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Protocol of clonal micropropagation of *Humulus lupulus* (Cannabaceae)

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Keywords: adaptation, explant, genotype, hops, *in vitro* culture, micropropagation, plant growth regulators, planting material, rooting.

Summary. Hops are a promising agricultural crop, in demand in the food, pharmaceutical and cosmetic industries. The problem of mass production of planting material for the establishment of industrial plantations and mother plantings can be solved using biotechnological methods. We have developed an effective protocol for clonal micropropagation of *Humulus lupulus*, including all stages from the preparation of mother plants and the introduction of explants into *in vitro* culture to the adaptation of regenerants to non-sterile conditions. The protocol was tested on 13 varietal and wild genotypes. For the first time, a well-growing aseptic culture of national cultivars was obtained. The dependence of the multiplication efficiency on the genotype and the number of passages was confirmed. Conditions were selected that ensure a high rate of reproduction of the *in vitro* hop culture, starting from passage IV. It was found that the maximum number of regenerated microcuttings can be obtained on the 24th–26th day of culturing the primary explants on the MS nutrient medium supplemented with 2 mg·L^{-1} BAP and 1 mg·L^{-1} GK3. For the first time, the possibility of combining the stages of micropropagation and rooting on the MS medium supplemented with 0.5 mg·L^{-1} IBA was demonstrated. With the availability of *in vitro* hop collection, the cloning process is reduced by one passage. To proceed to the adaptation stage, it is enough to form two nodes and a well-developed root system on the shoot. Two-stage adaptation to non-sterile growing conditions using hydroponics ensures plant survival of up to 100 %. The proposed protocol for clonal micropropagation of hops can be used to replicate valuable genotypes for scientific, breeding and production purposes.

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Протокол клонального микроразмножения *Humulus lupulus* (Cannabaceae)

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Ключевые слова: адаптация, генотип, культура *in vitro*, микроразмножение, посадочный материал, регуляторы роста растений, укоренение, хмель, эксплант.

Аннотация. Хмель является перспективной сельскохозяйственной культурой, востребованной в пищевой, фармацевтической и косметической промышленности. Решение проблемы массового получения посадочного материала для закладки промышленных плантаций и маточных насаждений возможно с использованием биотехнологических методов. В результате исследований разработан протокол клонального микроразмножения *Humulus lupulus*, включающий все этапы от подготовки маточных растений и введения в культуру *in vitro* до адаптации к нестерильным условиям. Протокол протестирован на 13 сортовых и дикорастущих генотипах. Впервые получена хорошо растущая асептическая культура отечественных сортов. Подтверждена зависимость эффективности размножения от генотипа и количества пассажей. Подобраны условия, обеспечивающие высокий темп размножения культуры хмеля *in vitro*, начиная с IV пассажа. Установлено, что максимальное число регенерировавших микрочеренков можно получить на 24–26 сутки культивирования первичного экспланта на питательной среде МС, дополненной 2 мг/л БАП и 1 мг/л ГК3. Впервые показана, возможность совмещения этапов укоренения и микроразмножения на среде МС, содержащей 0,5 мг/л ИМК. Это, при наличии биотехнологической коллекции *H. lupulus*, сокращает процесс клонирования до одного пассажа. Для перехода на этап адаптации достаточно формирования на побеге двух узлов и хорошо развитой корневой системы. Приём двухэтапной адаптации к нестерильным условиям выращивания с применением гидропоники обеспечивает приживаемость растений до 100 %. Разработанный протокол клонального микроразмножения хмеля может быть использован для тиражирования ценных генотипов в научных, селекционных и производственных целях.

Introduction

Hop growing, as a branch of agriculture, plays a leading role in providing the brewing industry with high-quality raw materials. Until recently, enterprises in the Russian Federation have worked mainly using imported hops. But the pressure of sanctions and the disruption of the established supply chains of raw materials and equipment have pushed brewing companies to search for new solutions that would ensure the industry's independence from imported hop products. Currently, the demand of national enterprises for hop raw materials amounts to 7–8 thousand tons per year. To meet this need, the production of the crop in Russia must be increased more than 40 times (Afanasyeva et al., 2022).

Humulus lupulus L. (Cannabaceae) is a perennial fast-growing vine with shoots that die off in winter. It synthesizes dozens of biologically active substances and has significant potential for the pharmaceutical, cosmetic and bakery industries (Astray et al., 2020; Chaplygina et al., 2020; Korpelainen, Pietiläinen, 2021; Pereira et al., 2022; Becker et al., 2023; Aubakirova et al., 2024; Rosa, Lannes, 2024). Recent studies have revealed new areas of its potential use as an anti-cancer and anti-SARS-CoV-2 agent (Harish et al., 2021; Bouback et al., 2023). Thus, an increase in demand for hop raw materials is expected.

One of the pressing problems of Russian hop growing is the limited range of varieties and the lack of high-quality planting material that meets international standards (Khlynovskiy et al., 2023). Increasing the diversity of national hop cultivars can be achieved by involving genetic resources from wild populations in breeding programs. For large scale cultivation, hops are propagated by stem and rhizome cuttings with one or more pairs of buds and much less frequently using etiolated or green shoots (Milosta, Lapa, 2010). Because of the difficulty of maintaining mother plantings and the accumulation of a complex of pathogens and pests as a result of long-term use of plantations (Pethybridge et al., 2008; Danilova et al., 2013; Gargani et al., 2017; Sastry et al., 2019), there is an urgent problem of developing effective methods for propagation and creating collections of valuable genotypes. In Russia

there is the only field collection of hops at the Chuvash Research Institute of Agriculture, which contains 250 samples of cultivar and wild hops from various regions of Russia and 17 foreign countries. The organization's production of a small number of cuttings cannot cover the huge market demand for seedlings to renew and establish new hop plantations. The mobilization and use of genetic resources of crops and their wild relatives, including the establishment of *in vitro* collections, is of strategic importance for sustainable crop production and the conservation of biological diversity (The Second Report…, 2010; Tyagi, Grawal, 2015; Mitrofanova et al., 2018; Panis et al., 2020). The problems of mass production of certified hop planting material can be solved using clonal micropropagation. Foreign literature describes various approaches, often applicable only to individual stages of this technology or to certain genotypes. National studies are represented only by a few publications by the authors of the article.

Various explants, which are isolated from plants most often grown in nurseries under field conditions, are used for *in vitro* micropropagation of hops. These are nodal stem segments (Kastritskaya et al., 2014; Machado et al., 2018; Liberatore et al., 2020), shoot tips (Popov et al., 1985; Roy et al., 2001), meristems (Adams, 1975; Svoboda, 1992; Patzak, 2003), stem or leaf segments (Batista et al., 1996, 2000; Škof et al., 2007), etiolated rhizome buds (Kastritskaya et al., 2014). In order to obtain regenerants identical to the original material during *in vitro* multiplication and to avoid somaclonal variability, it is recommended to put into practice cuttings and shoot buds as explants, using the natural ability of plants to produce already organized meristematic tissues (Mitrofanova, 2011; Kukharchyk, 2019; Liberatore et al., 2020). Significant variability is observed in sterilization protocols, nutrient media compositions, and adaptation to *ex vitro* conditions (Roy et al., 2001; Lagos et al., 2022; Iacuzzi et al., 2023). Different authors' data indicate that the reaction of hop samples *in vitro* varies depending on the varieties (Kastritskaya et al., 2014; Gashenko et al., 2019; Mafakheri, Hamidoghli, 2019; Myakisheva et al., 2024). This confirms the relevance of adjusting protocols for specific genotypes. First of all, this is important for wild-growing samples that are promising for breeding, and domestic varieties that have not been previously studied.

The purpose of this study was to investigate the features of clonal micropropagation of common hops and develop a protocol for creating an aseptic collection of varietal and wild genotypes.

Materials

Plant material

Humulus lupulus plants were used as the source material: four genotypes (Alt/29-1, Alt/29-2, Alt/15-1, Alt/14-1) from natural populations (Altai Territory), five Russian cultivars ('Civil'skij', 'Favorit', 'Flagman', 'Forvard', 'Sumer'') from the field collection of the Chuvash Research Institute of Agriculture – a branch of the Federal State Budget Scientific Institution "The Federal Research Center of the North-East", two Russian ('Smolistyj', 'Bryanskij') and and six foreign cultivars ('Magnum', 'Marynka', 'Columbia', 'Taurus', 'Spalter Select', 'Comet') from hop plantations in the lowlands of Altai (Fig. 1a, b). Wild hops were herbarized (Fig. 1c). The data have been entered into GBIF (Mironenko et al., 2024).

Reagents

1. Major elements: ammonium nitrate, calcium chloride, magnesium sulfate, potassium hydroorthophosphate, potassium nitrate (Russia).
2. Minor elements: Boric acid, Cobalt chloride, Copper (II) Sulfate, Manganese (II) sulfate, Potassium iodide, Sodium Molybdate, Zinc Sulfate (India).
3. Iron Chelate: Ethylenediaminetetraacetic acid (India), Iron (II) sulfate (Russia).
4. Organic elements: mesoinositol, Nicotinic acid, Pyridoxine HCL, Thiamin HCL (India), Glucose (Russia).
5. Plant growth regulators: 6-Benzylaminopurine (BAP), India Gibberellic acid (GA3), Indole-3-butyric acid (IBA) (India).
6. Agar-agar (Italy).
7. Hydrogen Peroxide 30 % (Russia).
8. Ethanol (70 % in distilled water, Russia).

Equipment

1. Laminar flow box BAVnp-01-"Laminar-C".-1,2 (Lamsystems, Russia).
2. Autoclave MLS-3020U (Panasonic, Japan).
3. pH meter Econix Expert (Econix, Russia).
4. Magnetic stirrer Heidolph RZR 2020 (Heidolph, Germany).
5. Hydroponics (Russia).
6. Water distillation unit GFL-30938 (GFL, Germany).
7. Dispensers Ekros PE 0,25-2,5 HF (EkrosChim, Russia).
8. Analytical scales AX224/E (Ohaus, USA).
9. Culture vessels – glass tubes (size 200 × 21 mm), medium volume – 10 ml.

Fig. 1. Stages of *in vitro* culture introduction and clonal micropropagation of *Humulus lupulus*: a – varietal accessions in a field collection; b – wild accessions in a natural environment; c – herbarium specimen of a accession from a natural population; d – mother plants in pots in a greenhouse; e – shoot at the stage of active growth; f – sterilization; g – passaging of primary explants on MS1 nutrient medium; h – primary explant with formed microshoots; i – separated microshoot on MS2 nutrient medium; j – secondary passaging of primary explant; k – development of the root system; l – microcuttings; m – regenerants ('Comet' variety) after 30 days of cultivation at passage II; n – regenerants ('Comet' variety) after 30 days of cultivation at passage XIV.

Procedures

Nutrient media and cultivation conditions. The preparation and sterilization of nutrient media were carried out according to standard methods. The Murashige-Skoog (MS) nutrient medium supplemented with 20 g·L^{-1} glucose and 7.3 g·L^{-1} agar-agar were used as the base medium. At the stage of introducing hop explants into the *in vitro* culture, MS + 2 mg·L^{-1} BAP + 1 mg·L^{-1} GK3 (MS1) was used. For cultivation at the multiplication and rooting stages, MS + 0.5 mg·L^{-1} IBA (MS2) was used. The pH of the nutrient medium was regulated within 5.8–5.9. The sterilization conditions for the nutrient medium were 121 °C for 20 min. *In vitro* plant cultivation was carried out in a culture room at a temperature of 21–23 °C, a photoperiod of 16 / 8 hours (day / night), and a light intensity of 2000–3000 lux.

Preparation of primary explants. H. lupulus mother plants were grown in a greenhouse (Fig. 1d). A soil-peat mixture was used as a substrate: neutral high-moor peat: sod soil: agroperlite (3 : 1 : 1). Cuttings from plants in the active growth stage were used (Fig. 1e).

Adaptation to non-sterile growing conditions. The hydroponic setup was filled with a nutrient solution according to the prescription of ¼ MS, modified by the content of KH_2PO_4 – 0.51 g·L^{-1}. The operating mode of the hydroponic setup: 5 min of solution supply / 20 min of break, illumination with blue and red LEDs 2000–3000 lux, a photoperiod of 16 / 8 hours (day / night).

Controlled morphometric parameters. Sterility is the number of aseptic (without visible lesions of bacterial and / or fungal infection) explants / total number of explants × 100 %.

Viability is the number of proliferating explants / total number of explants × 100 %.

Reproduction rate is the number of proliferating microcuttings obtained from one shoot of the previous passage.

Method implementation

Introduction to in vitro culture

1. Micropropagation was carried out by the axillary meristem activation method. Microcuttings isolated from donor plants were used as explants. The explants were 10–15 mm in size and had a pair of axillary buds.

2. To obtain a sterile culture, three-stage surface sterilization was carried out:

– pre-sterilization (carried out under non-sterile conditions): the explants were placed in a soap solution (30 min) and stirred using a magnetic stirrer. Then the explants were washed in running water (30 min);

– sterilization (carried out under sterile conditions): the explants were immersed in 70 % ethanol for 30 s, then transferred to hydrogen peroxide for 10 min (Fig. 1f);

– post-sterilization: the explants were washed in sterile distilled water (5 min × 3).

3. After sterilization, 2–3 mm of the basal part of the microcutting and stipules were removed from the explants with a scalpel.

4. The explants were vertically placed on the MS1 nutrient medium, deepening the basal part of the microcutting into the medium by 3–5 mm (Fig. 1g). The tubes were transferred to the cultivation room and incubated for 1 month.

5. After 20–22 days, the axillary buds proliferated and formed a shoot with a pair of true leaves. Such microshoots were separated from the primary explant and transferred to the MS2 nutrient medium (Fig. 1h).

In this way, the aseptic collection of *H. lupulus* genotypes was formed.

In vitro micropropagation and rooting stage.

1. *H. lupulus* regenerants were divided into microcuttings with 1 or 2 stem nodes and transferred to MS2 nutrient medium (Fig. 1l). The size of the microcuttings was at least 10 mm.

Adaptation to ex vitro non-sterile growing conditions.

1. Adaptation was performed in 2 stages: 1) in a hydroponic setup, 2) in a peat substrate in a greenhouse.

2. Regenerants that had at least 4 stem nodes and a formed root system (the number of roots was at least 5 pcs., the root length was at least 7 mm) (Fig. 2b) were transferred to seedling cassettes with a volume of 60–155 ml. The mixture of perlite and agrovermiculite was used as a substrate (1:1).

3. The seedling cassettes were placed in a hydroponic setup (Fig. 2c). To create conditions of high humidity, they were covered with polyethylene film for 3–5 days. The adaptation period lasted 18–20 days (Fig. 2d).

4. At the second stage of adaptation, the plants were transferred to plastic seedling pots (V = 0.5 l) with a peat-containing substrate (Fig. 2e). They were cultivated in a greenhouse for 20–23 days.

5. To improve shoot growth, supports were used to attach the vines. The supports were thin wooden sticks 0.4 m high (Fig. 2f).

Features of introduction into in vitro culture and clonal micropropagation of H. lupulus

1. The initial plant material grown in a greenhouse, when introduced into *in vitro* culture, provided 12–36 % more aseptic explants than shoots from the field. The maximum difference in sterilization efficiency was found in the 'Magnum' variety. When using cuttings from the field and from the greenhouse, the proportion of aseptic explants was 54.9 % and 90.0 %, respectively. No dependence of explant viability on the growing conditions of donor plants was revealed (Table 1).

Table 1

Effect of donor plant growing conditions on sterilization efficiency and viability of *Humulus lupulus* explants

Genotype	**Field conditions**		***Greenhouse conditions***	
	Sterility, %	***Viability, %***	***Sterility, %***	***Viability, %***
'Magnum'	54,9 ± 1,9	100 ± 0	90,0 ± 2,4	84,3 ± 2,0
'Marynka'	60,6 ± 1,8	92,7 ± 1,7	91,2 ± 2,1	84,8 ± 1,4
'Forvard'	52,8 ± 1,7	33,9 ± 2,3	83,3 ± 2,1	72,2 ± 1,7
'Sumer''	54,1 ± 2,0	81,2 ± 1,5	85,7 ± 1,2	72,6 ± 1,8
'Smolistyj'	63,7 ± 2,2	46,8 ± 2,7	75,3 ± 2,0	78,6 ± 1,9
Average	57,2 ± 1,5	71,8 ± 3,7	85,1 ± 1,7	78,5 ± 1,7

2. Cultivation of primary explants on the MS1 nutrient medium led to the formation of shoots with two nodes on the 18th–22nd day (Table 2). The formed shoots with two pairs of leaves (Fig. 1h) were transferred to fresh nutrient medium for propagation (MS2) (Fig. 1i). The primary explant was left on the MS1 medium for the development of additional buds (Fig. 1j). This approach led to an increase in the multiplication rate and the efficiency of introducing explants into *in vitro* culture.

Table 2

Number of shoots on primary explants of *Humulus* lupulus suitable for passage

Genotype	***Number of explants, pcs.***	***Growing time, days***							***Total***
		18	***20***	***22***	***24***	***26***	***28***	***30***	
'Columbia'	16	1	3	8	4	1	3	0	19
'Favorit'	11	–	–	7	9	2	0	0	18
'Flagman'	10	–	–	12	8	3	1	0	24

3. The maximum number of formed shoots suitable for passaging was observed on the 22nd–24th day with a subsequent decrease in regeneration capacity. For example, in 11 primary explants of the 'Favorit' variety, nine shoots suitable for passaging were formed by the 24th day of cultivation, then two shoots developed after two days, and by the 28th day, regeneration stopped.

4. When explants were kept for a long time on a medium enriched with growth regulators at the stage of introduction into *in vitro* culture, a decrease and cessation of regeneration, the appearance of chlorosis on the leaves, the death of the tops, and sometimes the death of the shoots or the explant were observed. Often, the death of an explant with already formed shoots was accompanied by visible infection of the medium (Fig. 3a). In this regard, it is recommended to cultivate primary explants on the MS1 nutrient medium for no more than 30–35 days.

5. Significant variability in the development of explants at the stage of introduction into *in vitro* culture was observed in different genotypes of *H. lupulus* (Fig. 4). Explants of the 'Flagman' variety formed 1–2 shoots, while those of the 'Magnum' variety formed 2–5 adventitious buds with subsequent uneven development of shoots, not all of which were suitable for passaging due to their small sizes. The 'Columbia' variety developed additional buds in the nodes during secondary cultivation of primary explants on the MS1 medium.

6. The reproduction rate of *H. lupulus* increased by passage IV in all studied genotypes (Table 3). For example, in the 'Bryanskij' variety, the reproduction rate at passages II and IV differed by 5 times, while at passage V and subsequent passages, the reproduction rate was stable (9.0–10.3). There are samples cloned up to the 14th passage and maintaining a high reproduction rate (Fig. 1n).

Table 3

In vitro reproduction rate of *Humulus lupulus* depending on the number of passages (pcs. / shoot)

Genotype	Passage				
	I	II	III	IV	V
'Flagman'	2,3 ± 0,34	2,1 ± 0,35	8,0 ± 0,49	8,0 ± 0,46	8,0 ± 0,30
'Favorit'	1,4 ± 0,53	1,9 ± 0,52	5,0 ± 0,54	10,0 ± 0,73	9,0 ± 0
'Bryanskij'	1,5 ± 0,49	2,3 ± 0,47	5,0 ± 0,77	10,0 ± 0,83	9,0 ± 0
Alt/14-1	1,0 ± 0,52	0,5 ± 0,67	2,5 ± 0,61	7,5 ± 0,67	8,0 ± 0,77
Alt/15-1	3,2 ± 0,78	1,0 ± 0,34	3,2 ± 0,63	6,0 ± 0,58	9,0 ± 0
Alt/29-1	1,0 ± 0,52	1,7 ± 0,39	5,1 ± 0,26	9,0 ± 0,52	9,0 ± 0
Alt/29-2	0,4 ± 0,55	2,2 ± 0,49	3,5 ± 0,54	7,5 ± 0,54	6,0 ± 0,30

Fig. 2. Adaptation of *Humulus lupulus* regenerants to *ex vitro* conditions: a – *in vitro* collection of genotypes; b – regenerant after *in vitro* rooting; c – stage I of adaptation in a hydroponic setup in cassettes; d – appearance after 20 days of adaptation; e – stage II of adaptation in a peat substrate; f – after 23 days of adaptation in a peat substrate.

7. *In vitro* development of *H. lupulus* on MS2 medium allows combining the stages of micropropagation and rooting. During the first 5–10 days of cultivation on MS2 medium, the root system is formed (Fig. 1k). Then one or two axillary buds develop.

8. To move on to the adaptation stage, it is sufficient to use regenerants with a developed root system and 2–4 stem nodes, which retain viability at the level of 90–100 % (Table 4). Only the 'Spalter Select' variety showed reduced survival. Two-stage adaptation using a hydroponic setup (Bychkova et al., 2024) allows maintaining the viability of *H. lupulus* regenerants up to 100 % and obtaining high-quality planting material.

Table 4

Efficiency of adaptation of *Humulus lupulus* regenerants to non-sterile conditions depending on the number of stem nodes (%)

Genotype	Number of stem nodes, pcs.					
	2	3	4	5	6	7
'Spalter Select'	75,0 ± 2,8	88,2 ± 1,8	92,9 ± 1	94,7 ± 1,8	80,0 ± 1,7	66,7 ± 1,8
'Taurus'	94,1 ± 2,5	85,2 ± 1,6	100,0 ± 0	100,0 ± 0	100,0 ± 0	100,0 ± 0
'Flagman'	92,0 ± 2,1	94,2 ± 1,8	100,0 ± 0	100,0 ± 0	100,0 ± 0	100,0 ± 0
'Civil'skij'	100,0 ± 0	100,0 ± 0	100,0 ± 0	100,0 ± 0	100,0 ± 0	100,0 ± 0
Average	**90,3** ± 2,3	**91,9** ± 1,8	**98,2** ± 1,3	**98,7** ± 1,2	**95,0** ± 2,2	**91,7** ± 2,9

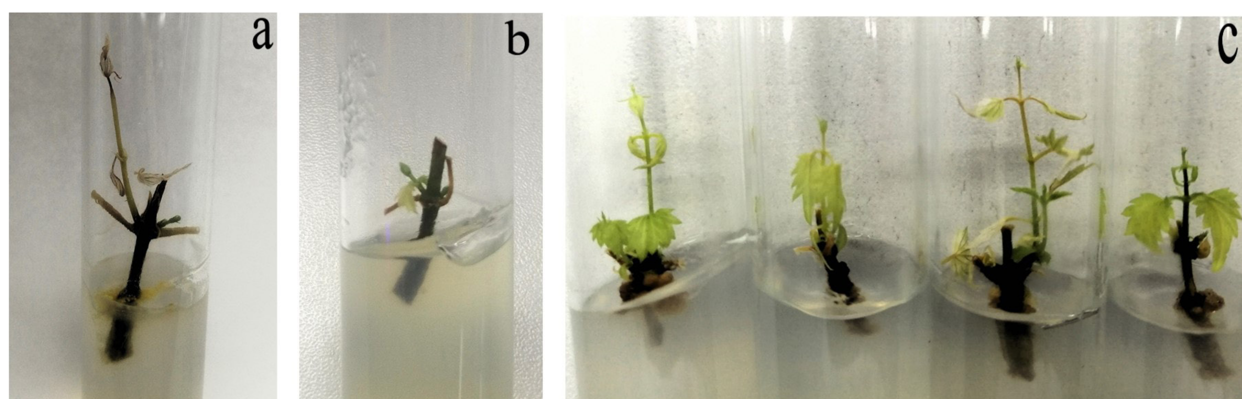

Fig. 3. *Humulus lupulus* explants cultivated for more than 35 days on MS1 nutrient medium: a – death of microshoot and development of infection; b – growth cessation; c – chlorosis.

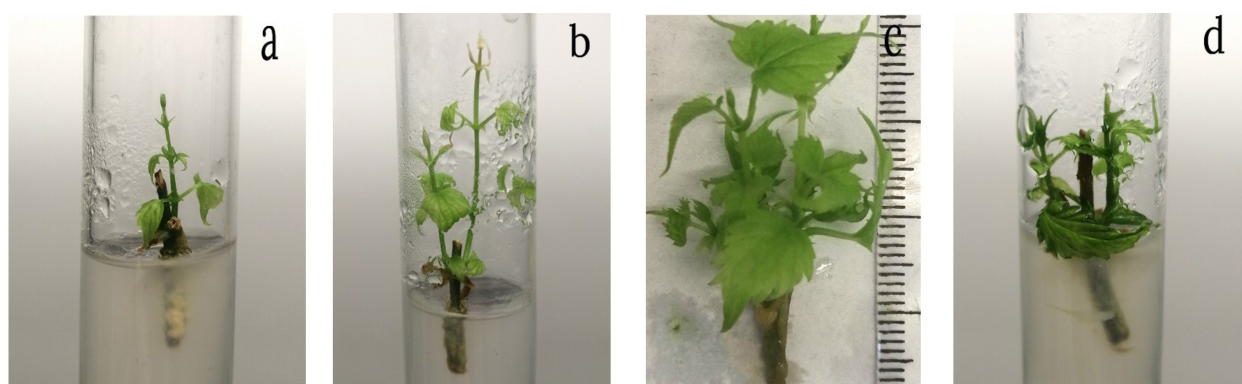

Fig. 4. Variability in shoot development of *Humulus lupulus* genotypes at the stage of introduction into *in vitro* culture: a – 'Flagman'; b – 'Magnum'; c, d – 'Columbia'.

Conclusion

A protocol for clonal micropropagation of *H. lupulus* has been developed, including all stages from the preparation of mother plants and introduction of explants into *in vitro* culture to adaptation of regenerants to non-sterile conditions, allowing for the production of seedlings with a closed root system. The protocol has been tested on 13 varietal and wild genotypes. For the first time, a well-growing aseptic culture of Russian cultivars has been obtained. The use of donor plants grown in greenhouse allows to avoid seasonality in the work, reduces the degree of contamination with fungal and bacterial spores. The efficiency of sterilization and the viability of explants increase by 48 % and 10.5 %, respectively. It has been established that the maximum number of regenerated microcuttings can be obtained on the 24th–26th day of cultivating the primary explants. The dependence of the multiplication efficiency on the genotype and the number of passages has been confirmed. A high rate of reproduction of the *in vitro* hop culture after the 4th passage has been revealed. It has been shown for the first time that the use of MS nutrient medium with 0.5 mg·L^{-1} IBA allows combining the stages of rooting and micropropagation. With the availability of *in vitro* hop collection, the cloning process is reduced by one passage. To move to the adaptation stage, it is enough to form two nodes and a well-developed root system on the shoot. The two-stage adaptation to non-sterile growing conditions using hydroponics ensures plant survival of up to 100 %. The developed protocol for clonal micropropagation of hops can be used to replicate valuable genotypes for scientific, breeding and production purposes.

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