Optimum conditions for primary callus production in microclonal propagation of *Aronia melonacarpa* (Michx) Elliott

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Abstract. The research describes the results of experiments aimed at determining the dependence of sterilization methods and the type of initial material on the effectiveness of primary callus production in microclonal propagation of *Aronia melonacarpa* (Michx.) Elliott. In the experiments, the optimal conditions for sterilization of biomaterials obtained from plants were initially selected. To increase the efficiency of callusogenesis, the composition of the MS medium was modified and used. Alternatively, microclonal shoots have been shown to be an effective initial material for primary callus production from *A. melonacarpa*. The results obtained in this work can serve to increase the efficiency of the technology of microclonal production in plants. Key words: *Aronia melanocarpa,* callus, callus induction, BAP, initial material, explant, cortex.

1 Introduction

A.melanocarpa, also known as black chokeberry, is a plant native to North America. But to date, this plant has been cultivated in various European and Asian countries. It has been cultivated in Uzbekistan since 2012. This plant belongs to the Rosaceae family and is a shrub. The fruit of this plant contains macro (Ca) and microelements (Fe, Mo, Mn, Cu, B, I, Co), vitamins (P, C, B2, B6, PP, E, proA), carbohydrates, cellulose, pectins, and is rich in anthocyanin, proanthocyanin, organic acids, and catechins. Also, the fruit contains valuable biologically active substances that are important for the pharmaceutical industry: anti-inflammatory, anti-microbial, anti-viral, beneficial for the gastrointestinal tract, has a positive effect on the cardiovascular system, delays the natural aging process, normalizes blood pressure, gastrointestinal, and blood. There are substances that improve the elasticity of the blood vessel wall. This plant can be propagated from seeds, but plants propagated by this method are not recommended due to their late germination, non-uniformity of growth, and inefficient mechanical harvesting. Scientific work on breeding *A. melanocarpa* by the microclonal method began in the 1990s. Root formation in the microclone of *A. melanocarpa* propagated in vitro was thoroughly studied by a number of scientists such as Zatyko JM, Molnar I., Velchev V, Mladenova O., and Ruzic D., [1, 2, 3, 4] W. Litwińczuk [5], microclonal production from the meristem of *A. melanocarpa* by Ionela Rusea and colleagues in 2019

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[6], microclonal from mature nodal stem Scientific research was carried out by scientists such as Mansoor H.M. and Pirlak L. [7]. Because the fruit of the black chokeberry plant contains a large amount of useful substances, extracting callus from its fruit and its development and use on an industrial scale have attracted great interest some scientists, and in 2014, research work on extracting callus from the epicarp and hypocarp parts of the fruit was carried out by T. Calalb and studied by his colleagues [8]. It was also possible to obtain a microclone from the meristem, petiole tissues of this plant in vitro[9,10]. In this case, *A. melanacarpa* cv. Nero was used, and meristem tissues were isolated from nodal shoots. After sterilization, some of the primary leaves in the shoot were removed and planted in MS medium supplemented with plant harmones BA and IBA, and the number of microshoots formed from each mikroshoots was 10.2 [11, 12]. Microclonal propagation from apical and nodal shoots in vitro by Borsai O. and colleagues [13]. Also, experiments on mikroclonal propogation from callus were conducted in several species of *A. melanocarpa* and achieved good results. [14-16].

In our following experiment, after studying the scientific and practical experiments on the *A. melanocarpa* i*n vitro* until now, it was found that the work carried out on which part of it can produce effective callus is insufficient and not well studied. Our research purposed to find out which part of this plant is more effective to use as a initial material for formation callus

2 Materials and methods

2.1. Plant materials

All necessary plant materials for the current experiment were obtained from the *A. melanocarpa* plant grown in the SAG AGRO in vitro laboratory area. The collection of initial plant material was carried out in the morning between 8 and 9:00 in April. The seeds were collected from fruits collected in autumn and stored in the laboratory. All required reagents were token from Duchefa (Netherlands).

2.2. Getting initial plant materials

Seed. *A. melanocarpa* seeds were treated in two different methods. In particular, a certain part of the seed is dried in the sun for 3 days after separating it from the mesocarp. For other seeds, the seed coat was removed and sterilized.

The whole cortex was removed from some seeds to study callus formation from dehusked seeds. The concentration and time of the sterilizing agent for dried and non-dried seeds were carried out in the same way. The concentration and time of the sterilizing agent were reduced in order not to damage the catylidon and inner part of the seeds without the cortex compared to the seeds with the bark.

Stem. Two different stems of *A melanocarpa* were selected. A one-year mature branch and newly formed one-month old stems with a length of 10-15 cm were selected.

Leaf. Newly formed, unripe leaves with a diameter of 2-2.5 cm were selected.

2.3. Sterilization

Leaf sterilization was carried out in two different ways. In the first method, the young leaves of the plant picked from the stem were first washed thoroughly with running tap water to remove dust particles, and then sterilized with a 10% domestose solution for 30 minutes. During this process, the sterilization solution containing the leaves was constantly shaken. Then it was washed seven times with distilled water and steeped in a 70% alcohol solution

for one minute in order to remove the remains of domestose. At the end of sterilization, it was washed again five times with distilled water. These works are performed in a laminar box under sterile conditions.

The second method used the same amount of reagents as the first method and additionally sterilized it in a fungicide solution for 1 minute. Sterilization of seed and a year-ld nodal segment of stem was carried out in separate containers with the same substance concentration at the same time. First of all, they were washed in running tap water, sterilized in a 10% solution of domestose for 30 minutes, in a fungicide (1 g/l) solution for 20 minutes, in a 70% ethanol solution for 2 minutes, and washed with sterile distilled water 4-5 times at each stage.

A newly formed one-month-old stem was also carried out in the same way, only it was kept in a fungicide solution for 15 minutes. Also, before sterilization, 25 cortexes of seeds were removed using special tweezers and sterilized in a 10% solution of domestose for 5 minutes, in a 1% solution of fungicide for 30 seconds, and in 70% alcohol for 2 minutes.

2.4 Media preparation and culture condition

In experiment, modified MS media was used. BAP plant hormone 2 g/l and IBA 1 g/l were added to this nutrient medium to improve callus induction. 7.5 g/l agar was added. Sucrose was used as a carbohydrate source. The ingredients of the media are given in table 1.

Table 1. Content of MS media

Chemical	MS (1962)	Modification MS
$CuSO_4 \cdot 5H_2O$	0.025 mg L-1	0.025 mg L^{-1}
FeEDDHA		12.5 mg L^{-1}
FeNaEDTA	36.70 mg L-1	
$MnSO_4 \cdot H_2O$	22.3 mg L-1	22.3 mg L^{-1}
H_3BO_3	6. 2 mg L-1	6.3 mg L^{-1}
$ZnSO_4 \cdot 7H_2O$	8.6 mg L^{-1}	8.6 mg L^{-1}
$Na_2MoO_4 \cdot 2H_2O$	0.25 mg L-1	0.25 mg L^{-1}
$MgSO_4 \cdot 7H_2O$	180.7 mg L-1	740.0 mg L^{-1}
$CaCl2 \cdot 2H_2O$	440 mg L-1	440 mg L^{-1}
NH_4NO_3	1650 mg L-1	1.650 mg L^{-1}
K_2SO_4		40 mg L^{-1}
KNO_3	1900 mg L-1.	
KH_2PO_4	170 mg L-1	170 mg L^{-1}
KI	0.83 mg L-1	
$FeSO_4 \cdot 7H_2O$	27.8 mg L-1	
$CoCl_2 \cdot 6H_2O$	0.025 mg L-1	
$Ca(NO_3)_2 \cdot 4H_2O$		1900.0 mg L^{-1}
Pyridoxine • HCl	0.5 mg L-1	

Tryptone	1.000 mg L-1	
myo-Inositol	100 mg L-1	0.1 mg L^{-1}
Glycine	2.0 mg L^{-1}	2.0 mg L^{-1}
Thiamine HCl	0.1 mg L^{-1}	0.1 mg L^{-1}
Nicotinic Acid	0.5 mg L^{-1}	0.5 mg L^{-1}
IAA	1-30 mg L-1	
Kinetin	0.04-10 mg L-1	
BAP		1.000 mg L^{-1}
IBA		2.000 mg L^{-1}

The necessary amount of media was first prepared in special 2-liter glass bottles. The pH value was adjusted to 5.6 before adding agar. 0.1 N KOH and HCl were used for pH adjustment. Sterilized under pressure in an autoclave at 121 °C for 15 minutes. Autoclaved media was poured into sterile petri dishes after cooling to the required temperature in a sterile laminar box.

Cultures were kept for 4 days at a temperature of 23 ± 2 °C, humidity of 40%, constant darkness, then cultures transferred other condition which a photoperiod of 16 hours of light and 8 hours of darkness. After 4 weeks, the formed calli were transferred to a new nutrient medium and kept in the growth chamber under unchanged conditions.

2.5 Culturing different parts of A. melanocarpa for callus formation

Sterilized seeds were first placed in a petri dish and dried in an autoclave on sterilized paper napkins for 2-3 minutes until there were no water droplets on the surface. Seed with 1/3 of the seed coat cut off (T1), whole seed (T2), seed without bark (T3) were all planted in petri dishes to modified MS nutrient using tweezers.

From one-year-old stems were cut from its peel and wood parts with a scalpel to a size of 5 cm and planted in the media. The surface of the leaves was dried for 5-7 minutes using filtered paper and cut into 5 cm squares and planted in the media. It was wrapped with strich to ensure sterility and wrapped in aluminium foil paper to encourage callus formation. The above processes were carried out in a sterile laminar box and placed in a growth chamber with a temperature of 23 ± 2 and a humidity of 40 ± 1. After 4 days in the dark, they were switched to a 16/8-h light/dark cycle, respectively.

2.6 Measuring callus fresh weight, dry weight, and humidity rate

To measure the total weight (fresh weight), the calli were first cleaned of media by rinsing with distilled water, and water droplets were removed using paper towels. It was placed in Petri dishes and weighed. To measure the dry weight, the Petri dishes were stored in a drying cabinet at 65 °C for 2 days and weighed on an electronic balance. The callus humidity level (ND) was calculated as follows: From total weight (UO), deduct dry weight (QO). UO-QO=ND

3 Results and Discussion

Callus formation took place at different times in different parts of the plant. Callus formation on the leaf started in 2 weeks. The results of sterilization performed on leaves by two different methods were different from each other (Table 2).

Table 2. Effect of sterilization on callusogenesis of biomaterial obtained from *A. melanocarpa* leaf (n = 4)

Leaf sterilization	Contamination (%)	No contamination (%)	Callus formation(%)
1 method	88,2±1,4	12,6±1,0	7,0±0,8
2 method	54,8±1,9	46,3±2,1	41,0±1,6

As a result of sterilization without fungicide, the bacterial contamination in leaves was equal to 88.2%. Bacterial and fungal contamination did not occur as a result of sterilization in the presence of fungicide. However, darkening of leaves was observed, and 50% of the total explants planted on the media had leaf tissue death. In intact leaves, callus induction was equal to 41% of indicators (Fig. 1, A). 5% of healthy leaves did not induce callus.

According to the results of callus formation in one-month-old young and one-year-old shoots of *A. melanocarpa*, no callus induction occurred as a result of darkening and death of leaf tissue in one-month-old young varieties. According to the results obtained from whole wood and bark parts of one-year old stems, contamination from the bark part of one-year stems was equal to the high index, and no callus formation was observed even from the 7% preserved part. Callus induction was carried out from wood and whole stem. In the wood, callus induction occurred in one week and the rate of callus formation was 20% (Fig. 1, D). Callus induction from one-year stems was 10%.

There was no difference between the results obtained from sun-dried and non-sun-dried seeds. Induction of callusogenesis in seeds was carried out in three weeks in half-cut samples. In whole seeds, plants were formed and the explants that germinated from the seeds were removed from the root and individual clones were planted in the medium. as a result, callus induction was carried out from the lower part of the microclone's shoot. Callus formation from the lower part of the stem of the microclone was 100%.

Table 3. Effect of *A. melanocarpa* seed coat on callus induction and contamination.

	Callus induction (%)	No callus induction (%)	Fungi (%)	Bacteria (%)
T1	100%	0	0	0
T2	15%	85	0	0
T3	0	90	0	10

T1–a microclone grown from a seed in which 1/3 of the cortex is cut, T2–seed which has cortex, T3–seed which without cortex.

The best callus was obtained from stem of microclones germinated from cut seeds (Fig. 1, C and E). The callus obtained from the stems of the microclone differed in size and growth rate compared to other calluses.

Induction of direct callusogenesis from initial materials planted in 3 different forms of seeds only resulted in 15% callus formation of uncut seed (fig.1, B).

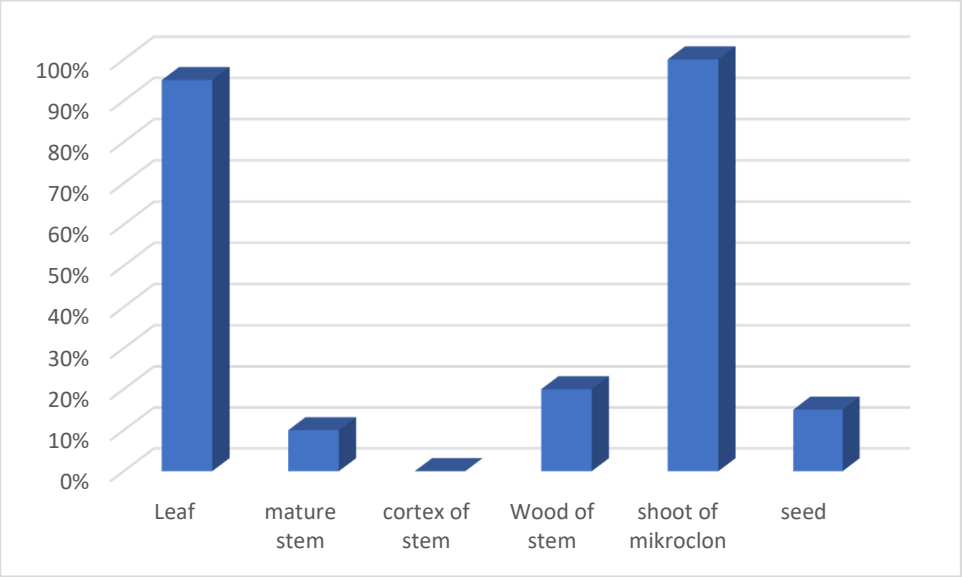

Fig. 1. Effect of initial material type on callus formation in *A. melanocarpa* (%).

Based on the obtained results, it was determined that the lower part of the leaf and stem *of A. melanocarpa* is the best initial material for callus formation. Experiments have shown that wood of stem and shoot of microclon are the best starting materials for obtaining hard, compact callus.

The color, density, and growth rate of callus from different vegetative organs and tissues of *A.melanocarpa* also differed (Fig. 2).

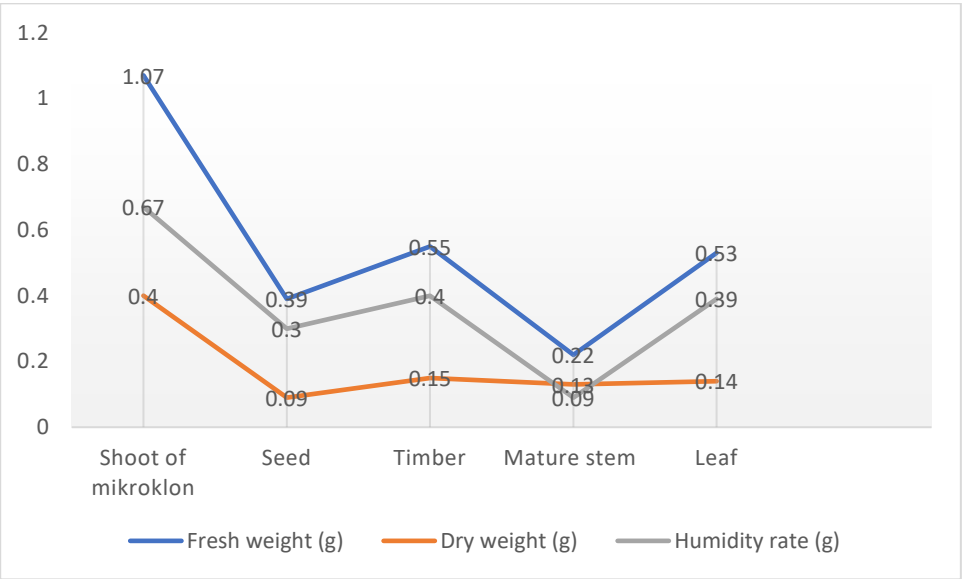

Fig. 2. Biometric indicators of calluses obtained from different parts of *A. melanocarpa*.

Callus from the leaf is very friable, pale yellow in color. Callus from the wood is very hard, dark green in color. Callus from the shoot of microclane is compact; the upper side is

violet, and the side that touches the media is green. Seed callus have root and leaf growths, medium compact, green, and violet patches.

When the humidity, dry weight, and fresh weight of callus obtained from different parts of *A. melanocarpa* were measured, the best result was obtained from callus obtained from a microclone shoot. The lowest indicator was obtained from the callus formed from the stem (Fig.3). The indicator of dry weight did not differ almost in wood, stem, and leaf. The moisture content of the callus formed from the stem of microclone was on average 0.67 grams, and the lowest indicator was equal to 0.09 grams in the callus formed from the stem.

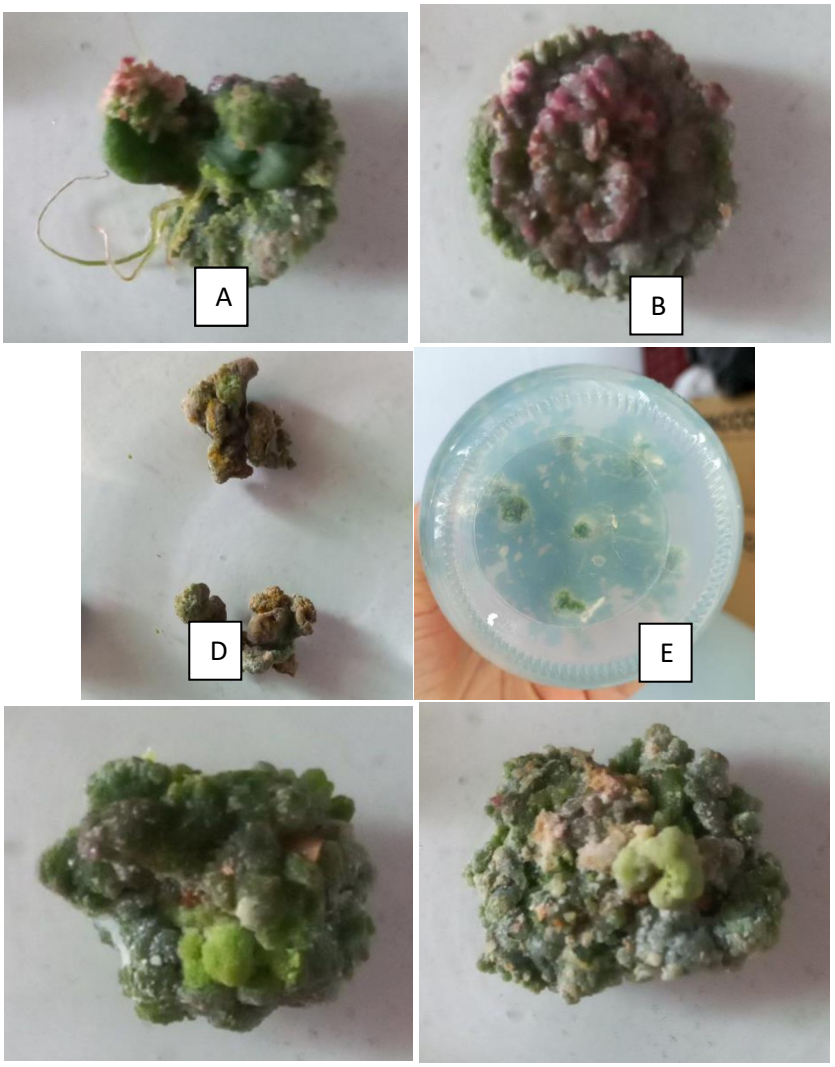

Fig. 3. Callusogenesis in *A. melanocarpa* (A–callus formed from a leaf, B–a callus formed from a seed, In C and D–callus forms from the lower part of the stem of a microclone. E–callus formed from wood. F–callus formed from a one-year-old stem.

4 Conclusion

Experimental results revealed that the leaf and microclonal stem of *A. melanocarpa* are the best vegetative organs for callus formation. In terms of callus growth rate, the callus obtained from the microclone stem differed from the callus obtained from other organs and tissues. It was found that the *A. melanocarpa* seed coat can delay the germination of seeds from the sterilization process, but no callus induction was observed as the seeds lost their ability to develop when the seed coat was completely removed. On the other hand, damage from bacterial contamination was observed in comparison with cortex and semi-cut cortex seeds. Callus obtained on the basis of different biomaterials of *A. melanocarpa* differed from each other in terms of color, growth rate, and strength.

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