

Dichotomomyces cejpíi — some characteristics of strains isolated from soil in the Slovak Republic

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The growth rate of vegetative hyphae of colonies of 22 *Dichotomomyces cejpíi* (Milko) Scott strains was determined on Sabouraud agar, on Sabouraud agar with 2 and 4 % NaCl, on Sabouraud agar with pH 4–11 and on a medium with 1 % peptone and 4–30 % saccharose. *Dichotomomyces cejpíi* strains are a new member of heat resistant fungi. In our experiments hyphal growth was limited only on Sabouraud agar with 4% NaCl. Chloroform-extractable metabolites which stopped the mobility of tracheal cilia of 1 day old chick *in vitro* were produced by eighteen (58.5%) out of 31 *Dichotomomyces cejpíi* strains in the mycelium and by 26 (83%) in the medium. One strain (No. 2268) had ciliostatic activity comparable with the activity of important known mycotoxins.

Key words: *Dichotomomyces cejpíi*, microfungi, hyphal analysis, soil, heat-resistance, mycotoxin.

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Stanovila sa rýchlosť rastu vegetatívnych hýf kolónií 22 kmeňov *Dichotomomyces cejpíi* (Milko) Scott na Sabouraudovom agare (IMUNA), na Sabouraudovom agare s 2 a 4 % NaCl, na Sabouraudovom agare s pH 4, 8, 9, 10, 11 a na médiu s 1 % peptónu a 4–30 % sacharózy pri teplote 25 a 37 °C. Kmene *Dichotomomyces cejpíi* patria do skupiny termorezistentných húb. Rast hýf bol v našich pokusoch obmedzený iba na Sabouraudovom agare so 4 % NaCl. Osemnásť (58,5%) z 31 kmeňov *Dichotomomyces cejpíi* produkovalo v mycéliu a 26 (83%) do média chloroformom extrahovateľné metabolity, ktoré zastavovali pohyb riasiniek priedušnice 1-dňových kurčiat *in vitro*. Jeden z kmeňov (č. 2268) mal ciliostatickú aktivitu porovnateľnú s aktivitou dôležitých známych mykotoxínov.

INTRODUCTION

A description of the strains of *Dichotomomyces cejpíi* (Milko) Scott — strains isolated from fruit in Moldavia near Tiraspol (former USSR), from soil in Kyoto (Japan) and from soil from The Hague and Copenhagen and in Varanasi, India — was represented in the year 1970 (Scott 1970). We isolated many strains of *Dichotomomyces cejpíi* during our investigation of the ecology of heat-resistant fungi in soil (Jesenská et al. 1992, 1993, Piecková et al. 1994).

The first strain from our isolated cultures (CCM 8085) was identified as *Dichotomomyces cejpíi* by Dr. L. Marvanová, CSc., Brno, Czech Republic.

The aim of our work was to study some characteristics of *Dichotomomyces cejpíi* strains, especially the effect of Sabouraud agar, Sabouraud agar with adjusted pH, and Sabouraud agar with incorporated NaCl and sucrose, at 25 and 37 °C on vegetative hyphal growth of colonies, and the ciliostatic activity of their metabolites resembling to mycotoxins.

MATERIAL AND METHODS

The strains of *Dichotomomyces cejpíi* were isolated from soil samples after heating at 70 °C/60 min. in Sabouraud agar (IMUNA) with Rose Bengal. The samples were taken from a garden, fields and forests in Bratislava and from a garden in Poprad, Slovak Republic. These strains were isolated from 18.8 % out of 32 (100 %) soil samples ($\times_{max} = 5$ colony forming units (CFU)/10g) in our previous study of the ecology of heat-resistant fungi (Jesenská et al. 1992). The investigated strains (22) were inoculated in the centre of the surface of 15 ml of Sabouraud agar (IMUNA) (pH 5.5-6.3), Sabouraud agar (IMUNA) with incorporated NaCl (2, 4 %), Sabouraud agar (IMUNA) with adjusted pH (4, 8, 9, 10, 11), and of agar (2 %) with peptone (1 %) and sucrose p.a. (4, 8, 16, 25, 30) in Petri dishes (ϕ 9 cm) and incubated at 25 and 37 °C. The colony diameter was measured at the 3rd, 4th or 5th day (t_1) (the first measure of the colonies was adapted according to the colony size) and once again at the 5th, 6th or 7th day (t_2). The growth rate of the vegetative hyphae of the colony in mm/24 hrs was calculated ($t_2 - t_1 : 4 = \times$ mm/24 hrs).

Thirty one isolated *Dichotomomyces cejpíi* strains from soil grew after the isolation and identification for 14 days on Sabouraud agar (IMUNA) slants in tubes at 25 °C. One of these strains was a strain requested and deposited as *Dichotomomyces cejpíi* ATCC 96464 at the American Type Cultures Collection. The culture of each strain growing in three tubes was scratched into a 200 ml liquid medium with yeast extract (2 %) and sucrose (10 %) in 500 ml Erlenmayer flasks and cultivated as a stationary culture at 25 °C. After 10 days of growth the culture fluid and the culture biomass, separately, were extracted twice with 200 ml of chloroform. The extracts were dried with anhydrous Na₂SO₄ and the chloroform was evaporated in a water bath. The extracts dissolved in dimethyl sulfoxide were added to the culture medium (20 mg/l) with tracheal rings of one-day-old chicks as was described by Jesenská and Bernát (1994). The ciliostatic activity of the extracts was observed after 24, 48 and 72 hours.

The morphology of *Dichotomomyces cejpíi* colonies (strain No. 2314) after 15 days of growing at 25 and 37 °C was observed on malt (MALT) and Sabouraud agar (SAB) [IMUNA], potato dextrose agar (PDA) [DIFCO] and on Czapek yeast autolysate agar — CYA (Pitt 1979). Micromorphology was documented by photos made with an OLYMPUS photomicrocamera.

Table 1. Growth rate (mm/24 h) of vegetative hyphae of colonies of 22 *Dichotomomyces cejprii* strains on Sabouraud agar (S) and on Sabouraud agar with NaCl (2 % and 4 % NaCl — SNaCl) at 25 and 37 °C.

Incubation temperature	Medium			
	S	SNaCl		
		2 % NaCl	4 % NaCl	
Growth rate in mm/24 h				
25 °C	abs. (\bar{x})	3.8	3.9	1.5
	rel. (%)	100.0	102	39
	x_{min}	2.7	3.0	0
	x_{max}	4.7	4.7	3.7
37 °C	abs. (\bar{x})	4.6	4.1	0.8
	rel. (%)	100.0	89	17
	x_{min}	2.7	2.7	0
	x_{max}	6.5	5.7	2.2

Table 2. Growth rate (mm/24h) of vegetative hyphae of colonies of 22 *Dichotomomyces cejprii* strains on Sabouraud agar with processed pH (4, 8, 9, 10, 11 — SpH) at 25 and 37 °C. For growth rate on Sabouraud agar (100 %) see Table 1.

Incubation temperature		SpH				
		pH 4	pH 8	pH 9	pH 10	pH 11
Growth rate in mm/24 h						
25 °C	abs. (\bar{x})	3.6	3.9	4.9	3.7	3.5
	rel. (%)	94	102	128	97	92
	x_{min}	2.7	3.0	3.0	2.0	2.0
	x_{max}	4.7	4.7	6.0	5.2	4.7
37 °C	abs. (\bar{x})	4.2	3.9	4.7	3.7	3.4
	rel. (%)	91	84	102	80	73
	x_{min}	3.0	2.7	2.2	1.7	1.5
	x_{max}	6.0	5.5	6.2	5.2	5.7

Table 3. Growth rate (mm/24 h) of vegetative hyphae of colonies of 22 *Dichotomomyces cejprii* strains on agar with peptone (1 %) and sucrose (4, 8, 16, 25, and 30 % — APS) at 25 and 37 °C. For growth rate on Sabouraud agar (100 %) see Table 1.

Incubation temperature		APS (sucrose)				
		4 %	8 %	16 %	25 %	30 %
		Growth rate in mm/24 h				
25 °C	abs. (\bar{x})	4.2	5.5	5.0	5.6	4.7
	rel. (%)	110	144	131	147	123
	x_{min}	3.2	4.0	4.2	4.5	2.7
	x_{max}	5.7	7.2	7.0	7.0	6.6
37 °C	abs. (\bar{x})	3.5	5.1	5.5	6.0	4.5
	rel. (%)	76	110	115	130	97
	x_{min}	1.5	4.2	4.0	3.2	2.5
	x_{max}	5.0	6.7	7.5	7.7	7.0

Table 4. The effect of chloroform-extracts from the mycelium and from the growing medium of 31 *Dichotomomyces cejprii* strains on the respiratory cilia movement of 1 day old chicks *in vitro*.

Dichotomomyces cejprii Number of strains		Chloroform extracts						
		mycelium			medium			
abs.	rel. in %	movement of cilia after hours						
		24	48	72	24	48	72	
2	6.5	+	+	+	+	+	+	
10 ^a	32.2	+	+	+	+	+	—	
1	3.2	+	+	+	+	—	—	
3	9.7	+	+	—	+	+	+	
11	35.4	+	+	—	+	+	—	
2	6.5	+	+	—	+	—	—	
1 ^b	3.2	+	—	—	+	+	—	
1 ^c	3.2	+	—	—	+	—	—	
Σ 31	100.0							
Strains with ciliostatic activity		abs.	—	2	16	—	4	22
		rel. (%)	—	6.5	52.0	—	13	70.0
		Σ 18 (58.5%)			Σ 26 (83.0%)			

Note ^a: 9 strains of *Dichotomomyces cejprii* and *Dichotomomyces cejprii* ATCC 96464

^b: strain No. 2056

^c: strain No. 2268

RESULTS

The average growth rate of vegetative hyphae of *Dichotomomyces cejpíi* colonies in mm/24 hrs was:

- 3.8 (100 %) on Sabouraud agar,
 - 3.9 (102 %) on Sabouraud agar with 2 % NaCl,
 - 1.5 (39 %) on Sabouraud agar with 4 % NaCl,
 - 3.6 (94 %) on Sabouraud agar with pH 4,
 - 3.9 (102 %) with pH 8,
 - 4.9 (128 %) with pH 9,
 - 3.7 (97 %) with pH 10,
 - 3.5 (92 %) with pH 11,
 - 4.2 (110 %) on agar with peptone and 4 % sucrose,
 - 5.5 (144 %) with peptone and 8 % sucrose,
 - 5.0 (131 %) with peptone and 16 % sucrose,
 - 5.6 (147 %) with peptone and 25 % sucrose
- and 4.7 mm/24 hrs (123 %) with peptone and 30 % sucrose at 25 °C.

At 37 °C the average growth rate in mm/24 hrs was:

- 4.6 (100 %) on Sabouraud agar,
 - 4.1 (89 %) on Sabouraud agar with 2 % NaCl,
 - 0.8 (17 %) with 4 % NaCl,
 - 4.2 (91 %) on Sabouraud agar with pH 4,
 - 3.9 (84 %) with pH 8,
 - 4.7 (102 %) with pH 9,
 - 3.7 (80 %) with pH 10,
 - 3.4 (76 %) with pH 11,
 - 3.5 (76 %) on agar with peptone and 4 % sucrose,
 - 5.1 (110 %) with peptone and 8 % sucrose,
 - 5.5 (115 %) with peptone and 16 % sucrose,
 - 6.0 (130 %) with peptone and 25 % sucrose
- and 4.5 mm/24 hrs (97 %) with peptone and 30 % sucrose (Tab. 1, 2, 3).

Two (6.5 %) out of 31 *Dichotomomyces cejpíi* strains in the mycelium and 4 (13 %) in the medium produced chloroform-extractable metabolites those stopped the movement of tracheal cilia *in vitro* after 48 hrs. Sixteen (52 %) strains in the mycelium and 22 (70 %) strains in the medium produced metabolites those stopped the mobility after 72 hrs. The most active were strains No. 2056 and 2268. Strain *Dichotomomyces cejpíi* ATCC 96464 was not active in this experiment (Tab. 4).

The most visible difference of colony morphology was seen on SAB: the colonies there were very lanose. The temperatures 25 °C and 37 °C on SAB,

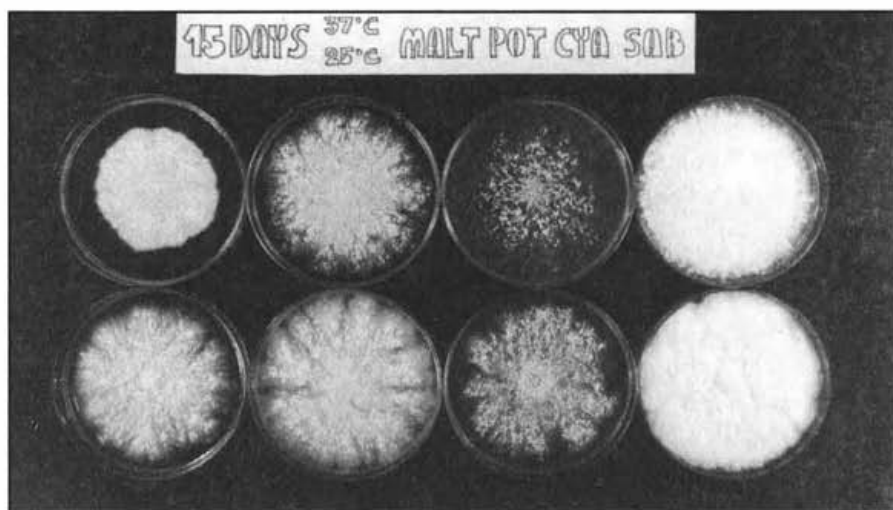


Fig 1. *Dichotomomyces cejpii* (Milko) Scott — colonies on malt agar (MALT), Sabouraud agar (SAB), potato dextrose agar (POT) and on Czapek yeast autolysate agar (CYA) after 15 days at 25 ° and 37 °C.

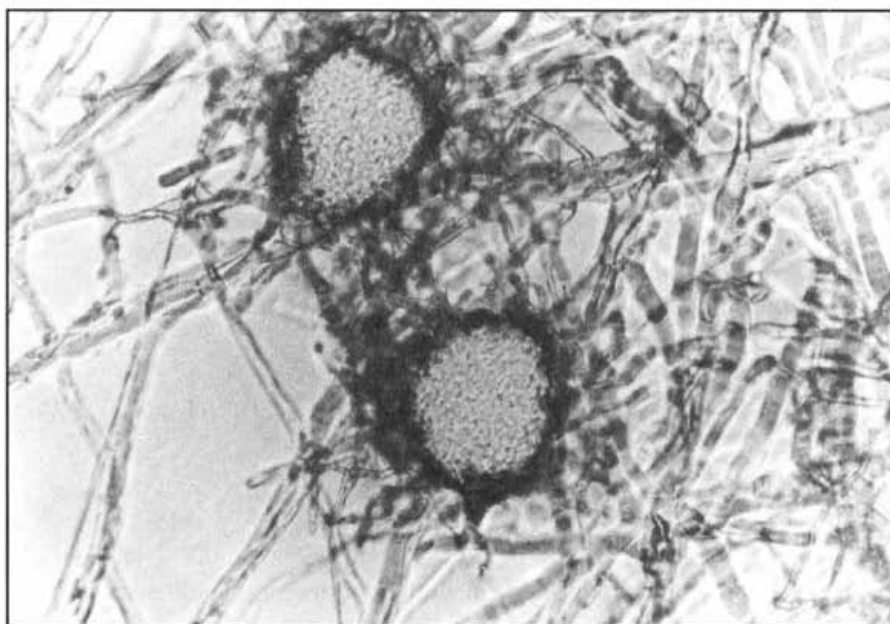


Fig 2. *Dichotomomyces cejpii* (Milko) Scott — sphaerical cleistothecia ($\times 40$).



Fig 3. *Dichotomomyces cejpīi* (Milko) Scott — conidiophore apice dichotomously branched with aleuriospores ($\times 1000$).



Fig 4. *Dichotomomyces cejpīi* (Milko) Scott — racquet hyphae ($\times 400$).

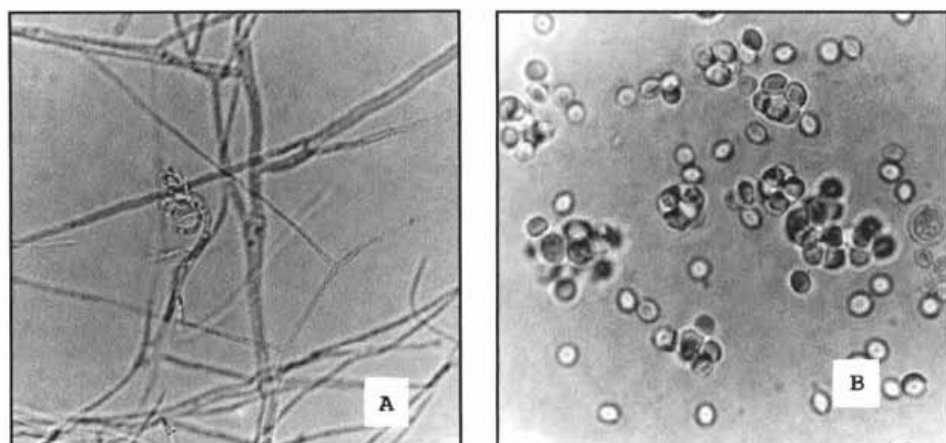


Fig 5. *Dichotomomyces cejpīi* (Milko) Scott — cleistothecial initials (A), asci, ascospores and one aleuriospore (B) ($\times 400$).

PDA, CYA were without any effect on the morphology, a difference was found on MALT (Fig 1). Sphaerical cleistothecia surrounded by a hyphal network (Fig. 2) were visible to the naked eye on PDA and CYA (Fig. 1). The racquet cells, the conidiophore apices dichotomously branched with aleuriospores, hyaline and lenticular ascospores with two closely appressed thin equatorial ridges (Figs 3, 4, 5) and other structures, as were described by Scott (1970), were seen microscopically.

DISCUSSION

It was clear from our previous results that *Dichotomomyces cejpīi* strains belong to the group of heat-resistant fungi, e. g. an artificially prepared suspension of *Dichotomomyces cejpīi* propagules *in vitro* survived the effect of a temperature of 70 °C for more than 300 min, and 80 °C for 8–10 min (dependent on number of CFU per 1 ml of the suspension). But they did not survive a temperature of 90 °C for 1 min (Piecková et al. 1994 and Jesenská and Piecková 1995). They need to be studied further, also because the strains of this genus were so rarely isolated (Scott 1970) and last not least because prof. Dr. Karel Cejp was a very well-known mycologist at Charles University in Prague, former Czechoslovakia.

Dichotomomyces cejprii var. *spinus* strains were isolated from samples of brackish water sediments and is a dominant species from river sediments between fresh and marine water areas in Japan (Ueda 1980 a, b).

The vegetative hyphae of the colonies of *Dichotomomyces cejprii* strains, investigated by us, were able to grow *in vitro* at pH 4, 8, 9, 10 and 11, on a medium with 4–30 % sucrose, on a medium with 2 % NaCl at 25 and 37 °C, with a limitation on a medium with 4% NaCl. This means they are osmotolerant fungi. The activity of the metabolites of *Dichotomomyces cejprii* strain No. 2268 on the respiratory tract cilia movement of 1-day-old chicks was comparable with the activity of diacetoxyscirpenol, T-2 toxin, aflatoxin B₁, M₁, G₁, patulin and ochratoxin A (Jesenská and Bernát 1994). Our *Dichotomomyces cejprii* strains are also available for further study as ATCC 96464, CCM 8085, and from our laboratory.

Conclusion: *Dichotomomyces cejprii* strains were characterized in our previous papers as a new member of a heat-resistant fungi group. The present study was made to provide more details on the environmental requirements of these fungi, since no data concerning *Dichotomomyces cejprii* were available. It was shown that hyphae of *Dichotomomyces cejprii* colonies *in vitro* were tolerant of temperatures of 25 and 37 °C, sucrose (4% – 30%) and pH (pH 4 – pH 11). Hyphal extension was limited by 4 % NaCl in these experiments. Some strains were able to produce metabolites resembling mycotoxins, as was detected in the experiment with the ciliostatic activity of tracheal cilia of 1 day old chicks *in vitro*. These interesting metabolites need to be chemically characterized.

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