Evaluation of physiological responses of hazelnut (*Corylus avellana* L.) explants to MS and WPM culture media

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In this study, the importance of culture medium and explant selection in microclonal propagation of hazelnut plants was investigated. The results showed that the success of microclonal propagation of hazelnut depends not only on the chemical composition of the culture medium, but also on the morphological and physiological characteristics of the explants. The results of the experiment on hazelnut microclonal propagation showed that the WPM culture medium has significant advantages over classical MS medium, especially in terms of stimulating the proliferation of single-node shoots and minimizing callus formation. The low ionic charge and balanced nitrogen profile of this medium promote stable growth and organogenesis of plants. In addition, single-node shoots were selected as the explant with the highest regeneration capacity. In the studies, the genotype dependence of the *in vitro* morphogenetic response of hazelnut plants was clearly observed. The "Giffoni" variety showed a particularly high regeneration capacity, while the "Galib" and "Sachaglı" varieties responded poorly. Also, while single-node shoots showed high regeneration capacity, leaf segments had poor regeneration potential. In conclusion, the WPM culture medium provides optimal conditions for *in vitro* propagation of hazelnut plants and emphasizes the importance of a personalized approach to increase the efficiency of microclonal propagation.

Keywords: *Corylus avellana, explants, WPM, MS, single-node shoot, regeneration potential*

INTRODUCTION

The Betulaceae family includes hazelnut (*Corylus avellana* L.) which serves as an important valuable and ancient fruit plant that reaches heights of approximately between 3 to 8 meters. This species which belongs to the Corylus genus, is a perennial deciduous plant characterized by alternately arranged simple, ovate, or rounded leaves, with serrated edges and it can grow up to 6-12 cm long. The natural habitat of hazelnut extends across Southern Europe (Italy, Spain, Greece), the Middle East (Turkey, Iran, Azerbaijan), and South Asia and it is also cultivated in North America.

The hazelnut fruit comprises different types of 50-70% fat acids together with 14-18% protein and 14-17% carbohydrates and 3-4% cellulose and 2-3% beneficial minerals. The nutritional profile of hazelnut consists of vitamins A, C (especially, ascorbic acid), E (tocopherol), and B-complex vitamins (B_1, B_2, B_6) along with significant minerals such as calcium, iron, magnesium, zinc, potassium, and phosphorus. The nutritional elements in hazelnut make them highly beneficial for medicine. The practical medicinal importance of the hazelnut is also high. For instance, the nutritional profile of hazelnut (*Corylus avellana* L.) helps to protect cardiovascular health by managing blood pressure and cholesterol levels. The vitamin E along with phenolic compounds in hazelnut demonstrates very strong antioxidant properties which can fight chronic diseases and delay natural aging. B vitamins together with omega-3 fatty acids in hazelnut promote brain health and preserve against age-related cognitive decline. The taxane metabolites found in hazelnut show apoptotic effects against different kinds of dangerous cancer cells (Hoffmann et al., 2008).

Due to its considerable economic and pharmaceutical value, the large-scale cultivation of hazelnut has become increasingly important. However, conventional propagation methods

commonly used for tree species are often inadequate and highly labor-intensive. To manage and solve these limitations, modern biotechnological approaches particularly, *in vitro* micropropagation are proposed. *In vitro* plant culture is a key branch of plant biotechnology and it is widely utilized for enhancing genetic diversity, conserving important rare species, multiplying economically plants, and developing disease-resistant varieties (Pua and Davey, 2013). Nevertheless, the application of *in vitro* techniques displays several challenges, including the maintenance of genetic stability, the risk of contamination, difficulties in explant adaptation, species-specific responses, and long-term culture sustainability (Bridgen et al., 2018).

In vitro plant culture constitutes a critical domain within modern biotechnology, playing a central role in enhancing genetic diversity, conserving rare and endangered plant species, propagating crops of economic importance, and developing disease-resistant cultivars (Pua and Davey, 2013). Despite its numerous advantages, the technique encounters several limitations, including concerns over genetic stability, susceptibility to microbial contamination, challenges in explant acclimatization, species-specific physiological responses, and the complexities associated with the long-term preservation of culture media.

Microclonal propagation technology emerged in the mid-20th century and has been extensively applied to a wide range of plant species since the 1960s and 1970s. It is a technique in which new plants are generated from small tissue explants under *in vitro* conditions, with the aid of plant growth regulators, to achieve rapid propagation. This method offers several advantages, including high multiplication rates, the production of disease-free plant material, maintenance of genetic stability, and independence from seasonal constraints.

Currently, microclonal propagation is widely employed in applications such as the conservation of specific plant species, pathogen-free production, and large-scale commercial multiplication. Due to advancements in biotechnology, genomics, and nanotechnology in the 21st century, this technique has become instrumental in enhancing plant quality, increasing uniformity in traits, and facilitating efficient mass production. Its cost-effectiveness and efficiency, when compared to traditional propagation methods, have made it a preferred technique.

In vitro plant culture involves growing and propagating plant cells, tissues, and organs under sterile conditions. In hazelnut, the technique is used extensively in clonal propagation, genetic transformation, and producing disease-free plants.

Micropropagation enhances the adaptability of hazelnut plants and plays a significant role in enabling their transplanting to new conditions, and therefore, it can be a cornerstone of sustainable agriculture (Kozak and Tatar, 2015). Hazelnut micropropagation has its own specific challenges, particularly in the sterilization process, where precision at each step is critical. Errors at any stage can inhibit plant growth or lead to productivity losses (Kumar and Reddy, 2011).

Although several protocols have been proposed for hazelnut micropropagation, most of them have been shown to be effective only for one or two cultivars (Bacchetta et al., 2008; Damiano et al., 2005; Mardani et al., 2020). The right balance of growth hormones is crucial for the production of healthier and faster plants (Güler and Polat, 2011). In a comparative study, Yu and Reed (1993) compared DKW, WPM, and Anderson media and stated that DKW gave superior results in shoot proliferation, elongation, and appearance. Bacchetta et al. (2008) developed an upgraded form of the MS medium (HM), which was successfully used in the propagation of six Italian traditional hazelnut cultivars. Silvestri et al. (2020) investigated the effect of NH_4^+/NO_3^- ratios in the MS medium and established that half-strength NH_4NO_3 improved internodal growth and chlorophyll content.

Hand et al. (2014) and Hand & Reed (2014) employed the RSM method in optimizing mineral nutrient concentration, while Akin et al. (2017a, 2017b) employed the CHAID data mining algorithm in modeling macronutrient concentrations in DKW.

Selection of explants is extremely crucial for the success of hazelnut *in vitro* culture. Juvenile nodal shoots are among the most popularly used explants and are especially suitable for research experiments. These shoots, typically cut from apical or lateral regions, possess high vitality and yield multiple buds and shoots due to high cytokinin content, characteristic of the active growth phase of the plant. These explants are generally highly viable and amenable to micropropagation. Buds, being the youngest and most active parts of the hazelnut plant, are best suited for *in vitro* propagation. Being dynamic and having a regenerative capability, they are most appropriate. BAP, a synthetic cytokinin, is one of the key hormones used to stimulate bud growth *in vitro*. It promotes cell division and also acts in synergy with auxins to promote plant growth (Huang et al., 2016; Zhang et al., 2020).

Although surface and leaf tissues can also be utilized for hazelnut micropropagation, their

viability tends to be lower. However, under appropriate hormonal formulations and environmental conditions, these tissues can be efficiently regenerated (Hemmati-Gougeh.et al., 2024). The aim of this study is to conduct a comparative analysis of the effectiveness of Murashige & Skoog (MS) and Woody Plant Medium (WPM) in the micropropagation of hazelnut (*Corylus avellana*) in vitro, in order to identify the optimal medium for this propagation technology.

MATERIALS AND METHODS

For conducting a scientific study on the microclonal propagation of hazelnut (*Corylus avellana L.*), plant materials were collected from a support station in the Zagatala region of Azerbaijan and transported to the research institute. Four local cultivars: "Ata-baba", "Galib", and "Sachaglı"- and one Italian cultivar, "Giffoni," were used as the subject of research. The cultivars were selected based on their genetic diversity, compatibility with local climatic conditions, and agricultural productivity. The key selection criteria were high potential for vegetative propagation, tolerance to *in vitro* conditions, and the potential for regenerating high-quality plantlets. Moreover, since these cultivars play an essential role in the hazelnut industry, their successful propagation under controlled laboratory conditions can be a worthwhile contribution to agricultural production.

For the study, 3 types of explants from each variety: bud, single-node shoot and leaf were taken and sterilized. The main purpose of sterilization is to completely clean the plant materials from both harmful microorganisms and diseases.

Sterilization Protocol. Considering the importance and precision involved in woody plant sterilization, the process was carried out in a series of steps: initially, the explants were washed by agitating them in soapy water in a magnetic stirrer for 40 minutes. The plant material was then rinsed thoroughly with normal water for 30 minutes in a sieve until it became clean. Soap particles-free explants were now prepared for the next step of sterilization.

The plant materials were then cleaned in a solution of $KMnO_4$ (potassium permanganate) for 5 minutes. The material was then thoroughly washed with distilled water after this treatment. The second step was conducted under a laminar flow hood where the explants were subjected to sterile air. They were then treated with 70% ethanol solution for 1 minute. When removed from the ethanol solution, the plant was washed using distilled water to eliminate any residual ethanol content. After that, the shoots were immersed for 18 minutes in a NaClO solution with 2-3 drops of Tween-20. This is an extra purification and sterilization step for the plant material.

In the last phase of the sterilization process, the plant material was exposed to antifungal agents for 20 minutes and then rinsed with fresh distilled water. Each washing step was carefully designed with special equipment and methodology to achieve microbiological safety and to avoid any recolonization of the contaminant. Thus, the washed plant materials were prepared for inoculation to the nutrient medium. After the sterilization process was completed, the planting materials were then transferred to the nutrient medium.

Cultivation Conditions. Two different nutrient media were used for laboratory -based cultivation: MS (Murashige and Skoog, 1962) and WPM (Lloyd and McCown, 1980) (Table 1). The mineral composition of both MS and WPM nutrient media was kept constant, while the hormonal composition consisted of BAP (1.0 mg/L) and IBA (0.5 mg/L). Three explants of every variety were taken, and 120 explants were placed in test tubes. The test tubes were kept at 22-25°C temperature, with light intensity being 3000-4000 lux, under the 16 hours light/8 hours dark photoperiod.

Table 1. Composition of MS and WPM nutrient media.

	MS (mg/L)	WPM (mg/L)
Macrominerals		
NH_4NO_3	1650	400
$Ca(NO_3)_2 \cdot 4H_2O$	-	556
$CaCl_2 \cdot 2H_2O$	440	96
$MgSO_4 \cdot 7H_2O$	370	370
KNO_3	1900	-
KH_2PO_4	170	170
K_2SO_4	-	990
Microminerals (mg/l)		
H_3BO_3	6.2	6.2
$CuSO_4 \cdot 5H_2O$	0.025	0.25
$MnSO_4 \cdot H_2O$	16.9	22.3
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25
$ZnSO_4 \cdot 7H_2O$	8.6	8.6
$FeSO_4 \cdot 7H_2O$	27.8	27.8
Na2·EDTA	37.3	37.3
$CoCl_2 \cdot 6H_2O$	0.025	-
Vitamins (mg/l)		
Thiamine (B_1)	0.1	1.0
Nicotinic acid (B_3)	0.5	0.5
Pyridoxine (B_6)	0.5	0.5
Glycine	2.0	2.0
Myo-inositol	100	100
Sucrose (G/L)	30	20
Agar (G/L)	8	6

RESULTS AND DISCUSSION

Sterilization is a complex technological process, and the meticulous performance of every step is essential for ensuring both the efficacy and safety of the microclonal propagation process. Specifically, the function of chemical substances such as NaClO (sodium hypochlorite) and $KMnO_4$ (potassium permanganate) is crucial, as these chemicals are responsible for effectively eliminating microorganisms from the plant material. In our experiment, each sterilization phase focused on preventing infection, thereby ensuring favorable outcomes in the microclonal propagation process. In addition to the above findings, the success of the sterilization process was rigorously analyzed in relation to the plant's vegetative season. Sampling the plant material in May and September demonstrated a clear impact on the infection rate. The material sampled in May exhibited an infection rate of 20%, whereas the samples collected in September showed a significantly higher infection rate of 90%. These results underscore the critical importance of the timing of material collection for effectively conducting the microclonal propagation process. Therefore, the data from material collected in May are presented in this manuscript, as the timing choice reduced infection levels and optimized the effectiveness of the sterilization technique. Due to the complexity of adapting hazelnut (*Corylus avellana* L.) plants to *in vitro* culture, a comparative study was conducted to establish the most suitable nutrient medium for the microclonal propagation process. In our study, both the general MS (Murashige and Skoog, 1962) medium, which is used for various plant species, and the WPM (Lloyd and McCown, 1980) medium, specifically optimized for *in vitro* culture of woody plants, were utilized. The results of this analysis have helped identify the most suitable medium for hazelnut microclonal propagation. During the comparative analysis of the two-nutrient media, it was observed that after two weeks of cultivation, the samples grown on the MS medium had a higher infection rate. This result can be attributed to the differences in the composition of the nutrient media, particularly the amount of carbohydrates, which serve as an energy source for the plants. Specifically, the high sucrose content (30 g/L) in the MS medium not only promotes plant cell proliferation but also stimulates the growth of microorganisms. For comparison, the sucrose concentration of the WPM medium is 20 g/L, which promotes less growth of microbes and accordingly a lower infection rate. This result highlights the significance of choosing appropriate conditions for plant cell culture and preventing infection based on the carbohydrate content of the nutrient medium. Two weeks after cultivation, changes began to be observed in the explants grown in both nutrient media (MS and WPM). These were varied based on the explant type and activities of the media (Table 2).

Table 2. Average multiplication rate and callus formation (%) of hazelnut explants on MS and WPM media (Mean±SD)

Variety	Medium	Explant	degree of proliferation (average±SD)	SE (degree of proliferation)	plant length (in cm) average±SD	SE (plant length)	The amount of callusing (%)
Ata-baba	MS	a	1.10±0.30	0.095	0.6±0.8	0.253	30
		b	2.60±0.60	0.190	2.5±3.5	1.111	40
	WPM	c	—	—	—	—	50
		a	1.60±0.60	0.190	0.8±0.8	0.253	10
		b	2.90±2.50	0.790	2.5±3.5	1.111	13
		c	1.70±0.60	0.190	2.0±3.0	0.950	20
Sachaglı	MS	a	—	—	—	—	—
		b	0.30±0.20	0.063	—	—	5
		c	—	—	—	—	—
	WPM	a	0.50±0.20	0.063	—	—	5
		b	0.80±0.40	0.126	—	—	7
		c	—	—	—	—	—
Galib	MS	a	—	—	—	—	—
		b	0.30±0.10	0.032	—	—	10
		c	—	—	—	—	—
	WPM	a	0.70±0.10	0.032	—	—	7
		b	1.10±0.10	0.032	—	—	10
		c	—	—	—	—	—
Giffoni	MS	a	1.40±0.30	0.095	0.8±0.8	0.253	20
		b	2.80±0.50	0.158	3.5±0.6	0.189	30
		c	—	—	—	—	34
	WPM	a	2.20±0.80	0.253	0.8±0.8	0.253	5
		b	4.10±3.50	1.107	3.5±0.6	0.189	8
		c	2.00±0.60	0.190	2.0±1.5	0.474	10

a- bud; b- single-node shoot; c-leaf; -- absence of callus; SD - mean deviation; SE - standard error

The average values of the propagation rate were calculated for 2-3 classes of explants (bud, single-node shoot, leaf), depending on the shoot growth. The standard error (SE) of the mean is taken as an expression of the reliability of the evaluation on the basis of biologically independent replicates. Data were omitted from analysis when no shoot formation was observed in the explants. Percentages of callus formation were evaluated separately for every explant type (single-node shoot, bud, leaf) on the MS and WPM mediums.

The results of the experiment on hazelnut (*Corylus avellana L.*) microclonal propagation demonstrated that the WPM nutrient medium offers significant advantages over the classical MS medium, particularly in promoting the proliferation of single-node shoots and minimizing callus formation (Fig. 1).

MS

WPM

Fig. 1. Degree of callus formation in different nutrient media

The findings suggest that the effectiveness of a nutrient medium is determined not only by the ratio of nitrogen forms but also by a balanced composition of minerals and vitamins tailored to the morpho-physiological characteristics of woody plant species. Originally developed for the cultivation of slow-growing trees such as fruit and nut-bearing species, the WPM medium contains lower levels of macronutrients, especially nitrogen, compared to the MS medium. This is particularly significant for plant species like hazelnut, which exhibit sensitivity to excessive nutrient concentrations, notably ammonium. The elevated NH_4^+ levels in the MS medium may trigger callus formation and induce physiological stress. In contrast, reduced ammonium concentrations and a nitrate-dominant nitrogen form in the WPM medium promote more stable growth and organogenesis, occurring without callus development. In addition to its optimized micro- and macroelement ratios, the WPM medium plays a significant role in enhancing *in vitro* culture outcomes. Its reduced ionic strength helps lower osmotic stress, thereby creating a favorable environment for the activation of apical meristems. Furthermore, the relatively high copper concentration in WPM contributes to the activation of several redox enzymes, boosting the metabolic activity of explants without causing toxicity due to its balanced formulation. The elevated thiamine (vitamin B_1) content in WPM may further enhance cellular metabolism, increase tissue resilience against *in vitro* stressors, and support improved morphogenetic responses. These combined properties make WPM particularly well-suited for the cultivation of woody species like hazelnut, especially during shoot induction and proliferation stages.

Fig. 2. The emergence of a leaf on a single-node shoot on WPM medium.

Besides the chemical properties of the culture medium, the type of explant used was also found to be a decisive factor in the success of microclonal propagation. The highest morphogenetic response was observed in single-node shoots, which contain active meristematic zones with strong regenerative potential under *in vitro* conditions (Fig. 2). Preservation of apical or subapical structures in the

shoot ensures a high potential for direct organogenesis, offering a distinct advantage over leaf segments, in which this ability is considerably less pronounced. In this study, leaf explants predominantly formed callus tissue and failed to generate shoots, confirming their restricted potential for regeneration.

In conjuction, the genotype-dependent nature of morphogenetic responses was clearly observed. The highest regeneration potential was the most pronounced in the "Giffoni" cultivar, which regularly demonstrated well-developed shoots, particularly in the WPM medium. This fact can be explained by its inherent physiological qualities as well as by its relatively high level of adaptation to *in vitro* culture conditions. On the other hand, the "Ata-baba" cultivar, which had relatively lower levels of morphogenic response, still produced satisfactory results. In contrast, the "Galib" and "Sachaglı" cultivars had a poor morphogenetic response, which indicates inherent genetic or physiological-metabolic constraints that limited their *in vitro* proliferation. The genotypic variation observed shows that the success of *in vitro* microclonal propagation is not only based on the chemical nature of the culture medium and the morphological nature of the explants but also on the biological nature inherent to the genotype. It is essential to take these aspects into consideration when developing microclonal propagation protocols for each cultivar (Hazrati et al., 2022).

In line with this, the results of the present study substantiate the fact that the WPM culture medium, having proportionately balanced mineral and vitamin constituents, and a nitrogen spectrum more in line with physiology, can be rated as ideal for *in vitro* microclonal propagation of hazelnut. The utilization of one-bud shoots possessing high regenerative potential considerably increases the effectiveness of this process. Moreover, the noted variation in the morphogenetic responses among the various hazelnut cultivars underscores the need to follow a genotype- and morphology-based strategy in developing micropropagation protocols.

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Fındıq (*Corylus avellana* L.) eksplantlarının MS və WPM qida mühitlərinə fizoloji cavablarının qiymətləndirilməsi

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Bu tədqiqatda, fındıq bitkisinin mikroklonal çoxaldılmasında qida mühiti və eksplant seçiminin əhəmiyyəti araşdırılmışdır. Nəticələr göstərmişdir ki, fındığın mikroklonal çoxalmasının uğuru yalnız qida mühitinin kimyəvi tərkibindən deyil, həm də eksplantların morfoloji, fizioloji xüsusiyyətlərindən də asılıdır. Fındıq mikroklonalçoxaldılması üzrə aparılan təcrübənin nəticələri göstərmişdir ki, WPM qida mühiti klassik MS mühitinə nisbətən xüsusilə bir buğumlu zoğların proliferasiyasını stimullaşdırmaq və kallus əmələ gəlməsini minimuma endirmək baxımından əhəmiyyətli üstünlüklərə malikdir. Bu mühitin aşağı ion yükü və balanslaşdırılmış azot profili bitkilərin sabit böyüməsini və orqanogenezini təşviq edir. Bununla yanaşı bir buğumlu zoğlar ən yüksək regenerasiya qabiliyyətli eksplant kimi seçilmişdir. Tədqiqatlarda fındıq bitkisinin *in vitro* morfogenetik reaksiyasının genotipdən asılılığı açıq şəkildə müşahidə edilmişdir. "Giffoni" sortu xüsusilə yüksək regenerasiya qabiliyyəti göstərmiş, "Qalib" və "Saçaqlı" sortları isə zəif cavab vermişdir. Həmçinin, tək buğumlu zoğlar yüksək regenerasiya qabiliyyəti nümayiş etdirərkən, yarpaq seqmentləri zəif regenerasiya potensialına malik olmuşdur. Nəticə etibarilə, WPM qida mühiti fındıq bitkisinin *in vitro* çoxaldılması üçün optimal şərait təmin edir və mikroklonal çoxalmanın səmərəliliyini artırmaq üçün fərdi yanaşmanın vacibliyini vurğulayır.

Açar sözlər: *Corylus avellana, eksplant, WPM, MS, bir buğumlu zoğ, regenerasiya potensialı*

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