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Research Article

Optimization of *In Vitro* Microcloning of *Lagochilus inebrians* Bunge

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Abstract
In the following years, introduction (*in situ*) of rare and promising medicinal plants, determination of morphological and ecological adaptability to selected soil and climate conditions, and obtaining pathogen-free seedlings through microclonal (*in vitro*) reproduction in areas affected by environmental stress factors creation of plantations are gaining importance. This research aimed at choosing the optimal conditions for obtaining pathogen-free seedlings of *Lagochilus inebrians in vitro* and acclimatization in arid conditions. The optimal amounts of added phytohormones were chosen to increase the efficiency of the nutrient media used in the microconal propagation of *L. inebrians*, based on the possibility of acclimatization in arid conditions. The obtained results serve to multiply *L. inebrians in vitro* and create plantations in arid regions based on the obtained seedlings.

Keywords: *Lagochilus inebrians*, explant, culture, phytohormones, in vitro, microclonal propagation, drought, ex vitro

1. INTRODUCTION

About 500,000 species of higher plants are known in the world. The medicinal properties have been determined in 5% of them. Today, 60% of pharmacological preparations are obtained from medicinal plants [1]. However, the raw material of medicinal plants is insufficient or their number is sharply reduced due to unplanned harvesting from nature [2]. Recently, breeding, preservation and use of medicinal plants on the basis of biotechnological methods are being considered as promising directions [3]. Accordingly, in order to supply the pharmaceutical industry with raw materials of medicinal plants, development of ways of their cultivation is one of the urgent issues. In this regard, making recommendations for breeding and production of medicinal plants, including *Lagochilus inebrians* Bunge, is of great scientific and practical importance.

L. inebrians is an intoxicating plant locally known as Bozulbang which grows in hot climates [4]. According to its life form, it is considered a semi-shrub. This unique type of plant grows in the foothills, in the mountains, on gravel and river tributaries, sometimes on the banks of canals and ditches, mainly in Samarkand, Jizzakh, Navoi, Surkhandarya regions of Uzbekistan, and distributed in some other republics of Tajikistan and Turkmenistan in Central Asia [5]. *L. inebrians* is an annual plant with a main root reaching 20–60 cm in height. The length of the main root is 25–35 cm. The stem is spiky, ascending, the base is rounded, four-sided, covered with hard glandular hairs. The leaf is simple, divided into three to five parts, arranged oppositely on the stem and branches. The length of green leaves is 2–3 cm. The flowers are reddish in color and are arranged in a semi-ring on the stem and branches. The fruit is 4 brown nuts and blooms in June-September. The time to harvest the fruits of the *L. inebrians* plant is July and August [6]. By studying the ontogeny of this plant in the conditions of Uzbekistan, it was determined that its lifespan is at least 25 years. Self-preservation of senororulations occurs only with seeds. A comparison of the ontogenetic structure of senororulations of *L. inebrians* in different ecological and phytocenotic living conditions showed that all senororulations secretory gives the highest result in middle-aged generative individuals. Its leaves and flowers are mainly collected by the local population [7]. In its natural habitat, *L. inebrians* grows only with seeds. The seeds germinate in mid-February to early March. Germination of seedlings does not exceed 26% [8].

Table 1. The effect of nutrient medium components on the recovery of shoots of *L. Inebrians*.

Components	Substance concentration (mg/L)		
	Introducing explants into *in vitro* conditions (MS1)	Multiplication (MS2)	Rooting (MS3)
NH_4NO_3	1650	1650	825
Kinetin ($C_{10}H_9N_5O$)	1900	1900	950
KH_2PO_4	170	170	85
$CaCl_2 \cdot 2H_2O$	340	340	170
$MgSO_4 \cdot 7H_2O$	370	370	185
$MnSO_4 \cdot 5H_2O$	22.3	22.3	11.2
H_3BO_3	6.2	6.2	3.1
$ZnSO_4 \cdot 7H_20$	8.6	8.6	4.3
KI	0.83	0.83	0.42
$CuSO_4 \cdot 5H_20$	0.025	0.025	0.013
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.13
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.013
$FeSO_4 \cdot 7H_2O$	27.8	27.8	13.9
$Na_2EDTA \cdot 2H_2O$	37.2	37.2	18.6
Inositol	100	100	50
Carantothenate	0.5	0.5	0.25
Nicotinic acid	0.5	0.5	0.25
Ryridoxine solution	0.1	0.1	0.05
Thiamine solution	2	2	1
Casein hydrolysate	-	-	-
Sucrose	30000	30000	10000
Agar “Difco”	6000	6000	6000
pH	5.8	5.8	5.8

The chemical composition of *L. inebrians* plant contains vitamin K, 0.60–1.97% lagoxilin, 0.67% flavonoid glycosides, 44–77 mg% ascorbic acid, 6–7% organic acids, 5–10 mg% carotene. It contains 9.66–12.42% tar, 2.58–2.78% slag and other substances, as well as calcium and iron salts. *L. inebrians* leaf contains lagoxilin, 0.03% essential oil, 11–14% digestive substances, organic acids, 7–10 mg% carotene, and 77–100 mg% vitamin C. From the tissues of *L. inebrians*, the physiologically active compound diterrenoid, tetrahydric acid - lagoxilin and its acetyl derivatives, which are very poorly soluble in water, are isolated and are used as the most important source in chemistry and pharmacology [9]. Based on the analysis of the above data, a promising medicinal plant was distributed in the territory of Uzbekistan is *L. inebrians* Bunge. The main goal of our research is to develop the biotechnology of microclonal propagation, preservation, and pathogen-free seedlings *in vitro*.

2. MATERIALS AND METHODS

2.1. Cultivation of Donor Plants

In the studies, the mother plant *L. inebrians* was grown in greenhouses under protected conditions. The collection of planting material was carried out according to standard technology for growing flower crops [10]. The medium selected for growing *L. inebrians* seedlings should have a saturated balanced nutrient composition [11]. The

Table 2. The effect of nutrient medium components on the recovery of shoots of *L. inebrians*.

Option	Concentration of growth regulators (mg/L)	
	BAP	NAA
1	0.5	0.01
2	1.0	0.10
3	1.5	0.10

Table 3. Regenerative performance of *L. inebrians*.

Variant	B_5		B_5+ 5.0 mkM BAP+2.0 mkM NSK		BDS+5.0 mkM BAP+2.0 mkM NSK	
	Regeneration rate (%)	Number of buds (pieces/ex.)	Regeneration rate (%)	Number of buds (pieces/ex.)	Regeneration rate (%)	Number of buds (pieces/ex.)
L. inebrians (greenhouse cultivation)	40	3.0±0.1	50	3.0±0.5	60	3.4±0.6
L. inebrians (field conditions)	60	2.0±0.5	70	2.5±0.3	-	-

substrate contained peat particles of 0–5 mm, dry matter was at least 10% of the substrate volume. The moisture capacity was 78–82%, the air capacity was 8–12%, and the pH was 5.5. To improve the structural properties, 15% of rellite was added to the peat [12][13]. In order to ensure rapid rooting of the rhizome and maintain high humidity, daily watering of the seedlings was carried out [14]-[16]. The rooted plant was planted in a larger container (0.7–1.0 L) with the same amount of substrate and nutrients as in the rooting stage, creating a ratio of N:P:K = 140:160:180 mg/L. During the studies, the air temperature was maintained between 16 and 18 °C for the first 2–4 weeks, then the temperature was lowered from 8 to 10 °C to complete the vernalization period, which lasted 3–6 weeks. During the cold season, regular but intermittent watering and periodic fertilization were carried out once a week with a complex fertilizer containing the main elements in the N:P:K ratio of 250:300:350. At the end of the vegetative period, the plants were treated once with a stimulant in a ratio of 10:1 in 15 mL to form a bush. The air temperature was gradually increased from 16 to 18 °C over 12–14 days. The temperature for growing the donor plants was then increased from 18 to 22 ° C. When the temperature reached the maximum value, the plants were illuminated with 9-10 KLK (120 W/m^2) lamps for 8–10 h/day for 14 days. During the warm season, it was observed that the number of upper crusts increased twice a week.

2.2. Sterilization of Nutrient Medium and Planting Materials

In accordance with the current recommendations, aseptic conditions were used to sterilize nutrient medium and planting materials. Murashige and Skoog (MS) nutrient medium used in *in vitro* culture was autoclaved twice for 15 min at 0.75 atm pressure and 117°C. The initial explants for *in vitro* culture were sterilized in the following sterilization media: 70% ethanol; 0.5, 1, 2% of calcium hydroxide solution with an exposure time of 5 to 15 min. Stages of sterilization of plant material to obtain sterile explants: 1) cleaning the plant from various contaminants; 2) holding in a soap solution for 30 min; 3) rinsing with running water; 4) sterilization with 70% ethanol for 5 min; 5) sterilization with 0.5, 1, 2% calcium hypochlorite

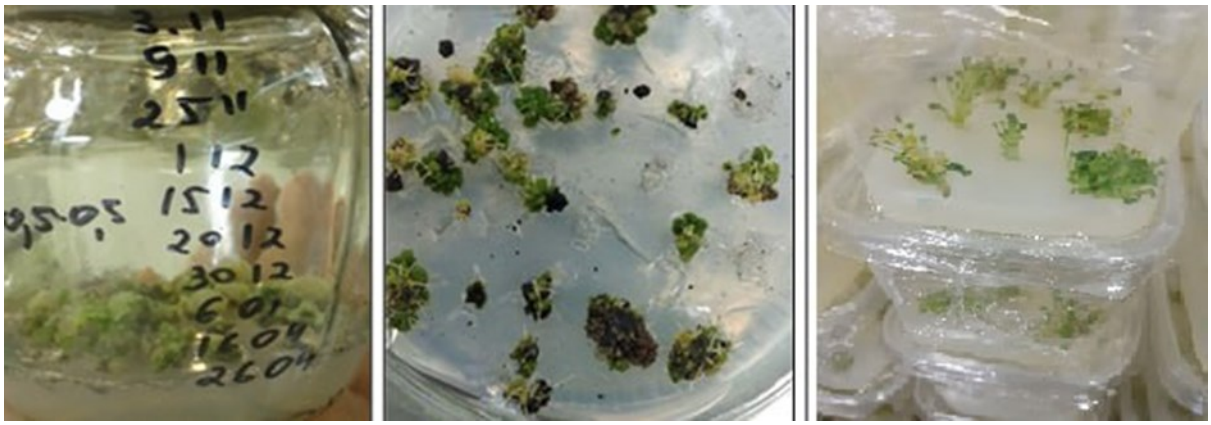

Figure 1. Regeneration of *L. inebrians* shoots on primary explant surface in BDS medium supplemented with 5.0 µM BAP and 2.0 µM NAA (47 days old).

solutions for 7–10 min; and 6) re-washing with distilled water 3–5 times.

2.3. Selection of Nutrient Medium and Growth Regulators for In vitro Propagation of L. inebrians Explants

The optimal time for grafting from donor plants was during the period of active growth. Plant sample explants were grown on MS medium (sucrose 30 g/L, agar 6 g/L; BAP 2.0 mg/L, NAC 0.1 mg/L). Morphogenetic activity was assessed by the number of explants formed during the observation period (17–21 days). The composition of the modified MS medium was changed stepwise during the studies (Table 1).

In vitro cultivation of explants was carried out in MS nutrient media with two different growth regulators: BAP diazonium from 2 to 6 mg/L and NSK from 0.01 to 0.2 mg/L. In the cultivation of explants, we used different ratios of BAR and NAA concentrations as growth regulators (Table 2).

In addition, the effect of different concentrations of BAP, kinetin and thidiazuron (TDZ) (0.1 and 0.5 µM) on the viability of cultures, shoot regeneration and stem number increase was also studied. At the initial stage of *in vitro* cultivation, cultures were fed with an agar medium supplemented with 5.0 µM BAP and 2.0 µM NAA according to the BDS and B5 recipe in addition to MS. *In vitro* culture of explants, ascorbic acid medium solution was used to reduce the negative effects of phenolic compounds released during the cultivation process [17]-[19]. After pretreatment, sterile axillary buds were dissected into 1 mm samples in a Retry dish under laminar flow box conditions. The isolated explants were transferred to a nutrient medium in 1 cm diameter flasks for *in vitro* cultivation [20].

2.4. Microcloning and Rooting of Explants

Microcloning was carried out in several stages. The rhizomes grown from the flasks were transferred to a nutrient medium with a reduced concentration of growth regulators in laboratory flasks for propagation. One transplant was carried out for 27–38 days. The living environment was the same as that in the cultivation of explants. Subsequently, the conglomerates that had grown and sprouted were divided into more sprouts under sterile conditions and planted for rooting. For rooting, shoots with 3–4 leaves, 1.5–2.0 cm long, were cut from the base and placed in a ½ MS hormone-free medium with a 2-fold reduced nutrient concentration and a fixed amount of sucrose (10 g/L). In the studied samples, the rooting indicators, average rooting frequency, 100% rooting frequency of plants planted for rooting or rooting in hormonal and non-hormonal media were observed, and in the presence of auxins in various concentrations - NAA, IAA, IBA - on ½ BDS and ½ MS mineral bases. The most effective concentration of the agent for stimulating rhizogenesis in *L. inebrians* was determined. Root formation began on average after 14 days. Rooted regenerated plants were ready for planting *in vitro* in 21–28 days. To gradually acclimate the regenerated plants to harsh *in vitro* conditions with high humidity and high infectious background, a small hole was drilled in the tops of the flasks,

Table 4. Effect of nutrient medium mineral content and growth regulators on regeneration of *L. inebrians* buds in *in vitro* cultures.

Growth regulators	Mineral base			
	BDS		B_5	
	Regeneration rate (%)	Random shoots (pcs./exr.)	Regeneration rate (%)	Random shoots (pcs./exr.)
Control (hormone-free medium)	33.3	2.5±0.3	52.9	1.6±0.3
BAP 0.1	-	-	25.7	1.8±0.3
BAP 0.5	-	-	67.0	1.8±0.3
BAP 5.0	45.8	2.1±0.3	37.5	2.0±0.9
BAP 5.0+NAA 2.0	57.9	2.3±0.5	36.6	2.4±0.6
BAP 5.0+NAA 5.0	-	-	16.1	2.1±0.3
TDZ 0.1	-	-	38.5	1.5±0.3
TDZ 0.5	18.2	1.8±0.5	29.0	2.3±0.3
TDZ 5.0	-	-	8.0	1.7±0.4
TDZ 5.0+NAA 2.0	-	-	78.6	2.0±0.6
Kinetin 0.5	21.4	1.6±0.5	-	-
BAP 5.0+IAA 5.0	57.9	2.8±0.9	-	-
BAP 0.4+NAA 3.2+IAA 2.3	-	-	80.0	2.1±0.5

Note: $p \leq 0.05$; "-" – no information

which was gradually enlarged over 3–5 days, and then the tops were completely removed and discarded. For 7–14 days, the plants were grown in pre-sterilized, moistened peat granules, where the living environment was maintained at a temperature of 23±1 °C and 100% humidity. Starting from the beginning of intensive growth, the regenerants were placed in normal conditions. The period from planting the explant to receiving the adapted regenerants was an average of 75 days.

3. RESULTS AND DISCUSSIONS

3.1. Getting Input Materials

In the initial stage of in vitro culture, agar nutrient medium supplemented with 2.0 µM NAA and 5.0 µM BAP was used for the cultures of BDS and B5 resert. The use of nutrient media containing plant growth regulators induced a regeneration process in the tissues of *L. inebrians* primary explants compared to media without control hormones [21]. In this study, *L. inebrians* segments were found to have the least regenerative performance among other species studied (Table 3).

The samples growing in field conditions, after 51 –56 days, the first changes on the surface of the buds, i.e., a slight growth of excretory tissues, were noted. In addition, it was noted that the regeneration of random shoots from the appearance of the tumor on the surface of the explant to the formation of primary vegetative organs lasted 12–17 days on average [22]. It should be noted that the development of callus tissue did not occur during the initial stages of cultures *in vitro*. *L. inebrians* bud regeneration proceeded directly through the process of organogenesis. Regeneration of buds was noted in the intact part of the tissue above the surface of the nutrient medium, but their formation occurred in the part closer to the original cut surface (Fig. 1).

At the same time, the process of regeneration occurred based on the accidental division of buds into segments, that is, tissue damage. Similar results caused by the positive effect of tissue damage during the regeneration process are explained by the activation of cell division [23][24]. In the course of research, stratification of buds in cold conditions (5±2 ºC), i.e., the influence of regeneration processes in the tissues of primary explants, was studied [25]. In the case of *L. inebrians* taken from shoots growing in greenhouse conditions, it was noted that morphogenic reactions occurred in 20–25 days with prior storage of the tissue at the right temperature. Regeneration began after 5–7 days in samples that were not subjected to stratification in cold conditions. In both cases, the average number of additional tissues was the same (2.4–2.6 units/ex). However, the absence of the initial suppression of cold differentiation resulted in the arrest of the growth of the primary vegetative organs formed in the secondary transition and the delay in the formation of subsequent shoots in the true seedling suppression. The formation of buds was not observed in the microclones obtained from the stratified plant tissue in cold conditions.

3.2. Effect of Nutrient Medium Mineral Content and Growth Regulators on the Morphogenesis of L. inebrians

Regeneration of *L. inebrians* microbuds proceeded synchronously during the visual stress. On 35–40 days of culture, the diameter of

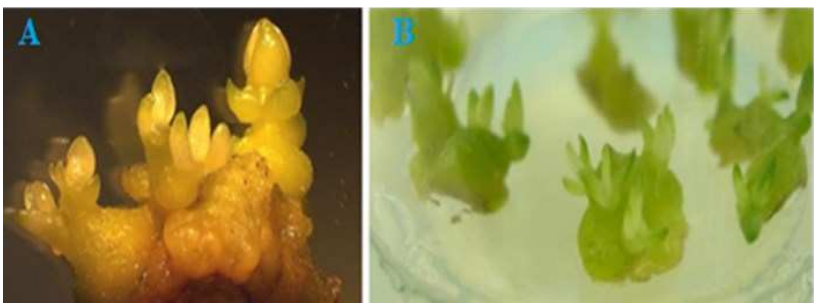

Figure 2. The influence of the mineral content of the nutrient medium and growth regulators on the morphogenesis of *L. inebrians* (A – shoot regeneration on the surface of morphogenic callus, BDS medium supplemented with 0.5 µM BAP, 31 days of cultivation; B – grown explants shoots formed in tissues, B5 medium supplemented with 0.1 µM BAP, day 23).

Table 5. Effect of mineral composition of nutrient medium and growth regulators on the regeneration of *L. inebrians* shoots *in vitro*.

Growth regulators	Mineral base			
	BDS		B_5	
	Regeneration speed (%)	Number of rows (pieces/ex.)	Regeneration speed (%)	Number of rows (pieces/ex.)
Control (hormone-free medium)	30	2.3±0.6	30	2.4±0.3
BAP 0.1	-	-	55	5.1±1.1
BAP 0.5	55	3.3±1.0	45	2.5±0.8
BAP 5.0	40	2.5±0.3	30	3.5±0.6
BAP 5.0+NAA 2.0	45	2.0±0.5	55	2.0±0.4
BAP 10.0+NAA 2.0	20	1.5±0.8	30	1.7±0.6
TDZ 0.1	50	3.3±1.5	20	3.5±1.0
TDZ 0.5	40	3.4±0.7	50	2.2±0.2
TDZ 5.0	25	2.5±0.1	25	3.5±1.4
TDZ 10.0+HUK 2.0	50	2.3±0.5	35	2.5±0.5
Kinetin 0.5	30	3.0±0.5	-	-
BAP 2.2+2,4-D 0,5	35	2.5±0.2	-	-
BAP 0.4+NAA 3.2+IAA 2.3	-	-	35	2.5±0.3

Note: $p \leq 0.05$; «-« – no information

Table 6. Effects of growth regulators on the regeneration of *L. inebrians* shoots in the *in vitro* cultures on B5 medium.

Growth regulators	Regeneration speed (%)	Number of buds (pieces/ex)
Control (hormone-free medium)	50	1.5±0.5
BAP 5.0+NAA 2.0	75	2.0±0.5
BAP 0.4 +NAA 3.2 +IAA 2.3	65	2.0±0.3

Figure 3. *L. inebrians* tissues with developed absorptive leaves on BDS medium supplemented with 0.4 µM BAP + 3.2 µM NAA + 2.3 µM IAA (33 days old).

microbuds reached 3.0–4.5 mm. They were transferred to a new hormone-free medium for further growth. It was not possible to determine the most important factors affecting the number of shoots formed in the actual field of vision [26]. However, BDS and B5 mediums were characterized by a similar rate of regeneration of *L. inebrians* microplants, and a decrease was noted in the MS medium. Taking this into account, further work was carried out using BDS and B5 mineral bases. Similar results were obtained in the cultivation of other studied species. In the control samples, in BDS medium without hormones (regeneration – 42.7%, the number of shoots – 4.1±0.2 units/ex.) during a long cultivation period (2 years), high pictures of shoot growth and development were observed. It was found that the addition of exogenous growth regulators to the nutrient medium in the growth medium *in vitro* slightly increased the activity of *L. inebrians* bud formation, and the regeneration activity in some samples was higher than in the control medium (Table 4).

Addition of the growth regulators 5.0 µM BAP and 2.0 µM NAA to BDS medium resulted in the formation of additional shoots on day 12–15 of cultivation, compared with 22–25 days on BDS medium without hormones did. The average number of buds in each explant was 4.6±0.4, the regeneration rate was 56.3%. Higher growth and development rates were also observed in BDS medium supplemented with 10.0 µM BAR and 2.0 µM NAA. The regeneration rate was 66.2% and 4.0 ± 0.8 random shoots were formed per explant. The use of rast concentrations (0.1 and 0.5 µM) of BAP, Kinetin and TDZ resulted in the formation of yellow, morphogenic callus, regardless of the mineral composition of the medium. At the same time, the rate of formation of morphogenic callus did not exceed 38.0%. The formation of buds on the surface of the callus was observed only after 5 weeks of cultivation, which occurred much later than in medium supplemented with cytokinins and auxin. From these shoots, new shoots emerged, that is, indirectly, organogenesis continued. This was especially evident in BDS medium supplemented with 0.5 µM TDZ. Although analysis of variance revealed no significant effect of environmental factors on the number of microbuds that developed, addition of 5.0 µM BAP and 2.0 µM NAA to BDS nutrient medium increased regeneration compared to control and medium supplemented with

cytokinins alone. It was found that it accelerates almost twice. Addition of 0.1 µM BAR to the medium of B5 resert resulted in the growth of explant tissue and the highest number of explants on its surface with a frequency of 56.5% (5.0 ± 1.5 units/led to the formation of exr.). This made it possible to consider this selected environment as optimal in the formation of buds (Figure 2).

At the same time, kinetin (0.5 µM) had the most accurate morphogenic activity among all used cytokinins. During the study, the influence of the mineral content of the nutrient medium (B5 and BDS) on the regeneration activity of *L. inebrians* buds was determined. At the same time, the effect of growth regulators on the number of shoots formed was analyzed (Table 5).

Analyzing the results, it was found that the highest concentration of BAP and TDZ (0.1 and 0.5 µM) was more effective. The combination of high concentrations of BAP (10.0 µM) and NAA (2.0 µM) reduced the visual factor by 19% with a regeneration frequency of 1.3±0.5 per explant. This combination of growth regulators was effective in the microarray of *L. inebrians*, and the obtained research results were analyzed as follows.

3.3. Regeneration of L. inebrians Buds

The development of *L. inebrians* larvae continued directly in the process of organogenesis. Cultivation in medium supplemented with growth regulators improved shoot formation and regeneration rate increased from 50.0 to 75.3% compared to control. The use of nutrient medium B5 containing 5.0 µM BAP and 2.0 µM NAA was considered the most effective. In this environment, the maximum speed of regeneration and the number of additional shoots per explant were obtained (Table 6).

It has been noted that the response of plant development in the *in vitro* conditions when using mineral media of different composition varies in different plants [27]. The combination of 5.0 µM BAP and 2.0 µM NAA and BDS of the mineral base was considered the most effective for *L. inebrians* microblooming. Such a combination of growth regulators in nutrient medium B5 led to the acceleration of shoot formation in *L. inebrians* cultures. When 0.4 µM BAP was combined with 3.2 µM NAA and 2.3 µM IAA, a high frequency of *L. inebrians* hemogenesis was observed in nutrient medium B5.

Both direct and indirect organogenesis were observed during cultivation of *L. inebrians.* Only indirect organogenesis was observed in *L. inebrians* in all types of nutrient media. This can be explained by species characteristics. The first visible changes on the surface of *L. inebrians* explants were observed in 25–31 days in nutrient medium supplemented with 5.0 µM BAP and 2.0 µM NAA. After 2.5–5.0 weeks in the food medium, all the microbuds of the studied species formed in the explants began to form in the form of 2–3 leaves (Figure 3).

3.4. Formation of the Root System of L. inebrians Regenerants and Adaptation to Ex Vitro Conditions

The formation of the root system of *L. inebrians in vitro* was observed mainly in nutrient medium with added auxin and partly without hormones. Seedlings obtained under *in vitro* conditions were

Figure 4. Transplantation of *L. inebrians* from *in vitro* conditions to growth.

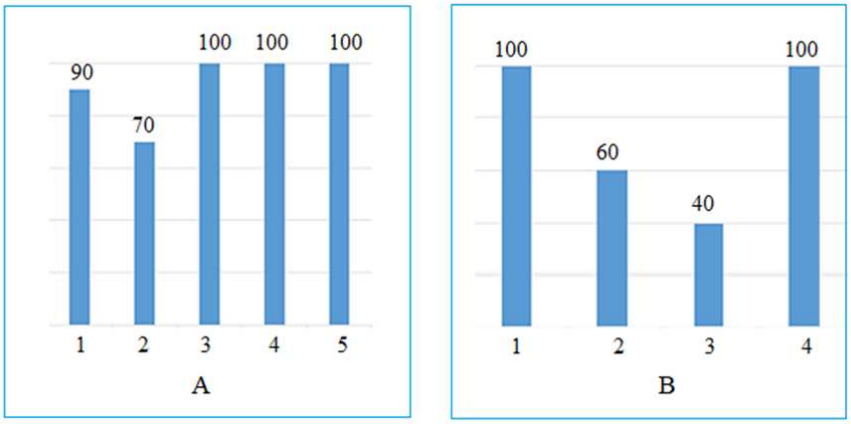

Figure 5. Rooting rate in *L. inebrians* explants obtained *in vitro*, in % (A-laboratory conditions: 1-1/2BDS; 2-1/2BDS+NSK5; 3-1/2BDS+NSK1.5; 4-1/2MS+IMK5; 5-1/2BSK5; B - in greenhouse conditions: 1-1/2BDS; 2-1/2BDS+NSK5; 3-1/2BDS+NSK1.5; 4-1/2MS+IMK5).

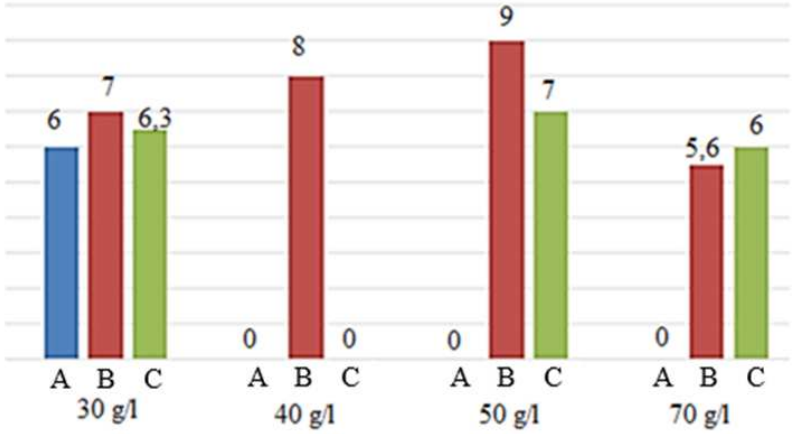

Figure 6. Effect of sucrose concentration on the formation of microflora in *L. inebrians* in laboratory (A), greenhouse (B), and natural (C) conditions, in piece (n = 3).

transplanted one by one. Therefore, the plant obtained *in vitro* was planted on the substrate in the greenhouse. The top of the substrate is a simple substrate, wood shavings were placed on the surface layer (Fig. 4).

It was found that *L. inebrians* callus tissue proliferation under *in vitro* conditions is optimally realized in the combination of BAP (1 mg/L)+NAA (1 mg/L) in MS nutrient medium. In the combination of BAP (2 mg/L)+NAA (0.1 mg/L) +GA_3 (0.5 mg/L), the intensity of root formation was relatively high. It should be noted that the plants in the studied samples took root easily in both hormonal and non-hormonal media [28][29]. Average rooting rate in all tested samples was 81.3%. A 100% rooting in L. inebrians microplants was observed only in the presence of auxins in ½ BDS and ½ MS mineral bases at a concentration of 5.0 µM - NAA, IAA, IBA. The most effective medium for inducing rhizogenesis in *L. inebrians* was ½ B5 supplemented with 5.0 µM NAA (Figure 5, Tables 7 and 8).

The type of auxins used did not affect rhizogenesis activity. It can only be shown that the presence of 5.0 µM NAA, IAA or IMK in the nutrient medium induced root formation compared to medium without hormones and medium containing NAA (1.5 µM). Table 9 shows the growth and development of the studied samples of *L. inebrians* in two temperature regimes. According to the obtained data, the length of the roots is 1.5–2.5 times shorter at a temperature of 23±2 ºC. At the same time, other growth indicators did not depend on the temperature regime.

Table 7. Indications of growth and development of *L. inebrians* regenerated plants (7 ºC).

The place where plant samples were taken	Nutrient medium	Number of roots	Root length (mm)
Samples grown in a sterile environment under laboratory conditions	½ BDS NAA 5.0 mkM	4.1±1.0	20.4±1.5
	½ MS IMA5.0 mkM	6.3±1.5	15.0±1.3
	½ MS IAA 5.0 mkM	3.4±1.1	15.2±2.2
Samples grown in greenhouse conditions	½ BDS	4.5±0.3	11.0±1.5
	½ MS IAA 5.0 mkM	8.1±1.5	16.4±2.5
Samples grown in natural conditions	½ B_5 NAA 5.0 mkM	4.0±1.1	25.5±2.7

Table 8. Growth and development of regenerated *L. inebrians* plants.

The place where plant samples were taken	Number of roots		Root length (mm)	
	7 ºC	20±2 ºC	7 ºC	20±2 ºC
Samples grown in a sterile environment under laboratory conditions	3.5±0.5	4.5±0.5	10.4±1.3	20.5±2.5
Samples grown in greenhouse conditions	4.4±0.2	4.0±1.5	11.5±0.5	15.5±1.5
Samples grown in natural conditions	2.5±0.7	3.0±1.2	8.5±2.5	20.5±2.5

Another phenomenon of interest in microplant cultivation is the formation of stolons in *L. inebrians*. The development of stolons at the rooting stage was recorded only in 3 microcuttings grown on ½ B5 medium with high sucrose content, but all of them had the same morphology. At the top of the formed stolon there is a bud of regeneration - a special vegetative part, which is formed from the maternal specimens and can later replace it. Adventitious roots also begin to form at the base of a special new vegetative part. Thus, under *in vitro* conditions, *L. inebrians* microplants develop the structural characteristics of underground organs in accordance with those grown in natural conditions. From the above observations, we can conclude that differences in the level of underground organs affect rooting. The size of roots obtained *in vitro* indicates the successful adaptation of regenerated plants to non-sterile conditions in the future [30]. To evaluate the effect of sucrose concentration on the growth and development of plants during rooting, we used 30.0 (control), 40.0; We obtained 50.0 and 70.0 g/L of sucrose. In the research, it was found that the increase in the concentration of sucrose (70.0 g/L gas) led to the acceleration of root growth in *L. inebrians* plants (Figure 6).

To adapt to non-sterile conditions, vegetative parts of at least 5.0–5.5 cm in size with a developed root system were selected. In this case, a mixture of sphagnum moss, peat and sand (3:1), crushed coconut fiber was used as a substrate [31]. The regenerants of the studied samples were kept in laboratory and greenhouse conditions at a temperature of 23±2 ºC and during the corresponding light period, the development of new leaves did not take place and caused a slowdown in growth. A similar result was observed in the transition step from the used substrate and root formation. In this case, a resting period was determined at a temperature of 23±2 ºC. The use of sphagnum moss as a substrate is suitable only as an initial step in adaptation. This is due to the need to re-transplant these regenerants to a more diverse substrate when growing in a greenhouse or in open ground [32]. In this substrate, an increase in the length of the roots and the appearance of root hairs were observed, but the process of leaf regrowth was not noted after 4–5 months of work. It was more effective to use a mixture of peat and sand, as well as crushed greens and sand. At the same time, adaptation to room conditions by alternation of high and low temperatures was carried out on the basis of adding growth-promoting hormones to transplanting of regenerated plants. Thus, keeping the planted plants in the substrate for 4–6 weeks in a dark place at a temperature of 5±2 ºC and transferring them to a temperature regime of 23±2 ºC, inducing them from the dormant period and accelerating the assimilation of the leaves came. It was developed only in samples with a developed root system. It should be noted that when grown in room conditions, even after cold stratification, the leaf quickly died and a second dormancy occurred. This can be explained by the relatively high temperature and dryness of the air. This adaptation made it possible to eliminate the secondary dormancy period by transferring the planted plants to the greenhouse.

Acclimatization of regenerated plants in a greenhouse (cold storage) was carried out by another research group [33] in the case of cold air temperature (October-December). In both options of the substrate, the growth of the first leaf was recorded in February-March. The average air temperature in the cold part of the greenhouse during this period was 8.5±1.4 ºC. Activation of growth processes of regenerative plants at positive

Table 9. Efficiency of adaptation of *L. inebrians* regenerated plants to *ex vitro* conditions.

Options	Adaptation (%)	
	Peat and sand (3:1)	Coconut fiber and sand (3:1)
Samples grown in a sterile environment under laboratory conditions	40	70
Samples grown in greenhouse conditions	40	75
Samples grown in natural conditions	35	80

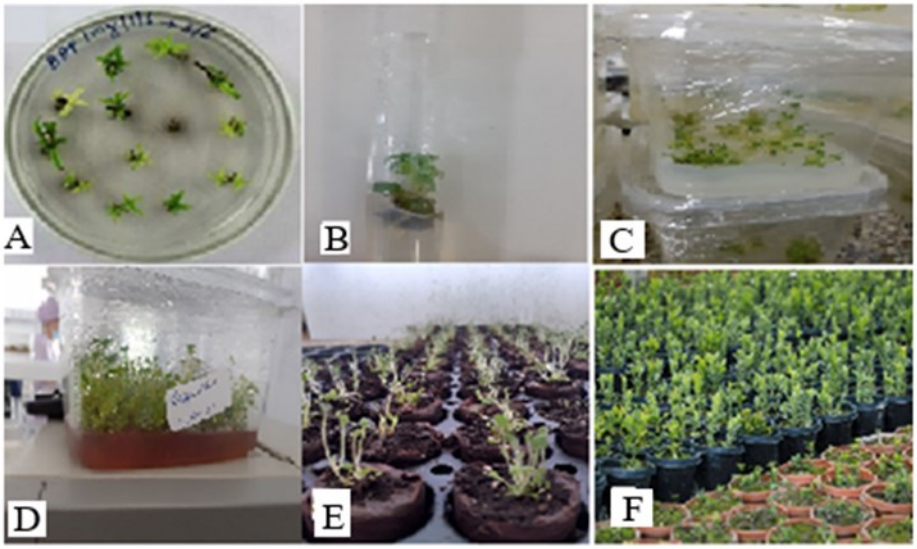

Figure 7. Microclonal breeding of *L. inebrians* in vitro and adaptation to species conditions (A-B - a callus formed from excrement, C - formation of a cyst; D - rooting process; E – air conditioning; F-ready planting).

temperatures (in the greenhouse) was observed, regrowth when the temperature exceeds 5 ºC [34]. In this, *L. inebrians* samples showed a normal seasonal rhythm of development. At the same time, the synchronous sprouting of tissues is characteristic of all studied samples. The degree of adaptation of plants was evaluated by the number of shoots with *ex vitro* formed leaves (Table 9).

According to the obtained data, the optimal substrate for adaptation of the regenerative plants of the studied samples of *L. inebrians* growing in different conditions is a mixture of coconut fiber and sand (3: 1). This made it possible to develop 82.7% hashish in plants adapted to a temperate climate with well-developed leaves and root systems. The type of substrate also affected the level of development of leaf organs of plants [35] [36]. Thus, when a mixture of peat and sand (3:1) was used, the presence of the first leaf in the studied samples of *L. inebrians* was recorded after 4 months of cultivation, and the presence of one leaf in each *L. inebrians* tissue was recorded. was characteristic. At the same time, the percentage of tissues successfully adapted with absorbent leaves did not exceed 41.6%. The vegetation period was 3 months from the growth of the first leaf to the death of the last one. At the end of the specified period, *L. inebrians* specimens growing in natural, laboratory, and greenhouse conditions were characterized by one large and one or two human tissues. When a mixture of peanut flour and sand (3:1) was used, the adaptation efficiency of all studied samples growing under different conditions varied from 71.9 to 82.7%. Depending on the sample, regrowth of the first assimilative leaf was noted 5–9 weeks after transfer of in vitro rooted *L. inebrians* tissues to non -sterile conditions (cold part of the greenhouse). The regenerants grown in the temperature regime of 8.5±1.4 ºC helped the development of 1–3 assimilatory leaves, depending on the samples grown in different conditions. The cultivation of plants in the next step biotechnological method was carried out in the experimental area of the laboratory. At the same time, 3.5–4.0 months have passed after the first leaf appears. During this period, the effect of cold stratification (7 ºC) on rooting from shoots *in vitro* was studied. The favorable temperature conditions had a positive effect on the development of *ex vitro* shoots of the plant. The growth of the first leaf in *L. inebrians* was observed in 48–56 days after the start of adaptation in a mixture of coconut fiber and sand. Regrowth occurred twice as slowly after 89–94 days in samples without Rast temperature treatment. Therefore, when the positive temperatures are transferred to *ex vitro* conditions, it helps to eliminate the dormant state of vegetative organs and their germination. The rest period is necessary for the normal development of geophytes, without it during the annual cycle, the growth of

tissues slows down and the development of generative organs does not take place. Choosing the optimal duration of the period with the right temperature helps the rapid development of leaves and roots [37]. Thus, the method of cultivation at a positive temperature (7 ºC) is the most effective in terms of rooting and tissue formation, as it allows to obtain renewed plants with a developed root system (3.5–8.0 per shoot, 0 root). At the same time, the use of nutrient medium supplemented with auxins (5.0 µM NAA or IBA) promotes the formation of a more developed root system in *L. inebrians*. Changes in the concentration of sucrose in the nutrient medium have almost no effect on the growth and development of micro-plants during the rooting stage.

The results of our research showed that plant development at the right temperature not only promotes more intensive rhizogenesis, but also promotes faster development of regenerating plants in *ex vitro* conditions. At the same time, the adaptation of regenerant *L. inebrians* plants grown in different conditions showed that the most optimal substrate was a mixture of walnut meal and sand (3:1), with the growth and development of the plant under the selected different conditions. A 82.7% increase in adaptability. A network of *in vitro* samples of *L. inebrians* under different conditions was created based on the microclonal display systems developed in the research. At the same time, the collection includes more than 10,000 micro-plants in the state of active growth and more than 1,000 samples of them in the state of slow growth. We have developed a schematic system related to microclonal display, maintenance and development of *L. inebrians*. The scheme includes the selection of primary explants, cultivation and preservation of tissues in vitro, rooting, adaptation to different climatic conditions and subsequent *ex vitro* conditions (Figure 7).

4. CONCLUSIONS

In vitro microcloning of *L. inebrians*, the intensity of the processes (callus tissue formation and proliferation; shoot formation, root formation) explant, explant sterilization method, depends on factors such as combinations of phytohormones used in the nutrient medium. In the option where leaf axil buds and leaf fragments were used as initial explants, MS medium containing a combination of BAP (5 mg/L)+NAA (0.4 mg/L), shoots and leaf fragments located at the tip of the stem when lakshas are used, the intensity of callus tissue formation is high in the combination of BAP (3–4 mg/L)+NAA (0.4 mg/L) in MS nutrient medium. The obtained information provides the possibility of effective reproduction of *L. inebrians* plant and serves to organize plantations of this plant.

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K. S., B. A., N. X., Y. A. and M. D. performed the experiments. K. S., H. H., and B. K. analyzed data. B. J. and K. S. statistically analyzed results. All authors wrote the draft of the manuscript. K. S., B. A. and N. X. conducted the critical revision of the manuscript. K. S. and B. A. worked out the concept and design, supervised and funded the experiments. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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