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SUSPENSION CULTURE OF THE RUBBER-BEARING PLANT *SCORZONERA TAU-SAGHYZ* LIPSCH. & G.G.BOSSE

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Annotation: *Scorzonera tau-saghyz Lipsch. & G.G. Bosse is an endemic species of Southern Kazakhstan, which leaves and roots synthesize rubber, a strategically important substance used in various sectors of the economy, beginning from the auto industry in the production of car tires to medicine in the production of latex products. Tau-saghyz suspension cell culture is the ideal system for continuous extraction of rubber from in vitro cultured cells. Friable callus tissues were used to obtain suspension culture of tau-saghyz. It was showed that 1.5 mg/l of indole acetic acid and 0.1 mg/l of gibberellic acid positively affected on the formation of morphogenic callus in leaf explants culture of S. tau-saghyz. The growth dynamics of a suspension cell culture of the wild-growing species tau-saghyz was determined. Among the studied tau-saghyz cell suspension cultures, cell line No.9 had high growth dynamics. The*

suspension was characterized as homogeneous and consisted of small 3-5-, 7-9-cellular aggregates, as well as single cells. For eleven days, active cell division was observed, with a maximum density of 5,8x10^6 cells/ml reached by the twenty-first day of cultivation. In the future, it is planned to extract cis-isoprenoids, i.e. the main structural component of rubber, from this cell line.

***Key words**: Scorzonera tau-saghyz, suspension cell culture, callus formation, rubber.*

Introduction

Rubber plants are trees, shrubs, and grasses that form rubber in certain organs and tissues. There are about 1,500 species of rubber plants belonging to more than 20 families [1-6]. But few of them have a derived meaning. Rubber is an elastic, resilient, wear-resistant material. It is mainly used for the production of rubber and the manufacture of rubber products. Automobile tires, cameras, and rubber products used in everyday life are produced on an industrial scale and are qualitatively identified by chemical analysis [2, 7]. In the production of synthetic rubber, the hydrocarbons contained in oil gases and the products of oil refining are used [8-10]. It needs to improve the methods of obtaining rubber in Kazakhstan. In the production of synthetic rubber, its physical properties are taken into account: resistance to water, acids, organic solvents and resistance to high temperatures. In the industry, a large role in the production of rubber is played not only by the collection of rubber, but also by the production of this product from it. One of the most important is the *Hevea brasiliensis* tree, which produces 90-96% of the world's production [11].

Rubber solvents are divided into latex, parenchymal, chloroenchymal, and mesenchymal solvents (depending on which organ and tissue they accumulate). In latex rubber plants, rubber accumulates in the latex tubes of the roots (*Hevea, Sapient, Maniota, Euphorbia, Ficus*) [12]. Among them, the *Hevea* tree has a derived meaning. It is grown in Southeast Asia, South and Central America of Africa. Rubber is obtained from the milky juice of the *Hevea* tree, for which the *Hevea* peel is collected and the milky juice is isolated - a colloidal solution of rubber. When exposed to or heated by the electrolyte solution and coagulation, rubber is released (Chow rubber-a tear of a tree). In parenchymal rubber plants, rubber is mainly formed in the cells of the leaf parenchyma (grass mycelium, hemp, chondrill), but due to its low quality, it has little industrial value. Chloroenchymal rubber is obtained in such a way that it is formed by mixing the rubber with the tissues of the parenchyma of the stem and root. For example, an evergreen semi-shrub that grows in Central and North America-Guatemala [11, 5]. In mesenchymal rubber-bearing plants, rubber accumulates in the tissues of the plant's assimilation organs. Some plants contain wet rubber, but with it, there are impurities of resins and others that require cleaning. This rubber contains gutta-percha.

In Kazakhstan, tau-saghyz, kok-saghyz (*Taraxacum kok-saghyz*) natural rubber is found in the roots and stems of plants [13, 14]. A relict species containing rubber-the common mountain gum (*S. tau-saghyz*) is an endangered endemic and Khan-tau-saghyz (*S. chantavica*) 2 species are also listed in the "Red Book" of Kazakhstan [14]. Carrying out research and selection work in order to obtain new varieties of rubber with a high content of natural rubber allows to obtain raw material of relative size. In addition, the technologies being developed allow the production of mountain gum supplied by rubber on an industrial scale. Due to the difficulties of vegetative reproduction of tau-saghyz, it is not limited in distribution in the nursery economy, it is almost absent, but is under threat of extinction, which leads to the fusion of the gene pool. In this regard, it is necessary to develop regulations for the general replication of population rare and endangered species, which will be universal for all varieties and will allow developing a cost-effective technology for high-quality growing material, as well as limiting the usual gene pool of reproduction and a high yield of sterile seed material. To create an *in vitro* collection of tau-saghyz samples of scientific and commercial interest, it is necessary to obtain a suspension

culture and plant cuttings. Suspension culture allows to extract latex from cultivated cells in a large scale.

Materials and methods

The initial plant material is samples of wild plants collected at the beginning of the spring growing season on the territory of the Karatau State Natural Park in Southern Kazakhstan. As the explants, size 0.5 cm fragments of leaves and roots of the 2-year-old plant were used. As a rule, friable embryogenic callus tissue is used to obtain a cell suspension [15]. It is easy to separate isoprenoids (the structural basis of rubber) from callus biomass and cells suspension of tau-saghyz. The explant leaves were sterilized by 0.1% mercury chloride solution for 5 min., after which they were rinsed with sterile distilled water. The proximal parts of the leaf are planted separately in a modified Murashige-Skoog culture medium. The modification was performed with PGRs: 1 mg/l BA (6-benzyladenine), 0.1 mg/l NAA (α-naphthalene acetic acid), 0.1 mg/l IAA (β-indole-3-acetic acid), and 1 mg/l 6-BA, 0.1 mg/l IAA, also 1 mg/l BA and 0.5 mg/l GA (gibberellic acid). To induce callus formation leaf explants were incubated in a dark place at a temperature of 24±2° C, and in diffused light. The obtained embryogenic callus was grown on MS culture medium supplemented with 1.5 IAA mg/l and 2 mg/l BAP, at a temperature of 25±2°C, in 16-hour photoperiod, under illumination of 2000 lux. Weekly screening was conducted for morpho-physiological parameters (growth of cultivated explants, increase of biomass, necrotic phenomenon). The explants were subcultivated every 20-25 days, on a fresh culture medium. After the callus biomass reached 3 g, it was moved to a liquid medium. In order for the cells to live in a floating state, without sinking into media, they must be continuously mixed with the apparatus. In this study, the mixing mode was 130 rev./min. The cells are supplied with oxygen by continuously rotating, shaking, or blowing sterile air through the liquid. The composition of the liquid medium was the same as that of the solid agar medium. The culture medium should not contain Ca^{2+} ion, as it affects the formation of calcium pectinate. Therefore, MS culture medium consumed 20 g/l of sucrose carbohydrates. With frequent shaking and stirring of loose corn in a liquid medium, it breaks down into individual cells and begins to break down into smaller cellular aggregates. The remains of dense calluses and large aggregates left without separation were filtered through 1-2 layers of nylon sieve and removed. With frequent transfer of the light fraction of the suspension to a new medium, the number of individual cells increases. Every 7-10 days after receiving the first cell suspension, a suspension subculture was performed. To determine the suspension density and to determine the growth dynamics of the cell culture, a Goryaev chamber was used.

Results and Discussion

Compared to cells grown in solid media, cells in suspension have some advantage. Cells need to provide certain favorable conditions for life. The creation of conditions for a uniform effect on all such cells is carried out by suspension cultivation. Another advantage of this method is that the cells easily get rid of harmful metabolites by releasing them, and it is easier to control the impact of various factors on the processes of metabolism and growth of these cells. Cells grown in suspension are suitable for biochemistry and molecular biology research. In the best growing suspension, individual cells make up no more than 50-60%, the rest are aggregates consisting of 2-10 cells, or even more, grouped into cells. In order to obtain a suspension with a large number of individual cells, i.e. dispersed, it is often attempted to evaluate the composition of culture medium. According to the available data, auxins have the positive effect on the cells being closed to each other, individualized (dissociated), and cytokines, on the contrary, suppress this process. Consequently, the conditions that favor the extended growth of cells and inhibit their division lead to the fact that the cells in the suspension are split and crushed as much as possible. In the embryogenic suspension, small-sized cells that are homogeneous in morphology

are combined into smaller aggregates (about 10 cell groups). To ensure intensive growth, the suspension must have a cell density 10^5- 10^6 in the same media. Explants were cultivated in MS medium containing 0.1 mg/l NAA, 1 mg/l BA, and MS with 0.1 mg/l IAA, 1 mg/l 6-BA. Despite this, the complex of auxins are widely used to stimulate the processes of organogenesis. In our practice, its influence on the tissue and organ development was not particularly observed. Analysis of the results of morphogenetic processes shows that the presence of IAA in the culture medium has a positive effect on the regeneration process. Another type of auxin (NAA), in the same concentration stimulates the process of callus formation. In particular, in the medium supplemented with 0.1 mg/l NAA, 1 mg/l 6-BA, the frequency of callus formation was 31.6±5.07%, and in medium with 0.1 mg/l IAA, 1 mg/l 6-BA callus formation was with frequency 28.3±2.2% and regenerating plants were formed. In this case the regeneration frequency was 0.34±0.001%, respectively. In the third type of culture medium, i.e. MS medium containing 1 mg/l 6-BA, 0.1 mg/l IAA, 0.5 mg/l GA, the rate of callusogenesis and regeneration were 42.6±1.2% and 2.7±0.64% respectively.

The auxin β-indole-3-acetic acid regulates the regenerative process of physiological activity with a morphogenetic effect, which is more effective for cell culture of endemic tau-saghyz; its analog is somewhat less than in α-naphthalene acetic acid. 6-benzyladenine exhibits a cytokinin type action that also promotes the formation of calli in cell culture in the proximal leaf. Poor light (scattered light) enhances the stimulating effect of cytokinin, which promotes the proliferation and development of callus tissue. Thus, the obtained results show that *in vitro* cultivation affects not only the physiological state of the explants, but also the composition of the culture medium, the balance of exogenous hormones, and the effectiveness of physical factors (especially light). The stimulating effect of gibberellic acid on morphogenetic processes was observed in tau-saghyz cell culture, as well as in the preparation of cell suspensions *in vitro*.
Comparative morphometric analysis of the suspension showed that in 3 culture media with different PGRs composition it was differed, especially in consistency and uniformity. Cell population was more homogeneous in suspension culture, which appeared in a medium containing 1 mg/l 6-BA, 0.1 mg/l IAA, 0.5 mg/l GA. The cells in suspension had an oval shape and actively multiplied (divided) in a medium containing gibberellic acid. Thus, the types and concentration of growth regulators in the cell culture of leaf explants contribute to the emergence of morphogenetic potential. Concentrations of plant growth regulators 1 mg/l 6-BA, 0.1 mg/l IAA, 0.5 mg/l GA in the MS culture medium are optimal for cell growth. The presence of gibberellic acid in the culture medium promotes the process of adventive organogenesis in cell culture of *Scorzonera tau-saghyz*.

Analysis of growth dynamics of the suspension culture showed that the best cell growth was observed in cell line № 9 (Fig. 1). This line was characterized by a short lag-phase (9 days) and rapid cell division within 11 days, with the maximum cellular density reaching 5.8×10^6 cells/ml on the 21st day of cultivation, followed by a long (for two weeks) phase of the stationary period. For cell line № 9, the growth curve was corrected, the suspension was homogeneous, no more than 7-9, 3-5 cell aggregates and single cells prevailed in it. Unlike the aforementioned cell line, for suspension cell line № 7, cell growth occurred in two stages: after a short lag-phase (3-day) with the first peak of the first maximum density at the level of $4x10^6$ cells/ml, active cell proliferation was observed, and stationary phase of growth culture, but on the fourth week of subcultivation the cells began to divide rapidly, and at this time the second peak of density reached the level of 5.2×10^6 cells/ml (Fig. 1). Then the number of viable cells decreased, and by 6 weeks the number of living and dead cells stabilized and reached a stationary level. For cell lines № 5, 13, 14, the growth of the suspension culture was relatively similar: the duration of the lag-phase varied from 5 days (line № 5) to 14 days (line № 13, No. 14), then there was a slight increase in cell biomass and on the fourth week the cells began to divide rapidly, reached their maximum density on the fifth week (cell line № 13 - $1.5x10^7$

cells/ml, cell line № 14 - 1.4x10^7 cells / ml), reached the maximum density in cell line № 5, only on the seventh week of cultivation - a cell density of 5.7x10^6 was achieved (Fig. 2-3). Cell lines №5, 13, 14 at this stage of the study have not reached the stationary phase, and the cell population continues to grow. For cell line № 12, in contrast to the above lines (№ 5, 13, 14), the exponential phase is clearly marked and is reached at the fourth week. Cell growth and proliferation occurs in the fourth week of cultivation, and at the sixth week, the proliferation rate slows down (Fig. 4). This cell line has not reached the stationary phase because the growth of the cell population has not yet stopped.

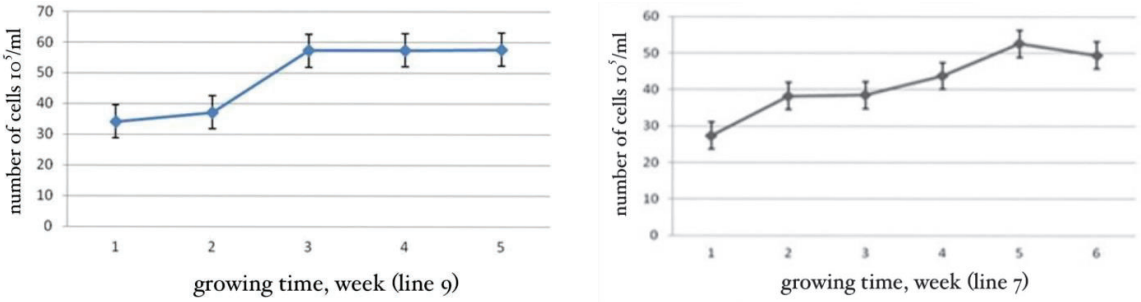

Figure 1. Growth dynamics of suspension culture in cell lines № 9 and № 7 of tau-saghyz

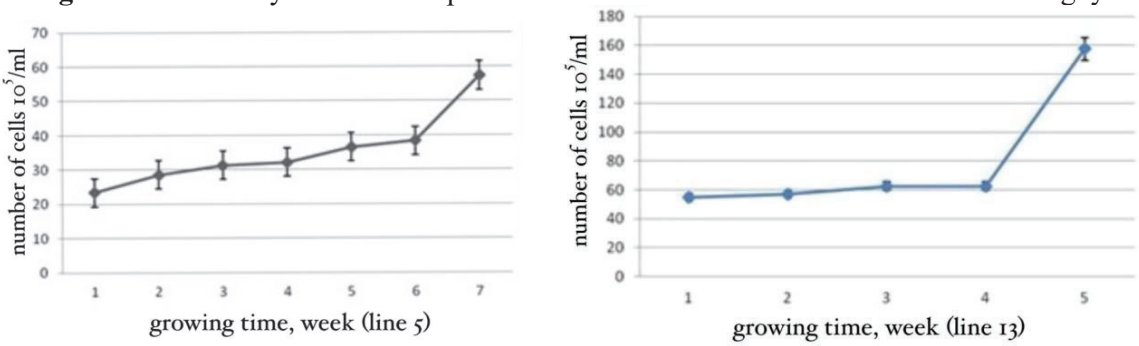

Figure 2. Growth dynamics of suspension culture in cell lines № 5 and № 13 of tau-saghyz

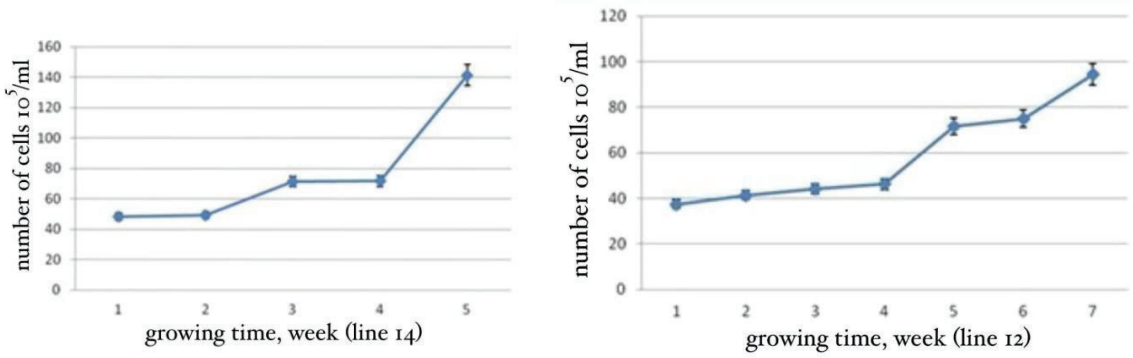

Figure 3. Growth dynamics of suspension culture in cell lines № 14 and № 12 of tau-saghyz

In general, the maximum density of the suspension culture varied from 49x10^5 cells / ml (line № 7) to 157x10^5 cells / ml (line № 13) for different cell lines. However, for line № 13, the lag-phase (28 days), which is characterize the cell population with a low density, is so large that it is ineffective for large-scale (industrial) cultivation. With the most optimal suspension in terms of

cell population growth, the suspension culture of cell line № 9 is recommended for further study and cultivation.

Conclusion

The developed technologies make it possible to extract rubber from the cultivated cells of rare rubber-bearing plant *Scorzonera tau-saghyz* Lipsch. & G.G.Bosse on an industrial scale. The presence of 0.5 mg/l of gibberellic acid (GA) in the culture medium, as well as the conversion of NAA into another auxin IAA (0.1 mg/l) stimulates the process of morphogenesis and regeneration plants in cell culture of *S. tau-saghyz*. The best growth dynamics from the analyzed suspension cell culture was in line № 9. Isoprenoids are expected to be isolated from this cell culture in the future.

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