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Saprobic microfungi in tea based on *Camellia sinensis* and on other dried herbs

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The quality of various types of tea from shops in Prague (Czech Republic) in respect to microfungal contamination was investigated. Altogether, 40 samples of tea were tested including black, green and herbal teas. Eighty-one species of microfungi were detected. *Aspergillus niger* agg. was found to be the most frequent. Great differences were recorded in microfungal species composition between various kinds of tea. However, when using the Monte Carlo Permutation test in CCA, we did not find any correlation between microfungi and type of tea. Aflatoxin production was not proven in any of the tested strains. Colony forming units (CFU) did not exceed the limits valid in the Czech Republic.

Key words: tea, *Camellia sinensis*, contamination, ascomycete anamorphs, toxigenic fungi

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Byla studována kvalita různých typů čajů pocházejících z pražských obchodů (Česká republika) z hlediska jejich kontaminace mikroskopickými houbami. Celkem bylo testováno 40 vzorků černých, zelených a bylinných čajů. Bylo v nich zjištěno 81 druhů mikroskopických hub. Nejčastějším druhem byl *Aspergillus niger* agg. Mezi různými typy čajů byly zaznamenány velké rozdíly v druhovém složení společenstev mikroskopických hub. Pokud byl ale použit Monte Carlo permutační test v CCA, nezaznamenaly jsme žádnou asociaci mezi mikromycety a druhem čaje. Produkce aflatoxinů nebyla prokázána u žádného z testovaných kmenů hub. Počet zárodků houbových organizmů (CFU) nepřekročil limity platné v České republice.

INTRODUCTION

Teas are one of the basic commonly used drinks. Their contamination with microfungi, which can be a possible source of mycotoxins, is therefore undesirable. Teas, as a dry herbal product, present a substrate, which contains a low amount of water as well as soluble sugars. This means that teas are a product which may be colonised first of all by xerotolerant and osmotolerant fungi comprising many potentially toxigenic species.

Data on fungal contamination of teas are sporadic. Only a few authors have dealt with the occurrence of microfungi in teas. For example, Jesenská (1987) investigated mycobiota of dried food including black teas in Slovakia. Abdel-Hafez and El-Maghraby (1992) examined microfungi in tea in Egypt. In the Sultanate of Oman, Elshafie et al. (1999) studied tea quality and fungi associated with black tea. In the Czech Republic, several authors (e.g. Kubátová et al. 2000, Ostrý et al. 2002) were interested in microbial, in particular microfungal, contamination of teas. Also in other works (e.g. Aziz et al. 1998, Halt 1998) microfungi contaminating herbal teas have been investigated.

The main aim of our study was therefore to discover the spectrum of microfungi in teas in the sale conditions of the Czech Republic and evaluate the quality of teas according to the Czech hygienic standard (ČSN ISO 7954). Further we aimed to compare the quality of different types of teas, i.e. black (fermented) versus green (non-fermented) teas and packaged (tea bags) versus unpackaged teas to know the effect of processing, and to carry out a simple test of aflatoxin production among the strains of the genus *Aspergillus* section *Flavi*.

MATERIALS AND METHODS

Forty tea samples used for mycological examination were obtained from local markets in Prague between 1999 and 2001. They came from different producers and belonged to the most widespread types of tea in the Czech Republic. They were in good condition and without visible growth of moulds. To make a comparison, ten black, fermented tea samples (*Camellia sinensis*), ten green, non-fermented ones, ten samples of herbal teas of plants from the temperate zone (*Matricaria chamomilla*, *Melissa officinalis*, *Mentha piperita*, *Sambucus nigra*, *Tilia cordata*, and *Urtica dioica*) and ten samples of herbal teas of subtropical or tropical plants (*Aspalathus linearis*, *Cassia senna*, *Foeniculum vulgare*, *Hibiscus sabdariffa*, *Ilex paraguariensis*) were processed. Each sample was represented by only one plant. Moreover the difference between packaged and unpackaged teas was investigated (Tab. 1).

For the qualitative study the direct inoculation method was used. A small amount, c. 0.2 g of each sample was equally distributed on a medium. The following three isolation media were used: dichloran 18 % glycerol agar (DG18) according to Pitt and Hocking (1997), soil extract agar with glucose and Rose Bengal (SEGA) and wort agar (WBA) according to Fassatiová (1986). Streptomycin was added to suppress bacterial growth (100 mg/l). Petri dishes were incubated at c. 25 °C for seven days. The method of heating samples at 75 °C for 30 min. according to Samson et al. (1996) was used to isolate heat resistant fungi. Growth of microfungi was examined during a period of two weeks.

Tab. 1. Types and numbers of tested teas.

		unpackaged teas	packaged teas
Camellia sinensis	Fermented (black) teas	5	5
	Non-fermented (green) teas	5	5
other herbal teas	Teas of temperate origin	5	5
	Teas of (sub)tropical origin	5	5

The quantitative study followed the ČSN ISO 7954 standard. The dilution plate technique was used for the isolation of microfungi. A portion of each sample (1 g) was shaken for 10 min. in 10 ml of sterile deionised water. One ml of each diluted suspension (1:10 and 1:100) was pipetted into a 90-mm Petri dish and mixed with the medium (yeast extract 5 g, glucose 20 g, chloramphenicol 0.1 g, agar 15 g and distilled water 1000 ml). Two Petri dishes were prepared per sample. Colony forming units (CFU) were counted after 5 days of incubation at ca 25 °C.

Microfungi were identified according to micro– and macro-morphological characteristics. For *Aspergillus*, *Penicillium*, *Paecilomyces*, and *Verticillium* strains, Czapek yeast extract agar (CYA) and malt extract agar (MEA) (Pitt 1979) were used. To identify *Eurotium* strains, dichloran 18 % glycerol agar (DG18) and Czapek yeast extract agar with 20 % sucrose (CY20S) according to Pitt and Hocking (1997) were used. Species of the order *Mucorales* were identified on malt extract agar (MEA) according to Pitt and Hocking (1997) and the other fungi on potato dextrose agar (PDA).

Microfungi were identified according to Domsch et al. (1980) and monographic works and reports (Arx 1981, 1982, Ellis 1971, 1976, Horn 1997, Kozakiewicz 1989, Pitt 1979, Pitt and Hocking 1997, Tzean et al. 1990, Váňová 1989).

Some physiological methods were used for the identification of *Penicillium* species (Frisvad 1981, 1983, 1985): the production of acids on creatine sucrose agar (CREA), growth on nitrite sucrose agar (NSA), production of a brown pigment on the reverse side of the colony on yeast extract sucrose agar (YES) and the ability to cause apple rot (according to Raper and Thom 1949). The following methods were used to differentiate species of the genus *Aspergillus* section *Flavi*: production of an orange or brown pigment on the reverse side of colonies on *Aspergillus flavus/parasiticus* agar (AFPA) at 30 °C according to Pitt et al. (1983) and the growth on Czapek agar (CZA) at 42 °C according to Kurtzman et al. (1987). Moreover, a rapid method for the detection of aflatoxin-producing strains was used by means of a 25 % solution of ammonia (Saito and Machida 1999).

Data analysis: effects of the type of tea on fungal species richness were assessed using the Sørensen index. It is described as: $SI = 2j/(a + b)$, where j = number of species in common at site A and B, a = number of species at site A, b = number of species at site B.

Effects of tea variables (fermented, non-fermented, unpackaged, packaged, origin in the temperate or (sub)tropical zone) on species composition were tested by means of the canonical correspondence analysis (CCA), using Canoco version 4.5. The significance of the relationship between fungal species and type of tea was assessed using the implemented Monte Carlo permutation test (499 permutations). Significance of the analyses was accepted at $p \leq 0.05$.

RESULTS

All the 40 samples of tested teas were contaminated with microfungi. A total of 81, mainly saprotrophic microfungal species from 31 genera, were identified in the tea samples in the period 1999–2001 (Tab. 3). A majority of the isolated microfungi were ascomycete anamorphs. Most of them are fungi typical of food or feed, many of them are xerotolerant or osmotolerant. Although a great number of species was recorded, many isolates are sporadic or accidental occurrences and seem to have very little significance from the viewpoint of quality. *Aspergillus niger* agg. (82.5 % of samples), *Rhizopus stolonifer* (40 %), *Eurotium repens* (32.5 %), *Penicillium chrysogenum* (32.5 %), *Eurotium amstelodami* (27.5 %), *E. chevalieri* (27.5 %) and *Penicillium aurantiogriseum* (25 %) were identified as the most frequent species.

Sørensen similarity index (Tab. 2) revealed great differences in species composition between all types of tea studied. Many more species (62) were found in packaged tea samples compared to the unpackaged tea samples (49 species; Tab. 2). Higher counts of species (37) were also recorded from fermented than from non-fermented tea samples (25 species). In herbal tea samples of local origin 48 species were detected compared to teas of other origin (25 species). There were no great differences between herbal teas and tea from *Camellia sinensis* in numbers of fungal species.

When the data were processed using the Monte Carlo permutation test in CCA, no correlation between microfungal species and type of tea was found. The only significant graphic evaluation is given in Fig. 1.

A large number of toxigenic fungi were found. The most important are the potentially aflatoxigenic *Aspergillus flavus*, ochratoxigenic *A. ochraceus* and *A. niger*, and *A. versicolor*, which can produce sterigmatocystin (see e.g. Frisvad and Samson 1991, Samson et al. 2004). But using the rapid screening method for the detection of aflatoxins according to Saito and Machida (1999), the production of aflatoxins was not demonstrated in any of the isolated *Aspergillus* section *Flavi* strains.

Using the method for isolation of heat resistant fungi some thermophilic or thermotolerant (e.g. *Rhizomucor pusillus*, *Aspergillus fumigatus*) and thermoresistant fungi (*Eurotium* species) were recorded; the ascospores of the latter can survive higher temperatures (Pitt and Hocking 1997).

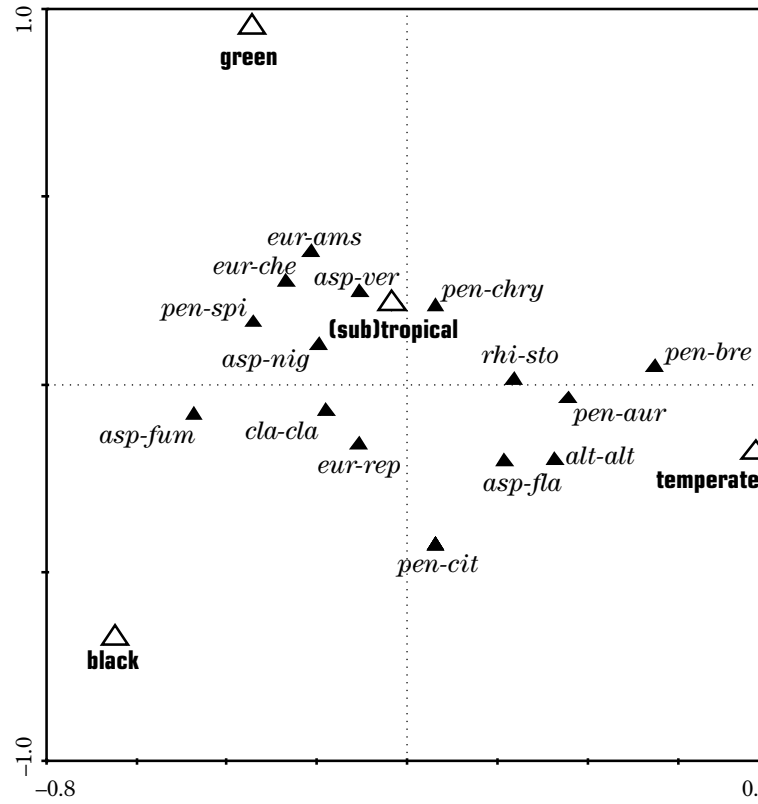

Fig. 1. Graphic evaluation (ordination biplot) resulting from canonical correspondence analysis CCA of fungal species data with kind of tea as an explanatory variable. All species data were included in the analysis, but only the most frequent ones (isolated at least from 5 samples) are shown here. The first axis explained 4.3 % (P-value = 0.002) and all axes together 15.9 % (P-value = 0.002) variability of the data package.

Abbreviations: fermented, black, teas (black), non-fermented, green, teas (green), teas from plants originated in temperate zone (temperate), teas from plants originated in subtropical or tropical areas (sub/tropical), *Alternaria alternata* (alt-alt), *Aspergillus flavus* (asp-fla), *A. fumigatus* (asp-fum), *A. niger* (asp-nig), *A. versicolor* (asp-ver), *Cladosporium cladosporioides* (cla-cla), *Eurotium amstelodami* (eur-ams), *E. chevalieri* (eur-che), *E. repens* (eur-rep), *Penicillium aurantiogriseum* (pen-aur), *P. brevicompactum* (pen-bre), *P. chrysogenum* (pen-chry), *P. citrinum* (pen-cit), *P. spinulosum* (pen-spi), *Rhizopus stolonifer* (rhi-sto).

Tab. 2. Species richness, mean values of colony forming units per gram and Sørensen similarity index (SI) calculated for microfungi obtained from different types of tea.

kind of tea	species numbers	SI	CFU/g
packaged	62	0.56	0.20×10^3
unpackaged	49		0.09×10^3
herbal tea	50	0.52	0.13×10^3
Camellia sinensis	57		0.15×10^3
fermented (black)	37	0.43	0.08×10^3
non-fermented (green)	25		0.22×10^3
local origin	48	0.46	0.09×10^3
other origin	25		0.06×10^3

Tab. 3. List of micromycetes isolated from two types of teas.
Abbreviations: black: fermented, black, teas, green: non-fermented, green, teas, temperate: herbs of temperate origin, (sub)tropical: herbs of subtropical or tropical origin, L: unpackaged, P: packaged

Fungal species	Numbers of contaminated samples								
	Camellia sinensis				other herbs				total
	black		green		temperate		sub/tropical		
	L	P	L	P	L	P	L	P	
Acremonium sp. 1		1							1
Acremonium sp. 2						1			1
Acremonium sp. 3					1				1
Acremonium strictum W. Gams						1			1
Alternaria alternata (Fr.) Keissl.						3		1	5
Aspergillus aculeatus Iizuka							1		1
Aspergillus cf. *caelatus* B. W. Horn		1							1
Aspergillus flavus Link	1	1			2	3			7
Aspergillus fumigatus Fresen.	1	3		2			1		7
Aspergillus niger agg. Tiegh.	5	5	5	5	2	3	3	4	32
Aspergillus ochraceus K. Wilh.			1			1			2
Aspergillus parasiticus Speare						1			1
Aspergillus penicillioides Speg.					1				1
Aspergillus sydowii (Bainier et Sartory) Thom et Church				1			1		2
Aspergillus tamarii Kita	2	1							3
Aspergillus versicolor (Vuill.) Tirab.	1		1	1		1	1	1	6
Basipetospora sp.		1							1
Byssochlamys nivea Westling					1	1	1	1	4
Chaetomium cf. *fusiforme* Chivers				1					1
Chaetomium globosum Kunze: Fr.		1							1
Chaetomium sp.		1							1

Tab. 3. Continuation.

Cladosporium cladosporioides (Fresen.) G. A. de Vries	1	1		1		1		1	5
Clonostachys rosea (Link : Fr.) Schroers, Samuels, Seifert et W. Gams				1					1
Emericella nidulans (Eidam) Vuill.					1	1		1	3
Epicoccum nigrum Link						1			1
Eupenicillium sp.		1							1
Eurotium amstelodami L. Mangin	1	2		4		1	1	2	11
Eurotium chevalieri L. Mangin	2	3		3		1		2	11
Eurotium cristatum (Raper et Fennell) Malloch et Cain						1			1
Eurotium repens de Bary	3	2		1	2	1	3	1	13
Eurotium rubrum Jos. König et al.		1				1			2
Eurotium sp.					1				1
Fusarium sp.				1					1
Gelasinospora cf. *retispora* Cain		1		1	1				3
Microascus brevicaulis S. P. Abbott					1	1		1	3
Microascus manginii (Loubiére) Curzi					1				1
Mucor dimorphosporus f. *sphaerosporus* (Hagem) Váňová				1	1	2			4
Mucor petrinsularis Naumov					1				1
Mucor plumbeus Bonord.					1				1
Mycocladus corymbifer (Cohn) Váňová	1				1			1	3
Paecilomyces variotii Bainier		1							1
Penicillium aurantiogriseum Dierckx		1			3	3		2	10
Penicillium bilaiae Chalab.								1	1
Penicillium brevicompactum Dierckx				1	2	2			5
Penicillium chrysogenum Thom		2	2	3	2	3		1	13
Penicillium citrinum Thom	2	1			2	1			6
Penicillium commune Thom			1	1		1			3
Penicillium cf. *coprophilum* (Berk. et M. A. Curtis) Seifert et Samson						1			1
Penicillium corylophilum Dierckx		1						1	3
Penicillium crustosum Thom				1	1		1	1	4
Penicillium expansum Link					1				1
Penicillium fellutanum Biourge		1							1
Penicillium glabrum (Wehmer) Westling					1			1	2
Penicillium miczynskii K. M. Zalessky		2							2
Penicillium miniioluteum Dierckx						1			1
Penicillium solitum Westling	1			2					3
Penicillium spinulosum Thom		2		1			1	1	5
Penicillium cf. *verrucosum* Dierckx		1			1				2

Tab. 3. Continuation.

Penicillium waksmanii K. M. Zalessky			2						2
Penicillium sp.1	1								1
Penicillium sp.2	1								1
Penicillium sp.3	1								1
Penicillium sp.4			1						1
Periconia byssoides Pers.						1			1
Phoma sp. 1						1			1
Phoma sp. 2		1							1
Rhizopus arrhizus A. Fisch.					1				1
Rhizomucor pusillus (Lindt) Schipper		2							2
Rhizopus microsporus var. *rhizopodiformis* (Cohn) Schipper et Stalpers	1				1				2
Rhizopus stolonifer var. *stolonifer* (Ehrenb.) Vuill.	2		1	1	4	4	1	3	16
Sordaria fimicola (Roberge) Ces. et de Not.				1	1	1			3
Stachybotrys sp.							1		1
Syncephalastrum racemosum Cohn ex J. Schröt.							1	1	2
Talaromyces flavus (Klöcker) Stolk et Samson						1			1
Talaromyces wortmanii (Klöcker) C. R. Benj.		2							2
Talaromyces sp.		1							1
Trichoderma longibrachiatum Rifai				2					2
Trichothecium roseum (Pers.: Fr.) Link					1				1
Ulocladium botrytis Preuss					2	1			3
mycelia sterilia 1					1				1
mycelia sterilia 2								1	1
Total no. of fungal species	**17**	**29**	**8**	**22**	**30**	**25**	**13**	**21**	**81**

The quantitative study according to ČSN ISO 7954 confirmed that all tested tea samples met the hygienic conditions relating to them by Act no. 294/1997 coll. The most contaminated sample of packaged non-fermented tea contained only 1.2×10^3 CFU/g, whereas according to the above cited notice teas can not contain more than 10^5 CFU/g. Mean values of colony forming units per gram (CFU/g) are in Tab. 2.

DISCUSSION

In the majority of the tested samples and in all tea samples (*Camellia sinensis*), *Aspergillus niger* agg. was found. Its occurrence was also reported by all the other authors who carried out mycological examination of teas (Dvořáková 1982, Jesenská 1987, Abdel-Hafez and El-Maghraby 1992, Březina et al. 1997,

Čutková 1997, Elshafie et al. 1999, Kubátová et al. 2000, Ostrý et al. 2000, Ostrý et al. 2002). This result could indicate that there is a relationship between the *Camellia sinensis* and *Aspergillus niger* agg. But when using the Monte Carlo Permutation test in CCA, we did not found out any correlation between particular microfungal species and a type of tea studied. In respect of the collateral high occurrence of this species in other herbal tea samples, it could be rather the substrate containing a low amount of water which *A. niger* agg. prefers.

Aspergillus niger and three other species of the section *Nigri* now belong to ochratoxigenic fungi (Abarca 1994, Samson et al. 2004). Therefore, the occurrence of *A. niger* in teas should not be underestimated. The very important aflatoxigenic fungus *A. flavus* was found in only 13 % of samples. We did not prove production of aflatoxin in any isolate. However, we used only a simple screening method. For achieving more exact results, other methods could be used.

There were quite some differences in species richness between all kinds of tea, which was reflected in a low value of the Sørensen similarity index. This result is interesting mainly in the case of fermented compared to non-fermented tea and in the case of local teas compared to teas of other origin. But we did not find any effect of fermentation or origin of plant on the species composition. Likely, most microfungi coloniaze the tea product later during processing. This hypothesis supports not only the fungal spectrum recorded (generally saprobic fungi preferring dried food) but also the great difference in species composition between our results and microfungal species isolated from soils under tea shrubs (*Camellia sinensis*) and directly from living tea plants (Agnihothrudu 1962, Farr et al. 1994). Unfortunately, data about the country of plant origin and packing hall could not be obtained. The amounts of colony forming units (CFU/g) and so the differences among various types of tea were too low to assess a significant effect of any type of tea.

The quantitative study according to ČSN ISO 7954 confirmed that the tested groups of green teas met all the hygienic conditions concerning them according to Act no. 294/1997 coll. But the quantitative study was also applied to the black teas even though they are considered as food without risk to human health and there are not any hygienic standards in the act. This result is very favourable, because the number of colony forming units (CFU/g) was much lower than 10^5 CFU/g which is the admissible maximum of CFU/g according to the above mentioned act.

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