საქართველოს მეცნიერებათა ეროვნული აკადემიის მოამბე, ტ. 17, №3, 2023
BULLETIN OF THE GEORGIAN NATIONAL ACADEMY OF SCIENCES, vol. 17, no. 3, 2023

Biotechnology

Goji Berry (*Lycium Barbarum* L.) Plant-Obtaining Regenerants through Adventitious Bud Formation *in vitro* Culture

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Goji berry (*Lycium Barbarum* L.) is a very popular crop in world horticulture. Plantations require large-scale production of high-quality, healthy seedlings. The purpose of the research was developing the stages of introducing Goji berry into *in vitro* culture and its micro clonal propagation. Branch cuttings with dormant buds and vegetative shoots were used as explants. 0.2% aqueous solution of dioside was used as a sterilizing agent to obtain sterile, viable cultures with 10-12 minutes exposure. Subculturing of explants was carried out on the medium area of Gamborg (B5). Hormones (BAP; Zn; NAA) and their concentrations were selected to optimize the reproduction ratio. Mass proliferation of buds was achieved by the formation of adventitious buds from the morphogenic tissue developed in the basal part of the main explant. An optimal method for rooting micro cuttings using IBA (indole butyric acid) has been developed; Plants-regenerants with high adaptability and good ability to grow and develop in the soil have been obtained. *© 2023 Bull. Georg. Natl. Acad. Sci.*

Gamborg (B5) medium, explant, adventitious shoot regeneration, hormones, plants-regenerants

Goji berry (lat. *Lycium Barbarum* L.) is a popular crop in world horticulture. Its fruit is characterized by unique taste and healing properties. Due to its unique properties, Goji berry fruit is biologically important. Traditional vegetative propagation of Goji berry by cuttings is impractical due to high demand on mother plants, limited seasonal growth and non-uniform progeny production as a result of generative propagation.

The development of Goji berry in *in vitro* propagation techniques is quite effective for rapid mass production of healthy planting material, large-scale cultivation, germplasm improvement, gene conservation and other research purposes.

Materials and Methods

The object of the research – the plant Goji berry belongs to the *Lycium barbarum* family, it is a

small bushy plant. Vegetative young shoots are yellowish in color and covered with small spines. The leaf has an elongated shape, the surface is green, and the lower side is light-gray in color [1]. The flower crown is purple or pink It blooms throughout the growing season, that is why Goji berry is used as an ornamental plant as one of the components of the landscape of gardens and parks. Goji berries are not consumed raw. Mostly dried fruits are used. Goji berries are characterized by a sweet-sour taste. The plant has strong root system, thanks to which the plant easily adapts to different types of soil and humidity [1].

Harvesting starts from May and lasts until October. Harvesting can be done several times during one season. The juvenile period of the plant is two to three years. Adult mother plants, from which the primary explants were isolated, are grown in the greenhouse at the department of agrotechnology of the faculty of technology of the same university.

Explant preparation: In the first stage, at the end of February and the beginning of March, the cuttings of the previous year's shoot with one dormant bud were isolated from the native mother plant as an explant and introduced into the culture. In the second stage, at the beginning of the vegetation, we used the apical buds of the vegetative shoots, which were isolated from the mother plant in April and May.

Apical shoots and axillary bud cuttings were washed with Tween-80, soaked under running water for 30min, then rinsed with distilled water several times. We performed the following works in a sterile environment – in a laminar box. For surface sterilization of explants we used 0.1% and 0.2% aqueous solution of diocide with 7-10-12 minute exposure. We washed the explants with sterile water 3-4 times, left them in the last portion of water for disintoxication for 15-20 minutes. After sterilization we sowed the explants in the nutrient area for the first time.

Nutrient area and growth regulators: For the cultivation of explants we used nutrient area prepared according to the formula of Gamborg (B5) [2], which was supplemented with 0.6% (w/v) agar, 3% (w/v) sucrose. At the zero stage of micropropagation we used the minimum concentration (3mol) of 6-benzylaminopurine (BAP) from cytokinins from the plant growth regulators, while at the micropropagation stage itself, six options were selected for the optimization of the process based on the content of growth regulators, namely BAP and Zeatin with 10; 15 and 20 mol concentrations. Together with cytokinins, naphthylacetic acid (NAA) from auxins was added to the nutrient area, in all variants with the same concentration (3 μM). The pH of the nutrient area was 5.6-5.8; Sterilization was done under 0.9-1±0.1 atmospheric pressure for 20-25 minutes. We chose several ways to root micro-shoots:

1. Ex-vitro/in vivo rooting by placing 15-20 mm long microshoots in 20 μmol indole butyric acid (IBA) solution for 20-30 minutes, followed by direct planting in 50 mm diameter pots filled with peat and soil.

2. In vitro rooting on ½ nutrient area of Gamborg (B5), added 10 mol IBA.

Physical conditions of cultivation: Incubation of cultures was carried out – on light, illumination 3000 lux, 16/8 hours photoperiod, temperature – 27±1°C, duration of zero subcultivation was 20 days and in different variants of the experiment, one subcultivation lasted 25-28 days.

Acclimatization of plant-regenerants: We used different methods to adapt plant-regenerants. 1) Due to the fact that a large part of plant-regenerants die during the acclimatization stage together with the laboratory staff. We developed an unusual way of acclimatization, which allowed us to make 85-90% of the plants viable after transfer to the ground. This method consists in the following: we placed rooted plant-regenerants in the same culture vessel in which we rooted under

temperature conditions of -25±1°C. At the same time of the day we opened the lid of the cultural vessel for 5 minutes on the first day and in the following days we increased it multiple times (10.15, for 20 minutes, etc.). During the next five days, we opened the lid of the cultures twice a day for 30 minutes. The next three days we left it in the ambient air for 4 hours, the next three days - for 8-10 hours, then 24 hours for two days and only after that we transferred it to the substrate. We used a mixture of soil, sand and perlite in the ratio of 2:2:1 as a substrate.

2) In the second case, we planted rooted plant-regenerants on a substrate of a mixture of soil, sand and perlite (2:2:1). We covered the plants with plastic jars and moved them to the greenhouse (temperature 25±1°C, relative humidity 80-90 We started reducing humidity after 8-10 days and continued for 30 days. We transplanted the acclimatized plant-regenerants into larger pots, left them for another 30-45 days in the greenhouse and transferred them to the ground only after intensive growth began. The acclimatization period was 50-60 days on average. The percentage of regenerated plants that survived was documented and recorded after 60 days.

The evaluation of the results of the experiment: was carried out daily after sterilization during the zero subcultivation period and we determined the effect of sterilization as a percentage on the 25th day. We determined the micropropagation coefficient before each subcultivation, the number of obtained sprouts compared to the number of sown explants. The rooting ratio was calculated as the number of rooted explants compared to the total number of explants. Statistically processed in excel, data evaluation was performed using statistical dispersive analysis of variance and multiple range analysis.

Experiment Results and Discussions

Effect of sterilizing substances on obtaining aseptic viable cultures. Sterilization and selection of concentrations of sterilizing substances is an important stage of successful *in vitro* cultivation of Goji berry. A comparative analysis of the obtained results confirmed that in the options where 0.2% aqueous solution of diocide was used, exposure for 12 minutes was more effective for cuttings with one dormant bud than exposure for 7-10 minutes. The percentage of sterilization was 82% and 62% with 0.1% aqueous solution, and most of the sterile explants (80%) were viable at both concentrations (Table 1).

Better surface sterilization results were obtained when the apical buds of vegetative shoots were introduced into the culture, in this case 0.2% aqueous solution of diocide was also more effective, but here the exposure was reduced to 10 minutes, increasing the sterilization exposure by 12 minutes when using 0.2% aqueous solution increased the sterilization effect, but reduced the

Table 1. Influence of concentrations of sterilizing substance on the reception of aseptic explants

Sterilants solution	Exposition (min)	Sleeping buds		Vegetative shoots	
		The number of sterile ex- plants %	The number of Viable ex- plants %	The number of sterile ex- plants %	The number of Viable ex- plants %
Diocide 0,1 % solution	7	42.8	70.2	58.0	90.0
Diocide 0,1 % solution	10	54.6	77.8	70.0	100.0
Diocide 0,1 % solution	12	62.0	80.0	80.5	78.6
Diocide 0,2 % solution	7	62.3	75.0	72.0	68.2
Diocide 0,2 % solution	10	74.0	76.2	83.4	100.0
Diocide 0.2 % solution	12	82.0	80.2	94.5	88.0

viable number of explants (88%). The sterilization effect of the 0.1% solution was 70%, and the number of viable cultures in this variant was 100% (Table 1). Thus, to obtain non-infected viable explants, the described mode of surface sterilization: 0.2% diocide aqueous solution for 10-12 minutes is effective for obtaining aseptic cultures of Goji berry at the zero stage of cultivation. And for introduction into the culture as an explant, it is better to use the apical buds of vegetative shoots.

The literature describes the effectiveness of the use of aqueous solutions of various sterilizing substances for the successful surface sterilization of Goji berry seedlings [3-5], in which sterility was achieved through a commercial bleach, including the active ingredient Sodium hypochlorite [6] or Calcium hypochlorite. In experiments on different cultures, scientists achieved high sterility of explants through aqueous solutions of silver-containing salts, the percentage of sterile cultures was maximal [7,8].

Bud regeneration through adventitious organogenesis. Adventitious bud formation is a widespread form of organogenesis in in vitro culture. It is regulated by phytohormones of different classes and concentrations [9]. Adventitious budding refers to propagation through buds that develop directly on explant tissue or on secondary callus. Adventitious buds are formed on both despecialized meristem tissue and tissue containing specialized cells. The genotype of the plant also has a dominant influence on this process. In our experiment, there was a different response of explants to the induction of organogenesis due to the nature and different concentrations of phytohormones included in the nutrient area. For the initiation and optimization of the micropropagation process, the hormones included in the nutrient area were a necessary condition for the induction of bud development, because in the hormone-free nutrient area, only the primary explant growth in height was observed without the induction of organogenesis processes.

The results of the experiment showed us that adding BAP to the nutrient area was more effective compared to Zeatin. Adventitious buds were formed under the influence of both hormones. They showed similar morphogenetic effects to induce organogenesis from explants.

Initiation of morphogenetic areas and regeneration of buds from them was carried out from the basal part of the main explant, this process was preceded by the development of asynchronously growing callusoid tissue in the hypocotyl area of the explant, which was facilitated by NAA included in the nutrient area. The formation of morphogenic reddish nodules on it was initiated during the II subcultivation period and continued intensively in the following subcultivations as well. At the same time, the emergence of primary leaves from the germinal buds was also taking place. At the end of IV subcultivation, the average number of buds per explant increased to 35-40 units in accordance with the concentrations of cytokinins included in the nutrient area. As can be seen from the Table, different concentrations of BAP affected the quantitative index of adventitious organogenesis with different efficiency. The micropropagation coefficient was maximal when using a concentration of 10μM. A higher concentration (15μM) resulted in the formation of morphogenetic nodes, primary leaves and buds, but inhibited the growth process of buds. A high concentration of cytokinins in the nutrient zone led to the removal of the apical dominance of the formed buds and, accordingly, the adventitious or axillary meristem was activated. The introduction of BAP at a concentration of 20μM into the feeding area was accompanied by a significant decrease in the reproduction coefficient. It prevented the induction of callus in the base of the explant and, accordingly, the intensity of its proliferation. The average number of adventitious buds per explant decreased to 8 units (Table 2) and all of them were characterized by apical dominant growth. The morphology of the formed buds also changed. The leaf stalk and plates were shortened

Table 2. Effect of hormones on micropropagation of Goji berry

Variant	BAP	Zn	NAA	Average number of adventitious buds	Average number of dominant buds	Average number of leaves per explant
Control	-	-	-	-	1.0±0.2	2.0±0.4
I	10	-	3	35,1 ± 4.5	30,0 ±3.8	3,5 ±0.4
II	15	-	3	40,0 ± 5.2	20,4 ±2.6	2,8 ±0.6
III	20	-	3	8,0 ±1.0	8.0 ±1.0	2,5 ±0.5
IV	-	10	3	25,0 ± 3.2	14.0 ±1.8	2,8 ± 0,6
V	-	15	3	28,0 ±3.6	10,1 ±1.2	2.2 ±0.4
VI	-	20	3	34,0 ±4.3	6.0 ±0.7	2.0 ±0.4

and thinned. At the point of contact of the leaf plates with the nutrient area, morphogenetic areas were formed, from which the direct regeneration of plants took place.

For Goji berry explants a concentration of 20 μM of BAP was the optimal upper limit, above which higher concentrations caused the development of buds with different anomalous morphology [7]. During clonal micro-propagation, methods based on cultivation of apically growing buds are preferred [10]. In these works, the proliferation efficiency of cultures was obtained directly from the explant tissue through different con centrations of BAP and 2-ip. In all cases, the advantage of BAP on the formation of organogenic cells and the regeneration of buds is described. In general, plant regeneration from callus tissue is much more difficult and limited by genetic factors than the induction of micropropagation in tissue culture [9]. Accordingly, the number of varieties that are characterized by callusogenesis reproduction is quite small. Different combinations of concentrations of growth regulators used for callus induction and plant regeneration from callus from different explants (leaf, root, hypocotyl and bud) of Goji plants were particularly successful. The percentage of regenerants formation (100% and the highest number of shoots (23.33±1.86) were obtained from callus obtained from bud explants on a nutrient area containing 0.5mg/L BA [11].

Our research has confirmed that the best way for in vitro reproduction of Goji berry is bud culture, and the effective phytohormone – BAP. The findings of our experiment correlate with Rania Taha's research [9] who states that BAP regulates cell division, new bud induction, apical bud dominance, as well as root formation along with NAA, this is due to the fact that BAP is more stable than other cytokines. It is less sensitive to light oxidation and can be easily synthesized in the plant body. Takeshi Hirakawa [12] reported in his study that cytokinin inhibits the formation of axillary buds of Humulus lupulus, but together with gibberellin causes the axillary buds to increase in length.

Rooting and acclimatization of microclones: for rooting, we selected buds higher than 25 mm. The results showed that among the options selected for rooting, in vitro rooting on of the nutrient area of Gamborg (B5) with 10mol of IBA was effective. Rooting percentage was 96%. Plant-regenerants developed a root system in a short time (18-25 days), at the end of subcultivation, the roots were well branched and rooted, which is important for the acclimatization process. In the process of rooting, the growth of regenerants also took place in parallel, the creation of green biomass increased the probability of adaptation of the plant in the ex vitro environment. Scientific papers described the effects of NAA and indolylacetic acid (IAA) on rhizogenesis, but the resulting root system was very weak [13]. In the same paper, it was shown that 20 ppm

of the biologically active substance – chitosan was better than auxins in initiating rhizogenesis of Goji plants, while the plants were characterized by a stronger habitus and a complete root system [13]. Like us, Taha also uses IBA for microbud rooting and before transplanting into the soil, the plants were characterized by a well-developed root system [9].

A well-developed root system produced in our experiment by ex- vitro/in vivo rooting of plant-regenerants, but the drawback of this method is that it takes a long time to develop the root system and the rate of adaptation to the soil was low, 50% of the buds died.

As for acclimatization – the first method used for adaptation of plant-regenerants turned out to be very effective and 85-90% of the plants were viable after being transferred to the ground. In the second case, the acclimatization period was 50-60 days on average. The percentage of surviving plant-regenerants was 65%.

Conclusions

The conducted experimental research showed us the possibility of successfully using the in vitro method for Goji berry micropropagation;

– vegetative buds are the best material for obtaining viable cultures in the form of primary explants;

– to obtain non-infected and viable cultures, the use of a 0.2% aqueous solution of diocide as a sterilizing substance was found to be the best, with an exposure of 10-12 minutes;

– to increase the reproduction coefficient of Goji berry in vitro culture, the best results were obtained by adding BAP with a concentration of 10-15μm to the nutrient area with 3μm of NAA;

– an optimal method for rooting micro cuttings using IBA was developed;

– regenerative plants are characterized by a fairly high adaptability. Acclimatization to non-sterile conditions allows us to grow and develop the resulting plants in soil.

ბიოტექნოლოგია

გოჯი ბერის (*Lycium Barbarum* L.) მცენარე-რეგენერანტების მიღება ადვენტური კვირტწარმოქმნის გზით *in vitro* კულტურაში

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გოჯი ბერი (*Lycium Barbarum* L.) მსოფლიო მემცენარეობაში მეტად პოპულარული კულტურაა. მისი ნაყოფი შეიცავს ორგანიზმისათვის აუცილებელ ბიოაქტიურ ნივთიერებებს, რის გამოც, გოჯი ბერის ნაყოფი ბიოლოგიურად მეტად საინტერესოა. გოჯი ბერის ტრადიციული ვეგეტაციური გამრავლება კალმებით არამიზანშეწონილია სადედე-მცენარეებზე დიდი მოთხოვნის, შეზღუდული სეზონური ზრდისა და გენერაციული გამრავლების შედეგად არაერთგვაროვანი შთამომავლობის წარმოქმნის გამო. გოჯი ბერის *in vitro* გამრავლების ტექნიკის შემუშავება საკმაოდ ეფექტურია გაჯანსაღებული სარგავი მასალის სწრაფი მასობრივი წარმოებისთვის. შემუშავდა გოჯი ბერის (*Lycium Barbarum* L.) *in vitro* კულტურაში შეყვანისა და საკუთრივ მიკროკლონური გამრავლების ეტაპები, ექსპლანტს წარმოადგენდა გამერქნებული კალმები მძინარე კვირტებით და ვეგეტირებადი ყლორტები, სტერილური სიცოცხლისუნარიანი კულტურების მისაღებად მასტერილებელი ნივთიერების სახით გამოყენებული იყო დიოციდის 0,2% წყალხსნარი 10-12 წუთით, ექსპლანტების სუბკულტივირება ხორციელდებოდა გამბორგის (B5) საკვებ არეზე. გამრავლების კოეფიციენტის ოპტიმიზაციისთვის შეირჩა ჰორმონები (BAP; Zn; NAA) და მათი კონცენტრაციები. მიღწეულ იქნა კვირტების მასიური პროლიფერაცია ადვენტური კვირტების წარმოქმნით ძირითადი ექსპლანტის ბაზალურ ნაწილში განვითარებული მორფოგენური ქსოვილიდან. შემუშავებულია მიკროკალმების დაფესვიანებისათვის ოპტიმალური ხერხი იემ-ს გამოყენებით; მიღებულია მცენარე-რეგენერანტები მაღალი ადაპტაციისა და გრუნტში ზრდა-განვითარების კარგი უნარით.

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Received May, 2023