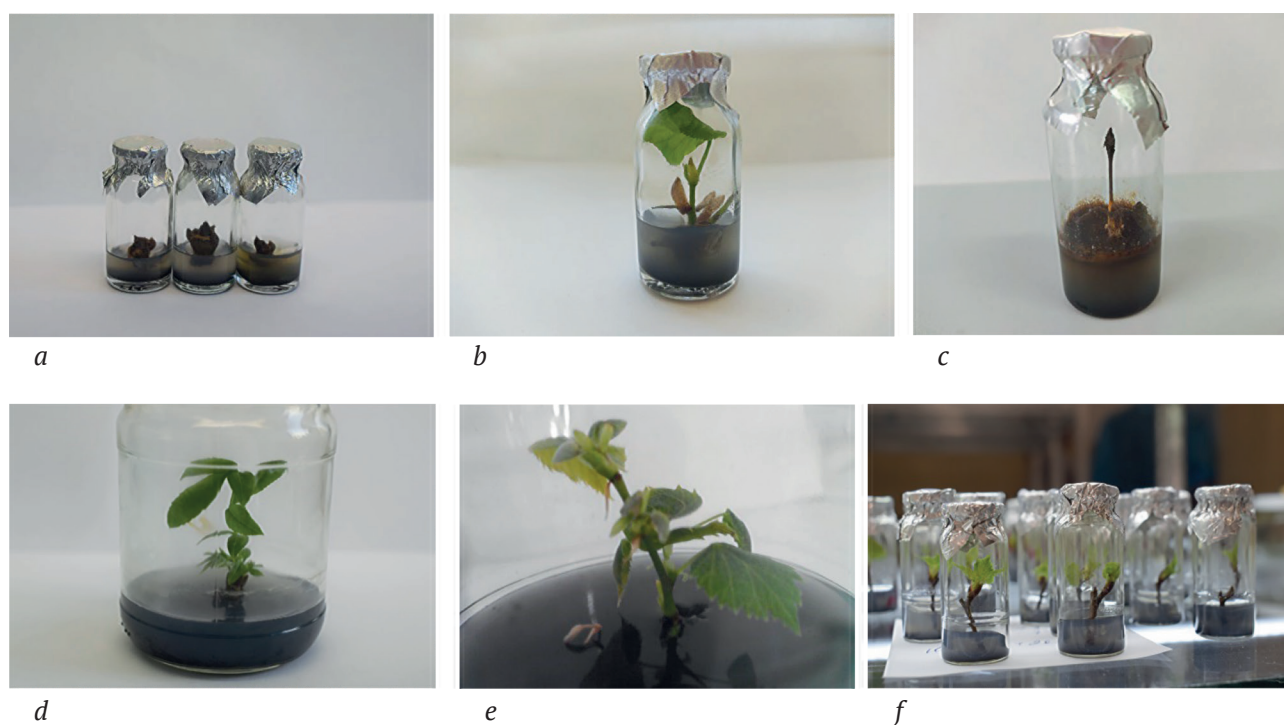
Variant	Species	Fungicide (pre-sterilisation)	Basic sterilisation mode	Explant sterilisation efficiency (mean ± standard error). %
1	<i>F. excelsior</i>	–	70% ethyl alcohol (1–2 min). 5% NaClO (10 min) followed by transfer to 2.0% AgNO <sub>3</sub> (9–10 min)	27.5±3.0
2		spraying 0.1% “Samshit”		52.3±4.3
3		soaking in 0.1% Samshit (24 hours)		70.3±2.8
4	<i>T. platyphyllos</i>	–	70% ethyl alcohol (1–2 min). 5% NaClO (6–7 min) followed by transfer to 2.0% AgNO <sub>3</sub> (9–10 min)	29.3±2.2
5		spraying 0.3% “Fundazol”		31.3±1.5
6		soaking in 0.3% “Fundazol” (24 hours)		65.0±2.6

Table 2, Continued

Variant	Species	Fungicide (pre-sterilisation)	Basic sterilisation mode	Explant sterilisation efficiency (mean $\pm$ standard error). %
7	<i>B. pendula</i>	-	70% ethyl alcohol (1-2 min). 2.5% NaClO (5-6 min) followed by transfer to 1.0% AgNO <sub>3</sub> (5-6 min)	22.0 $\pm$ 3.4
8		spraying 0.3% "Fundazol"		57.3 $\pm$ 2.2
9		soaking in 0.3% "Fundazol" (24 hours)		58.5 $\pm$ 1.9

On day 4, the growth of biota (fungi/bacteria) at the base of the explant on the surface of the nutrient medium was recorded. This was caused by insufficient aseptic intake during surface sterilisation

of plant material (Buckley & Reed, 1994). It was visually established that the basis of contaminants was fungi (over 80 %, Fig. 1, c) bacteriosis occurred occasionally (up to 20 %).



**Figure 1.** Results of explant sterilisation: aseptic explants of *F. excelsior* (a) and *T. platyphyllos* (b); infected with fungal mycelium *B. pendula* (c); viable micro-shoots *in vitro* *F. excelsior* (d), *T. platyphyllos* (e), *B. pendula* (f)

It is known that to minimise the toxic effects of sterilising agents and reduce the proportion of non-viable plants, their concentration and exposure should be minimal (Kalinin et al., 1980; Kataeva & Butenko, 1983). Responses of woody plant explants to decontamination regimes differed,

which is due to morpho-physiological features of genotypes. According to the results of univariate analysis of variance (ANOVA), the effect of the sterilisation regime of experimental plants on asepsis is statistically substantial at the level of 5% ( $F > F_{crit}$ ,  $P < 0.05$ ) (Table 2).

**Table 2.** Final results of univariate dispersion analysis for experimental plant tissues *in vitro*

<i>F. excelsior</i>						
Source of variation	SS	df	MS	F	P-value	$F_{crit.}$
Between groups	3731	2	1865.3333	39.2014	3.6077	4.2565
<i>T. platyphyllos</i>						
Between groups	3228	2	1614.0833	89.9489	1.1248	4.2565
<i>B. pendula</i>						
Between groups	3435	2	1717.5833	65.5705	4.3106	4.2565

where: *df* – number of degrees of freedom; *MS* - variances; *F* – calculated value of the Fischer criterion; *P-value* – calculated value of the minimum substantiality;  $F_{crit.}$  – critical value of the Fischer criterion

The experimental results showed the feasibility of keeping plant material in fungicide solutions and using step-by-step sterilisation, which is consistent with the data of other authors (Skálová et al., 2012; Payamnour et al., 2014; Gaidamashvili et al., 2015). Effective sterilisation of plant shoot fragments of *B. pendula* was achieved by using step-by-step sterilisation (70% ethyl alcohol – 1-2 min, 2.5% NaClO and 1.0% AgNO<sub>3</sub> – 6-7 min) with preliminary exposure for a day in 0.3% Fundazol solution to obtain 58.5±1.9% asepsis (Fig. 1, *f*). The highest percentage of efficiency (65.0 ± 2.6 %) for explants of *T. platyphyllos* was obtained in the case of using 70% ethyl alcohol (1-2 min), 5.0% NaClO (6-7 min) and 2.0% AgNO<sub>3</sub> (9-10 min) and pre-holding in 0.3% Fundazol solution (Fig. 1, *b*).

For effective neutralisation of exogenous biota *F. excelsior* (70.3±2.8 %), it is advisable to apply an asepsis regime that provided for the use of 70% ethyl alcohol (1-2 min), 2.0% AgNO<sub>3</sub>, and 5.0% NaClO (9-10 min) with preliminary exposure for a day in 0.1% “Samshit” solution (Fig. 1, *a*). On 30-50 of cultivation, viable microshoots with characteristic pigmentation without signs of vitrification and necrotisation were obtained on an MS nutrient medium with the addition of 0.25 mg\*1<sup>-1</sup> kinetin and 2.0 g\*1<sup>-1</sup> activated carbon (Fig. 1, *d, e, f*).

## Conclusions

Protocol for sterilisation of plant shoot fragments of *F. excelsior*, *T. platyphyllos*, and *B. pendula*, isolated in February-March was optimised, and aseptic viable microshoots *in vitro* were obtained. The expediency of keeping plant material in fungicides for a day (0.1% Samshit solution) is shown. – *F. excelsior*; 0.3% solution of “Fundazol” – *T. platyphyllos*, *B. pendula*) and the use of step-by-step sterilisation using ethyl alcohol, AgNO<sub>3</sub> and NaClO. It was identified that the effect of the sterilisation regime of experimental plants on asepsis is statistically substantial at the level of 5%. It is advisable to use the following protocols (with preliminary exposure in a fungicide solution and 70% ethyl alcohol for 1-2 min): 1) 2.5% NaClO (6-7 min) and 1.0% AgNO<sub>3</sub> (6-7 min) – for *B. pendula*; 2) 5.0% NaClO (6-7 min) and 2.0% AgNO<sub>3</sub> (9-10 min) – for *T. platyphyllos*; 3) 2.0 % AgNO<sub>3</sub> and 5.0% NaClO (9-10 min) – for *F. excelsior* to obtain a substantial amount of aseptic viable microshoots (over 50%). An MS nutrient medium with the addition of 0.25 mg\*1<sup>-1</sup> kinetin and 2.0 g\*1<sup>-1</sup> activated carbon is applied to initiate explants. Further research is aimed at developing a protocol for the direct regeneration of microshoots under the action of components of the nutrient medium *in vitro*.

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## Оптимізація протоколу стерилізації експлантатів деяких листяних деревних рослин у культурі *in vitro*

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**Анотація.** Одним із методів отримання садивного матеріалу листяних деревних рослин, зокрема ясеня звичайного (*Fraxinus excelsior* L.), липи широколистої (*Tilia platyphyllos* Scop.) та берези повислої (*Betula pendula* Roth), є мікроклональне розмноження. Асептичність експлантатів – передумова мікроклонального розмноження рослин. Здебільшого для цього використовують хімічну стерилізацію рідкими речовинами. На режим деконтамінації впливає низка чинників, зокрема генотип рослини. Мета дослідження – оптимізація протоколу стерилізації експлантатів *F. excelsior*, *T. platyphyllos* та *B. pendula* для мікроклонального розмноження. Для досліджень використовували 20-30 см частини пагонів, ізольовані із 12-річної *T. platyphyllos*, 10-річної *B. pendula* та 15-річного *F. excelsior* у лютому-березні 2021 р. Рослинний матеріал культивували за загальноприйнятою методикою на живильному середовищі MS (Murashige & Skoog, 1962). Для опрацювання дослідних даних використовували програмні засоби MS Excel, розраховано середнє та його основну помилку. Для аналізу впливу режиму стерилізації експлантатів на асептичність проведено однофакторний дисперсійний аналіз (ANOVA). Показано доцільність витримування рослинного матеріалу упродовж доби у фунгіцидних препаратах, зокрема у 0,1 % розчині «Самшит» – *F. excelsior* та 0,3 % розчині «Фундазол» – *T. platyphyllos* і *B. pendula*. Оптимізовано протокол стерилізації дослідних рослин (ефективність понад 50 %) шляхом використання ступінчастого способу із застосуванням 70 % етилового спирту, 1,0 % і 2,0 %  $\text{AgNO}_3$  та 2,5 % і 5,0 %  $\text{NaClO}$ . Установлено, що вплив режиму стерилізації дослідних рослин на асептичність є статистично значущим на рівні 5 %. Для ініціації експлантатів використовували живильне середовище за прописом MS із додаванням 0,25 мг·л<sup>-1</sup> кінетину і 2,0 г·л<sup>-1</sup> активованого вугілля. Подальші дослідження спрямовані на розроблення протоколу прямої регенерації мікропагонів рослин *F. excelsior*, *T. platyphyllos* та *B. pendula* за дії компонентів живильного середовища *in vitro*

**Ключові слова:** культура тканин рослин *in vitro*, *Fraxinus excelsior* L., *Tilia platyphyllos* Scop., *Betula pendula* Roth, асептичність, експлантати, регенерація, живильне середовище, мікроклональне розмноження