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HOW THE BASIDIOMYCETES RESPOND TO BIOGENIC ASPARTATE-BOUND METALS(II) OF VARIABLE VALENCY IN GROWTH MEDIA

O.M. TSIVILEVA[1], A.N. SHATERNIKOV[1], V.V. FADEEV[1], E.V. LYUBUN[1], V.G. AMELIN[2], S.P. VORONIN[3], A.P. GUMENYUK[3], V.E. NIKITINA[1]

[1]*Institute of Biochemistry and Physiology of Plants and Microorganisms RAS*, 13, pr. Entuziastov, Saratov, 410049 Russia, e-mail tsivileva@ibppm.ru (✉ corresponding author), andrejsh93@gmail.com, vvf2593@gmail.com, lyubun@ibppm.ru, nikitina_v@ibppm.ru;
[2]*Alexander and Nilkolay Stoletovs Vladimir State University*, 87, ul. Gor'kogo, Vladimir, 600000 Russia, e-mail amelinvg@mail.ru;
[3]*AO «Bioamid»* 27, ul. Mezhdunarozhnaya, Saratov, 410033 Russia, e-mail bioamid@yandex.ru
ORCID:
Tsivileva O.M. orcid.org/0000-0002-5269-349X
Shaternikov A.N. orcid.org/0000-0002-1364-1902
Fadeev V.V. orcid.org/0000-0002-9725-3439
Lyubun E.V. orcid.org/0000-0002-8814-6949
Amelin V.G. orcid.org/0000-0001-7477-7398
Voronin S.P. orcid.org/0000-0002-3530-1001
Gumenyuk A.P. orcid.org/0000-0002-1147-088X
Nikitina V.E. orcid.org/0000-0003-2687-7890
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Abstract

Current studies on artificial mushroom cultivation are aimed at optimizing mineral nutrition and the delivery of metals(II). Organically bound trace metals are superior to their inorganic precursors. Cu, Mn, Fe, Zn, and Co metal(II) complexation with essential amino acids seems to be a solution to the bioavailability problem, thus making amino acid chelates of biogenic metals relevant for study. In particular, aspartic acid salts potentially could improve cultivable mushroom growing due to use of bioavailable organic compounds of microelements. However, a comprehensive study on mineral nutrition of cultivated mushrooms using biogenic metal chelates has not been conducted previously. This paper is the first to discover and characterize the effect of metal(II) aspartates on growth, biochemical response, antibacterial activity of mycelium submerged cultures, and fruiting-body formation in basidiomycetes *Ganoderma lucidum* strain 1315, *Grifola umbellate* strain 1622, *Laetiporus sulphureus* strain 120707, *Lentinula edodes* strain F-249, and *Pleurotus ostreatus* strains 69, BK1702 and HK352. The work was aimed at elucidating and comparing action of the variable-valent metal(II) aspartates on the physiological and biochemical parameters of the basidiomycetes. Glucose- and wheat powder-based nutrient media supplemented with 1×10^{-4} mol/l Cu(II), Mn(II), Fe(II), Zn(II), and Co(II) aspartates were used to grow mycelia in submerged culture, comparing growth parameters and production of fruiting bodies. Media without any supplements or with 2×10^{-4} mol/l L-aspartic acid were control. Antimicrobial activity of the metal-containing biosamples against plant pathogenic bacteria *Micrococcus luteus* B-109, *Pectobacterium carotovorum* subsp. *carotovorum* (strains 603 and MI), *Pectobacterium atrosepticum* 1043, *Pseudomonas fluorescens* EL-2.1, *Xanthomonas campestris* B-610 was determined by agar well diffusion method. A pool of secondary metabolites was analyzed by high performance liquid chromatography/high resolution time-of-flight mass-spectrometry method. Metal levels in specimens were quantified by atomic absorption spectroscopy technique. The fruiting body formation was tested in the lab and upon commercial growing. In lab tests, it was established that amino acid chelates of biogenic metals(II) intensify mycelium growth in liquid-submerged culture and fruiting body formation. Chelates of copper, manganese, zinc, and to a lesser extent iron, exhibited the significant growth-promoting effect on the basidiomycetes' mycelium under the submerged culture conditions, especially in respect to lacquered polypore, umbrella polypore, and sulfur-yellow polypore. The additives of $Cu(Asp)_2$, $Mn(Asp)_2$, $Zn(Asp)_2$ showed only slight stimulation or even inhibition of *P. ostreatus* 69 growth. Aspartic acid caused a suppressing impact on mycelia accumulation, regardless of the basidiomycetes' taxonomic characteristics. At the oyster mushroom fermentation in the presence of biogenic metal aspartates, the interstrain distinctions occurred among rapidly and slower growing cultures in relation to the metal chelates' exogenic

action. Thus, in assays with $Cu(Asp)_2$ and $Zn(Asp)_2$, the strain *P. ostreatus* BK1702 had an advantage over others in accumulating biomass. Manganese chelate exerted the most profound positive effect on the fast-growing strain *P. ostreatus* HK352. The latter, however, was suppressed in its development to the greatest extent compared with BK1702 or 69, when the cobalt organic salt appeared in the liquid medium. Earlier we discussed in detail the items related to these substances' increased level resulted from the exogenic action of some compounds. As a biochemical response of cultures to the above aspartates occurrence in the starting nutrient media, the organic substances with double bond, which were not detected in the absence of the same additives, appeared in the growth liquid. These substances were aromatic alcohol 2-phenylethanol, as well as *para*-hydroxyphenylacetic acid, the latter's maximal extracellular concentration evaluated by the analytical signal being observed at $Mn(Asp)_2$ introduction. According to the data we gathered by physicochemical research, the metal(II) aspartates, notably $Mn(Asp)_2$, $Cu(Asp)_2$ in the growth liquid induced the increased level of 5-hydroxymethyl-2-furaldehyde, dihydropyrone (structurally similar to kojic acid), *para*-hydroxyphenylacetic acid, which antioxidative properties are important for mushroom culture. Positive impact of the certain combinations of Mn(II), Cu(II), Fe(II), Zn(II) chelate compounds on *P. ostreatus* vital functions could be efficiently used for elaborating upon scientific foundations and developing the technology of mushroom mineral nutrition, including wide-scale growing. Biogenic metal aspartates could serve as the active ingredient in biopreparations for commercial mushroom culture. Oyster mushroom fruit bodies and mycelium parameters provided by the aspartates implementation allowed us to propose manganese(II) chelate for put into practice.

Keywords: basidiomycetes, physiological and biochemical features, biometals, amino acid chelates, aspartates

Interest in studying the influence of microelements on the physiological, cultural, biochemical properties of edible and medicinal higher mushrooms is due, on the one hand, to wide practical use, on the other, to the uniqueness of basidiomycetes as objects of microbiological and biochemical studies [1, 2]. The cultivation of xylotrophic macromycetes is, in fact, a biotechnological utilization of lignocellulosic waste, effectively converted into human food or feed with high nutritional value and improved bioavailability [3, 4].

Significant efforts to improve production of functional food ingredients and natural nutritional supplements aim at production of microbial biomass enriched with biometals [5-7]. The enrichment of mushroom cultures with substances containing essential elements can be very effective [8]. Therefore, the optimization of mineral nutrition and delivery of metals(II), such as copper, manganese, iron, zinc, cobalt, is essential for artificial cultivation of mushrooms.

The organic form of trace elements has significant advantages over inorganic precursors [9-11], in addition, when used in the form of inorganic salts, metals are unavailable for utilization by organisms [12-14]. Chemical complexation of metals(II) (in particular Cu, Mn, Fe, Zn, Co) with essential amino acids seems to be a solution to the problem of bioavailability [15, 16].

Interaction of metal ions with amino acids at a molar rate of 1:(1-3) leads to chelation through the formation of covalent-coordination bonds [17]. Amino acids and products of enzymatic degradation of proteins. i.e. small peptides are ideal ligands, since they possess at least two functional groups necessary for the formation of a ring structure with a metal [18]. Metal ions bind to carboxyl groups, and in aspartic acid complexes, some metal ions are able to form a chelate bond with amino groups [19, 20]. Therefore, stability constants of a number of metal complexes of aspartic acid are mainly determined by the metal ion affinity for the amino group [21] followed by selective binding of metal ions and their transfer through building complexes with aspartate [22, 23]. Coordination complexes of trace elements increase the absorption of minerals by the body [24]. Bioconjugation of metals with amino acids is a valuable tool for the functionalization of natural proteins and peptides [25, 26].

Our previous work studied the biological activity of fungal substations when the nutrient medium contained inorganic salts of biometals(II) [27]. Min-

eral salts of biometals as an exogenous source of microelements in deep fungal cultures did not provide a positive biological effect. So far, systemic studies to optimize mineral nutrition of cultivated mushrooms using aspartates of biogenic metals have not been carried out.

This paper is the first to discover and characterize the effect of aspartates of metals(II) with variable valency on growth, biochemical response in submerged cultures, antibacterial activity, and fruiting body formation in basidiomycetes of genera *Ganoderma*, *Grifola*, *Laetiporus*, *Lentinula*, and Pleurotus.

Our work aimed to reveal and compare the action of aspartates of metals(II) with variable valency on physiological and biochemical parameters of basidiomycetes.

Materials and methods. Basidiomycetes used in the experiments were zizhi (*Ganoderma lucidum* 1315), umbrella polypore (*Grifola umbellata* 1622), chicken-of-the-woods (*Laetiporus sulphureus* 120707), shiitake mushroom (*Lentinula edodes* F-249), and tree oyster mushroom (*Pleurotus ostreatus*). Strains *P. ostreatus* BK1702 and HK352 were obtained from the collection of basidiomycetes of the Institute of Biochemistry and Physiology of Plants and Microorganisms RAS (IBPRM RAS), *P. ostreatus* 69 was provided by the Institute of Microbiology of the National Academy of Sciences of Belarus. Mushroom cultures were maintained on wort agar (4 degrees Balling) in the dark at 4 °C.

Bacterial test systems for antimicrobial activity assay were selected based on the World Federation for Culture Collection — WFCC, #975, World Data Center of Microorganisms — WDCM, #1021. Plant pathogenic bacteria *Micrococcus luteus* B-109, *Pectobacterium carotovorum* subsp. *carotovorum* (strains 603 and MI), *Pectobacterium atrosepticum* 1043, *Pseudomonas fluorescens* EL-2.1, *Xanthomonas campestris* B-610 were used to measure antibacterial activity of submerged cultures of mushroom mycelium grown in the presence of chelates.

Glucose-asparagine culture medium contained 9.0 g/l D-glucose (300 mM carbon concentration) and 1.5 g/l L-asparagine (20 mM nitrogen concentration); yeast extract culture medium contained 10 g/l D-glucose and 1 g/l yeast extract. For solid media, 1.8-2.0% (w/v) agar was added.

Oyster mushroom liquid inoculum was cultured in wheat flour medium based on decoction of wheat flour at 26 °C. For culture medium, 100 ml of cold water was added to 20 g of premium wheat flour, stirred until homogeneous. The suspension was poured into 900 ml of boiling water in a thin stream, boiled for 2-3 minutes, and autoclaved at a 1 atm extra pressure.

Micrococcus luteus, *Pectobacterium carotovorum* subsp. c*arotovorum*, *Pectobacterium atrosepticum*, and *Pseudomonas fluorescens* were grown on the medium containing 10.0 g/l meat extract, 10.0g/l peptone, and 5.0 g/l NaCl. The medium for *Xanthomonas campestris* had the following composition: 20.0 g/l glucose, 10.0 g/l yeast extract, and 20.0 g/l $CaCO_3$. For solid media, 18 g/l Bacto® Agar (Difco Laboratories Inc., USA) was added, pH was adjusted to 7.2-7.4. All bacteria were cultured at 28 °C.

Cu(II), Mn(II), Fe(II), Zn(II), and Co(II) aspartates added to glucose- or wheat decoction-based media had the general formula $M(Asp)_2$, where Asp is aspartic acid, and were used at a 1×10^{-4} mol/l metal concentration in the medium. A 2×10^{-4} mol/l aspartic acid was another additive. Aspartates were non-hygroscopic free flowing fine powders of lilac color for cobalt, blue for copper, beige for iron, slightly beige for manganese, and white for zinc. Aspartates of metals(II) were derived from direct interaction of metal sulfates with a stoichiometric amount of aspartic acid (Asp) via complexation in neutral conditions,

followed by thermal spraying to dry [28].

For mycelial culture, 14-day-old mycelium of *G. lucidum*, *G. umbellata*, *L. sulphureus*, *L. edodes*, and *P. ostreatus* grown on beer wort agar (4 degrees Balling) was used as an inoculum. A 5 mm wort agar disc taken with a sterile metal punch from one zone of mycelium in a Petri dish was the dose of inoculum. Three disks per 50 ml of liquid medium were put into flasks to grow liquid culture mycelium, and 1 disk was put in the center of a Petri dish to grow mycelium in solid medium. The flour medium was inoculated with mycelium grown on solid beer wort in a Petri dish for 7-10 days.

Concentrated stock solutions of organic and inorganic salts in 50% (v/v) aqueous ethanol were added, with an automatic pipette, into each flask or molten agar cooled to a temperature of ~ 40 °C after autoclaving, prior to pouring Petri dishes. Calculation of the resultant concentration of M^{2+} cations in nutrient media considered the specified dilution. Growth media without aspartates or aspartic acid served as a control for mushroom cultures, including oyster mushroom strains.

P. ostreatus inoculum for growing fruiting bodies was cultured according to the standard technology on durum wheat grain substrate [29]. The grain was exposed to hot water (90 °C) for 20 min and twice (with a 24 h interval) autoclaved at 1 atm for 30 min in 500 ml containers. The wheat grain substrate was inoculated with 14-day old mycelium from submerged cultures grown in flour medium with or without (control) of metal aspartates. Mycelial inoculum was also grown in flour medium with pairs of amino acid chelates added to sterile wheat flour decoction. On days 3, 5, 7, 9, and 14 of growth, the grain substrate was shaken to provide better colonization and inspect contamination by competing microflora. Cultivation lasted 2 weeks at 24-26 °C.

Sunflower husk, the most available and therefore popular lignocellulosic substrate for industrial mushroom farming in Russia, was used in the experiments. Fruiting bodies were grown in lab tests according to a standard technique with pasteurized substrate. After 2 weeks in the dark, containers with colonized substrate were exposed to light in a humid chamber. On day 15 after inoculation, all the studied strains produced primordia. Further, the growth of fruiting bodies was monitored daily. The intensity of the substate colonization by *P. ostreatus* when using myceliated grain (grain spawn) from inoculation with liquid cultures grown with and without $M(Asp)_2$ additive was estimated to assess the effect of metal(II) aspartates. Metal aspartates were also applied to sunflower seed husk lignocellulosic substrate. The production of fruiting bodies was first assessed in the lab tests and then in a mushroom farm.

Mycelium grown in submerged culture was separated using filters previously weighed on an analytical balance, then dried to constant weight, and weighed again. The increase in biomass compared to 3-hour culture was measured in the control (without aspartates or Asp) and in test samples. The growth rate in submerged culture was expressed as dry biomass accumulation per unit time during culturing. The effect of exogenous aspartates or aspartic acid on mycelium liquid culture was expressed as biomass accumulation in the presence and absence of L-aspartates or Asp.

Metal-containing biosamples of fungal origin were obtained as described for inorganic salts [27]. The sensitivity of plant pathogens to fungal bioagents was measured by agar well diffusion method as the radius of the zones of bacterial growth inhibition around the well minus the diameter of the well itself. If the zones were oval in shape, the largest and smallest radius of the zone was measured to calculate the average value. The estimates were deemed indicators

of bactericidal activity.

Metal levels in biosamples were quantified by atomic absorption spectroscopy on an iCE 3000 C093500037 v1.30 spectrometer (Thermo Fisher Scientific, USA) at the Symbiosis IBPRM RAS Center for Collective Use (CCU) of scientific equipment in physical and chemical biology and nanobiotechnology.

Effects of the chelates on the fungal secondary metabolic profile were assessed by comparing control growth media extracts and those with 10^{-4} mol/l metal(II) aspartate (an UltiMate 3000 liquid chromatograph, Thermo Fisher Scientific, USA, coupled with a maXis Impact quadrupole time-of-flight mass spectrometric detector, maXis 4G, Bruker Daltonics, Germany). Separation was carried out on a column (150×2.1 mm) Acclaim ™ 120 C18 (2.2 μm) (Thermo Fisher Scientific, USA) in the mobile phase gradient elution mode.

The mobile phases contained 0.1% formic acid in water with the addition of 5 mM ammonium formate (A) and 0.1% formic acid in acetonitrile (B). Gradient elution mode was as follows: 0 min — 98% A + 2% B, 15 min — 100% B, 20 min — 100% B, 30 min — 98% A + 2% B, with the flow rate of 0.3 ml · min^{-1}, the optimal temperature of the chromatographic column of is 35 °C, the injection volumes of 20 μl. An ionBooster device (Bruker Daltonics, Germany) was used for electrospray ionization. Selection of the optimal parameter values was described in previous works [30, 31].

The registered ion masses ranged within 200-500 Da. Sodium formate (10 mM) in aqueous isopropanol solution of (1:1) was a calibrant. Calibration was performed automatically when recording a chromatogram. The error in determining the ion masses did not exceed ±5 ppm (n = 3).

TargetAnalysis-1.3. software (Bruker Daltonics, Germany) was used for identification. Chromatograms of the total ionic current and chromatograms of extracted ion masses were processed using DataAnalysis-4.1 software (Bruker Daltonics, Germany), the isotope distribution of analytes was drawn up with IsotopePattern software (Bruker Daltonics, Germany).

Microsoft Excel software package was used for data statistical processing. The arithmetic mean values (M) and standard deviations (±SD) are given. The values of the parametric Student's t-test were found for the 95% significance level.

Results. Biogenic metal chelates of amino acida, in particular aspartates, allow the use of microelements in a bioavailable organic form for artificial cultivation of mushrooms. Aspartic acid is used as a substance that forms a compound with metals, while the molar ratio of aspartic acid:metal is 2:1.

In contrast to inorganic salts of biometals(II) in our previous study [27], aspartates which have low toxicity and high biological activity [28] showed a high potential to mycelial biomass production and fruiting body production in lab tests with 1×10^{-4} mol/l trace element level in the nutrient media.

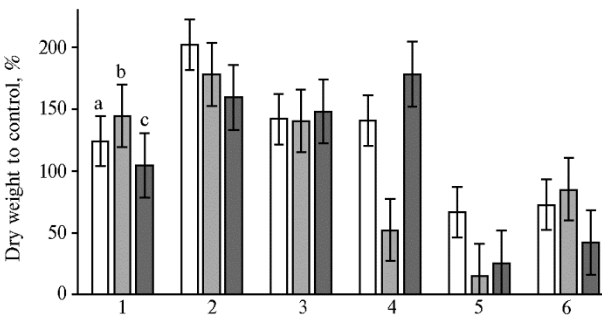

Fig. 1. Biomass accumulation in 21-day old submerged cultures of *Ganoderma lucidum* 1315 (a)**, *Grifola umbellata* 1622** (b)**, and *Laetiporus sulphureus* 120707** (c) **grown in a yeast extract-glucose nutrient medium with additives:** 1 — Cu(Asp)2, 2 — Mn(Asp)2, 3 — Fe(Asp)2, 4 — Zn(Asp)2, 5 — Co(Asp)2, 6 — Asp (M±SD).

In submerged cultures, most of the additives showed a significant growth-stimulating effect, especially for *Ganoderma lucidum* 1315, *Grifola umbellata* 1622,

Laetiporus sulphureus 120707 (Fig. 1). The dry biomass statistically significantly ($p < 0.05$) exceeded the control values without aspartates.

Stimulation of basidiomycetes by metal cations can be due to their ability to bind and stabilize key compounds for mycelium growth which molecules have various chemical structures, dimensional and charge characteristics, solubility, lipophilic properties, and reactivity. The distribution of charges plays a certain role. The negative charge on the mycelium surface, which promotes complexation during metal-ligand interactions, is provided by chitin, a component of the fungal cell wall [32], and carboxyl, amine, thiol, amide, imine, thioether, and phosphate functional groups [33]. There is not just a chemical reaction between the metal-containing compounds and the mycelium surface, in which the biomass passively binds metal ions according to known physicochemical mechanisms. The interaction with aspartates is metabolically dependent, which undoubtedly contributes to diverse responses of different fungal species to the same metal compound (see Fig. 1).

However, organic salts $Cu(Asp)_2$, $Mn(Asp)_2$, $Zn(Asp)_2$ showed very weak stimulation or even inhibited *P. ostreatus* 69 despite a significant positive effect on the growth other mushroom species (Fig. 2).

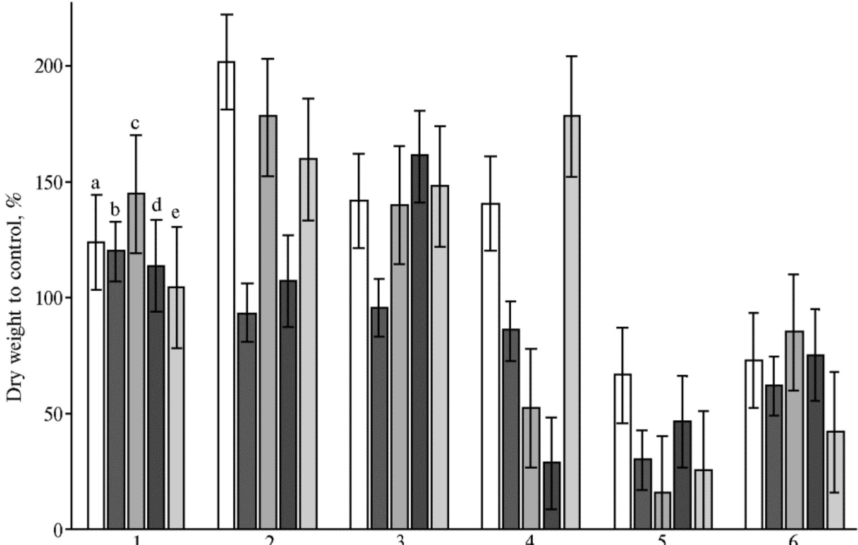

Рис. 2. Biomass accumulation in 21-day old submerged cultures of *Ganoderma lucidum* 1315 (a), ***Lentinula edodes* F-249** (b), ***Grifola umbellata* 1622** (c), ***Pleurotus ostreatus* 69** (d), **and *Laetiporus sulphureus* 120707** (e) **grown in a yeast extract-glucose nutrient medium with additives:** 1 — $Cu(Asp)_2$, 2 — $Mn(Asp)_2$, 3 — $Fe(Asp)_2$, 4 — $Zn(Asp)_2$, 5 — $Co(Asp)_2$, 6 — Asp (M±SD).

The pronounced dependence on the taxonomic characteristics of basidiomycetes during their artificial cultivation with inorganic metal salts has been experimentally confirmed. On the example of copper and zinc, the species-specificity of the effects of metals was shown for tens of cultures of edible mushrooms of different taxonomic positions [6, 34]. Eleven xylotrophic strains of *Trametes* fungi in submerged cultures significantly differed in the activity of lignin-modifying enzymes in the presence of copper, iron, and manganese salts [35]. Cu(II), Fe(II), and Zn(II) sulfates had different effects on the yield of biomass and polysaccharide metabolites of the fungus *Antrodia cinnamomea* [36].

Thence, the problem arose of selecting a *P. ostreatus* strain for more efficient use of amino acid chelates for spawn and fruiting bode production.

Fungiculture of *P. ostreatus* is promising primarily due to its high productivity, valuable nutritional composition, significant protein content [37, 38], and utilization of substrates unsuitable for any other purposes, namely the non-food waste of agriculture and industry [39-41]. Current studies of *P. ostreatus* growth in liquid media aimed at developing a technology for submerged mycelium culture to produce biomass for feed and food purposes, and as a source of various physiologically active drugs [42, 43] and biotechnologically valuable products [44-46]. Submerged culture is known as a fast and efficient method of producing liquid spawn in the cultivation of this mushroom [47, 48].

To reveal the effects of exogenous aspartates and aspartic acid on slow- and fast-growing strains of *P. ostreatus*, we compared growth of *P. ostreatus* 69, *P. ostreatus* BK1702 and *P. ostreatus* HK352 in a liquid synthetic nutrient medium with glucose and yeast extract supplemented or not supplemented with metal(II) (Cu, Mn, Fe, Zn, Co) L-aspartates and Asp. The dry weight of mycelium characterized the growth rate of *P. ostreatus* strains in the submerged culture. In 28 days after inoculation, the biomass in the absence of additives was 49.00±8.00, 124.86±6.38, and 244.7±15.77 mg/100 ml for the strains 69, BK1702 and HK352, respectively. Dry biomass accumulation in *P. ostreatus* HK352 was the highest.

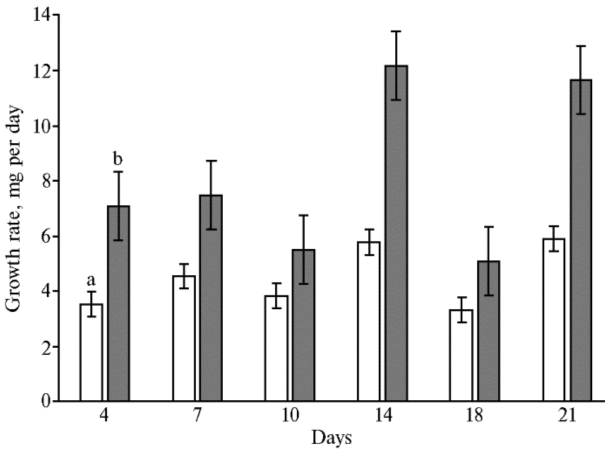

Fig. 3. Growth rate of *Pleurotus ostreatus* 69 (a) **and *P. ostreatus* HK352** (b) **in liquid yeast extract-glucose nutrient medium** (*M*±SD).

The high rate of mycelium growth in liquid culture is associated with advantages over contaminating species, significant competitiveness with extraneous microflora, the ability to utilize various carbon-containing compounds, including those hardly available, of plant waste from agriculture and timber processing industry. *P. ostreatus* HK352 grew up the fastest compared to other oyster mushroom strains, while *P. ostreatus* 69 grew slower than others (Fig. 3).

It turned out that growth characteristics significantly contribute to the sensitivity of *P. ostreatus* submerged cultures to the presence of metal(II) L-aspartates in the nutrient medium (Fig. 4). Aspartic acid showed a weak effect, and interstrain differences were practically not notable in the experiment.

Organic cobalt salt in the medium of the fastest growing strain HK352 suppressed the biomass accumulation to the greatest extent (see Fig. 4). Exogenous $Mn(Asp)_2$ and $Fe(Asp)_2$ caused changes in this parameter of the same or opposite direction compared to the controls. The sensitivity of slow- and fast-growing oyster mushroom strains to $Cu(Asp)_2$ and $Zn(Asp)_2$ was not the same. These treatments were favorable for *P. ostreatus* BK1702 (see Fig. 4). The revealed cultural characteristics should be taken into account when selecting strains for artificial culture.

Trace elements at physiological levels can be incorporated into the active site or act as active modulators of fungal enzymes [49]. It was shown that

the activity of *P. ostreatus* laccase, endo-1,4-β-glucanase, and 1,4-β-glucosidase increases in the presence of zinc and copper [50]. Zinc, copper and iron in the culture medium can have a strong effect on the composition of the fungal cell wall, as well as on the content of polyphenols and polysaccharides, which are involved in the antioxidant, antitumor, immunomodulatory, and other biological activities of basidiomycetes [51, 52].

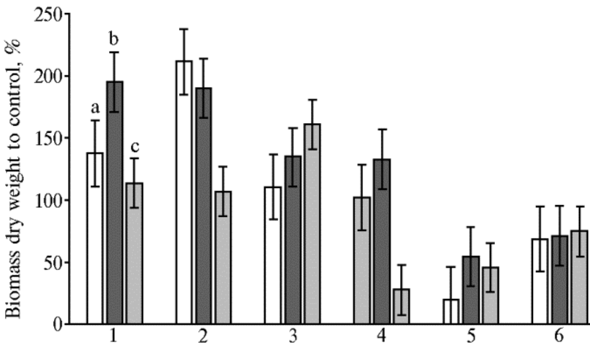

Fig. 4. Biomass accumulation in 21-day old submerged cultures of *Pleurotus ostreatus* HK352 (a), ***P. ostreatus* BK1702** (b), **and *P. ostreatus* 69** (c) **grown in a yeast extract-glucose nutrient medium with additives:** 1 — $Cu(Asp)_2$, 2 — $Mn(Asp)_2$, 3 — $Fe(Asp)_2$, 4 — $Zn(Asp)_2$, 5 — $Co(Asp)_2$, 6 — Asp (M±SD).

However, it should be borne in mind that only relatively low concentrations of trace metals are necessary for the growth and development of fungal cultures and the activity of various enzymes [6]. Cobalt has a rather high toxicity for fungi, and biomineralization of Co sulfate compounds is poorly studied even for metal-tolerant species [53], which explains the higher sensitivity to $Co(Asp)_2$ in all the strains we studied. Possibly, the synergistic effects of the fungal metallothionein compositions, which are responsible for binding metal cations of different toxicity [54], lead to an enhanced bioaccumulation of cobalt and, consequently, a slowed down accumulation of fungal biomass.

We have characterized the biochemical response of submerged cultures of basidiomycetes to the exogenous metal chelates. Analysis of the extracellular levels of metals in the chelates by atomic absorption spectroscopy reveled that during a 2-week submerged growth the amount of metal in the culture liquid decreased several times vs. the initial concentration in the nutrient medium (Table 1). Aspartates were involved in the growth and development of fungal mycelium.

1. General characterization of samples and metal detection in filtrates of fungal culture liquid at different culture ages (M±SD)

Samples	M^{2+} concentration, μg/ml	
	day 0	day 14
Lentinula edodes Cu 10^{-4} mol/l	25.6	3.78±0.05
Ganoderma applanatum Cu 10^{-4} mol/l	25.6	1.49±0.06
Ganoderma lucidum Cu 10^{-4} mol/l	25.6	3.58±0.07
Grifola umbellata Cu 10^{-4} mol/l	25.6	2.54±0.02
Pleurotus ostreatus Cu 10^{-4} mol/l	25.6	3.83±0.01
Laetiporus sulphureus Cu 10^{-4} mol/l	25.6	2.51±0.03
Lentinula edodes Fe 10^{-4} mol/l	22.4	8.14±0.02
Ganoderma applanatum Fe 10^{-4} mol/l	22.4	2.90±0.01
Ganoderma lucidum Fe 10^{-4} mol/l	22.4	2.50±0.02
Grifola umbellata Fe 10^{-4} mol/l	22.4	3.44±0.01
Pleurotus ostreatus Fe 10^{-4} mol/l	22.4	3.61±0.01
Laetiporus sulphureus Fe 10^{-4} mol/l	22.4	1.93±0.01
Lentinula edodes Mn 10^{-4} mol/l	22.0	7.45±0.07
Ganoderma applanatum Mn 10^{-4} mol/l	22.0	0.33±0.01
Ganoderma lucidum Mn 10^{-4} mol/l	22.0	4.30±0.01
Grifola umbellata Mn 10^{-4} mol/l	22.0	6.39±0.19
Pleurotus ostreatus Mn 10^{-4} mol/l	22.0	6.98±0.02
Laetiporus sulphureus Mn 10^{-4} mol/l	22.0	6.26±0.02
Lentinula edodes Zn 10^{-4} mol/l	26.0	3.82±0.03
Ganoderma applanatum Zn 10^{-4} mol/l	26.0	3.12±0.01

Continued Table 1

Ganoderma lucidum Zn 10^{-4} mol/l	26.0	4.04±0.02
Grifola umbellata Zn 10^{-4} mol/l	26.0	3.35±0.01
Pleurotus ostreatus medium1 Zn 10^{-4} mol/l	26.0	2.66±0.07
Laetiporus sulphureus Zn 10^{-4} mol/l	26.0	2.95±0.01
Lentinula edodes Co 10^{-4} mol/l	23.6	8.48±0.01
Ganoderma applanatum Co 10^{-4} mol/l	23.6	5.17±0.01
Ganoderma lucidum Co 10^{-4} mol/l	23.6	7.86±0.01
Grifola umbellata Co 10^{-4} mol/l	23.6	5.56±0.03
Pleurotus ostreatus Co 10^{-4} mol/l	23.6	8.89±0.01
Laetiporus sulphureus Co 10^{-4} mol/l	23.6	7.87±0.03

The effect of the added metal(II) chelates on the pool of secondary metabolites of fungal cultures was assessed by high-performance liquid chromatography/high-resolution time-of-flight mass spectrometry. The method combines simple and fast sample preparation with the identification and sensitive determination of low molecular weight secondary metabolites in biological samples.

2. Main characteristics of extracellular compounds in *Lentinula edodes* culture medium with 10^{-4} mol/l metal(II) aspartate (high-resolution mass spectrometry of positive ions $[M+H]^+$)

Analite	Empirical formula	$[M+H]^+$, m/z	Signals for samples with $M(Asp)_2$					
			Cu	Mn	Fe	Zn	Co	C
CH_2OH 2-hydroxymethylfuran	$C_5H_6O_2$	99.0441	500	nf	500	6000	nf	nf
$HOCH_2$ 5-hydroxymethyl-2-furaldehyde	$C_6H_6O_3$	127.0389	3000	4000	3000	nf	3000	н.о.
HO OH CH_3 3,5-dihydroxy-2-methyl-5,6-dihydropyran-4-one	$C_6H_8O_4$	145.0495	6000	600	8000	nf	nf	nf
CH_2CH_2OH 2-phenylethanol	$C_8H_{10}O$	123.0804	1000	nf	1000	nf	nf	nf
HO CH_2COOH 4-hydroxyphenylacetic acid)	$C_8H_8O_3$	153.0546	2500	5000	nf	nf	nf	nf

N o t e. C —no additives, nf — not found.

The extracts from the nutrient medium supplemented with 10^{-4} mol/l metal(II) aspartates were compared with the control (Table 2). The results showed that aspartates of some metals(II) increased the production of 5-hydroxymethyl-2-furaldehyde and dihydropyrone (see Table 2) in the culture. Our previous works discussed in sufficient detail the data on the increased content of these substances as a result of exogenous action of some compounds [55, 56]. Thus, the medium for submerged culture of basidiomycete *Lentinula edodes* in the presence of 1,5-diphenyl-3-selenpentanedione-1,5 $C_6H_5COCH_2SeCH_2COC_6H_5$, the diacetophenonyl selenide, bis(benzoylmethyl)selenide, DAFS-25 [57], which leads to an increase in the growth rate of the fungus and the activity of its extracellular lectins, and also serves as an antioxidant, contains 2-hydroxymethylfuran, 5-hydroxymethyl-2-furaldehyde, 3,5-dihydroxy-2-methyl-5,6-dihydropyran-4-one [55].

In the presence of transition metals (Cu, Mn, Fe, Co) [58] in the form of

aspartates, the conversion of carbohydrates into 5-hydroximethylfurfural [59] seems to occur via catalytic hydrolysis and dehydration of hexose-containing components of the nutrient medium leading to the formation of 5-hydroxymethyl-2-furaldehyde. The latter inhibits tyrosinase which is responsible for the synthesis of the fungal pigment melanin, that is, it serves as an inhibitor of melanogenesis [60]. The recognized importance should be mentioned for an innovative way of chemical utilization of hexose-containing raw materials to produce 5-hydroxymethyl-2-furaldehyde, a promising intermediate product for chemical industry (production of food additives, pharmaceuticals, polymeric materials, additives to motor oils and biofuel precursors) [55, 59, 60].

Some samples of culture liquid contained 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (see Table 2). Note the similarity of the structure of the discovered dihydropyranone and 5-hydroxy-2-hydroxymethyl-4H-pyran-4-one, also called kojic acid. Kojic acid is a known inhibitor of melaninogenesis in fungi, which is mediated by an increase in tyrosinase activity and occurs under conditions of oxidative stress [61, 62]. Enhanced synthesis of this substance as compared to the culture without additives indicates an increased antioxidant activity of submerged culture mycelium grown in the presence of aspartates we studied in the work. This is another evidence of an increase in the adaptive potential of a mushroom culture grown in the presence of aspartates, especially chelates of copper, iron, and manganese.

Organic substances with double bonds, including phenolic ones, not registered in the control, appeared in the culture medium as a biochemical response of fungal strains to aspartates. $Mn(Asp)_2$ caused the maximum extracellular concentration of para-hydroxyphenylacetic acid, as it followed from the intensity of the analytical signal (see Table 2). This phenolic acid was detected in the mycelium of some higher fungi, e.g. *Chroogomphus rutilus* [63], *Suillus granulatus* [64], and *Clitocybe nuda* [65].

It is believed that the antioxidant activity of mushroom extracts correlates with the total content of phenolic substances [66]. It was 4-hydroxyphenylacetic acid that contributed to the accumulation of phenols in the mycelium to the greatest extent than other exogenous para-hydroxy-substituted phenolic compounds [56].

The increase in the number of compounds with antioxidant properties that we found in the presence of a number of aspartates in submerged basidiomycete cultures is consistent with the data available for other organisms. It is known that the antioxidant properties of compounds can be enhanced when they are used in various synergistic compositions with other antioxidants [67] and with substances that do not in themselves exhibit this biological activity [68, 69]. The aspartates of Cu, Mn, Zn, and Mg were characterized by inhibitory activity against xanthine oxidase and NADPH oxidase, reducing the production of oxygen radicals by these enzymes. The most active inhibitors of oxidative stress were aspartates of the transition metals copper [70, 71] and manganese [72]. The effect of zinc [73] and magnesium [74] aspartates can be associated with the influence on the rate of spontaneous superoxide ion dismutation. It is reasonable to assume that $Cu(Asp)_2$, $Mn(Asp)_2$ and $Zn(Asp)_2$ act as biomimetics of Cu-, Zn-, or Mn-dependent superoxide dismutases. The antioxidant properties of zinc aspartate as an effective inhibitor of production of the most reactive hydroxyl radicals [75] were also demonstrated in experiments with laboratory animals [76].

The obtained results suggest that amino acid chelates of copper, iron, and manganese can affect the production of compounds important for mushroom culture adaptation.

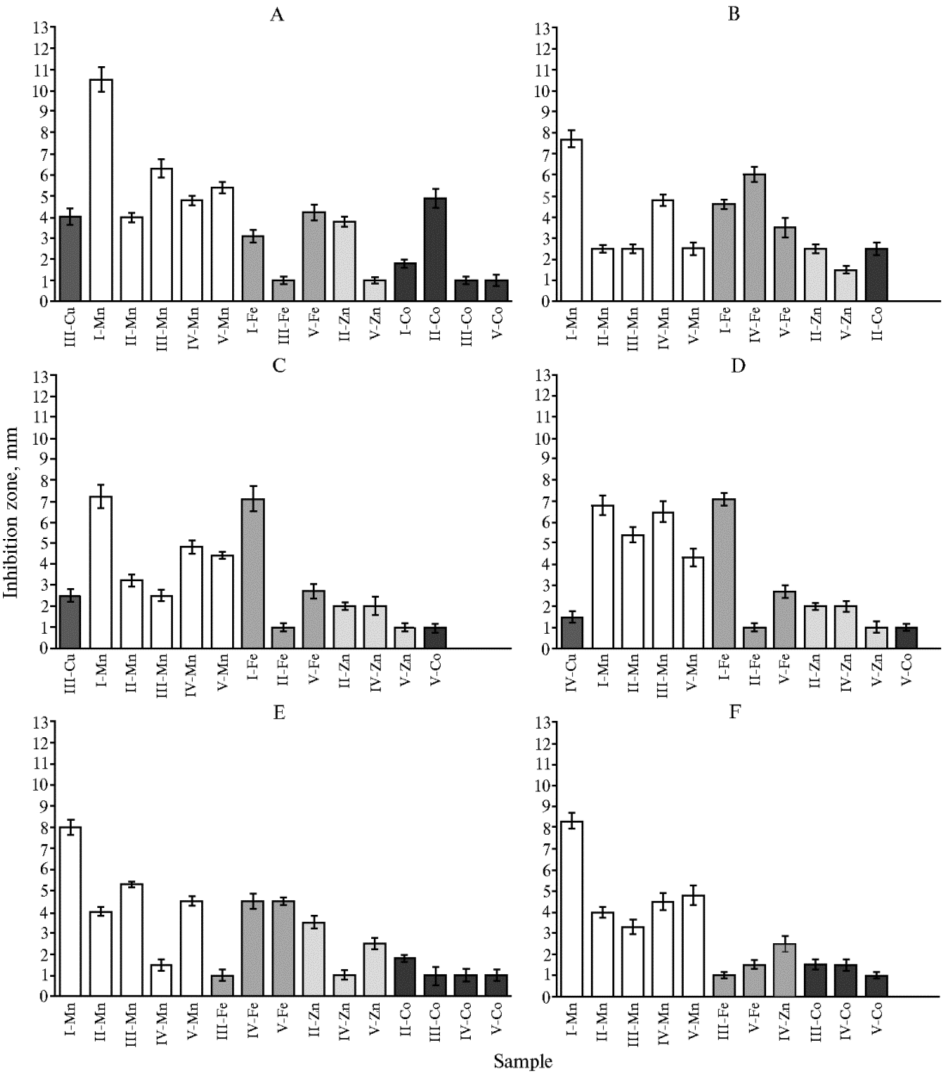

Рис. 5. Inhibitory activity of Cu-, Mn-, Fe-, Zn-, and Co-containing biocomposites based on *Pleurotus ostreatus* HK352 (I), ***Ganoderma lucidum* 1315** (II), ***Lentinula edodes* F-249** (III), ***Grifola umbellata* 1622** (IV), ***Laetiporus sulphureus* 120707** (V) **against bacterial cultures:** A — *Pectobacterium atrosepticum* 1043, B — *Pectobacterium carotovorum* subsp. *carotovorum* MI, C — *Xanthomonas campestris* B-610, D — *Pectobacterium carotovorum* subsp. *carotovorum* 603, E — *Micrococcus luteus* B-109, F — *Pseudomonas fluorescens* EL-2.1 (M±SD, agar diffusion method).

To better characterize the biochemical response of basidiomycetes to metal chelates with aspartic acid, we measured the antibacterial activity of products from submerged mushroom cultures grown in the presence of chelates against plant pathogenic bacteria *Micrococcus luteus*, *Pectobacterium carotovorum* subsp. *carotovorum* (two strains), *Pectobacterium atrosepticum*, *Pseudomonas fluorescens*, *Xanthomonas campestris*. A total pool of extracellular metabolites was extracted from submerged cultures of *G. lucidum*, *G. umbellata*, *L. sulphureus*, *L. edodes*, and *P. ostreatus*. The extracts contained products of biotransformation of the introduced organic metal(II) complexes by fungi. The bactericidal effect of the studied metal-containing biocomposites was assessed with plant pathogenic bacteria as a test culture by agar diffusion methods (Fig. 5).

At first glance, the discussed above property of cobalt(II) to inhibit the growth of microorganisms does not correlate with the low bactericidal activity of

its biocomposites of fungal origin. Only in 50% of cases, Co-containing biosamples showed at least minimal toxicity towards plant pathogens, and only *G. lucidum*-derived composites had a zone of inhibition of more than 2 mm in tests with *P. atrosepticum* (Fig. 5, A) and *P. carotovorum* MI (see Fig. 5, C). The M(II) concentration in potentially antibacterial samples with cobalt and other metals was the same. It can be assumed that the production of extracellular metabolites, reduced due to a slow increase in biomass in the presence of cobalt aspartate, does not make a sufficient contribution to the formation of Co-composites. In our opinion, production of fungal substances with low content of extracellular antimicrobial metabolites leads to a sharply reduced ability to suppress the studied plant pathogenic bacteria. Additional indirect confirmation of the importance of the chemical environment of the metal should be considered when using its compounds to combat plant pathogens.

Aspartates of other metals which stimulated growth of the basidiomycetes (see Fig. 1) differed quite significantly in the effect of metal-containing biosubstances on bacterial plant pathogens. Virtually no inhibition of bacterial growth occurred in the presence of copper biocomposites. Only from the culture liquid of *Lentinula edodes* it was possible to obtain an antibacterial sample characterized by a noticeable zone of growth inhibition for *P. atrosepticum* (see Fig. 5, A) and *X. campestris* (see Fig. 5, C). The biocomposite from *Grifola umbellata* was ineffective (see Fig. 5, D). Probably, the induction of copper-containing lytic enzymes in the presence of this cation, noted in a large number of microorganisms [44, 46] and necessary for assimilation of nutrient substrate by bacteria, favors the survival of plant pathogens. This is also facilitated by bioproduction of bacterial copper reductases [32] which catalyze the transformation of metal into less toxic chemical forms.

The manganese is believed to be responsible for the induction of manganese peroxidase in microorganisms, primarily higher fungi. However, studies [50] have shown a negative correlation between the maximum of Mn-peroxidase activity and the exogenous Mn concentration. It was found that this enzymatic activity in the presence of Mn is lower than in its absence. Possibly, this was partly due to the high antimicrobial activity of fungal substances that contain manganese in a bioavailable organic form as observed in our experiment (see Fig. 5). When they act on test bacteria, the indicated peroxidase activity may decrease. Simultaneously, upon stimulation with Mn^{2+} ions, the activity of laccases (and lignin peroxidases) may not be detected, as is the case in microorganisms with different taxonomic affiliation and ecological strategy [77]. Indeed, the highest quantitative characteristics of bactericidal action were found in the variant with $Mn(Asp)_2$. The effect of this manganese chelate as a component of the submerged culture medium used to produce potentially bactericidal samples was much more pronounced than that of $Zn(Asp)_2$ and especially of $Fe(Asp)_2$, and manifested itself against test strains of plant pathogens (see Fig. 5). The *Pectobacterium carotovorum* subsp. *carotovorum* 603 insensitive to the manganese-containing inhibitor from *Grifola umbellate* was the only exception (see Fig. 5, D).

In our tests, the highest antibacterial effect with the absolute maximum size of zone of *P. atrosepticum* growth inhibition was characteristic of the bioagents based on extracellular metabolites of *Pleurotus ostreatus* grown in the culture media with $Mn(Asp)_2$ (see Fig. 5, A). The same producer of the organic part of the biocomposite containing Zn(II) is in second place. However, this Zn-containing bioagent acted exclusively on the plant pathogenic *P. carotovorum* 603 (see Fig. 5, D).

Other Zn(II) fungal-based composites showed moderate bactericidal activity and were effective in less than half of the tests with $Zn(Asp)_2$. We failed to produce a Zn-containing antibacterial agent containing extracellular metabolites of one of the basidiomycetes, *Lentinula edodes*. That is, along with the selective growth inhibitor of *P. carotovorum* 603, all fungal metal-containing biosamples for which the size of bacterial growth inhibition zone was at least 8 mm were derived using $Mn(Asp)_2$ (see Fig. 5, E, F).

The iron aspartate-based bioagents in some tests were slightly inferior in bactericidal action (see Fig. 5, C) or even showed slightly greater efficiency (see Fig. 5, D) compared to $Mn(Asp)_2$, but these results were obtained using *Pleurotus ostreatus* only. Iron biocomposites of fungal origin had almost no effect on *Ps. fluorescence* (see Fig. 5, F).

Soil microorganisms, including members of the genera *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Azotobacter*, can be used to increase the phytoavailability of minerals, to improve plant health and for biocontrol [24, 32]. Such lab tests were successful. However, the practical use of the bacterial biocontrol method requires a comprehensive consideration of the originality and uniqueness of the plant protection strategy depending on the cultivar, the chemical properties of the soil, and the external environment. The ability of potentially biocontrol preparations to compete with resident rhizobacteria during root colonization in natural ecosystems varies greatly and is not always predictable. Nevertheless, the problem of the interaction of drugs based on the biocomposites we proposed with rhizobacteria-based formulations is relevant. The fact that in our experiments the bactericidal activity against *Pseudomonas fluorescens* turned out to be the least pronounced (see Fig. 5) is of interest to address the problem of negative impact of biocontrol drugs on resident rhizobacteria. In particular, biologicals based on biocomposites to which the *P. fluorescens* is tolerant or those compatible with useful rhizobacteria can be used. Thence further studies are promising to identify the fungicidal properties of metal-containing agents of fungal origin.

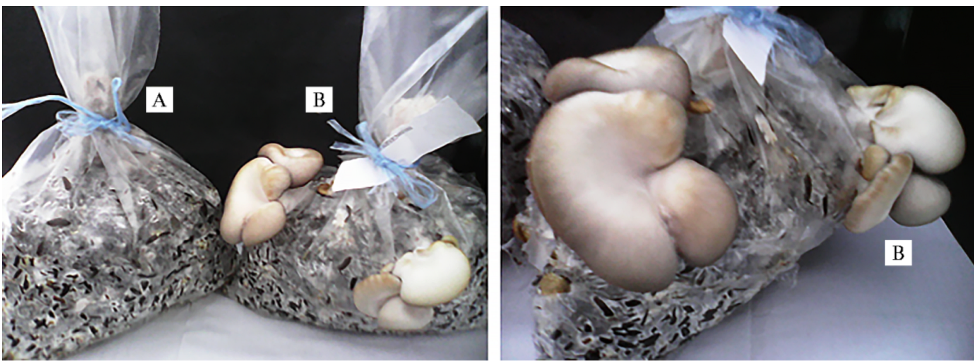

Рис. 6. Growth of *Pleurotus ostreatus* NK352 mycelium on sunflower husk substrate. The grain spawn was produced in liquid cultures without additives (A) and with 1.0×10^{-4} mol/l $Mn(Asp)_2$ (B).

Our experiments assessed the possibility of using metals(II) L-aspartates as an active ingredient of biopreparations with complex (growth-stimulating and adaptogenic) action for industrial cultivation of *P. ostreatus*. The accumulation of biomass of submerged mycelium of *P. ostreatus* strains under the influence of exogenous $Mn(Asp)_2$ (see Fig. 2) changed in the same mode as that in control, so an assumption can be made about the most pronounced positive effect of manganese chelate on the fast-growing *P. ostreatus* HK352 strain. The

liquid inoculum was obtained in the medium with a decoction of wheat flour, as well as in the media supplemented with Cu, Mn, Fe, or Zn aspartates. The colonization of grain substrate by *P. ostreatus* mycelium upon inoculation with liquid culture grown in the presence of the listed organic salts was more intensive than in the control.

The mycelium obtained on a wheat grain substrate was used as an inoculum for growing fruiting bodies on a sunflower seed husk substrate. Given the intensity of colonization of this lignocellulosic substrate by *P. ostreatus* mycelium grown in the presence of $Mn(Asp)_2$ (Fig. 6), the manganese(II) chelate had the most favorable effect on the formation of oyster mushroom fruiting bodies.

Oyster mushroom growth is peculiar depending on trace metal chelates of amino acids as additives, which is significant to select proper *P. ostreatus* strains for biotechnological cultivation. E.g., the growth index under the influence of exogenous $Fe(Asp)_2$ (see Fig. 2) changes in the opposite way to the growth parameters of the control strains (see Fig. 2), that is, the greatest tolerance to iron chelate, including its mixtures with other aspartates, may be expected in the slow-growing strain *P. ostreatus* 69. At the same time, the patterns of *P. ostreatus* sensitivity to $Cu(Asp)_2$ and $Zn(Asp)_2$ (see Fig. 2) shows a clear advantage of moderately growing strain BK1702.

The impact of mixtures of aspartates on the growth and development of cultures of three *P. ostreatus* strains was assessed based on combination of growth parameters of the cultures and the strain tolerance to the exogenous effect of the metal chelates.

We added metal aspartates to the liquid medium to produce liquid spawn, then to treat the grain substrate for the production of grain spawn, and finally to treat the lignocellulosic substrate to grow *P. ostreatus* fruiting bodies. It turned out that $Mn(Asp)_2 + Zn(Asp)_2$ and $Cu(Asp)_2 + Zn(Asp)_2$ have the greatest stimulating effect on the *P. ostreatus* submerged mycelium growth and the rate of grain substrate colonization, in addition, the fruiting occurs faster and is more intensive.

Our findings allow us to propose some principles for application of trace metal organic salts in growing oyster mushroom mycelium. Combination of manganese and zinc aspartates in an equimolar ratio provides the fastest growth of *P. ostreatus* cultures. In *P. ostreatus* strains with a moderate growth rate, combination of copper and zinc aspartates at a 1:2 molar ratio enhances submerged mycelium production in all nutrient media. In slow-growing *P. ostreatus* strains, treatment of the dense substrate with iron and zinc aspartates in a molar ratio of 1:2 leads to higher fruiting body formation. Chelated compounds of biogenic metals in the above combinations had a positive effect on the *P. ostreatus* vital activity.

Thus, amino acid chelated biogenic metals(II) are the factors for the intensification of spawn and fruiting body lab production of macrobasidiomycetes. Our findings reveal a significant growth-stimulating effect of chelates of copper, manganese, zinc and, to a lesser extent, iron on the submerged culture of basidiomycetes, especially *Ganoderma lucidum*, *Grifola umbelata*, and *Laetiporus sulphureus*. $Cu(Asp)_2$, $Mn(Asp)_2$ and $Zn(Asp)_2$ weakly stimulate or even inhibit the growth of *Pleurotus ostreatus* 69 though have a pronounced positive effect on the other fungal cultures. Aspartic acid exhibits an inhibitory effect regardless of the taxonomic characteristics of basidiomycetes. The oyster mushroom growing in the presence of biogenic metal aspartates revealed differences between fast- and slow-growing strains in the response to the exogenous metal chelates. $Cu(Asp)_2$ and $Zn(Asp)_2$ are favorable for the *P. ostreatus* BK1702 strain while $Mn(Asp)_2$ has

the most pronounced positive effect on the fast-growing *P. ostreatus* HK352 strain. The physicochemical results indicate that metal(II) aspartates, especially $Mn(Asp)_2$ and $Cu(Asp)_2$, affect the biosynthesis of 5-hydroxymethylfurfural, dihydropyrone (structural analogue of kojic acid) and para-hydroxyphenylacetic acid, i.e. the compounds with antioxidant properties which are important for mushroom culture adaptiveness. The positive effect of combinations of chelated Mn(II), Cu(II), Fe(II), and Zn(II) compounds on the vital activity of *P. ostreatus* should be studied to reveal fundamentals of mineral nutrition of higher mushrooms and can be used in practical mushroom farming. This work suggests a biotechnological scheme for the application of organic salts of microelements to culture oyster mushroom mycelium. The obtained results indicate high potential of mixtures of metal(II) chelates (aspartates) when used for production of mycelial biomass and mushroom fruiting bodies in lab conditions, as well as the prospects of commercial biologicals based on aspartates of biogenic metals. The patterns of oyster mushroom mycelial growth and formation of fruiting bodies in the presence of $Mn(Asp)_2$ allow us to recommend the manganese(II) chelate for practical use.

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