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Plant Growing

Peculiarities of Morphogenesis of Cherry Laurel *Laurocerasus officinalis* Ro. (*Prunus laurocerasus* L.) in Vitro Culture

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The possibility of using the plant tissue, cell and organ for microclonal propagation and massive regeneration of cherry laurel *Laurocerasus officinalis* Ro. (*Prunus*) in vitro is considered in the paper. The tissue culture method ensures rapid and massive propagation of genetically similar forms of a plant retaining morphological, biochemical and physiological characteristics of the donor plant that is determined by totipotency of the cell, i.e. realization of complete genetic information in optimum conditions. The advantage of the cell culture technology is that it provides growth and development of the plant throughout the year. It allows propagation of such plants whose vegetative propagation is difficult or impossible. The results of the study showed that in vitro method can be successfully used for microclonal propagation of cherry laurel. It is recommended to use Anderson's medium and to introduce cytokinin into it. The best raw material for obtaining the viable explants is the apical and axillary buds of the vegetative shoot. For the experiment the primary material was isolated from the adult, intact, field-grown plant and greenhouse intact plant in both juvenile and adult generative phases. Apical and axillary buds were used as the explants. The results of the experiments showed that the explants, which were isolated from the donor plant in the juvenile phase of development, are characterized by a faster growth rate than the explants isolated from the donor plant in the generative phase. Cherry laurel is characterized by a rather high acclimatization ability to non-sterile conditions and can be transferred to greenhouses at any time of the year. *© 2021 Bull. Georg. Natl. Acad. Sci.*

Cherry laurel, in vitro culture, explant, cytokinin, regenerative plants, callusogenesis

Nowadays, alternative methods of propagation are widely used. One of such unconventional methods is the isolated plant cell and tissue culture method. Tissue culture method ensures rapid and massive propagation of genetically similar forms of a plant retaining the morphological, biochemical and physiological characteristics of the donor plant, which is determined by totipotence of the cell or the realization of complete genetic information in optimum conditions.

Plant cell culture technology, as a non-traditional method of propagation, provides high propagation rate and allows modelling, implementation and control of morphogenetic

processes as well as the study of the changes occurring in the process of propagation of rare genetic forms, production of somaclonal variants and cultivation of regenerative plants, and also provides the "Bank" and "Gene Fund" of the valuable elite plants [1, 2]. The mentioned method is widely used in genetics and selection of plants such as cherry laurel *Laurocerasus officinalis* Ro. (*Prunus laurocerasus* L.).

The goal of the study was to introduce cherry laurel into in vitro culture in order to develop a microclonal propagation method and to study the characteristics of callusogenesis, embryogenesis and regeneration, and to obtain regenerative plants. Based on the goal of the present study, our task was: to select the explants, sterilizers and their exposition; to introduce them into in vitro culture; to develop and optimize the conditions for microclonal propagation of cherry laurel; to induce callusogenesis and meristems; to study the peculiarities of organogenesis; to study the influence of growth regulators and different components of nutrient medium at all stages of morphogenetic processes of microclonal propagation of cherry laurel.

The advantage of the cell culture technology is that it provides the plant growth and development throughout the year. It allows propagation of such plants whose vegetative propagation is difficult or impossible.

Material and Methods

Cherry laurel *officinalis* Ro. (*Prunus lauro-cerasus* L.) (Fig. 1) belongs to the Rosaceae family. It is an evergreen tree or shrub from 1 to 5 m in height with short-stemmed or large oval shiny leaves and white thin, short-stemmed aromatic flowers. The fruit is blackberry. There are about 25 species distributed in Eurasia, North America, the Antilles, western Georgia and Asia Minor. They are grown as orchards or ornamental plants. They occur in the forests of the Caucasus, at an altitude of 1700 meters above sea level. Unlike other evergreens, it is a frost tolerant plant that can withstand frosts up to -250℃.

The cherry laurel leaves contain glycosides, essential oils, tannins, fats, mucus. The content of amygdalin determines its anesthetic action. The extract of the leaves is used against gastrointestinal pain, skin rashes and nerve diseases.

Fig. 1. Chery Laurel in flowering period.

From the cherry laurel fruit some soft drinks are prepared and from the leaves the "Laurel Water", which is used in medicine. The leaves of cherry laurel contain tannins, which are used in leather manufacture. Ripe cherry laurel fruit contains 50-52 mg% vitamin C, 22.5% tannins, 15-20% sugars. The raw fruit is used for fever reduction. Cherry laurel fruit has nutritional, dietary and medicinal values. There are some preparations used in medicine, which are produced from the leaves and other parts of the plant. The substances contained in cherry laurel fruit can neutralize harmful radioactive agents in the human body.

The following cherry laurel species and forms are worth noting: common laurel, sweet black-fruit cherry laurel (Nakifu), sweet white-fruit cherry laurel; Form 14 (sweet black-fruit Chlow), Form 28 (large red-fruit), Form 29 (sweet black-fruit), Form 33 (pink), Form 40 (black-fruit Narazeni) [3].

The research methodology implies rapid and massive propagation of genetically similar forms of the donor plant by means of tissue culture method for receiving the high-quality seedlings.

Methods of sterilization of the source material. At the initial stage of introduction of the explant into in vitro culture, one of the most difficult and time-consuming processes is optimization of sterilization conditions and selection of proper sterilizing agents. We studied and tested the action of the following aqueous solutions as sterilizing agents:

a) Commercial chlorinated solution. Composition: sodium hypochlorite water solution in ratio of 50/50 and 25/75;

b) Preparation Diocide. Composition: Ethyl *alcohol-mercuric chloride*, cetylpyridinium chloride ½ in the ratio of 0.1/0.2 and 0.5 aqueous solutions.

c) 10-15% aqueous solution of chloramine B added by a few drops of Tween-80 as a surface sterilization activating agent [4-6].

Table 1 shows that the yield of uninfected material was low - 20% and 32%, respectively. The commercial chlorinated aqueous solution had relatively better result with 35-49% of uninfected material; the positive effect of surface sterilization was achieved by the use of the preparation Diocide. Of all the tested concentrations the best result was achieved by 0.5% solution, under the action of which 80% of material was disinfected. However, the viability of aseptic cultures was lower than in the case of other sterilizing agents, as the Diocide is highly toxic and its high concentration caused tissue intoxication.

It should be noted that it was not only the concentration of sterilizing agents that had an influence on the yield of uninfected viable material, but also the exposition, i.e. duration of the explants exposure in the sterilization solution.

The results of the surface sterilization procedure showed that the yield of viable aseptic material depended on the nature of the sterilizing agent, the explants exposure in the solution and the type of the explants.

In the open ground the plant is inhabited by a number microflora of various etiology, which do not have much influence on the viability of the plant in vitro. Most of them are in symbiosis with the host. For cultivation of plants in vitro it is necessary to remove the microflora, which is highly problematic because the sterilizing agents are chemicals causing different toxic effects on the living tissues. Therefore, they are selected empirically based on experiments and observations. Also, the anatomical-morphological structure of the explant is very important for selection of the sterilizing agent. Consequently, the explant was sterilized as follows: First, the material intended for transferring to the nutrition medium was thoroughly washed under the running water to receive the sterile and well-growing explants. Then 75%-household soap together with water was carefully shaken in the jar so that not to damage the

Table 1. Sterilizing Agents

Sterilizing solution	Concentration %	Exposition (min)	Non-infected, %	Viability
Chloramine B	10	20	20	82
	15	20	32.0	78
Commercial chlorinated solution	25	20	35.0	100
	50	20	49.0	100
Diocide	0.1	15	34.0	93.0
	0.2	15	58.0	92.0
	0.5	15	86.0	69.0

buds; the process took 10 minutes. Then it was rinsed two or three times with distilled water. The other manipulations were performed in aseptic conditions in a special device - the laminar box. The primary material was placed into 75% ethyl alcohol for 1-2 minutes and then transferred into a sterilization solution. We used thimerosal (Mercurous *nitrate*) as a sterilizing agent. The primary experimental material was obtained from the medicinal donor plant of cherry laurel found in both above the ground and under the ground.

From adult plants we used: a) the axillary and apical buds, leaves and stems of young shoots of the plant grown in the nursery plantations; b) the axillary and apical buds of the plants vegetated in the greenhouse.

From juvenile plants we used: a) dormant and apical buds, stem tissue and leaves of 3-4 months old plants grown in the greenhouse; b) the whole shoot without root of the in vitro cultivated seed; c) rejuvenated material from the in vitro cultivated adult plant - shoots, buds and leaves.

Composition and preparation of nutrient medium. Gamborg medium was used as the main culture medium. The culture medium was prepared with the following components: micro-salts, vitamins – thiamine, B_1, inositol, nicotinic acid, vitamin P, carbohydrates, which help regulate morphogenesis; hormones – gibberellin, which regulates the growth of the above-ground vegetative organs; cytokinin, which helps growth of the axillary buds and the plant development.

The culture medium was prepared as follows: the agar was weighed and added by micro- and macro salts and growth regulators in necessary amount and concentration, and a certain amount of distilled water to receive a viscous mass.

After that the pH of nutrient medium was defined and was regulated to the required norm (5.6-5.8). Then the nutrient medium was heated separately, and the agar was boiled together with the distilled water until it was completely melted. After that they were mixed. The prepared nutrient medium was poured into a culture vessel, was autoclaved and was placed in a laminar box. After superficial sterilization, the explants were introduced into the nutrient agar added by certain concentrations of appropriate growth regulators.

Primary material was isolated from an adult, intact, field-grown plant in both juvenile and adult phases, and from an intact greenhouse plant in both juvenile and adult phases. Apical and axillary buds were used as explants.

Research and Discussion

According to the results of the experiments, the explants isolated from the donor plants in juvenile phase of development were characterized by a faster growth rate than the explants isolated from the donor plant in the generative phase. In the former case, the formation of green mass in the first stage of micropropagation was much faster and more vigorous in vitro. Consequently, the duration of cultivation was short.

The juvenile, immature tissue of the explant rapidly developed the callus mass. The growth rates and, consequently, morphogenetic potential was also high resulting in formation of the callus mass and de novo buds. In the explants taken from the donor plant in the mature *phase* the process was delayed, yet the callus was developed. The daily delay cycle was 8-10 days. However, de novo formation of buds was typical for such type of explants.

Since in the tissue culture the explants can be rejuvenated at any age, the growth rate of the explants of generative phase isolated from the donor plant at the next stages is not much slower or different from that of the explants taken from the juvenile donor plants.

Comparison of the types of the primary implants showed that the axillary and apical buds differ from each other in regenerative ability and growth rate. The axillary buds wake up later, and form the shoots slowly compered to the apical buds.

The season of the year has an influence on their sterilization and regenerative ability. The worst period for superficial sterilization is found to be autumn, the last phase of vegetation of the intact plant when its above-ground parts are getting ready to stop growth, and there is going on accumulation of aging hormone of ethylene in it.

In every nutrient medium a morphogenic callus developed in the basal part of the explant. It had reddish morphogenetic nodules, from which the promordial buds were formed, but those buds were not characterized by stem morphogenesis.

Distribution of such buds in nutrient mediums was ineffective as they died in the next passage. It seemed that a high concentration of mineral salts hindered the normal growth of buds in height, so we completely excluded the Murashige and Skoog medium from the experiment, and halved the mineral salts and vitamins in the Anderson's medium. As a result, from the meristematic nodules the normal viable advent buds developed.

Out of the growth regulators we studied the effects of different concentrations of cytokinins, kinetin and zeatin on the bud activation and proliferation as well as the effect of joint action of cytokinins and GB_3, BAP and IBA, BAP and IAA.

It should be noted that although in the initial I and II passages the cytokinins action had different effects, it caused germination and formation of new axillary meristems on the main explant. The cultures growth in height was very slow or was not observed at all. Therefore, together with the above-mentioned cytokinins we introduced GB_3 into the nutrient medium in concentration of 15 Um in one passage, and during that passage (30 days) the germinated axillary meristems began to grow in height. Meanwhile, the de novo buds germinated, but in the process of the bud growth it was clearly observed that: several basic shoots with strong stems were simultaneously formed; formation of internodes on those shoots was reduced, which in its turn reduced the number of axillary buds. The branching occurred in the apical part of the shoots. There micro-propagation coefficient did not increase significantly, but the introduction of gibberellic acid into the nutrient medium caused the stimulation of cytogenetic and physiological processes in the culture. Transportation of the cultures back to the GB_3 containing nutrient medium was followed by morphological changes: internodes grew longer, the number of developing leaves on the main shoots decreased, leaves shrinked, the leaf plate area reduced, and the stem thickened too much. The morphological change was epigenetic and subjected to phenotype normalization in the non-hormon nutrient medium.

The results of the experiment showed that the propagation rate, the form and character of the buds and shoots, the activation of the axillary meristem and formation of the additional adventive buds depended on the action and concentration of the applied cytokinin (Fig. 2).

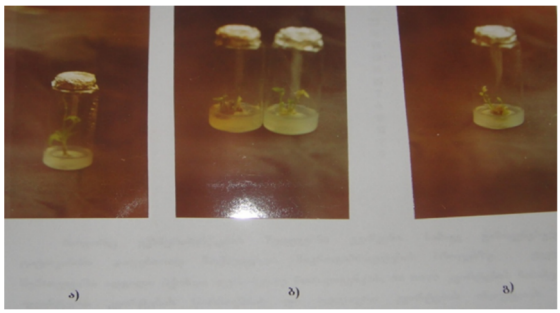

Fig. 2. A) The action of cytokinins: a) BAP (10 μM); b) Zeatin (10 μM); c) Kinetin (10 kM) on the bud formation process in the I passage.

The cytokinin used in the experiment had a positive effect on the micropropagation process. In all cases, the morphogenesis of the stem, germination of the de novo buds, formation of adventive buds and induction of axillary buds occurred. Activation of the latter depended on the concentration of hormones in the nutrient medium. Despite such a result, the effect of cytokinins action was different. The effects of zeatin and kinetin were significantly weak compared to BAP. For the

development of buds and shoots, it was necessary to prolong the cultivation (passage), or significant increase of concentrations. The optimum concentration of quinetin was 15 μm, whereas for zeatin it was 10 μM. The effect of BAP on morphogenetic processes was apparent even at low concentrations. The buds started reacion at a concentration of 2-3 μm and grew rapidly. After 30-35 days the length of the main shoot was 20 cm. Activation of the axillary meristems was not observed. The increase of BAP concentration caused the stem shortening and the development of the axillary meristem.

Thus, the proper supply of explants with mineral elements is an important stage in the process of micro-propagation and is the prerequisite for the high coefficient in the presence of exogenous phytohormones.

Conclusion

Experimental research showed the possibility of successful use of tissue, cell and organ in vitro method for microclonal propagation and massive regeneration of passion flower.

It was found that the best primary material for obtaining viable explants is the apical and axillary buds of the vegetative shoot. For clonal propagation of passionflower, it is necessary to introduce some hormones like cytokinin into the nutrient medium. We used the Anderson medium. To stimulate the micro- propagation process, it is advisable to introduce small amounts of hormones like auxins in the food along with cytokinins. In order to maintain a high intensity of micro-propagation, it is better to incubate the cultures for 16/8 hours. The dependence of the morphogenesis and regeneration ability of callus cultures on the nature and concentration of phytohormones is revealed and their optimal concentrations are selected. Cherry laurel is characterized by a fairly high accli-matization ability to non-sterile conditions and can be transferred to greenhouses at any time of the year.

მემცენარეობა

წყავის *Laurocerasus officinalis* Ro. (*Prunus laurocerasus* L.) მორფოგენეზის თავისებურებანი in vitro კულტურაში

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წარმოდგენილ ნაშრომში შესწავლილია ქსოვილის, უჯრედისა და ორგანოს in vitro მეთოდის გამოყენების შესაძლებლობა წყავის მცენარის მიკროკლონური გამრავლებისა და მასიური რეგენერანტების მისაღებად. ქსოვილური კულტურის მეთოდი უზრუნველყოფს დედა მცენარის გენეტიკურად მსგავსი ფორმების სწრაფ და მასიურ გამრავლებას დამახასიათებელი მორფოლოგიური, ბიოქიმიური და ფიზიოლოგიური ნიშან-თვისებების შენარჩუნებით, რასაც განაპირობებს უჯრედის ტოტიპოტენტურობა, ანუ ოპტიმალურ პირობებში მთლიანი, გენეტიკური ინფორმაციის რეალიზაცია. უჯრედული ტექნოლოგიის უპირატესობაა ის, რომ მცენარეთა ზრდა-განვითარება მიმდინარეობს მთელი წლის განმავლობაში, აგრეთვე ცვლის და ამცირებს სადედე ნაკვეთებს კალმებით გამრავლებისას, საშუალებას იძლევა გავამრავლოთ ის მცენარეები, რომლებიც ვეგეტატიურად ძნელად ან აბსოლუტურად არ მრავლდებიან. კვლევის შედეგებმა გვიჩვენა, რომ: in vitro მეთოდი შესაძლებელია წარმატებით იქნეს გამოყენებული წყავის მიკროკლონური გამრავლებისათვის. რეკომენდებულია ანდერსონის საკვები არის გამოყენება, ხოლო საკვებ არეში ციტოკინინური ბუნების ჰორმონების შეტანა. სიცოცხლისუნარიანი ექსპლანტების მიღებისათვის საუკეთესო პირველად მასალას წარმოადგენს ვეგეტირებადი ყლორტის აპიკალური და იღლიური კვირტები. პირველადი მასალის იზოლირება, წარმოებდა ზრდასრული, ინტაქტური, ველად მზარდი, პლანტაციური მცენარიდან და სათბურის პირობებში მზარდი ინტაქტური მცენარიდან, როგორც იუვენილურ, ასევე, ზრდასრულ გენერაციულ ფაზაში. ექსპლანტის სახით გამოყენებული იყო აპიკალური და იღლიური კვირტები. ექსპერიმენტების შედეგების მიხედვით ექსპლანტები, რომელთა იზოლირება მოხდა განვითარების იუვენილურ ფაზაში მყოფი მცენარე დონორიდან, ხასიათებოდნენ ზრდის უფრო სწრაფი ტემპით, ვიდრე გენერაციულ ფაზაში მცენარე დონორიდან იზოლირებული ექსპლანტები. წყავი ხასიათდება საკმაოდ მაღალი აკლიმატიზაციის უნარით არასტერილური პირობებისადმი და შეიძლება მათი სათბურებში გადატანა წლის ნებისმიერ პერიოდში.

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