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Screening of the Effect of Chloramine on the Mycobiota of *Ulmus Laevis* Pall. Plant Tissues *in vitro*

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Abstract. European white elm (*Ulmus laevis* Pall.) tissue *in vitro* is a donor material for obtaining cultures with stable resistance to pathologies of infectious origin, namely to Dutch elm disease. To solve this problem, it is necessary to develop an effective protocol for the regeneration of *U. laevis in vitro*. The purpose of this study is to investigate the effect of chloramine concentrations on the mycobiota of *U. laevis* plant tissues for propagation *in vitro*. 10-15 cm parts of shoots from 25-year-old *U. laevis* were used as plant material. The study was conducted in the autumn of 2021. Microshoots previously sterilized with chloramine (1.0%, 2.5%, 5.0%, 10.0%) for 10 min were cultivated on a solid nutrient medium according to the WPM recipe (McCown & Lloyd, 1981) with the addition of 0.2 mg·l^{-1} *2* - iP (6-(γ,γ- Dimethylallylamino)purine) and 2.0 g·l-^{1} of activated carbon. For microbiological analysis, sterilised plant material was cultured by accumulation in Petri dishes with a nutrient medium (sour potato agar) in a thermostat without lighting at +26 ± 1°C and a relative humidity of 68 ± 2%. Methods of biotechnological, mycological, and statistical research were employed in this study. Over 95% of the samples were found to be infected with microscopic fungi of the genus *Mucor* Fresen., *Penicillium* Link, *Chaetomium* Kunze and *Trichoderma* Pers. The effect of preparation concentration on the total number of infected explants is statistically insignificant at 5%. It was found that 5.0% preparation is effective for neutralising mycobiota of the genus *Chaetomium* and *Trichoderma*; 10.0% – for neutralising *Penicillium* mycobiota. If the concentration of chloramine increases, the intensity of infection of explants with mycobiota of various genera decreases. As a result of the research, a small amount of aseptic cultures were obtained from the shoots of *U. laevis* isolated in autumn. This study is relevant for biologists, biotechnologists, microbiologists, and biological scientists

Keywords: European white elm, Dutch elm disease, asepsis, infection, microclonal propagation

Introduction

European white elm (*Ulmus laevis* Pall.) is a valuable forest, decorative, soil protecting, forest improvement, honey-bearing, medicinal, and fodder plant. Medicinal features of the plant are conditioned upon the presence of valuable biologically active substances that have antimicrobial properties [1-3]. Elms are successfully used in settlement gardening and landscape design. According to the results of forest pathology examinations, plants are damaged by pests, namely the winter moth, the clouded magpie, the elm leaf miner, and *Exaereta ulmi* [4]. Currently, the Dutch elm disease (DED), which causes mass drying of European and Asian species, is particularly dangerous. The causative agent of the DED is sac fungi *Ophiostoma ulmi* Melin & Nannf. (*Ceratocystis ulmi*) and *Ophiostoma novo-ulmi* with the conidial stage of *Graphium ulmi*, which spread in the vessels, obstruct the flow of water, and cause the death of individual branches or the tree in general. The disease is mainly spread by bark beetles or insects (*Scolytus scolytus* F., *Scolytus multistriatus* Marsh., *Scolytus* pygmaeus F.) [4; 5]. However, many researchers have established that infectious vascular pathogens are not the main cause of the mass disease of oaks and other deciduous plants, their spread as a consequence or a secondary phenomenon [6; 7]. Furthermore, there is no consensus on pathogenicity of *Ophiostoma.* In a normal state, species of the *Ophiostoma* genus vegetate as saprophytes in dry branches of

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the crown, but in conditions of increased air in the vessels, they change to a parasitic state [6]. Therefore, the root cause of the mass drying out of *Ulmus* is still debatable.

During the decades of spread of DED in Europe and North America, various recommendations have been proposed and tested to prevent the drying out of these species and to minimize damage to forest and urban plantations (use of systemic insecticides by injection into wood or vessels, pruning of branches with the first symptoms of the disease, treatment of felled trees with insecticides and sanitation treatment). However, the proposed methods are currently ineffective. Innovative *in vitro* technologies, in contrast to conventional methods, allow obtaining artificial hybrids/somaclonal variants/mutants with disease resistance using protoplast culture, cell selection, and genetic engineering [8-10]. This strategy can only be initiated after the establishment of efficient plant regeneration protocols from *Ulmus* tissues [11].

The purpose of the study is to figure out the effect of chloramine concentrations on the mycobiota of *U. laevis* plant tissues for microclonal propagation. Tasks: 1) to find the total proportion of infected explants under the action of chloramine concentrations; 2) to find the mycobiota of the samples under study and the intensity of infection; 3) to investigate the effect of chloramine concentrations on the mycobiota. The originality of this study lies in the identification of mycobiota of shoots isolated in autumn from a 25-year-old *U. laevis,* after sterilisation with chloramine.

Literature Review

The important value of *Ulmus* plants predetermined several biotechnological scientific studies [12-14]. It was found that the combination of *Ulmus davidiana* and *Cornus officinalis* extracts has an inhibitory effect on osteoporotic bone loss and contains biologically active substances; *Ulmus pumila* plants have antimicrobial properties against *Escherichia coli*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* [1-3]. It was proved that the use of stem fungal endophytes from the *Cystobasidiomycetes*, *Eurotiomycetes*, and *Dothideomycetes* classes stimulates the induction of the protective metabolism of *Ulmus minor* plants to pathogens [10]. Aseptic elms *in vitro* are used for microclonal propagation, reintroduction of elite germplasm, creation of plant banks and long-term cryopreservation [5; 9; 11].

Asepsis of explants – is a prerequisite for microclonal propagation of plants. At the stage of obtaining an aseptic culture, it is important to choose the correct sterilisation mode. Currently, a wide range of sterilising substances is used to obtain aseptic culture of woody plants [15; 16]. One of the most common is chemical sterilisation with substances containing active chlorine. In different years, the culture of *Ulmus in vitro* was investigated by many scientists [17-19]. The authors of [14] disinfected nodal segments with buds of *Ulmus americana* L. plants in 70% ethanol (1 min), washed with sterile water (3 min), surface-sterilised in 15% commercial bleach (5.5% sodium hypochlorite) with the addition of Tween- 20 (2-3 drops, 10 min) followed by washing three times with sterile water. The study was conducted from April to September. Another group of scientists [5] sterilised buds of *U. laevis* and *Ulmus glabra* Huds. plants with the following regimes: 70% ethanol (10 min) followed by exposure to 10% hydrogen peroxide (3 h) / 70% ethanol (20 min) / hydrogen peroxide with constant stirring (10 min). PPM (Plant Preservative Mixture™), antibiotics, and sodium hypochlorite were also used. After sterilisation, the buds were kept on a filter with 100 mg·l^{-1} 1-vinyl-2-pyrrolidone (PVP, Polyvidone). For the use of sodium hypochlorite in early summer, the researchers obtained an aseptic culture from the tops of shoots of *Ulmus procera* Salisb. [17]. Researchers [20] observed the prominent efficiency of regeneration of *U. glabra* plants due to the effects of meta-Topolin (6-(3-hydroxybenzylamino)purine) in the nutrient medium. MS [21], WPM [22], and DKW [23] media with the addition of antibiotics were used for the proliferation of microshoots. Dormant buds collected in February were washed with running water and sterilised in 0.1% $NaClO_3$ (10 min), followed by immersion in 0.01% $HgCl_2$ and rinsed three times in sterile distilled water [19]. Other researchers sterilised seeds collected from two *Ulmus parvifolia* 'Pathfinder' lines, P-6 and P-10, in 15% Clorox with 15 drops of Tween 20 for 15 min followed by rinsing in sterile distilled water. Under such sterilisation conditions, 100% of aseptic viable explants were obtained. Plant material was cultivated on DKW nutrient medium with 3% sucrose, 1.0 mg·l-^{1} thiamine, 0.5 mg·l^{-1} pyridoxine, 0.5 mg·l^{-1} nicotinic acid, 100 mg·l^{-1} inositol, 0.1 mg·l^{-1} 1 BA (6-benzylaminopurine) and 0.2% Phytogel, the pH was adjusted to the level of 5.8 [9].

At the same time, studies have found that the sterilisation mode of *Ulmus* plant material depends on several factors. Therefore, the right type of explant is selected experimentally, considering morphological and physiological features.

Materials and Methods

For research, the authors used 10-15 cm parts of shoots isolated from 25-year-old *U. laevis* in the autumn of 2021. As explants, 1.0-1.2 cm parts of shoots with one bud were used. Sterilisation of plant material involved exposure to a soapy solution with a few drops of Tween-80 (20 min), washing in tap water (10-12 min), rinsing in distilled water, immersion in 70% ethanol (2-3 min), sterilisation in chloramine solutions (1.0%, 2.5%, 5.0%, 10.0%) for 10 min and subsequent three-time washing in sterile distilled water (5-6 min each). The content of active chlorine in chloramine is ⩾ 26 mg·l^{-1}. At the stage of introduction into *in vitro* culture, nutrient medium was used according to WPM prescription. 100 mg·l^{-1} of inositol, 0.2 mg·l^{-1} of 2 – iP (6-(γ,γ-Dimethylallylamino)purine), 2.0 g·l^{-1} of activated carbon, 30 g·l^{-1} of sucrose and 7.0-7.3 g·l^{-1} of microbiological agar were added to the nutrient media. The acidity index of the medium (pH) was adjusted to 5.6. The total share of infected explants (%) was determined on the 10th day of cultivation (the ratio of the amount of infected plant material to the total amount introduced is expressed as a percentage). Biotechnological methods were applied, namely microclonal reproduction using the culture of fragments of plant shoots *in vitro* [24-26].

For microbiological analysis, sterilised explants (Fig. 1a) were cultured according to the accumulation method in Petri dishes with acidic potato agar [27], 5 pieces in each. The plant material was cultured in a thermostat without lighting at +26 ± 1°C and a relative humidity of 68 ± 2%. The samples were examined under a KONUS

BIOREX-3 microscope according to the method adopted in mycological studies [28]. The genus of isolated fungi was figured out according to a generally recognised identifier [29]. The intensity of infection of samples after sterilisation was investigated on the 7th day of cultivation according to DSTU 7127:2009 [27]. MS Excel software tools were used to process the research data. The results are presented as M ± m (M is the arithmetic mean, m is the standard error). One-way analysis of variance (ANOVA) was performed to analyse the influence of chloramine concentrations on the infection of explants. The study was conducted in the Plant Biotechnology Laboratory of the Separate Subdivision of the National University of Life and Environmental Sciences of Ukraine "Boyarka Forest Research Station" and in the State Organisation "Ukrainian Forest Breeding Centre" (SO "UFBC").

Results and Discussion

Using all experimental concentrations of chloramine for 10 days, significant mycoses were detected in explants of U. laevis (over 95% infection) cultivated on a modified nutrient medium for WPM tree species. Low asepsis, according to the authors, is caused by insufficient techniques during surface sterilisation of plant material, which is consistent with the results of other studies by other authors [30].

To stimulate the growth and development of microscopic fungi, sour potato agar was used as a nutrient medium in the experiment. Studies on the 2nd day of cultivation recorded the growth of mycobiota near the top of the *U. laevis* explant on the surface of the nutrient medium after using 1.0% chloramine (Fig. 1b).

Figure 1. Plant material of *U. laevis in vitro* on a nutrient medium: a) fragments of shoots as explants on the first day of cultivation (1 cm line); b) infected explants on the second day (arrows show the mycobiota); c), d) general appearance of infection of explants for 10 days using 5.0% chloramine

In case of 5.0% and 10.0% concentrations, infection was observed somewhat later (on 4-5 days). On day 10, over 95% of tissue contamination was recorded using all experimental concentrations of chloramine (Fig. 1c, d; Table 1, 2).

Table 1. Results of sterilisation of microshoots of *U. laevis* plants using chloramine *in vitro*, %

No. seq.	Chloramine concentration, %	Total proportion of infected explants, %	Intensity of explant infection with mycobiota*, %			
			Mucor Fresen.	*Penicillium* Link	*Chaetomium* Kunze	*Trichoderma* Pers.
1	1.0 %	97.4 ± 1.9	Strong	Weak	Single	Single
2	2.5 %	96.4 ± 1.6	Strong	Weak	Single	Single
3	5.0 %	95.6 ± 1.7	Strong	Single	–	–
4	10.0 %	95.8 ± 1.0	Single	–	–	–

*__Note:__ Sporadic – up to 5% of infection, weak – up to 25%, medium – up to 50%, and strong – over 50%

Table 2. Final results of univariate analysis of variance

Variance analysis						
Variation source	*SS*	*df*	*MS*	*F*	P_{value}	$F_{critical}$
Between groups	9.8	3	3.2667	0.26081	0.8526	3.2389
In the middle of groups	200.4	16	12.525			
Total	210.2	19				

where α is the level of reliability; *SS* is the sum of squares; *df* is the number of degrees of freedom; *MS* is the variances; *F* is the calculated value of the Fischer criterion; P_{value} is the calculated value of the minimum significance; F_{crit} is the critical value of the Fischer criterion

Morphological and physiological features of plant material isolated in the autumn period are one of the reasons for the significant intensity of infection, which is consistent with studies by other authors [14]. At the same time, according to the data of some researchers [7], it is shown that in autumn, the number of cases of release of potentially dangerous fungi, namely *Ceratocystis sp*. from samples of *Quercus robur* L. increases, compared to the summer period.

As a result of mycological analysis, microscopic fungi belonging to the *Zygomycota* and *Ascomycota* divisions were found. A mycobiota belonging to four genera *Mucor* Fresen., *Penicillium* Link, *Chaetomium* Kunze, *Trichoderma* Pers. was isolated from the chloramine-sterilised plant material of *U. laevis*. A group of researchers [7] isolated 129 species of microscopic fungi of various taxonomic groups in samples of different organs of control and drying out *Q. robur*, namely genera were present and identified by the authors in the microshoots of *U. laevis*. It was also found that the species composition of fungi differed slightly from that on roots, bark, and wood of *Q. robur* [7]. Pathogenic fungi of the genus *Ophiostoma* were not recorded in the samples of *U. laevis* under study.

The authors of this paper, unlike other researchers [9; 14; 17], did not obtain enough aseptic explants when using a chlorine-containing preparation. Thus, other researchers have shown the expediency of using sodium hypochlorite for introducing microshoots of *U. americana* [14] and *U. procera* [17] in the spring and summer period. According to research results, it is advisable to use 15% Clorox with 15 drops of Tween 20 for 15 minutes to sterilise *Ulmus parvifolia* "Pathfinder" [9]. The difference in results, according to the authors, is caused by the different period of isolation and the type of plant material. According to ANOVA results, the effect of 1.0-10.0% chloramine on total explant infection is statistically insignificant at a 5% level ($F < F_{crit.}$, $P<0.05$). At the same time, it was found that the intensity of infection with exogenous mycobiota of different genera differed. Microscopic fungi of the genus *Chaetomium* and *Trichoderma* were effectively neutralised using 5.0% and 10.0% chloramine; using 10% – *Penicillium*. The presence of *Mucor* mycobiota was observed on the explants under different sterilisation variants. Its increased intensity of infection was recorded on plant material treated with 1.0% and 5% preparation. It was found that in case of an increase in the chloramine concentration (from 1.0% to 10%), the intensity of infection decreased (from strong to sporadic).

Conclusions

The total share of infected explants of *U. laevis* isolated in the autumn period after exposure to 1.0-10.0% chloramine was over 95%. Among them, microscopic fungi of the genus *Mucor* Fresen., *Penicillium* Link, *Chaetomium* Kunze, *Trichoderma* Pers. were found. Chloramine acted on the corresponding mycobiota of *U. laevis* plant shoots. It was found that 5.0% preparation is effective for neutralising mycobiota of the genera *Chaetomium* and *Trichoderma*; 10.0% – for neutralising *Penicillium mycobiota*. By using experimental concentrations of chloramine, the presence of *Mucor* mycoses was recorded. In case of an increase in the chloramine concentration (from 1.0% to 10%), the intensity of infection decreases (for *Mucor* – from strong to sporadic). The effect of 1.0-10.0% chloramine on total explant contamination is statistically insignificant at the 5% level. As a result of the research, a small amount of aseptic cultures were obtained from the plant shoots of *U. laevis* isolated in autumn. Further studies are aimed at establishing the best mode of introduction of *U. laevis* plants for microclonal propagation.

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Скринінг дії хлораміну на мікобіоту тканин рослин *Ulmus Laevis* Pall. *in vitro*

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Анотація. Тканини в'язу гладкого (*Ulmus laevis* Pall.) *in vitro* – донорний матеріал для одержання культур зі стійкою резистенстністю до патологій інфекційного походження, зокрема до голландської хвороби. Для вирішення цієї задачі необхідно розробити ефективний протокол регенерації *U. laevis in vitro*. Дослідження дії концентрацій хлораміну на мікобіоту тканин рослин *U. laevis* для розмноження в умовах in vitro – мета дослідження. Як рослинний матеріал використовували 10–15 см частини пагонів із 25-річного U. laevis. Дослідження виконували восени 2021 року. Мікропагони, попередньо простерилізовані хлораміном (1.0 %, 2.5 %, 5.0 %, 10.0 %) упродовж 10 хв, культивували на твердому живильному середовищі за прописом WPM (McCown & Lloyd, 1981) з додаванням 0.2 мг·л$^{-1}$ 2 – iП (N-ізопентеніламінопурин) та 2.0 г·л$^{-1}$ активованого вугілля. Для мікробіологічного аналізу простерилізований рослинний матеріал культивували методом накопичення у чашках Петрі з живильним середовищем (кислий картопляний агар) у термостаті без освітлення за температури +26 ± 1 °C та відносної вологості повітря 68 ± 2 %. Використовували методи біотехнологічних, мікологічних та статистичних досліджень. Виявлено понад 95 % зразків інфікованих мікроскопічними грибами роду *Mucor* Fresen., *Penicillium* Link, *Chaetomium* Kunze та *Trichoderma* Pers. Вплив концентрації препарату на загальну кількість інфікованих експлантатів статистично незначущий на 5 % рівні. Установлено, що 5.0 % препарат ефективний для нейтралізації мікобіоти роду *Chaetomium* і *Trichoderma*; 10.0 % – *Penicillium*. У разі зростання концентрації хлораміну зменшується інтенсивність інфікування експлантатів мікобіотою різних родів. У результаті проведених досліджень із пагонів *U. laevis*, ізольованих восени, одержали асептичні культури у незначній кількості. Матеріал статті актуальний для біологів, біотехнологів, мікробіологів та науковців біологічного профілю

Ключові слова: в'яз гладкий, голландська хвороба ільмових, асептичність, інфікування, мікроклональне розмноження