Relationships of heat resistant micromycetes from soil to sucrose, natrium chloride, and pH

Vzťahy termorezistentných mikromycét zo zeminy voči sacharóze, chloridu sodnému a pH

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Numbers of colonies of micromycetes , which had been isolated from three soil samples, exposed to 80 °C heat for 60 minutes in Sabouraud agar, were counted. The pH of the agar varied (from 4 to 8), and sucrose (10–50 %), or natrium chloride (2–8 %) were incorporated in the agar. The most resistant germs in the experiment seemed to be the ones of *Talaromyces avellaneus* (Thom & Turesson) C. R. Benjamin which were present in the soil. The germs of *Eupenicillium baarnense* (van Beyma) Stolk & Scott were relatively equally sensitive to the experimental conditions as the germs of the *Neosartorya fischeri* (Wehmer) Malloch & Cain.

Stanovovali sa počty kolónií mikromycét, ktoré sa izolovali z troch vzoriek zeminy, exponovaných v prostredí Sabouraudovho agaru počas 60 minút teplote 80 °C. V agare sa upravovali hodnoty pH (4 až 8) a inkorporovala sa sacharóza doň (10 až 50 %) resp. chlorid sodný (2 až 8 %). Ako najodolnejšie sa v podmienkach pokusu javili v zemine prítomné zárodky druhu *Talaromyces avellaneus* (Thom & Turesson) C. R. Benjamin. Zárodky druhu *Eupenicillium baarnense* (van Beyma) Stolk & Samson boli na podmienky pokusu relatívne rovnako citlivé ako zárodky *Neosartorya fischeri* (Wehmer) Malloch & Cain.

Introduction

Heat resistant micromycetes, having gone through certain latent period, cause moulding of canned fruit products (Baggerman 1981, Beuchat et Rice 1979, Beuchat et Toledo 1977, Hocking et Pitt 1984, Jesenská et al. 1984, King 1986, Scott et Bernard 1987, Splittstoesser 1978, and others), namely the strains *Byssochlamys nivea* Westling. *Neosartorya fischeri* (Wehmer) Maloch & Cain and *Talaromyces flavus* (Kloecker) Stolk & Samson (Jesenská et Petríková 1985) above all in Czecho-Slovakia. We were making research into ecology of heat resistant micromycetes and found considerable numbers of their germs in soil. We also isolated certain micromycetes whose heat resistance had not been known before (Jesenská et Piecková 1991, Jesenská et al. 1992).

The paper therefore centers on the relationships of micromycetes from soil to high heat (80 °C) in Sabouraud agar with varying pH, different sucrose and natrium chloride concentrations.

Materials and methods

Soil samples were taken from three different beds of a private garden which had been fertilized with organic fertilizer by an amateur gardener. For each sample the pH, phosphorus and potassium levels were specified in the Central Agricultural Monitoring and Testing Institute in Bratislava.

A: 5 grams of each sample were put into a Erlenmayer flask with 100 ml of Sabouraud agar (IMUNA) with 150 mg Bengal Rose/l. The 80 °C temperature of the agar was held in water bath for 60 minutes.

B: Sabouraud agar was put into a series of Erlenmayer flasks, Bengal Rose, and sucrose were added, so that Sabouraud agar contained 10, 15, 20, 30, 40, and 50% (w/v) of sucrose. The series of flasks was extended so that the pH values in separate agars with sucrose levels were adjusted from 4 to 8.

Similar procedure was followed at the preparation of Sabouraud agar with 2, 4, 6, and 8% (w/v) of NaCl, and pH was adjusted from 5 to 8.

Note: Further extreme pH values could not be employed due to technological problems at agar gel setting.

5 grams of each sample were added onto 100 ml of prepared Sabouraud agar, and exposed to 80 °C heat, as mentioned above.

60 minutes later the agar with soil was distributed onto 9 or 10 sterile glass Petri dishes of 9 cm diameter. The dishes were incubated at laboratory temperature for 7 - 10 days, the grown colonies were counted, and representative colonies selected on the basis of their macromorphology, were inoculated onto culture medium in tubes or on Petri dishes. The isolated strains were identified on the basis of morphological signs of cultures which had grown up on Sabouraud agar, malt extract agar (IMUNA), Czapek agar with yeast extract (Pitt 1979), potato-dextrose agar (Potato-dextrose agar DIFCO), and on agar poor in nutrients (Nirenberg 1976) at lab temperature, and at 37 °C temperature. For their identification the following keys were used: Ellis (1971), Fassatiová (1986), Pitt (1979 a, b), Pitt et Hocking (1979) and Raper et Fennel (1965).

Results

Despite the fact that all samples were obtained from a single private garden, there were differences not only in chemical specifications, but also in numbers and species representation of micromycetes whose germs in Sabouraud agar were able to survive the effects of 80 °C heat. The 15 grams of soil yielded alltogether 377 (100%) colonies of *Eupenicillium baarnense* (van Beyma) Stolk & Scott, most of which were from sample 2, 5 colonies of *Gilmaniella humicola* Barron, 18 (100%) colonies of *Neosartorya fischeri* (Wehmer) Malloch & Cain, 12 (100%) colonies of *Talaromyces avellaneus* (Thom & Turesson) C. R. Benjamin, and sample 3 yielded also colonies of *Botryotrichum piluliferum* Saccardo & Marchal, *Chaetomium sp., Nodulisporium sp.,* and *Talaromyces bacillisporus* (Swift) C. R. Benjamin (Tab. 1).

After having incubated Petri dishes with spilt modified Sabouraud agar and soil, the following facts were found out:

- The higher was NaCl concentration in Sabouraud agar, the lower was the number of isolated *Neosartorya fischeri* colonies (Tabs. 2 and 3). The number of isolated colonies also was decreasing with the level of sucrose incorporated into Sabouraud agar (Tab. 6), and higher numbers of colonies were isolated in sucrose environment in pH 6 and 7 than in pH 3 and 4 - a finding similar (although not as abvious) to what observed in case of NaCl environment (Tab. 6).

- Similarly, in Sabouraud agar with higher NaCl concentration, successively lower numbers of *Eupenicillium baarnense* colonies were isolated from the soil (Tabs. 2 and 4). Relatively high numbers of *Eupenicillium baarnense* colonies grew in agar with 10-40 % of sucrose. The influence of pH in conjunction with NaCl or sucrose upon the numbers of isolated colonies was not particularly obvious (Tabs. 4 and 7).

The numbers of colonies isolated in the experiment indicate that germs of the *Talaromyces avellaneus* species present in soil were the most resistant ones. If compared with the numbers of *Neosartorya fischeri* and *Eupenicillium baarnense* colonies, they were

relatively resistant to NaCl concentration in Sabouraud agar (Tabs 2 and 5), and to 10 – 40 % levels of sucrose (Tab. 8). They were more sensitive to the varying pH in sucrose agar (Tab. 8) than in NaCl agar (Tab. 5)

Discussion

Heat resistant micromycetes represent a considerably important group of fungi. Some of them are known as the classical originators of moulding of canned/preserved fruit products and juices. It is concerned mainly with *Byssochlamys nivea* Westling. *Byssochlamys fulva* Olliver & Smith, *Neosartorya fischeri* (Wehmer) Smith and *Talaromyces flavus* (Kloecker) Stolk & Samson and some other species (Pitt et Hocking 1985, and others).

Searching for sources of contamination of preserved fruit, first of all we focused on the occurrence of heat resistant micromycetes in soil. We found out that germs of heat resistant micromycetes which are able to survive the effects of 70, 80 or 90°C temperatures, can be found in soil. Their numbers and the representations of separate species were different. Besides the classical heat resistant species we isolated strains of other species whose ability to survive the effects of heat exposure has not been described yet (Jesenská et al., 1992).

The resistance of spores of heat resistant species *Neosartorya fischeri, Byssochlamys nivea, Byssochlamys fulva* or *Talaromyces flavus* to heat exposure was examined and in vitro experiments were conducted with cultures grown on culture medium. Thus it was found out that the heat resistance of these ascospores is influenced by the environment in which the cultures were grown as well as by the age of the culture, incubation temperature, and the like, and by the experimental environment in which the proper heat resistance testing was done. Saccharides seemed to have mostly protective effects on ascospore survival (Beuchat 1988 a, b, Casella et al. 1990, Conner et Beuchat 1987 a, b, Conner et al. 1987, Eckardt et Ahrens 1978, Hatcher et al. 1979, King et Whitehand 1990, Splittstoesser 1978, Splittstoesser et al. 1986).

In our experiment we estimated the numbers of micromycete colonies which had been isolated from the soil samples exposed to 60-minutes heat of 80°C in Sabouraud agar with adjusted pH, and with incorporated sucrose or natrium chloride. Relative values of the counted numbers of colonies were compared with numbers of colonies which had been isolated from soil samples exposed to 80°C heat in commercial Sabouraud agar and for the same time periods.

Micromycetes isolated in our experiment from the soil proved not only their ability to survive extreme conditions but also their ability to grow and make colonies over the surface of medium with high sucrose concentrations, and to a lesser degree over the surface of medium with high natrium chloride concentrations. The germs of strains of *Talaromyces*

avellaneus in soil samples, seemed to be the most resistant ones in the experiment. The resistance to experimental conditions of the germs of *Eupenicillium baarnense* strains from the soil samples was found to be relatively the same as the resistance of the traditionally known heat resistant *Neosartorya fischeri*. The germs of micromycete strains from soil exhibited higher tolerance to varying pH and sucrose values than to the varying natrium chloride concentrations in the medium.

Certain differences in the numbers of isolated colonies in separate experiments could be attributed to uneven distribution of heat resistant micromycete germs over the examined soil samples. Still, we have not found out yet in what morphological forms the heat resistant micromycetes occur in soil. They could occur in separate cleistothecia, gymnothecia, asci as well as separate ascospores, all these at different stages of maturity, and different degrees of resistance to environmental conditions which can result in different numbers of germs that would produce colonies on laboratory media. In our experiment, to the difference from laboratory conditions where one can adjust the number of germs in the inoculum, we had soil samples in which germs of certain species occurred sparingly. There also arised the question whether the conditions we made, had fungicidal, or just fungistatical effects on the heat resistant micromycetes.

The heat resistance mechanism in certain micromycete species has not been sufficiently explained yet. In cells the mechanism could be based on the composition of fatty acids, higher levels of certain substances or lower level of a in the cell, properties of the cell membrane, the effects of proteins of heat shock, and the like (Banner et al. 1979, Casella et al. 1990, Conner 1987). Questions related to halotolerance or osmotolerance of microorganisms are very complex and only partially explained (Gilmour 1990, Jennings 1990).

There is still much work to be done on research into micromycetes whose germs are able to survive extremely unfavourable conditions, and that would be helpful not only to the food industry.

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Table 1
Characterization of 3 soil samples from 3 various places in a private garden.

Sample No	Chemical parameters[a]			Micromycetes[b]			
	pH	P	K	Number of isolated colonies/5g of soil			
		mg/of soil		E.B.	G.H.	N.F.	T.A.
1	7,5	1780	7360	3	1	5	7
2	7,7	1690	5346	355	3	5	1
3[c]	7,5	610	479	19	1	8	4
			Σ	377	5	18	12

Note: a: P . . . phosphorus, K . . . potassium
b: number of CFU/5 of soil, surviving in Sabouraud agar (IMUNA) effect of temperature 80°C 60 minutes.
E.B. . . . Eupenicillium baarnense, G.H. . . . Gilmaniella humicola
N.F. . . . Neosartorya fischeri, T.A. . . . Talaromyces avellaneus
c: a rare occurrence of colonies of Botryotrichum piluliferum (2 colonies), Chaetomium sp. (3), Nodulisporium sp. (2), Talaromyces bacillisporus (1)

Table 2
Number of colonies of Neosartorya fischeri, Eupenicillium baarnense and Talaromyces avellaneus isolated from 3 soil samples (15 g of soil) on Sabouraud agar with 2% – 8% NaCl after effect of 80°C/60 min.

Micromycetes % NaCl	Neosartorya fischeri[a]	Eupenicillium baarnense[b]	Talaromyces avellaneus[c]
	Number of isolated colonies in %		
2	122%	106%	216%
4	127%	49%	200%
6	50%	18%	141%
8	0%	2%	50%

Note a: see Table 1 100% = 18 colonies
b: dtto 100% = 377 colonies
c: dtto 100% = 12 colonies

Table 3
Number of colonies of Neosartorya fischeri isolated from soil samples on Sabouraud agar with 2% – 6% NaCl and with modified pH values (pH 5 to 8) after effect of 80°C 60 min.

NaCl / pH	2	4	6	8
	Number of isolated colonies in %[a]			
5	200%	94%	22%	n
6	105%	83%	27%	n
7	177%	72%	5%	0%
8	122%	77%	0%	5%

Note: a: see Table 1 100% = 18 colonies
b: not done

Table 4
Number of colonies of Eupenicillium baarnense isolated from soil samples on Sabouraud agar with 2% – 8% NaCl and modified pH values (pH 5 to 8) after effect of 80°C 60 min.

% NaCl / pH	2	4	6	8	$\bar{X}$
	Number of isolated colonies in %[a]				
5	93%	50%	31%	0,2%	43%
6	80%	40%	22%	0,2%	35%
7	60%	45%	26%	7%	34%
8	85%	42%	15%	4%	36%
$\bar{X}$	79%	44%	23%	2%	

Note: a: see Table 1 100% = 377 colonies

Table 5
Number of colonies of Talaromyces avellaneus isolated from soil samples on Sabouraud agar with 2 % – 8% NaCl and modified pH values (pH 5 to 8) after effect of 80 °C 60 min.

% NaCl	2	4	6	8	
pH	Number of isolated colonies in %[a]				$\bar{X}$
5	125%	175%	41%	100%	110%
6	283%	150%	183%	150%	191%
7	241%	175%	158%	58%	158%
8	316%	166%	166%	75%	180%
$\bar{X}$	241%	166%	81%	95%	

Note: a: see Table 1 100% = 12 colonies

Table 6
Number of colonies of Neosartorya fischeri isolated from soil samples on Sabouraud agar with 10% – 50% succrose and modified pH values (pH 4 – 8) after effect of 80°C 60 min.

pH succrose	10%	15%	20%	30%	40%	50%	
pH	Number of isolated colonies in %[a]						$\bar{X}$
4	27%	138%	5%	5%	11%	0%	31%
5	100%	55%	55%	50%	0%	0%	43%
6	127%	94%	94%	111%	5%	44%	79%
7	122%	55%	100%	72%	111%	0%	76%
8	88%	138%	61%	44%	22%	0%	58%
$\bar{X}$	92%	96%	63%	56%	29%	8%	

Note: a: see Table 1 100% = 18 colonies

Table 7
Number of colonies of Eupenicillium baarnense isolated from soil samples on Sabouraud agar with 10% – 50% succrose and modified pH values (pH 4 – 8) after effect of 80°C 60 min.

pH succrose		10%	15%	20%	30%	40%	50%	
pH		Number of isolated colonies in %[a]						$\bar{X}$
4		44%	75%	51%	68%	84%	4%	54%
5		69%	84%	55%	77%	61%	54%	66%
6		48%	64%	82%	88%	74%	45%	66%
7		50%	58%	84%	59%	49%	9%	51%
8		18%	51%	22%	29%	76%	15%	35%
	$\bar{X}$	45%	66%	58%	64%	68%	25%	

Note: a: see Table 1 100% = 377 colonies

Table 8
Number of colonies of Talaromyces avellaneus isolated from soil samples on Sabouraud agar with 10% – 50% succrose and modified pH values (pH 4-8) after effect of 80°C 60 min.

pH succrose		10%	15%	20%	30%	40%	50%	
pH		Number of isolated colonies in %[a]						$\bar{X}$
4		100%	33%	16%	0%	16%	0%	27%
5		83%	100%	58%	58%	91%	8%	66%
6		50%	83%	50%	50%	33%	16%	47%
7		83%	108%	141%	91%	91%	0%	85%
8		75%	58%	50%	41%	83%	0%	51%
	$\bar{X}$	78%	76%	63%	48%	62%	4%	

Note: a: see Table 1 100% = 12 colonies