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ENDOGENOUS CYTOKININS IN MEDICINAL BASIDIOMYCETES MYCELIAL BIOMASS

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The aim of the research was to study the cytokinins production by medicinal basidial mushrooms. Cytokinins were for the first time identified and quantified in mycelial biomass of six species (*Ganoderma lucidum*, *Trametes versicolor*, *Fomitopsis officinalis*, *Pleurotus nebrodensis*, *Grifola frondosa*, *Sparassis crispa*) using HPLC. *Trans*- and *cis*-zeatin, zeatin riboside, zeatin-O-glucoside, isopentenyladenosine, isopentenyladenine were found but only one species (*G. lucidum*, strain 1900) contained all these substances. The greatest total cytokinin quantity was detected in *F. officinalis*, strain 5004. *S. crispa*, strain 314, and *F. officinalis*, strain 5004, mycelial biomass was revealed to have the highest level of cytokinin riboside forms (zeatin riboside and isopentenyladenosine). The possible connection between medicinal properties of investigated basidiomycetes and of cytokinins is discussed. *S. crispa*, strain 314, and *F. officinalis*, strain 5004, are regarded as promising species for developing biotechnological techniques to produce biologically active drugs from their mycelial biomass. As one of the potential technological approaches there is proposed fungal material drying.

Key words: medicinal mushrooms, mycelial biomass, cytokinins.

Mushrooms form a special kingdom of living creature — Fungi. They possess both plant and animal features in their cell structure and metabolism. The most important regulation system of plants and animals is a hormonal one, all vital functions of these organisms are under hormonal control. Hormones trigger all life programs without exceptions and control their running during ontogenesis. The action of such system in fungi remains unknown. More than 35 fungi species are able to synthesize the phytohormones, but a functional significance of these substances has not been investigated [1]. The main attention was concentrated on microscopic phytopathogenic fungi causing an abnormal growth of host plants tissues and tumours formation. One of the specific symptoms of fungal infection is a considerable cytokinins levels increase in these tissues (several times higher than those in healthy ones) [2, 3].

Cytokinins are polyfunctional phytohormones that are involved in plants growth and development regulation. They stimulate cells division and shoot apical meristems initiation and activity, delay leaves senescence, inhibit roots growth and branching, control sink/source relationships, seeds germination, nutrient uptake and response to biotic and abiotic stresses [4, 5]. Naturally occurring cytokinins are adenine derivatives which have the similar structure but not equivalent functions. Hormone molecules with the side chain structure variations are likely to mediate different biological signals. The participation of *trans*-zeatin and isopentenyladenine in long-distance signal transmission in acropetal and basipetal directions along plant stem correspondently is assumed [6].

Phytopathogenic fungi are shown to produce high concentrations of cytokinins in culture [7, 8]. The incorporation of ^{14}C-adenine into cytokinins in *Fusarium moniliforme* confirmed the ability of this fungus to synthesize hormone *de novo* [9]. Biotrophic fungus *Claviceps purpurea* produced substantial quantities of cytokinins in culture and expressed the cytokinins biosynthesis enzymes genes [2]. The general strategy of

parasitic fungi is to use cytokinins for host plant growth manipulations. They not only can apply hormones synthesized by themselves but change cytokinins biosynthesis genes expression in occupied plant [10, 11].

Until now there are few data on the cytokinins occurrence and functioning in basidial mushrooms. Cytokinins activity was for the first time detected in their fruit bodies by bioassays in the 70s of the XX century. Cytokinins activity corresponding to zeatin and zeatin riboside was detected in *Agaricus bisporus* (J. E. Lange) Imbach and *Pleurotus sajor-caju* (Fr.) Singer [12], in six species of genus *Rhizopogon* and twenty two species of genus *Hebeloma* [13]. The content of zeatin riboside in *Pleurotus ostreatus* (Jacq.) P. Kumm. basidiomes [14], zeatin — in *Lentinus tigrinus* (Bull.) Fr. and *Laetiporus sulphurous* (Bull.) Murrill [15], *trans*-zeatin — in eighteen macromycetes species, particularly in *L. tigrinus, Boletus impolitus* Fr., *Ptychoverpa bohemica* (Krombh.) Boud., *Volvariella speciosa* (Fr.) Singer, *Amanita gemmata* (Fr.) Bertill. etc. was determined by high performance liquid chromatography (HPLC) [16]. Recently, combination of HPLC with electrospray ionization tandem mass spectrometry allowed to determine seven cytokinins in twenty species of forest fungi [3].

Last years, a higher interest to growth regulating substances in edible mushrooms fruit bodies is connected with necessity to produce ecologically pure protein products and to increase cultivated mushrooms yield. Additionally, almost all basidiomycetes possess the medicinal properties and that is why medicinal mushrooms science devoted to study of these fungi characteristics is developing successfully in 21st century [17]. It is not yet completely known what specific components of biochemical mushrooms composition are effective therapeutic agents. At the same time, therapeutic properties of cytokinins, in particular, anticancer and immunomodulatory action, have been proved now [18–20]. The question about possible interconnection between medicinal mushrooms effects and cytokinins synthesized in their cells arises. We have previously established that cytokinins content in *Lentinus edodes* (Berk.) Singer mycelium is by an order in magnitude higher than that of fruit bodies (data not published). Taking in consideration all above mentioned we aimed to study the cytokinin production by mycelial biomass of basidial mushrooms and to determine the species with the higher productivity.

Materials and Methods

Biological material

The following basidial macromycetes were screened for cytokinins content: *Pleurotus nebrodensis* (Inzenga) Quel., *Grifola frondosa* (Dicks: Fr.) S.F.Gray, *Fomitopsis officinalis* (Vill.) Bondartsev et Singer, *Sparassis crispa* (Fr.) Fr., *Ganoderma lucidum* (Curtis) P. Karst and *Trametes versicolor* (L.) Lloyd. All of them are the valuable fungi species with long history of applying in oriental medicine and ethnoscience for many diseases treatment and prevention. We investigated the pure cultures of *F. officinales* 5004, *S. crispa* 314, *G. lucidum* 1900, *T. versicolor* 353, *G. frondosa* 976 and *P. nebrodensis* 2035 from the National Ukrainian Culture Collection of Mushrooms of Kholodny Institute of Botany of the National Academy of Sciences of Ukraine.

Mushrooms cultivation

To obtain the mycelial biomass of above mentioned strains we cultivated them in 250 ml Erlenmeyer flasks with 50 ml liquid medium at thermostat conditions (26 ± 1 °C) in darkness (Fig. 1). Inoculation with physiologically active mycelium in proportion 10% to total volume was carried out in accordance with method developed for basidiomycetes [21]. Microbiological control of nutrient medium and seeding material purity was fulfilled before inoculation. For cultivation of *F. officinales* 5004 and *S. crispa* 314 the following liquid nutrient medium was used (g/l): glucose — 30,0; NH_4NO_3 — 3,5; KCl — 0,5; K_2HPO_4 — 1,0; $MgSO_4 \cdot 7H_2O$ — 0,5; beer wort (15° in accordance with Baling method) — 115 ml; pH — 5,0. For cultivation of *G. lucidum* 1900, *T. versicolor* 353, *G. frondosa* 976 and *P. nebrodensis* 2035 such liquid nutrient medium was used (g/l): glucose — 25,0; peptone — 3,0; yeast extract — 3,0; KH_2PO_4 — 1,0; K_2HPO_4 — 1,0l; $MgSO_4 \cdot 7H_2O$ — 0,25; pH — 6,5. Mediums acidity was maintained at necessary pH levels by 1 N KOH and 1 N HCl solutions addition.

Cytokinins analysis

The cytokinins extraction and purification from mushrooms mycelial biomass and final determination in samples were based on the methods described for plants [22] with some modifications. Choice and combination of procedures and their adaptation to work with mushrooms mycelial biomass were realized for the first time. The sample (10 g of mycelial biomass) was homogenized in 80% ethanol solution. Homogenous material was mixed

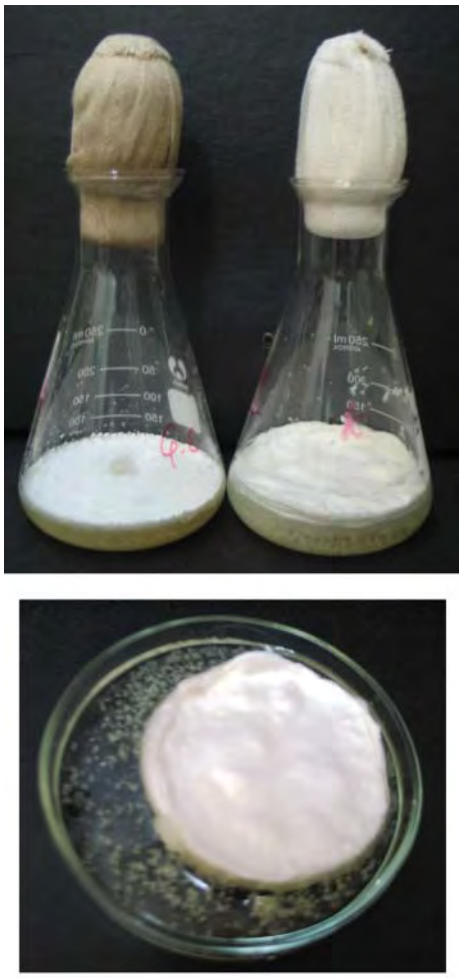

Fig. 1. **Basidial mushrooms mycelial biomass:** *A* — *Ganoderma lucidum*, strain 1900, and *Pleurotus nebrodensis*, strain 2035; *B* — *Sparassis crispa*, strain 314

with 80% ethanol (10 ml per 1 g) thrice during a day (2 h + 2 h +20 h). Every time extracts were filtrated under vacuum and dry debris was washed with new ethanol portion. All procedures were performed in cold conditions (+4 °C) to prevent hormones enzyme or oxidative degradation. Joined extract was evaporated under vacuum on rotor evaporator at +50 °C to water phase state. Water residue was kept in freezer at −20 °C. After 24 h it was fast defrosted to break cells and subcellular structure. Then extract was adjusted with 1 N HCl to pH 2,5 and centrifuged at 15 000 g and +4 °C during 30 min in K-24 centrifuge (Janetzky, Germany). Supernatant was adjusted with 1 N NaOH to pH 8,0 and fourfold fractionated with *n*-butanol (1:1 by volume). Joined butanol extract was dried under vacuum and kept at −20 °C. For further purification dry residue was dissolved in 0,1 N HCl and taken through a column 20×2 cm Bio-Rad (USA) containing ionexchange resin Dowex 50Wx8 (Serva, Germany) in H^+ form. The column was rinsed with water, the sample was eluated by 0,1 N ammonia and eluate was evaporated under vacuum. Then, sample was purified by thin layer chromatography (TLC). The dry residue was dissolved in 1 ml of 96% ethanol and applied on TLC plates Silicagel 60 F_{254} (Merck, Germany) 20×20 cm. The plates were run in solvent systems isopropanol:ammonia:water (10:1:1 by volume) and dried. Zones correspondent to the cytokinins standards were detected under UV 254 in chemoscope Desaga (Germany) and then eluated with 96% ethanol. The final cytokinins identification and determination were performed by HPLC (Agilent 1200 LC, USA) using Eclipse XDB-C 18 column (2,1×150 mm), particles size 5 μм. Elution was carried out with solvents system methanol:water (37:63 by volume). Data were analyzed and processed by software Chem Station, version B.03.01 on line. Standard solutions of *trans*- and *cis*-zeatin, zeatin riboside, isopentenyladenosine, isopentenyladenine and zeatin-O-glucoside (Sigma, USA) were used as a markers. The example of HPLC chromatogram of cytokinins determination is shown on Fig. 2 (illustrated by *Sparassis crispa* 314 micelial biomass purified sample).

Methods of statistical analysis

Cytokinins concentration and water content were determined from three replicates of each experiment. HPLC analysis was carried out with five analytical replications. The data was processed by standard methods of variation statistics using Microsoft Excel 2007 program. In the Tables the average values and standard errors ($M \pm m$) are presented. Values of $P < 0.05$ were considered to be significant.

Results and Discussion

Trans- and *cis*-zeatin, zeatin riboside, zeatin-O-glucoside, isopentenyladenosine and isopentenyladenine were determined in the mycelial biomass of six medicinal macromycetes species but only in one of them (*G. lucidum* 1900) all these forms were present (Table 1). Studied species contained mostly zeatin-type cytokinins that are normally dominant in higher plants [6]. Isopentenyladenosine was not detected in fungi mycelial biomass excepting *G. lucidum* 1900. Very low levels of isopentenyladenosine were found only in *S. crispa* 314 and *G. lucidum* 1900. This data indicate that a direct

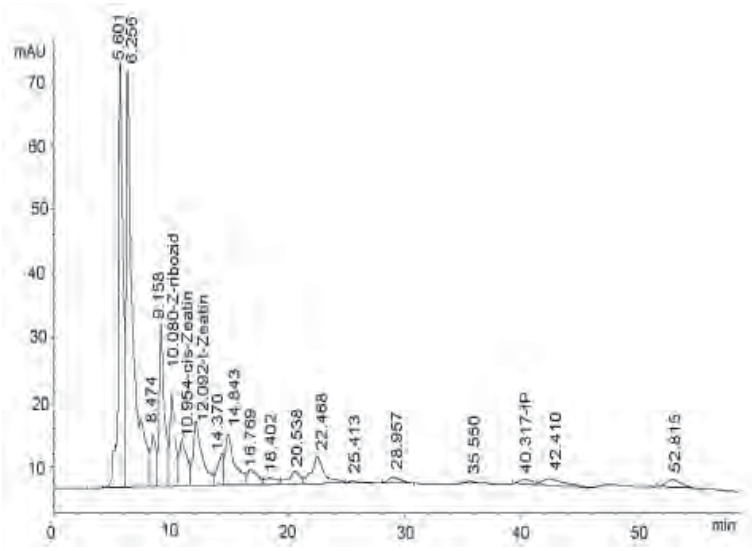

Fig. 2. **HPLC-analysis of of cytokinins, purified from:** *Sparassis crispa*, strain 314, mycelial biomass, $\lambda = 254$ nm

isopentenyl-dependent (mevalonate) pathway of cytokinins biosynthesis was suspended or its level was very low. Mevalonate pathway is appropriate to higher plants but it also occurs in animals, fungi and bacteria and is localized in the cytoplasm and mitochondria [4, 23]. However, presence of rather large amounts of *cis*-zeatin in all studied fungal species testifies to the possibility of a considerable contribution of tRNA degradation to the cytokinin pool because it is *cis*-zeatin that is produced in this way [24]. Thus, the obtained data demonstrated an insignificant level of cytokinins direct biosynthesis at the studied stage of fungal mycelial biomass development and the necessity to check the intensity of these hormones production at the other stages to provide a more significant yield of cytokinins.

Mycelial biomass of investigated fungi species differed both in a qualitative composition and in quantitative content of endogenous cytokinins (Table 1). In *P. nebrodensis* 2035 the amount of a conjugated form — zeatin-O-glucoside was the greatest. In *G. frondosa* 976 the level of free zeatin-type forms — *trans*- and *cis*-zeatin was relatively high. Mycelial biomass of *F. officinalis* 5004 was characterized by a considerable concentration of bound zeatin forms — riboside and O-glucoside. *Trans*-zeatin and zeatin riboside were dominant in *S. crispa* 314 while *cis*-zeatin — in *T. versicolor* 353 and *G. lucidum* 1900. In general, cytokinin qualitative composition in analyzed fungal mycelial biomass was, probably, species-specific like that in plants [25].

The largest total cytokinins content was detected in *S. crispa* 314 and the smallest one — in *G. lucidum* 1900 (Table 1). Amount of individual cytokinin forms in fungal mycelial biomass varied in most cases approximately in the range of 40–200 ng/g of fresh weight. Such concentrations of these hormones in fresh plant tissues are regarded as low and are typical for plant organs at the final stages of growth. Cytokinins levels in rapidly growing plant organs with a high mitotic index are by order of magnitude greater as a rule [5, 23]. Since it is highly possible that cytokinins biosynthesis and metabolism pathways differ in different organisms [7] and taking into account the fact that fungi belong to a separate kingdom of living creatures, comparison of concentrations of these hormones in mycelial biomass and plant tissues does not enable to make unambiguous conclusions concerning the correspondence of hormones accumulation to growth processes intensity in fungi. Unfortunately, literature data on the cytokinin dynamics in fungal tissues at the various developmental stages are not available.

The analysis of studied material has shown that mycelial biomass of all six medicinal macromycetes species had a great quantity of moisture (Table 2).

According to this the results about endogenous cytokinin concentrations were recalculated per gram of dry weight (Table 3). This data showed that the total amount of these hormones is considerably higher as compared with plants [5, 23]. The highest cytokinins content was detected in *F. officinalis*

Table. 1. **Cytokinins content in medicinal mushrooms mycelial biomass (ng/g of fresh weight)**

Sample	*t*-Z *	*c*-Z*	ZR*	iPa*	iP*	ZG*	∑
Pleurotus nebrodensis, strain 2035	88.66 ± 4.0**	71.46 ± 3.1**	42.34 ± 1.8**	0	0	213.85 ± 8.5**	416.31
Grifola frondosa, strain 976	95.31 ± 4.2**	111.04 ± 4.9**	0	0	0	51.79 ± 2.2**	258.14
Fomitopsis officinalis, strain 5004	81.41 ± 3.7**	130.4 ± 5.9**	146.21 ± 6.7**	0	0	167.34 ± 7.5**	525.41
Sparassis crispa, strain 314	212.03 ± 9.1**	95.78 ± 4.5**	212.66 ± 9.7**	0	20.21 ± 0.9**	0	540.68
Trametes versicolor, strain 353	81.45 ± 3.4**	180.16 ± 8.7**	55.70 ± 2.2**	0	0	2.27 ± 0.1**	319.58
Ganoderma lucidum, strain 1900	40.04 ± 1.8	95.34 ± 4.3	48.95 ± 2.1	15.44 ± 0.7	6.81 ± 0.3	34.24 ± 1.6	240.82

Notes. Hereinafter: * *t*-Z — *trans*-zeatin, *c*-Z — *cis*-zeatin, ZR — zeatin riboside; iPa — isopentenyladenosine, iP — isopentenyladenine, ZG — zeatin-O-glucoside; ** $P \leq 0{,}05$ in comparison with correspondent cytokinins content in *Ganoderma lucidum*, strain 1900, mycelial biomass.

Table. 2. **Water content in medicinal mushrooms mycelial biomass (%)**

***Pleurotus nebrodensis*, strain 2035**	***Grifola frondosa*, strain 976**	***Fomitopsis officinalis*, strain 5004**	***Sparassis crispa*, strain 314**	***Trametes versicolor*, strain 353**	***Ganoderma lucidum*, strain 1900**
95.2	95.3	95.7	90.6	96.3	95.2

Table 3. **Cytokinins content in medicinal mushrooms mycelial biomass (ng/g of dry weight)**

Sample	*t*-Z *	*c*-Z*	ZR*	iPa*	iP*	ZG*	∑
Pleurotus nebrodensis, strain 2035	1847 ± 90**	1489 ± 71**	882 ± 39**	0	0	4437 ± 218**	8655
Grifola frondosa, strain 976	2028 ± 95**	2363 ± 108**	0	0	0	1102 ± 53**	5493
Fomitopsis officinalis, strain 5004	1893 ± 87**	3034 ± 147**	3400 ± 161**	0	0	3892 ± 182**	12219
Sparassis crispa, strain 314	2256 ± 109**	1019 ± 46**	2262 ± 111**	0	215±9**	0	5752
Trametes versicolor, strain 353	2201 ± 105**	4869 ± 233**	1506 ± 71**	0	0	62 ± 3**	8638
Ganoderma lucidum, strain 1900	834 ± 38	1986 ± 92	1020 ± 45	322 ± 14	142 ± 6	713 ± 32	5017

5004 (more than 12 mkg/g of dry weight) and the lowest one — in *G. lucidum* 1900 (5,017 mkg/g of dry weight). Thus, considering high moisture of mycelial biomass, we can make conclusion about very high relative cytokinins concentrations in fungi tissues. Accordingly, it is possible to consider mushrooms mycelium as a perspective source of cytokinins.

All studied fungal species produce great amounts of cytokinin hormones. At the same time, all of them possess medicinal properties and are used in traditional east medicine, folk medicine, and homeopathy. Particularly, an edible mushroom *P. nebrodensis* contains essential amino acids, unsaturated fatty acids, microelements and vitamins thereby it is very important for human dietary nutrition [26]. Powder made from its mycelial mass or fruit bodies and their water extracts inhibit tumour growth in experimental animals both by oral intake and injections [27, 28]. Fruit bodies, mycelium and metabolites of *G. frondosa* show antibacterial, antiviral, antifungal, antitumour and immunostimulating effects, regulate blood pressure and have anti-diabetic properties [29, 30]. *F. officinalis* has been used in folk medicine of Eastern Slavs to treat insular diabetes, thyroid gland function, neurasthenia, asthma [17]. In the countries of the South-East Asia *F. officinalis* has been known for more than 2000 years and is included in the composition of many drugs [31]. *S. crispa* extracts have oncostatic, immunostimulating and antimetastatic effects [32–34]. Medicinal potential of *G. lucidum* and *T. versicolor* is expressed in antitumour, immunostimulating, antibacterial, antiviral, antiphlogistic action [35–37]. Medicinal properties of these fungi are usually connected with their biologically active substances — polysaccharides and specific thriterpenoid compounds. Our studies have shown that fungal mycelial biomass contains a considerable amount of cytokinin hormones. At the same time, cytokinins are known to possess therapeutic properties. Cytokinins are known to stimulate cells division in plants [38]. Contrary, in animal and human they induce apoptosis and block the cell cycle of a wide spectrum of tumour cells [39]. They change morphology and disorganize actin cytoskeleton of bladder carcinoma T24 cells [40], block DNA synthesis and increase the level of cycline-dependent kinase inhibitor [41] and induce genes involved in a negative regulation of the cell cycle [42] in tumour cells of epithelium. Cytokinins also inhibit a replication of human enterovirus [43], show immunostimulating effects promoting proliferation of natural killer cells [44]. Comparing the above data on medicinal fungal properties and information on biological effects of cytokinins on animal and human cells, it can be suggested with a high probability that there is relation between them. Since fungal mycelial biomass contains a great quantity of cytokinins, it is not excluded that these substances make a major contribution to a therapeutic effect of fungi. Analysis of interrelation between these hormones structure and their antiprolifiration and cytotoxic effects on tumour cells indicates that mostly cytokinin riboside forms possess therapeutic properties [39, 45, 46]. It was shown in our experiments the largest total content of cytokinins in terms of dry weight in mycelial biomass of *S. crispa* 314, and about 50% of this quantity were riboside forms — zeatin riboside and isopentenyladenosine (Table 1).The highest level of ribosides was found in *F. officinalis* 5004 (Table 3). Thus, these two basidomycetes species may be regarded as perspective to obtaining highly biologically active preparations from their mycelial biomass to be further tested for a therapeutic effect and use to make treating and prophylactic remedies.

Thus, for the first time cytokinins were qualitatively and quantitatively analyzed in mycelial biomass of six medicinal mushrooms species — *Ganoderma lucidum, Trametes versicolor, Fomitopsis officinalis, Pleurotus nebrodensis, Grifola frondosa, Sparassis crispa* — using HPLC. The widest cytokinins spectrum was shown in *G. lucidum*, strain 1900. The largest riboside-type cytokinins content was detected in *S. crispa*, strain 314 *and F. officinalis*, strain 5004. The endogenous cytokinin concentration per gram of dry weight in mycelial biomass was considerably higher as compared with plants. The fungi raw material exsiccation can be offered as one of the possible stages in biotechnology of high biological active preparations obtaining.

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ЕНДОГЕННІ ЦИТОКІНІНИ У МІЦЕЛЯРНІЙ БІОМАСІ ЛІКАРСЬКИХ БАЗИДІОМІЦЕТІВ

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Мета роботи — дослідити продукування цитокінінів лікарськими базидієвими грибами. Уперше методом високоефективної рідинної хроматографії було ідентифіковано цитокініни та визначено їх вміст у міцелярній біомасі 6 видів (*Ganoderma lucidum*, *Trametes versicolor*, *Fomitopsis officinalis*, *Pleurotus nebrodensis*, *Grifola frondosa*, *Sparassis crispa*). Виявлено зеатин (*транс-* і *цис*-форма), зеатинрибозид, зеатин-О-глюкозид, ізопентеніладенозин та ізопентеніладенін, проте всі ці форми були присутні лише в одного виду (*G. lucidum*, штам 1900). Найбільший сумарний вміст цитокінінів визначено у *F. officinalis*, штам 5004. У міцелярній біомасі *S. crispa*, штам 314, і *F. officinalis*, штам 5004, виявлено найвищий рівень рибозидних форм цитокінінів (зеатинрибозиду й ізопентеніладенозину). Обговорюється можливий взаємозв'язок між лікарськими властивостями досліджених базидіоміцетів та виявленими цитокінінами. *S. crispa*, штам 314, і *F. officinalis*, штам 5004, розглядають як перспективні види для створення біотехнології отримання з їхньої міцелярної біомаси препаратів із біологічною активністю. Одним із можливих технологічних підходів може бути висушування грибного матеріалу.

Ключові слова: лікарські гриби, міцелярна біомаса, цитокініни.

ЭНДОГЕННЫЕ ЦИТОКИНИНЫ В МИЦЕЛЯРНОЙ БИОМАССЕ ЛЕКАРСТВЕННЫХ БАЗИДИОМИЦЕТОВ

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Целью работы было исследование продуцирования цитокининов лекарственными базидиевыми грибами. Впервые методом высокоэффективной жидкостной хроматографии были идентифицированы цитокинины и определено их содержание в мицелярной биомассе 6 видов (*Ganoderma lucidum*, *Trametes versicolor*, *Fomitopsis officinalis*, *Pleurotus nebrodensis*, *Grifola frondosa*, *Sparassis crispa*). Выявлены зеатин (*транс-* и *цис*-форма), зеатинрибозид, зеатин-О-глюкозид, изопентениладенозин и изопентениладенин, однако все эти формы присутствовали только у одного вида (*G. lucidum*, штамм 1900). Наибольшее суммарное количество цитокининов определено у *F. officinalis*, штамм 5004. В мицелярной биомассе *S. crispa*, штамм 314, и *F. officinalis*, штамм 5004, обнаружен наиболее высокий уровень рибозидных форм цитокининов (зеатинрибозида и изопентениладенозина). Обсуждается возможная взаимосвязь между лекарственными свойствами исследованных базидиомицетов и цитокининов. *S. crispa*, штамм 314, и *F. officinalis*, штамм 5004, рассматривают как перспективные виды при разработке биотехнологии получения из их мицелярной биомассы препаратов с биологической активностью. Одним из возможных технологических подходов может быть высушивание грибного материала.

Ключевые слова: лекарственные грибы, мицелярная биомасса, цитокинины.