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Organic Acids Induce Tolerance to Zinc- and Copper-Exposed Fungi Under Various Growth Conditions

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Abstract Heavy metals, Zn and Cu, in high concentration (2 mM for Zn and 0.5 mM for Cu) have some inhibiting effect on the growth of *Aspergillus niger* and *Penicillium citrinum*. Toxic effects of these metals considerably depend on cultivation conditions including nitrogen sources, pH of nutrient media, and its consistency (presence or absence of agar). In general, nitrate media provides less inhibiting effect on fungal growth under heavy metal exposure than ammonium-containing media. Adding of Zn in nitrate media induces oxalic acid production by fungi. Importance of oxalic acid production in detoxification of heavy metals is confirmed by the formation of Zn-containing crystals in fungal cultures. Cu bringing to the cultural media had no stimulating effect on oxalic acid production as well as no copper-containing crystals were observed. But proceeding from essential increase in oxalic acid production during a long-term fungi adaptation to Cu, it may be proposed that oxalic acid plays some functional role in Cu tolerance of fungi as well. It may be concluded that the role of organic acids and oxalate, in particular, in fungi tolerance and adaptation to heavy metals can be determined by the nature of the metal and its ability to form stable complexes with an acid anion. Stimulating effect of metals on acid production is not universal for all species of fungi and largely depends on metal concentration, nitrogen form in a medium, and other cultivation conditions.

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Introduction

Heavy metals are natural components of the Earth's crust. Accumulation of significant concentrations of certain metals on earth surface may result from both natural processes and human activity. Metals emission from anthropogenic sources happens in a number of ways including fossil fuels extraction, waste burning, ore processing into non-ferrous metals, and extending of traffic [16]. A lot of metals in low concentrations stimulate the growth of fungi being involved in various metabolic processes as essential microelements. However, accumulation of metals in high concentrations in the environment may cause cell membrane damage, induce lipid peroxidation and formation of reactive oxygen species, suppress respiration, modify activity of some enzymes, and damage DNA and proteins structure [7, 9, 14]. Heavy metals in high concentrations under culture conditions of fungi often cause the slowdown of mycelium growth and suppression of generative structures formation [9, 22]. Ability of fungi to survive in the presence of potentially toxic metals depends on numerous internal and external factors, including physiological, biochemical and/or genetic adaptations, morphological modifications of mycelium and the form of the metal, and determining its biological availability and toxicity [7, 14].

Among mechanisms providing heavy metals tolerance of fungi, extracellular chelation of metal ions by excreted metabolites is of great importance. Low molecular weight organic acids (LMWOA), which may interact with metals both outside and within the cell, can play a special role in fungi adaptation to living in the environment with high concentrations of heavy metals [4, 7, 14]. Oxalic acid is the strongest chelating agent among LMWOA. It reduces significantly biological availability of metals, forming stable complexes and/or insoluble salts with a number of them,

thus, oxalic acid production is deemed to be a highly effective way of numerous metals immobilization [7, 14].

Oxalic acid excretion is well described for many fungi species from different ecological and taxonomic groups [4, 14]. A lot of studies demonstrate that addition of certain metals leads to activation of organic acids formation by fungi [4, 6, 11, 14]. However, mechanisms of these processes still remain unclear and require further examination. Importance of ecological factors, such as physical and chemical characteristics of substrates, is also underexplored in regard to mechanisms functioning of fungi heavy metals tolerance.

The aim of the work was to study the possible functional role of organic acids of *Aspergillus niger* and *Penicillium citrinum* as a factor of their tolerance to high concentrations of zinc and copper during fungi cultivation on liquid and agar-containing nutrient media with various nitrogen sources and different pH levels.

Materials and Methods

Organisms and Cultivation

The fungal strains used in the experimental study were *P. citrinum* (Thom) L 4/09 and *A. niger* (Tiegh) Ch 4/07 from Culture Collection of the laboratory of mycology of Saint-Petersburg State University. For fungi cultivation, two nutrient media differed in the nitrogen source were used: Czapek medium ($NaNO_3$—3.0 g/l) [3] and Rollen medium (NH_4NO_3—3.0 g/l). Content of other mineral salts was the same in both media: KH_2PO_4—1.0; $MgSO_4 \times 7\ H_2O$—0.5; KCl—0.5; $FeSO_4 \times 7\ H_2O$—0.015 g/l. Glucose content was 30.0 g/l in Czapek media and was 50.0 g/l in Rollen media to maintain the optimal C/N balance. Initial pH values in nutrient media were 4.2, 5.6, and 6.8 depending on the experiment specificity.

Metals were brought in the media as $ZnSO_4 \cdot 2H_2O$ and $CuSO_4 \cdot 5H_2O$ in concentrations 2 mM for Zn and 0.5 mM for Cu. In control media, no Zn or Cu was added.

Cultivation was carried out in the surface culture on liquid media, traditionally used for biochemical studies of fungi, and on agar-containing nutrient media, in order to model conditions very similar to natural ones. Micromycetes were cultivated at a temperature of 25 °C during 10 days. The biomass of mycelium was determined by the gravimetric method. Before weighing, a mycelium was separated from the agar or liquid media and dried till the absolute dry weight.

The organic acids content in liquid nitrate and nitrate–ammonium media was measured in experiment with initial pH 5.6 on the 10th day of growth. The acidity of the cultural fluid was determined with pH-meter pH-410. In case of cultivation on agar, the content of organic acids was measured in experiments with initial pH 5.6 and 6.8.

In order to get *P. citrinum* adapted to high Cu level, the culture was grown on Czapek medium with Cu concentrations gradually increased from 25 to 500 mM during 1 year.

Cultural Study and Analysis of Metals in Mycelium

Cultures micromorphology was studied using Axio Scope A1 light microscope, as well as Tescan MIRA3 LMU scanning electronic microscope with a R-BSE detector and Advanced Aztec Energy(IE350)/X-max80 energy-dispersive microanalysis system. Zn and Cu concentrations in fungi mycelium were measured for 10-day fungi cultures, grown in liquid media with pH 5.6, by MGA-915MD Zeeman atomic absorption spectrometer with electrothermal atomization. The determination of phase composition of crystallization products was carried out using Rigaku powder diffractometer (CuKα radiation).

Samples Preparation for Chromatography

To determine the total content of organic acids in the cultural fluid, test samples were treated with 0.1 M HCl up to pH 1.0 in order to dissolve oxalic acid salts. In case of cultivation on solid media, agar was preliminarily dissolved in hot water in the ratio of 1 ml of agar : 10 ml of water, then treated with 0.1 M HCl, and filtered. To determine soluble oxalate forms exclusively, no treating of media with HCl was done. Further an aliquot of a filtrate was passed through cationic exchanger (KU—2–8) to get free acids from their salts.

In order to eliminate excessive sugars, the samples after cationic exchanger were passed through anionic exchanger (AN-2FN), the bound acid anions were replaced with 0.2 M NaOH, and these samples were passed through the cationic exchanger again. The obtained water solution of organic acids was evaporated, and the dry residue was dissolved in pyridine. TMS-derivatives of organic acids were obtained using *N*,*O*-bis-(trymethylsilyl) trifluoroacetamide (BSTFA).

GC–MS Analysis

Carbon acids extracted from the cultural fluid were analyzed using chromatography and mass spectrometry (GC–MS) on Agilent device with MSD5975 mass selective detector, column HP-5MS, 30 m × 0.25 mm. Chromatography was carried out with linear temperature programing from 70 to 320° at a speed of 4 °C/min. Data were collected using Agilent ChemStation software. Mass spectrometric information was processed and interpreted

using AMDIS program (http://www.amdis.net/index.html) and standard NIST2005 and Wiley6 libraries. Quantitative interpretation of chromatograms was carried out with the internal C_{18} standardization using UniChrom program (http://www.unichrom.com/unichrome.shtml).

Statistical Analysis

Statistical analysis was performed using programs Origin Pro and Microsoft Excel.

Results

The obtained results showed that the growth rate of fungi being cultivated with NH_4NO_3 was slightly higher than with $NaNO_3$. When Cu and Zn were added in concentrations of 0.5 and 2.0 mM correspondingly, the fungi growth was suppressed on both liquid and agar-containing media. The extent of inhibition on a nitrate–ammonium medium reached 47 % for *A. niger* and 50 % for *P. citrinum*, on nitrate medium it was only 20–30 % (Figs. 1, 2). Experiment with Cu added to NH_4NO_3—containing agar medium with pH 6.8, where Cu has activated mycelium growth in both strains of fungi, was an exception. It was not observed in any other experiment including the one on a liquid medium with the same pH. The results of mycelium morphological study demonstrated that Zn and Cu in the used concentrations had no significant effect on the fungal hyphas growth on a nitrate-containing medium, while addition of metals into nitrate–ammonium medium led to the formation of swelled cells, often constituting a main part of mycelium. It follows from the results that the tolerance to Cu was higher in *A. niger* than in *P. citrinum.*

Analysis of organic acids content in the liquid cultural media showed that on nitrate media both species of fungi produce significant amounts of oxalic acid. In the control condition, oxalate production by *A. niger* was in average 2.3 times higher than by *P. citrinum.* When Zn was added, both species of fungi demonstrated an increase in oxalic acid production of 30–35 % (Table 1).

At the same time, production of succinic and citric acids, which was typical for *A. niger* and *P. citrinum* in the control group, has been completely suppressed in the presence of Zn, as well as production of gluconic acid by *P. citrinum.*

When Cu was added, the content of oxalic acid on the contrary was significantly less compared to the control group, especially for *A. niger* (from 57.8 to 2.2 mg/g), but at the same time, more intense production of succinic, malic, and, especially, citric acid by this strain was observed. As for *P. citrinum*, the production of citric acid in the presence of Cu was reduced, production of succinic acid was not detected, but the content of gluconic acid was found 4.2 times higher than at the control group.

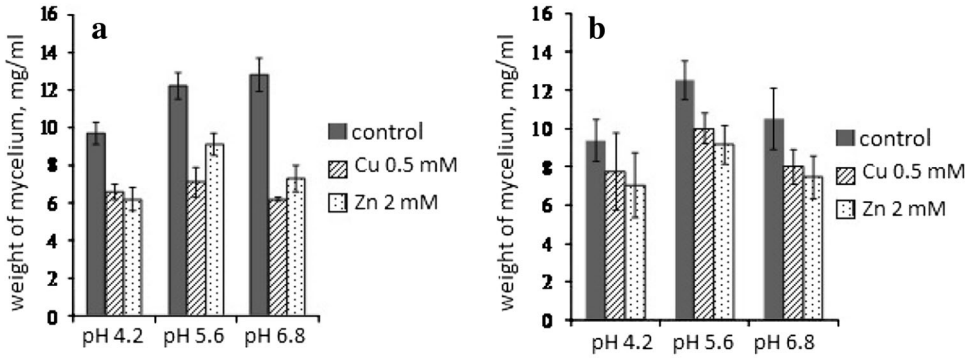

Fig. 1 Effect of zinc and copper on the growth of *A. niger* in liquid media at different pH levels: **a** nitrate Czapek medium; **b** nitrate–ammonium Rollen medium

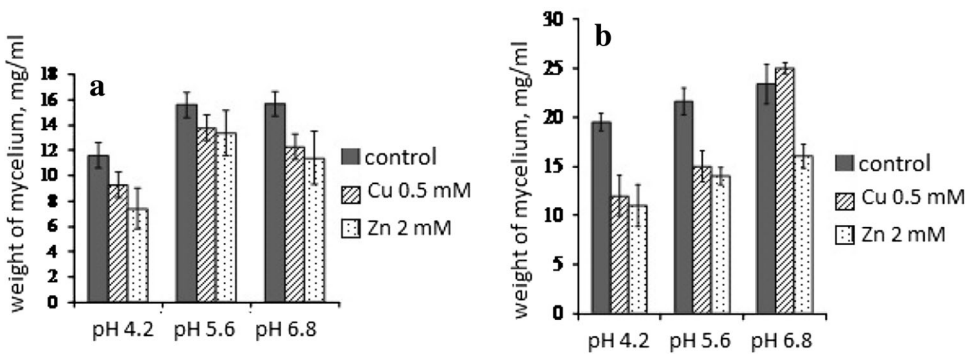

Fig. 2 Effect of zinc and copper on the growth of *A. niger* in agar media at different pH levels: **a** nitrate Czapek medium; **b** nitrate–ammonium Rollen medium

Table 1 Effect of Zn and Cu on organic acids production by *A. niger* and *P. citrinum* on liquid nutrient media with different sources of nitrogen

Species of fungi	Nitrogen sources	Added metal	Final pH	Content of organic acids, mg/g of mycelium					
				Oxalic	Citric	Succinic	Malic	Fumaric	Gluconic
A. niger	$NaNO_3$	Control	2.75 ± 0.05	57.8 ± 7.8	5.5 ± 1.2	1.0 ± 0.2	–	–	–
		$ZnSO_4$ 2 mM	3.07 ± 0.04	89.6 ± 116	–	–	–	–	–
		$CuSO_4$ 0.5 mM	3.58 ± 0.02	2.2 ± 0.4	12.1 ± 1.1	2.1 ± 0.4	0.70 ± 0.03	–	–
	NH_4NO_3	Control	1.69 ± 0.05	0.11 ± 0.02	6.6 ± 1.2	0.28 ± 0.03	0.45 ± 0.9	0.4 ± 0.04	–
		$ZnSO_4$ 2 mM	1.88 ± 0.06	0.16 ± 0.04	8.40 ± 1.20	0.29 ± 0.04	0.40 ± 0.07	0.04 ± 0.01	–
		$CuSO_4$ 0.5 mM	1.71 ± 001	–	1.87 ± 0.56	0.24 ± 0.06	1.02 ± 0.20	–	–
P. citrinum	$NaNO_3$	Control	6.95 ± 0.11	24.95 ± 5.74	5.4 ± 1.15	0.69 ± 0.10	–	–	5.14 ± 0.95
		$ZnSO_4$ 2 mM	7.09 ± 0.09	36.90 ± 3.72	–	–	–	–	–
		$CuSO_4$ 0.5 mM	7.28 ± 0.08	10.56 ± 2.30	1.43 ± 0.45	–	0.21 ± 0.02	–	21.95 ± 4.31
	$NH + NO_3$	Control	2.13 ± 0.26	0.16 ± 0.03	0.86 ± 0.09	0.84 ± 0.08	0.18 ± 0.05	–	–
		$ZnSO_4$ 2 mM	2.10 ± 0.22	0.12 ± 0.02	0.42 ± 0.12	0.04 ± 0.01	0.14 ± 0.01	–	–
		$CuSO_4$ 0.5 mM	2.29 ± 0.13	–	0.26 ± 0.04	–	0.31 ± 0.11	–	–

Table 2 Production of organic acids by Cu-resistant culture of *P. citrinum* in the presence of Cu on liquid Czapek medium (pH 5.6)

Added metal	Content of organic acids, mg/g of mycelium			
	Oxalic	Citric	Succinic	Malic
Control	55.8 ± 6.30	7.2 ± 0.15	1.5 ± 0.4	0.8 ± 0.2
$CuSO_4$ 0.5 mM	62.8 ± 5.7	5.9 ± 2.0	1.8 ± 0.4	1.1 ± 0 4

Production of oxalic acid by both strains in control group on nitrate–ammonium media was strongly reduced in comparison with nitrate media. The addition of Zn did not lead to statistically significant changes in oxalic or other organic acids production by both fungi species, whereas *P. citrinum* produced lower amounts of citric acid and no succinic acid as on the nitrate media. In the presence of Cu there was no production of oxalate on NH_4NO_3 media, however, content of citric and malic acid was found.

During the 10-day period of cultivation, pH level in *A. niger* culture decreased from 5.6 to 2.75 on a nitrate nitrogen source and to 1.69 on a nitrate–ammonium one, while in *P. citrinum* culture pH level increased to 6.95 in nitrate media and decreased to 2.13 in nitrate–ammonium media (Table 1). The presence of metals had no significant effect on pH values of a medium.

Production of oxalic and other organic acids of Cu-adapted culture of *P. citrinum* observed in the presence of Cu did not differ significantly from the production in the control group (Table 2), however, these amounts were significantly higher than amounts of oxalic acid produced by initial strain (Table 1). In this case, oxalic acid was found exclusively in insoluble form.

Analysis of the content of organic acids excreted by fungi on agar-containing medium, carried out for *P. citrinum* culture, showed that tendencies in acid production changes influenced by heavy metals were similar to those observed in a liquid culture (Table 3), and were poorly associated with the initial pH level (5.6 or 6.8). Intensity of organic acids production calculated per unit of mycelium biomass was lower than during cultivation on a liquid nutrient medium.

Analysis of oxalate forms in fungi cultural media revealed that in case of *A. niger* in control condition on Czapek nitrate medium oxalates were present both in soluble and insoluble forms in the ratio 1.2:1,0, while under Zn influence almost all oxalic acid, the total amount of which increased, was insoluble (Table 4). When Cu was added to the medium, only traces of insoluble oxalates were discovered, and the amount of soluble oxalate was as low as on the medium with zinc. As to *P. citrinum*, all of the oxalic acid was in insoluble form only, both in the control condition and in the presence of metals. Moreover, in the presence of Zn, its amount increased by a factor of 1.6, and in the presence of Cu it reduced by a factor of 3.

Quantitative analysis of Zn and Cu content in fungi showed that both metals were accumulated in the mycelium. Zn was accumulated much more intensively in comparison with Cu (Table 5). The concentration of zinc was much higher in mycelium grown on a nitrate medium compared to nitrate and ammonium one (for *A. niger*-times and for *P. citrinum*-2.4 times), while the inverse correlation was observed for copper.

Using the scanning electronic microscopy and X-ray phase analysis, it was established that during 10-day cultivation of *A. niger* and *P. citrinum* in the presence of Zn, zinc-containing crystals of various types were formed on fungal hyphas and in a medium (Fig. 3). In *A. niger*, such crystals were identified as dihydrate zinc oxalates. On hyphas of *A. niger* mycelium, plate crystals formed flower-like structures (Fig. 3a). Crystals formed on *P. citrinum* mycelium also had

Table 3 Effect of Zn and Cu on organic acids production by *P. citrinum* on agar media with different nitrogen sources and pH values

Nitrogen sources	Final pH	Added metal	Content of organic acids, mg/g of mycelium					
			Oxalic	Citric	Succinic	Malic	Fumaric	Gluconic
$NaNO_3$	5.6	Control	6.2 ± 2.8	1.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	50.8
		$ZnSO_4$ 2 mM	19.1 ± 4.6	–	–	–	–	–
		$CuSO_4$ 0.5 mM	0.8 ± 0.2	–	0.3 ± 0.1	0.7 ± 0.2	–	–
	68	Control	7.6 ± 2.3	–	–	–	–	–
		$ZnSO_4$ 2 mM	18.6 ± 3.4	–	–	–	–	–
		$CuSO_4$ 0.5 mM	1.5 ± 0.4	–	–	–	–	–
NH_4NO_3	56	Control	0.3 ± 0.1	0 4 ± 0.1	0 5 ± 0.1		–	3.4 ± 0.5
		$ZnSO_4$ 2 mM	0.9 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	–	–	2.9 ± 0.4
		$CuSO_4$ 0.5 mM	0.3 ± 0.1	0.3 ± 0.1	–	0.09 ± 0.03	–	8.9 ± 2.3
	6.8	Control	0.45 ± 0.09	0.26 ± 0.06	1.07 ± 0.11	1.62 ± 0.13	–	–
		$ZnSO_4$ 2 mM	0.7 ± 0.1		0.24 ± 0.02	0.11 ± 0.08	0.07 ± 0.01	–
		$CuSO_4$ 0.5 mM	0.07 ± 0.02	–	0.55 ± 0.10	0.24 ± 0.04	0.05 ± 0.01	–

Table 4 Content of different forms of oxalate in the culture fluid in liquid nitrate Czapek media

Experimental variant	Content of oxalate in cultural fluid, μg/ml of the medium			
	Aspergillus niger		*Penicillium citrinum*	
	Insoluble form	Soluble form	Insoluble form	Soluble form
Control	260.5 ± 32.2	312.1 ± 29.0	146.7 ± 28.4	–
$ZnSO_4$ 2 mM	618.4 ± 28.1	12.2 ± 2.4	239.9 ± 33.2	–
$CuSO_4$ 0.5 mM	1.3 ± 0.4	13.8 ± 3.4	51.5 ± 9.4	–

Table 5 Accumulation of metals in mycelium of *A. niger* and *P. citrinum* on different media

Media	Concentrations of metals, μg/g of mycelium			
	A. niger		*P. citrinum*	
	Zn	Cu	Zn	Cu
Czapek (with $NaNO_3$)	4,513 ± 80	55 ± 2	4,265 ± 51	24 ± 4
Rollen (with NH_4NO_3)	1,571 ± 33	353 ± 38	1,740 ± 390	884 ± 334

a plate crystal habit, but formed splices of other types (Fig. 3b). On media with copper, no Cu-containing crystals were identified for *A. niger* and *P. citrinum*. Cu-resistant *P. citrinum* culture did not form Cu-containing crystals in a media or mycelium similarly to the initial strain.

Discussion

Obtained data demonstrated that metals toxic effect, which makes itself evident in the fact that mycelium growth of fungi *A. niger* and *P. citrinum* is suppressed, is mostly expressed in a nitrate–ammonium (Rollen) medium, than in a nitrate (Czapek) medium. Zinc on a nitrate medium has in general less inhibiting effect than copper. It is likely connected to the active excretion of oxalic acid on a nitrate medium and its production is more intense in the presence of zinc. Oxalic acid chelation with metal ions leads to formation of its insoluble complexes. Zn oxalate crystals that have been observed on the mycelium also confirmed this fact. Almost complete absence of oxalic acid in the medium with NH_4NO_3 source of N may be determined both by direct influence of ammonium form of nitrogen and medium strong acidity. It is a well-known fact that ammonium consumption and assimilation by fungi lead to intracellular generation and efflux of protons, which in their turn reduce pH of extracellular solution [18]. In our experiments, the pH value in NH_4NO_3-containing media reduced significantly (up to 2.3–1.7) in comparison with its initial value (5.6). As far as production of organic acids in this case was very low, the observed acidification can rather be explained by protons efflux. In a number of works, it was established that high acidity prevents the accumulation of oxalic acid in a culture of fungi [1, 19]. The value of pH is one of the key regulators of oxaloacetase activity, an enzyme which hydrolyses oxaloacetic acid

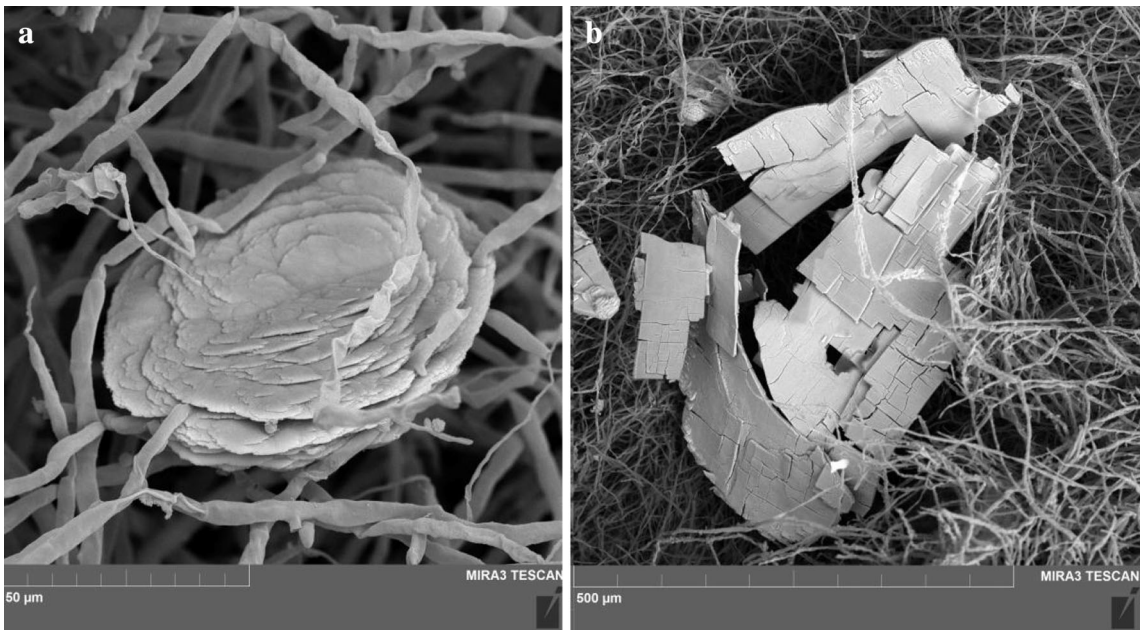

Fig. 3 Zn-containing crystals in cultures *A. niger* (**a**) and *P. citrinum* (**b**)

with the formation of oxalate and acetate. The activity of this enzyme is suppressed in response to pH decrease [11, 19].

Fungi and plants are considered to produce organic acids more intensively when nitrate, not ammonium nitrogen, is mainly used [4, 15]. Nitrate stimulation of organic acids production is explained by the increase of intracellular pH during NO_3^- assimilation, which activates functioning of carboxylating enzymes and oxaloacetate formation as an oxalate precursor in fungi and plants [5]. In our research, the presence of ammonium in a cultural medium suppressed oxalic acid production, while the amount of citric acid increased. It is known that low pH values facilitate biosynthesis of citrate by fungi unlike the case of oxalic acid. Besides, increased carbohydrate content is required for intensive production of citric acid as well as other acids of the TCA cycle [1]. Sometimes for biotechnological purposes, media with high glucose concentrations up to 150 g/l are used [10].

A number of research demonstrated that synthesis of oxalic, citric, malic, and several other acids by fungi is activated due to the effect of such metals as Zn, Cu, Cd, Ni, and Pb [2, 4, 6–8]. In our work, Zn on a nitrate medium stimulated production of oxalic acid by *A. niger* and *P. citrinum*. Cu at a statistically significant level stimulated production of malic acid by both fungi species, and also of citric acid by *A. niger*. These acids are known as effective metals chelators [13]. Production of other acids in the presence of Cu was reduced. According to Dutton and coworkers [4], accumulation of oxalic acid, in contrast to the TCA acids and gluconic acid, is carried out mainly at the late stages of a culture growth. As long as the process of oxalic acid formation by Cu-adapted culture of *P. citrinum* was not inhibited by Cu, we may suggest that low concentrations of oxalic acid in fungi cultures on media with copper are largely explained by the inhibiting effect of Cu on mycelium growth and have no direct influence on oxalate biosynthesis processes.

The total content of oxalate in the cultural fluid of *A. niger* was significantly higher than in the culture of *P. citrinum*. In the latter, 100 % of oxalate was bound in insoluble salts both in the control and in the metal-exposed experiments, and the pH level was higher than the initial one. We may suppose that the presence of insoluble oxalate in control as well as in *P. citrinum* culture under Cu exposure may be explained with its binding in the form of magnesium oxalate. Considering higher content of free oxalic acid in *A. niger* cultural fluid it can be explained by lower pH value of the medium.

It is believed that under natural environment the availability of metals for fungi increases significantly, when medium pH decreases. In this case, metals accumulate in the mycelium more rapidly [7, 8, 21]. In our research carried out in a liquid medium, there was no statistically significant change in fungi growth in the case of using metals with pH ranging from 4.2 to 6.8. However, on an agar-containing medium which allows to model partially fungal natural growth conditions in soil or other substrates, the increase of a medium pH value in the presence of Cu led to an increase of the growth rate, but only on a medium containing nitrogen in form of NH_4NO_3. As far as no increases in organic acid content were observed in this

case, it may be supposed that detoxication of metals was carried out through their chelating with aminoacids produced by mycelium or by means of metal ions absorption on agar polymer cation-exchange groups. Formation of such complexes may depend heavily on pH of a medium [12] and, thus, in this case, most part of copper is bound. The concentration of Cu in bioavailable form may reduce to the level, which has some stimulating effect of mycelium growth. Therefore, changes in metal availability depend on a number of medium factors, and the effect of each factor separately may not lead to significant changes of its toxicity for fungi.

According to the literature [11, 17, 20], the presence of oxalate crystals was often observed in cultures of fungi. Zinc oxalates may form splices, which are morphologically different. Splices similar to flower-like structures observed in our work were also found in a culture of entomopathogenic fungus *Beauveria caledonica* during cultivation on a medium containing zinc phosphate. These crystals were identified as zinc oxalate dihydrates as well [11]. Zinc oxalate crystal splices found by Sayer and Gadd in *Aspergillus niger* culture were significantly smaller and with different morphology, but also had a plate structure [20]. On the mycelium of ectomycorrhizal fungi, tetragonal zinc oxalate crystals were described [17]. Literature provides data that copper oxalate can be also formed in fungi cultures [11]. In the research of Manceau and Matynia [13], it was shown that copper chelates with oxalic acid were stronger that complexes with C_3 and C_4 acids, however, unlikely they are present in the natural organic material.

In our research, no copper oxalate crystals were found on the mycelium or in the cultural fluid of *P. citrinum* and *A. niger*. Oxalic acid production by Cu-adapted culture of *P. citrinum* was more intensive in comparison with the initial strain, but it was not stimulated with the presence of Cu. Obviously, in this case, oxalate is produced to bind excessive Cu amounts, but is still not a primary factor, which determines culture resistance to copper.

Therefore, the production of oxalic acid and fungal resistance to metals is enhanced on nitrate medium, in comparison with the nitrate–ammonium medium. Formation of oxalic acid on the nitrate media is induced by zinc and leads to formation of insoluble zinc oxalates on the surface of the mycelium, which determines lower Zn toxicity. Adaptation to copper is also associated with increased oxalic acid production and is fixed at the strain. Low production of chelating agents of nitrate–ammonium medium leads to excessive influx of Zn^{2+} and Cu^{2+} into the cells and their accumulation in the vacuoles. The presence of swelled cells on a nitrate–ammonium medium is, probably, a result of metal ions accumulation inside the mycelium. This assumption is confirmed by the high concentration of copper in the mycelium of *P. citrinum* on the nitrate–ammonium medium. In this case, *P. citrinum* accumulated more Cu, than *A. niger*, what, probably, explains its lower resistance to copper in comparison with *A. niger*. Accumulation of large amounts of zinc in mycelium of fungi on a nitrate medium is, probably, explained by its extracellular binding with produced oxalic acid and precipitation of zinc oxalate on the mycelium surface.

It may be concluded that the role of organic acids and, in particular, oxalate in fungi resistance to heavy metals, is not universal and can be determined by the nature of the metal and its ability to form stable complexes with an acid anion, which reduces metal toxicity for fungi. Stimulating effect of metals on acid production is obviously explained by adaptation. However, it is not universal for all species of fungi and largely depends on metal concentration, nitrogen form in a medium, and other cultivation conditions.

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