IRSTI 62.33.29

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IN VITRO CLONAL PROPAGATION OF REPAIRING HYBRIDS OF WILD STRAWBERRY *FRAGARIA ANANASSA* DUCH.

The features of the clonal propagation technology of repairing hybrids of wild strawberry (Fragaria ananassa Duch.) have been studied. Traditionally, the strawberry is vegetative propagated by grafting, but for the repairing varieties of strawberry, this method is less effective because plants form only 1–2 rosettes per plant during the growing season. The clonal micropropagation in vitro is the alternative method of reproduction to vegetative propagation. For the introduction of strawberry in vitro culture apical stolons and non-rooting rosettes collected from April to June have been taken. To obtain polyploid plants formed during callusogenesis process, stem and leaf explants were used. The main medium was Murashige-Skug agar medium (MS) supplemented with plant growth regulators (PGRs) and ascorbic acid (1.5 mg/l), the control medium was MS medium without PGRs. The influence of different concentration of cytokinin (0,3-1 mg/l 6-BAP) on the multiplication and auxin (0,5-1 mg/l IAA) on rooting of repairing hybrids of strawberry in vitro culture have been studied. The optimal concentrations of 6-benzylamino-purine (0.3 mg/l) were determined at the propagation stage and the same for α-indoleacetic acid (0,5 mg/l) on the rooting stage have been determined. Microrosettes with a well-developed root system obtained during in vitro cultivation were acclimatized and grown on hydroponics. Adapted to growth in the open ground plants were used as a planting material, which was propagated in a greenhouse.

Key words: Fragaria ananassa Duch.wild strawberry, clonal micropropagation in vitro, plant growth regulators, planting material.

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Fragaria ananassa Duch. бақты құлпынайдың ремонтантты будан түрлерін *in vitro* клондық микрокөбейту

Fragaria ananassa Duch. ремонтантты бақты құлпынайдың будан түрлерінің микроклондық көбейту технологияның ерекшеліктері зерттелінді. Дәстүрлі селекция бойынша құлпынайды вегетативті жолымен көбейтіп алады, бірақ ремонтантты құлпынай үшін вегетация кезеңі барысында әр 1-2 сабақтан бір ғана бұршақ (розетка) түзілетіні болғандықтан, бұл көбейту тәсілі

тиімді болмайды. Вегетативті көбейту әдістің альтернативасы in vitro клондық микрокөбейту әдісі болып келеді. Құлпынайды in vitro культураға еңгізу үшін сәуір айынан бастап маусым айына дейін жинап алынған тамырланбаған розеткалары және үсті столондар пайдаланылды. Каллусогенез үдерісі нәтижесінде пайда болатын полиплоидтарды алу үшін жапырақ және сабақ эксплантары қолданылды. Қоректік ортаның құрамына аскорбин қышқылы (1,5 мг/л) және өсу реттегіштері қосылған агарланған Мурасиге-Скуг (МС) негізгі орта болып келді, ал бақылау нұсқасы ретінде гормонсыз МС орта болып пайдаланылды. In vitro жағдайында ремонтантты бақты құлпынайдың көбейту коэффициентін және тамырландыруын арттыру мақсатымен әртүрлі концентрацияларда 6-бензиламинопуриннің (0,3-1 мг/л 6-БАП) және ауксин α-индолилсірке қышқылының (0,5-1 мг/л ИСҚ) әсері зерттелінді. Құлпынайдың нақты көбею кезінде 0,3 мг/л 6-бензиламинопуриннің және құлпынайдың тамырландыру кезінде 0,5 мг/л индолилсіркеқышқылдың оптимальды концентрациялары анықталынды. Жақсы дамыған тамырлары бар in vitro өсіру барысында алынған микророзеткаларды бейімделіп гидропоникада өсірдік. Ашық топырақ стерильды емес жағдайға бейімделген өсімдіктер көшет материал ретінде қолданылған. Сонымен қатар сауықтырылған көшет материал ары қарай жылыжайда өсіріп көбейтілді.

Түйін сөздер: Fragaria ananassa Duch. бақты құлпынай, in vitro клондық микрокөбейту, өсу реттегіштер, көшет материалы.

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Клональное микроразмножение *in vitro* ремонтантных гибридных форм земляники садовой *Fragaria ananassa* Duch.

Изучены особенности технологии клонального микроразмножения ремонтантных гибридных форм земляники садовой (Fragaria ananassa Duch.). Традиционно землянику садовую размножают вегетативно с помощью усов, однако для ремонтантной земляники такой способ размножения малоэффективный, так как за период вегетации она образует 1–2 уса на одну розетку. Альтернативным методом вегетативного размножения является клональное микроразмножение in vitro. Для введения земляники в культуру in vitro использовали верхушечные столоны (усы) и неукоренившиеся розетки, собранные в период с апреля по июнь. С целью получения полиплоидных форм, образующихся в процессе каллусогенеза, использовали стеблевые и листовые экспланты. Основной питательной средой была агаризованная среда Мурасиге-Скуга (МС), дополненная регуляторами роста и аскорбиновой кислотой (1,5 мг/л), контролем являлся безгормональный вариант среды МС. Изучено влияние различных концентраций цитокинина 6-бензиламинопурин (0,3-1 мг/л 6-БАП) на коэффициент размножения и ауксина α-индолилуксусной кислоты (0,5-1 мг/л ИУК) на укоренение ремонтантных гибридов земляники садовой в культуре in vitro. Определены оптимальные концентрации 6-бензиламинопурина (0,3 мг/л) на этапе собственно размножения и α-индолилуксусной кислоты (0,5 мг/л) на этапе укоренения земляники садовой. Полученные в процессе культивирования микророзетки с хорошо развитой корневой системой акклиматизировали и выращивали на гидропонике. Адаптированные к росту в открытом грунте растения использовались как посадочный материал, который далее размножали в теплице.

Ключевые слова: земляника садовая Fragaria ananassa Duch., клональное микроразмножение in vitro, регуляторы роста, посадочный материал.

Introduction

The strawberry (Fragaria ananassa Duch.) – one of the most popular berry crops grown in the Northern hemisphere of the temperate zone. This culture is traditionally propagated vegetatively with the whiskers. For everbearing strawberries this method of reproduction is inefficient, as over the period of the growing season it forms 1-2 runners on one socket. This is due to the feature of the structure of the rosette and the laying of vegetative buds in the everbearing strawberries. In addition, when breeding strawberries, many diseases are traditionally transmitted and, as a result, the characteristics of the variety are reduced (Lutov V. I., 2006: 23).

An alternative method of vegetative reproduction is clonal micropropagation in vitro. Biotechnological approaches and techniques contribute to the expansion of assortiment, accelerated introduction of new hybrids everbearing varieties of strawberries, fruiting period which is much longer than traditional varieties. The advantages of these techniques have long been known and among them can be distinguished as follows: obtaining improved planting material; rapid production of vegetative offspring difficult to reproduce forms; obtaining genetically homogeneous material; cultivation regardless of season and climatic conditions throughout the year; obtaining hybrid seedlings from embryos with distant hybridization; work at the polyploid level; long-term storage of the material in vitro.

Clonal micropropagation is one of the biotechnological methods, which fully shows the limitless potential of plants to reproduce.

There are several types of micropropagation:

1. the induction of axillary meristem development;

2. the development of adventitious shoots from the explant tissue;

3. the induction of organogenesis or somatic embryogenesis from callus tissues of plants.

The most common is the first model of reproduction, which is based on the removal of apical domination with cytokinin activity – 6-benzylaminopurine, kinetin. In most studies it is noted that low concentrations of 6-BAP are required for berry crops propagated by means of cellular technologies (Vysotsky V. A., 2011: 3; Dzhafarova V. E., 2010: 72; Dzhafarova V. E., 2015: 29). In the application of the technology of clonal micropropagation should take into account the influence of several factors, key of which is the composition of the nutrient medium and the balance of phytohormones at different stages of cultivation. For cultivation and passage of fruit and berry crops is often recommended Murashige-Skoog environment, Niche, Gamborg, Andersen, Lloyd-Maccoun and others. It is believed that the MS medium is universal for many crops or varieties, but in some experiments it was shown that to increase the rate of reproduction in blackberries and other berry crops, regardless of the form of growth, it is better to use the medium of Lee and de Fossard (Tashmatova L. V., 2014: 63). This environment ensured the formation of a larger number of buds and shoots, and also contributed to the growth of the shoots.

At the stage of rooting, many cultures, especially seed and berry crops have difficulties associated with the formation of a full-fledged root system that provides survival of microbreeds at the stage of adaptation. Usually used different rooting stimulants: IBA, NAA and IAA, as well as different ways of application. For example, in blackberry varieties with different forms of growth, when IMK was introduced into the nutrient medium at a concentration of 0.5-1.0 mg/l, rooting reached 90-100% (Tashmatova L. V., 2013: 20).

Another important factor determining the success of berry crops cultivation is the process of adaptation of micro-transfers to non-sterile conditions. One of the ways to increase the survival rate of microshops is the use of elicitors, which have immunomodulating properties and cause systemic resistance of plants to adverse factors. For example, the use of Russian-made preparations El-1 and Ecost 1/3 increases the survival rate of micro-shoots of strawberry varieties to 69% (Belyakova L. V., 2011: 200).

Despite the large number of works on reproduction and regeneration in vitro of strawberry, to date, remains relevant modification of the basic techniques of micropropagation, since each variety requires its own specific physico-chemical conditions of in vitro cultivation (Alekseenko L. V., 1998: 3; Belyakov, L. V., 2010: 38; Rastorguev L. S., 2010: 57; L. B. Tashmatova, 2015: 19).

Material and Methods

The strawberry (*Fragaria ananassa* Duch.) belongs to the family Rosaceae (Rosaceae). Fragaria ananassa is a natural hybrid of *Fragaria chiloensis* (L.) Duch. and *Fragaria virginiana* (Duch.). The object of these studies was a 3 hybrid everbearing strawberry foreign selection: ZH 15-3, 14-3 ZH, ZH 14-1-3. Donor plants were grown using drip irrigation technology on the black tape.

As a source of plant material for the introduction of strawberries into the culture in vitro were used apical pillars (runners) and unburdened rosettes collected in the period from April to June 2017. Taken in the nursery planting subjects the hybrid strawberry was sterilized according to the following scheme: free from soil mustache and outlets were exempt from the upper leaves, within 15 minutes, rinsed with water, using detergent and, subsequently, for 30 minutes, washed under running water. Then, in the laminar-box, the initial material was processed by step sterilization for 3 seconds with 70% ethyl alcohol, then with various disinfectant solutions for 5 minutes. As sterilizing agents were used: commercial household product «Domestos «(diluted with sterile distilled water in a ratio of 1:3); commercial household product «Belizna»

(5%); hydrogen peroxide, 3% solution. Treated vegetable material was washed several times with sterile distilled water.

After carrying out surface sterilization under aseptic conditions was performed to isolate the meristematic apex of buds and rosettes. Sheet and stem fragments were also used to produce callus. Explants were placed on agarized modified medium MS (pH 5,6-5,8). Modification of the environment was in addition to the main part of the standard medium 1.5 mg/l of ascorbic acid (AA), used as antioxidant, and growth regulators 6-benzylaminopurine (BAP) at a concentration of 0,3; 0,5; 1 mg/l. This nutrient medium was used for culturing explants at the stage of actually breeding of hybrid strawberry. At the stage of rooting applied nutrient MS medium containing indoleacetic acid (IAA) in concentrations 0.5 and 1 mg/l. In the control used the MS medium without any growth regulators (Murashige T., 1962: 489).

Cultivation of explants was carried out at 16-hour photoperiod for 3-4 weeks. Intensity of illumination ranged from 5 to 10 kLux in the culture room with air-conditioned maintained at temperature of 25 ± 2 °C and humidity of 70%. Accounting and monitoring were carried out at the end of each passage, after 10-15 days.

Rooted regenerates were grown on hydroponics different mineral composition, mg/l:

A – $Ca(NO_3)$ x $4H_2O$ 320; KNO_3 320; $5Ca(NO_{3)2}$ x $2H_2O$ 720; NH_4NO_3 720; Fe-EDTA 24;

B – $MnSO_4$ x $4H_2O$ 2,1; KNO_3 320; $MgSO_4$ x $7H_2O$ 380; KH_2PO_4 180; NH_4PO_4 20; K_2SO_4 10; H_3BO_4 2,8; $ZnSO_4$ x $7H_2O$ 1,44; $CuSO_4$ x $5H_2O$ 0,19; Na_2Mo_4 x $2H_2O$ 0,12.

All experiments were carried out in three repetitions. Statistical data processing was carried out using the Microsoft office Excel 2007 application package.

Results and Discussion

The results show that the sterilization of initial plant material is the most effective at sterilizing agent 5% solution of sodium hypochlorite (commercial name «Belizna»). The percentage of contamination in the treatment of sodium hypochloride was 40%, while sterilization with aqueous solution of commercial means «Domestos» led to infection 70.83% of isolated explants. Sterilization of the initial material with 3% hydrogen peroxide solution was the least effective – 89% of the explants were infected. Thus, among chlorine-containing means, at the identical mode and time of pretreatment, the commercial preparation «Belizna» was 1,7 times more effective, than «Domestos». A solution of hydrogen peroxide, which is the most common and frequently used sterilizing agent, proved to be less suitable for the sterilization of vegetative organs of strawberry (tab. 1).

Table 1 – Results of the sterilization of the initial plant material of strawberry garden with various sterilizing agents

Sterilizing agent	Time of pretreatment, min	The percentage of contamination, %
Commercial household preparation «Domestos» (1:3)	5	40,00±1,23
Commercial household product «Belizna» (5%)	5	70,83±6,47
Hydrogen peroxide (H_2O_2), 3%	5	89,00±7,17

The study of the impact of different concentrations of 6-BAP on the sprouting and increase in the breeding rate of strawberry genotypes showed that the modified nutrient medium Murashige-skuga, supplemented by 0.3 mg / l, noted a very rapid development of micro-shoots in the culture of axillary meristems (table. 2). The use of low concentrations of 6-BAP induced not only regeneration processes in the culture of meristems, but also the formation of morphogenic callus in the culture of stem explants (Fig. 1, 2).

The addition of 6-BAP in the minimum concentration resulted in the development of 1.5–2 axillary buds on the average on the microrosette. Separating the shoots and re-placing them on medium with 6-BAP, cycles can be repeated within a certain period of time, with the required amount of planting material. Increasing the concentration of 6-BAP to 1 mg/l led to suppression of growth and development of explants. Similar results were obtained in studies of Russian scientists, who also pointed out that high concentrations of 6-BAP (5-

10 μM) reduced the height of strawberry sockets from 2-3 to 0.5-1 cm, which further hindered their rooting. In addition, the high concentration of cytokinine in the medium (10.0 μM of 6-BAP) reduced the appearance of rosettes (Rastorguev L.S., 2010: 61).

Figure 1 – Formation of plants-regenerants in the culture of meristems of strawberry Fragaria ananassa Duch. on an MS medium supplemented by 6-BAP 0.3 mg/ l

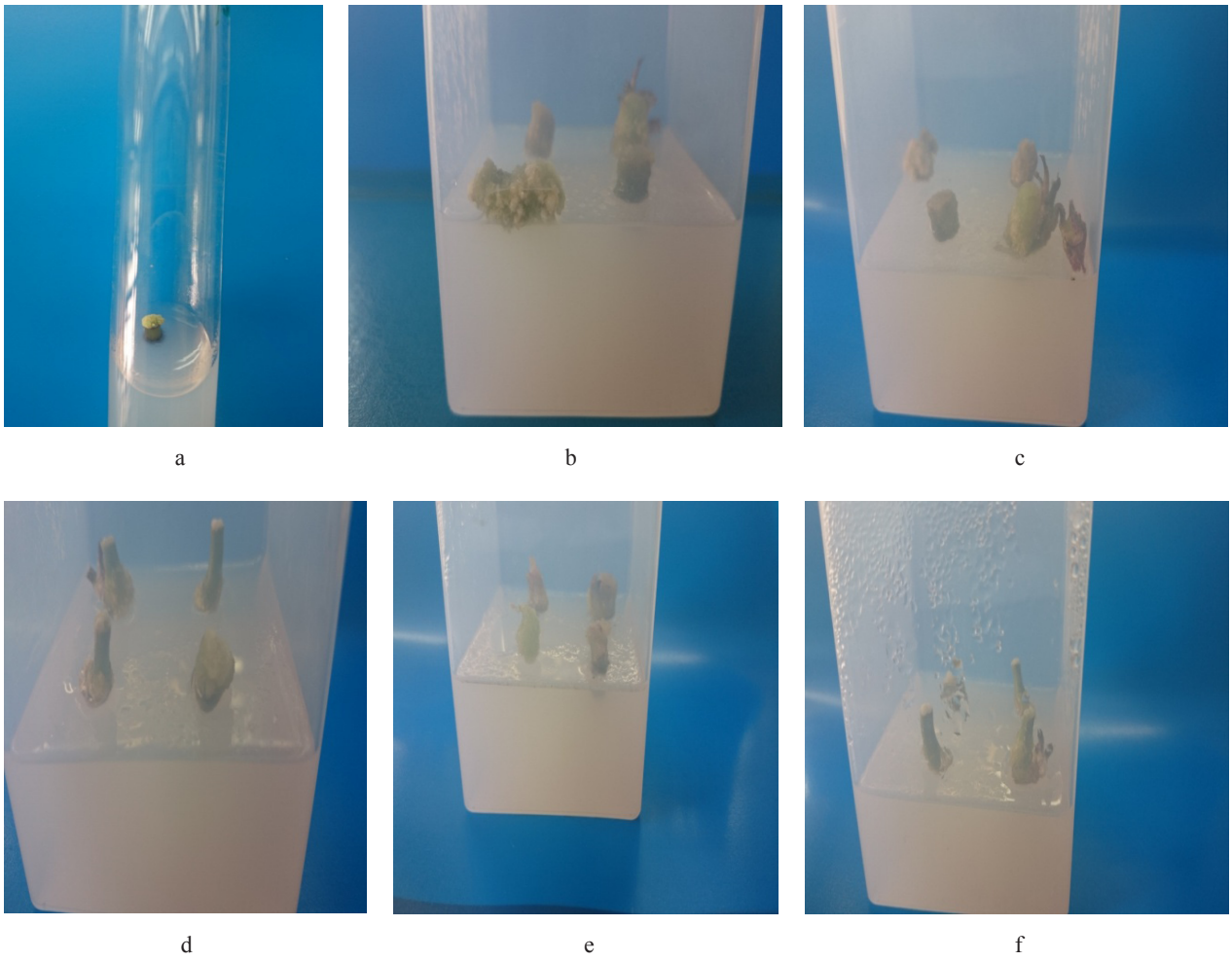
Table 2 – Frequency of regeneration and callus in the culture of apical meristem and stem explants of strawberry garden

Medium	Explant	Frequency of regeneration, %	Frequency of callus, %
MS + 0,3 mg /l 6-BAP +1,5 mg /l AA	stem	15,0±1,1	85,0±7,19
	apical meristem	10,0±1,8	0
MS (control)	stem	0	29,16±1,47
	apical meristem	0	0

Obtaining callus, and then regeneration of them polyploid forms of strawberry garden is of great interest to breeders. Among the polyploids of berry crops, triploids are the most popular, because they differ in a good taste of berries, regular fruiting and high adaptability.

Known studies, which were obtained polyploid plants in culture in vitro (Tashmatova L.V., 2014: 62; Tashmatov L.V., 2013: 18). The material for induced polyploidization was apical and adventive buds, which were affected by a colchicine. In our studies, the initial explants (leaves and stems) were not pretreated by mutagenic chemical agents, as spontaneous polyploidization of callus tissue took place in the process of callusogenesis.

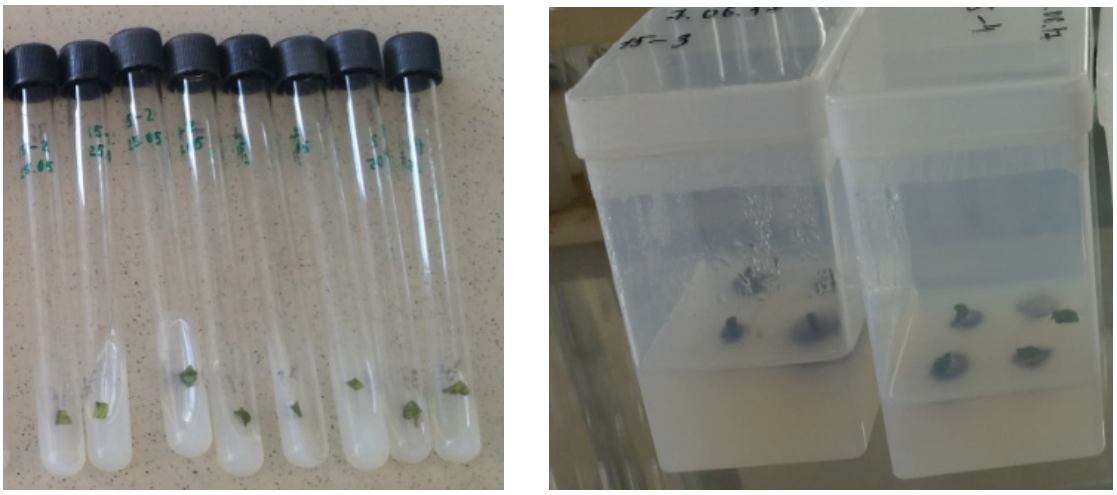
In the culture of leaf explants was observed a slight darkening of the medium around the explants, which testified to exogenous secondary metabolites secreted by the cells of the leaf. However, the presence of ascorbic acid as antioxidant in the medium, suspended minor necrotic processes observed in the cut of fragments of the leaf blade (Fig.3).



a b c

d e f

Figure 2 – Production of morphogenic callus in the culture of stem explants of strawberry garden (A, B, C, D, E,) and regenerants in callus culture Fragaria ananassa Duch. (C, F)



а в

Figure 3 – Leaf explants culture of garden strawberries on MS medium with growth regulators and ascorbic acid (a); on control medium with dark areas around the explants (B)

For rooting, regenerated plants were transferred to MS medium containing 0.5 mg/l and 1 mg/l IAA. On non-hormonal nutrient medium all studied genotypes of strawberries were not rooted. The use of IAA in the nutrient medium at a concentration of 0.5 mg/l did not affect the change in the frequency of regeneration, but stimulated the rooting of microrosettes. Formed roots were not thin, long enough and had root hairs (roots of the second order). Well-developed root system provides further better adaptation of strawberry plants in ex vitro conditions. The use of IAA in higher concentrations was inappropriate, since the process of rhizogenesis is significantly inhibited. Thus, low concentrations of auxin, in particular 0,5 mg/l of IAA are recommended for rooting of shoots of hybrid forms of strawberries.

As a result of the cultivation of exhibits in vitro microrostettes with a well-developed root system, which was acclimatized and grown on hydroponics (Fig.4). The hydroponic plant used solutions (A, B) with different composition of mineral salts. The results of studies have shown that the most optimal for the development of the root system is the B mineral composition, which is more enriched by the content of mineral salts necessary for the growth and development of strawberry roots and adaptation to the conditions of open ground.

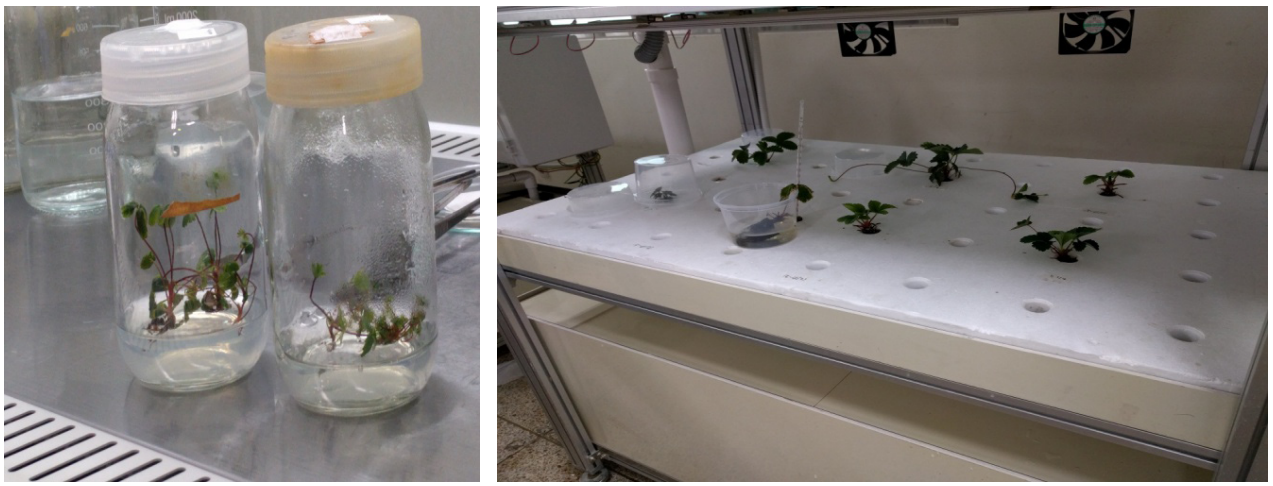


Figure 4 – Cultivation of microrosettes in vitro on the medium for rooting and growing on hydroponic installation

Plants adapted to non-sterile conditions were used as planting material, which was grown and propagated in a greenhouse.

Conclusion

Strawberry garden is the most profitable berry crop and now the question of laying of industrial plantings by the certified highly productive material in sufficient quantity is acute. It is known that biotechnological methods play an important role in improvement and replication of vegetable material on an industrial scale. Recommended Protocol of optimized nutrient media for cultivation of stem apex, leaf explants and microrosettes everbearing forms of strawberry allows recovery of the valuable genotypes of berry plants, create and maintain genetic collection, which ultimately will increase the efficiency of science and commercial production for obtaining high quality planting material of fruit crops.

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