PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.2915

HORIZON
e-Publishing Group
HePG

RESEARCH ARTICLE

Influence of different factors on *in vitro* multiplication and rooting of three local *Juglans regia* L. genotypes in Uzbekistan

Guljakhon Eshbekova [1]*, Ilyos Haydarov [2], Bakhtiyor Kadirov [2] & Zafar Ismailov[1]

[1]Molecular Biotechnology Lab, Department of Genetics and Biotechnology, Samarkand State University named after Sharof Rashidov, University Boulevard, 15, Samarkand, 140100, Uzbekistan
[2] *In vitro* laboratory of SAG-Agro "Bog'bon", Samarkand, 140100 Uzbekistan

*Email: guljakhonbio@mail.ru

OPEN ACCESS

ARTICLE HISTORY

Received: 02 September 2023
Accepted: 22 November 2023

Available online
Version 1.0 : 16 February 2024

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc
See https://horizonepublishing.com/journals/index.php/PST/indexing_abstracting

CITE THIS ARTICLE

Eshbekova G, Haydarov I, Kadirov B, Ismailov Z. Influence of different factors on *in vitro* multiplication and rooting of three local *Juglans regia* L. genotypes in Uzbekistan. Plant Science Today (Early Access). https://doi.org/10.14719/pst.2915

Abstract

The Persian walnut (*Juglans regia* L.) is one of the most lucrative and widely distributed nut crops. It is appreciated as a forestry and ornamental tree in addition to its benefits as a fruit crop. Although Central Asian countries, especially Uzbekistan, are among the origins of the Persian walnut; they are not considered as top industrial producers of walnuts. Uzbekistan possesses a wide range of walnut genetic resources and as a result of conducted research, several promising, fruitful, early-harvesting varieties and forms have been selected. The aim of this study is to optimize microclonal *in vitro* propagation of selected Uzbekistan local varieties and forms by evaluating concentrations of different plant growth regulators and genotype on multiplication and rooting stages. As mother plants, 2 forms and one variety were selected: the Ideal variety, 'Form PDM23' and 'Form 202YaKT'. On the proliferation stage, the growth rate of walnut microshoots on basal medium Driver and Kuniyuki Walnut Medium (DKW) with different concentrations of 6-benzylaminopurine (BAP) and Indole-3-butyric acid (IBA) (0.01 mg/L) was studied. The rooting stage was assessed in half strength macronutrient DKW medium containing different IBA concentrations (0.0, 2.0, 4.0 and 6.0 mg/L). The Ideal variety and 'Form PDM23' performed best in DKW medium supplied with 1.5 mg/L BAP and 0.01 mg/L IBA, whereas 'Form 202YaKT' performed best in DKW medium supplemented with 1.0 mg/L BAP and 0.01 mg/L IBA for the proliferation stage. For all genotypes, 6.0 mg/L IBA provided the best rooting results.

Keywords

BAP; DKW medium; IBA; *Juglans regia* L.; microclonal propagation; MS medium; multiplication; rooting

Introduction

Persian walnut (*Juglans regia* L.) is considered as one of the most valuable plants, with all of its parts having economic value and growing primarily in temperate climate. It is widely utilized as a forestry and decorative tree, in addition to its benefits as a fruit crop. Central Asia, particularly Uzbekistan, is one of the centers of origin of walnut (1, 2). Vast walnut forests have long existed on the slopes of the Karjontog, Ugom, Piskom, Chatkal, Nurota and Hisar mountain ranges and dense walnut groves could be found in the Bostanlik district mountain ranges (3). Despite this, essentially no scientific research has been conducted in the fields of walnut cultivation technique,

disease and pest management and seed breeding in Uzbekistan. Some scientific research has been carried out in walnut forests, but only in the form of expeditions and monitoring. Practical and innovative research on cultured walnut growing have not been conducted, except from some small walnut groves, walnut orchards have not been established on large areas (4).

However, in recent years, orchards have been established more than 15000 ha in Jizzakh, Samarkand and other regions (5). In these orchards, saplings of walnut varieties, which are world-famous for their productivity, rapid growth and valuable fruit that meet world standards, are being planted and their introduction features are being studied. In particular, in Jomboy, Bulungur, Payarik districts of Samarkand region, orchards were established based on California Chandler variety and Paradox cl.Vlach rootstocks. However, in these saplings, cases of late autumn and early spring frost damage and crown gall infection were high. However, late autumn and early spring frost damage and crown gall infection were severe in these saplings. This suggests that establishing walnut orchards on the basis promising local varieties and forms would be more effective (5).

Central Asian countries including Uzbekistan has an advantage in developing new varieties of walnut, since it has an unlimited set of genetic resources for breeding. They differ not only in good quality fruits and annual sustainable abundant fruiting, but also in resistance to pests and diseases, early spring frosts, extremely low winter temperatures, soil droughts and a variety of other benefits. Hundreds of forms have been identified in forests and household plots over the years that are appropriate for developing excellent varieties that are superior in quality to those cultivated in the rest of the world (6, 7). Some of them were already registered at the Research Institute of Horticulture, Viticulture and Winemaking (RIHVW, Tashkent, Uzbekistan), stored in the uterine garden, but not used for growing varietal seedlings (7, 8). Over the past 25 years, more than 100 forms of walnut have selected with high economically valuable qualities at the Research Institute of Forestry of Uzbekistan (RIFU). These forms need further research in order to use them on industrial scale (9).

In the walnut nursery industry, many various techniques of propagation are used to develop completed propagated trees for orchard planting, including sexual propagation, micropropagation, cuttings, budding and grafting. But, only tissue culture could provide for the production of large quantities of certified materials in a short period of time while keeping the genetic uniqueness of propagated clones (10). Despite the fact that several authors have reported micropropagation procedures for distinct genotypes (11, 12), walnuts are considered recalcitrant to tissue culture, making *in vitro* propagation of newly discovered genotypes challenging (13, 14). Furthermore, the low reproducibility of micropropagation protocols is another essential aspect that makes the reproduction of some varieties a more complex process, pushing them to adapt genotype-to-genotype and laboratory-to-laboratory. In addition, since the results of walnut micropropagation are strongly reliant on genotype, using the same procedure under various conditions, even if carried out by the same staff, may result in significant variances (15, 16). For this reason, the aim of this study was to examine the effect of different plant hormone concentrations and genotype on the process of microclonal propagation by selecting different promising local varieties and forms for the establishment of industrial orchards.

Materials and Methods

Plant material

Three Persian walnut (*Juglans regia* L.) genotypes were obtained on May, 2022 from RIHVW: variety Ideal (N41^0 36.157,E70^{0}05.767) and 'Form 202YaKT' (N41^{0}36.157; E70^{0}05,767), 'Form PDM23' (N39^{0}73.852; E66^{0}73,381) (Fig. 1). They have been recommended as a promising sources for establishing orchards by RIFU (7). Apart from delivering consistent high-quality harvests, these genotypes have been selected for their resilience to cold temperatures, pests and disease, making them valuable in regions characterized by freezing temperatures occurring during early fall and late spring (8). For *in vitro* introductions, sticks from healthy and vigorous grafted adult trees were selected as starting materials.

Fig. 1. Location of donor trees, which initiation materials were obtained.

General in vitro culture conditions

The formulations of DKW (11) and MS (17) were used as culture media. DKW was prepared from stock solutions and for MS a dry powder (code 5519, Sigma Aldrich; St. Louis, MO, USA) was used. The pH was adjusted to 5.7 with NaOH (0.1 N); afterward the culture media were sterilized by autoclaving during 20 min at 121 °C. All processes performed in Bogbon, SAG-Agro *In vitro* laboratory (SAG Agro Lab**,** Samarkand, Uzbekistan). Standard photoperiod (16/8 h) was utilized in the culture room, humidity was 40-60%, mean light intensity was 4500 lx (PH LED tube 1200 mm 2*36 W) and temperatures ranged from 22 to 24 °C. Chemicals were provided by Duchefa Biochemie (Haarlem The Netherlands). Culture mediums were gelled with industrial agar (5.5 g/L) for *in vitro* introduction, proliferation and root induction. Rooting was accomplished in 2 stages: root induction (5-7 days) and root expression (16/8 h photoperiod). For this stage, robust and vigorous microshoots from multiplication were chosen, with no sign of defoliation or wilting and a length of at least 20-30 mm, as recommended for clones of the walnut hybrid progeny Mj209xRa (15).

In vitro introduction

In vitro introductions were performed during 2022. Taking into account that the introduction stage is one of the critical stages, in order to increase the efficiency, the trees selected as mother plants were first treated with $CuSO_4$*5 H_2O in February. Then, in April, it was treated with fungicide and in May, the introduction material was taken. Materials were individually labelled and transferred to the laboratory within 12 h after being collected. Cuttings were washed profusely in tap water for 1 h, then washed in household soap and washed again to remove the soap residues. Then, they were washed in the carbendazim fungicide solution (code 378674, Sigma-Aldrich), for 10 min, in the solution of streptomycin as a bactericide (1 g/L) for 12 min, in ethanol (C_2H_5OH) for 2 min and in $AgNO_3$ solution (0.2%) for 1 min. After each process, the samples were rinsed 3 times in distilled water. All procedures were performed in a sterile laminar hood. Explants were individually inoculated in glass test tubes (150×20 mm) containing 10 mL MS culture medium supplemented with 2 mg/L isopentenyl adenine (2iP) and maintained under the standard photoperiod conditions. Contaminated or dead explants as well as those with profuse phenolic releasing were counted and discarded (14).

Multiplication

Cultures were inoculated in polypropylene containers (LA-Rectangular, 90/180 (A), Lab Associates, Bosschendijk, Netherlands). For *in vitro* multiplication, DKW formulation supplemented with BAP (0.0, 1.0, 1.5, 2.0 mg/L) and IBA (0.01 mg/L) concentrations was utilized. Ten explants were inoculated in 100 mL of culture medium each vessel. During the 4th week, subcultures were demonstrated. At the end of subculture, the number and the length of the microshoots, the number of leaves was evaluated for each treatment (18).

Rooting

Cultures were incubated during one week in the darkness in a culture medium with macronutrients of DKW reduced to 50% and different concentration (0.0, 2.0, 4.0, 6.0 mg/L) of IBA. Following that, microshoots were transferred to the expression sub-stage in the same culture medium with vermiculite, without IB nor agar for a 2 week. After 3 weeks, the number of rooted microshoots and the length of the roots were counted, and the general state of the rooted microshoots was recorded (16).

Experimental design and statistical data processing

The statistical significance of data was verified using an analysis of variance of the Microsoft Excel 2013 package. The least significant difference (LSD) test (P=0.05) was used to conduct mean comparisons. For all experiments, a randomized model was used. The container was the basic experimental unit; thus, the average of the containers was utilized as individual data for analysis. For all trials, each treatment was composed of at least 3 experimental units. During the proliferation stage, at least 3 subcultures were assessed, with each subculture considered a repetition.

Results and Discussion

The establishment is a crucial stage in the process of microclonal propagation of walnut and microbial contamination, phenol releasing, darkening of explants are the main problems (10, 19). Various contaminations have a great impact on the successful implementation of this process (18, 19), especially when the somatic organs of trees grown in the field are used as initiation material. Additionally, each plant contains microorganisms as endo- and ectosymbionts, each genotype has its own microbiome and it is noted that this stage also depends on the genotype (20, 21). Similar results were obtained at this stage when DKW or MS medium were utilized (10, 19). However, in most cases better result is observed when MS is used (13, 14). Therefore, MS medium supplemented with 2 mg/L 2iP was used for initiation in this study. Then, losses due to contamination and the number of successfully established microcuttings were determined depending on the genotype (Fig. 2). In our study, we have found that the highest

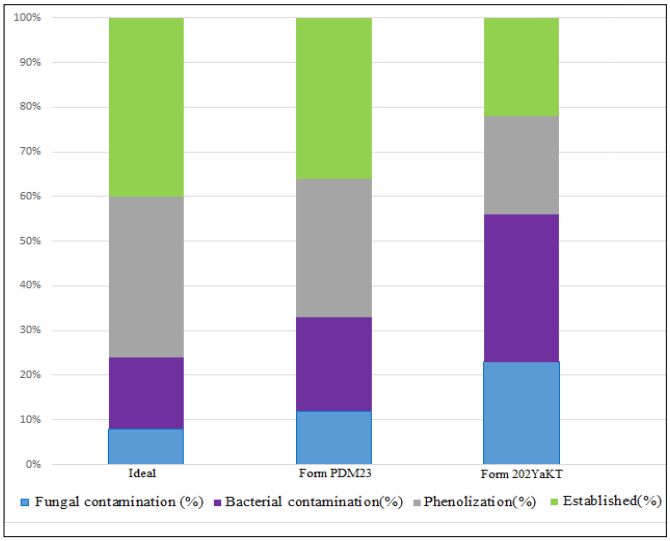

Fig. 2. Results of *in vitro* introduction on different genotypes.

loss in the number of initiation material is due to bacterial (33%) and fungal contamination (23%) was observed in Form 202YaKT, while it was 2-fold (16%) and 3-fold (8%) less in Ideal variety respectively. However, as a result of phenolization, 36% of Ideal variety and 31% of 'Form PDM23' microcuttings died. The rate of successfully established microcuttings was slightly higher in Ideal variety (40%) than, 'Form PDM23' (36%), but in 'Form 202YaKT' it was almost 2 times lower (22%). Results of several studies was consistent with our findings (22-24).

After 4 weeks of subculture, shoot number and callus size were investigated and they were different depending on the genotype. The shoot number of explants of Ideal variety (3.1) and 'Form PDM23' (2.9) showed similar results, but it was lower in 'Form 202YaKT' (2.2). Callus size showed the smallest value in 'Form 202YaKT' (1.4) in contrast to Ideal variety (2.01) and 'Form PDM23' (2.09) (Table 1). A similar study compared the performance of Chandler, Hartley and Z60 genotypes and their results were compatible with our study (25).

Table 1. Assessment of studied factors on different genotypes

Variety and forms	Shoot length (cm)	Mean shoot number	Leaf number	Callus size
Ideal	$3.10^{a} \pm 0.11$	$1.86^{b} \pm 0.10$	$14.13^{d} \pm 0.55$	2.01 ± 0.10
'Form PDM23'	2.91 ± 0.11	$1.79^{c} \pm 0.10$	$13.26^{e} \pm 0.42$	$2.09^{f} \pm 0.08$
'Form 202YaKT'	$2.21^{a} \pm 0.07$	$1.10^{b,c} \pm 0.11$	$10.41^{d,e} \pm 0.36$	$1.50^{f} \pm 0.08$

Values in each column represent means ± SE. $n = 20$; shoots of explant means with different letters are significantly different at $P < 0.05$.

Furthermore, shoot length, shoot number and callus size were studied to determine the relationship between plant hormone treatment (BAP) and genotypes. Results showed that, these parameters increased in all genotypes with increasing hormone concentration (Fig. 3). When the concentration of BAP was 2.0 mg/L, the average shoot length was 4.5 cm and 4.3 cm in the Ideal variety and 'Form PDM23' and the shoot number was 2.8 and 3.5 respectively. However, at this concentration, callus size in both genotypes showed a high index, 4.3 cm and 4.5 cm respectively. Also, yellowish leaves, vitrification and weakening of the main stem were observed (Fig. 4). At 1.5 mg/l concentration of BAP, callus size was significantly smaller in both genotypes, namely, 3.2 cm and 2.5 cm respectively. At 1.5 mg/L and 2.0 mg/L concentrations of BAP, there was no significant difference in shoot length and number on explants of Ideal variety and 'Form PDM23'. In addition, callus size was smaller and the overall condition of seedlings was good at 1.5 mg/L. For these 2 genotypes, 1.5 mg/L concentration of BAP was found to be optimal in the proliferation stage. In Form 202YaKT, BAP at 1.0, 1.5 and 2.0 mg/L concentrations did not significantly differ in shoot length (respectively, 2.8 cm, 3.2 cm, 3.2 cm) and number (respectively, 1.8, 2.1, 2.2), however, with increasing concentration, callus size also increased (2.5 cm, 3.5 cm, 3.8 cm). Therefore, BAP concentration of 1.0 mg/L was found to be optimal for Form 202YaKT.

Cell division and growth can be controlled in the proliferation step by the application of several cytokinins. Among different cytokinins, benzylaminopurine (BAP), zeatin, kinetin, isopentenyl adenine (2iP) and thidiazuron (TDZ) are the most extensively utilized in walnut micropropagation (26-28). Significant changes in the studied factors were noted in this study when explants were treated with BAP. Shoot length, number and leaf number increased with increasing hormone concentration, which is similar to the results reported previously (29-31). This supports our findings and indicates importance of cytokinins in walnut proliferation.

The success of rooting stage is determined by the induction method, the concentration of 3-indole butyric acid (IBA), the light and the quality of the explant (32, 33). For the rooting stage, high-quality, well-grown, 2-3 cm explants were chosen. After 3 weeks, the %, number and length of roots were determined (Table 2). There was a significant difference between the studied indicators in the control and hormonally treated variants, that is, they increased with the increase in the concentration of IBA. When the concentration of IBA was 6.0 mg/L, the % of rooting reached the highest level in all genotypes (58.3%, 63.2% and 45.7% respectively). At this concentration, the number of roots also increased 2-fold compared to the control in all 3 genotypes (4.2, 4.1, 3.5 respectively). The root length was the highest in 'Form PDM23' (4.4 cm), while Ideal variety and 'Form 202YaKT' showed a similar result (3.4 cm 3.1 cm). The results showed that the average rooting % was the highest in 'Form PDM23' (41.2%), followed by Ideal (37.8%) and 'Form 202YaKT' (32.4%). In addition, when the average number of roots for each genotype was calculated, the Ideal variety showed a higher result (3.6), in contrast to 'Form PDM23' (3.3) and 'Form 202YaKT' (3.1) (Fig. 5).

Table 2. Assessment of studied factors under different indole butyric acid (IBA) levels on rooting

IBA (mg/L)	Rooting rate (%)			Number of the roots			Length of the roots (cm		
	Ideal	PDM23	Form 202YaKT	Ideal	PDM23	Form 202YaKT	Ideal	PDM23	Form 202YaKT
0.0	$14.13^{a} \pm 1.10$	$13.41^{b} \pm 1.23$	$11.12^{d} \pm 1.41$	$2.32^{f} \pm 0.23$	$2.10^{f} \pm 0.15$	2.11 ± 0.21	2.52 ± 0.23	$2.95^{j} \pm 0.24$	$1.92^{h,j} \pm 0.19$
2.0	36.42 ± 1.30	$40.63^{a,b,c} \pm 1.12$	$31.28^{d} \pm 1.10$	$4.05^{e} \pm 0.18$	3.26 ± 0.22	$3.22^{f} \pm 0.18$	$2.88^{h,i} \pm 0.18$	3.87 ± 0.28	2.53 ± 0.22
4.0	$42.25^{a} \pm 1.20$	$46.32^{a} \pm 1.14$	$41,36^{a} \pm 1.202$	3.85 ± 0.15	$3.68^{f} \pm 0.18$	3.85 ± 0.23	3.24 ± 0.14	$4.21^{i} \pm 0.31$	$2.84^{j,i} \pm 0.12$
6.0	$58.37^{a} \pm 1.05$	$63.21^{b,c} \pm 1.30$	$45.72^{c,d} \pm 1.31$	$4.25^{f} \pm 0.19$	$4.15^{f} \pm 0.14$	$3.54^{f,g} \pm 0.26$	$3.43^{h,i} \pm 0.25$	$4.42^{h,j} \pm 0.15$	$3.12^{j} \pm 0.16$

Values in each column represent means ± SE. $n = 30$; shoots of explant means with different letters are significantly different at $P < 0.05$.

According to the results of this study, it can be concluded that IBA (6 mg/L) is the best hormonal treatment

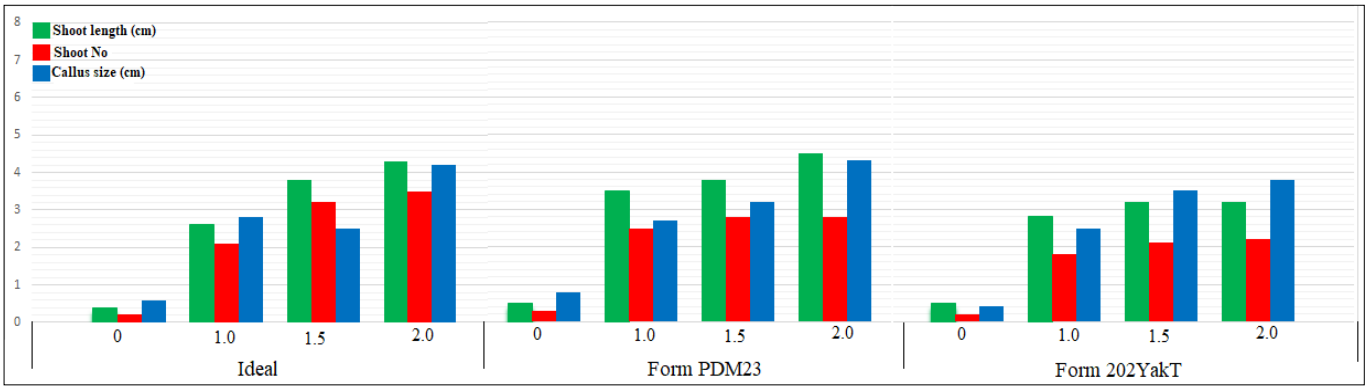

Fig. 3. Influence of BAP (0, 1.0, 1.5, 2.0 mg/L) and genotypes interaction on different factors.

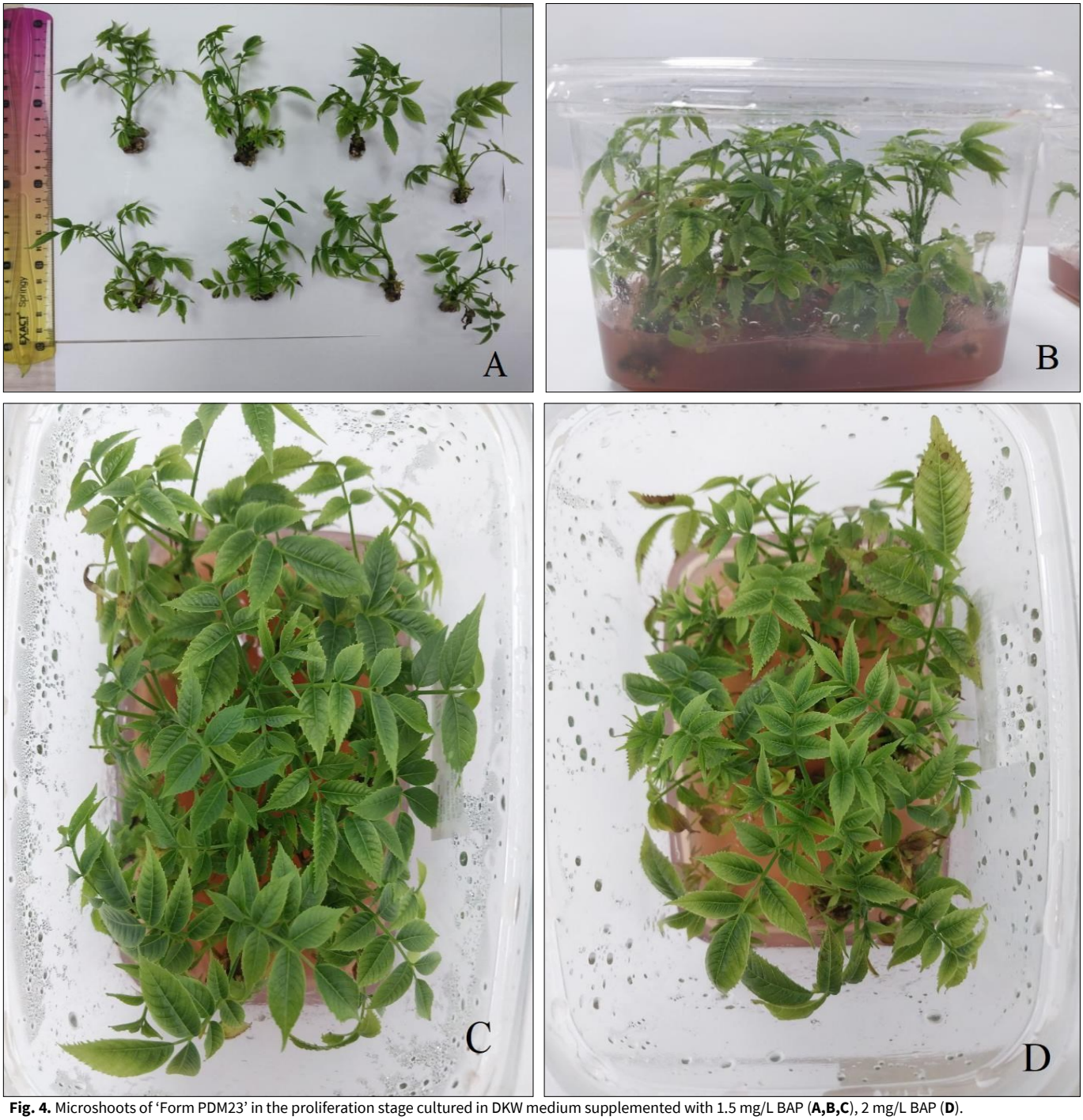

Fig. 4. Microshoots of 'Form PDM23' in the proliferation stage cultured in DKW medium supplemented with 1.5 mg/L BAP (**A,B,C**), 2 mg/L BAP (**D**).

for the rooting stage of the walnut genotypes mentioned above. In a similar experiment, when the concentration of IBA exceeded this value, a decrease in the % and number of rooting was observed (25). In addition, when IBA was used, the rooting % was higher than when NAA was used (32, 34). The influence of the genotype at the rooting stage has been studied in various experiments and corresponds to the results of this study (23, 32).

Conclusion

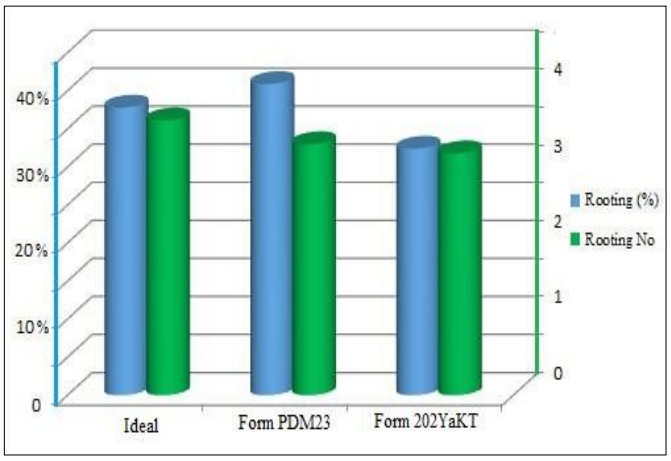

Fig. 5. Rooting percentage and root number on different genotype.

Persian walnut (*Juglans regia* L.) has economical and nutritional importance through worldwide, as well as Uzbekistan. Therefore, studying properties of different genotypes and creating protocols of their micropropagation would give a chance to utilize them in an industrial scale. The findings of our study revealed that Ideal variety and 'Form PDM23' performed better than 'Form 202YaKT' in the examined factors during the proliferation and rooting stages. BAP 1.5 mg/L was found to be optimal for Ideal variety and 'Form PDM23' for the proliferation stage, while 'Form 202YaKT' performed best in BAP 1.0 mg/L concentration. At the rooting stage, 6.0 mg/L IBA showed the best results for all genotypes. These local *Juglans regia* L. genotypes, especially the Ideal variety and 'Form PDM23', can be microclonally propagated on an industrial scale and orchards can be created based on them.

Acknowledgements

The authors would like to thank the SAG-AGRO, Bogbon in vitro Laboratory for providing the necessary equipments and reagents for the research.

Authors contributions

EG carried out the experiments, statistical analysis and drafted the manuscript. KB participated in analysing results of experiments. HI participated in the design of the study and conducted the critical revision of the manuscript. IZ conceived of the study and participated in its design and supervised the research. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

References

1. Bernard A, Lheureux F, Dirlewange E. Walnut: Past and future of genetic improvement. Tree Genetics and Genomes. 2018;14(1):1-8. https://doi.org/10.1007/s11295-017-1214-0
2. Shah RA, Bakshi P, Jasrotia A *et al.* Morphological to molecular markers: Plant genetic diversity studies in walnut (*Juglans regia* L.)- A review. Erwerbs-Obstbau; 2023. https://doi.org/10.1007/s10341-023-00892-x
3. Pollegioni P, Woeste KE, Chiocchini F *et al.* Landscape genetics of Persian walnut (*Juglans regia* L.) across its Asian range. Tree Genetics and Genomes. 2014;10:1027-43. https://doi.org/10.1007/s11295-014-0740-2
4. Umurzakov EU, Pulatov OA, Abdulloev KT. Walnut (*Juglans regia* L.). Samarkand: SamSIFL. 2023; p.37-43.
5. Pardaev G, Normamatov R. Economic and nutritional values of walnut: The main reason for development of walnut in Uzbekistan. 2021;12(2):103-12. https://doi.org/10.22034/jon.2021.1917649.1101
6. Mapelli S, Bertani A, Malvolti ME, Olimpieri I, Pollegioni P, Alexandrovski ES, Butkov EA, Botman EK. Resources of *Juglans regia* in Uzbekistan: A valuable step along the silk road. Acta Hortic. 2014;1032(7):55-62. https://doi.org/10.17660/ActaHortic.2014.1032.7
7. Butkov EA *et al.* Catalog of varieties and forms of walnut in Central Asia. Tashkent: Biodiversity International. 2018; p.190-97.
8. Butkov EA, Mamutov BKh, Nikolyai LV, Kasimkhodjaev A. Study on the selection of the best forms of walnut in Uzbekistan. IOP Conference Series: Earth and Environmental Science, Volume 614, 1st International Conference on Energetics, Civil and Agricultural Engineering; 2020 October 14-16; Tashkent, Uzbekistan. 2020;614:1. https://doi.org/10.1088/1755-1315/614/1/012107
9. Hamroev HF, Mashrapov KhT, Shaymatov OA, Tulaev DB. Efficiency of choosing promising walnut forms in the case of Uzbekistan. IOP Conference Series: Earth and Environmental Science, 2nd International Conference on Energetics, Civil and Agricultural Engineering. 2021; October 14-16; Tashkent, Uzbekistan. 2021;939:1. https://doi.org/10.1088/1755-1315/939/1/012043
10. Vahdati K, Sadeghi-Majd R, Sestras AF, Licea-Moreno RJ, Peixe A, Sestras RE. Clonal propagation of walnuts (*Juglans* spp.): A review on evolution from traditional techniques to application of biotechnology. Plants. 2022;11:3040. https://doi.org/10.3390/plants11223040
11. Driver JA, Kuniyuki AH. *In vitro* propagation of paradox walnut rootstock. Hortic Science. 1984;19:507-09. https://doi.org/10.21273/HORTSCI.19.4.507
12. McGranahan GH, Driver JA, Tulecke W. Tissue culture of *Juglans*. In Cell and Tissue Culture in Forestry. 1987;3:261-71. http://dx.doi.org/10.1007/978-94-017-0992-7_19
13. Lone IA, Misger FA, Banday FA. Effect of different growth regulator combinations on the percent media browning in walnut *in vitro* studies using MS medium. Asian J Soil Sci. 2017;12:135-42. https://doi.org/10.15740/HAS/AJSS/12.1/135-142
14. Licea-Moreno RJ, Contreras A, Morales AV, Urban I, Daquinta M, Gomez L. Improved walnut mass micropropagation through the combined use of phloroglucinol and FeEDDHA. PCTOC. 2015;123:143-54. http://dx.doi.org/10.1007/s11240-015-0822-3
15. Vahdati K, Najafian Ashrafi E, Ebrahimzadeh H, Mirmasoumi M. Improved micropropagation of walnut (*Juglans regia* L.) on media optimized for growth based upon mineral content of walnut seed. Acta Hortic. 2009;839:117-24. https://doi.org/10.17660/ActaHortic.2009.839.13
16. Vahdati K, Leslie C, Zamani Z, McGranahan G. Rooting and acclimatization of *in vitro*-grown shoots from mature trees of three Persian walnut cultivars. HortScience. 2004;39:324-27. http://

dx.doi.org/10.21273/HORTSCI.39.2.324

17. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum. 1962;15:473-97. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

18. Leslie C, McGranahan G. Micropropagation of Persian walnut (*Juglans regia* L.). In High-Tech and Micropropagation II. Berlin/ Heidelberg, Germany: Springer. 1992; p. 136-50. https://doi.org/10.1007/978-3-642-76422-6_7

19. Yegizbayeva TK, García GS, Yausheva TV, Kairova M, Apushev AK, Oleichenko SN, Licea-Moreno RJ. Unraveling factors affecting micropropagation of four Persian walnut varieties. Agronomy. 2021;11:1417. https://doi.org/10.3390/agronomy11071417

20. Akramov I, Axanbayev Sh, Alikulov B, Mukhtorova S, Ergashev A, Ismailov Z. Plant growth-promoting properties of endophytic bacteria isolated from some xerophytic plants distributed in arid regions (Uzbekistan). Plant Science Today. 2023;10(4):228-37. https://doi.org/10.14719/pst.2725

21. Ngoc TP, Andreas MD *et al*. Endophytic bacterial communities in *in vitro* shootcultures derived from embryonic tissue of hybrid walnut (*Juglans×intermedia*). Plant Cell Tiss Organ Cult. 2017;130:153-65. https://doi.org/10.1007/s11240-017-1211-x

22. Kushnarenko S, Aralbayeva M, Rymkhanova N, Reed B. Initiation pretreatment with plant preservative mixture increases the percentage of aseptic walnut shoots. *In Vitro* Cellular and Developmental Biology- Plant. 2022;58:964-71. https://doi.org/10.1007/s11627-022-10279-4

23. Kepenek K, Kolagası Z. Micropropagation of walnut (*Juglans regia* L.). Acta Physica Polonica A. 2016;130(1):150-56. https://doi.org/10.12693/APhysPolA.130.150

24. Leal DR, Sánchez-Olate M, Aviles F, Materan ME, Uribe M, Hasbun R, Rodríguez R. Micropropagation of *Juglans regia* L. In: SM Jain and H Häggman (Eds.), Protocols for Micropropagation of Woody Trees and Fruits. 2007;7:381-90. https://doi.org/10.1007/978-1-4020-6352-7_35

25. Zarghami R, Salari A. Effect of different hormonal treatments on proliferation and rooting of three Persian walnut (*Juglans regia* L.) genotypes. Pak J Biol Sci. 2015;18:260-66. https://doi.org/10.3923/pjbs.2015.260.266

26. Bosela MJ, Michler CH. Media effects on black walnut (*Juglans nigra* L.) shoot culture growth *in vitro*: Evaluation of multiple nutrient formulations and cytokinin types. *In Vitro* Cellular and Developmental Biology- Plant. 2008;44:4:316-29. https://doi.org/10.1007/s11627-008-9114-5

27. Lone IA. Effect of different growth regulator combinations on *in vitro* callusing of walnut (*Juglans regia* L.). An Asian Journal of Soil Science. 2017;12(1):203-09. https://doi.org/10.15740/HAS/AJSS/12.1/203-209

28. Tuan PN, Meier-Dinkel A, Höltken AM, Wenzlitschke I, Winkelmann T. Factors affecting shoot multiplication and rooting of walnut (*Juglans regia* L.) *in vitro*. In: M Beruto and EA Ozudogru (Eds.), Proceedings of the VI International Symposium on Production and Establishment of Micropropagated Plants, ISHS Acta Horticulturae. 2017;1155:525-30. https://doi.org/10.17660/ActaHortic.2017.1155.77

29. Stevens ME, Pijut PM. Rapid *in vitro* shoot multiplication of the recalcitrant species *Juglans nigra* L. *In Vitro* Cellular & Developmental Biology-Plant. 2018;54(3):309-17. https://doi.org/10.1007/s11627-018-9892-3

30. Cem D, Hacer K, Nurberat Ç, Senem Ş, Begüm G, Aynur G. Effects of different culture media compositions on *in vitro* micropropagation from paradox walnut rootstock nodes. 2022;9(4):500-15. https://doi.org/10.54287/gujsa.1194822

31. Meier-Dinkel A, Wenzlitschke I. Micropropagation of mature *Juglans* hybrids. In: M Beruto and EA Ozudogru (Eds.), Proceedings of the VI International Symposium on Production and Establishment of Micropropagated Plants, ISHS Acta Horticulturae. 2015;1155:85-92. https://doi.org/10.17660/ActaHortic.2017.1155.11

32. Dong P, Lichai Y, Qingming W, Ruisheng G. Factors affecting rooting of *in vitro* shoots of walnut cultivars. The Journal of Horticultural Science and Biotechnology. 2007;82(2):223-26. https://doi.org/10.1080/14620316.2007.11512223

33. Caboni E, Damiano C. *In vitro* propagation of walnut (*Juglans regia* L.): Critical factors for the induction of the rooting response. Acta Horticulturae. 2006;705:329-33. https://doi.org/10.17660/ActaHortic.2005.705.44

34. Ribeiro H, Ribeiro A, Pires R, Cruz J, Cardoso H, Barroso JM, Peixe A. *Ex vitro* rooting and simultaneous micrografting of the walnut hybrid rootstock 'paradox' (*Juglans hindsi* ×*Juglans regia*) cl. 'Vlach'. Agronomy. 2022;12:595. https://doi.org/10.3390/agronomy12030595