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Plant secondary metabolites as bioactive substances for innovative biotechnologies

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Abstract. Plants are natural sources of bioactive compounds, and the intensive use of wild plants to obtain them, in particular secondary metabolites, depletes natural biocenoses. Instead, modern biotechnological methods, especially cell and tissue culture in vitro, make it possible to get environmentally friendly, highly productive plant raw materials that are able to synthesize and accumulate specialized substances, which are valuable for pharmacology, cosmetology, and medicine. Regenerating in vitro-plants of different plant species such as *Acorus calamus* L., *Phalaenopsis* sp. were obtained in our research. It was proved that by changing the cultivation conditions it is possible to change the content of substances of secondary metabolites in explants and in the nutrient medium under aseptic culture.

1 Introduction

Constant changes in environmental conditions necessitate changes in the biochemical and physiological processes of all living organisms in order to increase their adaptive capacity. Numerous mechanisms of adaptation to the action of abiotic, biotic and anthropogenic factors specify unique properties of plants. Plants synthesize hundreds of thousands of organic compounds, which are divided into three main groups. The first group includes primary metabolites – compounds that are directly required for plant growth. The second one – phytohormones, which perform a regulatory function in the plant body and metabolism in general. To the third group, scientists attribute organic substances with relatively low molecular and high biological activity, namely substances of specialized metabolism. The other name for this group of substances is secondary metabolites [1, 2].

As early as the XX-th century, Nobel laureate in physiology and medicine, Albrecht Kossel firstly introduced the term «secondary metabolite», and Chapek described this kind type of substances as the final products of metabolism. Further development of science – the emergence of new research methods in molecular biology, biochemistry, physiology helped to establish that secondary metabolites play several roles in the growth and development of the plant organism, and are also synthesized in response to various stressors [3].

Plant tissues and organs synthesized a huge variety of compounds of secondary origin. Primary metabolites are formed in the cells of all plant species, but specialized substances may be unique to individual species. The plant usually synthesizes a complex of such substances, the individual components of which have an additional and enhancing effect on more than one molecular target. Secondary metabolism originates from different primary pathways, which indicates changes in the activity of enzymes of main metabolism during evolution. As a result of such changes, completely new compounds were formed that increased the plant resistance to the factors of a certain environment and the gradual transformation of primary compounds into specialized metabolites [4].

Scientists classify secondary metabolites by type of their chemical structure into the following classes:

- alkaloids;
- isoprenoids;
- saponins;
- glycosides;
- phenolic compounds etc.

Each of these groups includes from units to several hundred or thousands of individual compounds. Their chemical structure determines the specificity of the functions of such substances. For example, plants can synthesize secondary metabolites to protect itself against various pathogens (viruses, bacteria, fungi, etc.) or to neutralize toxic products of primary metabolism. Interestingly, some secondary compounds largely determine the nutritional and taste qualities of various plant products. All the plants' properties for the formation of multifunctional organic compounds with various properties helps to produce body care products, immune support, a great use in medicine (for example, as analgesics, antioxidants, blood pressure normalizers, etc.), use as food supplements, bioindications, and other [5,6].

1.1 Plant phenolic compounds

Phenolic compounds are included to one of the most diverse and numerous classes of secondary metabolites of the aromatic series, characterized by the presence of a benzene ring consisting of six Carbon atoms (C₆); and one or more hydroxyl groups (OH). The classification of phenols is based on determining the number of Carbon atoms and aromatic rings in molecules (from one or two benzene rings to many – polyphenols). Depending on the chemical structure and properties are distinguished [7]:

- simple phenols;
- coumarins;
- tannins;
- flavonoids;
- xanthenes;
- chromones;
- stilbenes;
- lignans.

In the plant body, the phenolic substances synthesis occurs with the involvement of enzymes of the three main ways of transformation of aminoacid phenylalanine:

- polyketide pathway (elongated side chains of phenylpropanoids, flavonoids are formed ($C_6 - C_3 - C_6$);
- shikimic pathway (synthesis of phenyl-propanoid derivatives ($C_6 - C_3$));
- acetate-mevalonate pathway (formation of aromatic terpenoids, in particular monoterpenes).

Natural phenols are synthesized and accumulated in all plant organs, for example in members of the *Orchidaceae*, *Theaceae*, *Gentianaceae* and other families, the content of which is 2-3% of the total mass of organic matter of plants, in some cases can reach 10% or more. These compounds have a strong effect on plant growth, elongation of stems and roots, inhibiting seed germination, play an important role in wound healing, cell division [8,9].

Phenolic compounds determine the color of leaves, flowers, fruits. They are also involved in the growth and reproduction of plants involved in the mechanisms of protection against ultraviolet radiation (UV-light), infection with pathogenic microorganisms, parasites or from being eaten by animals, and so on. Phenolic substances are synthesized when the plant cell receptors recognize potential pathogens by preserved pathogenic-associated molecular features, which leads to a response to a biological stimulus. Therefore, the development of infection is limited long before the spread of the pathogen throughout the body. They are also part of plant foods and beverages, such as vegetables, fruits, cereals, legumes, chocolate, tea, coffee and others. In addition, phenolic compounds determine the healing properties of plants, making them extremely valuable for cosmetology, pharmacology and medicine. The study of the content of certain phenols gives grounds to distinguish between plant species, i.e. to use as a marker for molecular taxonomy [10].

1.2 Sources of xanthenes phenolic compounds

Xanthenes are heterocyclic compounds belonging to secondary metabolites, namely polyphenolic substances with the molecular formula $C_{13}H_8O_2$. Xanthenes have been studied since the early 1900's and it has been established that the natural sources of these compounds are plants, as well as fungi, lichens and even bacteria.

The classification of these polyphenols is based on the number of benzene rings, as well as the type, number and position of substituents. The biosynthesis of plant xanthenes occurs by converting shikimic acid with the formation of aromatic rings, and acetate –in the piron ring. These substances are derivatives of diphenylketone and the simplest formula of the xanthone molecule has the form (Fig. 1) [11,12].

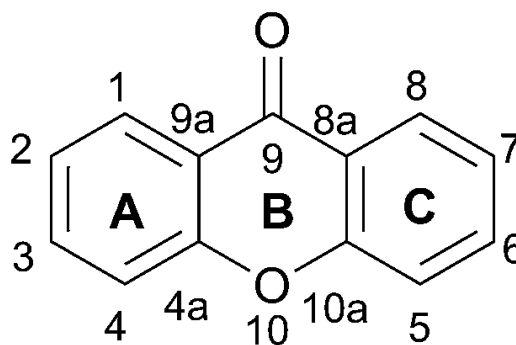


Fig. 1. Formula of xanthone with numbers of Carbon atoms

Xanthenes isolated from natural sources are classified into 6 main groups:

- simple xanthenes;
- xanthone glycosides;
- prenylated xanthenes,
- xanthanolignoids;
- bixanthenes;
- mixed xanthenes.

From ancient times to the present days, people use plants in folk and conservative medicine as a source of bioactive substances for the prevention and treatment of various diseases. It has been experimentally proven that these secondary metabolites act as lipid peroxidation inhibitors, metal chelators, free radical scavengers, ie as antioxidants. Thus carry out hepatoprotective, anti-inflammatory action and prevent the development of cancerous tumors. As for the representatives of phenolic compounds, xanthenes are characterized by other medicinal properties, namely: antiviral, antibacterial, antifungal, etc. [13,14].

Especially well-known plant sources of these polyphenols are representatives of the following plant families: *Moraceae*, *Polygalaceae*, *Gentianaceae*, *Hypericaceae*, *Iridaceae*, *Asparagaceae* and others. That is why these plants are very valuable for the pharmaceutical industry and there is a need for alternative methods of obtaining plant raw materials with advanced modern biotechnological measures [15-18].

1.3 Sources of flavonoids phenolic compounds

Flavonoids are another group of polyphenolic compounds that have the general formula $C_6 - C_3 - C_6$. They are also classified by the number of benzene rings and the type of substitution into the following main groups:

- halcones;
- catechins;
- flavonols;
- flavonones;
- anthocyanins;
- isoflavones;
- proanthocyanidins.

Natural flavonoid sources are algae, mosses, horsetails, ferns, as well as gymnosperms and angiosperms. Among the angiosperms representatives are the families *Lamiaceae*, *Rosaceae*, *Fabaceae* and others can be mentioned, which synthesize flavonoids different

in structure and properties. Flavonoids are accumulated in leaves, roots, fruits, seeds of plants. Like other phenolic compounds, they are derivatives of phenylalanine and malonyl-CoA. One molecule of flavonoid compound consists of two phenyl residues A and B combined with a propane link, which can close in an oxygen-containing heterocycle C (Fig. 2) [19-21].

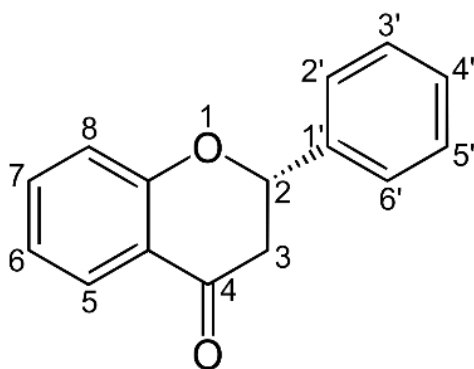


Fig. 2. Formula of flavonoid with numbers of Carbon atoms

Flavonoids are plant pigments that also play an important role in increasing the plant resistance to stress factors. In addition, they help determine the quality of food, acting as preservatives and have strong antioxidant, anti-inflammatory, antimutagenic and anticancer properties. These polyphenols can modulate key functions of cellular enzymes. Due to these properties, plants containing flavonoids are also used in the manufacture of medicaments, as well as nutraceutical, pharmaceutical, medical and cosmetic applications [22,23].

1.4 Biotechnological importance of secondary metabolites of plants

Due to the increased need in the use of unique in structure and properties of substances of specialized synthesis, the volume of extraction of plants-sources of secondary metabolites from natural sites is increasing, resulting in reduced biodiversity of natural phytocenoses. It also leads to a decrease in the number of individual plant species, which are becoming rare and some even endangered. Instead, the development of plant biotechnology allows it to be used as a raw material for the extraction of valuable compounds not only from wild or artificially grown plants, but also from microclonal propagation and culture of plant cells, tissues and organs *in vitro*. The use of these cultivation methods opens up the possibility of regulated synthesis of bioactive secondary metabolites, genetic transformation in order to obtain more productive regenerating plants, while preserving their natural sources. Regulation of the formation of valuable substances is carried out by selecting the composition of nutrient media, lighting, temperature under which plants are cultivated [24].

Growing plants in controlled conditions on artificial nutrient media allows to obtain plant biomass in almost unlimited quantities, which is actually used as a source of valuable metabolites. Plant raw materials obtained in this way are environmentally friendly, not contaminated with

chemical fertilizers, pesticides, herbicides, radioactive isotopes, heavy metals and the like. The use of cell cultures and painstaking selection work made it possible to get a wide range of bioactive substances (xanthones, terpenoids, alkaloids, glycosides, etc.) of plant origin both on a laboratory and industrial scale. Examples of applications of this technology are:

- cloning of plants;
- obtaining cell cultures of different types (meristem culture, callus production);
- cultivation of individual plant organs (lateral or apical buds, anthers, «hairy roots»);
- suspension cultures;
- bioreactors, etc.

The intensity of the process of formation of bioactive metabolites *in vitro* is influenced by: plant growth regulators; mineral composition of the nutrient medium; carbohydrate nutrition of explants; physical factors, such as light intensity and wavelength, artificial selection of producer cells. Thus obtained bioactive substances are widely used in pharmacological, medical, food, cosmetic and other industries for the production of goods to improve the quality of life and treatment [25,26].

That is why the aim of our research was to study different species of plants grown under aseptic conditions, callus production, as well as the use of nutrient media as additional environmentally friendly sources of pharmacologically valuable secondary metabolites. Growing plants in controlled conditions on artificial nutrient media allows to obtain plant biomass in almost unlimited quantities, which is actually used as a source of valuable metabolites.

2 Materials and methods

Cultivation material was obtained by different plant species microclonal propagation under aseptic culture conditions. Then cloned plants were grown *in vitro* for 3-5 months at 24 °C, 16-hour photoperiod on a modified agar nutrient medium Murassige-Skuga [27]. The chemical composition of the used nutrient medium:

- standart Murazige-Skuga nutrient medium (mix of micro- and macroelements) (Sigma, Germany);
- mesoinozit (China);
- composition of vitamins (LLC UKRCHIMEXPO, Ukraine): B₁ (thiamine), B₆ (pyridoxine), C (ascorbic acid), PP (nicotinic acid);
- Fe₂SO₄ (LLC «UKRCHIMEXPO», Ukraine);
- sucrose (LLC «UKRCHIMEXPO», Ukraine);
- agar (Spain);
- composition of plant growth regulators (Sigma, Germany): indolyl acetic acid, kinetin;
- casein hydrolyzate (Sigma, Germany) and yeast extract (LLC «UKRCHIMEXPO», Ukraine) were added to the nutrient medium prepared for *Phalaenopsis sp.*

The agar medium was also used as a source of bioactive substances. *In vitro* cultivation of plants was carried out in the research laboratory «Introduced and natural phytodiversity» of O. V. Fomin Botanical Garden of Taras Shevchenko National University of Kyiv.

Plant material and nutrient medium were quantitatively analyzed according to generally accepted methods: 96% ethanol solution was used for extraction and spectrophotometric analysis of phenolic compounds. Determination of the total content of phenolic compounds was performed using Folin-Chekolte reagent [28, 29], and the content of flavonoids – 0.2% solution of nitrate crystal hydrate of zirconyl chloride (IV) and rutin as a standard (with modifications) [30].

The content of xanthenes determined chromatographically with UV-light identification of spots (at $\lambda = 200\text{--}400\text{ nm}$) by our own modification. According to the literature, the extraction of xanthenes from plant material involves the use of solutions of toxic polar solvents of high concentration (70-96%): methanol, hexane, chloroform, butanol, ethylacetate or their different mixtures with 96% ethanol [31-33]. Hence, the presence of such solvents requires pre-purification of the extracts for further use in the analysis. Our modifications of the xanthone extraction method concerned the replacement of such a kind of solvents with environmentally friendly ethanol (DKP Pharmaceutical Factory LLC, Ukraine) only: 70% solution – for the extraction and 60% solution – for spectrophotometric analysis of xanthone content.

The approbation of methods covered in the literature, we noticed that repeated filtration (3-4 times) of extracts [34] instead of increasing the accuracy of the analysis, led to significant losses of the amount of test solution, which was absorbed by the filter components and it was difficult to drain the sediment. Accordingly, we noticed a negative effect on the accuracy of quantitative analysis. For that reason, we changed the extract to one stage of 3 hours duration. The chromatographic analysis involved the use of 40% acetic acid (LLC «UKRCHIMEXPO», Ukraine) – as a mobile phase for separation the xanthenes from other phenolic compounds.

The secondary metabolites content analysis in the nutrient medium is insufficiently mentioned in scientific papers only in liquid and suspension cultural medium but not in agar medium [35]. Therefore, we extracted secondary metabolites from agar medium by our own method using a low temperature staining a 70% ethanol solution, followed by centrifugation to remove residual particles of the agar medium [36]. The secondary metabolites content analysis of the medium was done by the same method as for the *in vitro*-plants. All extracts from plant material and nutrient medium were analyzed spectrophotometrically (SF SHIMADZU UV – 1800, Japan).

Biochemical analysis of plant material and samples of nutrient medium was carried out in the laboratory «Physiological basis of plant productivity» of the Department of Plant Biology at the Educational-Scientific Center «Institute of Biology and Medicine» of Taras Shevchenko National University of Kyiv.

Our experiment was performed in triplicate, results – statistically processed according to conventional methods using the standard program Microsoft Office Excel. In preparing, the method analyzed the average data obtained, the differences between which considered significant by Student's criterion (at $P \leq 0.05$).

3 Results and discussion

As a source of bioactive compounds, we used different types of plants. The explants of plants of the families *Orchidaceae* (Fig. 3a) [37] and *Acoraceae* (source plant material belonged to different localities formed in 2 groups for the experiment) (Fig. 3b) were obtained [38].

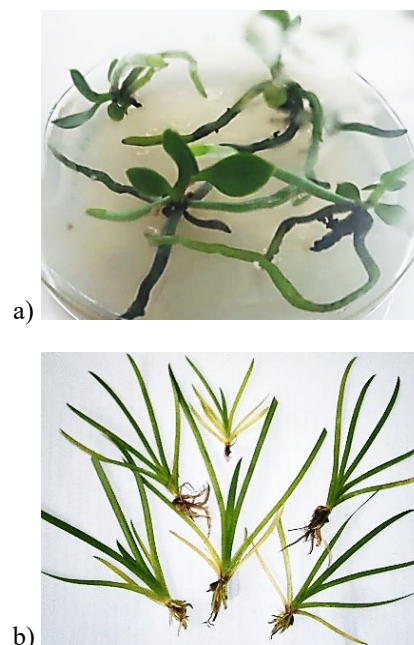


Fig. 3. *In vitro*-regenerated plants of *Phalaenopsis sp.* (a) and *Acorus calamus L.* (b)

Quantitative analysis of secondary metabolites content (total phenolic content) showed a steady increase in the content of phenolic in the nutrient medium used for cultivation of *Acorus calamus L.* (by around 30 %) (Fig. 4).

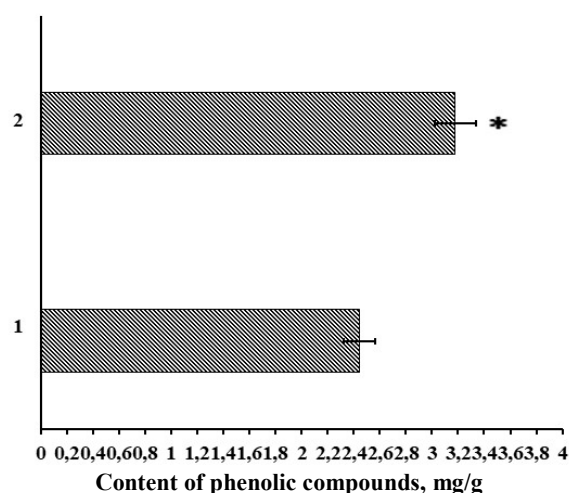


Fig. 4. Content of phenolic compounds in the nutrient medium used for cultivation of *A. calamus*, mg/g by DMW (significant at $P \leq 0.05$): 1) exposition for 3 months, 2) exposition for 5 months

It was also described the differences in phenolic compounds content in explants of the same plant during the experiment: increasing by 2 times in explants of Group

1 in leaves and roots (Fig. 5a) and of Group 2 – only in leaves (Fig. 5b).

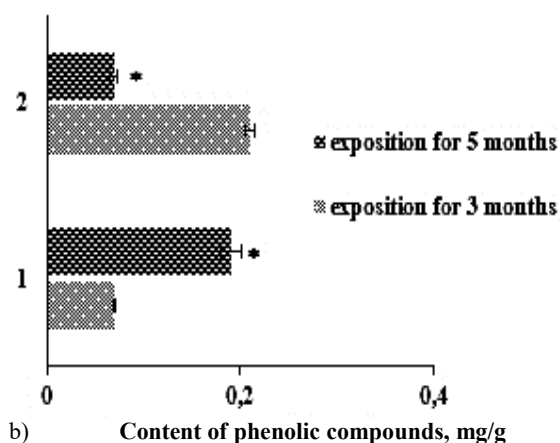
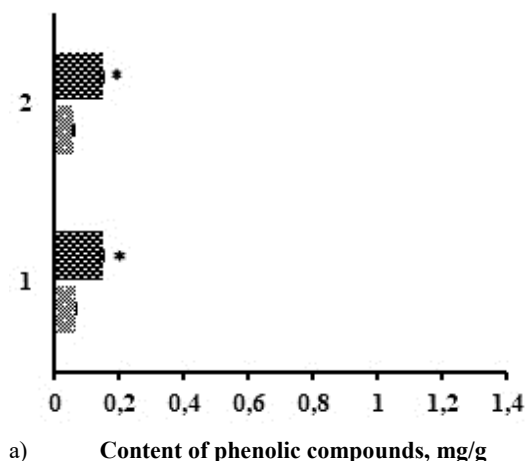


Fig. 5. Content of phenolic compounds in explants of *A. calamus* L., mg/g by DMW (significant at $P \leq 0,05$): a) Group 1, b) Group 2, 1) in leaves, 2) in roots

The obtained data on the flavonoid content in explants of *A. calamus* indicate an changing of their concentration during the experiment and also depending on the part of the explants: decreased in explants of Group 1 (Fig.6), increased in explants of Group 2 (Fig.7).

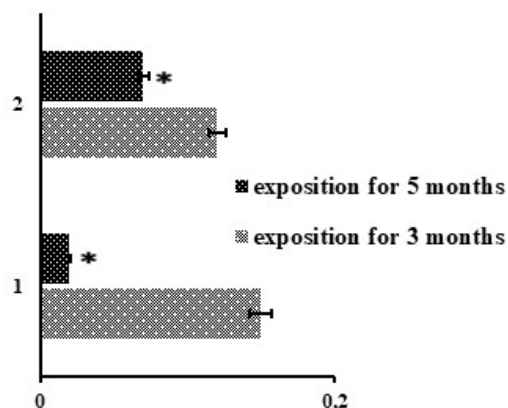


Fig. 6. Content flavonoids in explants of *A. calamus* L. (Group 1), mg/g by DMW (significant at $P \leq 0,05$): 1) in leaves, 2) in roots

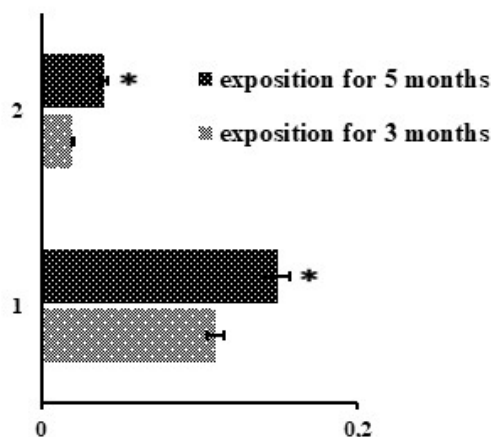


Fig. 7. Content flavonoids in explants of *A. calamus* L. (Group 2), mg/g by DMW (significant at $P \leq 0,05$): 1) in leaves, 2) in roots

We also identified variations in chemical composition xanthenes depending on the type of the explant. As an example, *A. calamus* explants of both groups synthesized and accumulated only one type of xanthenes – swerchirin. There were also differences in xanthone content among the explants of two different localities of *A. calamus*. Group 1 of explants accumulated compounds in leaves and roots (Fig.8).

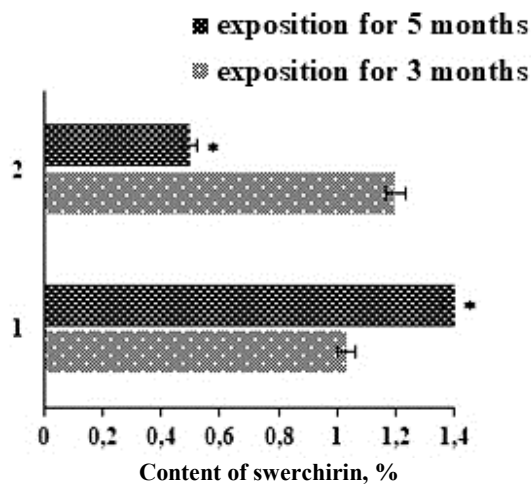


Fig. 8. Content of swerchirin in explants of *A. calamus* (Group 1), % by RMW (significant at $P \leq 0,05$): 1) in leaves, 2) in roots

However, explants of Group 2 accumulated swerchirin only in leaves and lost the starting amount of these compound in roots (almost by 50%) during the experiment (Fig.9).

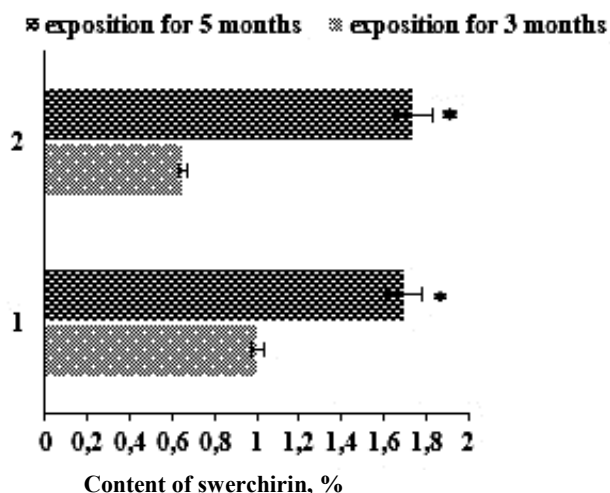


Fig. 9. Content of swerchirin in explants of *A. calamus* (Group 2), % by RMW (significant at $P \leq 0,05$): 1) in leaves, 2) in roots

The amount of xanthenes in the nutrient medium decreased significantly over the same period of time, and the content of flavonoids was too low to be detected by a quantitative analysis. We also identified a different composition of xanthenes depending on localities of explants that were cultivated on this medium (Fig. 10 and Fig. 11).

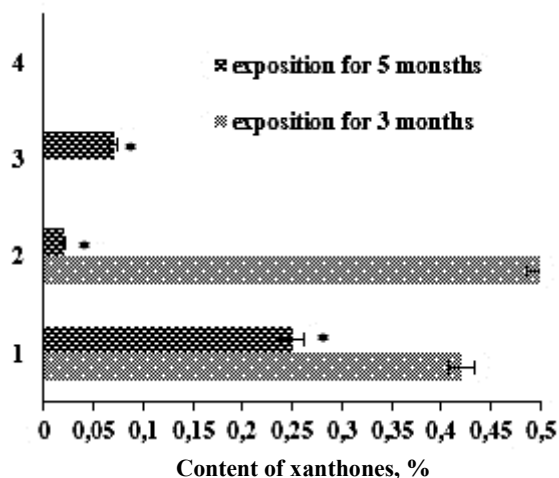


Fig. 10. Content of xanthenes in the nutrient medium used for cultivation of *A. calamus* (Group 1), % by RMW (significant at $P \leq 0,05$): 1) 1-hydroxy-2,3,5-trimethoxyxanthone, 2) swerchirin, 3) decussatin 4) mangostenone A

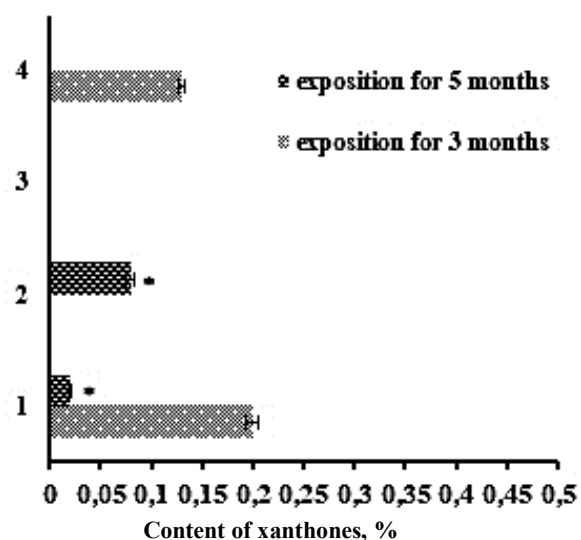


Fig. 11. Content of xanthenes in the nutrient medium used for cultivation of *A. calamus* (Group 2), % by RMW (significant at $P \leq 0,05$): 1) 1-hydroxy-2,3,5-trimethoxyxanthone, 2) swerchirin, 3) decussatin 4) mangostenone A

In contrast, *Phalaenopsis sp.* explants contained different xanthenes: 1-hydroxy-2,3,5-trimethoxyxanthone, decussatin, swertiaperennin, swerchirin, α -mangostin (Fig. 12).

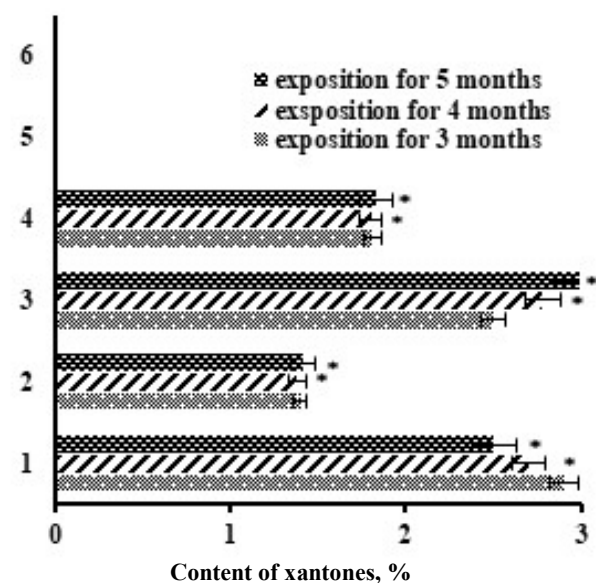


Fig. 12. Content of xanthenes in explants of *Plalenopsis sp.*, % by RMW (significant at $P \leq 0,05$): 1) 1-hydroxy-2,3,5-trimethoxyxanthone, 2) decussatin, 3) swertiaperennin, 4) α -mangostin 5) mangostenone A, 6) swerchirin.

The nutrient medium used for cultivation of *Plalenopsis sp.* explants contained decussatin, α -mangostin, mangostenone A and swerchirin (Fig. 13).

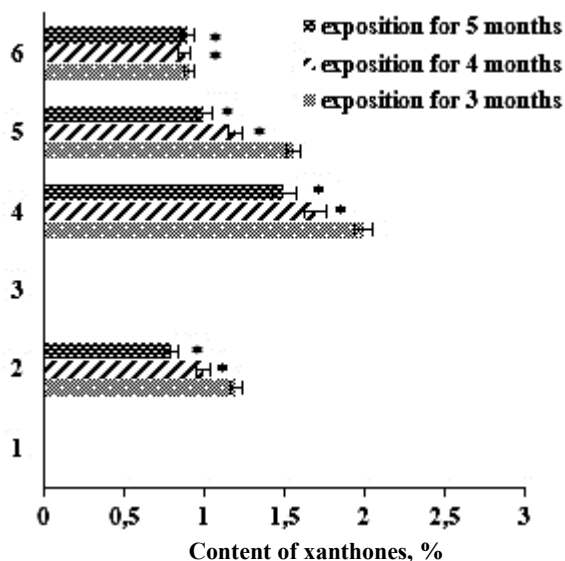


Fig. 13. Content of xanthenes in the nutrient medium used for cultivation of *Plalenopsis* sp. explants, % by RMW (significant at $P \leq 0,05$): 1) 1-hydroxy-2,3,5-trimethoxyxanthone, 2) decussatin, 3) swertiaperennin, 4) α -mangostin 5) mangostenone A, 6) swerchirin

All plants grew under the same conditions, so we assume that the differences in the metabolites content could be caused by genetical factors that influenced biochemical and physiological features of experimental plant material and the agar nutrient medium.

Given that the changing conditions of plant cultivation (duration of exposure, type of explants, chemical composition of the living environment), it is possible to regulate the content of special metabolism substances.

4 Conclusion

The unique properties of plants to synthesize a wide range of bioactive substances, in particular compounds of secondary origin, makes it possible to use these natural resources. In order to preserve the biodiversity of natural biocenoses and extract the necessary substances, biotechnological methods are used, especially plant cell, tissue and organ aseptically culture.

The results of our study fully confirm the data on the secondary metabolites extraction from the plant material and nutrient medium grown under *in vitro* conditions. It is also indicates the dependence of the synthesis of bioactive compounds on the species of plants, their origin and, accordingly, the type of explants obtained from them, as well as the exposure time and cultivation conditions.

We consider promising research of plants, cell cultures and various modifications in the chemical composition of the nutrient medium as environmentally friendly sources of pharmacologically valuable bioactive metabolites.

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