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The Effect of Ultrasound Irradiation on Induction of Callus Formation and Morphogenesis from the Leaf Discs of Apple Clonal Rootstocks

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Abstract

With the aim of increasing the intensity of callus formation and induction of morphogenetic processes in the cultivation of leaf discs of apple clonal rootstocks, the method of preliminary damaging plant tissues by treating them with ultrasound was used. Choosing optimal parameters of ultrasound treatment stimulated the process of callus formation on the leaf disks, and increased the frequency of regeneration of adventive shoots of apple clonal rootstocks 2.5-3.5 times.

Keywords: ultrasound, biotechnology, tissue culture, callus, morphogenesis.

INTRODUCTION

Development of methods for inducing morphogenesis from isolated plant tissues is the basis of all biotechnological methods aimed at widening the genetic diversity of agricultural crops. Without the development of efficient methods of whole plants' regeneration from isolated somatic tissues, clonal micropropagation and adaptation of the obtained micro plants to the natural growing conditions, works in any area of modern biotechnology, including tissue selection and genetic engineering, are impossible.

Morphogenesis is a complex process regulated at the cellular, tissue and organism levels. Establishing general regularities of inducing morphogenesis in the culture of plant tissues is complicated by the integrated nature of morphogenetic processes, their dependency on many internal and external factors, and their interaction [1, 2]. For many fruit and ornamental plants, morphogenesis in the tissues' culture remains an open issue due to the species- and grade-specificity of these plants that require individual optimization of the cultivation conditions. Despite the fact that each plant cell theoretically has the property of totipotency, in practice, one has to experimentally find the conditions of regeneration for each genotype.

A promising direction for improving the efficiency of biotechnological methods is the use of various stimulating biophysical factors, one of which is the ultrasound - an environmentally safe and cheap method. It is known that ultrasound irradiation may cause both reversible and irreversible changes in cell membranes' permeability. Under the action of US, the permeability of cells may increase due to changing the size and shape of stomatal pores, pores, tubules, loosening connections in the intercellular connective tissues, partial rupture of cells during cavitation. Exposure to ultrasound of certain power enhances enzymatic activity of a number of enzymes localized in the surface layers of cell membranes, and increases the sensitivity of the cells processed with ultrasound to a number of biologically active substances [3, 4].

In plant growing, ultrasound was used mainly to accelerate seed germination and for stimulation of seedlings' growth [5-8].

The principle of dispersing plant material using ultrasound is widely used for preparing foodstuffs and medicinal drugs enriched with biologically active compounds [9-11].

An interesting direction of research is the use of ultrasound irradiation for isolated plant tissues *in vitro*. Ultrasound was at the stage of introduction into the *in vitro* culture for sterilization of plant explants [12]. Treatment of plant tissues with ultrasound improved the efficiency of the *Agrobacterium*-mediated transformation [13].

Earlier experiments have shown that the choice of optimal parameters and methods of ultrasound irradiation increases the efficiency of horticultural crops microcuttings' rhizogenesis 1.5-2 times [14, 15], increases the intensity of callus formation 2-2.5 times, and contributes to the induction of morphogenetic processes in plant tissues [16].

Insufficient knowledge of the mechanism of ultrasound irradiation action on plant tissues in the *in vitro* conditions, and definitely promising nature of practical use of ultrasound in the field of plant biotechnology determine the need for expanding the research on this subject.

The research is aimed at developing efficient methods of improving the efficiency of callus formation and regeneration of adventive shoots from isolated leaf tissues of apple trees' clonal rootstocks using ultrasound irradiation.

MATERIALS AND METHODS

The biological objects of the research were clonal apple rootstock B9 bred at the Michurinsk State Agricultural University (Budagovsky's paradise apple-tree), and clonal rootstock 14-1 obtained as a result of spontaneous outbreeding of *Malus siboldy*.

In the experiments with inducing morphogenesis from isolated somatic tissues of apple clonal rootstocks, explants were cuttings of 4-5 upper leaves with well-developed shoots from the propagation medium. Each leaf was cut transversely to the central vein into 2 to 3 0.5-1.0 cm^2 pieces. Isosceles triangle-shaped cuts of the lamina with the leafstalk and middle parts of lamina were cultivated. For irradiation of explants, a UZDN-2T ultrasound installation with a cone nozzle emitter was used. Irradiation frequency was 22 kHz. Duration of exposure was 60 s. 20 to 25 chopped leaf disks were placed in a 100 ml beaker with 40 ml of liquid Murashige-Skoog medium [17] with the addition of 5 mg/l of 2,4-D, and treated with ultrasound. The following variants for ultrasound irradiation were used: 1 – 1.2; 2 – 2.6; 3 – 3.6; 4 – 6.0; 5 – 10.0; 6 – 12.6; 7 – 14.9 W/cm^2, and two of reference variants without ultrasound treatment (K – without cuttings, K1 - with cuttings made transversely to the central vein with a scalpel).

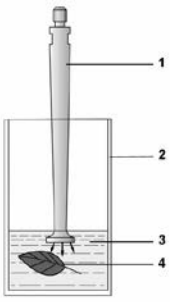


Fig. 1. Device for preparing mixtures from plant leaves: 1 – the ultrasound emitter; 2 – beaker; 3 – water; 4 – leaf.

After irradiation, leaf discs were placed onto regeneration nutrient medium based on the MS nutrient medium [17] with the addition of vitamins according to Murashige and Skoog, glucose - 30 g/l, 6-BAP in the concentration of 5 mg/l, and IAA in the concentration of 0.5 mg/l. The experiments lasted 3-3.5 months (3 passages, 4 or 5 weeks each). Initially, explants (two passages) were cultivated in a thermostat in the dark at the temperature of 24^0C, after which they were placed into the conditions of the in vitro chamber. Further cultivation continued with 16-hour long light day with the illumination of 2,000-2,500 Lux (fluorescent lamps Osram L36W Cool Daylight), the temperature of 24 ± 2^0C, and the humidity of 50-60%.

Regenerated shoots were cut from the leaf blades and grown to the end according to the standard scheme of plants' clonal micropropagation.

RESULTS AND DISCUSSION

As it follows from the literary data and the experience of the authors, efficiency of morphogenesis from somatic tissues is determined by several factors: plant genotype, mineral and hormonal composition of the media, composition and amount of sugars in the regeneration media, the type and the origin of the explant, its orientation on the culture medium, the nature of damage to the plant tissues, temperature and light conditions of cultivation, etc. [18].

Callus cultures are the material most frequently used for cell breeding and genetic engineering of plants, as well as the biomass that is the source of specific secondary metabolites. Callus is formed by disorganized proliferation of de-differentiated cells of plant organs. Callus is an amorphous mass of thin-walled parenchymal cells without strictly defined anatomical structures. Callus may be white, creamy yellow, or green. Calluses with high morphogenetic potential are usually matte, compact, structured with well-defined meristematic foci.

In the research, optimization of some factors that have a significant effect on regeneration of adventive shoots – medium composition and plant growth regulators - helped make the rate of shoots' regeneration from leaf explants of apple clonal rootstocks high enough. The optimal cytokinin to auxin ratio in the nutrient medium was 10/1 [19].

To increase the intensity of callus formation and regeneration of adventive shoots from isolated tissues, leaf blades were treated with ultrasound before placing them on the nutrient medium. Callus formation was assessed on a 5-point scale (1 point - one or two calluses with 1-2 mm diameter have been formed on the leaf disc; 5 points - the surface of the leaf blade is almost completely (80-100%) covered with calluses).

As a result of the research, it has been found that the exposure to ultrasound with the power density of 1.2 W/cm^2 to 14.9 W/cm^2 for 60 seconds results in microdamages of plant tissues, which can activate the process of callus formation. On leaf disks of apple clonal rootstocks, the intensity of callus formation increased at a maximum power of ultrasound irradiation (Fig. 2), increasing the number of disks with necrotic areas resulting from damage to plant cells.

The stimulating effect of ultrasound on callus formation manifested itself in varying degrees, depending on the genotype. The number of leaf discs with callus in apple clonal rootstock B9 in all variants of the experiment was 100%, the intensity of callus formation was 2.2 to 2.9 points at a low power of ultrasound irradiation, in the reference groups without additional damage to leaf blades - 2.1 points. The most intensive formation of callus – 4.8 points - was noted in the variant with the power of 14.9 W/cm^2; partial necrosis of the tissues being observed in 45.0% of the explants. This is due to the fact that with high ultrasound power density, irradiation causes irreversible damages to plant tissues. Making cuts on the leaf blade with a scalpel stimulated callus formation up to 3.4 points. The effect of callus formation stimulation after treating leaf blades with ultrasound has been shown before when working with cuts on the leaves of clematis [15].

Against the background of more intensive callus formation in the reference on leaf disks of apple 14-1 clonal rootstock, the stimulating effect of ultrasound damage was less pronounced.

Treating the leaf tissues with ultrasound stimulated morphogenetic processes. Microdamages to the leaf blades with ultrasound with optimal parameters of the exposure contributed to an increased adventive shoots' regeneration rate (Fig. 3-5).

Ultrasonication of leaf discs at the optimal power allowed to increase the adventive shoots' regeneration rate of apple clonal rootstock B9 3.5 times, compared to the reference with intact leaf blades (Fig. 5). The positive effect was obtained with the ultrasound power density of 6 W/cm^2 and 10 W/cm^2. With lower intensity of ultrasound exposure, the stimulation effect was not observed, due to insufficient leaf blade damage.

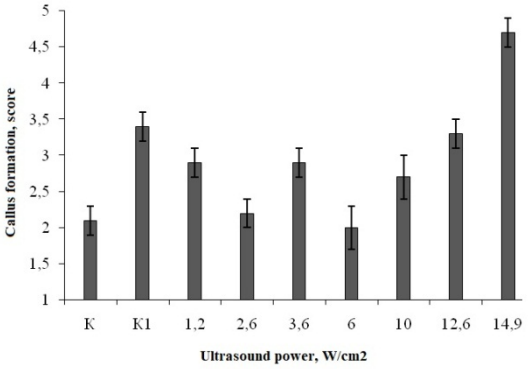


Fig. 2. The effect of leaf explants' ultrasound irradiation on callus formation from leaf explants of apple clonal rootstock B9

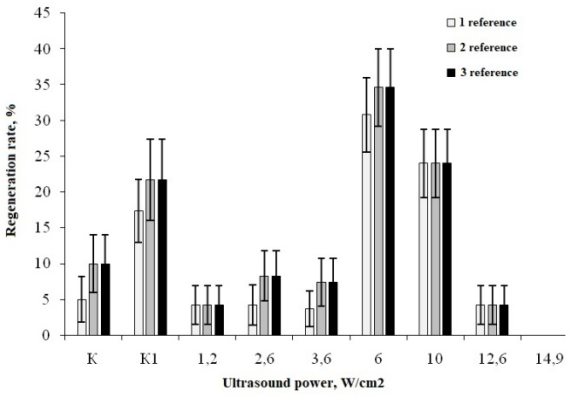


Fig. 4. The effect of ultrasound irradiation on the regeneration efficiency of shoots from leaf explants of apple clonal rootstock B9

In working with leaf cuts on apple clonal rootstock 14-1, the use of ultrasound was most efficient at lower intensity of exposure. This can be explained by the morphological features of the genotype, which has subtler and gentler epidermal and parenchymal leaf tissues, compared to rootstock B9. In all variants except one, when leaf discs had been exposed to ultrasound, regeneration efficiency of clonal rootstock 14-1 was higher than in the reference variants (Fig. 5). The best were the variants with the power of ultrasound irradiation of 1.2 W/cm^2, and 3.6 W/cm^2. Ultrasound irradiation (1.2 W/cm^2) allowed increasing the frequency of adventive shoots' regeneration rate of apple clonal rootstock 14-1 by 37.5%, compared to the reference with intact leaf blades, and by 33.9%, compared to the reference with leaf disks with scalpel notches. With the ultrasound power of

3.6 W/cm^2, the positive effect was even more pronounced. The efficiency of regeneration increased by 48.3%, compared to the reference with intact leaf blades, and by 44.7%, compared to reference 1 (notched with a scalpel) (Fig. 6).

The number of regeneration foci on a single regenerating explant also significantly (2-2.5 times) increased in the optimal variants with exposure to ultrasound (Fig. 6, 7). For apple clonal rootstock, direct regeneration from leaf tissues without formation of a significant number of calluses is typical. The use of ultrasound increased the number of the regenerants formed exactly from callus. This is an important condition for developing a method of genetic transformation and tissue breeding of fruit crops because in this case it is necessary to achieve regeneration of adventive shoots from callus cells sampled on media with selective agents.

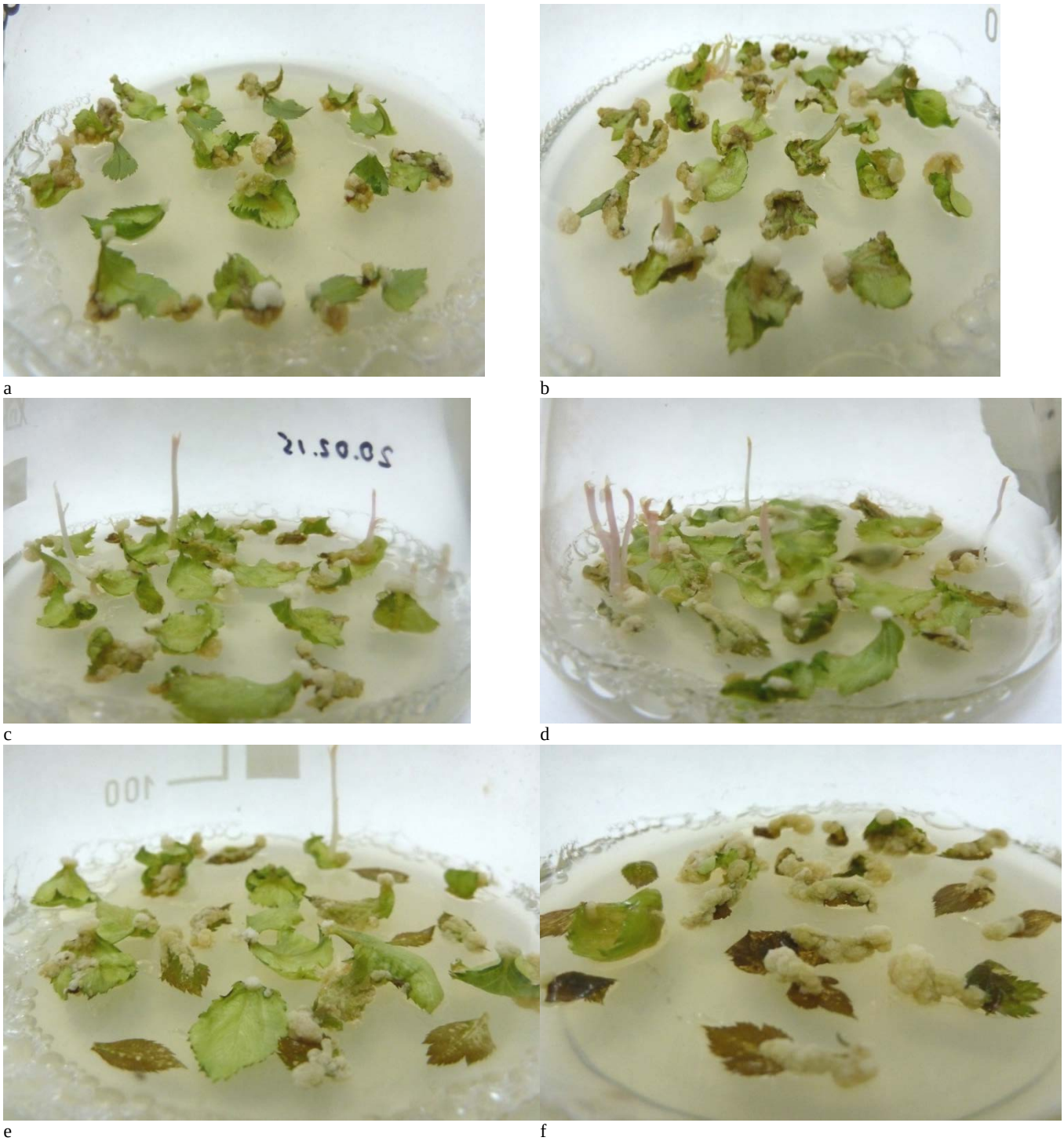


Fig. 3. The effect of leaf explants' ultrasound irradiation on callus formation and morphogenesis of apple rootstock B9: a – reference; b – 1.2 W/cm^2; c – 6.0 W/cm^2; d – 10.0 W/cm^2; e – 12.6 W/cm^2; f – 14.9 W/cm^2

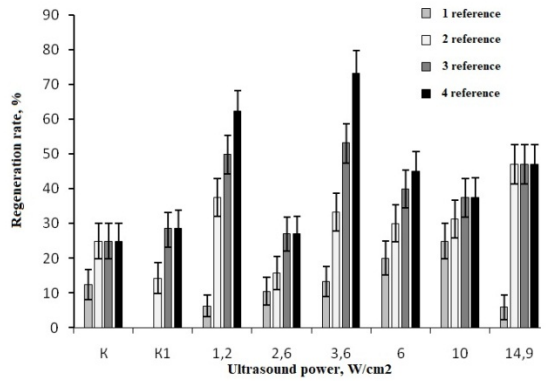


Fig. 5. The effect of ultrasound irradiation on the regeneration efficiency of shoots from leaf explants of apple clonal rootstock 14-1

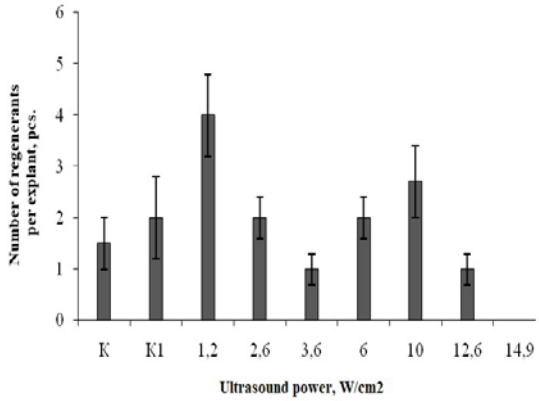


Fig. 6. The effect of ultrasound irradiation on the number of formed regenerants per one explant of apple clonal rootstock B9

Studying the dynamics of the process has shown that in the reference variants, the process of regeneration stopped after 10-11 weeks of keeping explants on nutrient media, while in variants with ultrasound irradiation, it continued after 14 weeks of cultivation. It is important to develop a method of tissue breeding for apple clonal rootstock since the process of sampling on selective media often lasts quite long.

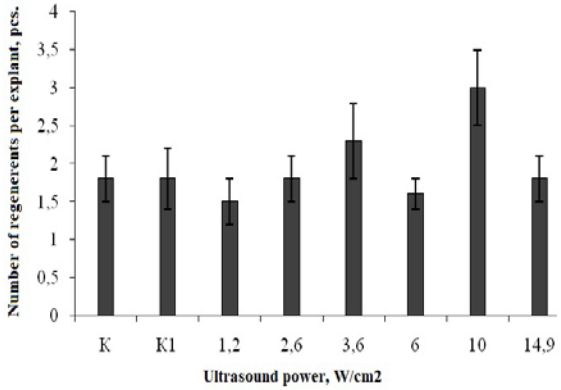
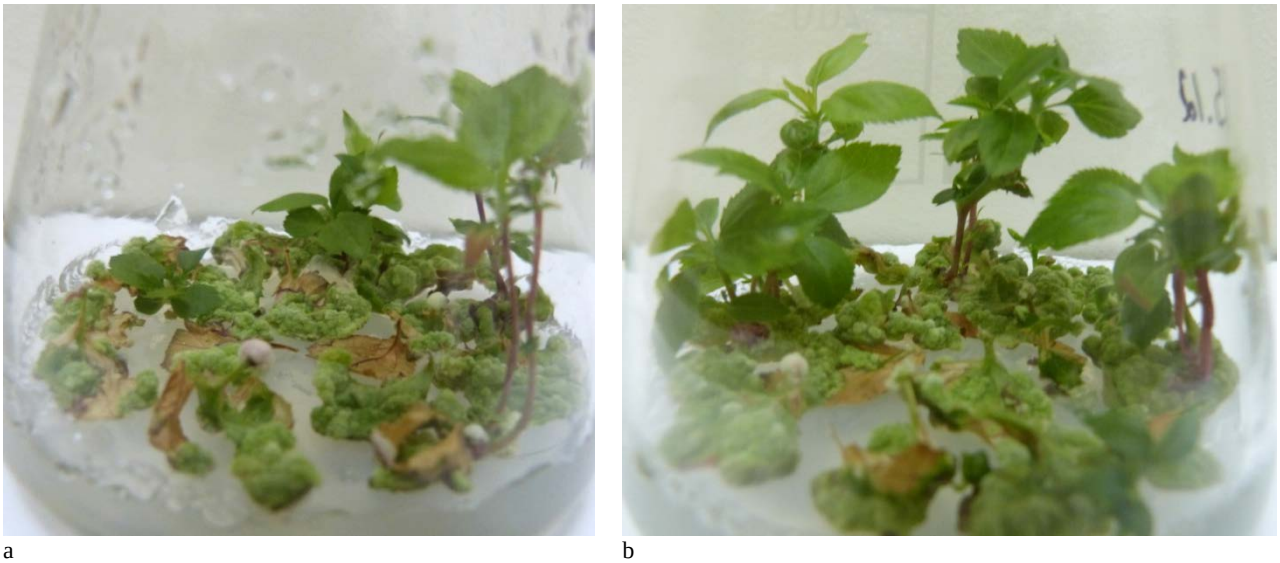


Fig. 7. The effect of ultrasound irradiation on the number of formed regenerants
per one explant of apple interspecies hybrid 14-1

After transferring explants into the in vitro chamber conditions, the formed regenerants developed into normal shoots with large leaves (Fig. 8). They were cut from leaf disks and grown according to the standard scheme of micropropagation for apple clonal rootstock.

The stimulating effect of ultrasound treatment on the regeneration process was shown earlier in other cultures. Ananthakrishnan [20] reported about 5 times increased regeneration rate, compared to the reference with cotyledon explants of *Cucurbita pepo* L. (c.v Ma'yan and Bareqet) when exposed to ultrasound for 0.5-2 minutes.

2-minute exposure of grape internodes to ultrasound in combination with adding amino acids tryptophan and Proline at the concentration of 100 µm into the nutrient medium contributed to callus formation and embryogenesis in grape [21].



a b

Fig. 8. Regeneration of adventive shoots from leaf explants of apple clonal rootstock 14-1 (a - leaf discs without damage, b - ultrasound irradiation 1.2 W/cm2)

CONCLUSION

Preliminary 60 seconds' exposure of leaf tissues to ultrasound of certain power has a positive effect on inducing the process of callus formation and using the morphogenetic potential of apple clonal rootstock. Intensity of callus formation increases with increasing the power of ultrasound, but not indefinitely, since excessive exposure results in the death of plant cells and necrosis of the plant tissues.

The integrated use of the methods of plant tissues' culture and ultrasound irradiation allows to increase regeneration efficiency of shoots 2.5-3.5 times and to increase 1.5-3 times the number of regenerants formed from callus of cultured leaf discs of apple clonal rootstock.

REFERENCES

1. Tran Tranh Van, K. M., Control of morphogenesis in vitro cultures, *Annu. Rew. Plant Physiol.* 1981, *33*, 291-311.
2. Butenko, R. G., Induktsiya morfogeneza v kulture tkanei rastenii [Induction of morphogenesis in plant tissue culture], in *Hormonal regulation of ontogeny of plants*, Nauka, Moscow 1984, pp. 42-54.
3. Elpiner, I. E., *Ultrasound. Fiziko-himicheskoe i biologicheskoe deistvie* [Physicochemical and biological action], State Publishing House for Physical and Mathematical Literature, Moscow 1963.
4. Golyamina, I. P., *Ultrasound. Small encyclopaedia*, Soviet encyclopedia, Moscow 1979.
5. Ruban, E. L., Dolgopolov, N. N., O vozdeistvii ultrazvukovih kolebanii na rannie fazi razvitiya rastenii [About the effect of ultrasound vibrations in the early plants development phases], *Proceedings of USSR Academy of Sciences* 1952, *84(3),* 623-626.
6. Grushvitsky, N. V., Limar, R. S., Uskorenie prorastaniya semyan zhenshenya pod vliyaniem ultrazvuka [Accelerating the ginseng seed germination rate under the influence of ultrasound], *Bot. Journal* 1961, *46(3)*, 1282-1285.
7. Limar, R. S., Vliyanie ultrazvuka na prorastanie semyan nekotorih bobovih kultur [The effect of ultrasound on seed germination of certain legumes], *Proc. in applied botany, genetics, and breeding* 1962, *34(1)*, 159-163.
8. Limar, R. S., Chuvstvitelost gibridov i ishodnih form kukuruzi k deistviyu ultrazvuka [Sensitivity of hybrids and initial forms of maize to ultrasound], *Bulletin of the Research Institute of Crop Production n.a. N. I. Vavilov* 1972, *27*, 15-18.
9. Arkhipova, T. N., Khabarov, S. N., Shelkovskaya, N. K, Progressivnie sposobi pererabotki plodov i ovoschei s pomosch'yu ul'trazvuka [Progressive methods of processing fruit and vegetables with the use of ultrasound], *Bulletin of RAAS* 2007, 2, 90-91.
10. Shepeleva, N. S., Karetkin, B. A., Intensifikatsiya videleniya inulina iz klubnei topinambura s pomosch'yu ultrazvuka [Intensification of inulin extraction from Jerusalem artichoke tubers with ultrasound], *Advances in chemistry and chemical technology* 2007, *XXI(5(73))*, 35-38.
11. Evdokimova, O. V., Issledovanie vliyaniya rezhimov ekstragirovaniya rastitelnogo sirya na izvlechenie fiziologicheski funktsionalnih pischevih ingradientov [Studying the influence of plant raw materials extraction modes on extraction of physiologically functional food ingredients], *Technology and merchandising of innovative food products* 2011, *2(7)*, 3-12.
12. Kosolapova, A. S., Lamberova, M. E., Issledovanie vliyaniya ultrazvuka na otdelnie stadii v tehnologii kulturi rastitel'nih kletok i tkanei in vitro [Studying the influence of ultrasound on certain stages in the technology of culturing plant cells and tissues in vitro], *Chemistry of vegetable raw materials* 2010, *2*, 179-180.
13. Klimek-Chodacka, M., Baranski, R., A protocol for sonication-assisted Agrobacterium rhizogenes-mediated transformation of haploid and diploid sugar beet (*Beta vulgaris* L.) explants, *Biochimica polonica* 2014, *61(1)*, 13-17.
14. Papikhin, R. V., Muratova, S. A., Vliyanie ultrazvukovogo izlucheniya na protsess rizogeneza mikrocherenkov in vitro [The effect of ultrasound irradiation on the process of rhizogenesis of microsprigs *in vitro*], *Horticulture and viticulture* 2009, *4*, 18-21.
15. Papikhin, R. V., Muratova, S. A., Vliyanie ultrazvukovogo izlucheniya na kallusoobrazovanie i morfogenez v kulture rastitelnih tkanei in vitro [The effect of ultrasound irradiation on callus formation and morphogenesis in the culture of plant tissues *in vitro*], in *Fruit and berry growing in Russia*, Moscow 2011, vol. XXVI, pp. 179-185.
16. Papikhin, R. V., Muratova, S. A., *Povishenie effektivnosti otdalyonnoi gibridizatsii semechkovih plodovih kultur* [Improving efficiency of remote hybridization of pomaceous crops], Publishing House of the Michurinsk State Agrarian University, Michurinsk 2011.
17. Murashige, T., Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol. Plant.* 1962, *15(95)*, 473-497.
18. Muratova, S. A., Solovykh, N. V., Terekhova, V. I., *Induktsiya morfogeneza iz izolirovannih somaticheskih tkanei rastenii* [Induction of morphogenesis from isolated somatic plant tissues], Publishing House of the Michurinsk State Agrarian University, Michurinsk, 2011.
19. Muratova, S. A., Bocharova, T. E., Papikhin, R. V., Potentsialnie vozmozhnosti adventivnogo organogeneza iz listovih visechek klonovih podvoev yabloni [Potential possibilities of organ genesis from leaf cuts of apple clonal rootstock], *Bulletin of MichSAU* 2012, *1(1)*, 54-58.
20. Ananthakrishnan, G., Xia, X., Amutha, S., Singer, S., Muruganantham, M., Yablonky, S., Ultrasounic treatment stimulates multiple shoot regeneration and explant enlargement in recalcitrant squash cotyledon explants in vitro, *Journal of Cell Biology and Morphologensis* 2007, *26*, 267-276.
21. Farokhzad, Y., Pourmand, S., Rezaei, A., Sharafi, Y., Effects of Ultrasound, Tryptophan and Proline on embryogenesis and regeneration of grape (*Vitis vinifera* L.), *Azarian Journal of Agriculture* 2016, *3(5)*, 112-118.