RESEARCH ARTICLE

SABRAO Journal of Breeding and Genetics
57 (4) 1480-1490, 2025
http://doi.org/10.54910/sabrao2025.57.4.13
http://sabraojournal.org/
pISSN 1029-7073; eISSN 2224-8978

STANDARDIZATION OF THE MICROCLONAL PROPAGATION PROCESS IN *ARONIA MELANOCARPA* FROM SEEDS

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SUMMARY

Black chokeberry (*Aronia melanocarpa*) is a shrubby plant with a root system of 0.5 m in the soil. Its fruits are rich with antioxidant properties, making it a valuable plant in the pharmaceutical, food, and perfumery industries. The following study comprised the microclonal propagation of *A. melanocarpa* acclimatized under the conditions of Uzbekistan. The efficacy of its seed coat in sterilization and germination underwent evaluation, and the germination was higher (32%) in husked seeds. Based on the analysis of clone stages, the study determined that starting from Mk3, the length of microshoots (3.2 cm) and other quality indicators were ready for the rooting stage. During the rooting of *A. melanocarpa*, applying different concentrations of IBA (1, 3, 5, 7, and 10 mg/l) served for the evaluation. Based on the results, 7 mg/l IBA emerged as an optimal dose of IBA for root formation in *A. melanocarpa*. During the black chokeberry's acclimatization, plant survival was 100% in a 3:1 peat-perlite substrate.

Keywords: Chokeberry (*A. melanocarpa*), seeds, ex vitro, rooting, in vitro, acclimatization, microclonal stages, hormone concentrations

Key findings: The presented research comprised microclonal propagation of *A. melanocarpa* acclimatized under the conditions of Uzbekistan. The IBA (7 mg/l) emerged to be an optimal dose for root formation in *A. melanocarpa*.

Communicating Editor: Dr. Himmah Rustiami

Manuscript received: February 06, 2025; Accepted: March 27, 2025.

Citation: Abduganiyeva D, Alikulov B, Ruziyev K, Eshbekova G, Sultonova K, Ismailov Z, Gulyamova T, Mamadiyarov M 2025). Standardization of the microclonal propagation process in *Aronia melanocarpa* from seeds. *SABRAO J. Breed. Genet.* 57(4): 1480-1490. http://doi.org/10.54910/sabrao2025.57.4.13.

INTRODUCTION

Black chokeberry (*Aronia melanocarpa* [Michx.] Elliot) is an underutilized and highly nutraceutical plant belonging to the family Rosaceae, recognized for its potential health benefits. Black chokeberry is native to North America before its transport to Europe almost a century ago. Black chokeberry became highly valuable in the Soviet Union and Eastern European countries in the late 20th century and had wide distribution in Europe and Asia (Szopa *et al*., 2018), mostly for the production of juices, jams, and wines on a large scale, as well as a rich source of natural food pigments (Denev *et al*., 2018). Its fruits, rich in polyphenols, also have the supreme content of anthocyanins and proanthocyanins (Jurikova *et al*., 2017; Borsai *et al*., 2021).

The plant can go through wide exportation due to its mechanical strength, inflicting minimal damage to its fruits. Growing black chokeberry can serve the food industry, pharmaceuticals, herbal medicines, and decorative purposes (Rustamova *et al*., 2024). The fruit's consumption can come straight from the can or in various processed forms (Szopa *et al*., 2017). It can also serve as an additive to increase the content of anthocyanins and flavonoids in other beverages (Szopa *et al*., 2017). Its commercial growing in Russia, Denmark, and Eastern European countries mainly supports the preparation of various beverages (Valentina and Catita, 2022).

Currently, chokeberry cultivars, such as Viking, Nero, Marton, and McKenzie, are the most widely used for commercial cultivation. This plant's propagation usually utilizes the seeds, and for commercial purposes, recommendations state its cultivation receive good sunlight and a distance of up to 3.04 m between plants to obtain a good harvest (Şahin *et al*., 2019). However, when propagated from seeds, studies do not recommend their plants due to their late germination, uneven growth, and problems with harvesting (Litwińczuk, 2013; Borsai *et al*., 2021). In *Aronia*, the research work on microclonal propagation began in the last decades of the 20th century (Velchev and Mladenova, 1992; Ruzic, 1993).

With the great importance of *A. melanocarpa* fruits for health and their effective use in various diseases, the last 20 years saw numerous research studies conducted on their propagation (Mansoor and Pirlak, 2018; Rusea *et al*., 2019; Toprak and Alan, 2020; Borsai *et al*., 2021). The in vitro callus production and its industrial development and use are currently of greater interest. The study on the production of callus from the epicarp and hypocarp parts of the fruits started in *A. melanocarpa* (Calalb *et al*., 2014). Later, scientific studies continued on the optimal growth conditions using microclones propagated in vitro using the apical part of *A. melanocarpa* (Damirel *et al*., 2023).

However, work on the microclonal propagation of this plant from seeds in vitro is yet to occur. Therefore, we conducted the research on in vitro propagation of black chokeberry (*A. melanocarpa*) from seeds and evaluated the differences in clone stages and the effect of hormone concentrations on its acclimatization.

MATERIALS AND METHODS

Plant material

Research work on chokeberry (*A. melanocarpa*) commenced in the laboratory located in the territory of the Bog'bon Agro-complex of SAG Agro LLC, Uzbekistan (39°42'29.8" N 67°04'57.9" E). The isolation of seeds of *A. melanocarpa* came from fruits grown in vitro in the laboratory in the said territory after their harvest in the fall and then stored.

Surface sterilization

For surface sterilization of the chokeberry seeds, the protocol followed has the process in Table 1. The sterilization process took place in a sterile laminar flow hood. In each step, the sterilizing agent's rinsing with distilled and autoclaved water continued until completely removing the residue (Eshbekova *et al*., 2024).

Table 1. Sterilization agents and time.

No.	Sterilization agents	Time (min)
1	Running water	60
2	Domestose (15%)	30
3	Fungicide	30
4	Ethanol (70%)	2

Before inoculating in the media, the drying of seeds used sterile paper towels to remove water droplets from the seeds. During the inoculating process, cutting a specific part of the seed coat with a tweezer knife comprised half-seed coat seed (T1), the seed with seed coat (T2), and seed without seed coat (T3) (Abduganiyeva *et al*., 2024).

Media

A modified version of Driver-Kuniyuki (DKW) media served as the initial media (Driver and Kuniyuki, 1984). The initial media contained the plant hormones isopentenyl adenine (2iP) and gibberellins (GA3) in a ratio of 10:1. These plant hormones' application stimulates the seed growth. Modified versions of MS media succeeded as media at the microclonal stage (Murashige and Skoog, 1962).

Growth conditions in the experiments

The glass bottles used in planting the seeds remained in a special growth chamber with a humidity of 60%, a temperature of 25 °C ± 2 °C/16 h of light, and 17 °C ± 1 °C/8 h of darkness. The light intensity was 2400 lux. Using white fluorescent lamps served as the light source, with the recommendations accepted and edited (Eshbekova *et al*., 2024).

Establishment stage

DKW media containing phytohormone (2 iP) with the property of forcing buds was the agent used. The transfer of each seed that started to germinate to new media ensued when it had formed 3–4 leaves and was 0.5–1 cm tall. After one month, calculating seed germination and damage index followed (Abduganiyeva *et al*., 2024).

Multiplication stage

MS media containing BAP was the agent used at this stage. Each explant that germinated from the seed incurred planting in separate glass vessels. Every 21 days, the microclones' transfer to fresh media transpired, recording the length of the microclone and microshoot, the number of microshoots per microclone, and the number of leaves per microshoot for further analysis (Nas *et al*., 2023; Eshbekova *et al*., 2024).

Rooting stage

The selection of healthy microshoots (3–4 cm long) with good quality indicators proceeded for rooting. The use of the IBA hormone with different concentrations served to evaluate its effect on root formation in *A. melanocarpa* microclones and determine the optimal amount of the hormone. After one month, determining the percentage of rooted shoots, the average number of roots per shoot, and the average root length (cm) succeeded (Valentina and Catita, 2022).

Acclimatization

The acclimatization comprised four stages. In the first stage, the rooted microclones, washed in running water to remove media residues, received washing with a fungicide solution for three minutes. The explants' planting continued in 104-well cassettes filled with a 3:1 mixture of peat and perlite before keeping for three days at 100% humidity, 23 °C ± 1 °C, 16 hours of light and eight hours of darkness, and 10 days at 90% humidity. Its transfer to the next acclimatization stage followed. At this time, the transfer of explants took place in 10 cm × 10 cm × 20 cm pots at

60%–70% humidity and 25 °C ± 2 °C temperature. Each stage had the calculation of shoot length, the number of leaves per shoot, and the number of surviving explants recorded.

Statistical analysis

All the collected data's analysis used the OriginPro (2021) and Excel programs based on mean and SE indicators (Alikulov *et al*., 2022).

RESULTS

Culture establishment

For the initial phase, the hormones' selection depended on their function. In particular, a small amount of gibberellins used in the initial media interrupted the dormancy period of the seeds and stimulated cell division and germination (Flasinski and Wydro, 2014). The seeds inoculated in DKW media had their germination monitored for one month. In the three categories of the seeds—the seeds with a seed coat, without a seed coat, and half-seed coat seeds—no germination was visible in the seeds without a seed coat. Germination in the seed with the seed coat was faster than in the seeds with a certain part of the seed coat cut off. However, late germination was evident in the whole-husked seeds, and the germination percentage was 32% higher than in the seeds with a certain part of the husk cut off. Bacterial contamination was notable in 10% of the seeds without a husk; although, no fungal contamination appeared. No growth resulted in any other seed, except the germinated ones (Table 2).

Multiplication stages

The multiplication stage focused on increasing the number of microclones of the plant, with each explant transferred to a fresh media proceeding to the next clone stage. In assessing the differences among the clone stages, data recording for the initial clone stage occurred. Initially, month-old explants from seeds succeeded in counting and transferring to a modified MS media containing BAP from cytokinins, designed for the propagation of microclones, with these explants considered the beginning of the clone stage. The assessment of variation in each clone stage in microclonal propagation had the counting of the initial four clone stages. These stages received labeling as microclone 1 (Mk1), microclone 2 (Mk2), microclone 3 (Mk3), and microclone 4 (Mk4). Each Mk stage continued for 21 days, and at the end of each stage, the length of microclones, the average length of the microshoots in each microclone, the number of leaves in the microclone, and the differences among them underwent evaluation.

The Mk1 was the first microclone plant to proceed in transferring from the initial media to the clonal media. The Mk2 obtained came from the transfer of Mk1 to the new media. Since the explants in Mk1 had many microshoots, their dividing into 2–3 pieces occurred before planting. In the microclonal stage, the calculation for the traits, such as microclone length, length of microshoot, the number of leaves per microshoot, and callus size, ensued.

Table 2. Results of surface sterilization of *A. melanocarpa* seed (n=5).

No.	Regeneration (%)	No regeneration (%)	Callus induction (%)	Fungal contamination (%)	Bacterial contamination (%)
T1	20	65	15	0	0
T2	32	67	0	0	0
T3	0	90	0	0	10

Note: Half-seed coat seed (T1), with seed coat seed (T2), seed without seed coat (T3).

When propagating different plants in vitro, microclone propagation rates vary, and some plants produce numerous microshoots, while others develop leaves and callus well. Reports have stated that microclone stages in vitro usually last up to 17 stages. Estimating the total length of the *A. melanocarpa* microclone and the length of the microshoots relied on the results of the four stages of the clone and the average length values. The total length estimation of the microclone was the length from the lowest callus to the highest growth point of the longest microshoot. The difference between the initial stage of the microclone, Mk1 (1.58 cm), and the Mk4 stage (5.08 cm) was 3.58 cm. By examining the differences in length of various stages, one can find that the length difference between Mk2 and Mk3 was equal to the highest value (1.56 cm). The microclone differed from Mk3 and Mk4 in total length by 0.76 cm.

The length of microshoots was also important at the clone stage, where the length of microshoots from the callus to the lower part separated from the callus, and taking the distance to the highest growth point was the length of the microshoot. The selection of the shoots was random. The average length of the initial microshoots was 1.22 cm, having increased by 1.22, 0.86, and 0.79 cm in length at each stage, respectively. Although, the average length of the microshoots enhanced gradually from Mk1 to Mk4, and the growth ratio between each microclone decreased compared with the previous clone stage. The results revealed microshoots from the Mk3 stage were well-developed and can have the best quality indicators for rooting (Figure 1).

The difference between the average length of microclones and microshoots was due to the callus formed below the microclone. This indicator's use helped calculate the callus size at the microclonal stage of *A. melanocarpa*. The highest callus size obtained came from Mk3 as compared to MS1 and Mk4 (Figure 2). The number of microshoots' definition was the number of microshoots produced in a single microclone and exceeding 2 mm in length (Banjac *et al*., 2023). The number of microshoots was two times higher in the Mk2 than in the Mk1, while the Mk3 stage produced six times more microshoots, becoming the highest value among the microclonal stages. The number of microshoots appeared to decrease in the Mk4 compared with the Mk3. This indicator had an average of 10.8 microshoots per microclone. Although the number of microshoots was fewer at this stage, and the traits, such as microshoot length and leaf size, enhancement was much better in MK4 than other microclonal stages.

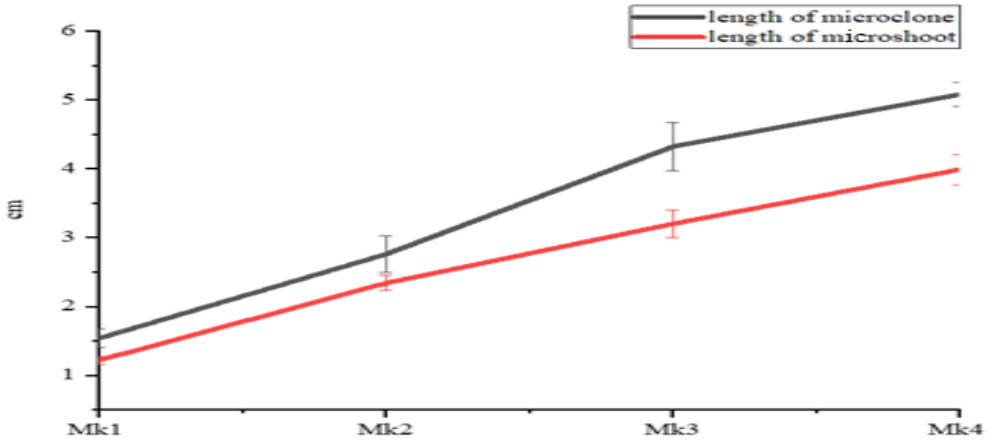

Figure 1. Effect of microclonal stages on microclone and microshoot length (n=10).

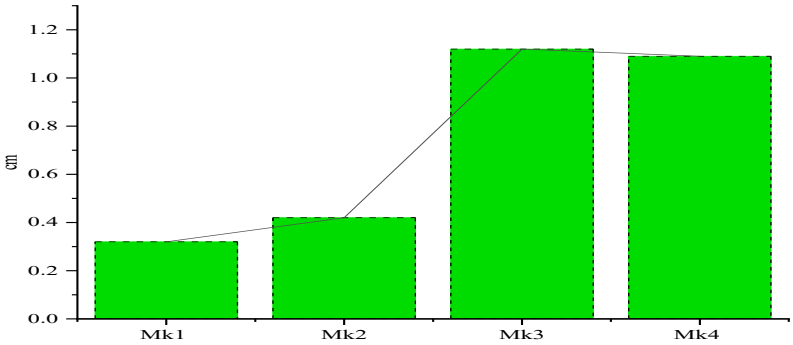

Figure 2. The size of callus formed from the bottom of the *A. melanocarpa* microclone, cm (n=10).

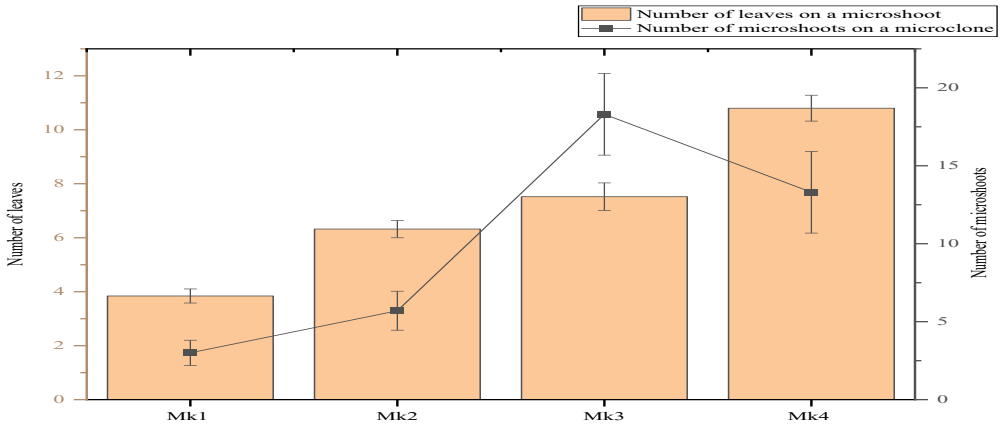

Figure 3. Effect of microclonal stages on the number of leaves and microshoots (n=5).

The leaf indices in the microshoots attained evaluation when the leaves were well-opened. In this case, five microclones of the same average size were the options. The assessment of results relied on the average indicator with five repetitions. In parallel with the increase in the height indicators of the microshoots in each microclone and at each stage, a boost in the average number of leaves also surfaced. Compared to the average leaf index in the initial microshoot, Mk4 recording had three times more results (10.8). Although it was noticeable that the number of leaves on the microshoots increased steadily, the rise in the number of microshoots was not stable, and the highest indicator for the number of microshoots corresponded to Mk3 (18.3) (Figure 3).

Rooting stage induction

For rooting, well-grown 3–4 cm long microshoots proceeded to the rooting media. The IBA's selection from auxins served as the root media, with different concentrations of IBA (1, 3, 5, 7, and 10 mg/l) used. The control was the media without phytohormone (0 mg/l). After 21 days, evaluating explant indicators in media containing IBA with different concentrations continued. The *A. melanocarpa* microshoots obtained from hormone-free media had an average number of roots (3 ± 0.4) at the rooting stage and formed a good root system with lateral roots. The average root length in hormone-free media was 5.6 ± 0.4 cm. In the media with 1 mg/l IBA, the number of roots was 3.3 ± 0.2,

and their length was 10 times smaller than in the media without the phytohormone (0.3 ± 0.0 cm).

In other variants, no root formations occurred; however, better callus formation was remarkable. Between 3 and 10 mg/l phytohormone concentrations, callus size increased from low to high. The largest callus was visible at the IBA concentration (10 mg/l). The explants grown for root formation at different concentrations of IBA differed not only in the formation of roots but also in the number of leaves and internodes. The leaf size of explants grown in the media without the phytohormone was larger than that of explants grown in the media with different concentrations of IBA. Moreover, the leaf size in media with the IBA (10 mg/l) hormone was bigger than that of other hormone concentrations in the media. Higher elongation of internodes was more prominent with the IBA (in 7 mg/l) than other experimental variants.

Acclimatization

Acclimatization can consist of several stages. In the first stage, since the explants grown in vitro were not well-adapted to the external environment and were using ready-made nutrients, for them to adapt relatively well, the humidity and temperature remained the same as in vitro in the first three days. Afterward, the explants that formed better roots and adapted proceeded to transfer to the next stage for enlargement. At this stage, the relative humidity of the air was lower than in the adaptation stage, conducting watering 3–4 times a week. The main aim was to gradually balance the humidity and temperature inside and the temperature and humidity of the external environment.

The first stage of acclimatization was the planting in pots filled with a 3:1 mixture of peat and perlite in special containers. For three days, the room temperature of the explants remained at 26 °C ± 2 °C and 70% humidity using special lidded containers and received watering every day for the first three days. In the following days, less watering occurred according to the humidity. At the next stage of acclimatization, opening special holes in the lid ensued, with the humidity reduced to 90%. After 20 days at this stage, separating explants with well-formed roots and no well-formed roots transpired. The two types of explants received care separately. The explants that did not form good roots acquired microelements and vitamin complexes to stimulate the root formation. In this case, keeping the plants in special acclimatization containers at the fourth day had 90% humidity and a temperature of 26 °C ± 3 °C. The explants that formed better roots when selected succeeded in transferring to the next stage of acclimatization.

The explants rooted at different concentrations of IBA sustained assessment for shoot length and number of leaves per shoot at subsequent stages of acclimatization. The *A. melanocarpa* explants grown on a hormone-free media, upon removal from the rooting media, succeeded in planting in the substrate. The average length of the explants was 2.7 cm, which increased by 2.5 times during the middle of the acclimatization (Mf) period. During the end of the acclimatization (Ef) period, acclimatization entered the adaptation phase. A nonsignificant increase in shoot length appeared during the growth period with relatively low temperature and humidity. The explants obtained from IBA (7 mg/l) showed the highest shoot length from all the stages of acclimatization. The increase in shoot length was due to an increase in internodal spacing, and most of the growth occurred between the initial planting in a peat-perlite mixture substrate (If) and Mf. Between the Mf and Ef periods, the shoot growth slowed length measurement transpired at the If, in the Mf, and at the Ef (Figure 4).

The growth rate of explants' evaluation determined the variations caused by the time of phytohormone effect. The growth rate of explants obtained at IBA (5 and 7 mg/l) during the acclimatization phase was 4.3 and 8.3 times higher during the primary growth period than during the secondary growth period, respectively. In IBA (1 mg/l), the total growth length was the same between the primary and secondary growth. When assessing the total growth value for the primary and secondary growth periods, the main growth in explants obtained from samples with different

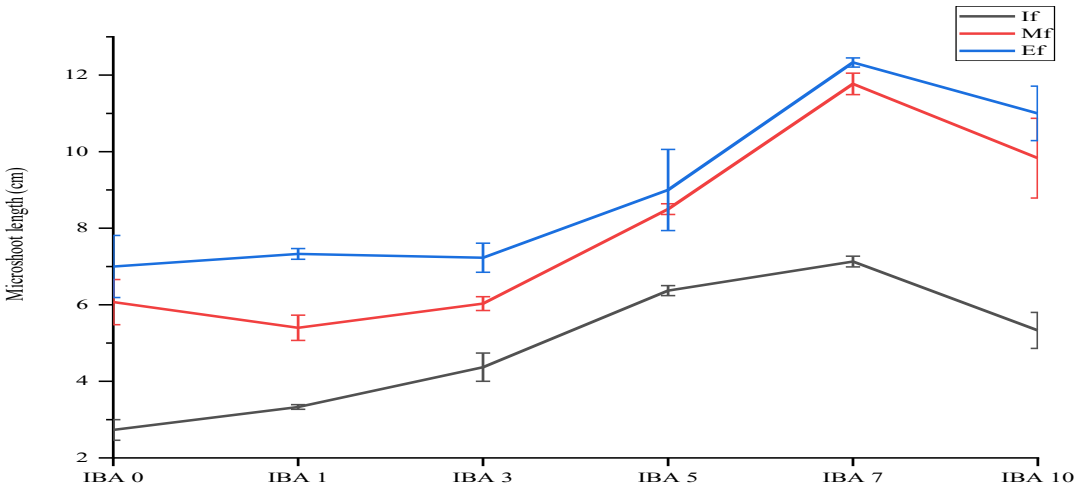

Figure 4. Effect of different IBA concentrations on microshoot length at acclimatization (n=5).

Table 3. Explants that rooted in media with different IBA concentrations for leaf length, leaf width, the number of leaves, and survival rate during acclimatization (n=5).

No.	Leaf length (cm)		Leaf width (cm)		Number of leaves		Surviving explants (%)	
	If	Ef	If	Ef	If	Ef	If	Ef
IBA 0	1.37±0.06	1.70±0.12	0.63±0.02	2.43±0.38	12±0.41	10.67±0.24	100	100
IBA 1	1.33±0.11	3.23±0.02	0.70±0.04	2.10±0.04	10±0.41	11.67±0.85	100	100
IBA 3	1.47±0.06	3.27±0.10	0.80±0.04	2.13±0.02	8.3±0.23	13.00±1.08	100	100
IBA 5	1.93±0.10	3.03±0.02	0.90±0.04	1.77±0.06	8.7±0.47	13.00±1.08	100	100
IBA 7	2.63±0.13	4.10±0.04	1.17±0.06	1.87±0.06	10±0.41	13.67±0.85	100	100
IBA 10	2.37±0.14	4.23±0.27	1.17±0.12	2.13±0.09	12±0.41	13.67±0.24	100	100

concentrations of IBA (0, 3, 5, 7, and 10 mg/l) coincided with the primary growth period. Based on the obtained results, the rooting media with IBA (7 mg/l) tended to be an optimal dose for *A. melanocarpa*.

By evaluating the leaf number traits based on the initial and final results of the acclimatization, a decrease of 1.33 in the average number of leaves was visible in the hormone-free media. The leaf indicators were the same in the IBA (1 and 10 mg/l), and an increase of 1.67 emerged. The highest value and an increase of 4.67 were notable with IBA (3 mg/l). As a result of the acclimatization, in various treatments the survival rate of explants was 100% (Table 3, Figure 5).

DISCUSSION

Past studies reported the shell had no effect on seed germination in peach; however, in other plant species, the shell had a significant effect on seed germination (Jimenez *et al*., 2020). In these presented experiments, the seed coat affected germination. Half-seed coat seed (T1), the seeds with seed coat (T2), and the seeds without seed coat (T3) showed different results. In particular, no germination resulted in the seeds without seed coats. However, the seeds with seed coats retained the best growth characteristics. Germination in the seeds with half-seed coats began earlier than other types of seeds, and the callus formation was remarkable, along with the formation of the initial microclone.

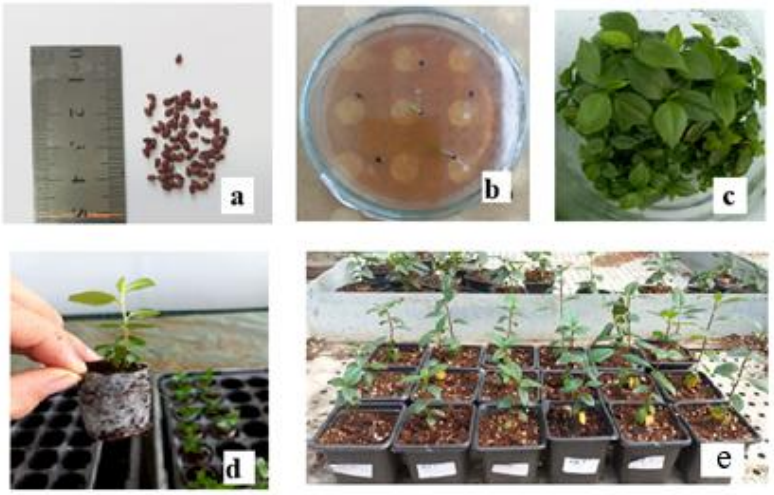

Figure 5. Microclonal propagation process in *A. melanocarpa* from seeds: a) seed of *A. melanocarpa;* b) primary explants sprouted from seed on initial media; c) microclone; d) well-formed root explants in the primary stage of acclimatization; and e) the regenerants at the end of acclimatization.

It is a well-known fact based on various studies that the seed coat plays a selective role in germination. This may be due to nutrients and phytochemicals reaching the interior parts of the seed late because of the seed coat, and they germinate later than in seeds with a certain part of the seed coat cut off (Borsai *et al*., 2021). When the effect of the seed coat on contamination and germination bore scrutiny in vitro, the germination and bacterial contamination observed may, in turn, correlate to the function and composition of the seed coat. The 10% bacterial contamination was noteworthy in the seeds without seed coats. In particular, the detection of antibacterial properties of the rice seed husk resulted in past studies (Shamsollohi and Partovinia, 2019).

The initial microclonal stages of *A. melanocarpa* microclones reached evaluation at the microclone stage, and the study determined that starting from the Mk3 stage, microshoots had the necessary quality indicators for rooting. Callus formation appeared at the bottom of the microclones at all clone stages. By the Mk4, the average number of microshoots was 18.3, the length of the microshoot was 3.99 cm, and the total length of the shoot was 5.08 cm. Rusea *et al*. (2019) reported the average number of newly formed microclones was 10.2, and the average microclone length was 2.24 cm in the cultivar Nero of *A. melanocarpa*. Previous studies displayed that in vitro propagation of three different *Aronia* cultivars (Eastland, Viking, and Nero), the average number of microclones in the cultivar Eastland was 5.04, and the microclone length was 7.01 cm (Toprak and Alan, 2020).

Past studies reported the highest concentration of phytohormones could cause vitrification during the acclimatization process (Bayhan and Yucesan, 2024). In the presented experiments, although using high concentrations of phytohormone, no vitrification was evident. As a result of their experiment, the average microshoot length in 31-day-old explants was 5.5 cm, which was equivalent to the 21-day-old experiment in the latest study. Previous research revealed 94% rooting of microclones in *A. melanocarpa* with IBA (1 mg/l) media (Borsai *et al.*, 2021), while Ionela *et al*. (2022) achieved 95% rooting in MS media supplemented with IBA (0.7 mg/l).

Although in these experiments, the highest concentrations of IBA provided better results for shoot length and leaf number, and this may refer to the strength of the IBA concentration in the plants. The effect of the hormone was high in the early stages of the

acclimatization, and later, the growth rate decreased. The current results for the rooting stage were lower than in other experiments. However, in the hormone-free media, the rooting was 100%. The roots were tiny at the IBA (1 mg/l), and with other IBA concentrations, the microshoots were rootless, but the callogenesis was high. A report stated that during the acclimatization phase, 80%–97% rooting of microclones and 90%–93% acclimatization at 70%–80% humidity were successful in the cultivars Nero and Melrom of *A. melanocarpa* (Valentina and Catita, 2022).

CONCLUSIONS

In *A. melanocarpa*, the in vitro propagation proved a very effective method. In obtaining a microclone from the seeds, it was successful to sow the seeds with the seed coat and to obtain callus by cutting off 1/3 of the seed coat. A little contamination in the seed propagation occurred, and the microshoots at the third clone stage had the necessary quality indicators and could be favorable for rooting in the rooting media. Using IBA (1 mg/l) results in a better average of microshoots and microclone length. However, at the rooting stage, IBA (7 mg/l) was superior for root formation.

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