IN VITRO RESISTANCE CELLS TISSUES OF SUNFLOWER TO WHITE ROT *(SCLEROTINIA SCLEROTIORUM)* AND STUDY THE ROLE OF PHENOLIC METABOLITES IN THE ADAPTATION MECHANISMS OF CELL CULTURES TO SELECTIVE FACTOR

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Abstract

The media for reception callus tissues and regeneration shoot of three different sunflower genotypes was optimized. The highest shoot regeneration was observed using hypocotyl explants on MS media supplemented with 2mg/l kinetin and lmg/l NAA. Receiving of in vitro resistance cells tissues of sunflower to exometabolite of white rot (Sclerotinia sclerotiorum), on the basis of cultivation callus cells on media containing culture filtrate of fungus (Sclerotinia sclerotiorum) with different concentrations (5-35% from final volume of media). The study of the role of phenolic metabolite in the adaptation mechanisms of cell cultures to selective factors.

Key words: Biotechnology, cell cultures, resistance, sunflower, selective factor, phenolic metabolites

Introduction

Sunflower is one of the most important oil crops in the world economy. This brings the attention to develop suitable measures to protect it from complex diseases. White rot *(Sclerotinia sclerotiorum*) and phomopsis (*Phomopsis helianthi),* are the most damaging sunflower diseases. These diseases are the basic causes of significant shortage of a crop and reductions in commodity and sowing qualities of seeds [3,14].

The specific problem is difficult to solve, using conventional practice of plants and crops protection, for example agrotechnical, chemical and biological, as these methods are not efficient and ineffective.

One of new perspective ways to increase the efficiency of selection process is the use of modem methods of biotechnology, allowing to expand a spectrum of a genetic variety (somaclonal variation, the somatic hybridization, induced mutagenesis, genetic engineering) and to reduce the period of plant breeding. Significant decisions on tissues cultures selection are based on selection of tissue populations which are resistant to selection factor and regeneration from the tissue population plants [7].

The search of sunflower genotypes which are resistant to various pathogens, in particular to *Sclerotinia sclerotiorum,* is feasible *in vitro* at cultivating explants on the mediums contents producing toxins pathogen.

Based on the objectives of this paper, the study was done on the cell and tissue cultures of sunflower, resistant to *Sclerotinia sclerotiorum* and adaptation mechanisms of cell cultures to stress factors.

Materials and methods

The objective of the research is to experiment the seed of three sunflower genotypes (VK 580, VK 653, Kuban 93), which are possessed by various resistant to *Sclerotinia sclerotioram.* Seeds were supplied by research station of oil cultures. During research, sterilization and *in vitro* techniques were used, which were developed by the department of Agricultural Biotechnology of the Russian State Agrarian University - MAA K.A.Timirjazeva [2].

The stress factor, culture filtrate pathogen *(Sclerotinia sclerotionm),* was studied. Culture of fungus (*Sclerotinia sclerotiorum)* grew on agar and the liquid mediums. Culture filtrate (CF) pathogen was received by cultivating fungus in 300 ml flasks filled with 200 ml volume of liquid medium on shaker with speed of rotation with 100 rotation /min. In each flask 10^8 pieces conidium of fungus were added. Culture filtrate received by filtering suspension of a fungus through a filter paper and autoclave.

Callus tissues were cultivated at medium with addition of CF in concentrations (5%, 15%, 25%, 35%) from final volume of a medium.

Callus tissues increment and the phytotoxic of CF were determined by formulas from "Practical for agricultural biotechnology" [2].

The total soluble of phenolic connections and polyphenol oxidase (PPO) defined in primary explants (hypocotyls) and callus tissue, which were cultivated in standard and stress conditions by N.B.Pronina method [4].

The experiment was repeated in three biological and 2-3 analytical times. The results of experiments were statistically analyzed with Straz and Excel program [1,8]. Means and standard deviations are shown in graphics and in tables.

Results and discussion

During the development of the cell tissue selection methods *in vitro,* this shows a need to develop technologies that increases morphogenesis isolated explants. It is well known that the process of morphogenesis depends on set of factors, for example genetic, physiological, hormonal and physical. These factors are necessary for certain taxonomic groups of plants. Morphogenesis reaction of the isolated cells, callus and organs of plants can be improved by changing conditions of cultivation, mineral and hormonal contents of medium. This allows solving different problems of cells biotechnology [11,12,13].

The research have shown that the best explants for reception of good proliferous callus tissues, which have morphogenesis ability, are hypocotyls segments of seedlings 5^{th} days. These explants cultivated in MS medium with addition kinetin (2mg/l) and NAA (lmg/1). The reaction depends on genotypes [10,11,12,13]. The study also shows that genotypes, Kuban 93, of which the process morphogenesis (10,2%) was more efficient in comparison with other genotypes (tab.l).

Table 1

Influence of morphogenesis potential from primary explants on genotypes

Genotype	Morphogenesis, %	Regeneration shoots on 1 explants, pieces	Callusogenesis
Kuban 93	10,20 + 0,21	1,80 ±0,75	+
VK 580	7,84 ±0,15	1,50 ±0,5	+
VK 653	6,38 ±0,15	1,67 ±0,47	+

At cultivation hypocotyls explants or callus tissues on medium with kinetin and NAA have developed regeneration shoots, which have been transferred to soil culture (fig.l). Hence, the cultivation callus tissues were optimized, which have regeneration ability.

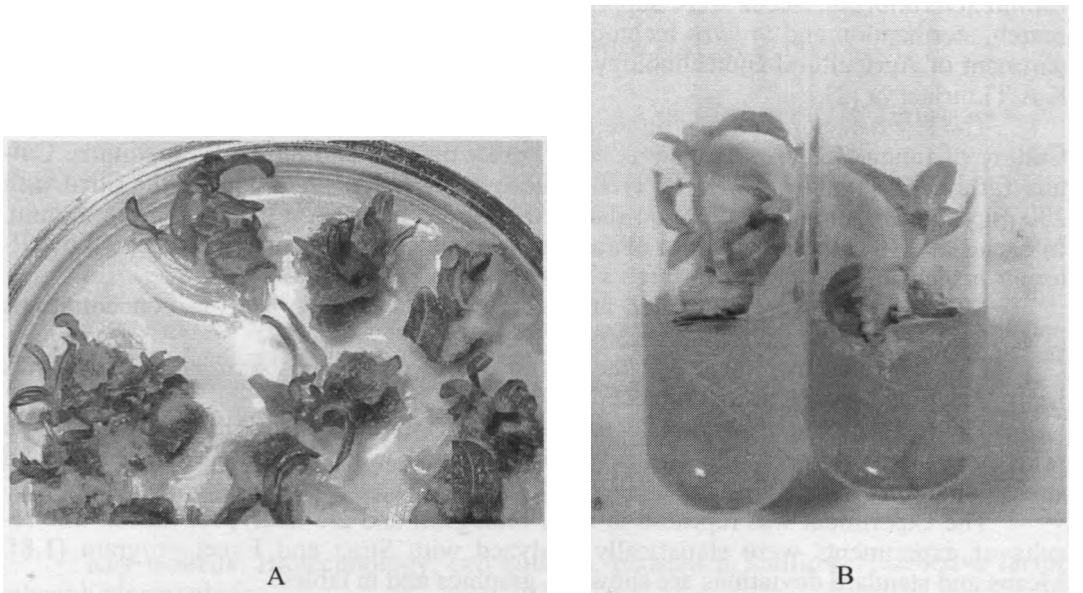

Fig.1. Morphogenesis of callus tissues: A - regeneration shoots, B - plant regeneration

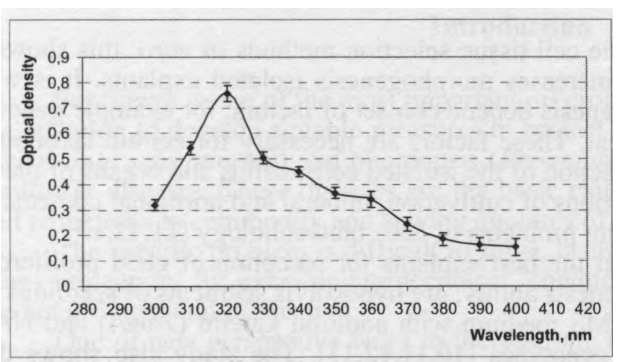

Fig. 2. Influence of optical density CF on wavelength

For cells selection of plants in conditions *in vitro* often use callus tissues. To carry out cells selection it is necessary to choose the correct stress-factor which has an effect on the cultivated callus tissues. CF pathogen *(Sclerotinia sclerotiorum)* was used in selecting factors. It is also necessary to estimate phytotoxic of CF. By this method, there is a need to define cultivation duration of CF pathogen in a liquid medium and show terms of its cultivation at which the maximal accumulation exometabolite a fungus in cultivation medium. The study shows that the wavelength (320 nm), CF has the maximal optical density (0,750 units) (fig. 2). Therefore the wavelength was used in further experiments.

In the second series of experiment duration of pathogen cultivation in a liquid medium was defined. Measurements of optical density were defined in each 5 days (fig. 3). The figure shows that the active allocation of exometabolite in medium is necessary for 20 days (0,345 units) from the beginning of pathogen cultivation. A further cultivation of fungus had shown a substantial increase of optical density cultivation liquids which has achieved a maximum of 45 days (0,750 units). However, from the 35th day, the increase

in optical density did not significantly change. The results indicated that after 35 days of pathogen cultivation in medium, exometabolite synthesis, which is shown in preservation of optical density of a solution during the next days cultivation *in vitro* has slowed down or stops, an indication of the inexpediency of further cultivation fungus.

The phytotoxic of CF was tested on various explants: seeds, hypocotyls segments isolated from 5-days seedlings, and on callus tissues. Researches have shown, that CF *Sclerotinia sclerotiorum* in various concentration influences to some extent on seeds germination, ability hypocotyl segments to form callus tissues and its increment.

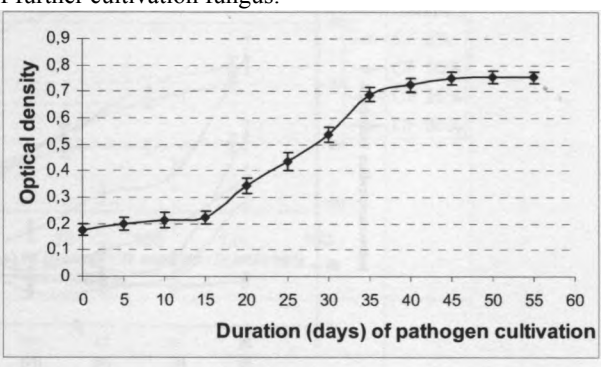

Fig. 3. Influence of optical density CF on duration of pathogen cultivation (Л =320 nm)

The study demonstrates that CF pathogen at 25% concentration for genotype VK 580 and Kuban 93 had stimulate seeds germination, and at 5% concentration for genotype VK 580 had stimulate the formation of callus tissues from hypocotyls segments. At high concentration, it has inhibition effect, where callus tissues of individual genotypes were destroyed. It have been established that cells selection can be carried out on the medium containing CF pathogen (5-35 %).

Cells selection was carried out on callus tissues, which were cultivated on medium with CF pathogen in the concentration of 5%, 15%, 25% and 35%. The research has shown the increase in concentration of CF in medium, which decreases callus tissues increment. However, due to longevity of cultivated callus tissues in stress conditions, the increment of cell biomass eventually decreases to IV passage and from V passage stabilization were observed. This shows the adaptation of callus tissues to the selective factor (fig-4).

The important moment in researches of cell selection on stability of biotic factors, is studying the changes in the metabolism of phenolic connections in callus cultures, which were cultivated in stress condition. The role of phenolic substances in protecting cells against the stress factor is well known. Therefore research was done on changes of quantitative and qualitative structure of phenolic connections in callus tissues of difference sunflower genotypes, which were cultivated in standard and stress conditions.

The research has shown that a difference in the total soluble of phenolic connections in the primary explants (hypocotyls segments) of the studied genotypes. In cells of genotype VK 580 was more efficient (about 30%) in synthesized polyphenols in comparison with other genotypes. The study also shows that the quantity total soluble of phenolic connection of callus tissues *in vitro* of the studied genotypes decreases by 2-4 times in comparison within the primary explants.

Earlier researches has shown that biosynthesis of phenolic connection changed in the presence of the stress factor [5,6,9]. The result of this study also shows the increase in biosynthetic activity of callus tissues of all studied genotypes, at cultivation callus tissues in stress conditions (CF pathogen in various concentrations). Possibly, long cultivation of callus tissues of sunflower in stress conditions leads to "start" of the raised program protective reaction, which show increase of phenolic connections synthesis [5,9]. The changes of quantitative contents of soluble phenolic connections in callus tissues of geno-

types Kuban 93 and BK 580, which were cultivated in medium with high concentration CF (25 and 35%), were higher in comparison with other concentration CF. For genotype BK 580 these changes were in proportional to concentration of the stress-factor (fig.5).

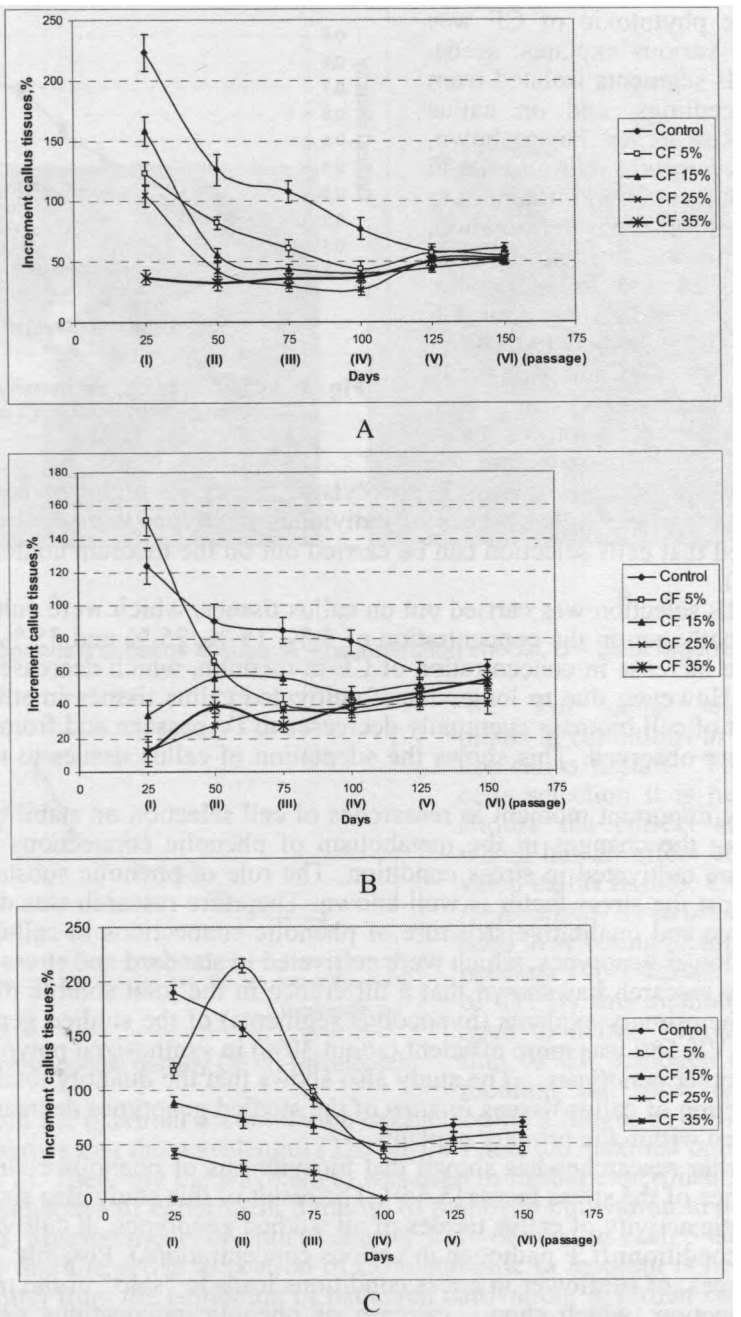

Fig. 4. Increment callus tissues of sunflower, cultivation in stress condition (CF *Sclerotinia sclerotiorum).* A- genotype Kuban 93; B- genotype VK 580; C- genotype VK 653

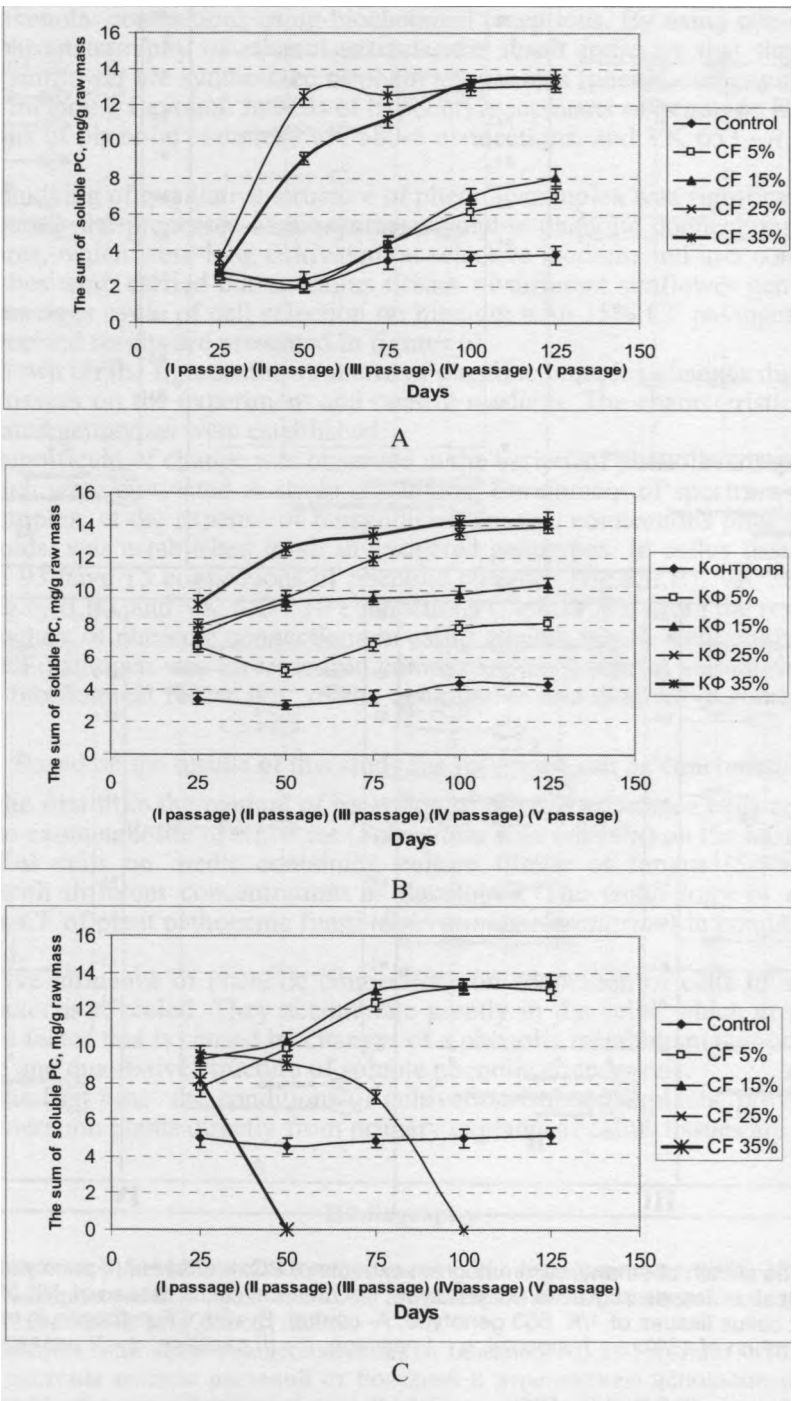

Fig. 5. The content of PC in callus tissues of sunflower in long cultivation: A - genotype Kuban 93; B - genotype VK 580; C- genotype VK 653

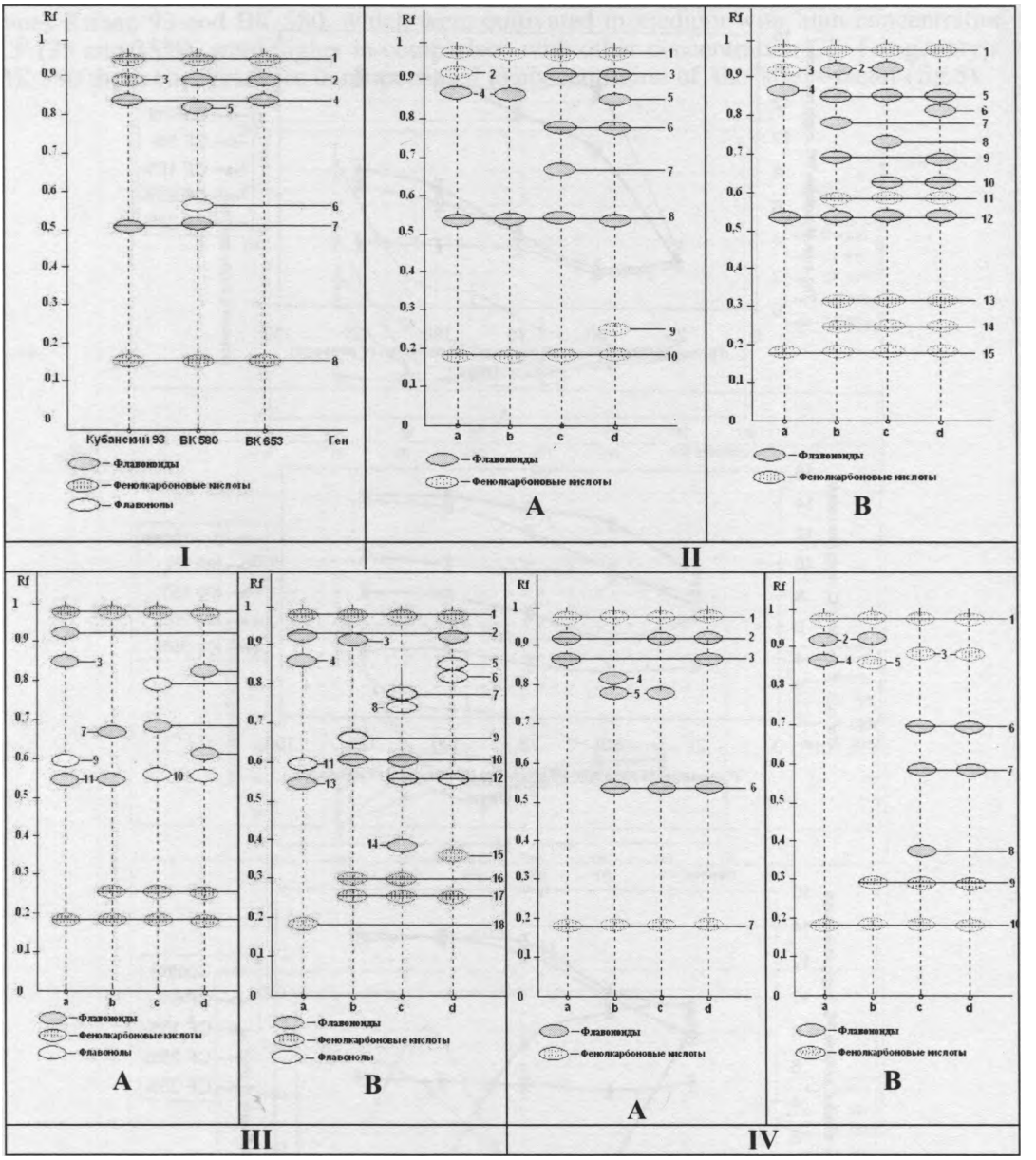

Fig-6. The sketch of ethanol chromatogram extracts of PC: **I-** different hypocotyls segment; II- different callus tissues of Kuban 93 genotype; III - different callus tissues of VK 580 genotype; IV- different callus tissues of VK 653 genotype. A- control; B- with CF pathogen in media concentration of 15% (a - hypocotyl, b - I passage, c - III passage, d - V passage)

The studying of qualitative structure of phenolic complex was significant important for investigating the processes of biosynthesis soluble phenolic connections changes in cells of primary explants. Therefore, the following experiment was done on the structure

of soluble phenolic connections using biochemical receptions. By using one-dimensional thin-layer chromatography of ethanol extracts, the result indicates that the hypocotyls segment of sunflower are synthesized as phenylpropanoids (phenol-carboxylic acids) and flavonoids, including flavonol. In cells of hypocotyls segments of genotype Kuban 93 has 5 connections of phenolic complex, VK 580-6 connections, and VK 653 - 4 connections (fig-6,1).

The studying of qualitative structure of phenolic complex was significant important for investigating the processes of biosynthesis soluble phenolic connections changes in callus cultures, which were long cultivated on selective medium and in a control variant. The researches were carried out on callus tissues of different sunflower genotypes on I, III and V passages cycle of cell selection on medium with 15% CF pathogen concentration. The received results are presented in figures 6.

As shown on the figures, the structure of phenolic complex changes during cultivation callus tissues on the experiment and control medium. The characteristic changes of all investigated genotypes were established.

The significant of change was observed in the variety of phenolic complex in callus tissues, which were cultivated in stress conditions. Enrichment of spectrum of synthesis phenolic complex, at the expense of biosynthesis *de novo* connections phenylpropanoids and flavonoids, was established in all investigated genotypes. In callus tissues of genotype Kuban 93 have 15 connections of phenolic complex (fig.6,II,B), VK 580 - 18 connections (fig.6,III,B), and VK 653 - 10 connections (fig.6,IV,B). From the results follows that the structure of phenolic connections in callus tissues, which were cultivated in medium with CF pathogen was increased in comparison with control variant that proves to be true the biochemical researches of the quantitative and qualitative contents of polyphenols.

Based on the results of this study the following can be concluded:

For the first time the method of reception of *in vitro* resistance cells and tissues of sunflower to exometabolite of white rot (*Sclerotinia sclerotiorum*) on the basis of cultivation of callus cells on media containing culture filtrate of fungus (*Sclerotinia sclerotiorum)* with different concentrations is developed. The technology of reception of highly toxic CF of plant pathogenic fungi (*Sclerotinia sclerotiorum*) in conditions *in vitro* is developed.

Positive influence of phenolic connections on adaptation of cells to action of the selective factor is revealed. They accumulate greatly in the cells, which are resistant to the selective factor that is caused by changes of a phenolic metabolism that occurs both in quantitative and qualitative structure of soluble phenolic connections.

For the first time the conditions of cultivation isolated explants, providing reception of regeneration plants directly from primary explants or callus tissues are optimized.

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