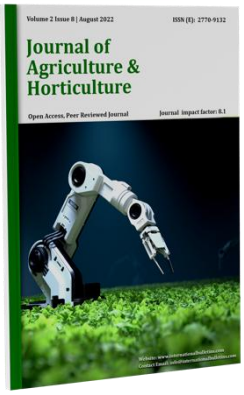

OPTIMIZATION OF MICROCLONAL PROPAGATION METHODS OF JUGLANS REGIA L.

Khaydarov Ilyos Abdurashitovich
Independent Researcher, Scientific-research institute of horticulture, viticulture and winemaking named after academician Mahmud Mirzayev
Allayorov Abdurakhmon Nazaraliyevich
Head of Laboratory
https://doi.org/10.5281/zenodo.18240001

Abstract. This scientific article shows that, in the propagation of *Juglans regia* L., the composition of the culture medium is the main source of variability in the proliferation process. Regardless of the genotype used, the growth of microshoots was better in DKW medium than in MS medium. The results indicate that growth occurred mainly due to the elongation of internodes rather than an increase in the number of nodes. In addition, the size of calli formed on DKW medium was larger than those on MS medium. Values in each column are presented as mean ± standard error (SE). $n = 20$; mean values of explant shoots marked with different letters differ significantly at $P < 0.05$.

Keywords: *in vitro*, nutrient medium, *Juglans regia* L., proliferation, DKW, MS, standard error (SE).

Introduction

Today, food security has global significance and is one of the key issues for the sustainable development of humanity. Population growth, climate change, and the depletion of natural resources are increasing the risk of food shortages. According to available data, in recent years the challenges associated with ensuring stable food security worldwide have had a serious impact on human health and economic development.

In order to ensure food security at the global level, consistent research is being carried out to develop disease-resistant varieties of crops and fruit trees and to introduce them on a large scale. In this regard, the selection, propagation, and acclimatization of disease-resistant varieties of fruit plants with high nutritional value, including walnut, are considered among the most important tasks. Special attention is being given to studies aimed at substantiating scientific solutions for establishing walnut orchards composed of healthy planting material by selecting local varieties and forms resistant to bacterial blight using highly virulent bacterial isolates obtained from infected trees, determining optimal conditions for their in vitro propagation, and developing microclonal propagation technologies for disease-resistant local varieties and forms.

One of the main problems in microclonal plant propagation is contamination of explants by various microorganisms, which can lead to the death of callus tissue and the obtained clones (Abdalla et al., 2022). Therefore, a critical stage in introducing explants into in vitro culture is their sterilization.

To prevent external contamination of explants by epiphytic microorganisms, their surfaces can be washed under running water (with or without detergents) and sterilized using chemical agents (ethanol, mercuric chloride [$HgCl_2$], sodium hypochlorite [NaOCl]) as well as plant preservative mixtures [1], [13]. In addition, to prevent external contamination, certain substances that inhibit microbial growth—such as plant preservative mixtures [1], [13] or the fungicide benomyl—may be added to the culture medium [1]. Moreover, antibiotics can be used

as antimicrobial agents to eliminate endophytic bacteria present in plants under in vitro conditions [6].

Based on the above, at the first stage, studies were conducted to select and optimize explant sterilization methods. A review of the literature showed that sterilizing agents can be divided into three main groups:

Weak disinfecting agents – for example, Tween-20; **Moderate disinfecting agents** – for example, sodium hypochlorite [NaOCl]; **Strong disinfecting agents** – for example, mercuric chloride [$HgCl_2$] [7], [15].

According to this classification, combinations of these agents are applied in various protocols for the sterilization of walnut explants [8], [15], [3], [17].

Based on the literature analysis, the sterilization procedure for explants in Protocol I was selected as follows: In study [8], the use of hypochlorite (1.5 g/L active chlorine) was recommended for explant sterilization. However, since this compound has strong oxidative properties and may cause browning of explants, we decided not to use hypochlorite as a sterilizing agent in our work.

In the study by [15], Tween-20 was used for the preliminary washing of walnut explants, while subsequent disinfection was carried out using hypochlorite, carbendazim, and captan solutions. For chemical sterilization, an $HgCl_2$ solution was applied. However, because $HgCl_2$ is toxic not only to plants but also to human health, we decided to отказаться from this method.

In the work of Yegizbayeva et al. [17], [16], explants were first sterilized by washing with water and dishwashing detergent. Subsequently, in the protocol, the explants were disinfected for 3 minutes in a solution containing 0.1% $HgCl_2$ with the addition of Tween-20.

Materials and Methods

This study was conducted during 2022–2025 at the Biotechnology Laboratory of the Academic Mahmud Mirzayev Research Institute of Horticulture, Viticulture and Winemaking, as well as at the Cell and Tissue Culture Laboratories of SAG Agro LLC, focusing on the development of in vitro clonal micropropagation technologies for walnut (Juglans regia L.) plants.

Modified DKWC (Driver & Kuniyuki, 1984) and MS (Murashige & Skoog, 1962; Murashige & Tucker, 1969) formulations were used as culture media (Table 1). The DKWC medium was prepared from stock solutions, whereas the MS medium was prepared from a dry powder (code 5519, Sigma-Aldrich; St. Louis, MO, USA). The pH was adjusted to 5.7 using 0.1 N NaOH. The culture media were solidified with industrial agar (5.5 g/L) for in vitro establishment, proliferation, and root induction. Subsequently, the media were sterilized in an autoclave at 121 °C for 20 minutes. The media were stored in a dark place and used within 7 days after preparation.

The laboratory was maintained under a standard photoperiod (16/8 h light/dark), relative humidity of 40–60%, average light intensity of 4500 lux (PH 1200 mm^2 × 36 W LED tubes), and temperature ranging from 22 to 24 °C. Chemicals were supplied by Duchefa Biochemie (Haarlem, The Netherlands). Rooting was carried out in two stages: root initiation (5–7 days) and root development under a 16/8 h photoperiod.

Results and Discussion

After the rooting stage, a general decline in culture vigor was observed, which was not associated with microbiological contamination, particularly on the MS nutrient medium. Similar phenomena have previously been reported in several walnut species. Even Driver and

Kuniyuki emphasized that certain deficiencies may become evident when MS medium is used [9], [4], [17].

The symptoms of culture decline included a sharp reduction in the vitality of microshoots, which led to decreased multiplication rates and the development of dark yellow–brown coloration of the leaves.

Loss of vigor has been described as one of the most critical causes of failure in walnut in vitro culture. In fact, this problem was a primary reason for the development of the DKW formulation [12], [9], [4]. Prior to this, microshoots were characterized by small size or the absence of basal callus formation, which may have resulted in the gradual slowdown of growth—an effect that was also observed in Mj209 × Ra clones [10]. It should also be noted that a decline in viability becomes particularly pronounced when mature trees are used as donor plants. This phenomenon has likewise been reported in American black walnut clones [14].

The composition of the culture medium proved to be the main variable affecting the proliferation process. Regardless of the genotype used, microshoot growth was superior on DKW medium compared with MS medium.

The results indicate that growth occurred mainly due to elongation of internodes rather than an increase in the number of nodes (as shown in Table 1). In addition, the callus formed on DKW medium was larger in size than that produced on MS medium.

Table 1. Evaluation of the studied factors in different genotypes

Variety	Culture medium	Shoot length (cm)	Average number of shoots	Number of leaves	Callus size
Ideal	DKWC-Fe	$3.10^{a} \pm 0.11$	$1.86^{b} \pm 0.10$	$14.13^{d} \pm 0.55$	2.01 ± 0.10
	MS	2.89 ± 0.1	1.62 ± 0.09	11.05 ± 0.47	1.79 ± 0.08
Form PDM23	DKWC-Fe	2.91 ± 0.11	$1.79^{c} \pm 0.10$	$13.26^{e} \pm 0.42$	$2.09^{f} \pm 0.08$
	MS	2.54 ± 0.09	1.48 ± 0.06	10.89 ± 0.4	1.85 ± 0.06
Form 202YaKT	DKWC-Fe	$2.21^{a} \pm 0.07$	$1.10^{b,c} \pm 0.11$	$10.41^{d,e} \pm 0.36$	$1.50^{f} \pm 0.08$
	MS	1.94 ± 0.06	0.89 ± 0.06	8.84 ± 0.36	1.32 ± 0.07

Values in each column are presented as mean ± standard error (SE). $n = 20$; mean values of explant shoots marked with different letters indicate statistically significant differences at $P < 0.05$. Rooting percentage was analyzed using the Kruskal–Wallis test ($p \leq 0.05$). These results are consistent with those of Driver and Kuniyuki (1984), who demonstrated that walnut proliferation on DKW medium was more successful than on B5, Cheng, MS, and WPM culture media.

Although the DKW formulation has been primarily used for walnut micropropagation, other researchers have reported comparable results on MS medium, and in some cases even better outcomes than on DKW. For example, lateral shoot elongation of *J. cinerea* was greater on MS medium than on DKW medium (Pijut, 1997).

Similarly, Bosela and Michler (2008) found that microshoot length of American black walnut was similar on both media; however, although the percentage of elongated explants was lower on MS medium, a higher number of hyperhydric microshoots were formed there compared with DKW medium.

The absence of a significant interaction between clone and culture medium for all evaluated variables based on ANOVA indicates that DKW medium was superior to MS medium for the growth of all genotypes studied (Table 2).

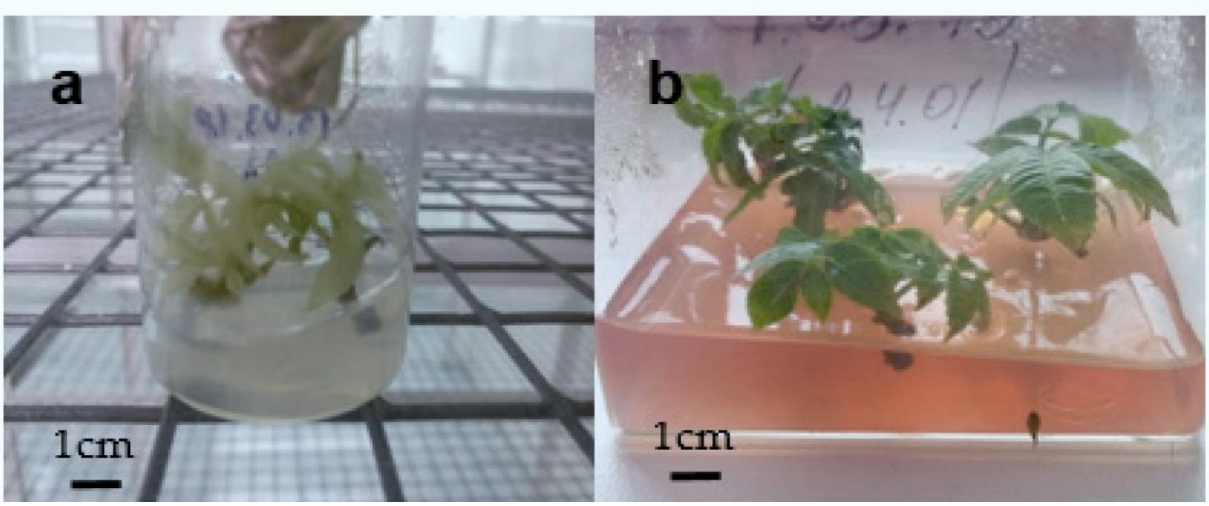

Figure 1. Microshoots at the proliferation stage: **a** — microshoots cultured on DKWS medium; **b** — microshoots cultured on DKWS-Fe medium.

In addition, it should be emphasized that the proliferation process is influenced not only by the culture medium but also by the genotype of the explants used. As shown in **Table 4.1**, the main parameters—on DKWS-Fe and MS media (shoot length, mean number of shoots, number of leaves per shoot, and callus size)—were highly dependent on the variety (genotype) of the explants.

Table 2. Variables evaluated during the proliferation process, main interaction effects, and results of ANOVA for each source of variation.

Variable	Genotype	Culture Medium
Shoot length	**	***
Proliferation rate	***	***
Callus diameter	**	***
Rooting (a)	***	***
Roots / microshoots	ns	***
Survivability	**	***

Because the rooting data did not meet the assumptions of ANOVA, the **Kruskal–Wallis test** was applied.

ns — not significant; * $p \leq 0.01$; ** $p \leq 0.001$.

Thus, in both media, the explants belonging to the Ideal variety showed the highest values for shoot length, mean number of shoots, and number of leaves, reaching 3.10 ± 0.11, 1.86 ± 0.10, and 14.13 ± 0.55, respectively, on the DKWC-Fe medium. The largest callus size (2.09 ± 0.08 on DKWC-Fe medium) was observed in the Form PDM23 variety. In both nutrient media, the lowest values for all parameters (shoot length, mean number of shoots, number of leaves per shoot, and callus size) were recorded for the Form 202YaKT variety.

Conclusion

For *de novo* organogenesis of walnut shoots, the composition of the nutrient medium and the concentrations of phytohormones varied depending on the genotype used: for the Ideal and Form PDM23 varietys, 2.0 mg/L BAP was added to the DKWC-Fe medium, whereas for the Form

202YaKT variety, 1.5 mg/L BAP was applied to the DKWC-Fe medium. At the same time, the optimal concentration of IBA for explants of all varietys was determined to be 6 mg/L.

At the in vitro introduction stage, the main factors affecting the process were identified as the explant genotype, time of plant material collection, accuracy of the sterilization procedure, and composition of the nutrient medium.

References:

1.Abdalla, N., El-Ramady, H., Seliem, M. K., El-Mahrouk, M. E., Taha, N., Bayoumi, Y., Shalaby, T. A., & Dobránszki, J. (2022). An academic and technical overview on plant micropropagation challenges. Horticulturae, 8(8), 677. https://doi.org/10.3390/horticulturae8080677

2.Ahmadpoor, F., Zare, N., Asghari, R., & Sheikhzadeh, P. (2022). Sterilization protocols and the effect of plant growth regulators on callus induction and secondary metabolites production in in vitro cultures of Melia azedarach L. AMB Express, 12(1), 1–12.

3.Vahdati, K., Sadeghi-Majd, R., Sestras, A. F., Licea-Moreno, R. J., Peixe, A., & Sestras, R. E. (2022). Clonal propagation of walnuts (Juglans spp.): A review on evolution from traditional techniques to application of biotechnology. Plants, 11(22), 3040.
https://doi.org/10.3390/plants11223040

4.Vahdati, K., & Khalighi, A. (2001). Persian walnut stooling in Iran. Acta Horticulturae, 544, 531–535.

5.Driver, J. A., & Kuniyuki, A. H. (1984). In vitro propagation of Paradox walnut rootstock. HortScience, 19(4), 507–509.

6.Jugreet, B. S., & Mahomoodally, M. F. (2020). Essential oils from nine exotic and endemic medicinal plants from Mauritius show in vitro antibacterial and antibiotic-potentiating activities. South African Journal of Botany, 132, 355–362.

7.Kutas, E., & Ogorodnik, L. (2011). The influence of sterilizing compounds on the yield of viable explants of Rhododendron L. (Ericaceae). International Journal of Biodiversity and Conservation, 3(1), 24–26.

8.Leal, D. R., Sánchez-Olate, M., Avilés, F., Materan, M. E., Uribe, M., Hasbún, R., & Rodríguez, R. (2007). Micropropagation of Juglans regia L. In S. M. Jain & H. Häggman (Eds.), Protocols for micropropagation of woody trees and fruits (pp. 381–390). Springer.

9.Leslie, C., & McGranahan, G. (1992). Micropropagation of Persian walnut (Juglans regia L.). In Y. P. S. Bajaj (Ed.), High-tech and micropropagation II (pp. 136–150). Springer.

10.Licea-Moreno, R. J., Contreras, A., Morales, A. V., Urban, I., Daquinta, M., & Gomez, L. (2015). Improved walnut mass micropropagation through the combined use of phloroglucinol and FeEDDHA. Plant Cell, Tissue and Organ Culture, 123(1), 143–154.

11.Murashige, T., & Tucker, D. P. H. (1969). Growth factor requirements of citrus tissue culture. Proceedings of the First International Citrus Symposium, 1155–1161.

12.McGranahan, G., Leslie, C. A., & Driver, J. A. (1988). In vitro propagation of mature Persian walnut varietys. HortScience, 23(1), 220.

13.Singh, C. R. (2018). Review on problems and their remedies in plant tissue culture. Asian Journal of Biological Sciences, 11, 165–172.

14.Stevens, M. E., & Pijut, P. M. (2018). Rapid in vitro shoot multiplication of the recalcitrant species Juglans nigra L. In Vitro Cellular & Developmental Biology – Plant, 54(3), 309–317.

15.Sharma, S., Sharma, S., & Kumar, P. (2025). In vitro establishment of walnut (Juglans regia L.) from the embryonic axis with customized nutrient media and synergy of growth regulators. South African Journal of Botany, 177, 598–603.
16.Pijut, P. M. (1997). Micropropagation of Juglans cinerea L. (butternut). In Y. P. S. Bajaj (Ed.), High-tech and micropropagation V (pp. 345–357). Springer.
17.Yegizbayeva, T. K., García-García, S., Yausheva, T. V., Kairova, M., Apushev, A. K., Oleichenko, S. N., & Licea-Moreno, R. J. (2021). Unraveling factors affecting micropropagation of four Persian walnut varieties. Agronomy, 11(7). https://doi.org/10.3390/agronomy11071407