Effect of heavy metals on the individual development of two fungi from the genus *Penicillium*

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The influence of cadmium, nickel and zinc on the development of fungi *Penicillium atramentosum* 25SL and *P. funiculosum* 6AL was investigated. All tested stages of fungal development (spore germination, germ tube growth, radial growth rate and conidiogenesis) were affected. The both fungi at their tube emergence stage were most susceptible to the metals than at the other periods of their development. *P. funiculosum* 6AL was more resistant towards the tested metals at all stages of development.

Key words: fungi, *Penicillium*, heavy metals, development, spore germination, germ tubes, radial growth rate, conidiogenesis

INTRODUCTION

Heavy metals are indicated to be harmful pollutants in soils affecting negatively the species composition and functions of the indigenous microorganisms including fungi [1–3]. Heavy metals can exert a harmful effect in many ways depending on environmental factors and metal species [4–7]. Metals can variously influence soil fungi: to diminish their populations, impoverish the diversity and to change fungal morphology and physiological activity: to affect the growth rate, reproduction processes, enzyme production, etc. [8–10].

Fungi of the genus *Penicillium* are widespread in the soil. They are known to produce a large variety of enzymes, organic acids, antibiotics and other metabolites and take an active part in soil microbiological processes [11]. It has been reported that *Penicillium* fungi response to heavy metals varies in a very wide range. There were detected rather sensitive and extremely resistant fungi of this genus, *e.g.*, *P. ochrochloron* is reported to grow in a saturated solution of copper sulfate [12]. In fungi, metal effects can vary not only among organisms, but also among different vegetative and reproductive forms of the same organisms [13].

The aim of this work was to assess the influence of heavy metals (nickel, cadmium and zinc) on the development of two fungi from the genus *Penicillium*. Micromycetes were selected by a previous investigation, and the most resistant – *Penicillium funiculosum* 6AL and quite sensitive – *P. atramentosum* 25S were chosen [14]. The following periods of fungal development were examined: spore germination, growth of germ tubes, colonial growth rate and sporulation peculiarities.

MATERIALS AND METHODS

Spore germination was tested in the liquid Czapek medium, where cadmium and nickel were added at rates of 0.2, 0.5, 1, 2 and 5 mM Cd^{2+} and Ni^{2+} and zinc at 0.2, 0.5, 1, 2, 5, 10, 20 and 40 mM Zn^{2+}. The metals were used as salts: $CdCl_2$, $NiCl_2 \cdot 6H_2O$ and $ZnCl_2$. Fungal spores from 2-week-old stock cultures were transferred into the medium containing the metals, and after 24 h spore germination was checked. Each treatment was made in 3 replicates. Germinating spores were detected using a light microscope at x150 magnification. Ten microscope fields were checked, and the average of germinated conidia was calculated. The germination index (percentage) was calculated as a ratio of germinated spores in a metal-containing medium *versus* germinated spores in a metal-free medium. The length of germ tubes was found by measuring 40–50 tubes of each sample, and an average was determined.

In order to reveal the colonial growth rate of the fungi, a radial extension of the colonies was measured every 24 hours for 7 days, and a growth rate average was calculated. Spore formation specificity was examined taking into account the spore occurrence time and spore abundance.

RESULTS AND DISCUSSION

Spore germination is affected by many factors such as humidity, temperature, some nutritive elements, the age of a fungus, etc. [15–18]. This investigation shows the metal effect on spore germination of *Penicillium funiculosum* 6Al, which in the previous investigation was most tolerant to metals, and P. *atramentosum* 25SL, one of the most sensitive fungi. It was intended to assess if spores of the more resistant fungus have a higher germination index than have spores of the sensitive fungus in heavy metal surroundings.

Of all the metals tested, differences in the effect of nickel and cadmium on spore ability to germinate was not considerable in general, but nickel exerted a more negative influence particularly at higher metal concentrations added into the medium (Fig. 1). Inhibition of spore germination of *P. funiculosum 6AL* by nickel was noticed at a concentration of 0.5 mM (95% of the conidia germinated in comparison with the control). When nickel concentration was increased to 1 mM, a sharp decrease was noted in the number of germinated spores: only 30% of the conidia germinated. At the highest, 5 mM, nickel concentration used, the germination index did not change significantly and corresponded to 21.1%. Conidia of *P. atramentosum* 25SL were affected by nickel more negatively – inhibition of spore germination was noticed from 0.5 mM nickel concentration in the medium (65%). Suppression of spore germination at 1 and 2 mM nickel in the medium almost did not differ (24 and 22%), but 5 mM of the metal reduced the number of germinating spores even to 6%.

Cadmium, like nickel, at a concentration of 0.2 mM did not exert a negative effect on spore germination of the fungi. *P. funiculosum* 6AL was positively stimulated also at a 0.5 mM cadmium concentration. Differences in the number of germinated spores between these two fungi became more evident when cadmium was added at a concentration of 1 mM. The germination index of *P. funiculosum* 6AL decreased gradually and at 5 mM in the medium fell down to 26.2%. A sharp decrease in the number of germinated spores of *P. atramentosum* 25SL was noticed from 1 mM to 5 mM cadmium in the medium – the germination index was reduced from 49% to 20%, respectively.

Spore germination was influenced considerably less by zinc. Even up to 5 mM for *P. funiculosum* 6AL and 2 mM for *P. atramentosum* 25SL, zinc had a positive effect on spore germination. A more visible difference in conidial sensitivity was noticed at 10–40 mM concentrations, where the germination index for *P. funiculosum* 6AL spores decreased from 90.3% (at 10 mM zinc concentration) to 21% (40 mM), while for *P. atramentosum* 25SL – from 45.9% to 14.8%, respectively.

Examination of spore germination showed that the metals influenced this development stage. Conidia of *P. funiculosum* 6AL were less sensitive to all the metals tested than were spores of *P. atramentosum* 25SL, especially at higher metal concentrations used. Low metal contents even promoted spore germination of the both fungi, while higher metal levels had a negative effect, mostly proportional to their concentrations used. Zinc exhibited a considerably less harmful effect on germination; only 10-fold concentrations when compared to those of nickel and cadmium had a negative influence.

Investigation on the sensitivity of germ tubes to heavy metals showed that the metals not only reduced the number of germinating spores, but also affected the growth capacity of germ tubes. As in the spore germination experiment, more sensitive was *P. atramentosum* 25SL. Even at the lowest nickel concentration used (0.2 mM), an average of the germ tube length was 4.7 μm af-

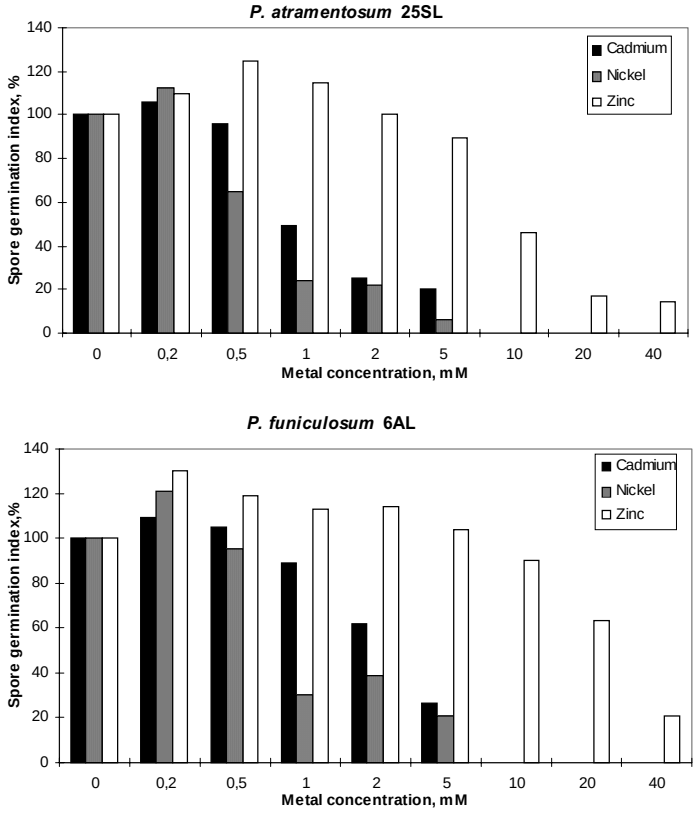

Fig. 1. Effect of cadmium, nickel and zinc on germination of *P. atramentosum* 25Sl and *P. funiculosum* 6AL spores

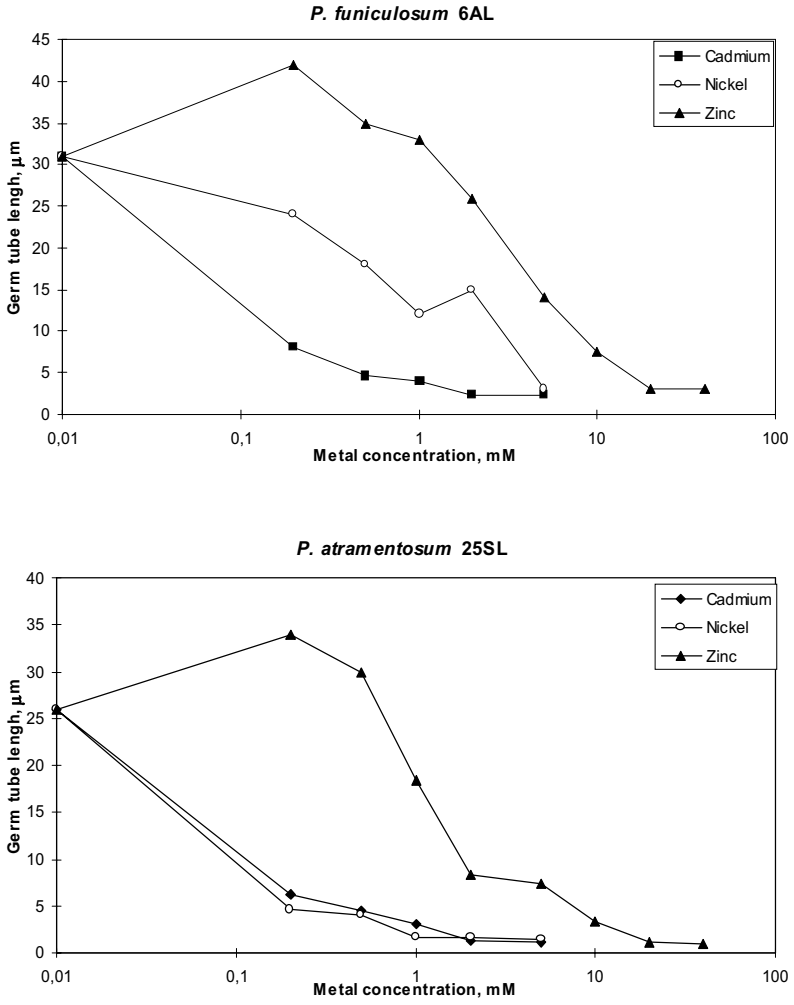

Fig. 2. Effect of cadmium, nickel and zinc on growth of germ tubes of *P. atramentosum* 25Sl and *P. funiculosum* 6AL

ter 24 hours, whereas in the metal-free medium the germ tube length was 26 µm (Fig. 2). When the metal content in the medium increased, the germ tube growth became slower, and at a concentration of 5 mM the tube length was only 1.5 µm (5.8% in comparison with the tube growth in the metal-free medium). Growth of germ tubes of *P. funiculosum* 6AL was not so much affected by this metal. The most evident differences in tube length were from 0.2 to 2 mM of the metal – the length of germ tubes of *P. funiculosum* 6AL corresponded to 77.4% and 48.4%, respectively, when compared to the control, while that of *P. atramentosum* 25SL – to 18.1% and 6.2%.

The response of *P. atramentosum* 25SL germ tubes to cadmium was quite similar as to nickel. In the presence of 0.2 mM cadmium in the medium, germ tube length was 6.2 mm (23.8%). The increasing metal content inhibited more evidently the emerging tubes – at the highest cadmium concentration the germ tube length exceeded only 1.2 µm (3.9%). Cadmium had a much more negative influence than nickel on the initial hyphal growth of *P. funiculosum 6AL*. The metal at a concentration of

Table. **Radial growth rate of the fungi in the medium supplemented with metals**

Metals added in the medium	Fungi			
	P. atramentosum 25 SL		*P. funiculosum* 6AL	
	mm · h^{-1}	%	mm · h^{-1}	%
Cadmium, mM				
0	167 ± 4.3	100	208 ± 6.8	100
0.2	79 ± 3.4	47.3	179 ± 7.2	86.1
2	18 ± 1.8	10.8	53 ± 2.3	25.5
5	0	0	30 ± 1.6	14.4
Nickel, mM				
0	167 ± 4.3	100	208 ± 6.8	100
0.2	104 ±3.8	62.3	205 ± 6.2	98.6
2	36 ± 1.7	21.5	142 ± 4.8	68.3
5	0	0	35 ± 1.1	16.3
Zinc, mM				
0	167 ± 4.3	100	208 ± 6.8	100
0.2	145 ± 4.0	86.8	230 ± 7.1	110.8
2	125 ± 3.2	78.1	213 ± 5.9	102.3
5	115 ± 2.4	68.9	155 ± 6.2	74.4
10	108 ± 3.0	64.9	132 ± 4.7	63.6
20	62 ± 1.2	37.1	124 ± 4.4	59.7
40	38 ± 0.8	22.5	61 ± 2.5	27.4

0.2 mM reduced germ tube length to 26.1%, and when metal content reached 5 mM in the medium the mean length of germ tubes was only 7.4%.

Zinc had a positive effect on the growth of *P. atramentosum* 25SL (conc. 0.2–0.5 mM) and on *P. funiculosum* 6AL – (conc. 0.2–1 mM), and only higher zinc contents reduced the germ tube length. While the growth of germ tubes of *P. funiculosum* 6AL decreased more gradually with increasing the zinc content from 26 μm (83.9%) at 2 mM zinc to 3.0 μm (9.7%) at a concentration of 40 mM, *P. atramentosum* 25SL tubes were affected much more severely: the germ tube length was reduced from 18.4 μm (70.8%) at 1 mM zinc to 8.3 μm (31.9%) at 2 mM metal concentration in the medium. Then, the germ tube outgrowth was observed to decrease to 1.2 μm (3.8%) at a sinc concentration of 40 mM.

The further investigation was focused on the growth rate of fungal colonies and the spore formation specificity under the influence of the metals. Evaluation of the colonial extension rate revealed that did *P. funiculosum* 6AL developed much faster than *P. atramentosum* 25SL. In the medium supplemented with 0.2 mM nickel, the growth rate of P. *funiculosum* 6AL did not differ at all from the control growth (Table 1). When the nickel concentrations increased, the growth rate of this fungus slowed down and at a 5mM content in the medium it was equal to 35 μm · h^{-1} (16.3%). The development of the *P. atramentosum* 25SL in the medium with the same content of nickel was inhibited much more significantly. Even 0.2 mM nickel slowed down the growth of the fungus: its growth rate corresponded to 62.3% in comparison with its growth in the metal-free medium. When the nickel concentration increased up to 5 mM in the medium, development of the fungus was not detected at all.

Cadmium had a more negative effect than nickel on the growth rate of *P. funiculosum* 6AL. The lowest cadmium concentration, 0.2 mM, slightly slowed down the growth rate of the fungus to 86.1%, and the more significant decrease in its development was observed at 2 mM cadmium content in the medium: the growth rate equalled 25.5% and fell down to 14.4% when 5 mM nickel were used. The growth of *P. atramentosum* 25SL under the influence of cadmium was more significantly suppressed, and its development was absolutely inhibited by 5 mM cadmium in the medium.

The negative influence of zinc on the growth rate of the both fungi as well as in the previous stages of the fungal development was not so severe. The development of *P. funiculosum* 6AL was even enhanced with up to 2 mM zinc in the medium. When the concentration of the metal increased, the growth rate of the fungus fell gradually down, and at 40 mM zinc added into the medium it reached 61 μm · h^{-1} (27.4%). A positive effect on the colonial growth rate of *P. atramentosum* 25SL was not noticed, but its growth rate decreased slowly, and at 40 mM zinc in the medium it was 38 μm · h^{-1} (22.5%).

Investigation of sporulation specificity showed that in most cases conidiogenesis was delayed when the metal concentrations increased. Figures 3 and 4 show the spore occurrence in *P. funiculosum* 6AL and *P atramentosum* 25SL, when they were grown in cadmium- and nickel-amended media. Spores were formed usually 1–3 days later than in a metal-free medium, depending on concentrations used and individual fungal tolerance to the metal. When the fungal growth inhibition was extremely high, spore formation was not observed at all, *e.g.*, growth of *P. funiculosum* 6AL in the medium with 5 mM and *P. atramentosum* with 2 mM cadmium was very slow, the morphology of the colonies was atypical (hypha were almost yeast-like and grew deeply into the agar), and sporulation was not detected at all. In some cases, slowly growing colonies formed spores, but the sporulation was scarce, conidia-bearing struc-

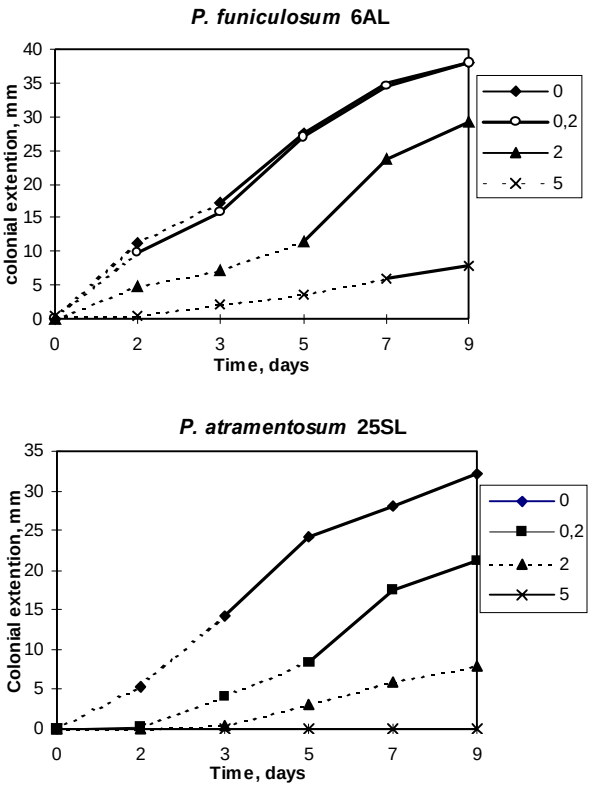

Fig. 3. Response of colonial growth and conidiogenesis of *P. atramentosum* 25Sl and *P. funiculosum* 6AL to nickel. The dotted line represents colonial growth when spore formation did not occur, the continuous line shows the presence of conidiogenesis

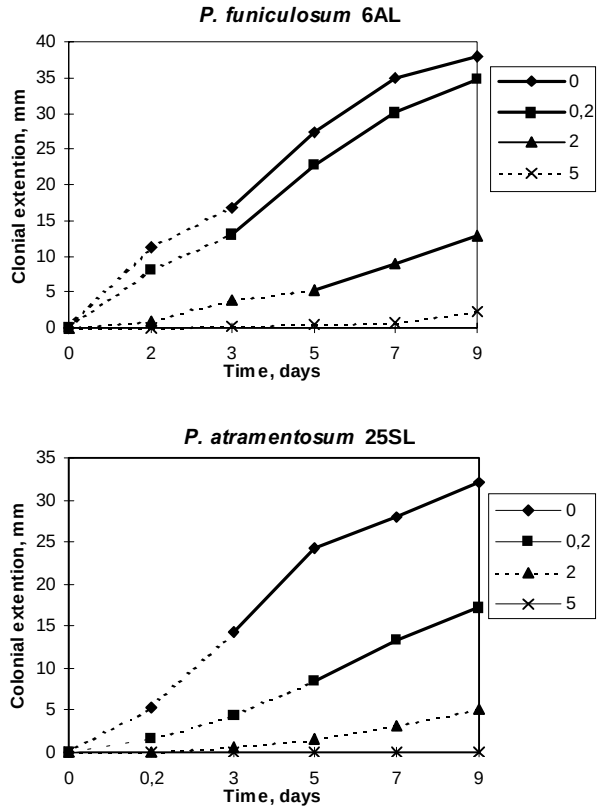

Fig. 4. Response of colonial growth and conidiogenesis of *P. atramentosum* 25Sl and *P. funiculosum* 6AL to cadmium. The dotted line represents colonial growth when spore formation did not occur, the continuous line shows the presence of conidiogenesis

tures were reduced and spore chains were much shorter. That type of the development was noticed for *P. funiculosum* at 5 mM nickel and 2 mM cadmium concentration and for *P. atramentosum* at 2 mM nickel. Additionally, *P funiculosum* 6AL at 0.2 mM nickel, where the fungal growth did not differ much from the control, started to form conidia faster (on day 2) than the colonies on the metal-free medium (on day 3). Zinc effect on spore formation at 0.2–10 mM had no negative influence. When the metal concentrations increased up to 20–40 mM, the conidiogenesis of the both fungi was weaker and slower, similarly as in the cases with 0.2–2 mM concentrations of the previous metals. The colonial pigmentation and appearance at these zinc concentrations in the medium was also affected: the colonies became atypically pink, more rugose and umbonate. Thus, the results obtained show that the test metals influenced considerably such periods of fungal ontogenesis as the growth rate and sporulation. Increasing concentrations reduced significantly the growth rate, and the slower colonies developed the later sporulation occurred or even was inhibited absolutely.

The results demonstrated that the metals influenced all stages of fungal development. *P. funiculosum* 6AL in all periods of its growth was more resistant than *P. atramentosum* 25SL. The negative response to zinc was considerably less evident than to nickel and cadmium by the both micromycetes. The sensitivity of *P. atramentosum* 25SL to cadmium and nickel did not differ significantly. *P. funiculosum* 6AL, however, was much more sensitive to cadmium than to nickel, with exception of spore germination. This could suggest that fungal tolerance of metals greatly depends on their individual properties. A particular organism may directly or indirectly rely on several survival strategies. It has been reported that individual defence reactions of fungi could include a variety of reactions: extracellular precipitation, complexation, transformation by oxidation, reduction and methylation, biosorption to cell walls, intracellular compartmentation, decreased transport and efflux, etc. [9, 19–23].

The investigation showed that the germ tube growth was the most susceptible stage of the fungal development. Spore germination was not so sensitive as germ tube outgrowth. The lowest concentrations of nickel and cadmium even stimulated spore germination, whereas the same concentration of the both metals inhibited strongly tube growth. Spores contain a variety of nutrient reserves, which could be used as a supply of energy and metabolic precursors for growth [24–26]. Thus, spore germination seems to rely greatly on endogenous reserves and is not so sensitive to exogenous stress factors. Small concentrations hence acted as a signal for spore germination of the both micromycetes. The both fungi at their tube emergence stage were more sensitive to the metals than at the extension of more mature hyphes, especially to cadmium. More evident differences in the sensitivity of the different developmental stages to nickel and cadmium was observed at 0.2 mM, when the tube growth was suppressed greatly. *P. funiculosum* 6AL tubes were very susceptible at this concentration to cadmium and *P. atramentosum* 25SL – to nickel and cadmium. Zinc also exerted a more negative effect on tube emergence, particularly at a concentration of 10–40 mM, compared to its effect on spore germination and colonial extension.

To conclude, the results of the investigation sugest that under a high metal stress in the environment the fungal development could be inhibited starting from spore germination up to spore formation.

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SUNKIŲJŲ METALŲ POVEIKIS DVIEJŲ *PENICILLIUM* GENTIES GRYBŲ INDIVIDUALIAM VYSTYMUISI

S a n t r a u k a

Tirtas kadmio, nikelio (0,2–5 mM) ir cinko (0,2–40 mM) poveikis grybų *Penicillium atramentosum* 25SL ir *P. funiculosum* 6AL vystymuisi. Nustatyta, kad visos tirtos grybų vystymosi stadijos – sporų dygimas, pradinis hifų vystymasis, radialinis kolonijų augimas bei konidiogenezė buvo veikiamos tirtų metalų. Mikromicetai buvo jautriausi metalų poveikiui pradinio hifų vystymosi stadijoje. Net 0,2 mM kadmio koncentracija šiame vystymosi etape ryškiai slopino hifų augimą. – hifų ilgis sudarė tik 23,8–26,1%, tuo tarpu kitose vystymosi etapuose mikromicetai nebuvo tokie jautrūs šiai metalų koncentracijai ar net jų vystymasis buvo stimuliuojamas. *P. funiculosum* jautriausiai reagavo į kadmį beveik visose vystymosi stadijose, o *P. atramentosum* 25SL reakcija mažai skyrėsi kadmio ir nikelio atžvilgiu. Cinkui abu tirti mikromicetai buvo žymiai atsparesni visais vystymosi laikotarpiais. Nustatyta, kad *P. funiculosum 6Al* buvo žymiai atsparesnis tirtiems metalams nei *P. atramentosum* 25SL.