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Peculiarities of determining the morphogenesis of plants *Corylus avellana* L. and *Prunus dulcis* (Mill.) D.A.Webb *in vitro* culture

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Abstract

The purpose of the research was to identify the physiological–biochemical and anatomical–morphological peculiarities found during *in vitro* cultivation of *Corylus avellana* L. and *Prunus dulcis* (Mill.) D.A.Webb, which occur as a result of the action of trophic and phytohormonal determinants. The research was conducted on three hazelnut varieties (Trapezund, Jefferson, Tonda Gentile Romana) and four almond varieties (E5 Borozan, M41 Alex, Georgia, Louise). A theoretical and experimental rationale for their use in the microclonal propagation of these cultures has been developed. The effectiveness of the preparatory stage before introduction into aseptic conditions for growing mother plants in closed soil conditions with scattered artificial lighting and microbiological protection has been proven. This reduced self-intoxication by oxidation products with phenol-like substances and microflora contamination of primary explants. Among the nutrient media compared, the best was Nas and Read (NRM) for hazelnuts and Nas Almond Medium (NAM) for almonds. Passaging on the same media leads to a decrease in regeneration indicators. In regenerants of almonds, the rosette of the shoots was noted, and in hazelnuts, the death of apical buds was also found. To prevent this, alternating NRM and driver and kuniyuki walnut (DKW) media for hazelnuts and NAM and Quirin and Lepoivre (QL) for almonds was effective. Long-term cultivation on media with a high content of synthetic analogues of phytohormones leads to the accumulation of phytotoxic effects with each subsequent passage. The phytotoxic effect of cytokinins was manifested in hyperhydration of shoots and of auxins in callus fertilisation. On comparing the ontogenesis of regenerants from explants isolated from mother plants aged 30–180 days, it was found that the optimal age for hazelnut and almond was 90 days. To reset the trophic and hormonal determinants, the introduction of explant donors into a state of dormancy has been successfully used. At the final fourth stage of microclonal propagation, in order to adapt plants simultaneously with an increase in the number of regenerants, it is effective to use the photoautotrophic method of microclonal propagation with intensive lighting and air enriched with carbon dioxide. For the transition of plants from conventional heterotrophic propagation to autotrophic cultivation, an intermediate stage of pre-adaptation of regenerants in wet chamber conditions is effective.

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Key words

cytokinin–auxin index, explant, heterotrophic and autotrophic nutrition, microclonal propagation, nutrient medium, regeneration, self-intoxication with phenol-like substances

Introduction

Growing demand for nut crops requires large-scale production of high-quality, disease-free planting material of new varieties. A reliable method of obtaining such plants is micropropagation. For the successful management of plant objects in these biotechnologies, it is necessary to perfectly use the determinants of ontogenesis. Firstly, these are trophic and hormonal factors, regulating growth and development, which affect the physiological–biochemical and anatomical–morphological features of plants *in vitro*.

Nut growing is a new branch of modern agricultural production of Ukraine. The main nut-forming species *Juglans regia* L. is cultivated throughout the territory of the country (Ishchuk et al. 2021). *Corylus avellana* L. is also common in most regions, and it grows successfully on irrigated lands in southern steppe. Almond (*Prunus amygdalus* (Mill.) D.A.Webb) has been cultivated successfully in the south and west of the country (Dubetska 2020). As niche crops, the possibility of microclonal reproduction and cultivation of sweet chestnut (*Castanea sativa* Mill.), common pecan (*Carya illinoinensis* (Wangenh.) K. Koch) and common pistachio (*Pistacia vera* L.) is being investigated (Tilkat et al. 2013; Gammoudi et al. 2022). In recent decades, nut growing has developed from an amateur level to a commercial one. In 2020, Ukraine ranked fourth in the world in terms of walnut exports and is the leader in the continent (Ishchuk et al. 2021). State support programmes are being developed for the creation of nut plantations (Satina et al. 2011).

Domestic research is mainly concerned with the problem of microclonal propagation of hazelnuts (Andrievsky et al. 2019; Tarasenko et. al 2020; Kızılkaya et al. 2022). A lot of work has been done in European countries regarding the microclonal propagation of hazelnuts and almonds, but in Ukraine, the scientific searches and industrial interest in these crops owing to increased demand for their fruits are developing rapidly. Simultaneous with the expansion of hazelnut production, taking into account the high soil and climatic potential and the market demand, the transition to new varieties, new technologies and the expansion of areas are induced and the need for seedlings increases accordingly. As for almonds, almost all of them are imported, despite the favourable conditions for their cultivation in the south and west of Ukraine (Dubetska 2020). For the rapid introduction of large-scale quantities of high-quality planting material of hazelnuts, the use of various modifications of microclonal propagation is relevant: classic on gel media, bioreactors with periodic flooding (TiS) and photoautotrophic methods (Matskevych et al. 2022). For the rapid introduction of hazelnuts and almonds, large-scale production and high-quality planting material are necessary. Modern technologies in the nursery of these crops involve the use of *in vitro* improvement and propagation. Biotechnological production uses living objects, determining the paths of ontogenesis according to technological needs.

Extensive research was carried out by Damiano et al. (2005), who made a protocol for effective micropropagation of hazelnuts of the Italian varieties ‘Montebello’ and ‘Tonda Gentile Romana’. It allows obtaining high-quality micro-plants without the phenomena of necrosis or hyperdigestion, which can be easily acclimatised. The aim of research by Yahyaoui et al. (2021) was to carry out sanitary treatment of varieties and clean plants from the most important viral pathogen – the apple mosaic virus. This research makes it possible to create *in vitro* true and virus-free hazelnut seedlings using apex encapsulation technology. In addition, the viability, regrowth and conversion rates of both conventional meristem tip cultures (MTC) and unconventional (MTC combined with encapsulation technology) sanitation methods were evaluated (Yahyaoui et al. 2021). The studies of Gao et al. (2008) were related to the propagation of five genotypes derived from *Corylus heterophylla* × *C. avellana* hybrids. The authors recommend using driver and kuniyuki walnut (DKW) as the main medium for microclonal propagation of this culture. At the same time, the survival rate was 90% when the plants were transplanted into a medium con-

sisting of vermiculite and peas. Bacchetta et al. (2008) elaborated the steps of sterilisation and culture propagation. In order to study genetic diversity and harmonise morphological and biochemical descriptors, scientists offer this procedure for a specific composition of the medium. The stages of rooting, which turned out to be one of the most important steps in achieving large-scale commercial application of hazelnut micropropagation, were studied (Bacchetta et al. 2015). Many studies have been devoted to diseases of the cultures. In particular, Hand et al. (2014) found out that *Brevundimonas* sp. and *Pseudomonas* sp. bacteria are most common in hazelnuts. Reed et al. (1997) and Yahyaoui et al. (2021) believe that persistent bacterial and fungal contamination is a serious problem in microclonal reproduction of the crops. The effect of carbon sources on the propagation of hazelnut shoots was studied by Yu et al. (1993).

The purpose of the research was to determine the peculiarities of determining the morphogenesis of plants *C. avellana* L. and *Prunus dulcis* (Mill.) D.A.Webb *in vitro* at various stages of microclonal propagation and develop theoretical and expected substantiation of microclonal cultivation.

Material and methods

Plants of three hazelnut varieties (Trapezund, Jefferson and Tonda Gentile Romana) and four almond varieties (E5 Borozan, M41 Alex, Georgia and Louise) were involved in the experiments.

Donors of explants were grown in the conditions of a closed soil depository according to Matskevych et al. (2019, 2022). Donors grown in the field were used as controls. Aseptic cultivation was carried out in transparent containers with a total volume of 250 ml. One explant was planted in one container at the stage of introduction under aseptic conditions, and five explants at other stages. The average values in one container were counted as one biological replicate. The number of repetitions at the first stage during decontamination was 50 primary explants and was 10 per each subsequent stages. Repeatability in time was three sessions.

Explant donor mother plants were prepared under artificial diffused lighting without UV radiation and with decapitation of the tops because under such conditions, less phenols are accumulated (Matskevych et al. 2019; Christofi et al. 2022). Phenol-like substances were isolated based on the principle that when phenolic compounds are oxidised to quinones, brown spots (coffee colour) are formed, which are visible (Filipova and Matskevych 2013).

Nutrient media (Tab. 1) were prepared based on the classical recipes of Murashige and Skoog (MS), Quirin and Lepoivre (QL), DKW (Driver and Kuniyuki 1984; Kushnir and Sarnatska 2005), Nas Almond Medium (NAM) and Nas and Read (NRM) (Nas and Read 2004; Nas et al. 2013). The acidity was adjusted to 5.8–5.9 for hazelnuts and 5.9–6.0 for almonds before autoclaving. The lighting period was 16 h per day. Lighting intensity was 2200 lx. The cultivation temperature was 24.0± 2.0°C.

The height of the regenerants was determined by the highest shoot in the conglomerate of shoots. In the part of the studies related to multiplication, hormones were added to 6-benzylaminopurine (BAP) 1.5 mg/l and indolyl butyric acid (IBA) 0.3 mg/l. At the third stage of microclonal reproduction, the following were added to induce rhizogenesis: BAP 0.3 mg/l and IBA 1.5 mg/l. A state of dormancy was entered at a temperature of 2.0°C–4.0°C.

Results and discussion

At the first stage of microclonal reproduction, along with decontamination, it is problematic to adapt the metabolism and change the hormonal status according to the new conditions of existence. In particular, it relates to the type of nutrition changes from autotrophic to mixotrophic with heterotrophic dominance. The addition of exogenous hormones analogues is compatible with other factors, for example, a change in correlations is a prerequisite for a change in the synthesis and action of endogenous hormones.

In the case of isolation of primary explants from donor plants, in addition to the disruption of relationships that existed in the regulation system of a whole organism, the formation of wounded surfaces occurs. As a protective reaction, phenol-like substances are oxidised to quinones on slices, in surface tissues and in growth points. In nature, these substances protect against damage by pests and oxidative stress. Also, in closed containers with a small volume of air and nutri-

Table 1. Composition of modified artificial nutrient media

Component	Medium composition, mg/l				
	MS_{mod}*	QL_{mod}	DKW_{mod}	NAM_{mod}	NRM_{mod}
NH_4NO_3	1650.0	400.0	1416.0	900	530
KNO_3	1900.0	1800.0	-	250	550
KH_2PO_4	170.0	270.0	265.0	1550	1300
$MgSO_4 \times 7H_2O$	370.0	360.0	740.0	2050	1650
K_2SO_4	–	–	1600.0	-	-
$Ca(NO_3)_2 \times 4H_2O$	–	833.8	1365	1050	700
$CaCl_2 \times 2H_2O$	440.0	–	150	45	90
$FeSO_4 \times 7H_2O$	27.80		33.8	–	
$Na_2MoO_4 \times 2H_2O$	37.3		45.4	–	
Ferrilene 4.8 Orto-Orto	–			114.63	
$MnSO_4 \times 4H_2O$	22.3	0.76	33.5	6.0	20.0
H_3BO_3	6.2		4.80	11.0	6.5
$ZnSO_4 \times 7H_2O$	8.6		–	11.0	8,8
$Zn(NO_3)_2$	–		17.0	–	–
$CuSO_4 \times 5H_2O$	0.025		0.25	3.2	2.5
$Na_2MoO_4 \times 2H_2O$	0.25		0.39	0.1	2.5
$CoCl \times 6H_2O$	0.025		–		
KJ	0.83	0.08	–		
$NiSO_4 \times 6H_2O$	–		0.0053	–	
BAP	1.5 during introduction and multiplication and 0.3 during rhizogenesis				
IBA	0.3 during introduction and multiplication and 1.5 during rhizogenesis				
Vitamin B_1	1.0				
Vitamin B_6	0.6				
Vitamin PP	1.0				
Vitamin C	3.0				
Meso-inositol	100.0				
Glycine	1.0				
Sucrose/glucose	Sucrose 30×10^3 almond; glucose 15×10^3 hazelnut				

Note: 'mod' – means to 'modified'

ent medium, self-poisoning occurs in non-adapted primary explants (Kim et al. 2006; Filipova and Matskevych 2013).

During the primary cultivation, the influence of *in situ* plant growing conditions and trophic determinants of different mineral-modified artificial nutrient media was established (Tab. 2).

First of all, the influence of biological features of primary explants, namely species and variety, on poisoning by oxidation products of phenol-like substances was identified. The number of losses was higher in hazelnut explants compared to almonds. In most explants, the entire object or point of bud growth had a 'phenolic colour'. In almonds, the oxidation of phenol-like substances prevailed in the basal part of the explants, and a part of such explants survived due to the proliferation of buds.

Differences between varieties were also noted. Among the three hazelnut cultivars compared, Tonda

Gentile Romana had the most explants with phenol poisoning, and Jefferson the least. In almonds, the most explants with phenolic secretions were noted in the E5 Borozan variety and the least in the Louise variety.

Table 2. Self-intoxication of primary explants depending on the medium and growing conditions of donor plant varieties, %

Variety	Nutrient medium				
	MS_{mod}	QL_{mod}	DKW_{mod}	NAM_{mod}	NRM_{mod}
Hazelnut					
Trapezund	100/16*	98/11	83/4	70/2	24/1
Jefferson	98/19	91/12	77/9	63/1	22/0
Tonda Gentile Romana	100/32	98/26	90/11	66/3	29/2
Almond					
E5 Borozan	23/12	6/2	21/18	6/1	8/3
M41 Alex	19/12	5/2	12/9	0/0	7/2
Georgia	14/10	3/1	11/9	0/0	4/0
Louise	8/6	-	6/5	0/0	1/0

*Numerator: self-intoxication of explants isolated from mother plants grown in the field (control); denominator: grown in the conditions of the depository

Phenol formation and control of this phenomenon were dependent on the species and measures taken to prepare the mother plants. For example, on the MS_{mod} medium, in the hazelnut explants isolated from grown donor explants in the depository, intoxication decreased by 3–5 times depending on the variety (from 98%–100% to 16%) and in almonds from 8%–23% to 6%–12%, that is, less than twice.

It should be noted that glucose was added to the media for hazelnuts as a source of carbohydrates, and for almonds, sucrose was added to exclude the influence of carbohydrates, and primary explants were also planted on media with sucrose. On such media, the number of explants with self-intoxication was 3%–5% higher compared to media with glucose. Hazelnut regenerants on the medium with sucrose were also characterised by relatively slower growth, the presence of callus in the basal part and partial tissue hyperhydration.

The composition of the medium also influenced the appearance of toxic compounds. Both almond and hazelnut varieties produced the most phenol-like substances on the MS_{mod} medium. The lowest activity of toxin formation in both species was on NAM_{mod} and NRM_{mod} media.

Nutrient media differed among themselves in the content of mineral elements, which had a trophic-determining effect on the ontogenesis of regenerants, in particular, on biometric indicators (Tab. 3). The highest hazelnut regenerants on the 90th day of cultivation were on the NRM_{mod} medium, on average for three varieties; 10% smaller were the regenerants on the DKW_{mod} medium. This difference was found in both the total length of the shoot and the number of internodes.

For almond regenerants of all four varieties, the NAM_{mod} medium was the best. MS_{mod} and DKW_{mod} media were technologically unacceptable for these varieties of plants.

Table 3. The height of regenerants on the 90th day of *in vitro* cultivation on different nutrient media

Variety	Nutrient medium				
	MS_{mod}	QL_{mod}	DKW_{mod}	NAM_{mod}	NRM_{mod}
	Height of regenerants, mm				
Hazelnut					
Trapezund	59/23*	63/27	98/79	86/74	116/91
Jefferson	41/16	58/32	108/87	88/75	109/101
Tonda Gentile Romana	79/29	72/35	111/86	99/70	121/97
Almond					
E5 Borozan	84/21	93/73	39/12	123/98	97/69
M41 Alex	78/18	84/71	37/13	134/94	96/66
Georgia	71/13	69/63	33/15	103/84	81/61
Louise	45/12	67/64	23/12	101/85	77/63

* In the numerator is the height of regenerants during the first cultivation; in the denominator is the height of regenerants during the fifth cultivation.

Based on the results of these comparisons, NRM_{mod} was used for hazelnut in further studies as the main medium and DKW_{mod} as the auxiliary unloading medium.

In the process of long-term vegetative reproduction, which is *in vitro* passage, the negative impact of a lack of some and/or an excess of other nutrients is constantly accumulated on one variant of the medium (Terek and

Patsula 2011; Matskevych et al. 2022). In the case of our studies with grafting, the overlay method led to a decrease in the biometric dimensions of the regenerants for the same period of growth (90 days).

Cultivation of explants was on an 'unloading' medium every five passages (Tab. 4). For hazelnuts, the main medium was NRM_{mod}, and DKW_{mod} was used as an unloading medium. For almonds, NAM_{mod} was the main one and QL_{mod} was used as an unloading medium.

For all three varieties of hazelnuts on DKW_{mod}, the main effect of unloading medium (NRM_{mod}) allowed the seventh passage regenerants to have higher shoot height rates compared to the fifth passage regenerants. However, for 12th passage, in the varieties of Trapezund and Jefferson, the rate was reduced from 111 and 110 to 77 and 83 mm, respectively. In the 'Tonda Gentile Roman' variety, the height of the shoots at the fifth and seventh passages was within 91–122 mm due to the use of the NRMmod unloading medium.

Table 4. The height of regenerants on different unloading media on the 90th day of propagation, mm

Variety/media	Nutrient medium					
	DKW_{mod}	ULM*	DKW_{mod}	NRM_{mod}	ULM	NRM_{mod}
	Passage					
	5	6	7/12	5	6	7/12
Hazelnut						
Trapezund	79	100	111/77	91	109	133/98
Jefferson	87	106	110/83	101	112	139/104
Tonda Gentile Romana	86	113	122/91	97	108	151/ 99
Variety/media	QL_{mod}	ULM	QL_{mod}	NAM_{mod}	ULM	NAM_{mod}
	Passage					
	5	6	7/10	5	6	7/10
Almond						
E5 Borozan	73	106	109/65	98	112	114/95
M41 Alex	71	102	106/54	94	116	109/81
Georgia	63	91	98/57	84	94	103/69
Louise	64	97	101/50	85	90	/94/69

* ULM corresponds to the 'unloading medium', which was different in the composition of mineral elements

In the case of the combination NRM_{mod} as the main medium and DKW_{mod} as the unloading medium, the height of regenerators' shoots on the 12th passage was more than that of those on the fifth passage. Thus, this combination of rotation nutrient media is effective for maintaining a sustainable growth of regenerants.

The use of QL_{mod} and NAM_{mod} combinations was not effective for maintaining sustainable regeneration potential during the passaging till 10th internal passage for the almond varieties involved in the study. Transplanting to another medium (sixth, seventh passages) gave an increase in height. However, in the subsequent passages, there was a decrease in this indicator in regenerants. The QL_{mod} medium of more than half of the plants was inherent in rosette, that is, shortening of the shoot.

NRM for hazelnut and NAM for almond with changed cytokinin and auxin contents were also used as unloading media. The essence of such combinations was that the sixth passage was made on the medium with a changed hormonal balance (Tab. 5). For example, at the multiplication stage, the main medium contained BAP 1.5 mg/l and IBA 0.3 mg/l and the unloading medium contained BAP 0.3 mg/l and IBA 1.5 mg/l. That is, the cytokinin–auxin index in this medium composition (ratio of cytokinin BAP and auxin IBA) was changed in the unloading medium with 1.5/0.3 by 0.3/1.5. Also, in the case of induction of rhizogenesis, the cytokinin–auxin index varied on the contrary from 0.3/1.5 to 1.5/0.3.

The regenerants of both cultures were bigger height under the predominance of auxins over cytokinins (1.5/0.3). But plants usually formed one shoot. However, prolonged cultivation in such predominance of auxins leads to a decrease in the regeneration potential, decrease in the height of regenerants and the germination factor (Fig. 1). We believe that during vegetative germination by passaging, there is an accumulation of excess of both exogenous and endogenous phytohormones, including auxins and cytokinins (Terek and Patsula 2011; Matskevych et al. 2022).

The use of a medium with a changed hormonal balance was effective, but over time, the growth of the shoot as well as the regenerative performance decreased. The most sensitive among the variants under study were all four varieties of almonds in the third stage of microclonal propagation – multiplication.

Table 5. The height of regenerants on the 90th day of propagation with different selection of passages and cytokinin–auxin index, mm

Variety	Multiplication			Induction of rhizogenesis		
	Passage					
	5	6	7/10	5	6	7/10
	Cytokinin–auxin index					
	1.5/0.3	0.3/1.5	1.5/0.3	0.3/1.5	1.5/0.3	0.3/1.5
Hazelnut						
Trapezund	91	114	109/90	109	129	130/99
Jefferson	101	121	97/94	117	132	139/103
Tonda Gentile Romana	97	124	111/96	103	121	132/97
Almond						
E5 Borozan	98	103	100/91	102	107	103/104
M41 Alex	94	100	97/89	108	112	107/101
Georgia	84	91	89/83	94	99	97/91
Louise	85	93	86/74	96	97	98/90

The effect of accumulation was analysed on regenerants of fifth, seventh and 10th passages with different concentrations of the synthetic cytokinin BAP against a background of 0.3 mg/l IBA in the following concentrations: 0.5, 1.0, 1.5 (control), 2.0 and 2.5 mg/l (Tab. 6).

Table 6. The height of the shoot of regenerants at different concentrations of BAP for fifth, seventh and 10th passages by the overlay passage method, mm

Variety	BAP concentration, mg/l				
	0.5	1.0	1.5	2.0	2.5
Hazelnut					
Trapezund	92/90/90*	98/94/96	91/83/79	71/66/62	61/51/-
Jefferson	100/102/99	105/107/99	98/95/83	89/76/74	70/65/61
Tonda Gentile Romana	98/97/100	96/98/97	93/88/85	73/58/53	59/52/50
Almond					
E5 Borozan	76/74/78	99/98/98	98/64/52	51/43/41	36/-/-
M41 Alex	71/73/75	102/103/96	94/61/52	46/38/36	33/-/-
Georgia	69/66/68	97/98/96	84/55/51	41/32/30	30/22/-
Louise	67/66/64	94/92/95	85/82/56	44/33/33	18/-/-

* 5/7/10 – indicators in the fifth, seventh and tenth passages

For all varieties of hazelnuts and almonds, it was found in the experiment that the tallest plants in vitro were on the version with the addition of 1.0 mg/l of BAP. Here the plants were

Figure 1. Reduction of biometric dimensions of regenerants of the Jefferson variety during long-term cultivation on a medium with an excess of auxins on 30th day of cultivation (the figures correspond to the passage number)

10–15 mm taller than the control plants in the first five passages. But in the following passages, their growth and reproduction ratio decreased (Fig. 2). Also, such plants had one shoot and, accordingly, a lower reproduction ratio.

This ratio initially increased the variants with higher concentrations of cytokinin, but the regenerants showed signs of hyperhydration. Leaf plates were elongated and reduced in size. In subsequent passages, both the length of the shoots and their number of conglomerates decreased. In addition, such plants did not form a root system even when transplanted to a medium with a predominance of auxins over cytokinins (BAP 0.3 mg/l and IBA 1.5 mg/l).

Long-term passaging on media with a combination of 0.3 BAP and 1.5 IBA (combination for the induction of rhizogenesis) also led to the loss

Figure 2. The condition of hazelnut regenerants of the Tonda Gentile Romana variety on a medium with 1.0 mg/l BAP and 0.3 IBA on the 30th day of propagation (the figures correspond to the passage number)

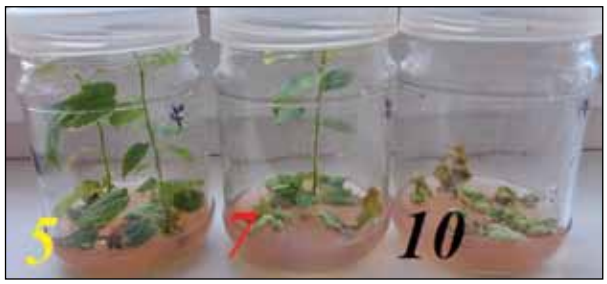

Figure 3. The condition of regenerants of the hazelnut variety Tonda Gentile Romana on a medium with 0.3 mg/l BAP and 1.5 IBA on the 30th day of propagation (the figures correspond to the passage number)

of the ability to root and morphogenesis with the formation of callus after the fifth passage (Fig. 3).

According to several studies, phytotoxic hormonal imbalance *in vitro* with high concentrations of cytokinins can be levelled by the addition of gibberellins (Kushnir and Sarnatska 2005; Terek and Patsula 2011; Filipova and Matskevych 2013). We tested the effectiveness of using the following combination: BAP 2.5 mg/l, IBA 0.3 mg/l and gibberellin 1.5 mg/l. With the specified combination of synthetic hormones, conglomerates of five to seven microshoots were formed in the first cultivation of hazelnut and almond regenerants. However, in subsequent passages on the medium with the specified combination of synthetic hormones, signs of hyperhydration, e.i the biometric parameters of the shoots decreased: a stems became thinner and the leaf plates were narrowed and twisted (Fig. 4).

The addition of synthetic analogues of both classes of hormones in high concentrations led to the formation of callus and the loss of tissue differentiation of both individual organs (e.g. the edge of the leaf blade) and the entire explant. Simultaneous addition of high concentrations of BAP cytokinin and IBA auxin led to intense callus formation (Fig. 5). These hormones stimulate division of the nucleus and the cell as a whole. As a rule, dedifferentiation occurred in the basal part of the stem of the cutting explant and in the places of contact of leaves with the nutrient medium.

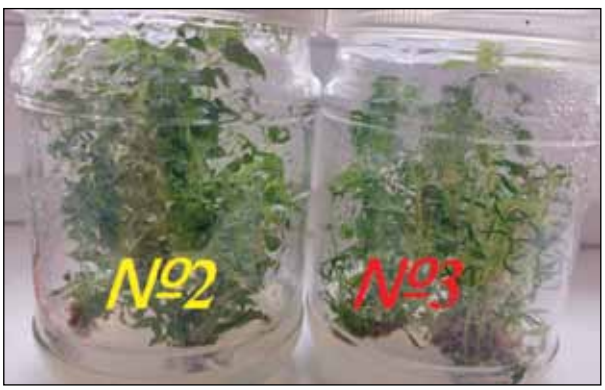

Figure 4. Hyperhydration of hazelnut regenerants of the Tonda Gentile Romana variety during passaging on media with the addition of 2.5 mg/l BAP and 1.5 mg/l gibberellin (the figures correspond to the passage number)

All four almond varieties in the study were more sensitive to excess hormones compared to the three hazelnut varieties. In almonds, concentrations above 1.5 mg/l formed a dark callus with necrosis of 60% or more. Light green callus was formed in hazelnut explants when the highest concentration (2.5 mg/l) was added. After transplanting to a medium with 1.0–1.5 mg/l gibberellin, indirect morphogenesis occurred in callus tissues. Thus, the addition of 1.5 mg/l BAP and IBA can be used to induce callusogenesis in almond explants. Also, in hazelnut explants, dedifferentiation processes are successful at concentrations of 2.5 mg/l of each hormone.

In our opinion, the different response of two botanical plant species to exogenous hormones is related to the activity of cambial tissues. Hazelnuts have lower cambium activity compared to almonds. So, they respond more positively to the addition of exogenous hormones, particularly cytokinins.

The development of regenerants by phytohormones is the result of the interaction of endogenous hormones and exogenous synthetic analogues or substances with prohormonal activity. Their quantitative and qualitative states, that is, the number and ratio between active, bound and destroyed oxidoreductase enzymes,

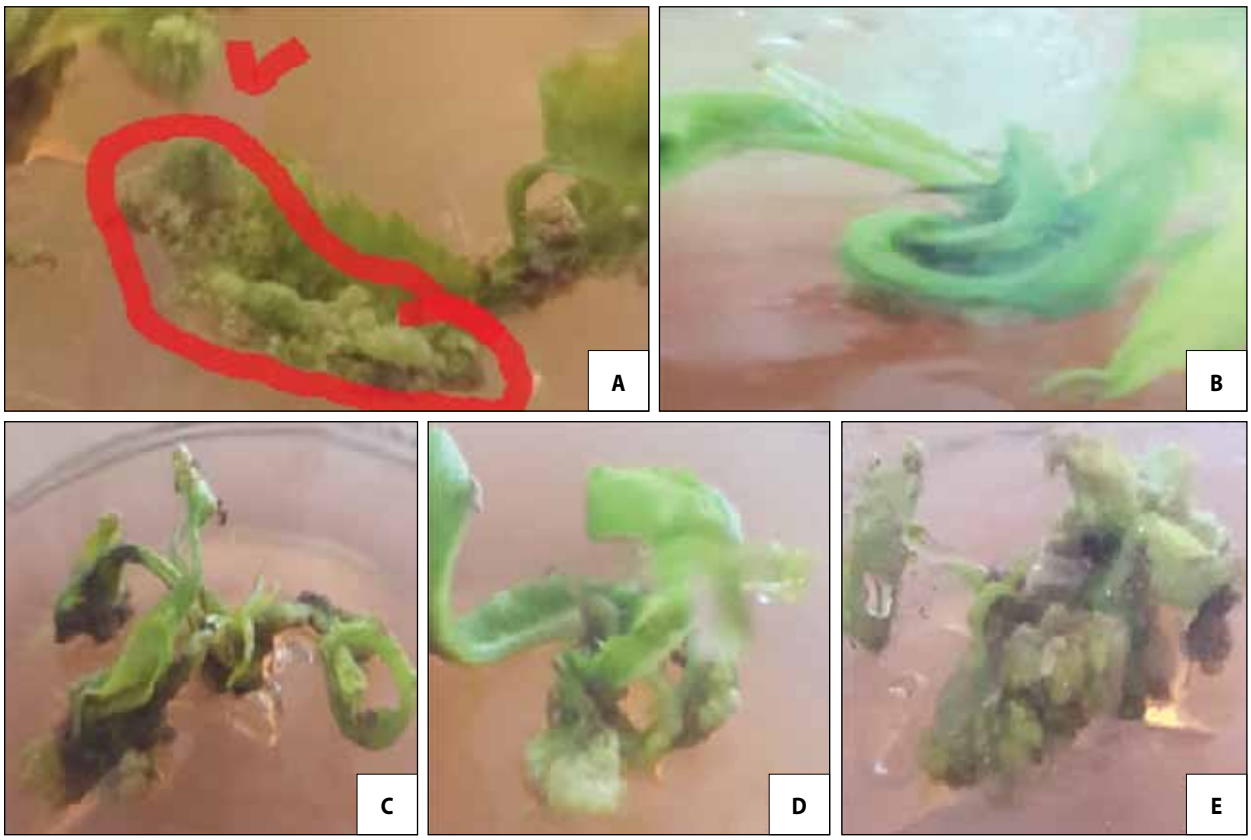

Figure 5. Callus formation *in vitro* in hazelnut and almond explants with the simultaneous addition of high concentrations of cytokinin BAP and IBA to the nutrient medium: (a) callus formation of hazelnut regenerants of the Tonda Gentile Romana variety on the medium with the addition of 2.5 mg/l BAP and 2.5 mg/l IBA; (b) rosette stem of almond regenerants of variety M41 Alex on the medium with the addition of 1.0 mg/l BAP and 1.0 mg/l IBA; (c) rosette stem and callus formation of almond regenerants of variety M41 Alex on the medium with the addition of 1.5 mg/l BAP and 1.5 mg/l IBA; (d) callus formation of almond regenerants of the M41 Alex variety in the basal part of the cutting explant on the medium with the addition of 2.0 mg/l BAP and 2.0 mg/l IBA; (e) callus formation of almonds of the M41 Alex variety over the entire surface of the cutting explant on the medium with the addition of 2.5 mg/l BAP and 2.5 mg/l IBA

change during the period of *in vitro* cultivation. We investigated the influence of the age of the mother plants of explant donors on the ontogeny of regenerants.

We compared the ontogenesis of regenerants from explants isolated from mother plants at the following ages: 30, 45, 90, 120 and 180 days. In particular, the influence of the age of donors of scion explants on the height of regenerants was determined (Tab. 7). In aseptic cultures of both botanical species, increasing the age of the original mother plants to 120 and 180 days had a positive effect on the height of the regenerants. The height of regenerated plants at the fifth, seventh and 10th passages remained without significant changes.

Spontaneous root formation was noted in some of the regenerants from explants of donor plants aged 180 days, even on the medium for multiplication. Visually, such regenerants had a thickened stem and leaves with a wider leaf blade. They had no signs of tissue hyperhydration. However, in cases of weak aeration (e.g. in case of technical failures of the ventilation system) the symptoms of ethylene poisoning were observed in regenerants aged 120–180 days: interveinal chlorosis, general chlorosis and leaf fall. It is known that old plants can not only self-poison with ethylene, but also be a source of this volatile hormone for other plant objects in the laboratory (Matskevych et al. 2019).

Table 7. Shoot height of regenerants depending on the age of mother plants of explant donors at different periods of passage, mm

Variety	Age of donors, days				
	30	45	90	120	180
Hazelnut					
Trapezund	35/24/-	49/41/32	97/84/81	112/109/115	116/114/119
Jefferson	36/31/29	41/32/29	98/95/83	123/127/119	122/127/124
Tonda Gentile Romana	39/30/-	44/35/31	93/89/85	126/119/129	129/133/130
Almond					
E5 Borozan	28/-/-	54/52/36	98/84/52	101/111/113	143/148/149
M41 Alex	17/-/-	47/43/26	94/81/52	98/95/103	141/146/145
Georgia	-/-/-	36/33/-	84/65/51	89/91/92	103/105/104
Louise	24/-/-	39/37/30	85/72/56	96/99/99	107/109/110

At the same time, during mass reproduction with long intervals between passages (120–180 days), there are problems of a non-biological nature: relatively low reproduction rate during the year, cost increase, drying of the nutrient medium and an increase in secondary contamination, which is difficult to detect on old nutrient media.

However, the use of such donors is effective in cases where there is a possible loss of regeneration potential in cultivated plants, which decreases with each subsequent passage.

The use as donors of mother plants aged less than 90 days, namely 45, 30 days, is technologically not expedient due to the loss of the ability of explants to morphogenesis, increased phytotoxic effect of an excess of cytokinins and hyperhydration of tissues.

Not all varieties of both hazelnuts and almonds on the 30- and 45-day options were able to obtain plants capable of direct morphogenesis up to the 10th passage. Almond varieties were more vulnerable to age reduction to 45 days or less. In the Georgia variety, direct morphogenesis was absent by the fifth passage. The loss of regeneration potential during such passages began in the following sequence: shortening and thickening of the shoot, outlet quality, hyperhydration, callus formation; necrotisation. During the passaging of hazelnuts, the sequence is follows: shortening of the shoot, hyperhydration, death of growth points.

Thus, in the process of long-term *in vitro* cultivation under the influence of suboptimal trophic and hormonal factors, the determinant undergoes a partial disorganisation of the processes in ontogenesis over time. Entering a state of rest is one of the methods of restarting the system of determinants, in particular, changing the hormonal status (Podhaetskyi et al. 2020). When the bud of the future explant regenerated from it enters a dormant state, it again goes through the stages of organogenesis and ontogenesis. The peculiarities of the stages of organogenesis and the life cycle of a plant regenerated from such an explant will be determined by the new conditions. Therefore, there is a high probability of restarting the systems of trophic, phytohormonal determinants, which will eliminate the factors that lead to the accumulation of phytotoxic substances.

In order to put plants into a dormant state *in vitro,* regenerants aged 90 days were placed in refrigerating chambers with a gradual decrease in temperature over 10 days from 24°C to 2.0°C–4.0°C. Removal from this state was carried out by reverse temperature increase. The influence of the length of stay of the mother plants for the periods 45, 90 and 120 days (Tab. 8) on the height of the progeny was studied. The progeny of plants that were not put into a state of dormancy (0 days) was taken as control.

Table 8. The height of the progeny shoots with different duration of the dormant state of explant donors, mm

Variety	Age of donors, days			
	0	45	90	120
Hazelnut				
Trapezund	99	114	139	131
Jefferson	98	114	154	152
Tonda Gentile Romana	96	107	133	138
Almond				
E5 Borozan	95	102	109	110
M41 Alex	91	100	103	101
Georgia	87	93	108	105
Louise	84	98	112	121

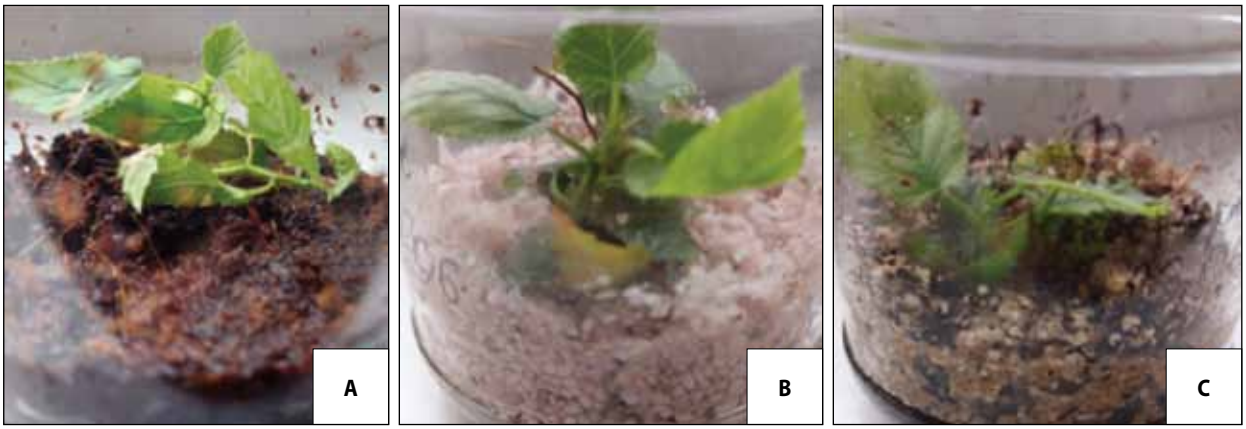

Figure 6. *In vitro* cultivation of Tonda Gentile Romana hazelnut explants during pre-adaptation: (a) coconut; (b) pearlite; (c) a mixture of pearlite and coconut (1:1)

The height of the shoots was higher in variants with the introduction of explant donors in a resting state compared to the control. Hazelnut plants on variants with duration of dormancy of 120 days had covering scales formed on the buds and a change in the colour of the stem, which is characteristic of semi-lignified shoots of this species in nature. The option with a dormant period of 45 days was inferior to the ones with a dormant period of 90 and 120 days. The mother plants had herbaceous stems and relatively smaller buds in the leaf axils. Also, the regenerated progeny had smaller growths.

According to the shoot height index, the regenerated progeny on the 90 and 120 variants did not differ significantly. However, regenerants from the variant with a dormancy period of 120 days for mother plants had a relatively thicker shoot and a more branched root system. In almonds, the trend was similar to that of hazelnuts.

A significant reboot of the determinant occurs at the fourth stage of the microclonal propagation. Along with the activation of genes that are carriers of processes such as xylem and root formation, the expression of genes encoding later stage of ontogenesis is necessary. This includes, in particular, the formation of thickened membranes of covering tissues, increased resistance to factornonstatic *ex vitro* conditions. Santos et al. (2009) found that in almonds, active genes encoding protective proteins were found only at a later stage of ontogenesis compared to genes associated with processes of structural protein synthesis, carbon and nitrogen metabolism. In the case of the last stage of microclonal propagation, there should be a transition from a deep juvenile state with heterotrophic nutrition to one in which genes encoding later stage of ontogenesis are expressed.

Photoautotrophic methods of microclonal propagation make it possible to obtain regenerants with simultaneous adaptation to conditions *in vitro*. Accumulation of organic substances occurs exclusively autotrophically with the intensification of photosynthesis. This is confirmed anatomically, as mother plants (explant donors) and regenerated offspring are closer to natural conditions (Kozai and Kubota 2005; Matskevych et al. 2019). Previously, we proved the effectiveness and feasibility of propagation and adaptation using this method on blackberries and hazelnuts (Kozai and Kubota 2005; Matskevych et al. 2022). However, in the first days of cultivation, there is partial damage to the photoassimilating apparatus by intense lighting (11,000 lx). Therefore, we believe that there should be an intermediate pre-adaptation period at the transition from 'classical' *in vitro* conditions (2–3 thousand lux lighting, mainly heterotrophic nutrition).

To select the conditions for intermediate adaptation (pre-adaptation), we compared the growth efficiency of regenerants on three types of substrates (Fig. 6): coconut, pearlite and their mixture in the same ratio by volume in a moistened state.

Plants were cultivated in one group of variants in aseptic conditions for 90 days with autotrophic nutrition (culture containers with a volume of 250 ml), and in the second group of variants, 90-day-old plants grown in aseptic conditions with heterotrophic nutrition were transplanted into micro greenhouses with

Table 9. Peculiarities of the photoautotrophic microclonal propagation of hazelnut plants with different pre-adaptation of the Tonda Gentile Romana variety

Indicator	Mediumт					
	coconut		pearlite		mixture of pearlite and coconut (1:1)	
	Germination conditions					
	in vitro	micro greenhouse	*in vitro*	micro greenhouse	*in vitro*	micro greenhouse
Survival rate, %	58	67	60	69	69	94
Damaged leaf area, %	12	27	15	19	12	14
The height of plants on the 14th day of growing FMP, mm	189	213	162	184	221	267

a film wet chamber for 6 days. Thus, adaptation to autotrophic nutrition in one case occurred during regeneration from a cutting explant under sterile conditions. And in the second case, regenerants from heterotrophic nutrition were temporarily (6 days) transferred to the substrate in a humid chamber. Then, plants from both groups were transferred to a photoautotrophic module with intense lighting and air with enriched carbon dioxide content. Differences in adaptation were found depending on the pre-adaptation of regenerant plants in conditions of photoautotrophic microclonal propagation (Tab. 9).

In this way, the transition of plants *in vitro* took place during the regeneration of the plant from cutting. In the second case, adaptation took place at the level of the formed plant. On all three types of substrates, the plants that were pre-adapted in vitro had lower survivability. However, the leaf plate burns were smaller. In our opinion, this is connected with the transition from juvenile to older periods of ontogenesis, during which the adaptive potential increases but the regenerative potential decreases.

Although the plants regenerating after heterotrophic nutrition after pre-adaptation in a humid chamber lose part of the photoassimilating apparatus, due to their juvenile state, they had higher biometric indicators. In particular, in terms of height, they were superior compared to the variants of pre-adaptation under *in vitro* conditions.

Among the substrates, both in *in vitro* culture vessels and in wet chamber cassettes, the option using a mixture (1:1) of coconut and pearlite was the best.

Conclusion

The determination of the processes of juvenilization, adaptation to *in vitro* and *ex vitro* conditions and the transition to the generative stage of ontogenesis have been established. The biochemical processes in plant objects occurring due to a change in metabolism were visually manifested in a change in the hydration of tissues and in the synthesis of phenol-like substances. Environmental components (nutritional elements, hormones) determined the following anatomical–morphological changes: apical dominance, branching, rhizogenesis and the size of vegetative organs.

To prevent poisoning by oxidation of phenol-like substances, which are products of primary hazelnut explants, it is advisable to apply the proposed measures for the preparation of donors of primary explants.

It was found that among the media compared, for hazelnut, the most suitable were alternating modified media DKW and NRM and for almond, the most suitable were NAM and NRM.

Among the compared donors of hazelnut and almond explants, the best results were obtained with the use of 90-day-old plants *in vitro*.

Periodic introduction of donor explants into a state of dormancy is one of the measures to preserve long-term and stable passage of hazelnuts and almonds.

The photoautotrophic method of microclonal propagation is effective for hazelnuts. The effectiveness of this method increases under conditions of intermediate adaptation in humid chambers.

References

Andrievsky, V., Vrublevsky, A., Filipova, L., Matskevych, V., Matskevych, O. 2019. The problems of hazelnut microclonal propagation. *Agrobiology*, 1 (149), 74–84. https://agrobiologiya.btsau.edu.ua/en/content/problems-hazelnut-microclonal-propagation

Bacchetta, L., Aramini, M., Bernardini, C., Rugini, E. 2008. *In vitro* propagation of traditional Italian hazelnut cultivars as a tool for the valorization and conservation of local genetic resources. *HortScience*, 43 (2), 562–566. DOI: https://doi.org/10.21273/HORTSCI.43.2.562

Bacchetta, L. et al. 2015. A multidisciplinary approach to enhance the conservation and use of hazelnut *Corylus avellana* L. *Genetic Resources and Crop Evolution*, 62, 649–663. DOI: https://doi.org/10.1007/s10722-014-0173-7

Christofi, M. et al. 2022. Profiling carotenoid and phenolic compounds in fresh and canned fruit of peach cultivars: Impact of genotype and canning on their concentration. *Journal of Food Composition and Analysis*, 114. DOI: https://doi.org/10.1016/j.jfca.2022.104734

Damiano, C., Caternaro, J., Giovinazzi, J., Fratarelli, A., Caboni, E. 2005. Micropropagation of hazelnut (*Corylus avellana* L.). *Acta Horticulturae*, 686 (1), 221–226.

Driver, J., Kuniyuki, A. 1984. *In vitro* propagation of paradox walnut rootstock. *HortScience*, 19 (4), 507–509. DOI: https://doi.org/10.21273/HORTSCI.19.4.507

Dubetska, M. 2020. Almond : restoration of powerful roots (in Ukrainian). *Horticulture and Viticulture. Technologies and Innovations*, 3 (22), 90–93.

Filipova, L., Matskevych, V. 2013. Formation of phenolic substances by regenerants during the first subcultivations concerning the conditions and plant species (in Ukrainian). *Journal of Lviv National Environmental University: Agronomy*, **17 (2), 233–239.**

Gammoudi, N., Nagaz, K., Ferchichi, A. 2022. Establishment of optimized in vitro disinfection protocol of *Pistacia vera* L. explants mediated a computational approach: multilayer perceptron–multi−objective genetic algorithm. *BMC Plant Biology*, 22, 324. DOI: https://doi.org/10.1186/s12870-022-03674-x

Hand, C., Maki, S., Reed, B. 2014. Modeling optimal mineral nutrition for hazelnut micropropagation. *Plant Cell, Tissue and Organ Culture*, 119, 411–425. DOI: https://doi.org/10.1007/s11240-014-0544-y

Ishchuk, H., Shlapak, V., Ishchuk, L., Bayura, O., Kurka, S. 2021. The introduced North American species of the genus *Juglans* L. in the Right-bank forest-steppe of Ukraine and their use. *Trakya University Journal of Natural Sciences*, 22 (1), 77–92. http://lib.udau.edu.ua/handle/123456789/8090

Kim, J., Mahoney, N., Chan, K., Molyneux, R., Campbell, B. 2006. Controlling food-contaminating fungi by targeting their antioxidative stress-response system with natural phenolic compounds. *Applied Microbiology and Biotechnology*, 70 (6), 735–739. DOI: https://doi.org/10.1007/s00253-005-0123-6

Kızılkaya, R., Dumbadze, G., Gülser, C., Jgenti, L. 2022. Impact of NPK fertilization on hazelnut yield and soil chemical-microbiological properties of Hazelnut Orchards in Western Georgia. *Eurasian Journal of Soil Science*, 11 (3), 206–215. DOI: https://doi.org/10.18393/ejss.1060314

Kosenko, I.S. et al. 2017. Hazelnut breeding in the National Dendrological Park «Sofiyivka» of the NAS of Ukraine. *Plant Varieties Studying and Protection*, 3 (13), 245–251.

Kozai, T., Kubota, C. 2005. *In vitro* aerial environments and their effects on growth and development of plants. In: Photoautotrophic (sugar-free medium) micropropation as a new micropropagation and transplant production system (eds. T. Kozai, F. Afreen, S.M.A. Zobayed), 31–52. DOI: https://doi.org/10.1007/1-4020-3126-2_4

Kushnir, H., Sarnatska, V. 2005. Microclonal propagation of plants, theory and practice (in Ukrainian). *Scientific Opinion*, 270.

Matskevych, O., Kimeichuk, I., Matskevych, V., Karpuk, L. 2022. Microclonal propagation of hazelnuts (in Ukrainian). *Bulletine of Uman NUH*, 1, 105–114. DOI: https://doi.org/10.31395/2310-0478-2022-1-105-114

Matskevych, V., Podhaetskyi, A., Filipova, L. 2019. Microclonal propagation of certain plant species (technology protocols): a scientific and practical guide

(in Ukrainian). *Bila Tserkva National Agrarian University*, 84. DOI: http://rep.btsau.edu.ua/handle/BNAU/3247

Nas, M., Bolek, Y., Sevgin, N. 2013. Shortcut to long-distance developing of a tissue culture medium: micropropagation of mature almond cultivars as a case study. *Turkish Journal of Botany*, 37 (6), 1134–1144. DOI: https://doi.org/10.3906/bot-1302-49

Nas, M., Read, P. 2004. A hypothesis for the development of a defined tissue culture medium of higher plants and micropropagation of hazelnuts. *Scientia Horticulturae*, 101 (1/2), 189–200. DOI: https://doi.org/10.1016/j.scienta.2003.10.004

Podhaetskyi, A., Matskevych, V., Filipova, L., Kravchenko, N., Hnitetskyi, M. 2020. Adaptivity of plants in stage *in vitro-ex vitro* (in Ukrainian). *East European Scientific Journal*, 4 (56), 25–33.

Reed, M., Mentzer, J., Tanprasert, P., Yu, X. 1997. Internal bacterial contamination of micropropagated hazelnut: Identification and antibiotic treatment. *Developments in Plant Pathology*, 12, 169–174. DOI: https://doi.org/10.1007/978-94-015-8951-2_20

Santos, A. et al. 2009. An integrated strategy to identify key genes in almond adventitious shoot regeneration. *Journal of Experimental Botany,* 60 (14), 4159–4173. DOI: https://doi.org/10.1093/jxb/erp250

Satina, G.M. et al. 2011. Scientific foundations and components of the sectoral program for the nut farming development in Ukraine. Logos, Kyiv, 100.

Tarasenko, G., Nebykov, M., Balabak, O. 2020. Application of differential sterilization methods of explants of represantatives of genus *Corylus* L. *in vitro*. *Journal of Native and Alien Plant Studies*, 12, 229–235. DOI: https://doi.org/10.37555/.12.2016.173404

Terek, O., Patsula, O. 2011. Growth and development of plants: educational manual (in Ukrainian). Lviv University, 328.

Tilkat, E. et al. 2013. A rapid and effective protocol for surface sterilization and *in vitro* culture initiation of adult male pistachio (*Pistacia vera* L. cv. "Atlı"). *Academia Journal of Scientific Research*, 1 (8), 134–41. DOI: https://doi.org/10.15413/ajsr.2013.0109

Yahyaoui, E., Marinoni, D.T., Botta, R., Ruffa, P., Germanà, M. 2021. Is it possible to produce certified hazelnut plant material in Sicily? Identification and recovery of Nebrodi genetic resources, in vitro establishment, and innovative sanitation technique from *apple mosaic virus*. *Frontiers in Plant Science*, 12, 1–14. DOI: https://doi.org/10.3389/fpls.2021.778142

Yu, X., Reed, B. 1993. Improved shoot multiplication of mature hazelnut (*Corylus avellana* L.) *in vitro* using glucose as a carbon source. *Plant Cell Reports*, 12, 256–259. DOI: https://doi.org/10.1007/BF00237130