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Establishment of an In Vitro Micropropagation Protocol for *Hibiscus moscheutos* L. 'Berry Awesome'

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Abstract: *Hibiscus moscheutos* L. 'Berry Awesome' is a complex hybrid of the new Proven Winners Summerific series of varieties with highly ornamental characteristics. Micropropagation of highly ornamental varieties is important for mass production of planting material for commercial purposes. The traditional methods for propagating *Hibiscus* varieties, such as cuttings or seed propagation, however, do not guarantee high rates of production of high-quality seedlings. To solve this problem, an attempt was made to develop protocols for micropropagation of *Hibiscus moscheutos* L. 'Berry Awesome' in vitro on agar and liquid medium using a bioreactor system, followed by ex vitro adaptation of the regenerants. The optimal method for sterilization of nodal explants as well as the optimal composition of the initiation medium for shoot proliferation and rooting were determined. For micropropagation on a liquid medium, a rocker-type bioreactor was used, and its advantages over micropropagation on an agar medium were demonstrated. The results showed that the best sterilization method for nodal segment explants was as follows: pretreatment by rinsing with running tap water, sterile water, and distilled water for 70 min and soaking for 5 min in a mixture of solutions of ethyl alcohol (96%), hydrogen peroxide (38%), and water in a ratio of 1:1:2. In this case, live and sterile explants accounted for 62.6%. The optimal initiation medium for axillary buds in nodal segments was the Murashige and Skoog (MS) medium supplemented with 0.1 mg L^{-1} N-(2-chloro-4-pyridinyl)-N'-phenylurea (CPPU), which resulted in 73.3% of axillary buds being induced. The optimal solid proliferation medium was MS medium supplemented with 0.1 mg L^{-1} CPPU with a proliferation coefficient of 5.8. In a liquid medium, the optimal concentration of CPPU was 0.05 mg L^{-1} with a proliferation coefficient of 9.2. The best medium for rooting/shoots with agar and in bioreactors was MS medium with the addition of 0.1 mg L^{-1} indole-3-butyric acid (IBA). The highest rooting rate was 99.0% in both types of media, and the survival rate of plantlets was 88.7% in solid media and 98.7% in the bioreactor.

Keywords: ornamental plants; nodal segments; liquid medium; CPPU; BAP; IBA; bioreactor

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1. Introduction

Hibiscus moscheutos L. 'Berry Awesome' is a complex hybrid native to North America. It belongs to the Proven Winners Summerific series, which comprises hybrids of pink mallow (*Hibiscus moscheutos*) and other *Hibiscus* species, such as *H. grandiflorus*, *H. coccineus*,

H. dasycalyx, and *H. laevis*. The Summerific varieties are more densely branched and compact than older varieties. *Hibiscus moscheutos* L. 'Berry Awesome' has erect, leafy, woody stems that can grow up to a height of 2 m. Unlike older varieties, the flowers of this plant develop along the entire stem. The blooms are large, up to 25 cm in diameter, and are lavender pink with a corrugated texture and a red center. This plant blooms from mid-summer to early autumn. The leaves are dark green, shiny, and divided into three broad lobes with a purple tinge (www.gardenia.net: 5 October 2023). Various forms and varieties of this *Hibiscus* are used for urban landscaping, with annual sales estimated to be worth USD tens of millions [1]. *Hibiscus moscheutos* is typically propagated through seed or cuttings, with encapsulation being a rare method [2–4]. Of particular interest is the in vitro micropropagation of these cultivars, which allows the efficient production of large volumes of planting material and ensures long-term genetic stability.

The concentration of macro- and micronutrients, organic matter, vitamins, sugars, and growth hormones has a significant influence on the probability of success in *Hibiscus* micropropagation [5,6]. Improper medium composition leads to plant death or improper development [7,8]. Most of the above authors used a modified version of Murashige and Skoog's medium (MS) for micropropagation of *Hibiscus moscheutos* [9]. Determination of the concentrations of growth hormones and their derivatives is one of the most important steps in establishing a micropropagation protocol. Different cytokines have different effects on the culture, and their selection should be made by taking into account the varietal characteristics of *H. moscheutos*. For example, TDZ at concentrations above a certain value has been found to affect shoot growth and lead to chlorosis of tissues, slow elongation, and necrosis of the meristem [3]. Significant effects of CPPU on the morphogenic responses of the apical meristem in vitro and enhanced regenerative functions of explants with increasing CPPU concentrations have been observed [10–12]. The best rates of in vitro plant development are achieved at low concentrations of BAP, with shoot formation decreasing significantly with increasing concentration [5,13].

Recently, there has been successful micropropagation of plants on a liquid medium in bioreactors, which enhances the processes of propagation and growth of shoots in vitro [14]. Currently, various models of bioreactors are used for micropropagation of plants, each with its own advantages, as described in the relevant literature [15]. In this work, we used a simple scheme of the Rocker Systems BIOMINT bioreactor. Rocker or rotary systems employ a mechanical platform to tilt the culture boxes at a given angle, allowing the medium to move from one end of the box to the other and back [16]. According to Krol et al. [17], the rocker system offers several advantages, including the ability to place numerous culture boxes on a single rack, the simplicity of the culture box, and relatively low cost. However, no data on the micropropagation of *H. moscheutos* 'Berry Awesome' or any *H. moscheutos* cultivars on a liquid medium in bioreactors could be found in the literature search.

The aim of this study was to develop an in vitro micropropagation protocol for *H. moscheutos* 'Berry Awesome' on solid and liquid media using a bioreactor. The plants were then acclimatized ex vitro. The present study could provide useful information for the sustainable large-scale production of *H. moscheutos* 'Berry Awesome' plantlets.

2. Materials and Methods

2.1. Sterilization and In Vitro Culture Establishment

The research was conducted in 2020–2021 at the laboratory of the Botanical Garden of the Southern Federal University and Don State Technical University, Rostov-on-Don, Russia. The uterine specimens of *Hibiscus* were obtained from a private collection. Plants without visible signs of disease or damage were selected.

In the laboratory, the shoots of the current year were removed from the mother plant and washed with tap water and liquid hand soap. Using a scalpel, leaves and part of the internodes were removed, and the shoots were cut into small nodular explants measuring 1.0 to 1.5 cm in length. The nodal explants were then placed in a 500 mL

glass and rinsed with running tap water for 40 min to remove any impurities. The nodal explants were washed with sterile water containing 1–2 drops of surfactant (Twin 20) and shaken for 15 min. Subsequently, the nodal segments were washed three times with distilled water for 5 min each in the same glass and placed in a laminar flow cabinet for further sterilization treatment. The total time for washing the explants before working in the laminar flow cabinet was 70 min. To achieve the highest yield of sterile explants, various sterilizers, including sodium hypochlorite (NaOCl), mercury chloride ($HgCl_2$), ethyl alcohol (CH_5OH), and hydrogen peroxide (H_2O_2), were tested. The following six sterilization schemes were used:

E96H38W5 = a solution of ethyl alcohol (96%), hydrogen peroxide (38%), and water in a ratio of 1:1:2 for 5 min;

E96H38W10 = a solution of ethyl alcohol (96%), hydrogen peroxide (38%), and water in a ratio of 1:1:2 for 10 min;

E70S10 = ethyl alcohol (70%) for 30 s, then sulema chloride (1.0%) for 10 min;

E70S15 = ethyl alcohol (70%) for 30 s, then sulema chloride (1.0%) for 15 min;

E70SH5 = ethyl alcohol (70%) for 30 s, then sodium hypochlorite (1.0%) for 5 min;

E70SH10 = ethyl alcohol (70%) for 30 s, then sodium hypochlorite (1.0%) for 10 min.

In each variant, after treatment with sterilizers, the explants were washed three times in sterile distilled water for 15 min. Sterilization and washing were carried out in 300 mL glasses, in which 25 explants were placed and 100 mL of sterilizing solution or water was added at the washing stage. After that, the base of the explant was cut off by 1 mm using a scalpel and cultured on a base medium of MS with the addition of 30 g L^{-1} sucrose and 7 g L^{-1} agar at a pH of 5.8. To obtain the maximum number of germinated explants in combination with the base medium, BAP (0.1–0.5 mg L^{-1}) and CPPU (0.05 mg L^{-1}) were tested. Plant growth regulators were purchased from Sigma-Aldrich, St. Louis, MO, USA. A variant with an MS medium without phytohormones was used to control and test sterilization modes. The medium was poured into 20 mL tubes with a volume of 2 mL each. One explant was placed in each tube. The autoclaving mode was set at 121 °C for 15 min. Culture vessels were placed at 25 ± 2 °C in a 16 h photoperiod with PPFD 50 µmol m^{-2} s^{-1} using cold white fluorescent tubes (6500 K). Each variant of the experiment included 25 explants in three repetitions. The number of clean, germinated nodal explants was recorded after 4 weeks of cultivation. The following indicators were also calculated: days to bud sprouting, shoot number, shoot length, and leaf number per axillary shoot.

2.2. Culture Proliferation

Shoots that formed from axillary buds after four weeks of cultivation at the in vitro induction stage were transferred to a multiplication medium. The shoots were then cut into single-node explants, and the leaves were partially removed (Figure 1A).

The study investigated the possibility of growing *Hibiscus* on agar and liquid medium using a bioreactor. Both variants used MS base medium with the addition of 30 g L^{-1} sucrose, at pH 5.8, autoclaved at 121 °C for 15 min. Agar medium was prepared by adding 7 g L^{-1} of agar to the base medium. Each culture vessel containing 40 mL was then inoculated with 5 nodal segments.

A rocking bioreactor system was used, consisting of polyethylene boxes measuring 180 mm × 160 mm × 70 mm mounted on rocking platforms. Each box contained 160 mL of agar-free medium, and 25 nodal segments were transferred.

Culture boxes were placed in a growth room at 25 ± 2 °C in a 16 h photoperiod with PPFD 50 µmol m^{-2} s^{-1} using cold white fluorescent tubes (6500 K). The bioreactors operated in the following mode: flooding of shoots every 6 h and period of flooding of 15 min. The choice of the flooding mode was selected in accordance with the work published earlier [14,15].

Variations of medium composition with the addition of BAP (0.1–0.5 mg L^{-1}) and CPPU (0.05–0.2 mg L^{-1}) in three replicates were tested on both solid and liquid media. Each variation of the agar medium experiment included 5 culture vessels with 5 shoots

each and 1 culture box with 25 shoots each on a liquid medium. The following indicators were also calculated: culture proliferation, proliferated shoot number, microshoot length, number leaves on a proliferated shoot, and callus induction.

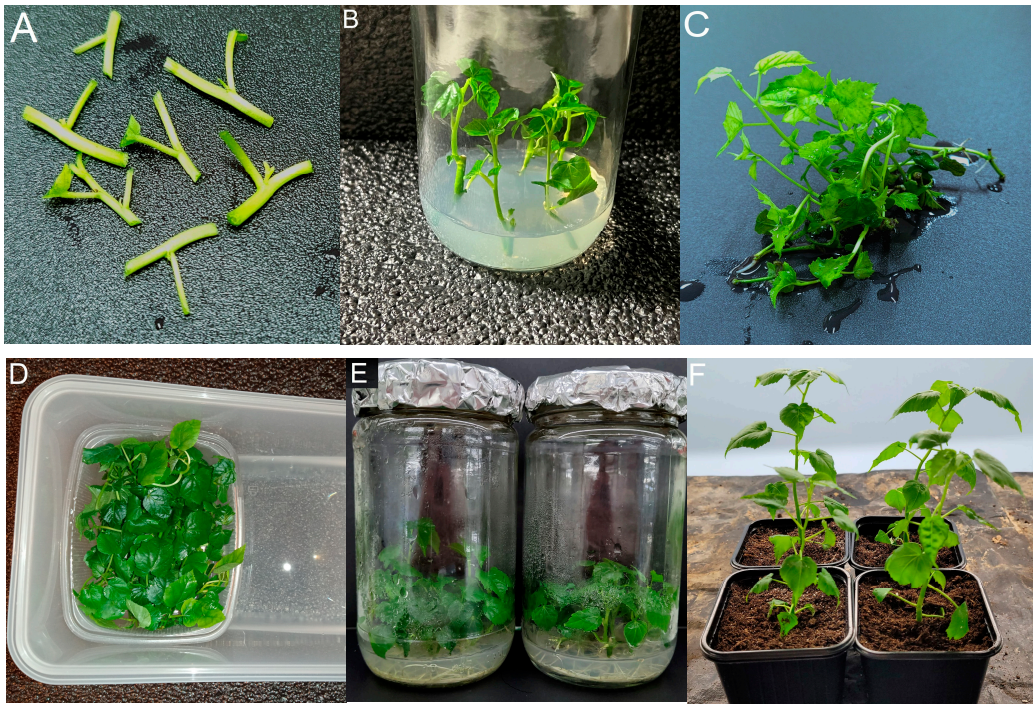


Figure 1. (**A**) Single-node explants for culture proliferation. (**B**) Axillary shoots on nodal explants on MS + 0.1 CPPU mg L^{-1}. (**C**,**D**) Proliferation and induction of shoots in a bioreactor after 4 weeks. (**E**) Rooting of shoots on MS + 0.1 IBA mg L^{-1}. (**F**) Adaptation of plantlets in peat–perlite mixture.

2.3. Rooting

Shoots that formed at the multiplication stage after 4 weeks of cultivation were transferred to the rooting medium. The possibility of rooting *Hibiscus* on an agar and liquid medium using a bioreactor was investigated. In both variants, MS base medium was used with the addition of 30 g L^{-1} sucrose, at pH of 5.8, with an autoclaving mode of 121 °C for 15 min.

The agar medium was prepared by adding 7 g L^{-1} of agar to the base medium. Then, the medium was poured into 250 mL culture vessels of 40 mL each. The 5 shoots were planted in each culture vessel.

Rooting was carried out on a liquid medium in rocking bioreactors (rocking bioreactor system) in polyethylene boxes measuring 180 mm × 160 mm × 70 mm. Then, 160 mL of agar-free medium was added to each box, and 25 shoots were transferred.

Culture boxes were placed in a growth room at 25 ± 2 °C in a 16 h photoperiod with PPFD 50 µmol m^{-2} s^{-1} using cold white fluorescent tubes (6500 K). The bioreactors operated in the following mode: flooding of shoots every 6 h and period of flooding of 15 min. The choice of the flooding mode was selected in accordance with [14,15].

Variants of the composition of the medium with the addition of auxins NAA (0.1–1.0 mg L^{-1}) and IBA (0.1–1.0 mg L^{-1}) in three repetitions were tested on both solid and liquid media. Plant growth regulators were purchased from Sigma-Aldrich, USA. Each variant of the experiment with an agar medium included 5 culture vessels with 5 shoots in each, while the liquid medium included 3 culture boxes with 25 shoots in each. After 4 weeks of cultivation, the following indicators were recorded: period of root formation, proportion of rooted shoots, number of roots per shoot, length of the roots, and proportion of shoots with callus induction.

2.4. Acclimatization

In vitro rooted seedlings obtained on solid and liquid media were thoroughly washed with distilled water from the remains of the medium and planted in 200 mL containers filled with a peat–perlite mixture (peat + perlite = 5:1). This modification of the peat–perlite mixture was chosen as the most versatile [18]. The containers were incubated for 20 days under inverted, transparent 5 L polypropylene containers, which acted as microparticles. Growth room conditions were maintained at 70% humidity, 25 ± 2 °C, 16 h photoperiod with PPFD 250 µmol m^{-2} s^{-1} using cold white fluorescent lamps (6500 K). Regenerants were sprayed with distilled water daily in the morning and evening. The microparticles were opened twice daily for 15 min during the first days of incubation; then, this time was gradually increased until they were completely open. After 4 weeks of ex vitro cultivation, the proportion of regenerates that successfully passed acclimatization was estimated by solid medium and liquid medium. Each variation of the experiment included 25 regenerants and was carried out in three replicates.

2.5. Statistics

In sterilization experiments, the indicator culture asepsis was calculated by dividing the number of uninfected explants, including dead ones, by the number of inoculated explants × 100. Explant survival was calculated by dividing the number of only living uninfected explants by the number of inoculated explants × 100. At the establishment stage of nodal explants, the indicator culture establishment was calculated by dividing the number of nodal segments on which the axillary buds were activated by the number of inoculated stem segments × 100. The number of days to bud sprouting was determined by subtracting the shoot number at the beginning of the development of the axillary bud from the number of new shoots formed on one explant taking into account the cases of adventive shoots. The shoot length was considered as the length of new shoots from the base to the apical bud, while the leaf number per shoot was the number of leaves formed on new shoots.

In the shoot proliferation experiments on agar medium and liquid medium, the culture proliferation index was calculated by dividing the number of nodal segments on which new shoots were formed by the number of inoculated stem segments × 100. The proliferated shoot number (proliferation coefficient) was calculated by dividing the number of formed axillary and adventitious shoots by the number of inoculated segments stem × 100. The shoot length was considered as the length of new shoots from the base to the apical bud, and the leaf number per proliferated shoot was the number of leaves per new shoot. Callus induction was calculated by dividing the number of shoots with signs of induction of callusogenesis at the base by the number of inoculated stem segments × 100. In experiments with rooting shoots on solid and liquid media, root initiation was calculated by the number of days before the appearance of the first root. Rooting was calculated by dividing the number of shoots with roots by the number of inoculated shoots × 100. Primary roots per shoot was calculated by dividing the number of primary roots by the number of inoculated shoots × 100. Root length was considered as the length of primary roots at the base of the root before its termination. Callus induction was calculated by dividing the number of shoots with signs of induction of callusogenesis at the base by the number of inoculated shoots × 100. In an experiment with acclimatization of regenerants obtained on solid and liquid media, plantlet survival during acclimatization was calculated by dividing the number of regenerants with signs of growth by the number of regenerants planted.

Completely randomized design with 3 replications was used for various experiments. Analysis of variance treatment was applied to all the data using Duncan' multiple range test. The data were analyzed using OPSTAT, a free online agriculture data analysis tool created by O.P. Sheoran, a computer programmer at CCS HAU, Hisar, India.

3. Results

3.1. Aseptic and Surviving Cultures

The sterilizing treatment had a significant impact on the asepsis of the culture. An increase in culture asepsis was noted when sterilizing agents were used in combination or when their concentration was increased (Table 1). The sterilization scheme in the E70S15 variant turned out to be the least effective. A total of 21.4% sterile explants were obtained, of which 21.2% survived. The E70SH5 and E70SH10 circuits performed slightly better with 33.4% and 37.8% of sterile explants, respectively, of which all explants survived. The best treatment result was shown by the E96H38W5 scheme, where the nodal explants were soaked for 5 min in a mixture of solutions of ethyl alcohol (96%), hydrogen peroxide (38%), and water in a ratio of 1:1:2. In this case, the maximum percentage of live and sterile explants was noted as 62.6%.

Table 1. Effect of different sterilization treatments on explant sterility and survival.

Treatment	Sterilant Used	Treatment Duration	Culture Asepsis (%)	Explant Survival (%)
E96H38W5	Solution of ethyl alcohol (96%), hydrogen peroxide (38%), and water in a ratio of 1:1:2	5 min	63.4 ± 1.25^{b}	62.6 ± 1.21^{a}
E96H38W10	The same as above	10 min	68.0 ± 1.14^{a}	50.0 ± 0.63^{b}
E70S10	Ethyl alcohol (70%) Mercuric chloride (1.0%)	30 s 10 min	44.4 ± 2.29^{c}	44.4 ± 1.63^{c}
E70S15	Ethyl alcohol (70%) Mercuric chloride (1.0%)	30 s 15 min	21.4 ± 0.98^{e}	21.2 ± 0.98^{e}
E70SH5	Ethyl alcohol (70%) Sodium hypochlorite (1.0%)	30 s 5 min	33.4 ± 0.68^{cd}	32.6 ± 0.51^{cd}
E70SH10	Ethyl alcohol (70%) Sodium hypochlorite (1.0%)	30 s 10 min	37.8 ± 0.37^{cd}	37.0 ± 0.32^{cd}

Data noted at the end of 4 weeks of culture on MS medium. Data are the mean of three replicates ± SE, each with 25 explants. Same superscript letters in a column do not differ significantly when compared by Duncan's MRT at a significance level of 5%.

3.2. Culture Establishment

Sterile explants were introduced into the culture on MS medium without the addition of phytohormones and with the addition of BAP and CPPU separately (Table 2). As a result of the experiment, the optimal variant of phytohormone concentration for the introduction of *Hibiscus* into the culture in vitro was found. The successful introduction of nodal segments of *Hibiscus* was primarily evaluated by the proportion of surviving explants (%) in the shortest time after introduction on the medium (days of bud sprouting). Indicators such as shoot number, shoot length (cm), and leaf number per shoot had a clarifying character and helped to identify the most optimal concentration of the required hormones. The maximum values of these indicators were signs of the most successful concentrations.

Thus, the most optimal variant of the experiment turned out to be a medium with the addition of 0.1 mg L^{-1} CPPU (Figure 1B), which resulted in 73.3% of axillary buds being induced. The addition of this hormone at a concentration of 0.2 mg L^{-1} at the injection stage did not lead to a significant increase in indicators. On the medium with BAP, the observed indicators were significantly lower. The most successful option with BAP was the medium supplemented with 0.1 mg L^{-1}. At the same time, induction of axillary shoots was observed in 67.2% of nodal segments. The activity of axillary buds on media with other concentrations of BAP was even lower.

Table 2. Establishment of nodal explants under different growth regulator treatments and medium composition after 4 weeks of culture.

Growth Regulators	Concentration (mg L^{-1})	Culture Establishment (%)	Days to Bud Sprouting	Shoot Number	Shoot Length (cm)	Leaf Number/ Shoot
Free	0	48.9 ± 0.41^{e}	13.8 ± 0.49^{a}	1.2 ± 0.20^{b}	1.7 ± 0.12^{c}	1.5 ± 0.08^{c}
BAP	0.1	67.2 ± 0.74^{bc}	11.2 ± 0.20^{b}	1.2 ± 0.20^{b}	2.1 ± 0.13^{b}	1.6 ± 0.09^{c}
BAP	0.25	52.5 ± 1.04^{d}	13.2 ± 0.20^{a}	1.4 ± 0.24^{ab}	1.9 ± 0.12^{b}	2.5 ± 0.14^{b}
BAP	0.5	52.5 ± 1.35^{d}	13.2 ± 0.20^{a}	1.5 ± 0.24^{a}	1.7 ± 0.07^{c}	2.8 ± 0.06^{ab}
CPPU	0.05	69.9 ± 0.42^{bc}	10.6 ± 0.40^{b}	1.6 ± 0.24^{a}	2.1 ± 0.04^{b}	3.1 ± 0.1^{ab}
CPPU	0.1	73.3 ± 1.05^{a}	11 ± 0.32^{b}	1.6 ± 0.24^{a}	2.4 ± 0.12^{a}	3.3 ± 0.13^{a}
CPPU	0.2	73.1 ± 1.24^{a}	11.3 ± 0.20^{b}	1.6 ± 0.24^{a}	2.3 ± 0.07^{a}	3.5 ± 0.10^{a}

Data noted at the end of 4 weeks of culture on MS medium. Data are the mean of three replicates ± SE, each with 25 explants. Same superscript letters in a column do not differ significantly when compared by Duncan's MRT at a significance level of 5%.

3.3. Culture Proliferation in Solid Media and in Bioreactors

3.3.1. Culture Proliferation in Solid Media

At this stage, the nodal segments of axillary shoots were planted on a solid medium with different concentrations of phytohormones (Table 3). The highest proliferation value (95.2%) and proliferation coefficient (6.2) were obtained on the medium with a CPPU concentration of 0.2 mg L^{-1}. The largest number of leaves on new shoots (5.2) were recorded on media with CPPU concentration of 0.05 mg L^{-1} and CPU concentration of 0.2 mg L^{-1}. In most cases, callus formed at the base of the shoots. The highest percentage of callus formation (15.2%) was observed at CPPU concentration of 0.2 mg L^{-1}. On the medium with BAP, the most acceptable option was the one with a concentration of 0.5 mg L^{-1}, where the proliferation value was 82% and the proliferation coefficient was 4.4. Variants of the medium with other concentrations showed lower results.

Table 3. Shoot proliferation of nodal segments under various growth regulator treatments on solid medium.

Growth Regulators	Concentration (mgL^{-1})	Culture Proliferation (%)	Proliferated Shoot Number	Shoot Length (cm)	Leaves/ Proliferated Shoot	Callus Induction (%)
free	0	44.0 ± 4.30^{d}	1.9 ± 0.19^{e}	2.4 ± 0.14^{d}	2.6 ± 0.24^{e}	0
BAP	0.1	49.0 ± 3.33^{c}	1.8 ± 0.12^{e}	3.2 ± 0.08^{b}	3.0 ± 0.32^{d}	3.0 ± 0.37^{b}
BAP	0.25	80.0 ± 3.53^{bc}	3.8 ± 0.37^{d}	3.0 ± 0.08^{b}	3.6 ± 0.24^{c}	3.0 ± 0.37^{b}
BAP	0.5	82.0 ± 1.22^{b}	4.4 ± 0.51^{c}	2.8 ± 3.32^{c}	3.8 ± 0.37^{c}	5.0 ± 0.72^{b}
CPPU	0.05	84.0 ± 1.87^{b}	4.8 ± 0.37^{c}	3.2 ± 0.11^{ab}	5.2 ± 0.37^{a}	0
CPPU	0.1	95.0 ± 3.32^{a}	5.8 ± 0.37^{b}	3.6 ± 0.14^{a}	4.8 ± 0.37^{b}	15.0 ± 0.40^{a}
CPPU	0.2	95.2 ± 2.32^{a}	6.2 ± 0.12^{a}	3.4 ± 0.08^{a}	5.2 ± 0.37^{a}	15.2 ± 1.20^{a}

Data noted at the end of 4 weeks of culture on MS medium. Data are the mean of three replicates ± SE, each with 25 explants. Same superscript letters in a column do not differ significantly when compared by Duncan's MRT at a significance level of 5%.

3.3.2. Culture proliferation in Bioreactors

In a liquid medium, nodal segments developed more successfully than in a solid medium. The variant containing CPPU at a concentration of 0.05 mg L^{-1} demonstrated the most efficient propagation of shoots in the bioreactor (Figure 1C,D). The proliferation value was 95.2%, and the proliferation coefficient was 9.2. Callusogenesis was not observed in this variant (Table 4). A higher concentration of CPPU (0.1 mg L^{-1}) influenced the increase in the proliferation value (98.5%) and multiplication factor (9.5); however, in this variant, shoots with signs of callus formation were found (7.1%). Media with the addition of BAP also gave high results. A medium with BAP 0.5 provided a proliferation value of 86.0% and a proliferation coefficient of 8.3; however, in this case, induction of callus formation was also observed (5.0%).

Table 4. Shoot proliferation of nodal segments under various growth regulator treatments in liquid medium by a bioreactor.

Growth Regulators	Concentration (mg L^{-1})	Culture Proliferation(%)	Proliferated Shoot Number	Shoot Length (cm)	Leaves/Proliferated Shoot	Callus Induction (%)
Free	0	56.0 ± 4.3^{e}	3.1 ± 0.27^{d}	3.7 ± 0.11^{e}	3.5 ± 0.62^{e}	0
BAP	0.1	71.0 ± 3.33^{d}	6.0 ± 0.13^{c}	4.1 ± 0.10^{c}	4.1 ± 0.32^{d}	0
BAP	0.25	85.1 ± 3.53^{c}	7.8 ± 1.23^{b}	4.2 ± 0.23^{c}	4.6 ± 0.66^{c}	5.0 ± 0.15^{c}
BAP	0.5	86.0 ± 1.22^{c}	8.3 ± 0.32^{b}	4.8 ± 1.80^{bc}	4.9 ± 0.77^{c}	5.0 ± 0.70^{c}
CPPU	0.05	95.2 ± 1.87^{b}	9.2 ± 0.12^{b}	5.3 ± 0.20^{b}	5.8 ± 0.32^{b}	0
CPPU	0.1	98.5 ± 3.32^{a}	9.5 ± 0.47^{b}	5.6 ± 0.31^{a}	5.8 ± 0.11^{b}	7.1 ± 0.30^{b}
CPPU	0.2	98.1 ± 2.32^{a}	10.1 ± 0.3^{a}	5.8 ± 0.11^{a}	6.3 ± 0.43^{a}	9.4 ± 2.10^{a}

Data noted at the end of 4 weeks of culture on MS medium. Data are the mean of three replicates ± SE, each with 25 explants. Same superscript letters in a column do not differ significantly when compared by Duncan's MRT at a significance level of 5%.

3.4. Rooting and Acclimatization

3.4.1. On a Solid Medium

New shoots obtained during the propagation process needed to be rooted. Growth regulators from the auxin group, NAA and IBA, were used for the development of adventitious roots on the shoots. In addition, the control version of the experiment used a hormone-free environment. The most successful rooting occurred on a solid medium containing 0.1 mg L^{-1} IBA (Figure 1E). The beginning of rooting was already recorded on the 15th day. At the same time, 99.0% of the shoots formed roots, and an average of 4.2 primary roots were formed on one shoot. On this medium, a minimal proportion (4.5%) of shoots with callus at the base was recorded (Table 5). On the medium with the addition of NAA, root formation was less successful. A rather intense process of callus formation (29.0%) was observed despite the high values of root formation (up to 96.0%) on a medium containing 0.1 mg L^{-1} NAA. On the other hand, a medium without hormones did not promote root formation, with 87% of rooted shoots observed on average on day 22.

Table 5. Rooting of in vitro raised shoots with different types of auxins on solid media.

Growth Regulators	Concentration (mg L^{-1})	Root Initiation (day)	Rooting (%)	Primary Roots/ Shoot	Root Length (mm)	Callus Induction (%)	Plantlet Survival during Acclimatization (%)
free	0	22.4 ± 1.20^{e}	87.0 ± 2.00^{d}	2.0 ± 0.31^{d}	31.2 ± 1.24^{b}	1.0 ± 1.1^{e}	86.2 ± 0.46^{b}
NAA	0.1	15.6 ± 0.40^{b}	98.0 ± 1.30^{a}	3.8 ± 0.37^{b}	43.4 ± 3.14^{a}	10.4 ± 1.58^{c}	78.3 ± 1.23^{d}
NAA	0.5	16.0 ± 0.63^{a}	91.0 ± 2.45^{c}	3.2 ± 0.37^{c}	32.0 ± 2.55^{b}	23.0 ± 2.55^{b}	83.1 ± 0.82^{c}
NAA	1.0	15.2 ± 0.37^{c}	96.0 ± 1.87^{b}	2.2 ± 0.58^{d}	17.6 ± 1.12^{d}	29.0 ± 1.10^{a}	84.7 ± 0.90^{c}
IBA	0.1	15.2 ± 0.37^{c}	99.0 ± 1.00^{a}	4.2 ± 0.37^{a}	44.0 ± 3.67^{a}	4.5 ± 0.63^{d}	88.7 ± 0.37^{a}
IBA	0.5	14.8 ± 0.37^{cd}	98.0 ± 1.22^{a}	3.6 ± 0.51^{b}	41.8 ± 1.93^{ab}	8.8 ± 1.24^{cd}	89.1 ± 1.26^{a}
IBA	1.0	14.6 ± 0.40^{cd}	98.0 ± 1.22^{a}	3.0 ± 0.44^{c}	24.0 ± 1.05^{c}	11.8 ± 0.92^{c}	90.3 ± 1.12^{a}

Data on in vitro rooting were noted at the end of 4 weeks of cultivation on MS medium. Acclimatization data were noted at the end of 4 weeks of cultivation on a mixture of substrates (peat + perlite = 5:1). The data are the average value of three repeats ± SE, each with 25 shoots. The same superscript letters in the column do not differ significantly when compared using Duncan's MRT at a significance level of 5%.

Rooted regenerants adapted to ex vitro conditions. A mixture of peat and perlite (1:5) was used as a substrate for adaptation. As a result of adaptation, the maximum plant survival rate was 88.7%, which was obtained on a medium containing 0.1 mg L^{-1} IBA. Acclimatization of rooted shoots that were obtained on a medium with NAA was worse. The maximum number of plantlets underwent adaptation with an NAA concentration of 1.0 mg L^{-1} (84.7%). In a medium without hormones, 86.2% of rooted shoots underwent adaptation.

3.4.2. On Bioreactors

Rooting in bioreactors also showed the best results in the variant with 0.1 mg L^{-1} IBA. The proportion of rooted shoots was 99.0%, and the average number of primary roots per shoot was 4.8; however, unlike the variant on a solid medium, no callus was found. The variant with 0.1 mg L^{-1} NAA showed high rooting results. In this case, the proportion of rooted shoots was 98.0%, and the average number of primary roots per shoot was 3.7. Callusogenesis was also not noted here. Increasing NAA concentrations induced callusogenesis. It should be noted that under bioreactor conditions, rooting on a medium without phytohormones also gave an acceptable result. The proportion of rooted shoots was 90.0%, the average number of primary roots per shoot was 2.5, and callusogenesis was not recorded (Table 6).

Table 6. Rooting of in vitro raised shoots with different types of auxins in bioreactors.

Growth Regulators	Concentration (mg L^{-1})	Root Initiation (Day)	Rooting (%)	Primary Roots/ Shoot	Root Length (mm)	Callus Induction (%)	Plantlet Survival during Acclimatization (%)
free	-	13.3 ± 1.90^{a}	90.0 ± 1.80^{c}	2.5 ± 2.333^{d}	36.1 ± 0.92^{e}	0	90.4 ± 1.65^{e}
NAA	0.1	12.1 ± 1.50^{b}	98.0 ± 2.10^{c}	3.7 ± 0.21^{bc}	45.1 ± 2.14^{c}	0	97.3 ± 1.49^{b}
NAA	0.5	12.5 ± 2.43^{bc}	98.0 ± 0.49^{a}	3.2 ± 0.29^{c}	43.1 ± 2.76^{d}	15.0 ± 1.85^{b}	98.1 ± 0.89^{a}
NAA	1.0	12.7 ± 1.77^{c}	99.0 ± 2.79^{d}	3.1 ± 0.10^{c}	42.9 ± 2.39^{d}	20.0 ± 1.15^{a}	95.6 ± 1.7^{c}
IBA	0.1	12.3 ± 0.72^{b}	99.0 ± 1.30^{b}	4.8 ± 0.49^{a}	49.0 ± 2.83^{a}	0	98.7 ± 0.27^{a}
IBA	0.5	11.3 ± 0.20^{d}	99.0 ± 0.59^{a}	4.2 ± 0.66^{b}	47.1 ± 1.44^{b}	0	98.7 ± 0.48^{a}
IBA	1.0	11.3 ± 0.10^{d}	99.0 ± 2.93^{d}	4.4 ± 0.29^{b}	45.2 ± 0.69^{c}	10.1 ± 0.71^{c}	97.8 ± 2.12^{ab}

Data on in vitro rooting were noted at the end of 4 weeks of cultivation on MS medium. Acclimatization data were noted at the end of 4 weeks of cultivation on a mixture of substrates (peat + perlite = 5:1). The data are the average value of three repeats ± SE, each with 25 shoots. The same superscript letters in the column do not differ significantly when compared using Duncan's MRT at a significance level of 5.0%.

After cultivation in a bioreactor, the plantlets successfully adapt to in vitro conditions in a peat–perlite mixture (1:5). The best result was shown by the medium with 0.1 mg L^{-1} IBA, where the proportion of shoots that had undergone adaptation was 98.7% (Figure 1F). Plantlets obtained on media with NAA also gave good results. On a medium with 0.1 mg L^{-1} NAA, the proportion of shoots that underwent adaptation was 97.3%. On the medium without phytohormones, the proportion of adapted plantlets reached 90.4%, which was slightly higher than in the case of the solid medium without phytohormones.

4. Discussion

The sterilization protocol is the first step in determining the success of micropropagation [19]. Exogenous as well as endogenous bacterial contamination remains a major challenge in plant tissue cultures. The explants are initially surface sterilized to remove most of the exogenous contaminants. Some organisms remain internally in the living tissues [20]. Nodal segments of *Hibiscus* subjected to surface sterilization are fragments of herbaceous shoots of the current year. They have an underdeveloped cuticle layer and a thin epidermis. Additional requirements require sterilization of the softest tissues [21]. In this work, a number of sterilizing agents were prepared from mercury chloride, as a substitute for a hard sterilizer, and a milder solution of ethyl alcohol (96.0%), hydrogen peroxide (38.0%), and water in a ratio of 1:1:2. [22,23].

Sterilization with mercury chloride (0.1%) in a two-stage treatment process did not provide a high percentage of sterile explants, although all surviving explants turned out to be sterile. This suggests that mercury chloride successfully copes with microorganisms. However, the relatively low percentage of surviving explants indicates the high toxicity of this sterilizer [24]. Treatment with sodium hypochlorite solution (1.0%) also did not provide a large number of sterile explants. In this case, a relatively low percentage of sterile and surviving microbeads was recorded. Chlorine-containing drugs often exhibit

phytotoxicity [25]. The maximum effect of sterilization was achieved with the use of hydrogen peroxide in the E96H38W5 variant. This sterilizer has the least phytotoxicity, unlike other drugs, and therefore provides the largest number of sterile and surviving explants. In other works, the MS medium or DKW medium were used at all stages of micropropagation for *H. moscheutos* [3,26]. At the multiplication stage, cytokinins such as BAP, KIN, and TDZ were used to activate the axillary and adventitious buds [27,28]. In order to reduce the cost of multiplication, we used the most common MS medium in combination with the hormones BAP or CPPU. CPPU has been quite successfully used at the stage of multiplication of *H. rosa-sinensis* [10] and other crops [29,30].

The micropropagation of *Hibiscus* species and varieties occurs mainly as a result of the laying of axillary and adventitious buds, which give rise to shoots [3,13]. At the stages of initiation and multiplication of shoots, we were guided by the following principles. For in vitro propagation, concentrations of nutrient medium components are optimal if they provide a sufficiently high reproduction coefficient in one passage, minimal tissue dedifferentiation, and secondary formation of shoots from it. From several values of concentrations of growth hormones that provide a high propagation rate, the smallest was chosen. When using relatively low concentrations of cytokinins, callus formation is minimized and vitrification of regenerants is not observed on such media, which is especially important for the stages of rooting and adaptation to nonsterile conditions [18]. Based on the above, the best option for the media at the stage of culture establishment was the option with 0.1 mg L^{-1} CPPU. Shoot proliferation, following the same principles, was best performed on a solid medium with 0.1 mg L^{-1} CPPU.

At the stage of induction of the axillary buds, we received a certain number of shoots. We used them for further propagation of *Hibiscus* in a bioreactor. In the literature, information on the cultivation of *Hibiscus* in TIS systems is not found. But other crops have been successfully grown by this method and, as a rule, with higher productivity than on a solid medium [31,32]. In most cases, the propagation of plants in TIS is carried out by intensive formation of axillary and adventitious shoots [15,33]. The shoot proliferation of *Hibiscus* depends on the composition of the nutrient medium and the concentration of cytokinins [12]. For the purpose of comparison, the same nutrient media were used in the bioreactor as in the case of agar. It was found that *Hibiscus* showed better growth results in the variant with 0.05 mg L^{-1} CPPU compared to other variants in TIS, and the results also surpassed all variants of the experiment on a solid medium.

When cultivating plants in TIS, it is necessary to take into account the period and frequency of explant flooding. We chose a mode suitable for a large number of crops grown in TIS, i.e., 4-fold immersion per day, every 6 h. The time of each flooding was 15 min. More frequent flooding usually leads to vitrification of shoots and then to difficult adaptation [34]. Rhizogenesis is the final stage of micropropagation of *Hibiscus* in vitro. At this stage, it is necessary to induce the formation of subordinate roots. *Hibiscus moscheutos* forms adventitious roots quite easily under the action of exogenous auxins or without them, which has been repeatedly noted in the literature [3]. In our study, adventitious roots were formed in all variants of the experiment. However, the presence of roots does not always lead to successful results of further adaptation [18]. Under the influence of certain concentrations of auxins, rhizogenesis is accompanied by the formation of a callus, which further complicates or slows down the adaptation ex vitro.

At the same time, it was found that in the absence of auxins, fewer roots were formed over a longer period than in the case of auxins. Thus, the variant with 0.1 mg L^{-1} IBA turned out to be optimal, where the maximum number of adventitious roots was fixed in the absence of callus formation. Plants rooted in this medium showed the highest rates of adaptation to ex vitro conditions. Up to 98.0% of the plants had adapted, which were then transferred to the open ground.

5. Conclusions

In this study, a protocol for mass propagation of *Hibiscus moscheutos* L. 'Berry Awesome' on solid and liquid media for in vitro cultivation was successfully developed. Cultivation on a liquid medium was carried out in a rocker-type bioreactor and showed better reproduction results than on a solid medium. The reproduction coefficient under bioreactor conditions was 9.2 shoots per planted shoot, while this indicator was 5.8 on a solid medium. Shoot rooting was good on both types of media. However, rooting in a bioreactor was less complicated and ensured the production of more viable seedlings. The acclimatization of regenerating plants was successfully carried out in a peat–perlite mixture.

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