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FEATURES OF MORPHOGENESIS OF *Actinidia arguta* LEAF TISSUES AT MICROCLONAL PROPAGATION

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ABSTRACT

The methods of microclonal propagation of *Actinidia arguta* (Siebold et. Zucc.) Planch ex Mix. (cv. ′Oryginalna′ and cv. ′Don-Juan′) plants by leaf tissues morphogenesis has been proposed. Leaves of aseptic actinidia plants from *in vitro* culture and leaves of plants grown in the garden were used as explants. When introduced into aseptic culture, the optimal period for the isolation of stem explants was the phase of active growth of actinidia shoots, and the medium - ½MS + 2 mg l^{-1} IBA and 0.5 mg l^{-1} BAP. The best culture medium for the morphogenesis of shoots from leaf explants of actinidia was a ½MS nutrient medium with the addition of 3.0 mg l^{-1} of BAP, B_1, B_6 vitamins and 12 mg l^{-1} of iron sulfate $Fe_2(SO_4)_3x9H_2O$. It was set that the frequency of shoot regeneration depends on the cultivar, plant sex, condition of their cultivation and the part of leaf from which the explant was taken. The maximum regeneration efficiency was 83% with the formation of an average of 4–6 shoots and was achieved for leaf explants of actinidia from aseptic culture. Perlite-based substrate was used for the adaptation of the obtained plants and the efficiency of plant adaptation was 65–80%.

Keywords: *A. arguta*, cultivars, callus, proliferation, plant regeneration, morphogenesis

INTRODUCTION

A. arguta (Siebold & Zucc.) Planch. ex Miq., commonly known as “baby kiwi”, “hardy kiwi” or “mini kiwi” – is a representative of the genus *Actinidia* Lindl. (Actinidiaceae Hutch. family), which according to the latest systematic revisions has 54 species and 21 subspecies (**Huang, 2016, Li *et al.*, 2007**). *A. arguta* is a valuable fruit, medicinal and decorative plant. Its fruit are characterized by high nutritional, dietary and medicinal properties, which are due to the high content of biologically active substances: vitamins C, E, K, organic acids, tocopherols, carotenoids, flavonoids and rich mineral composition. Actinidia berries are known for their immunomodulatory, analgesic, antibacterial, antioxidant, antitumor and other pharmacological effects (**Niu *et al.*, 2019**).

Today, *A. arguta* is a new commercial berry crop in many countries around the world, including New Zealand, China, United States, Japan, Belgium, Germany, Poland, and others. However, the selection of new large-fruited and high-yielding cultivars of this culture remains an important task, because it is the cultivar is the main element in the setting of high-yielding garden complexes. Significant selection work with actinidia is carried out in the M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine (NBG) (**Skrypchenko, 2017**), where the largest collection of actinidia in Ukraine is collected and 18 cultivars of *A. arguta* were created, which are promising for widespread horticulture.

The vast majority of actinidia cultivars are propagated with the shoots, but *in vitro* culture methods are used for accelerated propagation of new cultivars. In addition, the cultivation of plant cells and tissues *in vitro* is accompanied by the emergence of significant cytomorphological and genetic diversity, which allows the use of regenerating plants in subsequent selection.

Introduction to *in vitro* culture and biotechnological studies of actinidia are carring out since the 1970s **(Harado, 1975)**. At present, considerable experience has been gained in the introduction into culture and microclonal propagation of *A. arguta* plants **(Arteta *et al.*, 2018, Najaf-Abadi *et al.*, 2010, Calugaru-Spataru, 2013, Hameg, 2017, Tut', Upadyshev, 2008, Peticila *et al.*, 2012).** Murashige and and Skoog (MS) media was used in most studies. Authors (**Molkanova *et al.*, 2014**) successfully used *in vitro* cultures from shoots of *A. arguta* on MS medium with the addition of 2iP (2-isopentenyladenine) (5 mg l^{-1}). **Tut' (Tut' *et al.*, 2008)** also used nutrient medium with macronutrients according to MS, microelements according to Harad, casein hydrolyzate (500 mg l^{-1}), mesoinositol (100 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), sucrose (30 g l^{-1}) and agar-agar (7 g l^{-1}).

Regeneration of plants of different Actinidia species through organogenesis has been the subject of selected studies **(Prado *et al.*, 2007, Mitrofanova, 2000)** - *A. deliciosa*, **(Takahashi *et al.*, 2004)** - *A. polygama* Miq., **(Wu *et al.*, *2011)*** - *A. eriantha*. However, information on the regeneration of shoots from leaf explants of *A. arguta* actinidia is practically absent. Shoot regeneration is necessary for the genetic improvement of plants using biotechnological approaches such as somatic hybridization and gene transfer. The likelihood of somaclonal variability and the appearance of various gene variants increases at regeneration from leaf tissues increases, which is successfully used in breeding work with different crops **(Tut et al., 2008; Zagoskina, 2009**)...

The growth and development o f plants *in vitro* depends on many parameters, such as plant species, medium composition, growth conditions and so on **(George *et al.*, 2008).** Therefore, the aim of this work was the investigation of the peculiarities of microclonal propagation of *A. arguta* cultivars of NBG selection by morphogenesis of leaf tissues and the determination of the optimal physiological conditions for regeneration and cultivation of micro shoots.

MATERIALS AND METHODS

Initial plant material and disinfection

The research was carried out in NBG during the spring-summer season 2018-2019 using cuttings and leaves of ten-year-old plants *A. arguta* (cultivars ′Don-Juan′ and ′Oryginalna′). Introduction into culture and microclonal propagation was carried out according to the N.V. Zagoskina’s method **(Zagoskina, 2009**). To obtain the primary cultivation material, explants were selected during the dormant period and in different phases of vegetation of plants from February to July, using cuttings of *A. arguta* woody and green shoots 3–4 cm long with 1–3 axillary buds, which were sterilized using alcohol, sodium merthiolate and a solution of "Belizna" with different duration of processing. Three sterilization options were tested:

- 2 min with 70% alcohol and 3 min with 0.05% sodium merthiolate solution (thimerosal $C_9H_9HgNaO_2S$);
- 3 min with 70% alcohol and 3 min with 0.05% sodium merthiolate solution;
- 1.5 min with 70% alcohol and 30% solution of "Belizna", then rinsed thoroughly 2 times for 2 min with sterile distilled water.

For regeneration, young leaf plates from *in vitro* culture and leaves from vegetative actinidia plants were used, which were pre-washed in a detergent solution to reduce the amount of surface microflora, and then subjected to sterilization according to the scheme: 1 min with 70% alcohol and 1 min with 0.05% sodium merthiolate solution.

Nutritive media and conditions of cultivation

The basis for all experiments was Murashige-Skoog medium with reduced by half the content of macro- and microelements (½MS). The nutrient medium ½MS and

the medium ½MS with the addition of growth regulators in different combinations and concentrations were used for introduction into aseptic conditions: ½MS+0.5 mg l^{-1} 6-benzylaminopurine (BAP); ½MS+2.5 mg l^{-1} indole-3-butyric acid (IBA); ½MS+2.0 mg l^{-1} IBA+0.5 mg l^{-1} BAP. Nutrient mediums based on ½MS modified by the addition of growth regulators, mineral salts and vitamins were used for morphogenesis:

-3.0 mg l^{-1} BAP and ferrous sulfate $Fe_2(SO_4)_3x9H_2O$ in three concentrations (12.0, 22.0 and 52.0 mg l^{-1});

-0.2 mg l^{-1} thidiazuron (TDZ) and 12 mg l^{-1} ferrous sulfate $Fe_2(SO_4)_3x9H_2O$;

-0.4 mg l^{-1} TDZ and 12 mg l^{-1} ferrous sulfate $Fe_2(SO_4)_3x9H_2O$.

The explants were kept in the darkness at the initial stage of regeneration. The obtained micro shoots were transferred to a nutrient medium ½MS, modified by the addition of growth regulators and kept under illumination with fluorescent lamps (2.0–2.5 klx) for a 16-hour photoperiod at a temperature of +26 °C and a humidity of 70%.

Methods for determining of the content of flavonoids and catechins in plant leaves

The flavonoid content was determined by differential spectrophotometry method, extracting them with 70% ethanol in a ratio of raw material and extractant 1:100, by further extraction two times in a boiling water bath for 45 minutes and 35 minutes. A 2% solution of aluminum chloride was used as a complexing agent. The optical density was determined at a wavelength of 400 nm. The calculation of the amount of flavonoids was performed using the specific absorption of a standard sample of cinaroside **(Andreeva, 2000).** The amount of catechins was determined by the Kriventzov's method **(Kriventzov, 1982).**

Statistical analysis

The results from individual experiments were processed with statistical methods using Microsoft Excel 2010.

RESULTS AND DISCUSSION

The percentage of sterile material during introduction in culture *in vitro* depends on the sterilization regime, the type of explant and the genetic characteristics of the cultivar. According to studies **(Tut et al., 2008)**, the loss of actinidia explants during microclonal reproduction is due to bacterial and fungal infections and reaches (35–65%) and (25–42%), respectively. Sterilization of primary explants is an important step for the success of the process of introducing them into the culture. According to our research, the best option for sterilization of explants of the studied cultivars of *A.arguta* was as follows: sterilization for 2 min with 70% alcohol and 3 min with 0.05% sodium merthiolate solution. The leaves were pre-rinsed in detergent solution before sterilization to reduce contamination. The cultures capable to proliferation of callus tissues and the development of existing meristems were obtained with this method of sterilization.

The efficiency of microclonal propagation is largely determined by the composition of the nutrient medium. The several variants of the nutrient medium with a mineral base according to the recipe of MS medium were used when introducing actinidia into aseptic culture, which differed in the content of biologically active substances. It was found that the optimal nutrient medium is ½ MS with the addition of 2 mg l^{-1} IBA and 0.5 mg l^{-1} BAP (Fig. 1), in other variants of the medium ½ MS with 2 mg l^{-1} IBA and ½ MS with 0.5 mg l^{-1} BAP the survival of explants was lower (by 4–8% for explants isolated during the period of active growth and by 4–20% for explants isolated during the period of rest). The results in this study are in agreement with the finding of other authors who also successfully used BAP for shoot multiplication of different *A. arguta* cultivars. According to studies **(Najaf-Abadi et al., 2010)**, the best results of proliferation of shoots from the axillary buds of actinidia are achieved on a nutrient medium MS+2 mg l^{-1} BAP. Arteta with colleagues **(Arteta *et al.*, 2018)** also believe that the presence of BAP in medium of high concentrations (>0.6 mg l^{-1}) is necessary for successful micropropagation of *A. arguta*.

The dependence of explant engraftment in culture *in vitro* on the terms of their isolation has been established. Significantly higher results were obtained during the period of intensive growth of plant shoots (May-July), which may be due to the dynamics of hormones in the plant during the year (Figure 1). The viability of actinidia explants selected during this period increases on average by five to six times, compared with explants selected at the beginning of the growing season (February-April). Some authers point on the phases of rest as more favorable terms of isolation of explants, which contributed to better engraftment and development of explants (**Tut' et al., 2008).** The best results of engraftment of cuttings were observed in the period of deep and forced dormancy of plants. It was found that the regenerative capacity of actinidia explants at the stage of introduction into the culture depends on the sex of plants - there is a higher percentage of survival of female plant explants, compared to males: the excess at the beginning of the vegetation was 15–25% and in May-July - 5–8%.

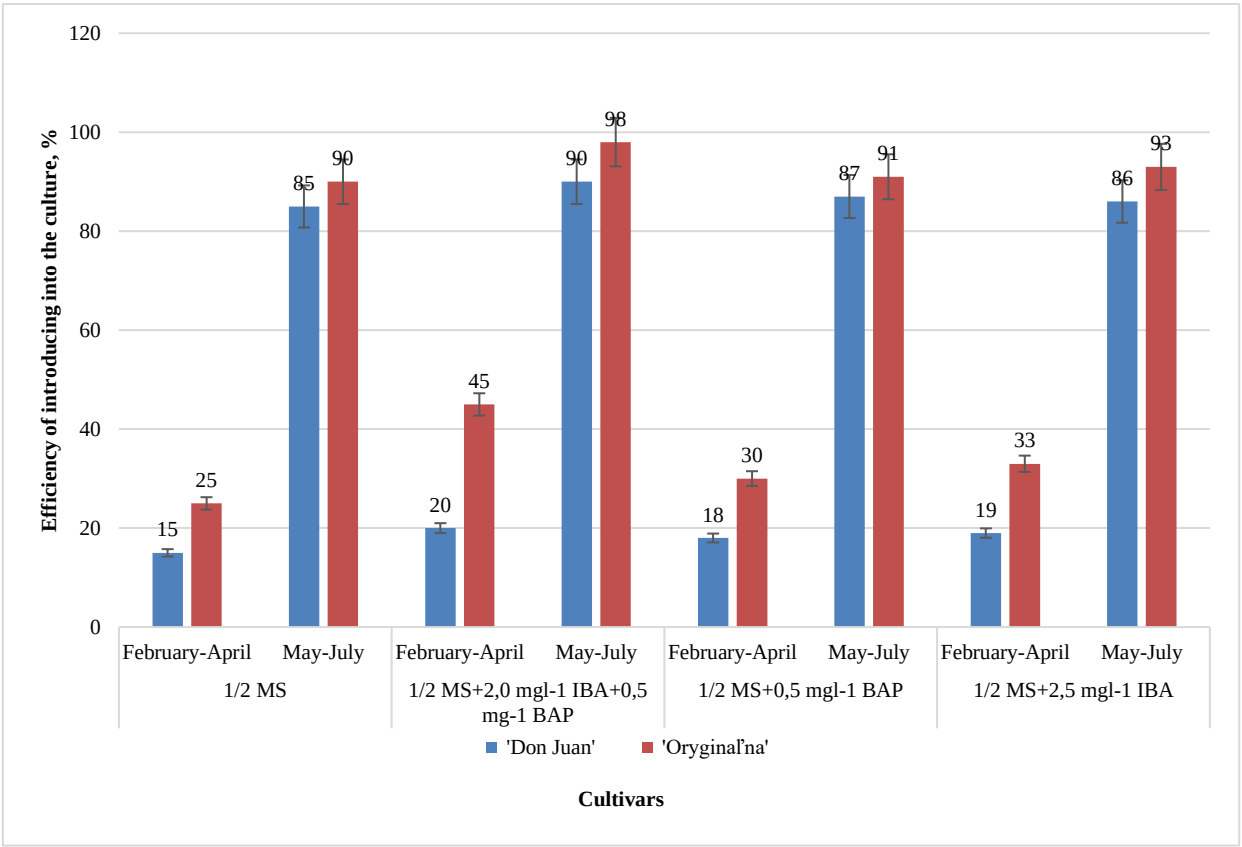


Figure 1 The efficiency of introducing into the culture of some *A. arguta* cultivars 'Don Juan' and 'Oryginalna' depending on the terms of their isolation and nutrient medium, %

Bud burst and the beginning of the development of shoots were observed already on the 11th day, which were transferred to a nutrient medium and then used to obtain sterile leaf explants (Figure 2).

According to the literature, one of the obstacles to the regeneration of plant tissues of woody plants is the content of a large number of secondary compounds (phenols, terpenes), which are activated in isolated tissues and can inhibit cell growth and division, which weakens the ability of tissues to regenerate **(Kushnir, Sarnatska, 2005, Charles et. al., 2010**, **Podhaietskyi *et .al.*, 2020**). According to **(Oliinyk *et al.*, 2016)**, A significant amount of phenolic compounds is synthesized in cells during traumatic stress in the initial stages of introduction of primary explants in culture *in vitro*, which are rapidly oxidized and create biochemical barriers to access of possible pathogens and viruses.

A

B

Figure 2 Plants of *A. arguta* cv. ′ Oryginalna ′ in *in vitro* culture: *a* - medium ½MS+2 mg l^{-1} IBA and 0.5 mg l^{-1} BAP (40 days); *b* - plants after long cultivation, which were used as uterine (medium ½ MS)

At the same time, oxidized and polymerized phenols and catechins complicate tissue nutrition, inhibit the growth and development of apical meristems of explants, cause tissue necrosis due to partial or complete death in most cases. Therefore, a comparative analysis of *A. arguta* leaf explants for the content of phenolic compounds under different conditions of cultivation was performed. There is a certain relationship between the origin of explants and content of phenolic substances it have (Table 1). It was found that the leaves selected from vegetative plants in the garden had a significantly higher content of flavonoids and catechins, compared to the leaves of aseptic actinidia plants. A much larger number of them was found in the leaves of the pollinator cultivar 'Don-Juan', compared with the leaves of female plants cv. ′Oryginalna′ under different cultivation conditions.

Table 1 The content of biologically active substances in plant tissues of *A. arguta* depending on the method of cultivation

Cultivar	Organ	Total content of flavonoids, mg/g of dry matter	Total content of catechins, mg/100 g of dry matter
′Oryginalna′ (*in vitro*)	Leaves	0,740±0,009	446,00±7,21
′Oryginalna′ (*in vivo*)	Leaves	0,933±0,009	668,00±14,42
′Don Juan′ (*in vitro*)	Leaves	0,810±0,005	1380,00±14,84
′Don Juan′ (*in vivo*)	Leaves	1,116±0,011	1510,00±36,06

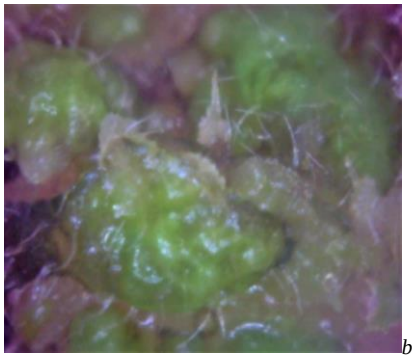
Five modifications of the culture medium ½ MS with different ratios of auxins and cytokinins, vitamins and sulfuric acid to stimulate callus and organogenesis were tested in order to develop an optimal environment as to content of mineral salts for micropropagation of actinidia by tissue regeneration.

Table 2 Dependence of *A. arguta* regeneration on the origin of explants and the composition of the nutrient medium

Medium ½ MS with the addition of:	Explants from culture *in vitro*		Explants from culture *in vivo*	
	Explants initiating shoots, %	Number of microshoots	Explants initiating shoots, %	Number of microshoots
3,0 mg l^{-1} BAP + 12 mg l^{-1} $Fe_2(SO_4)_3x9H_2O$	83.32±8.10	5.26±2.06	44.51±2.51	2.42±1.25
3,0 mg l^{-1} BAP + 22 mg l^{-1} $Fe_2(SO_4)_3x9H_2O$	78.01±7.39	4.75±1.81	41.71±2.43	1.21±0.53
3,0 mg l^{-1} BAP + 52 mg l^{-1} $Fe_2(SO_4)_3x9H_2O$	75.63±5.36	4.05±1.77	32.43±5.24	1.15±0.32
0,2 mg/l TDZ + 12 mg l^{-1} $Fe_2(SO_4)_3x9H_2O$	76.15±4.17	3.27±1.54	36.21±4.51	1.09±0.24
0,4 mg/l TDZ + 12 mg l^{-1} $Fe_2(SO_4)_3x9H_2O$	80.33±8.01	2.78±1.36	42.39±4.92	1.17±0.35

It was found that the most pronounced stimulating effect on the regeneration process provided the medium with the addition of 3.0 mg l^{-1} BAP in combination with 25 mg l^{-1} B_1 and B_6 vitamins and 12 mg l^{-1} $Fe_2(SO_4)_3x9H_2O$. The beginning of callusogenesis was noted in leaf explants (Figure 3, A, B) in 2–3 weeks (after etiolation). The callus resembled a loose green mass, which due to mechanical loading disintegrated into conglomerates in the first stages of formation. The callus tissue hardened with further shoot morphogenesis (Figure 3, C, D) gradually.

a



b

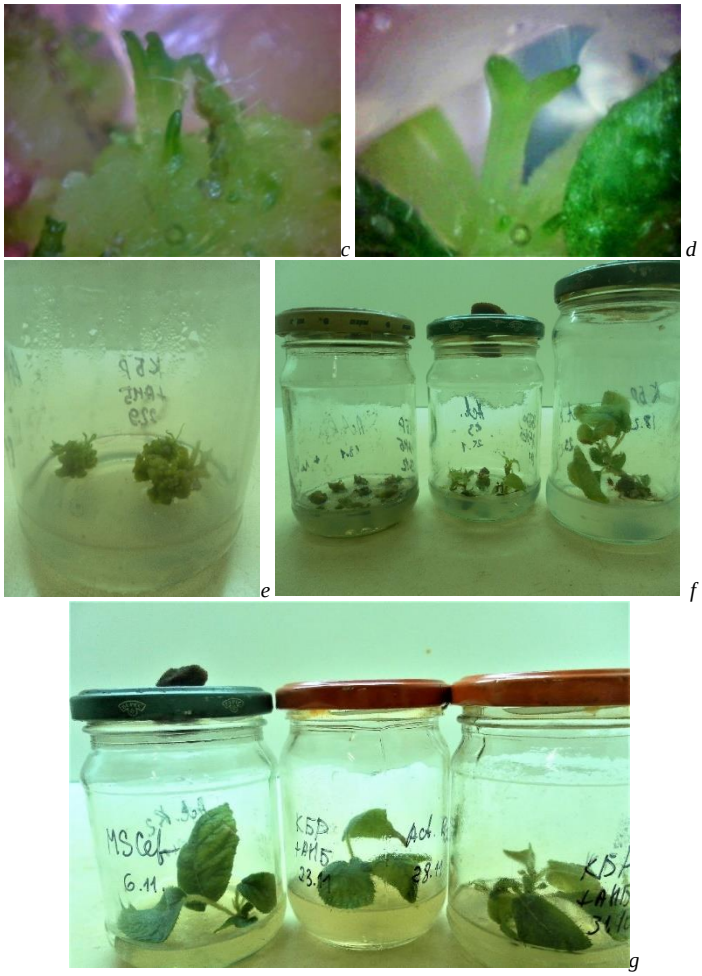


Figure 3 Organogenesis and plant regeneration from leaves of *A. arguta* (cv. ′Oryginalna′) *a* - leaf explants on nutrient medium ½MS with the addition of 3.0 mg l^{-1} BAP; *b* - callus; *c*, *d* - formation of shoots from callus; *e* - callus with rosettes of leaves; *f* - stages of rhizogenesis (nutrient medium ½MS with the addition of 2.0 mg l^{-1} IBA+0.5 mg l^{-1} BAP); *g* - regenerating plants

The maximum of shoot regeneration was observed in 83% of leaf explants from culture *in vitro* with the formation of an average of 4–6 microshoots per leaf explant. The regeneration efficiency was significantly lower and averaged 57% with the formation of 1–3 shoots when using leaf explants from culture *in vivo*. In our opinion, this may be due to the increased content of phenolic compounds in plant explants from the plants grown in the garden.

Regenerative shoots can be induced from different parts of the leaf blade, but the tissues of the leaf base of experimental plants have the greatest ability to regeneration, because the most active meristematic cells are in this area of the leaf blade. In addition, smaller segments of the leaf provided a higher level of its callusogenesis and shoot morphogenesis. Our results are in agreement with results of research **(Wu, 2011)**. The author has showed that the highest shoot initiation was obtained from leaf blades 0–3 cm long. If the leaf blades were less than 6 cm, no shoots were obtained.

The microshoots 1–1.5 cm long were separated from the microbush and transplanted them to the nutrient medium (½ MS) with the addition of 2.0 mg l^{-1} IBA+0.5 mg l^{-1} BAP (Figure 3, F) to induce rhizogenesis. On this medium, microshoots were rooted by 88–93%, although a significant advantage of the use of IAA (indolyl-acetic acid) (at an optimal concentration of 1 mg l^{-1}) over IBA (indolyl-butyric acid) was found at the rooting of *A. arguta* microshoots **(Molkanova, 2014).**

The final stage of microclonal propagation is the gradual adaptation of regenerating plants to the substrate in greenhouse conditions. The young plants (Fig. 3, G) were transferred to soil after 30 days of cultivation. A conditionally sterile mixture of perlite with sand in a ratio of 1:1 was used during the transfer. Plants with two or three leaves and a developed root system were planted in containers with the mixture (Figure 4).

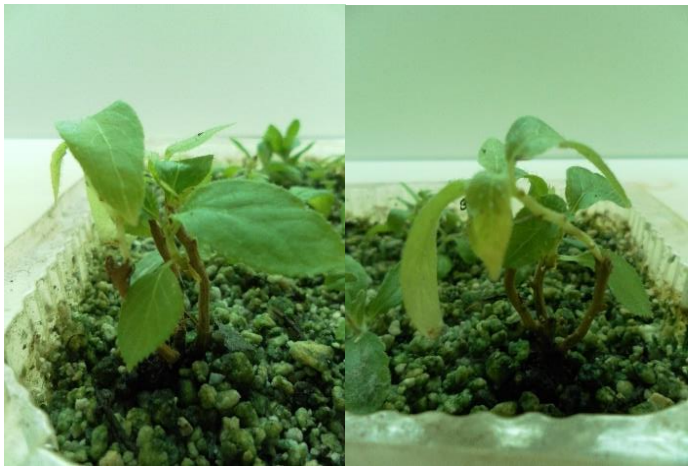


Figure 4 *A. arguta* plants in a soil mixture with perlite

According to our research, the adaptation period of *A. arguta* cultivated plants is 3 weeks with an efficiency of 65–80%, with the most viable plants were the plants with a root length of 10.2 ± 2.0 cm and 4–5 lateral roots. The plants with a less developed root systems, which did not exceed 5.0 ± 1.5 cm, were unviable.

CONCLUSIONS

The methods of microclonal propagation of *A. arguta* (cv. ′Oryginalna′ and cv. ′Don-Juan′) plants by leaf tissues morphogenesis has been proposed. The best nutrient medium is ½ MS with the addition of 3.0 mg l^{-1} BAP, 25 mg l^{-1} B_1 and B_6 vitamins and 12 mg l^{-1} $Fe_2(SO_4)_3x9H_2O$, and for rhizogenesis: ½ MS, supplemented with 2.0 mg l^{-1} IBA+0.5 mg l^{-1} BAP for the morphogenesis of shoots from leaf explants.

The decisive role of plant genetic factor, conditions of their cultivation in realization of morphogenetic potential were proved. The maximum regeneration efficiency was 83% with the formation of an average of 4–6 shoots and was achieved for leaf explants of actinidia from aseptic culture.

It is advisable to use a substrate based on perlite for plant adaptation, while the efficiency of plant adaptation was 65-80%.

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