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METHODOLOGY & APPLICATIONS

Elaboration the high mechanical strength medium for *in vitro* cultivation of lilacs under microgravity conditions

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ABSTRACT

In the modern world, outer space is an object of research in various fields of scientific activity. One of these spheres is plant breeding, i.e. cultivation of higher plants, as they are the most promising from the side of use as photosynthetic organisms. The use of nutrient media in which agar-agar, basalt wool, microcellulose can be used as a thickener is considered for plant life support. Also, 1.5 ml of 2-ip hormone was added to all substrates to stimulate plant growth. It was found that using agar-agar as a nutrient thickener in an amount of 15 g/l, allows the best growth and development of plants obtained by microclonal propagation under microgravity conditions. In addition, mechanical tests of nutrient media with agar-agar as a thickener determined the resistance to external factors, because the resulting substrate does not yield to indentation, deformation and fluidity.

Keywords: microgravity, nutrient medium, agar-agar, lilacs, plants in space, microclonal propagation of plants

РЕЗЮМЕ

Никулин И.С., Журавлёва Е.В., Тохтарь Л.А., Тохтарь В.К., Ткаченко Н.Н., Воропаев В.С., Бородаева Ж.А., Кулько С.В., Алфимова Н.И., Никуличева Т.Б., Титенко А.А. Моделирование среды высокой механической прочности для культивирования сирени *in vitro* в условиях микрогравитации. В современном мире космическое пространство является объектом исследования в различных сферах научной деятельности. Одной из этих сфер является растениеводство в общем и выращивание высших растений в частности, так как они являются наиболее перспективными со стороны использования в качестве фотосинтезирующих организмов. Для жизнеобеспечения растений рассмотрено использование питательных сред, в которых в качестве загустителя могут использоваться агар-агар, базальтовая вата, микроцеллюлоза. Так же во все субстраты добавляли 1.5 ml гормона 2-ip для стимулирования роста растений. Установлено, что использование в качестве загустителя питательной среды агар-агара в количестве 15 g/l, позволяет обеспечить наилучшие показатели роста и развития растений, полученных микроклональным размножением, в условиях микрогравитации. Кроме того, проведенные механические испытания питательных сред, которые в своем составе в качестве загустителя имели агар-агар, определили устойчивость к внешним факторам, т.к. полученный субстрат не поддается вдавливанию, деформации и текучести.

Ключевые слова: микрогравитация, невесомость, питательная среда, агар-агар, сирень, растения в космосе, микроклональное размножение растений

Cultivation of plants in space is currently a priority in long-term space exploration, because plants are an integral part of bioregenerative life support systems and an important component in the creation of large-scale controlled extraterrestrial ecosystems (Zheng et al. 2015).

Since the distinctive traits and characteristics of higher plants on Earth are the result of a long process of evolution and adaptation to conditions, the entire potential for plant survival and reproduction in extraterrestrial environments in general and in microgravity in particular is determined by the same factors as those operating on Earth, as well as additional factors such as altered gravity and ionizing radiation (de Pascale et al. 2021).

Analysis of experiments conducted at the end of the 20th century showed that microgravity, as well as other factors in space conditions, affect plants at different levels, leading to changes in the development, metabolism and growth of these plants (Dutcher et al. 1994). Consequently, for the growth and development of plants in microgravity it is necessary to think of a system for controlling the supply of water, obtaining in the necessary amounts of light as well as nutrients, which is an obvious problem for the use of plants in space, especially considering the need to minimize or eliminate the system expendables in microgravity conditions (Ferl et al. 2002).

Soil-free technologies should be used in weightless conditions. The nutrient solutions for these technologies will differ according to the properties of the particular crop (Savvas et al. 2018).

At present, the study of higher plants in microgravity has not considered the autonomy of their growth and development process, and is carried out only under human supervision and with his direct participation (Metzger 2016). However, in the future, when considering the possible urbanization of near space and nearest space objects to Earth, whether satellites or other planets, it is necessary to develop not only a system for the controlled care of plants for their future use, for example, by using phytotrons, but also a fully autonomous system that allows both the long-term transport of already germinated plants and the creation of space greenhouses for observing and studying the effects of microgravity on plants in the long term (Ferretti et al. 2020).

In order to study the long-term effect of microgravity conditions of outer space on the growth and development of higher plants *in vitro* in the growth phase without direct human participation, it was necessary to select a suitable life form of plants.

Woody plants, especially conifers, are characterized by slow growth and difficult rooting. They contain a large number of secondary compounds that are oxidized in isolated tissues by various phenolases. The formation of phenol oxidation products results in inhibition of cell division and growth. This can lead to the death of the primary explant or to a decrease in the ability of tree species tissues to regenerate adventitious buds. As the donor plant ages, the ability to regenerate adventitious buds gradually disappears completely. All this together complicates their microclonal propagation.

Shrubs have a higher *in vitro* reproduction rate than trees, therefore we decided to focus on the selection of the characteristics of shrub plants. The research work was carried out on the basis of the laboratory of physico-chemical methods of plant research, the staff of which has extensive experience in microclonal propagation of lilac shrubs under *in vitro* conditions. However, the choice of variety must necessarily be based on the conditions in which lilacs will need to survive, and these include the success of long-term cultivation of lilacs at reduced temperatures, which depends on the genotype.

Cultivation of lilacs at +10ºC for 5 months has no negative effect on the microplants. However, if the duration of cultivation at this temperature is longer, it may lead to death of plants of lilac cultivars with no increase of total anthocyanins in their leaves. Lilac cultivars with purple-blue coloring of flower corolla when grown *in vitro* under the conditions of low temperature show increase of total anthocyans in leaves from 1.5 to 10 times (Churikova et al. 2018). This indicates a possible correlation between the ability of plants to accumulate anthocyans in leaf plates and shoots at low temperature and corolla coloration. It is these sprouts of the variety 'Velikaya Pobeda' [Great Victory], selected in the process of cloning, and were selected for further research.

The purpose of this study was to select a nutrient medium that has not only high biological indicators affecting plant growth and development, but also certain mechanical characteristics, since it will be used under extreme conditions and must withstand the initial high loads of launching into open space, as well as retain its properties in the future.

MATERIAL AND METHODS

At present, a large number of nutrient media of various compositions is known, but the most commonly used for cultivation of isolated plant tissues under *in vitro* conditions is the medium developed by Murashige & Skoog (1962). During the experiments, we used a mineral base of Murashige-Skoog nutrient medium with the addition of 30 g/l sucrose to cultivate lilac microclones. 2-isopentyladenine (2-iP) at a concentration of 1.5 mg/l was used as a growth regulator.

The nutrient medium was prepared in the following sequence: sucrose was placed in a 1-liter glass and topped up with distilled water to 400 ml. After sucrose dissolution, the necessary amounts of mother solutions of macro- and microsalts, mesoinositol, glycine, and vitamins were added. The pH of the solution was adjusted using 0.1 N NaOH or HCl solution to 5.7–5.8. Next, the medium was transferred into a measuring cylinder or flask of 1 l, agar-agar was added, and the volume was brought to the mark of 1 l with distilled water. The medium was poured into glass test tubes, which were closed with aluminum foil covers. The tubes with the medium were autoclaved for 30 minutes at 120ºC and 1 atm operating pressure.

Based on the fact that the vessels with plants inside will be subjected to high mechanical stresses during the space launch, our research objectives were to create a nutrient medium with such characteristics that would allow to firmly hold plants in the vessels under extreme conditions, preserving their properties as a source of substances necessary for plants.

The additions of the following components were studied as components to create a dense nutrient medium: agar-agar, microcrystalline cellulose, and mineral wool.

In our studies, we tested media containing agar-agar as a thickener in the amounts of 7.5 (control), 10, 12.5, 15, 20 g/l, and basalt wool additives in amounts of 10, 25, 50, and 100 g/l. Also, 7.5 g/l agar-agar was added to all versions of media with basalt wool. We also experimented adding 10, 25, 50, and 100 g/L microcellulose to the nutrient media. In all media with microcrystalline cellulose, 7.5 g/l agar-agar was also added (Table 1).

Studies of the mechanical properties of nutrient media included determining the deformation of depression, fluidity (in the upside-down position in test tubes), resistance to indentation of a steel ball.

To carry out tests to determine the deformation of the recess (Fig. 1A), the prepared nutrient medium 3 was introduced into the flask 2 installed on the base 1, and then the rod 5 (in this case a quartz rod) was placed in it,

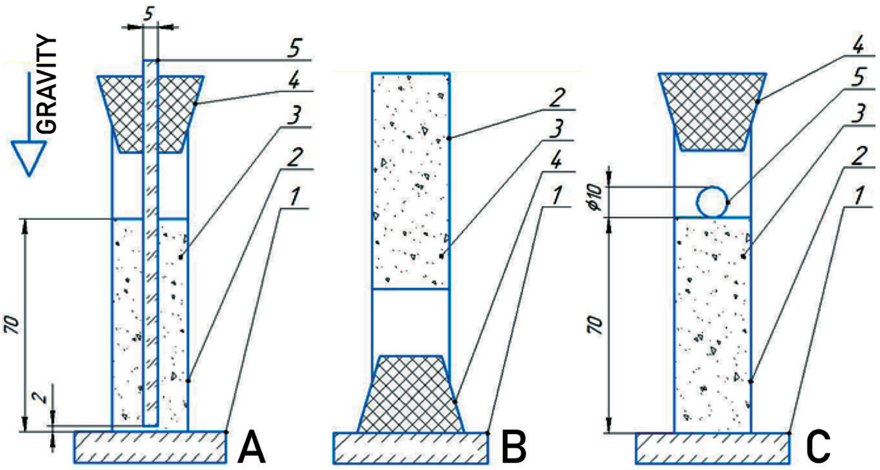

Figure 1 Scheme of the experiment to determine the deformation of the deepening of nutrient media (A), to determine the fluidity of nutrient media (B), to determine the resistance of nutrient media to the indentation of a steel ball (C). 1 – base, 2 – test tube, 3 – substrate, 4 – cap, 5 – rod, 6 – steel ball

Table 1. Dense nutrient media with additives of various components

Nutrient medium	Agar-agar, g/l	Basalt wool, g/l	Microcrystalline cellulose, g/l
Control	7.5	–	–
A1	10	–	–
A2	12.5	–	–
A3	15	–	–
A4	20	–	–
B1	7.5	10	–
B2	7.5	25	–
B3	7.5	50	–
B4	7.5	100	–
C1	7.5	–	10
C2	7.5	–	25
C3	7.5	–	50
C4	7.5	–	100

which had a free passage in the stopper 4. The zero point of the rod in the flask was marked, and the magnitude of its shrinkage in the nutrient medium under the force of the Earth's gravity was monitored.

When studying nutrient media for fluidity in the inverted position (Fig. 1B), the prepared nutrient medium 3 was introduced into flask 2, closed with cork 4 and fixed in the base 1 so that the composition was in the upper part of the flask relative to the cork. The bottom point of the medium in the flask at the beginning of the experiment was documented, after which observation of its mechanical changes under the influence of the Earth's gravitational force was carried out.

In order to determine the resistance of the obtained nutrient media compositions to indentation (Fig. 1C), medium 3 was placed in flask 2, on the surface of which a steel ball 5 was placed, the mass of which was 6.889 g, then the flask was closed with a cork 4 and fixed on the base 1. After fixing the flask marked the location of the steel ball in it and observed its change under the influence of the Earth gravity.

To study the effect of the obtained nutrient media on the growth processes of plants under *in vitro* conditions as biological objects in the experiments we used plants of the lilac variety 'Velikaya Pobeda' bred by L.A. Kolesnikov and V.D. Mironovich in 1986 and cultivated *in vitro* in the plant biotechnology laboratory of scientific and educational center "Botanical garden of NRU "BelGU". During the experiment, the following morphometric plant parameters were monitored: shoot height, number of internodes, and number of leaves. The measurements were performed 1 and 2 months after planting the plants on nutrient media.

RESULTS

The results of the mechanical tests of the nutrient media are presented in Table 2.

It was found experimentally that the use of microcrystalline cellulose as a thickener did not produce a dense nutrient medium. In experiments to determine the deformation of the deepening when removing the quartz rod from the medium, the formed niche was filled with water on all samples of media with MCC (C1, C2, C3, C4). In the tubes turned upside down, the water was collected at the base, and during the experiment on indentation the metal ball was displaced almost 2 centimeters in the direction of the bottom of the tube (Fig. 2A,B).

Testing the mechanical properties of nutrient media with 7.5 (control), 10 (A1), and 12.5 (A2 g/l) agar-agar additives showed their insufficient strength. For example, the depression from the quartz rod after its extraction was filled with water (from 5 to 34 mm), the metal ball was pressed deep into the medium (from 3 to 10 mm) under the force of gravity, and when the tube was turned over, water accumulated towards its base, Table 2.

When adding 15 (A3) and 20 (A4) g/l agar-agar, the mechanical characteristics of the nutrient media were the best among all the options studied. When tubes were turned upside down, there was no accumulation of water in the tube toward the base, the metal ball was not pressed in more than 2 mm, and when the quartz rod was removed, the cavity was not filled with water (Table 2, Fig. 2C,D).

Table 2. Mechanical characteristics of nutrient media

Mechanical characteristics	Nutrient medium								
	control	A1	A2	A3	A4	C1	C2	C3	C4
Depth of metal ball indentation, mm	10	3	3	2	1	14	4	2	1
Amount of liquid in the well after extraction of the quartz rod, mm	34	7	5	0	0	39	22	14	10

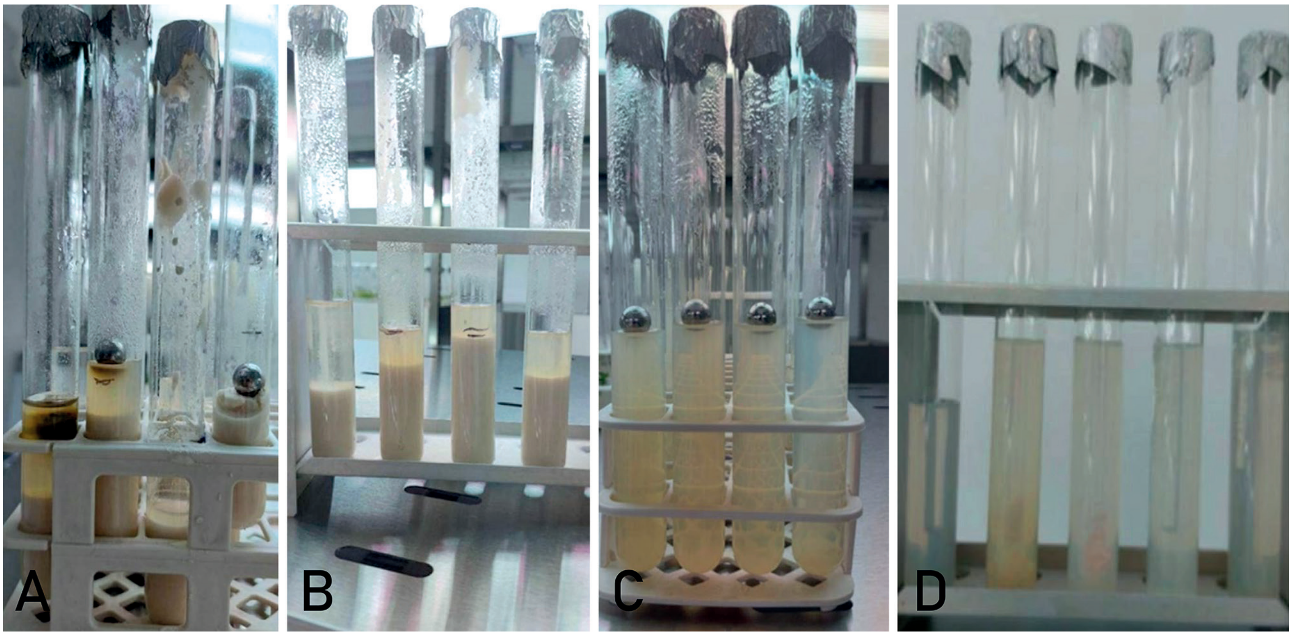

Figure 2 Testing the mechanical properties of microcrystalline cellulose-added nutrient media, where A – indentation resistance tests, B – indentation strain tests, and of nutrient media supplemented with 15 mg/L agar-agar, where C – indentation resistance tests; D – indentation strain tests

Due to the unsatisfactory results of morphometric indicators of plant growth and development on nutrient media with mineral wool additives, it was decided not to conduct their mechanical tests.

Comparative analysis of morphometric characteristics of lilac plants after one month of cultivation on nutrient media showed that the lowest plant height, the lowest number of leaves and internodes were observed on nutrient media with the maximum content (100 g/l) of basalt wool (B4) and microcrystalline cellulose (C4) in their composition (Table 3, Fig. 3).

At the concentration of basalt wool in the medium of 25 g/l (B2) morphometric indicators of growth processes were the best among the medium variants with this component. On media containing MCC at a concentration of 10 (C1) and 25 (C2) mg/l, the best morphometric indices of plants were observed on media with this additive.

On media with agar-agar, the height of plants decreased with increasing agar-agar concentration. The lowest rates of growth processes on media containing agar-agar were observed at its concentration of 20 (A4) g/l.

After two months of cultivation, growth processes on media with MCC and basalt wool decreased with increasing concentrations of both components in their composition. On medium with the addition of 100 g/l of basalt wool (B4), all plants died, Table 3. When agar-agar was used, the maximum plant height was observed at its concentration of 7.5 g/l (control), and the greatest number of leaves and internodes at 10 g/l (A1). Addition of 20 g/l agar-agar significantly inhibited growth processes up to plant death. On a medium containing 15 g/l agar-agar (A3), plant growth processes, although slowed compared to the control (7.5 g/l), but the plants were not normally developed.

It should be admitted that the use of basalt wool in the creation of nutrient media for use in microgravity conditions is inexpedient due to the fact that during autoclaving it formed lumps, as a result of which cracks appeared on the media surface later on, while plants were not firmly fixed in tubes with the medium. Analysis of morphometric indicators of growth processes on media with basalt wool additives also showed a negative effect of these additives plants.

Table 3. Effect of dense nutrient media on morphometric parameters of lilac plants *in vitro*

Nutrient medium option	Plant height, cm		Number of internodes, pcs.		Number of leaves, pcs.	
	1 month	2 months	1 month	2 months	1 month	2 months
Control	2.19±0.83	2.48± 0.56	6.8±2.10	9.1± 1.35	9.3±0.26	16.3 ±0.81
A1	2.03±0.38	2.04 ±0.46	7.3±1.63	9.4± 0.73	13.0±1.01	17.1 ±1.84
A2	1.92±0.64	2.23 ±0.62	6.4±1.40	7.8± 1.94	9.2±0.82	13.9 ±2.14
A3	2.13±0.58	2.28 ±0.77	6.3±1.37	9.0± 1.17	12.0±0.62	16.2 ±0.82
A4	1.77±0.34	1.85 ±0.37	6.1±0.94	7.3 ±0.72	11.0±1.01	13.7 ±0.59
B1	1.92±0.98	2.07 ±0.31	6.0±1.61	8.6 ±0.66	10.9±1.09	15.6 ±1.54
B2	2.05±0.68	2.10 ±0.48	6.4±1.42	8.1 ±1.26	11.3 ±0.75	15.0 ±2.02
B3	1.79±0.50	1.9 ±0.28	5.2±1.35	6.2 ±1.26	8.8±1.09	10.7 ±1.08
B4	1.30±1.10	-	3.3±1.41	-	5.0±0.61	-
C1	1.91±0.41	2.12 ±0.59	6.8±1.41	8.0 ±0.65	11.7±1.78	14.1 ±1.42
C2	1.72±0.35	2.26 ±0.91	7.0±1.33	6.4±2.80	11.9±1.06	12.4 ±2.62
C3	1.85±0.22	2.18± 0.45	5.2±1.24	7.1 ±1.84	9.6±1.10	12.3 ±1.42
C4	1.71±0.41	2.16 ±0.54	5.0±0.83	6.1 ±2.55	8.3±0.47	10.5 ±2.14

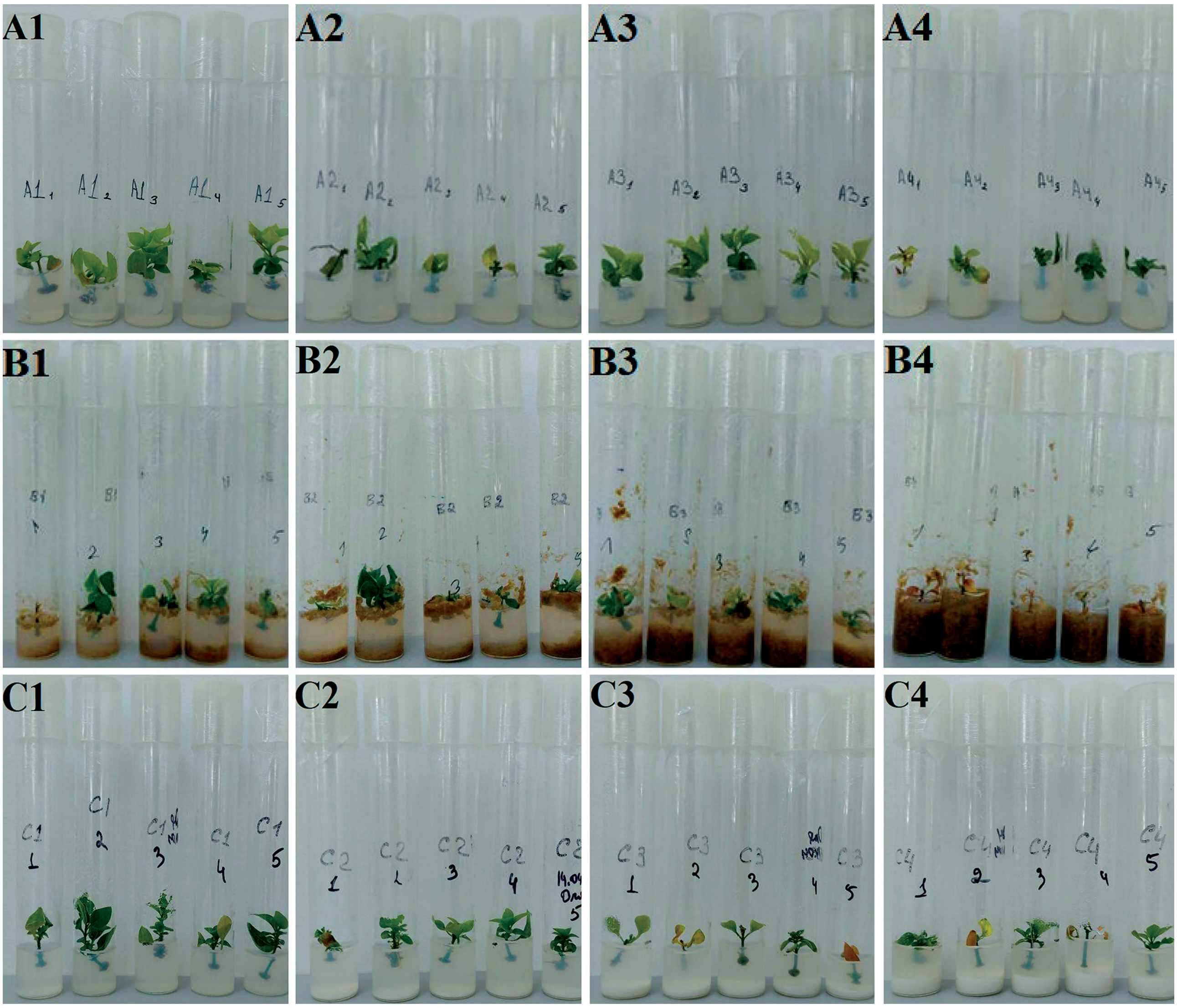

Figure 3 Lilac microclones after 1 month of cultivation on nutrient media

Despite the satisfactory results of tests of morphometric indicators of growth processes in lilac plants on media with microcrystalline cellulose in concentrations of 10 (C1) and 25 (C2) mg/l, its use as a thickening agent was also inexpedient, since during solidification of media most of it precipitated at the bottom of test tubes. At the same time, tests of mechanical properties showed that MCC additives did not produce dense nutrient media.

Based on the above, it is most appropriate to add agar-agar as a component to obtain a dense nutrient medium. During the experiment, it was found that when agar-agar was added in an amount of 7.5 g/l, which is the standard for the preparation of nutrient media for microclonal propagation, good indicators of plant growth processes were observed, but unsatisfactory results in mechanical tests of media strength were observed. When 20 g/l agar-agar was applied, a negative effect of its high concentration on morphometric indicators of plant development was noted, while the mechanical characteristics confirmed the receipt of a dense nutrient medium.

Analysis of the correlation between the morphometric indices of plant growth processes and mechanical strength tests of nutrient media showed that the most suitable composition for growing plant microclones in weightlessness is the composition with the addition of agar-agar in an amount of 15 g/l (A3).

Upon termination of this study, the obtained composition of nutrient medium was used in the payload module of the satellite launched on August 9, 2022 from Baikonur cosmodrome as a substrate for cultivation of micro clones of lilac variety 'Velikaya Pobeda'. Observations of plant growth and development in microgravity continue to this day, indicating the correctness of the selected nutrient medium.

CONCLUSIONS

According to the results of the experimental study, we can conclude that the use of an increased concentration of agar-agar (15 g/l) as a nutrient media thickener makes it possible to obtain a nutrient medium resistant to external factors, which is not destroyed in the conditions of simulated microgravity. The resulting composition was resistant to indentation, deformation, and flow. In addition, this composition maintained the necessary conditions for growth and development of lilac plants obtained by microclonal propagation and allowed predictable control of their growth.

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