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Methods for determination of enzymatic activities of marine fungi

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The physiology, particularly enzymatic activities of marine fungi are little investigated and their role in the marine habitat is understood unsufficiently. This is due to difficulties with cultivation and determination of the enzymes to the fact that mostly not standardized methods are used. Current methods for determination of enzymatic activities in marine fungi are reviewed and the problems in cultivating marine fungi and determining their enzymatic activities are discussed. Twentytwo commonly used qualitative, semiquantitative and quantitative methods for the determination of 26 enzymatic activities of different metabolic pathways after cultivation in solid and liquid media are presented and the references given.

Key words: marine fungi, enzymes, methods, metabolism, artificial seawater medium

Molitoris H. Peter (2000): Metody stanovení enzymatické aktivity mořských hub (Fungi) – Czech Mycol. 52: 97–124

Fyziologie a zejména enzymatické aktivity mořských hub (Fungi) jsou málo prozkoumané a jejich role v mořském prostředí je nedostatečně pochopena. To je důsledkem obtíží při kultivaci a stanovení enzymů a zejména pak při používání většinou nestandardních metod. Probírají se současné metody na stanovení enzymatické aktivity mořských hub a diskutují se problémy spojené s kultivací mořských hub a se stanovením jejich enzymatické aktivity. Je uvedeno 22 v současnosti používaných kvalitativních, semikvantitativních a kvantitativních metod na stanovení 26 enzymatických aktivit různých metabolických drah po kultivaci na pevných a tekutých půdách a jsou připojeny odkazy na literaturu.

Contents

"If the seas are to be viewed as dynamic "ecosystems", ... there is, however, a basic prerequisite: understanding the physiology of the organisms in that biota."

"Until such time, however, as the enzyme systems of marine fungi are carefully determined and critically analysed, such phenomena as host-parasite relationships, substrate specificity, vitamin syntheses, phosphate reduction and cellulose and lignin decomposition will remain largely unknown." (Johnson and Sparrow 1961)

1. INTRODUCTION

The oceans and their adjoining areas such as estuaries, mangrove swamps and brackish water regions are among those environments least known on our planet. This is true in particular for their microbiota, especially the marine fungi.

In order to understand an ecosystem, it is necessary to understand the organisms living in it and the role they play in it. A good approach in this direction is to study the substrate requirements of the organisms and the degradation processes involved. This in turn requires knowledge of the presence and identity of the participating enzyme systems.

Furthermore, the oceans are among the last unexploited resources on our planet, but on the other hand, are becoming increasingly the waste dumps of our "civilisation". For industrial exploitation, but also for the protection of the environment, the study of the physiological (enzymatic) potential of the organisms in this environment is essential.

Finally, since marine fungi in culture often grow slowly or do not show the characteristic morphological features necessary for identification, enzymatic and molecular characteristics are becoming increasingly important for taxonomic and systematic purposes and recently also for patenting strains in biotechnology.

Marine fungi, until now, have been mainly investigated with regard to their presence, isolation, cultivation, morphology, taxonomy and systematics. Interest in their physiological properties and capabilities has arisen only recently and is documented in only a few comprehensive publications such as Jones 1976, Kohlmeyer and Kohlmeyer 1979 and Moss 1986.

For the purpose of this chapter and because of the space available, papers dealing with enzyme studies in marine fungi have been considered only from the last decade. For this review marine fungi following the definition of Kohlmeyer and Kohlmeyer 1979 were taken from a list (Molitoris, unpublished) based on publications such as Hughes 1975, Kohlmeyer and Kohlmeyer 1979, Kohlmeyer and Volkmann-Kohlmeyer 1991 and Jones 1991 for higher marine fungi, on publications such as Margulis et al. 1989 for lower marine fungi, and on Kohlmeyer and Kohlmeyer 1979 and Moore 1980 for marine yeasts. However, to be more precise, in many cases one should refer rather to "marine isolates" because no data exist on whether they are "obligate" or "facultative" marine organisms in the sense of Kohlmeyer and Kohlmeyer 1979. This in particular applies to marine yeasts which in general are ecologically not well defined.

2. Special problems in testing enzyme activities of marine fungi

2.1. Cultivation

For most of the qualitative and semiquantitative enzyme assays the marine fungi are grown (and investigated for enzyme activity) on solid (agar) medium. This certainly in most cases does not correspond with the situation in nature. But even if the fungi are cultivated in liquid medium, usually a batch culture is employed, in other words, the liquid substrate in a confined vessel is inoculated and harvested after a certain incubation time. This again does not correspond with the natural environment which can be considered as an open system, similar to a continuous culture. In nature, moreover, we do not have very marked fluctuation of conditions, caused by of depletion of nutrients, accumulation of staling products and confined space. For the reasons mentioned above, the enzyme activities recorded in such experiments should always be considered only as potential activities (under the experimental conditions used).

2.2. Physical conditions

The distribution of marine fungi in nature is defined by Hughes 1974, according to the following temperature-determined biogeographical zones, arctic, temperate, subtropical, tropical and antarctic with correspondingly different (upper and surface) water temperatures. In contrast to that, the temperature of the ocean floor at greater depths is between 1.5 and 4 °C.

The hydrostatic pressure increases with depth to a maximum of 1200 bar at the greatest oceanic depth (Mariana trench).

Solar radiation with visible and UV light penetrates only the upper layers with green light reaching here a maximum depth of about 200 m. Whereas light (and

temperature) change during the day and between the seasons at the surface, in particular in estuarine and coastal areas, whereas these factors are fairly constant at greater depths.

The pH of ocean waters lies between 7.5 and 8.4, in surface waters between 8.1 and 8.3, but may reach lower values in brackish water zones (Johnson and Sparrow 1961).

The values for dissolved gases such as oxygen and carbon dioxide vary widely depending on temperature, movement of the water bodies and depth. These values are difficult to simulate in culture.

Generally, conditions in the sea are not met when experimenting with marine fungi in the laboratory because of technical problems or the fact that marine fungi in culture grow too slowly under such conditions. There are few exceptions to this (see Lorenz and Molitoris 1991) where deep sea conditions (hydrostatic pressure, temperature and oxygen pressure) were simulated. It therefore has to be kept in mind that most experimental conditions do not coincide with the conditions found in the natural environment.

2.3. Chemical conditions

The salinity, expressed as g salt/kg seawater, has in most oceans a value between 33 and 37 ‰ (Johnson and Sparrow 1961), with an average around 35 ‰, whereas in estuaries and brackish water regions it gradually reaches freshwater values.

To simulate natural conditions in this respect, either natural seawater or artificial seawater has been used for investigations of marine fungi, mostly, however, only for growth, but not for the enzyme assays.

Some artificial seawater salt mixtures are commercially available, such as RILA MARINE MIX (Rila Products, Teaneck, N. J., USA) and INSTANT OCEAN (Carolina Biological Supply Co., Burlington, N. C., USA).

Out of about 100 recent papers on enzyme studies in marine fungi, natural seawater was used in only 12 publications (4, 5, 29, 54, 63, 67, 77, 78, 79, 81, 98, 99), RILA MARINE MIX in 12 papers (31, 59, 63, 74, 77, 78, 79, 81, 82, 93, 94, 95) and other salt mixtures in 8 further papers. A comparison of the effect of freshwater on growth and enzyme production against seawater media (natural or artificial) was conducted only in a few cases (4, 5, 63, 77, 78, 79, 81).

The effect of and the problems involved in using different types of seawater in such experiments is discussed in extenso in Provasoli et al. 1957 and also in Molitoris and Schaumann 1986 and Rohrmann et al. 1992. In particular, care has to be taken that no precipitation of salts occurs when enzyme tests are used where clarification or precipitation indicates enzyme activity. It has also been found that water quality (seawater or freshwater) influences growth, morphology and enzyme

production in marine fungi (Johnson and Sparrow 1961; Kohlmeyer and Kohlmeyer 1979; Molitoris and Schaumann 1986; Rohrmann et al. 1992).

One particular problem is the substrates used for growth, enzyme biosynthesis and enzyme tests. Often the quality of the substrates used does not correspond with those occurring in nature, in particular when complex substrates such as cellulose, hemicellulose and lignin are used. Also the concentration of substrates used in experimentation normally exceeds by far that found in the oligotrophic environment of the ocean waters. The results obtained from experiments conducted under such unnatural conditions therefore have to be viewed with caution.

3. Available techniques

3.1. Types of substrates used

Corresponding with the present primary interest in elucidating the general role of marine fungi in the marine environment, a number of complex natural and other substrates have been used in experiments where growth and degradation of the substrates is taken as an indication of the presence of the respective enzymes (enzyme systems). This is shown in Table 1.

Table 1: Degradation of complex natural and other substrates as proof of the presence of the requisite degradative enzymes in marine fungi

Substrate	References
Wood, bark, cell-walls, leaves	17, 50, 65, 86, 96, 100, 105
Lignin (and related compounds)	27, 89, 96, 105, 106, 109
Cellulose	50, 86, 96, 105
Pectin	89, 109
Polysaccharides	102, 109
Chitin	37, 69, 99
Keratin	72, 99
Proteins	32, 89, 109
Plastic (polyhydroxyalkanoates)	58
Alcohols	4, 5, 8
Hydrocarbons	36, 98
Nucleic acids	21, 22,
Collection of individual C-sources	4, 5, 8
Collection of individual N-sources	4, 5, 8

3.2. Indirect determination of enzymes

In many cases synthesis and function of enzymes in marine fungi has been shown only indirectly. This has been accomplished e.g. by showing fungal growth on the appropriate substrates, by following the decrease in substrate concentration, by proof for typical degradation products or by other methods such as observation using light microscopy. The primary methods used are shown in Table 2.

Table 2: Indirect determination of degradative enzymes in marine fungi by methods indicating the use/degradation of certain substrates.

Parameters and methods used	References
Growth	
biomass (weight) increase	4, 5, 36, 72, 98,
microscopy (of hyphal growth)	36, 65, 99
colony diameter	89, 98
spectrophotometry	98, 109
superficial growth on cross-linked gels	109
turbidity	8, 58
oxygen consumption	98
Decrease in substrate concentration	
weight loss (direct, differential extraction)	37, 50, 86, 96, 100, 105, 65, 102
spectrophotometry (lignin)	98, 105
enzymatic determination	37
Typical degradation products	
reducing sugars (polysaccharides)	17, 18, 93, 94
N-acetyl glucosamine (chitin)	37, 69
acid production (carbohydrates)	4, 5
gas production	8
Liquefaction of cross-linked gels	48, 89, 102, 109
Clearing of turbid agar columns	50, 67
Viable counts	98
Test/identification kits	
colour changes by use of substrates	10

3.3. Specific enzymes

In the last decade about 100 papers have appeared dealing with enzyme activities of marine fungi. More than 60 different enzymes identifiable by their catalytic action and Enzyme Commission (EC) number were involved. The following Table 3 lists these enzymes, giving their EC number, the type of assay used and the references.

Table 3: Enzymes tested in marine fungi and type of assays used.

EC no1	Recommended name[1] ("commonly used name")	Assays used[2] and References
1.	OXIDOREDUCTASES	
1.1.	Acting on CH-OH groups of donors	
1.1.1.1	Alcohol dehydrogenase	s 107
1.1.1.37	Malate dehydrogenase ("Malat-DH")	e 59; s:98
1.1.1.42	Isocitrate dehydrogenase (NADP+) ("NADP+ isocitrate DH")	s 73
1.1.1.43	6-phosphogluconate-dehydrogenase ("6-P-gluconate-DH")	e 59
1.1.1.49	Glucose-6-phosphate-dehydrogenase ("Glucose-6-P-DH")	e 59; s 98
1.3.	Acting on CH-CH groups of donors	
1.3.99.1	Succinate dehydrogenase ("Succinate-DH")	s 29, 98
1.6.	Acting on NADH or NADPH	
1.6.6.2	Nitrate reductase (NAD(P)H)	s 1, 61, 107, 107
1.6.6.4	Nitrite reductase (NAD(P)H)	c 74; s 26
1.10.	Acting on diphenols and related substances as donors	
1.10.3.1	Catechol oxidase ("Polyphenoloxidase")	c 31, 50, 106
1.10.3.2	Laccase	c 8, 31, 50, 63, 67, 77, 78, 79, 81, 82, 89, 106
1.11.	Acting on H2O2 as acceptor	
1.11.1.6	Catalase	c? 89; s 17, 107
1.11.1.7	Peroxidase	c 8, 50, 63, 67, 77, 78, 79, 81, 82, 89, 106
1.14	Acting on paired donors with incorporation of molecular oxygen	
1.14.18.1	Monophenol monooxygenase ("Tyrosinase")	c 8, 50, 63, 67, 77, 78, 79, 81, 82, 89, 106
1.15	Acting on superoxide radicals as acceptors	
1.15.1.1	Superoxide dismutase	e 59
2.	TRANSFERASES	
2.4.	Glycosyltransferases	
2.4.1.16	Chitin synthase ("Chitin synthetase")	r 6, 15, 18, 73
2.4.1.34	1,3-β-Glucan synthase	r 33, 73
2.6.	Transferring nitrogenous groups	
2.6.1.16	Glutamine-fructose-6-phosphate-aminotransferase (isomerizing) ("L-Glutamine:D-fructose-6-P-aminotransferase")	s 15
2.7.	Transferring phosphorus-containing groups	
2.7.1.1	Hexokinase	e 59
2.7.1.11	6-Phosphofructokinase	s 73

EC no1	Recommended name[1] ("commonly used name")	Assays used[2] and References
2.7.1.40	Pyruvate kinase	s 73
2.7.1.59	N-acetylglucosamine kinase	r 73
3.	HYDROLASES	
3.1.	Acting on ester bonds	
3.1.1.1	Carboxylesterase	s 89
3.1.1.2	Arylesterase	s 89
3.1.1.3	Triacylglycerol lipase ("Lipase")	h 31, 32, 106; p 8, 21, 22, 31, 63, 77, 81, 82
3.1.1.11	Pectinesterase	c 21, 25, 31, 81; f 25, p 21, 25, 31, 81; t 25, 53, 80; v 53
3.1.3.1	Alkaline phosphatase	e 59; s 19, 73
3.1.3.2	Acid phosphatase	c 21, 22, 32; s 73
3.1.21.2	Deoxyribonuclease ("DNase")	h 21, 22, 32; p 21, 32
3.1.27	Ribonuclease ("RNase")	h 21, 22, 32; p 21, 22, 32
3.2.	Glycosidases	
3.2.1.1	α-Amylase ("Amylase")	c 4, 5, 8, 21, 22, 24, 31, 33, 63, 71, 77, 79, 81, 82, 89, 106
3.2.1.3	Glucan-1,4-glucosidase	c 24, 48, 89; m:109; s:24
3.2.1.4	Cellulase ("Endoglucanase")	c 8; s 18, 31, 54, 55, 86, 87, 93, 94, 105
3.2.1.6	Endo-1,3(4)-β-glucanase ("Laminarinase")	c 8, 82; h 8, 63, 77, 79, 81, 82; l 48, 89, 102, 109;
3.2.1.14	Chitinase	c 106; f 12, 49; h 4, 5, 8, 21, 22, 32, 63, 77, 79, 81, 82, 99, 106; r 6, 18, 19, 49, 85
3.2.1.15	Polygalacturonase ("Pectinase", "Pectin depolymerase")	h 8, 20, 31, 63, 77, 81, 10 l 20, 21, 22; p 8, 20, 31, 63, 77, 81, 106; s 20, 56, 80, 86, 87, 93, 94; v 20, 23, 80, 93 paperchromatography 93
3.2.1.20	α-Glucosidase	m 89, 109; s 19, 89
3.2.1.21	β-Glucosidase	c 8, 78, 82; m 09; s 31, 54, 55, 68, 73, 87, 89, 93, 94, 105
3.2.1.22	α-Galactosidase ("α-D-Galactosidase")	s 87, 93, 94
3.2.1.23	β-Galactosidase ("β-D-Galactosidase")	s 87, 93.94
3.2.1.24	α-Mannosidase	f 49, 49
3.2.1.25	β-Mannosidase	m 109
3.2.1.37	Xylan 1,4-β-xylosidase ("β-D-Xylosidase")	l 109; m 109; s 87, 89, 93, 94
3.2.1.55	α-L-Arabilnofuranosidase ("α-L-Arabinosiadase")	s 86, 87, 93
3.2.1.73	Lichenase	l 44
3.2.1.74	Glucan 1,4-β-glucosidase	m 48, 89, 109

EC no1	Recommended name[1] ("commonly used name")	Assays used[2] and References
3.2.1.78	Mannan endo-1,4-β-mannosidase ("Mannanase")	l 109
3.2.1.81	Agarase	l 81
3.2.1.83	k-Carrageenase ("Carrageenase")	c 109; l 81, 109; m 109
3.2.1.89	Arabinogalactan endo-1.4-β-galactosidase ("Galactanase") and	s 93
3.2.1.90	Arabinogalactan endo-1,3-β-galactosidase ("Galactanase")	s 93
3.2.1.91	Cellulose 1,4-β-cellobiosidasec ("Exoglucanase")	s 34, 54, 55, 87, 105; f 49
3.4.	Acting on peptide bonds (peptide hydrolases)	
3.4.21.48	Yeast proteinase B	s 19
3.5.	Acting on carbon-nitrogen bonds, other than peptide bonds	
3.5.1.5	Urease	c 21, 22, 82, 83, 89
3.6.	Acting on acid anhydrides	
3.6.1.3	Adenosinetriphosphatase ("ATPase")	s 29, 73
4.	LYASES	
4.2.	Carbon-oxygen lyases	
4.2.1.2	Fumarate hydratase ("Fumarase")	e 59; s 29
4.2.2.2	Pectate lyase ("Pectate transeliminase")	h 8, 22, 63, 77, 81, 82, 106 p 8, 22, 63, 77, 81, 82, 106; s 23, 56, 86, 93, 94; v 3, paperchromatography 93
4.2.2.3	Alginate lyase ("Alginase")	h 77, 81; m 102; p 77, 81; s 101, 102
4.2.2.10	Pectin lyase	s 23, 87
5.	ISOMERASES	
5.3.	Intramolecular oxidoreductases	
5.3.1.9	Glucose-6-phosphate isomerase	e 9
5.3.1.10	Glucosamine-6-phosphate isomerase ("Glucosamine-6-P-deaminase")	s 73
5.4.	Intramolecular transferases	
5.4.2.2	Phosphoglucomutase	e 59

[1] Enzyme Commission number (Webb 1984), where identifiable.

[2] ASSAY TYPE: c = colorimetric (appearance, disappearance, diffusion of clour, qualitative or semiquantitative); e = electrophoretic; f = fluorescence; h = halo, clearing; l = liquefaction; m = biomass, growth; p = precipitation; r = radiometric; s = spectrophotometric (mostly quantitative); t = titrimetric; v = viscosimetric; ? = no details given; double numbers cited for an assay type indicate two different methods.

3.4. Less defined enzymes or enzyme mixtures

In addition to those enzymes listed in Table 1, for marine fungi a number of further enzymes have been investigated which are less defined or constitute complex enzymes or enzyme mixtures. They are shown in Table 4.

Table 4: Less defined enzymes, complex enzymes or enzymemixturestestedin marine fungi and type of assays used.

Enzyme (common name)	Assays used[1] and Reference
Arabanase	s 93, 94
Caseinase	h 4, 5, 8, 21, 22, 63, 77, 79, 81, 82; s 7
Cellulase	c 48, 78, 82; ch 31; h 3, 4, 5, 21, 22, 32, 50, 63, 67, 71, 77, 78, 79, 81, 106; l 89; w 50
Dehydrogenases	s 98
Gelatinase	h 4, 5, 8, 32, 63, 77, 79, 81, 82, 106; l 21, 22; p 4, 5, 8, 32, 63, 77, 79, 81, 82
Glucanase	s 18
Glucomannanase	h 50; m 50
Mannanase (yeast-mannose)	l 48, 89, 102; m 102
Nitrate reductases	c 4, 5, 8, 26, 63, 74, 77, 79, 81, 82
Pectinase	l 20, 89, 109; m 109; p 20
Peptidase, Protease, Proteinase	c 89, 109; l 59; f 7; h 32; l 109; p 32; s 87
Polyhydroxyalkanoate-depolymerase	h 58
Phosphatase	c 89
Protopectinase	s? 80
Succinate oxidase	polarographically 98
Xanthanase	m 109
Xylanase	c 8, 106; h 8, 32, 50, 67, 79, 82; l 48, 89, 102, 109; p 8, 79, 82; s 31, 86, 87, 93, 94, 86, 95

[1] ASSAY TYPE: c = colorimetric (appearance, disappearance, diffusion of colour, qualitative orsemiquantitative), ch = cellophane hydrolysis, f = fluorescence, h = halo, clearing, l = liquefaction, m = biomass, growth, p = precipitation, s = spectrophotometric (mostly quantitative), w = weightloss of substrate; double numbers cited for an assay type indicate two different methods.

From Tables 3 and 4 it follows that almost the whole range of assay types from qualitative to quantitative tests, from clearing an agar medium to radio-labelling studies is already used in the work with marine fungi.

Only 12 enzymes have been investigated in more than 10 papers as is evident from Tables 1 through 4. These enzymes represent mostly hydrolases, mainly exoenzymes, acting more or less on complex, natural substrates. The preference for these enzymes indicates thatat present the main interest is more concerned with the general role of marine fungi in the oceans than with specific catalytic activities which could be exploited for biotechnological or systematic purposes.

This is substantiated by the fact, that presently about 3 times more qualitative/semiquantitative than quantitative methods are used for work with marine fungi.

Nevertheless, with increasing interest in this new and promising group of almost unexplored microorganisms, it is to be expected that the interest in their metabolism and their potential in the synthesis of important enzymes and metabolites will increase. Biotechnology and medicine are expected to be major areas of interest but the field of environmental studies, pathology, chemotaxonomy and chemosystematics might also be involved as it is already the case with terrestrial fungi.

This means that in future more specific enzyme activities, in particular those of endoenzymes will have to be studied and that increasingly quantitative assays will be employed. Therefore the following chapter lists in addition to the qualitative and semiquantitative enzyme assays which are used now, a number of methods for quantitative determinations including intracellular enzymes involved in major metabolic pathways.

4. Widely used and recommended techniques

Owing to the multitude of enzymes investigated in fungi and the multitude of different assays available, for the purpose of this chapter only assay methods used in recent literature have been selected. Methods that have been found suitable and have been used widely in the study of marine fungi and which cover the major types of enzyme assays generally employed, are outlined in the following section.

The assays given below are separated into qualitative/semiquantitative and quantitative methods. The former group, in addition, is divided into direct and indirect tests. For direct tests all necessary ingredients, including substrate and test reagent, are included in the solid medium and after inoculation and appropriate growth, the test may be regularly inspected. For indirect tests, the medium is inoculated and incubated first, and the reaction appears only when, after appropriate cultivation time, the substrate/test reagent has been added.

Medium: The GPY (agar) medium (glucose 1g/l, peptone 0.5 g/l, yeast extract 0.1 g/l, and in the case of solid medium agar 16 g/l, made up with deionized water, natural seawater or artificial seawater, pH 6.0 (before autoclaving) proved suitable for experiments with marine fungi in most cases (Molitoris and Schaumann 1986). For inducible enzymes, however, the appropriate substrate/inducer has to be added to the medium, whereas in other cases, e.g. for detection of cellulase activity, glucose or other substances as possible inhibitors (catabolite inhibition, repression) have to be omitted from the medium.

Temperature: An incubation temperature between 20 °C (minimum) and 28 °C (maximum) proved to be satisfactory in most cases.

In the following short descriptions of the assay methods, the following abbreviations have been used: Type of assay (T), principle of assay (P), medium (M), reagent (R), procedure (Pr), remarks (Rm), representative references (Rf).

4.1. Qualitative/semiquantitative

Method 4.1.1.

Semiquantitative determination of nitrate reductase (EC 1.6.6.) on solid medium in an indirect test with the Griess-Ilosvay reagent (Bresinsky and Schneider 1975).

T: Colorimetric, indirect.

P: The nitrate in the medium is reduced by nitrate reductase to NO^2- which gives a pinkish red colour upon addition of solution A and B of the Griess-Ilosvay reagent.

M: Agar medium containing 15 g/l sodium nitrate (control without nitrate) in slants.

R: Solution A: 0.05 g sulfanilic acid, dissolved in 15 ml 5N acetic acid. Solution B: 0.1 g naphthylamine (toxic!) dissolved in 20 ml boiling distilled water, decanted and with the supernatant made up to 150 ml with 5N acetic acid.

Pr: Addition of a few drops (about 0.1 ml each) of solution A and B to the surface of the culture. Mixed in a Whirlmix, read after 10 min and 60 min, respectively. The difference in pigmentation between the test and the control is taken as activity (in arbitrary units).

Rf: Bahnweg and Bland 1980; Molitoris and Schaumann 1986; Rau and Molitoris 1991.

Method 4.1.2.

Semiquantitative determination of laccase (EC 1.10.3.2) on solid medium in a direct test with a) α-naphthol, b) guaiacol or c) in an indirect test with benzidine or syringaldazine (Harkin and Obst 1973).

a) α-naphthol (Higuchi 1953; Higuchi and Kitamura 1953)

T: Colorimetric, direct.

P: The colourless medium turns blue due to oxidation of 1-naphthol by laccase.

M: Agar medium with 0.05g α-naphthol/l, Petri plates.

R: None, direct test.

Pr: Reading of colour intensity (arbitrary units) and of diameter of reaction zone.

Rm: Decoloration may occur. Growth may be inhibited by α-naphthol. Lignin or its derivatives may induce laccase.

Rf: Molitoris and Schaumann 1986; Mouzouras 1986; Rohrmann and Molitoris 1992.

b) Guaiacol (Boidin 1951)
T: Colorimetric, direct.
P: The colourless medium turns reddish-brown due to oxidation of guajacol by laccase.
M: Agar medium with 0.05 g guaiacol/l, Petri plates.
R: None, direct test.
Pr: Reading of colour intensity (arbitrary units) and of diameter of reaction zone.
Rm: Decoloration may occur. Growth may be inhibited by guajacol. Lignin or its derivatives may induce laccase.
Rf: Molitoris and Schaumann 1986; Rohrmann and Molitoris 1992

c) Benzidine (Lyr 1958)
T: Colorimetric, indirect.
P: The colourless medium turns blue due to oxidation of benzidine by laccase.
M: Agar medium, Petri plates.
R: Benzidine 0.1 g/100 ml acetate buffer pH 4.4. Benzidine is carcinogenic!
Pr: Addition of a few drops of benzidine solution to the underside of the colony. Reading of colour
intensity (arbitrary units) after 10 min and 60 min, respectively.
Rm: Alternatively, a solution of syringaldazine (0.1 % in 96 % ethanol; Harkin and Obst 1973) may be used resulting in a pink colour.
Rf: Gessner 1980; Molitoris and Schaumann 1986; Zare-Maivan and Shearer 1988; Rohrmann and Molitoris 1992.

Method 4.1.3.
Semiquantitative determination of peroxidase (EC 1.11.3.2.) on solid medium in an indirect test with benzidine and H_2O_2 (Lyr 1958)

T: Colorimetric, indirect.
P: The colourless medium turns blue due to the oxidation of benzidine and H_2O_2.
M: Agar medium, Petri plates.
R: H_2O_2 3 % aqueous and benzidine 0.1g/100ml in acetate buffer pH 4.4. Benzidine is carcinogenic!
Pr: Addition of a few drops of the H_2O_2 and the benzidine solution to the underside of the colony. Readings of colour intensity (arbitrary units) after 10 and 60 min, respectively.
Rm: If laccase activity is also present, peroxidase activity is indicated only if the blue colour appears more quickly or more intense. Syringaldazine may be used instead of benzidine (see also under laccase).
Rf: Gessner 1980; Molitoris and Schaumann 1986; Rohrmann and Molitoris 1992.

Method 4.1.4.

Semiquantitative determination of tyrosinase (EC 1.14.1.8.) on solid medium in an indirect test with p-cresol-glycine (Lyr 1958).

T: Colorimetric, indirect.

P: The colourless medium turns reddish-brown due to the oxidation of the p-cresol solution.

M: Agar medium, Petri plates.

R: p-cresol 1.08 g/l and 0.05 % glycine in distilled water.

Pr: Addition of a few drops of the reagent to the surface of the colony. Reading of colour intensity (arbitrary units) after at least 12 hrs.

Rm: Tyrosinase activity appears usually relatively late (> 2 weeks of incubation).

Rf: Leightley 1980; Molitoris and Schaumann 1986; Mouzouras 1986; Rohrmann and Molitoris 1992

Method 4.1.5.

Semiquantitative determination of lipase (EC 3.1.1.3.) on solid medium in a direct test with Tween 80 and Ca^{2+} ions (Trigiano and Fergus 1979, modified: without saponin, in Petri plates and in test tubes).

T: Precipitation, direct.

P: Production of a white precipitate of Ca^{2+} salts with the fatty acids split off from Tween 80 by lipase activity.

M: Agar medium with 1 % Tween 80 (sorbitan polyoxyethylene mono-oleic) and 0.1 g $CaCl_2 \times 2\ H_2O$/l. Petri plates, test tubes.

R: None, direct test.

Pr: Readings of precipitation intensity (arbitrary units) and diameter (Petri plates) or length (test tubes) of precipitation zone.

Rm: Lipase activity sometimes appears relatively late. If growth is too quick, saponin for growth retardation may be added here. Since the precipitate is difficult to observe below the colony in Petri plates, a parallel experiment in test tubes is recommended.

Rf: Gessner 1980; Federici 1983; Molitoris and Schaumann 1986.

Method 4.1.6.

Semiquantitative determination of amylase (EC 3.2.1.1.) on solid medium in an indirect test with soluble starch and Lugol solution (Trigiano and Fergus 1979).

T: Colorimetric, indirect.

P: Residual starch is coloured blue by addition of Lugol solution, resulting in a colourless halo in the blue amylase activity zone around the colony.

M: Agar medium with 0.2 % soluble starch. Petri plates.

R: Lugol (iodine) solution (KI 15.0 g/l and I_2 3.0 g/l) diluted 1:4 for use.

Pr: Flooding the plate with reagent. Readings (arbitrary units) for residual intensity of blue colour and determination of diameter of activity zone.

Rm: In the case of yeast colonies which prevent access of the reagent to the surface of the plate, part of the colony was removed immediately before adding the reagent.

Rf: Gessner 1980; Pugh and Beeftink 1980; Federici 1983; Molitoris and Schaumann 1986.

Method 4.1.7.

Semiquantitative determination of laminarinase (EC 3.2.1.6.) on solid medium in a direct test with insoluble laminarin (Chesters and Bull 1963).

T: Clearing of agar column, direct.

P: The medium, made turbid by insoluble laminarin, is cleared by action of laminarinase.

M: Agar medium with 0.5 % insoluble laminarin (prepared after Thiem et al. 1977) in test tubes.

R: None, direct test.

Pr: Reading of clearing intensity (arbitrary units) and length of activity zone.

Rm: Omit glucose (inhibition).

Rf: Molitoris and Schaumann 1986; Schaumann et al. 1986; Rohrmann et al. 1992.

Method 4.1.8.

Semiquantitative determination of chitinase (EC 3.2.1.14) on solid medium in a direct test with colloidal chitin (Hankin and Anagnostakis 1975).

T: Clearing of agar column, direct.

P: The medium, made turbid by colloidal chitin is cleared by action of chitinase.

M: Agar medium containing 2 % colloidal chitin (prepared after Campbell and Williams 1951) in test tubes or in double layer technique in Petri plates (Hankin and Anagnostakis 1975).

R: None, direct test.

Pr: Reading of agar clearing (arbitrary units) and length/diameter of activity zone.

Rm: Omit glucose from medium (inhibition). Using chitin-induced inoculum increases test sensitivity.

Rf: Bahnweg and Bland 1980; Ulken 1980; Federici 1983; Molitoris and Schaumann 1986; Rohrmann et al. 1992.

Method 4.1.9.

Semiquantitative determination of polygalacturonase (EC 3.2.1.15.) in a buffered (pH 5.0), solid medium with pectin in an indirect test (Hankin and Anagnostakis 1975).

T: Clear zone, indirect.

P: Residual substrate is precipitated leaving a clear zone indicating polygalacturonase activity.

M: Buffered (pH 5.0) agar medium containing 0.5 % pectin (apple or citrus) as sole carbon source. Petri plates.

R: 1 % aqueous solution of hexadecyltrimethylammonium bromide.

Pr: Flooding the plate with the reagent. Reading of clarity (arbitrary units) and of diameter of activity zone after 1 and 12 h, respectively.

Rm: Glucose inhibits. Seawater may interfere.

Rf: Molitoris and Schaumann 1986; Schaumann et al. 1986; Zare-Maivan and Shearer 1988.

Method 4.1.10

Semiquantitative determination of cellulolytic activity (EC 3.2.1.) in solid medium with cellulose in direct and indirect tests.

a) Direct test (Rautela and Cowling 1966; Tansey 1971).

T: Clear zone.

P: The agar medium, made turbid by cellulose, is cleared by the action of cellulase(s).

M: Agar medium with 0.5 % acid-swollen Avicel (Tansey 1971) or 0.5 % acid-swollen Whatman powdered cellulose (= Walseth cellulose) (Rautela and Cowling 1966). Test tubes or Petri plates.

R: None, direct test.

Pr: Reading of clearing intensity (arbitrary units) and length/diameter of activity zone.

Rm: Substrate-inducible enzyme; glucose inhibits by repression and feed-back.

Rf: Leightley 1980; Bahnweg and Bland 1980; Molitoris and Schaumann 1986; Mouzouras 1986; Zare-Maivan and Shearer1988; Rohrmann et al. 1992.

b) Direct test (Smith 1977; Collett 1984).

T: Colorimetric test, direct

P: Diffusion of pigmented cellulose particles, liberated by action of cellulases, into the lower, colorless agar layer.

M: Double-layered agar medium, the upper layer containing 1.25 % cellulose azure (cellulose uniformly dyed with Remazol brilliant blue R = RBBR; Smith 1977) or RBBR-Avicel (Collett 1984), the lower layer without the substrate.

R: None, direct test.

Pr: Reading (arbitrary units) of appearance of blue colour in lower agar layer.

Rm: Decoloration of the blue pigment may occur by oxidative, ligninolytic enzymes. Substrate-inducible enzyme; glucose inhibits by repression and feed-back.

Rf: MacDonald and Speedie 1982; Rohrmann and Molitoris 1992.

c) Indirect test. (Teather and Wood 1982)
T: Colorless zone, indirect.
P: Residual cellulose is dyed with Congo red/NaCl, leaving a clear, colorless zone indicating activity of cellulase.
M: Agar medium containing 0.5 % Carboxymethylcellulose (CMC). Petri plates.
R: 0.2 % aqueous Congo red, 1 M aqueous NaCl solution.
Pr: Flooding with Congo red, decant after 15 min, destain by flooding with NaCl, decant after 15 min, read colour intensity (arbitrary units) and diameter of active zone.
Rm: Substrate-inducible enzyme; glucose inhibits by repression and feed-back.
Rf: Schimpfhauser and Molitoris 1991; Rohrmann and Molitoris 1992.

Method 4.1.11.
Semiquantitative determination of xylanolytic activity (EC 3.2.1.) in a solid medium with xylan in a direct and an indirect test.

a) Direct test.
T: Clear zone, direct.
P: The agar medium, made opaque by xylan, is cleared by the action of xylanase(s).
M: Agar medium, containing 0.125 % or 0.25 % larch xylan (instead of cellulose, Rautela and Cowling 1966). Test tubes.
R: None, direct test.
Pr: Reading of clearing intensity (arbitrary units) and length of activity zone.
Rm: Substrate-inducible enzyme.
Rf: Leightley 1980; Mouzouras 1986.

b) Indirect test (Flannigan and Gilmour 1980).
T: Clear zone, indirect.
P: The agar medium, made opaque by xylan, is cleared by action of xylanase(s). Precipitation of residual xylan by ethanol enhances the effect.
M: Agar medium, containing 0.25 % xylan (larch). Petri plates.
R: Ethanol 96 %.
Pr: Reading of clearing intensity (arbitrary units) and diameter of active zone after 3 h (and 12 h) after addition of ethanol.
Rm: Substrate-inducible enzyme.
Rf: Schimpfhauser and Molitoris 1991; Rohrmann et al. 1992.

Method 4.1.12.
Semiquantitative determination of gelatinase (EC 3.4.) in a solid medium with gelatine in an indirect test (Hankin and Anagnostakis 1975).

T: Clear zone, indirect.

P: Residual substrate is precipitated leaving a clear zone indicating proteolytic (gelatinase) activity.

M: Agar medium containing 0.4 % gelatin. Petri plates.

R: Aqueous saturated aqueous solution of Ammoniumsulfate (ca. 75 g/100 ml).

Alternatively (Weyland et al. 1970): $HgCl_2$ 15 g, 20 ml HCl conc., 100 ml distilled water.

Pr: Flooding the plate with the reagent. Reading of clarity (arbitrary units) and of diameter of activity zone after 1 h.

Rf: Molitoris and Schaumann 1986; Rohrmann et al. 1992.

Method 4.1.13.

Semiquantitative determination of caseinase (EC 3.4.) in a solid medium opaque by casein (skim milk powder) in a direct test (Anderson 1962).

T: Clear zone, direct.

P: The medium, made opaque by the skim milk powder, is cleared by caseinase action.

M: Agar medium containing 10 % casein (skim milk powder), Petri plates.

R: None, direct test.

Pr: Reading of clearing intensity (arbitrary units) and diameter of activity zone.

Rf: Bahnweg and Bland 1980; Molitoris and Schaumann 1986; Rohrmann et al. 1992

Method 4.1.14.

Semiquantitative determination of pectate transeliminase (EC 4.2.2.2.) in a buffered (pH 7.0) solid medium in an indirect test with pectin (Hankin and Anagnostakis 1975).

T: Clear zone.

P: Residual substrate is precipitated leaving a clear zone indicating pectate transeliminase activity.

M: Buffered (pH 7.0) agar medium containing 0.5 % pectin (apple or citrus) as sole carbon source. Petri plates.

R: 1 % aqueous solution of hexadecyltrimethylammonium bromide.

Pr: Reading of clarity (arbitrary units) and of diameter of activity zone after 1 and 12 h, respectively.

Rm: Glucose inhibits. Seawater may interfere.

Rf: Federici 1983; Molitoris and Schaumann 1986; Zare-Maivan and Shearer 1988.

4.2. Quantitative

Below, a selection of quantitative enzyme assays is given which often are used in the determination of enzyme activity in marine fungi. Whereas in a number of

papers for the cultivation of the fungi seawater medium was used (see under 3. Available Techniques), the assays for enzyme activity were mostly conducted without seawater medium.

Method 4.2.1.

Quantitative spectrophotometric assay for endoglucanase (EC 3.2.1.4.) by determination with the dinitrosalicylic acid (DNSA) reagent of reducing sugars liberated from carboxymethylcellulose (CMC).

T: Spectrophotometric.

P: The reducing sugars liberated by endoglucanase from soluble cellulose (as CMC) are quantitatively determined by measuring their reaction product with the DNSA reagent at 575 nm (Miller 1959) or 540 nm (Robertson and Koehn 1978).

M: Preincubation of fungal cultures or enzyme with CMC.

R: DNSA reagent: 1 % dinitrosalicylic acid, 0.2 % phenol, 0.05 % sodium sulfite, 1 % sodium hydroxide (Miller 1959).

Pr: Incubation of 1 ml reaction mixture with buffered substrate (0.5 % carboxymethylcellulose in 0.025 M acetate buffer, pH 5.0) and enzyme (culture filtrate or mycelial extract) for 3 h at 30 °C, addition of 1 ml DNSA reagent (Sumner and Sisler 1944, minus K/Na-tartrate), incubation for 5 min in boiling water, addition of 0.3 ml 40 % K/Na-tartrate solution (Miller 1959), cooling to ambient temperature, reading of absorption at 575 nm. Standard: glucose.

The alternative method (Robertson and Koehn 1978) uses the DNSA reagent of Gascoigne and Gascoigne, 1960. Incubation of enzyme sample with 1 % CMC solution in sodium citrate buffer, pH 5.2, for 15 min at 55 °C, addition of DNSA reagent, incubation for 5 min in boiling water, cooling to ambient temperature, determination of absorption at 540 nm.

Rf: Torzilli and Andrykovitch 1980; Torzilli 1982; Zare-Maivan and Shearer 1988; MacDonald and Speedie 1982; MacDonald et al. 1985.

Method 4.2.2.

Quantitative radiometric assay for chitinase (EC 3.2.1.14) by determination of the release of soluble labelled material from radioactive chitin (Molano et al. 1977)

T: Radiometric.

P: Chitinase liberates from water-insoluble tritiated chitin the labelled water-soluble diacetylchitobiose whose radioactivity is determined in a scintillation counter.

M: Cultivation of fungal strains in liquid culture for enzyme solution (culture filtrate or mycelial extract).

R: Preparation of radio-labelled chitin following Molano et al. 1977.

Pr: Buffered reaction mixture containing 3H chitin, incubation for hydrolytic chitin cleavage, reaction stopped addition of 10 % trichloroacetic acid or by boiling, removal of insoluble labelled chitin by filtration or centrifugation, determination of radioactivity of soluble hydrolysis products of chitin in liquid scintillation spectrometer (Molano et al. 1977, with respective modifications).

Rm: Chitin induces, glucose inhibits.

Rf: Correa et al. 1982; Elango et al. 1982; Smucker 1982; Barrett-Bee and Hamilton 1984; Kuranda and Robbins 1987.

Method 4.2.3.

Quantitative fluorimetric assay for chitinase (EC 3.2.1.14) by determination of the release of fluorescing 4-methylumbelliferone from 4-methylumbelliferylglycosids of N-acetylglucosamins

T: Fluorimetric.

P: Chitinase is measured by determination of the fluorescence of 4-methylumbelliferone which is released by the glycosidase activity from 4-methylumbelliferylglycosids of N-acetylglucosamins.

M: Fungal culture filtrate or mycelial extracts are used as enzyme samples.

R: Commercially available 4-methylumbelliferylglycosids of N-acetylglucosamins such as 4-methylumbelliferyl-ß-D-N,N',N"-triacetylchitotrioside.

Pr: Incubation of 27 μmole substrate in 0.1 M citrate/0.2 M phosphate buffer, pH 6.0 and 10 μg enzyme sample with a total volume of 0.3 ml for 10–30 min at 28 °C; reaction stopped by heating to 100 °C for 5 min; cooling to room temperature; addition of 2.5 ml distilled water plus 0.2 ml 5 M NaOH; fluorescence of released 4-methylumbelliferone measured in a fluorescence spectrophotometer (excitation at 360 nm, emission at 440 nm) (Butler et al. 1991; Alternative method: Kuranda and Robbins 1987).

Rm: Chitin induces, glucose inhibits. Principally the same method may be used for determination of pectinesterase (EC 3.1.1.11.), α-mannosidase (EC 3.2.1.24), exoglucanase (EC 3.2.1.91) and proteases.

Rf: Butler et al., 1991.

Method 4.2.4

Quantitative spectrophotometric assay for polygalacturonase (EC 3.2.1.15) by determination with the dinitrosalicylic acid (DNSA) reagent of the reducing sugars released from the pectic substrate.

T: Spectrophotometric.

P: The reducing sugars liberated from the pectic substrate by polygalacturonase are determined by measuring their reaction product with the DNSA reagent at 575 nm.

M: Preincubation of plant tissue or pectins with fungal cultures for enzyme production.

R: DNSA reagent: prepared from 8,8 g dinitrosalicylic acid, 7 g phenol, 7 g sodium anhydrous bisulfite, 588 ml 1.25 N sodium hydroxide and 255 g Rochelle salt (K/Na-tartrate) in a total volume of 1400 ml distilled water, following Sumner and Sisler 1944.

Pr: Incubation of 1 ml reaction mixture with buffered substrate (0.1 % pectin or Na-polypectate in 0.025 M citrate buffer, pH 5.0) and enzyme (culture filtrate or extract) for 3 h at 30 °C, addition of 1 ml DNSA reagent (Sumner and Sisler 1944, minus K/Na-tartrate), incubation for 5 min in boiling water, addition of 0.3 ml 40 % K/Na-tartrate solution (Miller 1959), cooling to ambient temperature, reading of absorption at 575 nm. Standard: glucose.

Rf: Torzilli and Andrykovitch 1980; Torzilli 1982.

Method 4.2.5.

Quantitative spectrophotometric assay for ß-glucosidase (EC 3.2.1.21) by determination of the production of p-nitrophenol from p-nitrophenol-ß-D-glucose (Jones et al. 1972).

T: Spectrophotometric.

P: Production of p-nitrophenol from p-nitrophenol-ß-D-glucose by ß-glucosidase is determined as increase in absorption at 400 nm.

M: Fungal culture filtrate or mycelial extract plus p-nitrophenol-ß-D glucose.

Pr: Incubation of 2.0 ml reaction mixture containing enzyme solution, buffer and 0.5 mg p-nitrophenol-ß-D-glucose for 3 h at 30 °C, reaction stopped by adding 1 ml 1 N NH4OH containing 2 mM disodium-EDTA, absorption read at 400 nm. Activity determined with standard curve of p-nitrophenol.

Rf: Torzilli and Andrykovitch 1980; Torzilli 1982; Suberkropp et al. 1983.

Method 4.2.6.

Quantitative spectrophotometric assay for exoglucanase (EC 3.2.1.91) by determination with the dinitrosalicylic acid reagent (DNSA) of the reducing sugars released from crystalline cellulose.

T: Spectrophotometric.

P: Glucose released from crystalline cellulose by exoglucanase is determined quantitatively by measuring the reaction product with the DNSA reagent at 540 nm.

M: Preincubation of fungal liquid culture with crystalline cellulose.

R: DNSA reagent: 1 % dinitrosalicylic acid, 0.2 % phenol, 0.05 % sodium sulfite, 1 % sodium hydroxide (Miller, 1959 = 60).

Pr: Incubation of 0.5 ml reaction mixture with buffered substrate (1 % crystalline cellulose in 0.2 M citrate-phosphate buffer, pH 7.0) and enzyme (culture filtrate or mycelial extract) for 1 h at 65 °C, addition of 0.5 ml DNSA reagent, incubation in boiling water for 15 min, addition of 0.5 ml Rochelle salt solution (40 % K/Na-tartrate), cooling to room temperature and reading absorption at 540 nm (Robertson and Koehn 1978). Standard: glucose.

Rf: Zare-Maivan and Shearer 1988; MacDonald and Speedie 1982; MacDonald et al. 1985.

Method 4.2.7.

Quantitative spectrophotometric assay for "xylanase" (EC 3.2.1.) by determination with the dinitrosalicylic acid reagent (DNSA) of the reducing sugars released from xylan.

T: Spectrophotometric.

P: The reducing sugars released from xylan by "xylanase" are determined by measuring their reaction product with the DNSA reagent at 575 nm.

M: Incubation of plant tissue or xylan (larch) with fungal cultures for enzyme production.

R: DNSA reagent: prepared from 8.8 g dinitrosalicylic acid, 7 g phenol, 7 g anhydrous sodium bisulfite, 588 ml 1.25 N sodium hydroxide and 255 g Rochelle salt (K/Na-tartrate) in a total volume of 1400 ml distilled water, following Sumner and Sisler 1944.

Pr: Incubation of 1 ml reaction mixture with buffered substrate (0.5 % xylan in 0.025 M Tris pH 7) and enzyme (culture filtrate or mycelial extract) for 3 h at 30 °C, addition of 1 ml DNSA reagent (Sumner and Sisler 1944, minus K/Na-tartrate), incubation for 5 min in boiling water, addition of 0.3 ml 40 % K/Na-tartrate solution (Miller 1959), cooling to ambient temperature, reading of absorption at 575 nm. Standard: glucose.

Rm: Xylan induces.

Rf: Torzilli and Andrykovitch 1980; Torzilli 1982; Torzilli et al. 1985.

Method 4.2.8.

Quantitative spectrophotometric assay for pectate transeliminase (EC 4.2.2.2.) by determination with the dinitrosalicylic acid (DNSA) reagent of the reducing sugars released from pectins.

T: Spectrophotometric

P: The reducing sugars liberated from the pectic substrate by pectate transeliminase are determined by measuring their reaction product with the DNSA reagent at 575 nm.

M: Preincubation of plant tissue or pectins with fungal cultures for enzyme production.

R: DNSA reagent: prepared from 8,8 g dinitrosalicylic acid, 7 g phenol, 7 g anhydrous sodium bisulfite, 588 ml 1.25 N sodium hydroxide and 255 g Rochelle salt (K/Na-tartrate) in a total volume of 1400 ml distilled water, following Sumner and Sisler, 1944 = 88.

Pr: Incubation of 1 ml reaction mixture with buffered substrate (0.1 % pectin or Na-polypectate in 0.025 M Tris pH 8 containing 0.001 M $CaCl_2$) and enzyme (culture filtrate or mycelial extract) for 3 hrs at 30 °C, addition of 1 ml DNSA reagent (Sumner and Sisler 1944, minus K/Na-tartrate), incubation for 5 min in boiling water, addition of 0.3 ml 40 % K/Na-tartrate solution (Miller 1959), cooling to ambient temperature, reading of absorption at 575 nm. Standard: glucose.

Rf: Torzilli and Andrykovitch 1980; Torzilli 1982.

As well as the above-mentioned trends towards more quantitative enzyme assays, testing well-defined enzymatic activities and using defined substrates, other future developments are likely. Thus one envisages the use of test kits, including the necessary equipment for multiple enzyme determination, such as the API MICROBIAL IDENTIFICATION SYSTEM (bioMérieux, Marcy-L'Étoile, France (Bremer 1988). One also envisages that there will be a greater emphasis on the evaluation and documentation of the results by computer programmes as used by Böhm 1991 for the identification of marine yeasts based on absence or presence of specified metabolic (enzymatic) activities. The latter methods are now used widely in the major culture collections for characterization and identification of yeasts. The use of such methods with well chosen groups of filamentous marine fungi is long overdue.

Generally speaking, future enzymatic and physiological research work in the field of marine mycology will have to use the techniques and methods already used in the study of general microbial metabolism. We need to do much more to elucidate the biochemical and physiological activities of marine fungi and thereby to clarify the role of this interesting and long neglected group of microorganisms in the sea.

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Fungal association and deterioration of oil-type safflower (Carthamus tinctorius) seed during storage

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Hasan H. A. H. (2000): Fungal association and deterioration of oil-type safflower (*Carthamus tinctorius*) seed during storage – Czech Mycol. 52: 125–137

Different fungal species viz. *Aspergillus flavus, A. fumigatus, A. niger, A. sydowii, Emericella nidulans, Mucor circinelloides* and *Penicillium chrysogenum* were isolated from commercial safflower seeds. The total number of fungi increased with raising the moisture content of the seeds from 15 to 25 % and reached a maximum level after 3 months of storage. *Aspergillus* was the dominant genus at 25 °C. However, *Penicillium* was dominant at 10 °C. Germination of seeds significantly decreased with increasing moisture content and length of storage. Where no germination in seed at 25 % moisture content was observed after 5 months of storage at 25 °C, there was a perceptible increase in free fatty acids content, especially at high moisture and temperatures. This increase in free fatty acids positively correlated with the infestation of seeds by *P. chrysogenum* at 10 °C, and *A. flavus* and *A. niger* at 25 °C. In artificially inoculated seeds (25 % moisture content) by *A. flavus*, aflatoxin production reached their maximum level after 1 month and then decreased. Seed deterioration was accompanied by a corresponding rise in free fatty acids, free amino acids and soluble protein. The high levels of autolytic enzymes (amylase, cellulase, lipase and protease) detected in seed fungi clearly indicate the important role of these enzymes in seed deterioration. Also, the carcumin and turmerol oils of safflower promoted the growth and lipase activity of *A. flavus*. Thus, the oils encourage seed infestation and did not make the safflower safe from deterioration.

Key words: safflower, mycoflora, mycotoxin, oil, protein, sugars, enzymes, germination

Hasan H. A. H. (2000): Houbová společenstva a rozklad semen olejné odrůdy světlice barvířské (*Carthamus tinctorius*) v průběhu skladování. – Czech Mycol. 52: 125–137

Různé druhy hub, jako *Aspergillus flavus, A. fumigatus, A. niger, A. sydowii, Emericella nidulans, Mucor circinelloides* a *Penicillium chrysogenum* byly izolovány z prodávaných semen světlice barvířské („safloru") – *Carthamus tinctorius* L. Celkový počet hub vzrostl, když byla zvýšena vlhkost semen z 15% na 25% a počet dosáhl maxima po tříměsíčním skladování. Druhy rodu *Aspergillus* byly dominantní při teplotě 25 °C. Na druhé straně, *Penicillium* převažovalo při teplotě 10 °C. Klíčivost semen výrazně poklesla se stoupajícím obsahem vody a délkou skladování. Při úplné ztrátě klíčivosti (při skladování po dobu 5 měsíců při teplotě 25 °C za 20% obsahu vody) bylo zřetelné zvýšení obsahu volných mastných kyselin. Toto zvýšení obsahu volných mastných kyselin bylo v pozitivní korelaci s napadením semen *Penicillium chrysogenum* při teplotě 10 °C a *Aspergillus flavus* a *A. niger* při teplotě 20 °C. U uměle naočkovaných semen druhem *Aspergillus flavus* (25% obsah vody) produkce aflatoxinu dosahovala nejvyšší úrovně po 1 měsíci a pak poklesla. Znehodnocení semen bylo spojeno se současným nárůstem obsahu volných mastných kyselin, volných aminokyselin a rozpustných proteinů. Vysoká hodnota autolytických enzymů (amyláza, celuláza, lipáza a proteáza) zjištěná v těchto semena napadajících houbách jasně ukazuje významnou roli těchto enzymů při rozkladu semen. Také oleje světlice jako kurkumin a turmerol podporovaly růst a také lipázovou aktivitu *Aspergillus flavus*. Takto oleje povzbuzují napadání semen a nechrání semena světlice před rozkladem.

Introduction

Problems concerning fungal deterioration of seed in storage have received considerable attention in recent years. Safflower (*Carthamus tinctorius* L.) is grown in Upper Egypt and in warm and tropical areas as an oil cultivar. Its seed obtains about 35 % oil (containing carcumin and turmerol as constant oils), 20 % protein and 10 % carbohydrates. The oil is used in foods, and the oil cake produced after oil extraction is used as sheep and chicken feed. The seed is stored in humid tropics, which may lead to their fungal infestation, deterioration and often to contamination by mycotoxins.

There are different studies on the mycoflora associated with oil seeds such as sunflower, soybean and flaxseed (Robertson et al. 1985; Patil et al. 1986; White and Jayas 1991). However, no studies were made on safflower seeds. This may be due to the ancient belief that safflower carcumin and turmerol oils possess preservative and medicinal properties. So, no attention was paid to preserving the seed under controlled conditions.

This investigation was conducted to study the mycoflora in safflower seeds and their toxigenic and degrading enzyme potentialities. Also, the factors affecting safflower deterioration during storage were evaluated periodically for fungal invasion, viability and oil changes. The ability of toxigenic *A. flavus* strains to produce aflatoxin and cause biochemical changes in seed components was studied. Finally, the effect of carcumin and turmerol oils on *A. flavus* activity was also studied.

Materials and methods

Thirty samples of local production safflower (*Carthamus tinctorius* L.) seed were collected from retail markets in Assiut City (Egypt).

Isolation and identification of fungi

The dilution-plate method described by Christensen (1963) was employed. 20 g of each sample were blended for 1.5 min with 40 ml sterile distilled water in a sterile blender. Five ml of the suspension was immediately transferred into 95 ml sterilised distilled water, to form about 20–40 fungal colonies per plate. One ml of the suspension was transferred to each of 5 sterile Petri-dishes, and 15–20 ml of glucose-Czapek's agar medium, cooled to just above the solidifying temperature, were added to each dish. The dishes were rotated by hand in a broad swirling motion so that the suspension was dispersed in the agar medium. The dishes were then incubated at 28 °C for 7 days. The growing colonies were identified according to Raper and Fennel (1965) for *Aspergillus*, Booth (1977) for *Fusarium*, Christensen and Raper (1978) for *Emericella* and Pitt (1985) for *Penicillium* species. The

average number of colonies per dish was multiplied by the dilution factor to obtain the number of colonies per g of seed.

Mycotoxin potentialities of safflower seed-borne fungi

Isolates belonging to *Aspergillus flavus, A. fumigatus, A. niger, A. ochraceus, A. sydowii, Penicillium chrysogenum, Emericella nidulans* and *Mucor circinelloides* were subjected to mycotoxin screening. Twenty ml of a yeast extract-sucrose medium in a 100 ml Erlenmeyer flask was sterilised, inoculated with a 1 ml spore suspension of a 1 week-old culture of each tested isolate and incubated at 25 °C for 7 days. Three cultures were used for each isolate. The content of each flask was extracted with chloroform and concentrated by vacuum to near dryness and analysed by thin layer chromatography on silica gel 60 plates under long wave UV irradiation (UV 6 LC-12W lamp). The developing solvent chloroform-methanol (97:3 v/v) was used for aflatoxin B_1 and G_1 and identified as blue and green spots at R_f 0.56 and 0.48, respectively (Bullerman 1979). Toluene-ethyl acetate-acetic acid (6:3:1 v/v/v) was used for sterigmatocystin and identified as red-brown at R_f 0.8 (Josefsson and Moller 1977). Toluene-ethyl acetate-formic acid (6:3:1 v/v/v) was used for ochratoxin A and identified as green spot at R_f 0.55 (Nesheim 1976). Chloroform-acetone (9:1 v/v) was used for fumitremorgen and identified as blue-green at R_f 0.28 after spraying with $AlCl_3$-ethanol (Land et al. 1993). Standard aflatoxins were purchased from Sigma and the other toxins were prepared in our laboratory from IMI isolates.

Storage of seeds at various levels of moisture and temperature

Samples of 100 g safflower seed highly contaminated by fungi were adjusted to the desired moisture contents (15, 20 and 25 %) by adding the required amount of sterile distilled water to sterile polyethylene bags. The samples were shaken thoroughly and placed in a refrigerator at 5 °C for 24 h to imbibe eventually all the seeds. Thereafter, the bags were incubated at 10 ° and 25 °C. Weekly, the water content was readjusted and after 1, 3 and 5 months, the samples were assayed for their fungal content, germination rate and fatty acids content.

Germination of seed

Seed samples were surface sterilised by shaking in 5 % NaOCl for 5 minutes, rinsed in sterile distilled water, and allowed to germinate on moist sterile filter paper (Whatman No. 1) with 5 ml sterilised distilled water in a Petri-dish. The dishes (10 seed per dish) were incubated in the dark at 25 °C, and the germination rate was determined after 5 days of planting. Three dishes were used for each sample.

Oil determination

Seeds were dried and ground to a fine powder in a blender to pass through a 35-mesh screen (Canadian standard sieve series No. 40). Oils were extracted using chloroform-methanol (2:1 v/v) at 25 °C for 72 h and then the samples were filtered, evaporated and weighed. Free fatty acids were determined according FAO (1986).

Fungal degrading enzymes

The extracellular enzyme profiles of *A. flavus*, *A. niger* and *P. chrysogenum* were achieved using the methods of Eggins and Pugh (1962) for cellulase using cellulose agar medium and Hankin and Anagnostakis (1975) for amylase, lipase and protease using amylose, tween, and gelatin agar medium, respectively, with modification. The cellulose agar medium contained: 0.5 g/l $(NH_4)_2$ SO_4, 0.5 g/l L-asparagine, 1 g/l KH_2PO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_4$, 0.5 g/l yeast extract, 10 g/l microcrystalline cellulose, 15 g/l agar. The amylose agar medium contained: 5 g/l peptone, 3 g/l yeast extract, 5 g/l amylose, 15 g/l agar. The gelatin agar medium contained: 5 g/l peptone, 3 g/l yeast extract, 5 g/l gelatin, 15 g/l agar. Tween agar medium contained: 5 g/l peptone, 0.5 g/l NaCl, 0.1 g/l $CaCl_2.2H_2O$, 10 ml/l tween 20, 15 g/l agar. After autoclaving, the media were poured on plates (3 plates for each isolate for each medium). An inoculum was placed at the center of the plates, incubated at 25 °C for 5 days and then the enzymes were assayed. The degradation of cellulose and tween was observed as clear zones around the growing fungus. The degradation of amylose and gelatin was observed after adding iodine or saturated ammonium sulphate solutions, respectively. The diameters of clear zones around the growing colonies in otherwise blue or opaque media indicated the extent of amylase and protease activity, respectively.

Inoculation of seeds by toxigenic *A. flavus*

Samples of the 250 g of safflower seed were surfac sterilised by a 5 % NaOCl solution for 5 min. and rinsed in three changes of sterile distilled water. Thereafter, each sample was placed in a sterile polyethylene bag and inoculated with a spore suspension of aflatoxigenic *A. flavus*. Sterile distilled water, sufficient to raise the moisture content of the seeds to 25 %, was added. The samples were placed in a refrigerator at 5 °C for 2 h and shaken thoroughly. The inoculated seeds were incubated for 5 months at 25 °C in a desiccator at 100 % relative humidity (over distilled water). Periodically, the water content was readjusted and after 1, 3 and 5 months samples were taken for assaying germination, aflatoxin, sugars, protein and oil contents.

Quantitative analysis of aflatoxin

The seeds inoculated with *A. flavus* were extracted with chloroform and concentrated by vacuum to near dryness, and diluted to 1–5 ml with chloroform. 10 μl of the dilution (aflatoxins) was separated and qualitatively determined as mentioned above. The potential lower limit of visual detection for both toxins is 0.4 μg. Aflatoxin B_1 and G_1 spots were removed from the plates by chloroform/acetone, evaporated and estimated spectrophotometrically (Nabney and Nesbitt 1965).

Sugars, free amino acids and protein determination

Seeds were dried and ground to a fine powder as mentioned above. A particular weight of the ground samples was hydrolysed by distilled water (soluble components), 3 M HCl (total sugars) and 1 M NaOH (total protein) for 2 h in a boiling water-bath. After cooling the hydrolysate was neutralised to pH 7, filtered and the filtrate completed to a definite volume. The method of Nelson (1944) modified by Naguib (1965) was employed for the determination of soluble and insoluble carbohydrates using Nelson's and Arsenomolybdate reagents. The absorbance was measured at 700 nm with a spectrophotometer. Free amino acids were determined according to Lee and Takahashi (1966) and the absorbance was measured at 570 nm. The method described by Lowry et al. (1951) was employed for the determination of soluble and insoluble proteins and the absorbance was measured at 700 nm. D-glucose, glycine and egg-albumin, respectively, were used as standards.

Aflatoxin degradation in a liquid broth

Fifty ml of a liquid medium (10 g/l glucose, 2 g/l KNO_3, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4$, 0.5 g/l KCl, 5 g/l yeast extract) were dispensed in 250 ml Erlenmeyer conical flasks. After autoclaving, the flasks were inoculated with 1 ml of spore suspension (approx. 10^6 spores) of a 7-day old culture of aflatoxin B_1 and G_1 producing *A. flavus* and incubated at 25 °C for 14 days statically. Three flasks were used for aflatoxin estimation after 2, 4, 6, 8, 10, 12 and 14 days of incubation. Aflatoxin was extracted by chloroform and determined as mentioned above.

Effect of safflower oil on *Aspergillus flavus* activity in a liquid broth

Twenty five ml of the above liquid medium in 100 ml Erlenmeyer conical flasks was used for studying the activity of *A. flavus* in the presence of 1 and 5 % safflower oil. After autoclaving, the flasks were inoculated with 0.5 ml of spore suspension (approx. 10^6 spores/ml) of a 7-day old culture and incubated at 25 °C

Table 1: Fungi associated with safflower seeds and their mycotoxin potentialities[1]

Fungal genera and species	Colonies (count/g seeds)	Occurrence	Mycotoxin production
Total count	601		
Alternaria alternata (Fr.) Keissler	3	L	–
Aspergillus	397	H	
A. flavus Link	96	H	Aflatoxin B_1 and G_1
A. fumigatus Fresenius	14	M	Fumitremorgen
A. niger Van Tieghem	252	H	NTP
A. ochraceus Wilhelm	7	L	Ochratoxin A
A. sydowii Thom and Church	26	M	Sterigmatocystin
A. tamarii Kita	2	L	–
Emericella nidulans (Eidam) Vuill.	14	M	Sterigmatocystin
Gibberella fujikuroi (Sawada) Ito	3	L	–
Mucor circinelloides van Tieghem	19	M	NTP
Penicillium	161	H	
P. chrysogenum Thom	154	H	–
P. duclauxii Delacroix	2	L	–
P. funiculosum Thom	5	L	–
Rhizopus stolonifer (Ehrenb.) Vuill.	4	L	–

[1]H = high occurrence (>50 % of seed samples) M = moderate occurrence (50–25 % of seed samples); L = low occurrence (<25 % of seed samples); NTP = no toxin production.

for 10 days statically. Three flasks were used for the estimation of oil residue in filtrate, mycelial lipid, aflatoxin and lipase activity.

Lipase activity determination

Lipase (glycerol ester hydrolase, EC 3.1.1.3) activity in the media was determined by the method suggested by Urs et al. (1962) with some modifications. The reaction mixture, consisting of 2 ml of 1 % glycerol triacetate, 5 ml of a citrate-phosphate buffer (pH 8), 1 ml of culture filtrate and 1 ml of toluene, was incubated at 37 °C for 1 h. The assay mixture containing 1 ml distilled water served as the control. The reaction was terminated with 10 ml absolute ethanol and titrated against 0.05 M NaOH using phenolphthaleine as indicator. The activity was expressed in unit per 1 ml of culture broth (1 unit corresponds to 0.1 ml of 0.05 M NaOH being required to neutralise free fatty acids liberated from triacetin in 1 h).

Statistical analysis of the results

The least significant difference analysis (L. S. D.) adopted by Ostle (1963) was used.

Results and discussion

Safflower mycoflora and mycotoxin potentialities

Before this work no reports on the incidence of mycoflora in safflower seeds had been made. *Aspergillus flavus, A. niger* and *Penicillium chrysogenum* contaminated 20–30 samples of safflower seed (Table 1). 8–15 seed samples were contaminated with *A. fumigatus, A. sydowii, Emericella nidulans* and *Mucor circinelloides*. The moulds have potentialities for producing their respective toxin as aflatoxin B_1 and G_1, fumitremorgen, ochratoxin A and sterigmatocystin (Table 1). The data proved that potential hazard to human, sheep and chicken may exist due to the presence of toxigenic fungi in safflower seeds.

Factors affecting the deterioration of safflower seeds during storage

The changes in mycoflora of safflower seed stored under different conditions are illustrated in Table 2. The results show that the total fungal count increased with raising the moisture content of the seeds from 15 to 25 %. This reached its maximum level after 3 months of seed storage but then declined. *Penicillium* was the dominant genus at 10 °C. However, *Aspergillus* was the dominant at 25 °C during all periods. *A. niger* represented a more than a 10-fold stronger infestation at 25 °C compared to 10 °C. *A. flavus* increased 15–50 fold at 25 °C compared to 10 °C at high moisture content after 3 and 5 months of storage. In contrast, the *Penicillium chrysogenum* count increased 5-fold in seed stored at 10 °C compared to storage at 25 °C for long periods. Germination of seed was reduced over time by both relative moisture content and temperature.

The amount of free fatty acids in commercial safflower seed was 0.9 %, still at an acceptable level. Storage of safflower seed at different levels of moisture content and temperature induced critical changes in free fatty acids content (Table 2). In seed with a 15 % moisture content, free fatty acids increased at 10 °C and reached an unacceptable level (>1.8 %) at 25 °C after 5 months of storage. It was positively correlated with the high count of *Penicillium chrysogenum* at 10 °C, and *Aspergillus flavus* and *A. niger* at 25 °C. Increases in moisture content encourage activity of endogenous seed and seed-borne fungi enzymes which cause degradation of triglycerides and produce new short chain fatty acids. This study suggested that free fatty acids content, germination rate and fungal species are indicative

Table 2: Changes in common fungal species (count/mg seed), % germination and % free fatty acids of safflower seed stored at 3 moisture levels and 2 temperatures for 5 months.

Storage temp.	Fungal species, seed viability and free fatty acids	Zero Time	Moisture content								
			15 %			20 %			25 %		
			Storage periods (months)								
		0	1	3	5	1	3	5	1	3	5
10 °C	Total count	0.9	7.7*	23.0*	18.8*	29.3*	64.1*	30.8*	35.1*	55.4*	22.6*
	Aspergillus	0.6	6.9*	11.0*	2.3*	8.0*	4.1*	0.4	5.0*	5.4*	0.6
	A. flavus	0.1	6.5*	5.0*	2.2*	8.0*	0.8	0.4	5.0*	2.0*	0.6
	A. niger	0.5	0.4	6.0*	0.1	0.0	3.3*	0.0	0.0	3.4*	0.0
	Penicillium chrysogenum	0.3	0.8	12.0*	16.5*	20.0*	60.0*	30.0*	25.0*	50.0*	20.0*
25 °C	Total count	0.9	23.0*	44.6*	29.6*	28.0*	70.0*	30.0*	20.2*	70.0*	45.0*
	Aspergillus	0.6	23.0*	44.0*	9.6*	28.0*	60.0*	30.0*	20.2*	60.0*	45.0*
	A. flavus	0.1	3.0*	4.0*	1.6*	3.0*	40.0*	15.0*	0.2	30.0*	25.0*
	A. niger	0.5	20.0*	40.0*	8.0*	25.0*	20.0*	15.0*	20.0*	30.0*	20.0*
	Penicillium chrysogenum	0.3	0.0	0.6	20.0*	0.0	10.0*	0.0	0.0	10.0*	0.0
10 °C	% Germination	100	100	95	90	90	85	80	80	78	75
	% Free fatty acids[1]	0.9	1.0	1.2	1.5	1.4	1.6	2.0*	2.3*	2.8*	3.2*
25 °C	% Germination	100	100	85	77	90	40	12	60	10	0
	% Free fatty acids[1]	0.9	1.3	1.6	1.9*	1.8	2.4*	3.1*	2.7*	3.3*	4.9*

* Mean significant increase compared to the control (at zero time) at 5% of L. S. D.

[1] In good quality safflower oil the acid value is about 0.6 mg KOH/g oil (Codex Alimentarius Commission in FAO, 1986).

Table 3: Biodegradation of inoculated safflower seeds by A. flavus and aflatoxin production at 25% moisture content, 100% relative humidity and 25 °C.

Storage periods (months)	Germination %	Sugars (mg/g dry seed)			Oils (mg/g dry seed)		FAA[1] (mg/g seed)	Protein (mg/g dry seed)			Aflatoxin (μg/g seed)	
		Soluble	Insoluble	Total	Total	%FFA[1]		Soluble	Insoluble	Total	B_1	G_1
0	100	13	74	87	355	0.9	3	64	94	158	–	–
1	0	9	75	84	330	4.3*	5*	70	89	159	160	120
3	0	5	76	81	310	11.8*	7*	83*	77	160	45	0
5	0	1	77	78	260	18.0*	9*	98*	63	161	20	0

[1] FFA = free fatty acids; FAA = free amino acids.

* Mean significant increase compared to the control (at zero time) at 5% of L. S. D.

of safflower seed quality, and the extent of seed deterioration confirms the earlier report by Robertson et al. (1985) on sunflower seeds.

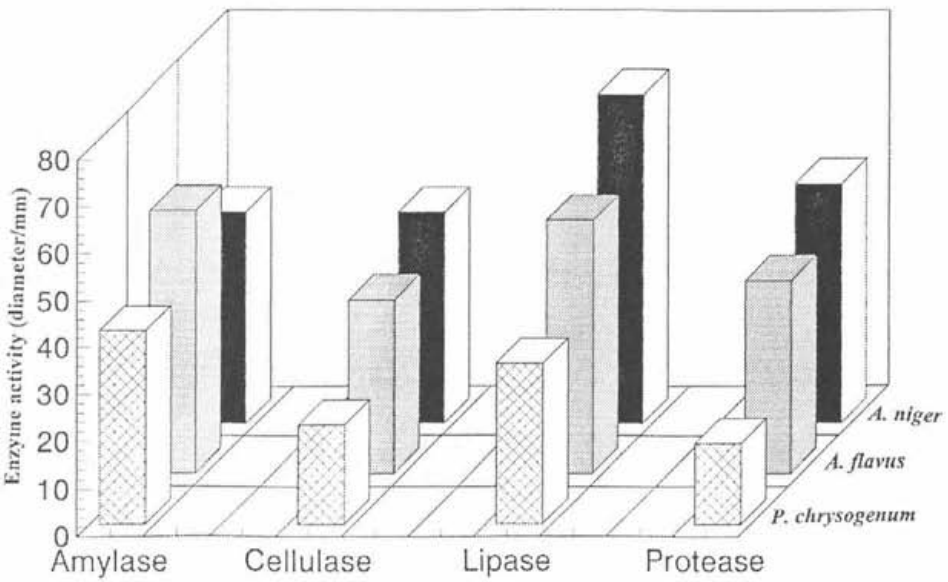

Fig. 1: Production of degrading enzymes by common fungal species on solid media after 5 days of incubation at 25 °C.

Fungal degrading enzymes

Aspergillus flavus, *A. niger* and *Penicillium chrysogenum* have the ability to produce degradation enzymes viz. amylase, cellulase, lipase and protease (Figure 1). Lipase and protease are secreted in relatively high amount into the culture media of all fungal species.

Aflatoxin and biochemical changes in seeds inoculated with *A. flavus*

The changes in aflatoxin and the content of sugars, proteins and oils in safflower seeds inoculated with aflatoxigenic *A. flavus* at 25 % moisture content, 100 % relative humidity and 25 °C are presented in Table 3. The degradation of safflower oils and insoluble proteins by *A. flavus* lipase and protease raises free fatty acids, free amino acids and soluble protein. These changes increased with the length of incubation period. Aflatoxin B_1 and G_1 yield of *A. flavus* reached the maximum level after 1 month of seed inoculation and then gradually decreased. Ciegler et al. (1966) reported that G_1 is very susceptible to rapid oxidative degradation. This might explain why production of G1 was completely eliminated after 3 months of safflower seed storage. Previously, Fernandez et al. (1991) demonstrated that production of aflatoxin B_1 in soybean seed increased with water content up to a certain period of incubation and then decreased.

Aflatoxin degradation in liquid broth

Aflatoxin production in a synthetic medium was detected 2 days after inoculation and increased steadily thereafter to reach the maximum accumulation on the 10th day, then started to decline, just as the mycelium growth on further incubation (Figure 2). This may be due to the degradation of aflatoxin by organic acids especially in the late phase where the biosynthesis of aflatoxin stopped and some chemicals (as organic acids) leached away from the vacuole. This confirms the decrease in its production on inoculated seeds with increasing length of storage. Previously, Varma and Verma (1987) concluded that aflatoxin may be degraded by the mycelium of *A. flavus* itself and recently Hasan (1996) found that some organic acids as ascorbic, propionic, malic and benzoic acids effectively destroy aflatoxin B_1 in cereals.

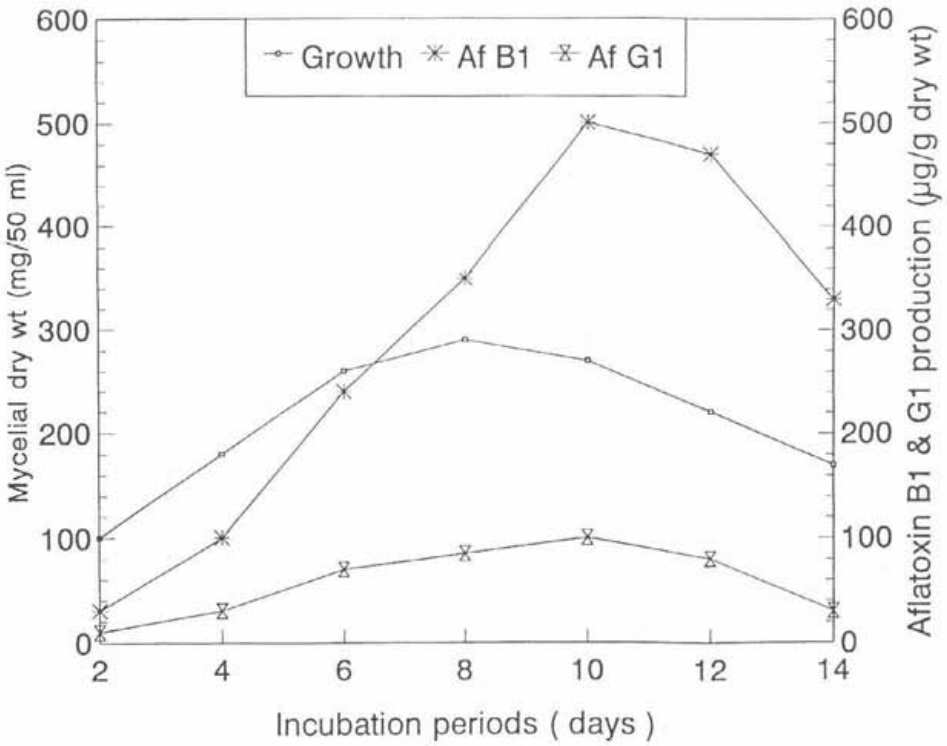

Fig. 2: Biodegradation of aflatoxin after 12 days of *A. flavus* inoculation in liquid broth at 25 °C.

Effect of safflower oil on *Aspergillus flavus* activity in liquid broth

Safflower seed contains constant oils (known as carcumin and turmerol), which promoted *A. flavus* growth and lipase activity as well as aflatoxin accumulation

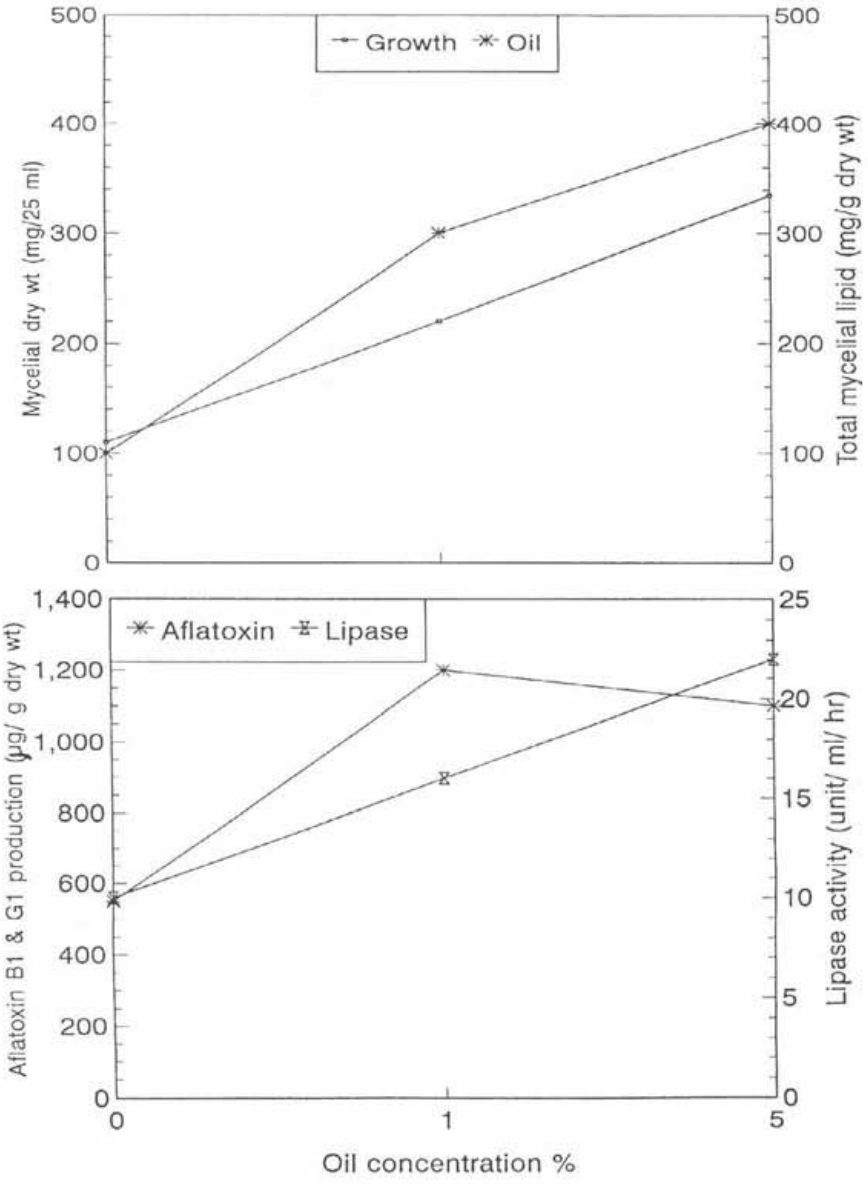

Fig. 3: Effect of safflower oil on *A. flavus* activity in liquid broth.

(Fig. 3). The oils were hydrolysed by *A. flavus* lipase with increasing the free fatty acids in the filtrate (Fig. 4). Thus, the oils encourage seed infestation and did not make the safflower safe from deterioration contrary to the ancient belief.

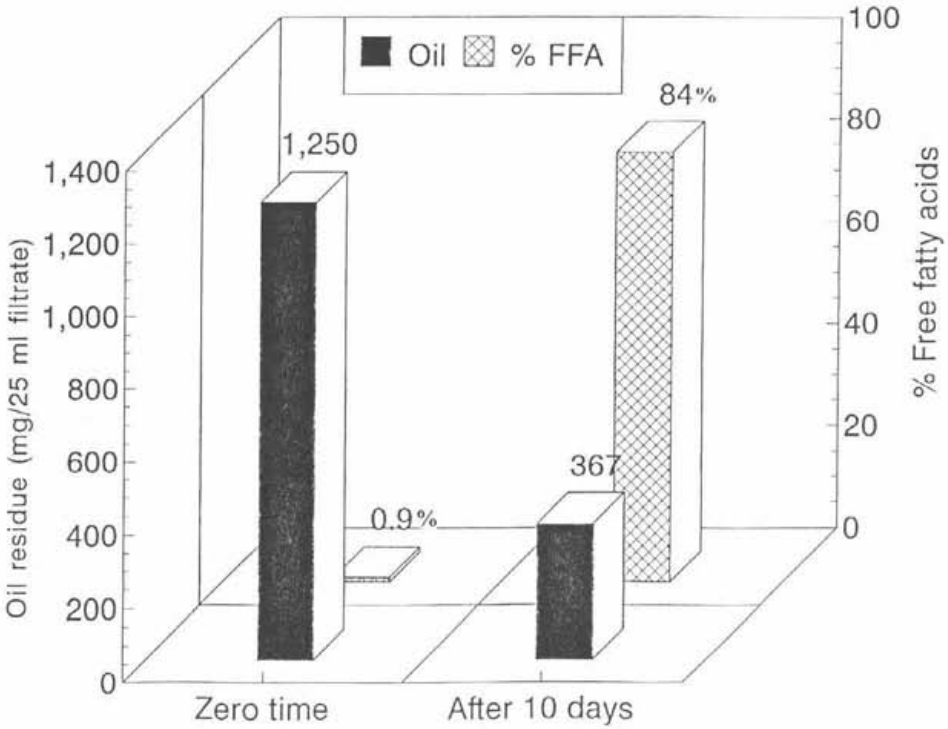

Fig. 4: Percentage of free fatty acids liberated from 5 % safflower oils (carcumin and turmerol) – treated *A. flavus* culture medium.

CONCLUSION

Carcumin and turmerol oils of safflower seed promoted the growth and lipase activity of *A. flavus*. Thus, the oils encourage seed infestation and did not make the safflower safe from deterioration. Seeds should be stored under controlled conditions just like other seeds. Seeds with a 15 % moisture content, even if moderately infected by fungi, remain sound and high in viability for as long as 5 months when stored at 10 °C. Based on findings in this experiment, measurements of free fatty acids and germination rates as well as number and species of fungi are valid indicators of safflower seed quality.

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Colloquium "Fungi as Model Organisms in Research and Biotechnology", Olomouc September 14th, 1999

The colloquium was organized by the joint Commission for Experimental Mycology of the Czechoslovak Microbiological Society and the Czech Scientific Society for Mycology. The purpose of the colloquium was to provide a platform for a broad discussion on the use of fungi as model organisms in both basic and applied research. The programme of the colloquium was divided into four parts dealing with the following topics: phytopathology, biotechnology and ecology, physiology and biochemistry, and medical mycology. Each topic was opened with a plenary lecture (30 min.), followed by short communications (10 min.) and accompanied by poster presentations. Besides four plenary lectures, 15 short communications and 21 posters were presented. In total 38 researchers took part in the colloquium, who discussed various questions of importance for experimental mycology until late in the evening. Abstracts of the contributions are given below.

Jiří Kunert and Václav Šašek

Structure and variability of plant pathosystems

Struktura a variabilita rostlinného patosystému

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Biological diversity (biodiversity) is defined as the total variability within all living organisms and the ecological complexes they inhabit. The diversity displayed among plant pathogens and their host plants is very large. More than 250,000 recently known vascular plants could be considered as potential hosts of pathogens (especially parasitic fungi). However, according to current estimates about 8.000 species of fungi are parasitic, i.e. cca 5% of known fungi (Allen et al. 1999). A plant pathosystem represents any subsystem of an ecosystem which involves parasitism or pathogenism. On the basis of origin there are two pathosystems, natural (wild pathosystem) and artificial (crop pathosystem). In general, these terms may be used at any system level (e.g. ecosystem, population, individual, tissue, physiological, molecular). Analysis of the pathosystem could be defined as the study of pathosystem structure and behaviour at all systems levels. Within the pathosystem, the host-pathogen interactions are governed by the laws applicable to different systems levels (Zadoks and Schein 1979). Both defined pathosystems must be considered as dynamic systems, however, with various level of stability.

As an example, in this paper some known and questionable features related to the pathosystem *Lactuca* spp. (lettuce) – *Bremia lactucae* (lettuce downy mildew) are summarized.

A wild plant pathosystem is autonomous. The control of this pathosystem is primarily based on communication between the three basic components: the host, the pathogen and the environment. Current knowledge of the structure and variation of this pathosystem is still limited (Burdon 1993). There is only rare information on the structure, variability and function of these interactions between naturally growing wild *Lactuca* spp. and *Bremia lactucae*. Some knowledge is available on host resistance and parasite virulence variation, resistance mechanisms and their genetics (Lebeda et al. 1999).

A crop pathosystem is more or less deterministic and has a fourth component which represents man – a highly influential factor in this system because under his control are: individual plants or plant populations (cultivars), the cultivation system and the control of pathogen populations. The stability of the crop pathosystem is extremely low because of the speed of co-evolutionary changes (Simms 1996). The interactions on various levels of biological hierarchy are known in more detail and illustrated by the host-parasite interaction *Lactuca sativa* – *Bremia lactucae*.

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Dynamics of enzymes during pathogenesis of Fusarium oxysporum on pea seedlings

Dynamika enzymů během patogeneze Fusarium oxysporum na rostlinách hrachu

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Plants have formed various defense mechanisms during their co-evolution with pathogenic fungi. Enzymes as biocatalyzators participate in all reactions of living organisms. Enzymes involved in plant defense responses have a direct antimicrobial activity or participate in synthesis of defense substances. Only a limited number of enzymes have been studied in relation to the infection process (Lebeda et al. 1999a). Thanks to an investigation of enzymes enhancing their activity during pathogenesis, new defense mechanisms have been found and utilized for practical purposes, e.g. as a markers in resistance breeding (Lebeda et al. 1999b).

In the host-pathogen interaction *Pisum sativum* – *Fusarium oxysporum* we have focused mainly on the role of enzyme metabolisms (peroxidase, catalase and superoxide dismutase) included in the active oxygen metabolism. Especially peroxidases, which occur in many isoforms in cells and extracellular matrix, are important for plant defense (Buonario and Montalbini 1993). They were found responsible for the process of lignification in healthy plants (Vidhyasekaran 1997) and probably play the same role in defense lignification of peas. According to our results the rhizodermis cells in infected pea roots show a high degree of lignification. Diamine oxidase and proteases have an important function in plant protein degradation. Diamine oxidase catalyze the oxidative deamination of biogenic amines and produce also an important secondary product, hydrogen peroxide (Peč and Frébort 1990). It is evident that hydrogen peroxide serves as a substrate for peroxidases in infected pea plants (Jančová et al., unpubl. results). It was found that diamine oxidase is also important in biochemical pathways including defense lignification and synthesis of phenolic compounds.

Our recent research is focused on a detailed comparative study of enzyme dynamics in susceptible and resistant genotypes of peas. However, practical use is also stressed on using of these data for the detection of efficient biochemical markers for disease resistance breeding.

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Suitable immunochemical methods for detection of Fusarium sp. in winter wheat

Vybrané imunochemické metody pro identifikaci Fusarium sp. v ozimé pšenici

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Fusarium culmorum is the dominant pathogen of winter wheat heads in the Czech Republic. The detections of pathogens were evaluated with PTA-ELISA, immunoprinting, dot blot and immunofluorescence, and realized in vitro, in artificially infected swelled grains and in seedlings. Inoculum was injected into swelled grains using syringe. Monoclonal and polyclonal antibodies were used in all methods. Antibodies were prepared and tested in the laboratories of RICP, Prague, Czech Republic. Specifity of antibodies was established by the reactions with antigens from other species of *Fusarium* (*oxysporum, graminearum, solani, avenaceum, tricinctum, moniliforme*) and with antigens from other pathogens of winter wheat (*Pseudocercosporella herpotrichoides, Gaeumannomyces graminis, Rhizoctonia solani*). The antibodies (mono- and polyclonal) were genus specific and did not cross-react with other fungal pathogens of winter wheat.

The tissues of host or mycelial mats were extracted in PBS pH 7,2 for ELISA and dot blot. A nitrocellulose membrane was used for immunoprinting and dot blot. Fluorescein was conjugated with special antibodies for immunofluorescence. The optimal method for the detection of pathogens in host tissues was dot blot. ELISA and immunoprinting were less suitable. The antibodies (mono- and polyclonal) were similarly specific and sensitive.

Relation between field resistance to the powdery mildew of cucurbits, development stage and plant habit of Cucurbita pepo L.

Vztah mezi polní odolností k padlí tykvovitých, vývojovým stadiem a habitem rostlin Cucurbita pepo L.

Eva Křístková[1] and Aleš Lebeda[2]

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The growth and development of plants are associated with changes on various levels of the plant organism, e.g. tissues, cells, intracellular structures, enzymes and hormones. These processes are linked very often with changes of reaction to pathogens. Changes of resistance to the powdery mildew of cucurbits during plant development have been studied on cucumber (*Cucumis sativus* L.) (Zijlstra and Groot 1992). The current study is aimed at a similar phenomenon in squash (*Cucurbita pepo* L.).

A set of 170 accessions of *Cucurbita pepo* L. genetic resources (PI introductions) was evaluated for the resistance to the powdery mildew of cucurbits (*Erysiphe cichoracearum* and *Sphaerotheca fuliginea*) under conditions of natural field infection. Plant genotypes were represented by local varieties and land races (zucchini, vegetable marrow and pumpkin morphotypes) with a combination of resistance genes and plant habit (i.e. bush and/or vine stem) resulting from a long-lasting natural evolutionary process. During vegetative growth, the infection degree was assessed four times by evaluating leaf surface covered by mycelia of powdery mildew. Disease infection progress on each accession was expressed as the Area below curve (ABC) and the resulting data were processed statistically.

The whole set of 170 *C. pepo* accessions was highly susceptible to the infection of powdery mildew (ABC = 3070.5), while two control cultivars (scallop morphotypes) were highly tolerant (ABC = 978.1). These results support the previously obtained information that the *C. pepo* morphotypes zucchini, vegetable marrow and pumpkin are highly susceptible to powdery mildew (Lebeda and Křístková 1994). Accessions with vine plant habit were more resistant to powdery mildew than bush types. Similarly, accessions with late female flowering were more resistant as compared to accessions with early pistillate flowering. Different plant habit, i.e. vine and/or bush type of stem and a beginning of female flowering existing among *Cucurbita pepo* genotypes reflect key physiological differences which could be connected with their resistance to pathogens. The ontogenetic stage of plants could thus influence their resistance to pathogens. It is probable that several genes are involved in conferring *C. pepo* resistance and that they are activated or suppressed during plant growth and development. These results could

confirm the hypothesis of Leibovich et al. (1996) that the resistance of *C. pepo* to powdery mildew is encoded oligogenically and becomes more susceptible to the infection during development the plants.

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Variability of interactions between morphotypes of squash (Cucurbita pepo L.) and two groups of obligate biotrophs – cucumber downy mildew (Pseudoperonospora cubensis) and powdery mildew of cucurbits (Sphaerotheca fuliginea, Erysiphe cichoracearum)

Variabilita interakcí morfotypů tykve obecné (Cucurbita pepo L.) a dvou skupin obligátních biotrofů – plísní okurkové (Pseudoperonospora cubensis) a padlí tykvovitých (Sphaerotheca fuliginea, Erysiphe cichoracearum)

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Cucumber downy mildew (*Pseudoperonospora cubensis* (Berk. et Curt.) Rostow) and powdery mildew of cucurbits (*Erysiphe cichoraccarum* DC.: Fr. and *Sphaerotheca fuliginea* (Schlecht.: Fr.) Pollaci) are the most important foliar fungal pathogens of cucurbitaceous vegetables. Both microfungi are obligate biotrophs but they belong to different groups of fungi – Oomycota and Ascomycotina – with different phylogeny, metabolism and ecological requirements (Hawksworth, 1995). Squash (*Cucurbita pepo* L., family Cucurbitaceae) shows a broad variability of fruit shapes which corresponds with their place and way of development. The aim of this study was to compare the interaction of a group of morphotypes of *Cucurbita pepo* with both pathogens on the level of individual genotypes.

Eight commercial varieties of *C. pepo* representing all groups of morphotypes were evaluated for their resistance to downy and powdery mildew after artificial inoculation in a cultivation chamber. The infection degree of the downy mildew on the leaf discs was measured visually, the yield of conidia of powdery mildew from a leaf unit was counted in a hemocytometer. Data were processed statistically.

The morphotypes zucchini, cocozelle and vegetable marrow were highly resistant to the downy mildew, the morphotypes acorn, scallop, crookneck and straightneck were very susceptible. On the contrary, the morphotypes cocozelle, zucchini and pumpkin were susceptible to the powdery mildew and the morphotypes acorn and scallop showed a high level of resistance. These results support the previously obtained information about the negative correlation between the reaction to both pathogens on hundreds of accessions of different morphotypes of *C. pepo* (Lebeda and Křístková 1993, 1996). It is very probable that the interaction of *C. pepo* morphotypes with downy and powdery mildews is influenced by the place and way of their evolution. In contrast with some other cucurbitaceous vegetables (cucumbers, melons) (Munger 1996) the genes encoding resistance to both pathogens are probably not linked in *Cucurbita pepo*.

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The resistance of Venturia inaequalis to fungicides

Rezistence Venturia inaequalis vůči fungicidům

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Venturia inaequalis (Cooke) Winter (anamorph *Spilocaea pomi* Fr.) is a bitunicate Ascomycete species, which is provisionally placed in the order Dothideales Lindau, family Venturiaceae E. Müll. et Arx.

Venturia inaequalis only attacks representatives from the *Rosaceae* family, especially the genus *Malus* Mill.. This pathogen causes the economically most important disease of produced cultivars of *Malus domestica* Borkh. and *M. sylvestris* Mill. It can attack the wild and ornamental apples *M. baccata* Borkh., *M. dasyphylla* Borkh., *M. purpurea* (Barbier) Rehder and *M. floribunda* van Houtte too.

One hundred and twenty-six monoconidial strains of *Venturia inaequalis* were collected in 1995 and 1996 from orchards either treated or not treated with different

fungicides. Ninety strains of *V. inaequalis* were collected from commercial orchards in Horoměřice. The remaining 36 strains were obtained from the experimental orchard of RICP Ruzyně, that had been sporadically treated with DMI fungicides (Topas 1996), and from a private garden that had never been treated with fungicides.

All strains were tested to flusilazole and benomyl. Most of isolates, except three resistant ones, were highly sensitive to benomyl at a concentration of 0.1 μg/ml. For flusilazole, strains collected from treated orchards showed 2–3 fold increase in frequency in the category of 20 to 50% inhibition of growth. Resistant strains with an ED_{50} – value up to 1.668 μg/ml were obtained only from treated orchards. For both fungicides, the level of strain sensitivity did not depend on the cultivars the strains were collected from.

Fifteen selected strains were tested to fenarimol, dodine and difenoconazole. A broad spectrum of strain sensitivity was found for dodine and difenoconazole. The ED_{50} – value for difenoconazole fluctuated from 0.0023 μg/ml to 0.42 μg/ml with a resistance factor of 88.

The ED_{50} – value for dodine varied between 0.005 μg/ml and 23.2 μg/ml, with an excessive resistance factor of 9,283.

For fenarimol, only one strain was found to be partly sensitive. The remaining 14 strains were resistant with ED_{50} – values from 0.46 μg/ml to 26.37 μg/ml (resistance factor 659). The calculated resistance factor markedly exceeded the value of 100, meaning a high level of resistance. Absence of sensitive strains was clearly demonstrated by the variability factor, which was approximately 10 times lower than the resistance factor.

Co-evolution of host-parasite interactions: Case study Lactuca sativa – Bremia lactucae

Koevoluce interakce hostitel-parazit: příčinná studie Lactuca sativa – Bremia lactucae

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Co-evolution, i.e. reciprocal evolution in interacting species, has become the major paradigm under which a great deal of research is carried out on the evolution of interactions between plants and pathogens. These interactions represent a very dynamic and flexible system. In a host-parasite interaction that follows gene-for-gene relationships, the expression of resistance or susceptibility to a particular pathogen is conditional on the pathogen genotype, and the degree of pathogen

virulence is conditional on the host genotype (Crute and Pink 1996). The level of genetic variance in resistance and virulence can strongly influence population dynamics and equilibrium of the interacting species. Understanding the co-evolution of hosts and parasites is crucial to understanding their epidemiology and ecology (Simms 1996). The use of resistant cultivars often results in genetic changes in parasite populations. During the last decade there has been a growing appreciation of the importance of understanding the processes which bring about these changes, which is essential for the development of effective disease control strategies. Most of these studies were focused on powdery mildews and rusts. However, a limited number of these studies is concentrated on peronosporaceous fungi (Lebeda and Schwinn 1999).

Probably the best known system (in peronosporaceous fungi) from the viewpoint of co-evolution is represented by the interaction *Lactuca sativa – Bremia lactucae*. The microevolutionary shift on the level of host genotypes and pathogen races was well documented and summarized by Lebeda and Schwinn (1994). From the history of lettuce breeding and occurrence of new *Bremia lactucae* races, interesting data were derived. It was demonstrated that the durability of resistance of newly introduced lettuce cultivars with race-specific resistance was rather short. Recent more detailed population genetic studies showed (Lebeda and Zinkernagel 1998) that the *Lactuca sativa – Bremia lactucae* system is enormously changable. On the example of a German host-parasite population it was demonstrated that changes in the frequency of R-genes in the host population were followed by a substantial shift of v-factors in the pathogen population. Occurrence of new v-factors (e.g. v18), considerable increase of some v-factors (e.g. v3, v7, v11, v16), and a complexity of v-phenotypes in the pathogen population was recorded. The recent data on changes of virulence structure very well exemplified the process of host-pathogen co-evolution, however, also the enormous instability of race-specific R-genes (Lebeda and Zinkernagel 1999).

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Comparative study of Oidium lycopersici development on wild representatives of Lycopersicon spp.

Srovnávací studium vývoje Oidium lycopersici na planých zástupcích rodu Lycopersicon

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Oidium lycopersici (Cooke et Massee, 1888, emend. Nordeloos et Loerakker, 1989) is an obligate biotroph of Erysiphaceae, which causes serious damage mainly to glasshouse tomatoes. Although nearly all recent tomato cultivars are highly susceptible, valuable sources of resistance to this pathogen were found only among wild *Lycopersicon* species (Mieslerová and Lebeda 1999).

Totally seven more or less resistant accessions of wild *Lycopersicon* spp. and two newly released oidium resistant tomato lines (OR 4061, OR 960008 /Rijk Zwaan/) were used for a detailed histological study. The obtained data were compared with the results of a parallel experiment in a glasshouse. *L. esculentum* cv. Amateur served as a susceptible control. The Czech isolate (C-2) of *Oidium lycopersici* was used for inoculation. The methodology of microscopic evaluation was described by Lebeda and Mieslerová (1999). Leaf discs of tested plants were artificially inoculated with *O. lycopersici* (isolate C-2). In various intervals after inoculation (6 h, 24 h, 48 h and 72 h) the following data were recorded: % of germinated conidia, number and length of germ tubes, formation of papillae, hypersensitive response and after 9 days conidiophore production. The same set of accessions (5 plants per genotype) was placed in a glasshouse together with artificially inoculated plants (= spread rows) serving as a source of inoculum. In two-week intervals the infection degree (% max ID) was assessed based on powdery mildew occurrence and sporulation on leaves. The dynamics of the infection process was quantified as area below curve (ABC) based on ID.

Comparison of *O. lycopersici* development revealed reduced germ tube growth on resistant and moderately resistant accessions. Hypersensitive response was detected to a large extent in highly resistant and moderately resistant accessions of wild *Lycopersicon* species and in oidium resistant tomato lines. The highly resistant *L. hirsutum* (LA 1347) showed a different response not based on hypersensitivity.

In glasshouse experiments the responses of the studied accessions were slightly different. Only moderately susceptible and highly susceptible accessions showed some symptoms of disease development under infection pressure. Even if the highly susceptible control was completely destroyed, highly resistant accessions such as *L. hirsutum* (LA 1347, LA 1738), *L. hirsutum* f. *glabratum* (LA 2128) and the moderately resistant *L. chmielewskii* (LA 2663) and *L. parviflorum* (LA

1322) were without disease symptoms. Newly released oidium resistant tomato lines showed a slower rate of disease development (ABC = 2685 for OR 96 0008, resp. ABC = 1328 for OR 4061) in comparison with the highly susceptible control (ABC = 5919).

Regression analysis between ABC and length of *Oidium lycopersici* germ tubes 48 hpi of all tested accessions revealed a non-linear relationship. The linear model explained only 35.8% of variability in ABC. For example, in *L. parviflorum* (LA 1322) a relatively high rate of *O. lycopersici* germ tube development was detected in contrast with no symptoms of disease found in conditions of natural infection. Thus, glasshouse experiments could reveal other sources of resistance (field resistance).

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Microtubular arrangement in epidermal cells of lettuce and wild Lactuca spp. during infection process of Bremia lactucae

Uspořádání mikrotubulů v epidermálních buňkách salátu a planých druhů locik během infekce Bremia lactucae

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Cytological studies in several pathosystems have revealed the connection between cytoskeleton and plant cell response to pathogen attack, hypersensitive cell death included. During the infection process the cytoskeletal network of host cells undergoes dramatic reorganization, which extent differs in various plant-pathogen interactions (Škalamera and Heath 1998). The interaction between *Lactuca sativa*, the closely related *L. serriola* and *Bremia lactucae* confirms a "gene-for-gene" relationship, i.e. race-specificity. A similar reaction pattern and resistance mechanism was recognized also in *Lactuca saligna* and *L. virosa* (Lebeda and Pink 1998). The experimental set of nine genotypes of *Lactuca* spp. (three of *L. sativa*, two of *L. serriola*, *L. saligna* and *L. virosa*) used in this study represented various types of resistance reaction to *Bremia lactucae* isolate NL16.

Early stages of cell pathogenesis and alignment of microtubules (MTs) in epidermal cells of *Lactuca* spp. susceptible genotypes and those with different mechanisms of resistance were investigated 24 and 48 h after inoculation by *Bremia lactucae*. Visualization of MTs was based on immunofluorescence detection of α-tubulin (Sedlářová et al. 1999).

In uninfected cells the cortical array of MTs was organized into a net in transverse orientation with respect to the long axis. In infected and adjacent (*Lactuca virosa*) epidermal cells special configurations of cortical MTs occurred. In epidermal cells of genotypes susceptible to race NL16 (*L. sativa* cv. Cobham Green and UCDM 2, *L. serriola* LSE/18) the pathogen developed quickly and induced formation of a microtubular "basket" surrounding primary and secondary vesicles. Hypersensitive reaction was rare with only one cell participating.

In cells of genotypes with different mechanisms of resistance the pathogen developed slower (esp. *L. saligna*) but caused rapid changes of the microtubular net already during penetration. Later a microtubular basket appeared just as in the cells of susceptible genotypes, with the exception of *L. virosa*, where HR occurred immediately after penetration. The expression of hypersensitivity varied between genotypes. Cv. Mariska (*L. sativa*) and PIVT 1309 (*L. serriola*) were characterized by a prompt elimination of *Bremia lactucae* with aggregation of cytoplasm, and clustering and depolymerization of MTs in infected cells. In *L. saligna* (CGN 05147, CGN 05271) large changes occurred later after the development of primary infection structures and led to microtubular depolymerization during cell death. *L. virosa* genotypes (esp. CGN 04683) exhibited a very fast response to infection. Thick microtubular cables incidental to penetration and MTs depolymerizing later during HR in infected and 1–2 adjacent cells were detected. A correlation of speed and extent of microtubular reorganization with development of pathogen infection structures and susceptibility/resistance mechanism of the genotype was proved.

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Biological treatment of winter wheat seed against phytopathogens transferable by seed and soil

Biologická ochrana osiva ozimnej pšenice proti fytopatogénom prenosných osivom a pôdou

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In 1997 and 1998 the effect of biopreparations in comparison with a chemical preparation for the protection of seed and young plants against phytopathogens transferable by seed, soil and crop residues was tested in field experiments at Borovce near Piešťany. The test comprised these variants: control – non-treated seed; seed treated with Ekovert biopreparation; Trichomil biopreparation applied to straw and the seed treated chemically with Vitavax 200 FF. The incidence of *Fusarium* spp. and *Pseudocercosporella herpotrichoides* was monitored in 32 and 75 DC phase in plant samples – 25 plants × 4 replications. Winter wheat was represented by the Ilona variety.

At both evaluation phases the highly significantly lowest number of infected plants was found in the variants to which biopreparations were applied in the form of Ekovert dressing (28 plants and 24 plants in 1997 and in 1998) and in the form of Trichomil spraying treatment for straw (1997–32, 1998–33 infected plants), compared with the control (1997–60, 1998–57 infected plants), and with chemical treatment with Vitavax 200 FF (1997–48, 1998–42 infected plants). In the variants with biopreparations a significantly higher yield was recorded in 1997 as compared to the control variants and chemical treatment with Vitavax 200 FF. In 1998 the yield was insignificantly higher in variants with biopreparations.

The results show that seed treatment against harmful phytopathogens can be realized by means of using antagonisms among fungi while natural microbial life in the soil remains well-preserved.

The effect of Basagran 600 on the phytotoxicity of Fusarium equiseti and Alternaria alternata isolated from pea seeds

Vliv Basagranu 600 na fytotoxicitu Fusarium equiseti a Alternaria alternata izolovaných z osiva hrachu

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Pesticides do not affect only target organisms. There are a lot of studies concerning this problem. The influence of the herbicide Basagran 600 (bentazon)

on the growth of the mycelium of the two fungus species *Fusarium equiseti* and *Alternaria alternata*, and the effect of their metabolites on germination and growth of pea (variety Komet) were investigated. The mycelium was cultivated on a potato liquid medium, then filtered off, dried and weighed.

Six types of filtrates were tested: (1) sterile water (control), (2) filtrate with fungus metabolites, (3) filtrate with Basagran 600 (0.5 %), (4) – (6) mixture of the filtrate containing fungus metabolites and a herbicide at given concentration (0.1, 0.5 and 1 %). Pea seeds were cultivated in Petri dishes or on sandy soil both treated with filtrates. Seed germination and length of stems and roots of pea seedlings were observed.

The herbicide Basagran did not affect mycelium growth at all. Solutions including Basagran inhibited germination and seedling growth. Metabolites of *Fusarium equiseti* had a similar effect as the herbicide. Filtrates containing both components caused a stronger inhibition of germination and growth. Filtrate including metabolites of *Alternaria alternata* had no effect. Metabolites in a mixture with Basagran increased germination and length of roots compared with the effect of the herbicide only.

We conclude that metabolites of *Fusarium equiseti* are much more toxic to pea seedlings than metabolites of *Alternaria alternata*. In a mixture with Basagran 600 the toxicity of metabolites even increased.

Leaf spot diseases of wheat – Pyrenophora tritici-repentis (Died.) Drechsler

Listové skvrnitosti na pšenici – Pyrenophora tritici-repentis (Died.) Drechsler

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Leaf spot diseases of wheat are caused by various pathogenic fungi. This study deals with *Pyrenophora tritici-repentis*, the causal agent of the wheat leaf spot disease known as tan spot. This disease occurs in all major wheat growing areas of the world. *Pyrenophora tritici-repentis* was also detected in the Czech Republic and its incidence has increased very significantly the last years. The fungus has probably expanded from western Europe to our country.

Tan spot on wheat can cause significant losses under suitable epidemic conditions. It is dependent on the stage of leaf damage and the loss of assimilation leaf area. Losses are manifested in total yield, kernel weight and number of grains per head.

Four factors are important for the development of the disease: presence and amount of primary inoculum, duration of leaf wetness after inoculation, stage of crop development at the time of infection and host genotype. The primary disease

cycle on wheat is initiated by ascospores, borne on infested plant residues. The secondary infection is spread by conidia, which can be transmitted very far by wind. Only a little amount of inoculum is enough for infection.

We studied the resistance of selected winter wheat cultivars to *Pyrenophora tritici-repentis* this year. The fungus was cultivated on sterile oat grains and this inoculum was applied on experimental field plots with different cultivars of winter wheat in November and in March. The first symptoms were detected at the end of April. Periodic assessment was pursued from June (every week). Diseased leaf area, total leaf area, stage of crop development and other features were observed. For the assessment of diseased leaf area the percentage scale was used.

The analysis of variance proved the differences between affected leaf area of selected winter wheat cultivars. The most resistant cultivars were Rialto, Estica, Astella, Alana, Vlasta, Arina, the most sensitive cultivar was Samanta.

Differences in the pathogenicity of Fusarium spp. on seeds and young of pea plants (Pisum sativum L.)

Rozdíly v patogenitě izolátů druhů rodu Fusarium na semenech a mladých rostlinách hrachu setého (Pisum sativum L.)

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Species of the genus *Fusarium* have mostly been studied from the viewpoint of their ability to cause diseases of many economically important crop plants (Haglund 1984). *Fusarium oxysporum* (Schlecht.) f. sp. *pisi* (Van Hall) Snyd. et Hans. and *Fusarium solani* (Mart.) Sacc. f. sp. *pisi* (Jones) Snyd. et Hans. have been reported as the most common pathogens of pea (*Pisum sativum* L.). *F. oxysporum* f. sp. *pisi* is known to cause a vascular wilt, *F. solani* f. sp. *pisi* is an important component of the complex of soil-borne pathogenic fungi causing dry root rot in pea (Tu 1994).

The pathogenicity of five monosporic isolates of *Fusarium* spp. from soil (*F. oxysporum, F. solani*) and pea roots (*F. oxysporum* f. sp. *pisi, F. oxysporum* v. *redolens, F. solani* f. sp. *pisi*) was tested on seeds and young plants of three pea cultivars (Tyrkys, Radim, SG-L-70). Two modified screening methods (inoculation of germinating seeds in Petri dishes, inoculation of seedlings grown in pots) were used. The degree of infection on the upper part of plants and on roots was evaluated according to Lebeda and Buczkowski (1986).

Significant differences in the pathogenicity in seeds and young plants were found among the isolates. The highest degree of pathogenicity of *F. solani* f. sp. *pisi* and *F. solani* was confirmed by both methods. *F. oxysporum* v. *redolens* showed to be weekly pathogenic. There is no good correlation between pathogenicity on seeds and young plants. Germinating seeds could be considered as more susceptible in comparison with young plants. No significant variations were found among isolates of the same *Fusarium* spp. obtained from soil samples and from host roots.

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Application of white rot fungi in biotechnology

Použití hub bílé hniloby v biotechnologii

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White rot fungi is a group of basidiomycetes that under natural conditions grow on wood; removing predominantly its lignin moiety they cause so-called white rot of the wood. The ability to degrade both lignocelluloses and man-made aromatic xenobiotic pollutants has brought these fungi into the focus of biotechnological application.

Cultivation of selected lignin-degrading fungi has a long history. However, during the last decades, mushroom growing has become a sophisticated and economically important food production biotechnology. It is an excellent model of closed-circle recycling biotechnology – waste of one production process (straw, saw dust, manure etc.) becomes the resource for another technology (production of edible fungi), and its possible waste – the spent substrate after mushroom collection – can serve as soil fertilizer, cattle fodder, and in some cases also as biological material for soil remediation.

Intensive research has been carried out to utilize the lignin-degrading capability of white rot fungi in pulp and paper industry. It was found that fungal pretreatment of wood chips (biopulping) results in lower energy demand and higher

quality of cellulose. White rot fungi have been introduced into the bleaching of paper pulp, and good results have been obtained in decolorization and detoxification of waste water.

White rot fungi are prospective microrganisms for the clean-up of soil polluted with different aromatic xenobiotics (chlorophenols, PCBs, PAHs, synthetic dyes, nitroaromatics, different pesticides, etc.). For the selection of fungal strains usable in soil remediation, we used a multi- step process that included: screening for the activity of ligninolytic enzymes using decolorization of model synthetic dyes, evaluation of degrading capability in liquid media, artificially contaminated soil and soil samples originating from polluted sites, and finally, testing the ability to colonize soil to be remediated.

The study was supported by grant No. 526/99/0519 of the Grant Agency of the Czech Republic.

Oyster mushroom (Pleurotus ostreatus) – its occurence, composition, active substances, and some of its physiological properties

Hliva ústricovitá (Pleurotus ostreatus) – výskyt, obsahové a účinné látky a ich niektoré fyziologické vlastnosti

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The authors investigated the occurrence of oyster mushroom (*Pleurotus ostreatus*) under natural conditions and discussed the possibilities of large-scale artificial growing of this mushroom on various lignocellulose-based substrates (straw, maize leaves, cotton, etc.). They deal with some optimal growth factors such as temperature, air humidity and proper growing technology providing maximal fructification of oyster mushrooms. We further describe the determining characteristic traits as well as the botanical classification of this mushroom species. We investigated the hypocholesterolaemic activity of the substances contained in oyster mushroom along with the specific factors capable of interfering with the metabolism of cholesterol and other fatty substances. These biologically active substances, after their application to the organism, are clearly able to decrease the level of two main risk factors of artherosclerosis, namely the concentration of cholesterol in plasma and blood pressure. Besides oyster mushroom we also deal in this study with other fungi having a similar composition and effects, such as *Lentinus edodes*, honey fungus *(Armillaria mellea)* and *Ganoderma lucidum*. We characterised and defined, as to its chemical structure, the isolate Eritadenin

and its derivatives, and also substances with strong hypocholesterolaemic activity. Besides these active substances from higher fungi we also studied some isolates of lower fungi, such as *Penicillium citrinum* and *Monascus ruber*. These substances are known as Compaktin and Monacolin K. Using chemical modifications, substances of the Lovastatin type were prepared (Mevinolin, Mevacor) that were highly effective for the investigated features and showed no serious side-effects. In the study we also discuss two of the assumed possible mechanisms of the effect of lowering the cholesterol level in blood due to the activity of biologically active substances from higher and lower fungi. It is presumed that both mechanisms work in mutual connection and in a complex way.

Degradation of polycyclic aromatic hydrocarbons by white rot fungi in soil

Degradace polycyklických aromatických uhlovodíků v půdě houbami bílé hniloby

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Polycyclic aromatic hydrocarbons (PAHs) represent a group of serious pollutants of the environment. Bioremediation is an environment friendly and cost-effective way to remove these pollutants from soil. In practice, mostly bacteria have been used for the clean-up. Over last two decades, white rot fungi have been focused on for their ability to degrade, besides lignin macromolecules, also other aromatic compounds like PCBs, PAH, chlorophenols, synthetic dyes, nitroaromatics, different pesticides etc. However, most studies have been carried out using *Phanerochaete chrysosporium*, which has become a model organism for the studies of both lignin and organopollutant degradation.

The present study was directed at the search for a fungus capable to remove PAHs efficiently from contaminated soil. The capability to produce ligninolytic enzymes was pre-screened in a set of white rot fungal cultures using a dye-decolorizing method (Šašek et al., Czech. Mycol. 50:303, 1998). Further selection was carried out on the basis of PAH degradation efficiency in liquid media. Out of six fungal species (*Phanerochaete chrysosporium, Bjerkandera adusta, Trametes versicolor, Pleurotus ostreatus, Pleurotus tuber-regium* and *Irpex lacteus*), *I. lacteus, T. versicolor* and *P. tuber-regium* significantly removed phenanthrene, anthracene, fluoranthene and pyrene. These fungal species were tested for their potential to colonize both tyndallized and untreated soil. *I. lacteus* efficiently colonized both soils. This fungus was used also for the treatment of a soil artificially contaminated with phenanthrene, anthracene, fluoranthene and pyrene (each 50

ppm). After 12 weeks of incubation, compared to the heat-killed control, the removal rates of anthracene and pyrene were close to 90 %, in the case of phenanthrene and fluoranthene they were around 50 %. In the really contaminated soil (originating from a former gas station area), *I. lacteus* removed low molecular PAHs up to 50 % during 5 weeks of incubation.

The study was supported by grant No. 526/99/0519 of the Grant Agency of the Czech Republic.

Phanerochaete magnoliae – a promising white rot fungus for decolorization of synthetic dyes

Phanerochaete magnoliae – perspektivní houba bílé hniloby pro dekolorizaci syntetických barviv

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Decolorization of model synthetic dyes has been used for an estimation of the activity of ligninolytic enzymes in white rot fungi. The low specificity of lignin-degrading enzymes suggests their possible involvement in the degradation of organopollutant xenobiotics. For this reason we screened the decolorizing ability of a set of white rot fungi. *Phanerochaete magnoliae* demonstrated quite an efficient decolorization capability. Since synthetic dyes themselves represent one of the pollutants entering the environment mostly as textile industrial effluents, we studied the ability of *Phanerochaete magnoliae* to decolorize synthetic dyes belonging to different chemical groups.

The dyes included in this study were: Methyl Orange (azo dye), Congo Red (diazo dye), Remazol Brilliant Blue R (RBBR, anthraquinone dye), Bromophenol Blue (triphenyl methane dye), Fluorescein (heterocyclic dye), and an unknown dye (extracted from industry-polluted soil). A dye mixture was prepared using all the mentioned dyes.

Agar media (malt-extract-glucose and N-limited Kirk's medium) containing all the dyes (150 ppm) were completely decolorized within 4–6 days. A further study in liquid media was made under both submerged (shaken) and static conditions at two temperatures (26 and 35 °C). Under static conditions at both temperatures a substantial decolorization (60 – 100 %) of all the dyes was observed within 10 days; at 35 °C the decolorization was more efficient and faster. In the case of the dye mixture at 26 °C, the different dyes were decolorized to a lesser extent. However, under the submerged condition no decolorization was observed. This indicates the involvement of ligninolytic enzymes in the decolorization process,

since static culture conditions have been reported to be necessary for the activity of ligninolytic enzymes.

The study was supported by grant No. 526/99/0519 of the Grant Agency of the Czech Republic.

Testing petrification solutions on selected species of micromycetes

Testovanie petrifikačných roztokov na vybraných druhoch mikromycét

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Fungal contaminants of various sculptures, canvases and frames located in the exterior or interior belong to the ubiquitous terrestrial organisms. While micromycetes are considered as universal destruents of organic matter, some of them have specific requirements, and the presence of certain chemical components in the substrate influences their growth inhibition. The physiological properties of micromycetes are utilized in practice to prevent undesirable extension, in restoration as well as protection.

In the restoration of a wooden sculpture (St. Nicolas – 18th century) after the application of three petrification solutions we registered considerable decrease in the growth of micromycetes from ten (*Aspergillus ochraceus, A. versicolor, A. ustus, Aureobasidium* sp., *Cladosporium cladosporioides, Chrysosporium* sp., *Monilia* sp., *Paccilomyces* sp., *Penicillium expansum*, *Verticillium* sp.) to five species. The micromycetes *Penicillium chrysogenum, P. glabrum, Aspergillus versicolor, A. flavus* and *Cladosporium* sp. belonged to the resistant species to the solutions used for petrification. This was also confirmed in our tests of inhibitory effects of the solutions on mixed culture of micromycetes. The species *Aspergillus versicolor* is very expansive in colonization of objects situated in depositories (polychrome wooden sculptures, wooden sculptures, canvases, frames, air and walls) and is the most resistant to the petrification solutions.

The following solutions for petrification were tested: solakryl BT-55 (used in the process of solidification, to arrange the surface and as a sticker); cloves oil (used in the process of solidification, for conservation and material impregnation); bee wax in toluene (added as a fungicide to the restoratory chalk).

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The CCBAS collection of basidiomycetes – its aims and use

Sbírka basidiomycetů CCBAS – účel a využití

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Culture collections have fundamental and growing importance not only for the preservation of the endangered gene pool, but also as a basic source of material for biotechnological processes, research and teaching. CCBAS (Culture Collection of Basidiomycetes) at the Institute of Microbiology AS CR is the only integral collection of hymenomycetes in the Czech Republic, the keeper of some endangered and rare species, the protector of patented strains. It is a basis for screening of strains possessing important metabolic activities and the supplier of cultures to different institutions in this and foreign countries. CCBAS is a member of the World Center for Culture Collections of Microorganisms listed under the number 558. The collection was founded in 1959, contains about 700 fungal strains belonging to 260 species and is continually extended. Most of the cultures belong to *Agaricales, Aphyllophorales* and *Gasterales*. The main method of the culture maintenance in CCBAS is cryopreservation in liquid nitrogen. This method seems to be the most reliable, safe and perspective for a long-term maintenance of most fungal species, especially those not amenable to freeze-drying. An electronic version of the CCBAS catalogue has been published. We have also created a home page on the Internet (http://www.biomed.cas.cz/ccbas/fungi. htm). Basic research concerning culture storage is an integral part of our work. Here is one example. A principal requirement for successful maintenance of production strains is the ability to retain their important properties (e.g. production of antibiotics, enzymes and other metabolites) unchanged. Fourteen strains of white-rot basidiomycetes belonging to eight species of two genera (*Inonotus* and *Pholiota*) were tested for their ability to keep the production of laccase, peroxidase and manganese-dependent peroxidase (enzymes involved in lignin biodegradation) after a short-time preservation in liquid nitrogen with different cryoprotectives (glycerol, dimethyl sulfoxide). No negative effect of cryopreservation or the used cryoprotective on the production of ligninolytic enzymes was found in the fungi tested.

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Micromycetes as a food resource for mites (Acari: Oribatida & Acaridia) – a survey

Mikromycety jako potrava pro roztoče (Acari: Oribatida a Acaridia). Přehled problematiky

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Micromycetes belong to the major food resource for oribatid and acarid mites in the soil and are also an important food resource for storage mites. This survey recapitulates observations on mites' fungal food preferences. Mites can utilise two fungal sources of nutrition: (1) polysaccharides in the hyphal cell walls, (2) storage polysaccharides, disaccharides and lipids. Digestive enzymes splitting these compounds are necessary for utilising fungal walls or their contents. Correlations between the enzyme production, microanatomy of the digestive tract and the ability to utilise fungal food resources are studied. Chitin and β-D-glucans are the main polysaccharides in fungal cell walls. Although the enzymes splitting these fungal polysaccharides occur in some mites, neither spores nor parts of the hyphae are digested. The hyphal walls are mechanically damaged during the ingestion, therefore an enzymatic degradation of hyphal walls is not necessary for releasing the fungal cell contents. The pieces of hyphae occur in the mesenteron. There is probably no correlation between hyphae digestion and presence or absence of chitinase. Thus, classification of feeding guilds based on this enzyme is discutable. The fungal storage polysaccharides and lipids are probably utilised by more species than fungal cell polysaccharides. The enzymes splitting storage compounds have not been studied intensively. The hyphae produce a lot of secondary metabolites, which strongly influence mites. The micromycetes preferred by mites in food preference tests need not be suitable for reproduction or individual growth of mites. The energetical contribution of micromycetes as food resources for mites is usually not examined due to methodical problems. Although the mites' food preferences are studied intensively under laboratory conditions, their interpretation as for natural conditions is difficult.

Can the genus Fusarium serve as a model organism of trichothecene production?

Může sloužit rod Fusarium jako modelový organismus produkce trichothecenů?

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Species of the genus *Fusarium* are mainly considered to be facultative necrotrophs. They are mostly organisms surviving in nature as saprotrophs which can adapt to parasitic life. We studied in particular barley where *Fusarium* caused systemic infection (the mycelium of the fungus was growing through the plants, not forming conidiophores). The genus *Fusarium* is a producer of different mycotoxins, e.g. zearalenon, culmonarasmin, trichothecene mycotoxins etc. Trichothecenes (scirpenes) cause primary toxic aleukia in man and animals and are potentially carcinogenic. Therefore we were interested in trichothecene mycotoxins, which we evaluated by gas chromatography.

We used the gas chromatograph HP-5890, serie II, which was equipped with the fused silica capillary column HP-35. In the grain and in fungal cultures the following mycotoxins were evaluated: DON (deoxynivalenol), NIV (nivalenol), DAS (diacetoxyscirpenol), T-2 toxin, HT-2 toxin and FUS-X toxin. Trichothecenes were evaluated separately in grain, malt agar with the mycelium, in macroconidia, and in the mycelium. We reached the following results, presented in the table.

Table: Trichothecene mycotoxins in *Fusarium poae* and *Fusarium culmorum* (the most frequent species of the genus *Fusarium* detected in the season 1997 — 1998). Barley — var. Rubín

Toxin (mg/kg)	NIV	DON	FUS-X	DAS	T-2	HT-2
F. poae myc.	0.058	–	0.913	–	–	–
F. poae macroconidia	0.026	–	–	0.769	–	–
F. poae grain	–	–	–	–	–	0.027
F. culm. myc.	0.058	0.696	–	–	–	–
F. culm. macroconidia	0.135	–	–	1.046	–	–
F. culm. grain	0.130	0.089	–	0.161	–	–

Detection limit: 2 mg/kg

These values represented the average of 20 measurings. It is clear from the table that the values of trichothecene mycotoxins in the examined samples were under the detection limit. It seems that the different variable and low values of these mycotoxins are not suitable for long-term model experiments. The main question for phytopathology is how these mycotoxins influence the pathogenic properties of parasitic fungi, as low amounts of trichothecenes are not accumulated in macroorganisms (vertebrates and man).

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The effect of Lactobacillus acidophilus CH5 on the genus Kluyveromyces

Vliv Lactobacillus acidophilus CH5 na rod Kluyveromyces

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The ability of neutralized and heated supernatant containing acidocin CH5 (SAC), a bacteriocin produced by the strain *Lactobacillus acidophilus* CH5, to inhibit the growth of yeast strains of *Kluyveromyces marxianus* var. *lactis* LCC 255 and *Kluyveromyces marxianus* var. *marxianus* LCC 269 in a complex medium was investigated. In a second experiment the effect of antimicrobials (NaCl, $NaNO_3$ and $NaH_2PO_4 \times 12\ H_2O$) alone or together with SAC on the yeast growth was tested.

The inhibition of the growth of both yeast strains (*Kluyveromyces marxianus* var. *lactis* LCC 255 and *Kluyveromyces marxianus* var. *marxianus* LCC 269) with the smallest used concentration of 0.1 % v/v SAC was detected (8 % to 9 % inhibition). Up to a concentration 2 % v/v of SAC the sensitivity of both strains was practically the same. At higher concentrations the sensitivity of *Kluyveromyces marxianus* var. *lactis* LCC 255 to SAC was higher than that of *Kluyveromyces marxianus* var. *marxianus* LCC 269. At 5 % v/v of SAC the first strain was inhibited by 52 % while the second one by 36 %.

Yeast strains were sensitive to antimicrobials in the concentration range applied (NaCl in a concentration of 0.1–5.0 % w/v, $NaNO_3$ in a concentration of 0.001–0.03 % w/v and NaH_2PO_4 . 12 H_2O in a concentration of 0.1–2 % w/v). The inhibition effect was the same or slightly higher with both antimicrobials and SAC (3 % v/v) than with antimicrobials only.

This work was supported by the Czech Grant Agency (Grant No. 104/98/P058).

Interactions between lactic acid bacteria and moulds in a milk environment

Interakce bakterií mléčného kvašení a plísní v mléce

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The contamination of fermented dairy products such as fermented milk and cheeses with undesirable yeasts and moulds is a serious problem. Mould contamination is responsible for causing technological problems during production

and therewith causes substantial economical losses. Additionally, it may even constitute a health risk as the result of mycotoxin production. Many fungal genera including *Penicillium*, *Aspergillus, Cladosporium, Alternaria*, *Geotrichum, Mucor, Rhizopus*, and *Fusarium* were found to be toxic to biological systems[1,2,3].

The antibacterial effects of lactic acid bacteria (LAB) have been well documented[4,5]. However, less has been published about their antifungal characteristics. The relative low number of studies on the antifungal properties of LAB thus emphasises the need for further research. Therefore, the main goal of this study was to screen LAB of different origins for their antifungal activity and to observe their interactions with moulds in a milk environment in view of their potential application in the dairy industry.

Regarding the experimental work done in the Department of Dairy and Fat Technology, 77 LAB strains of different origins were screened for their antifungal properties. The milk agar plate method appeared to be a suitable method for testing the interactions between LAB strains and moulds in a milk environment, simulating the real surfaces of fermented dairy products.

The strongest inhibition of fungal growth and spore production was exhibited by the following strains: *Lactobacillus reuteri* CCM 3625, *Lactobacillus rhamnosus* VT1, and *Lactobacillus rhamnosus* LBK7.

Both tested *Fusarium* sp. strains were more sensitive than *Penicillium* sp. and *Aspergillus* sp. to the inhibition effects of lactobacilli. The growth of *Fusarium* sp. DMF 0101 was totally suppressed by the most active lactobacilli, while only the inhibition of spore production was observed for *Penicillium* sp. and *Aspergillus* sp. strains.

The highest antifungal effect was found for lactobacilli actively growing in milk with a 0.5 % w/w yeast extract, but some effect (mainly for *Lactobacillus reuteri* CCM 3625) was demonstrated even after a 20 % v/v addition of cell-free MRS broth supernatants into the milk.

The addition of 0.5 % w/w N-sources (especially yeast and beef extracts) stimulated the antifungal effect of lactobacilli in milk.

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Soil micromycetes – biosorbents of heavy metals

Pôdne mikromycéty – biosorbenty ťažkých kovov

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The results of a model experiment are presented the objective of which was to evaluate the ability of soil micromycetes to absorb heavy metals (Pb, Hg, Cd) from humic acid as natural substrate. Biosorption of the mentioned metals by micromycetes and their influence on micro- and macro-morphological mycelium changes was studied. The cosmopolitan species *Trichoderma viride* isolated from soil was selected as a model micromycete. Microscopic fungi as universal destruents of organic matter are considered part of terrestric and aquatic ecosystems as well as other natural systems where they participate in chemical elements mobility. The humic acids as the essential component of humic substances were obtained from turf using alcaline extraction. Under experimental conditions the metals under study – Pb, Hg, Cd – remained bound to the humic substances during purification and sterilization.

A certain part of the metals was transferred into various parts of the culture where biosorption took place by the species *Trichoderma viride*. The presence of the observed metal had a negative effect in the order Hg < Cd < Pb, which corresponds with the content of individual metals analytically determined in the mycelium of *Trichoderma viride* (23.97 ppm Hg; 0.49 ppm Cd and 0.28 ppm Pb). Due to the presence of Hg a very weak air mycelium was formed, the conidiophores were branching and sporulation considerably suppressed compared to the control. The weak air mycelium and sporulation was the result of a negative Cd effect compared to the control. Addition of Pb had a weak negative effect. The cover of airy mycelium was dense and sporulation at the same level compared to the control.

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Biochemical and physiological changes during photo-induced conidiation of Trichoderma viride

Biochemické a fyziologické zmeny počas fotoindukovanej konidiácie Trichoderma viride

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Fungi of the genus *Trichoderma* are important industrial organisms mostly used for the production of polysaccharide-degrading enzymes and antibiotics. Furthermore, they are increasingly being applied as biocontrol agents against various fungal plant diseases. An interesting property of *Trichoderma* spp. is that their sporulation can be induced by light and/or starvation. Among the primary biochemical and physiological changes following illumination of the mycelia of *Trichoderma* grown in the dark are the increase in the intracellular levels of ATP and cAMP, the increased rate of respiration, phosphorylation of sporulation-specific proteins, hyperpolarization and depolarization of plasma membrane and degradation of glycogen reserves. Exogenously added cAMP as well as compounds inhibiting cAMP-phosphodiesterase or drugs stimulating adenylyl cyclase can mimick the light effect to a certain extent and induce conidiation of the mycelia also in the dark.

Comparison of secreted aspartic proteinases of four pathogenic Candida spp.

Srovnání extracelulárních aspartylových proteas čtyř patogenních druhů rodu Candida

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Candida infections cause diseases ranging from superficial disorders to invasive, mostly fatal infections in a host with reduced immunity. The major human pathogen classified in the genus *Candida* is *Candida albicans*. However, other species of this genus have been recently shown to cause an increasing incidence of mycoses. Secreted aspartic proteinases (SAPs) produced by *Candida* are believed to act as one of the virulence factors. Most of the efforts of characterization of SAPs were focused on aspartic proteinases of *C. albicans*. Studies of proteinases secreted by other *Candida* spp. are less frequent.

To examine and compare SAPs of different *Candida* spp., we collected a series of isolates of *Candida* spp. from Czech patients. The strains identified as *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. famata* were cultivated and proteinases secreted into the medium were tested for proteolytic activity with hemoglobin or peptide substrates. Cultivation conditions of *Candida* spp. were optimized and proteinases isolated using ion exchange chromatography. The stability and pH optima of the SAPs were tested. In order to design subnanomolar inhibitors of secreted aspartic proteinases of different pathogenic *Candida* spp., we designed, synthetized and tested in vitro a series of inhibitors varying in the type of scissile bond replacement as well as N- and C-terminal modification. We found inhibitors with K_i in the range of $10\text{–}9\text{–}10\text{–}1^0$ M for all the proteinases tested here. We also prepared an inhibitor containing a cyclohexylstatine residue with K_i differing by several orders of magnitude for individual SAPs. We further tested HIV proteinase inhibitors used clinically for inhibitory activity of the proteinases tested here.

Submerged preparation of sodium gluconate by a strain of the fungus Aspergillus foetidus var. pallidus

Submerzní příprava glukonátu sodného pomocí plísně Aspergillus foetidus var. pallidus

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An industrial strain of the filamentous fungus *Aspergillus foetidus* var. *pallidus*, which has a 6 to 8 -fold higher glucose oxidase activity as compared with the original strain, was improved and patented.

By submerged cultivation on synthetic glucose media, with a continuous regulation of pH by means of sodium hydroxide, glucose was nearly completely oxidatively converted into sodium gluconate. The improved strain was capable of growing also on highly concentrated substrates.

Further experiments showed, that this strain contains, in addition to the high glucose oxidase level, also a highly active invertase (β-fructofuranosidase) in spite of not being able to ultilize fructose. In fermentations on sucrose substrates, sucrose was first inverted and the resulting glucose was almost immediately converted to sodium gluconate. After depletion of glucose from the medium, the supernatant contained practically only fructose and sodium gluconate, the latter very simply isolated from the supernatant by using polar solvents.

Fermentations on highly concentrated sucrose substrates were monitored with liquid chromatography. The results showed that in certain stages of the fermentation the medium contains besides sucrose, glucose, sodium gluconate and fructose also trisaccharides and tetrasaccharides, whose level then gradually decreases so that only trace amounts remain in the medium by the end of the fermentation.

Induction of Candida albicans extracellular proteinase production by oligopeptides

Indukce tvorby extracelulární proteinasy Candida albicans oligopeptidy

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Secreted aspartic proteinases (SAPs) produced by *Candida albicans* are believed to act as one of the virulence factors playing an important role in *Candida* infection. In vitro, the SAPs are produced in presence of an exogenous protein as the sole nitrogen source, during cultivation in liquid media. The isoenzyme called SAP 2 is the major extracellular proteolytic enzyme present under these conditions. Although proteinase secretion is induced by a variety of protein substrates, the precise molecular mechanisms controlling this phenomenon are unknown.

We used a panel of peptides as putative inducers of *C. albicans* SAPs. We synthesized a series of 6-mer to 15-mer peptides derived from a fragment of epidermal cystatin A, which is a substrate of SAP 2. The extracellular proteolytic activity was examined also in the presence of random tetra-, octa- and nonapeptides. The aim of this study was to investigate if (and how) SAP production is affected by the length of peptide backbone and particular amino acid residues.

We found that the best SAP inducers were 9-, 13- and 15-mer peptides of the series tested. Simultaneously, all of these peptides were cleaved by SAP at the expected sites. However, extracellular proteinase production was induced even by peptides that are not substrates of SAP. Nevertheless, our observations indicate that SAP induction does not depend only on the presence of certain peptide or protein molecules, but rather on the level of exogenous ammonium ions.

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Antifungal activity of new Cu(II) compounds with some bio-active ligands

Antifungálna aktivita nových meďnatých komplexov s bioaktívnymi ligandami

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Many metal cations play an active role in a great number of various biological processes, especially the six-membered ring system, being a component of several vitamins and drugs. The CuX_2L_2 – X = clofibrinate (clof), 2,6-pyridinecarboxylate (2,6-pyr), salicylate (sal) or nicotinate (nic) and its derivates (isonicotinate – inic, methylthionicotinate – mnic, bromnicotinate – Brnic); L = nicotinamide (nia), ronicol (ron), caffeine (caf) – have been prepared and characterized mainly by elemental analysis, infrared, electronic and EPR spectra. Their antifungal effects have been tested on various strains of yeast and filamentous fungi. Inhibitory concentration IC_{50} and MIC were determined by microdilution methods with vigorous shaking (yeast) and by macrodilution methods during static culturing (filamentous fungi). The mutagenicity assay with *Salmonella typhimurium* TA 97 and *S. typhimurium* TA 100 was performed with $Cu(clof)_2(caf)_2$. Also its effect on energy requiring processes (biosynthesis of nucleic acids and proteins) in *Pseudomonas aeruginosa* was studied. Some of the newly synthesized Cu(II) compounds inhibited the growth of tested yeast and filamentous fungi. The strongest effects on *Candida albicans, C. parapsilosis, Rhizopus oryzae* were magnifested by $Cu(clof)_2(caf)_2$, $Cu(mnic)_2 . H_2O$, $Cu(5Brnic)_2 . H_2O$ (IC_{50} = 1.5–2.1 mmol.l^{-1}). The same compounds also induced morphological changes in growing colonies of *Alternaria alternata* at concentrations, which partially inhibited growth. The most effective compounds against *Microsporum gypseum* were copper clofibrinates and $Cu(5Clsal)_2$, IC_{50} = 1.7 mmol.l^{-1}.

$Cu(clof)_2(caf)_2$ influenced the incorporation rate of [^{14}C] adenine and [^{14}C] leucine into the biomolecules. Biosynthesis of nucleic acids indicated by the incorporation of [^{14}C] adenine was approximately twice as "sensitive" (IC_{50} = 0.380 mmol.l^{-1}) than proteosynthesis indicated by the incorporation of [^{14}C] leucine (IC_{50} = 0.642 mmol.l^{-1}). $Cu(clof)_2(caf)_2$ demonstrated no mutagenic activity.

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Occurrence and induction of β-*N*-acetylhexosaminidase in filamentous fungi

Výskyt a indukce β-*N*-acetylhexosaminidasy u vláknitých hub

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β-*N*-Acetylhexosaminidases can be used for the preparation of many glycosidic structures, e.g. Lewis antigen, selectines, glycoconjugates of aminoacids and modified chitooligomers having immunostimulatory activity.

As glycosidases of fungal origin have better transglycosidation capabilities than bacterial enzymes, we have started an extensive screening to obtain β-*N*-acetylhexosaminidases from different fungi. In the course of the screening we found slight extracellular production of this enzyme in many fungi. This production can be frequently improved by induction. An excellent inductor for β-*N*-acetylhexosaminidase is crude chitin hydrolysate (chitooligomers containing 2–10 glycosyl units). In some strains β-*N*-acetylhexosaminidase is specifically induced even by free *N*-acetylglucosamine. Further improvement of induction (almost 2×) was reached by alkali catalyzed ($Ca(OH)_2$) epimerization of chitooligomers (Lobry de Buyn-Alberda van Ekestein reaction) giving derivatives of *N*-acetylmannosamine. It was proved that *N*-acetylmannosamine and its derivatives serve as non-metabolizable inductors.

Differences in the production and inducibility of β-*N*-acetylhexosaminidase and other glycosidases led, in cooperation with the Culture Collection of Fungi at Charles University, Prague, to the re-determination of six fungal strains.

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Structural characterization and biological activity of mannans of pathogenic Candida species

Štruktúra a biologická aktivita manánov patogénnych druhov rodu Candida

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On the basis of structural analysis of cell wall polysaccharides of pathogenic *Candida* species it was found that they are mannans with highly branched structures containing α-(16)-linked D-mannosyl units in the main chain, and α-(12) and α-(13)-linked mannosyl units in the side chains. Highly pathogenic *Candida* species such as *C. albicans, C. tropicalis, C. glabrata* have a high degree of branching and

possess long side chains (penta- to hepta-mannooligosaccharides), while species showing low pathogenicity such as *C. krusei* or *C. pseudotropicalis* have low branched mannans with short side chains (tri- and tetra-mannooligosaccharides). Immunochemical cross-reactivity of the yeast strains corroborated the established structures of the surface mannan antigens.

Surface mannans and mannoproteins of pathogenic yeasts are important constituents of the adhesins that mediate specific attachment to the host cells. A correlation was found between the adherence and pathogenicity of the pathogenic yeast strains and the structure of their surface polysaccharides. Adherence of the yeast cells to host tissue is an important initial step in the pathogenesis of candidal infections. We have found that several mannans show a strong inhibition of adherence of *C. albicans* to human epithelial cells. Some mannans were investigated for inhibition of plant viruses and revealed high protective activity.

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Changes in glucan- and chitin synthase activity in Trichoderma viride caused by illumination

Zmeny v aktivite glukán- a chitinsyntázy Trichoderma viride spôsobené osvetlením

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In *Trichoderma viride* a number of biochemical and developmental responses are controlled by blue light. We have investigated the response of the cell-wall biosynthesizing enzymes glucan synthase and chitin synthase to illumination.

We found that a 10 min. illumination of mycelia with white fluorescent light stimulated glucan synthase activity by about 230 % in relation to the dark control and caused a decrease of chitin synthase activity by about 50 % in relation to the dark control. These changes were probably caused by *de novo* synthesis of

corresponding synthases and their regulatory components since they were not observed in the presence of 50 μg/ml cycloheximide.

Preparation, characterization and antimutagenic properties of chitin-glucan from Aspergillus niger

Príprava, charakterizácia a antimutagénne vlastnosti chitín-glukánu z Aspergillus niger

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In recent years increased attention has been paid to β-glucans isolated from the cell walls of the yeasts and moulds that act as non-specific modulators of the immune system and are applied as immunoadjuvants, antitumor agents, etc. The cell wall of *Aspergillus niger* contains predominantly glucan, chitin and galactomannan. The alkali-soluble fraction obtained from the cell wall is composed of α-(13)-D-glucan, galactomannan and glycoprotein. The alkali-insoluble fraction contains a covalently linked complex of β-(13)-D-glucan and chitin which probably plays a critical role in fungal morphogenesis.

The crude, water-insoluble chitin-glucan complex was isolated from cell walls of the industrial strain *Aspergillus niger* by hot alkaline (1 m NaOH) extraction and after derivatization with monochloroacetic acid, carboxymethylated chitin-glucan (CM-CG) with M_w 220 kDa was obtained. A protective effect of the high molecular CM-CG complex (220 kDa) against cyclophosphamide (CP)-induced incidence of micronuclei in reticulocytes of mice was demonstrated after intravenous and intraperitoneal administration. However, this effect was not observed when CM-CG was administered orally. Oral pre-treatment of mice with original high-molecular CM-CG did not decrease the clastogenic effect of CP. The reason for such ineffectiveness of the orally administered CM-CG probably lies in its inability to pass to the bloodstream from the gastro-intestinal tract. In order to overcome this hindrance, a CM-CG$_I$ fragment with lower molecular weight (19 kDa) was prepared. The anticlastogenic effect of CM-CG$_I$ with ultrasonically lowered molecular weight against CP also by oral administration was confirmed.

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DNA vaccines – new strategic possibilities in the prevention and prophylaxis of mycotic infections

DNA vakcíny – nové strategické možnosti v prevenci a profylaxi mykotických infekcí

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The therapy of many mycotic diseases is time-consuming and often imperfect. Although fungi are not frequent infectious agents, in consequence of the advance in medicine their importance increases together with the number of long-term immuno-suppressed patients. Also rising migration of people increase the frequency of patients infected with endemic infectious agents (e.g. *Histoplasma capsulatum*) normally eliminated through natural resistance in local populations.

At present the treatment of mycoses is based on the application of some antimycotics (either locally or systematically) according to the type of mycose. Another method is based on the construction of vaccines derived from proteins which play an important role in the pathway of infection. Promising results are reached for example in the application of recombinant Heat Shock Proteins HSP60 or HSP70 or their fragments derived from *H. capsulatum* in mice. Some efforts to prepare a multispectral vaccine based on surface glycoproteins have not been successful, but this option is presently discussed as a promising immunomodulation. Modern molecular biology offers an alternative in the prevention of particular infections – DNA vaccination. Because resistance to mycoses is supposed to be based on the activity of cytotoxic lymphocytes, DNA vaccination seems an optimal option.

A lot of tests with DNA vaccination confirmed the hypothesis of stimulation of specific cytotoxic T-lymphocytes after vaccination in mice, rabbits, but also some non-primate monkeys. DNA vaccine is a plasmid carrying target protein coding gene suspected to be an important factor of pathogenity. For the vaccine function of this plasmid it is important that after administration into a vaccinated organism the gene inserted into the plasmid is expressed by the expression apparatus of the host cells. The particular protein product is presented on the cell surface to the immune system as main histocompatibility complex I – restricted and stimulates immune response by cytotoxic T lymphocytes.

Our target organism was the filamentous fungus *Trichophyton mentagrophytes*, a causing agent of dermatophytoses. We were concerned with the possible role of the HSP60 based cDNA vaccine and its protective effect in mice. According to the above mentioned presumptions a 800 bp long DNA fragment suspected to be HSP60 gene part was prepared and inserted into a cloning vector. We then tested the optimal vaccination vector for an immunological response test in mice.

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Molecular epidemiology of nosocomial candidiasis – the use of PFGE and RAPD

Molekulární epidemiologie nozokomiální kandidózy – využití PFGE a RAPD

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Candida yeasts cause life threatening mycoses in immuno-deficient patients. Hospital units where these patients are treated may be in danger of mini-epidemics. In such cases powerful and fast typing techniques are essential for the discrimination between true epidemics and coincidental clusters of infection. Further surveillance of the health care personnel and environment makes eradication of potential causes of infection possible. DNA-fingerprinting is evaluated as the most powerful typing method, whereas Pulsed Field Gel Electrophoresis (PFGE-typing, karyotyping) and Random Amplification of Polymorphic DNA (RAPD) are classified as more cost-effective and less time consuming alternatives. It is recommended to use at least two typing techniques to increase the reliability of results.

We typed 51 isolates from 30 patients using PFGE, 25 of which were *C. albicans*, 9 *C. glabrata*, and 17 *C. lusitaniae*. For comparison, 18 of these isolates were typed using RAPD. Optimization of PFGE parameters allowed us to obtain very well-defined differences between strains of *Candida* yeasts, even in the case of *C. albicans* isolates, for which some authors question the discriminative power of PFGE. There were no discrepancies between the results of PFGE-typing and RAPD-typing. Colonization by different strains was detected in some cases where isolates from different sites of the same patient were typed. Our results demonstrate very well the discriminative power of PFGE-typing.

Thie research was supported by grants CEZ J14/98: 15110004 and 15110002 and by the internal grant of the Faculty of Medicine, Palacký University No. 11701102.

The effect of the optical brightener Rylux BSU on spore germination rate and spore survival after UV irradiation in Trichophyton mentagrophytes.

Vliv optického zjasňovače ryluxu BSU na klíčení a na přežívání spor po působení UV záření u Trichophyton mentagrophytes.

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We tested the effect of the optical brightener Rylux BSU on the germination rate of spores of *Trichophyton mentagrophytes*. Spore suspensions were exposed to various concentrations of brightener in distilled water and incubated on a reciprocating shaker in darkness at laboratory temperature for 24 hours. The suspensions were then diluted and spread on the surface of SGA. The stimulation effect of Rylux was confirmed. The spore germination rate was increased to 105 % of the control at 0.1 % (w/v) and to 129 % of the control at 0.2 % (w/v) Rylux BSU concentration. Higher Rylux BSU concentrations decreased spore germination rate. We further tested the effect of Rylux BSU on the killing effect of UV radiation. A UV dose which decreased the germination rate of the spore suspension to 18 % of the control was applied. If the spore suspensions were treated with Rylux BSU prior to UV irradiation, considerably more colonies were formed. The decrease in killing effect and/or the increase in spore germination rate if compared with the control was as follows: 1.6 at 0.1 % (w/v) Rylux BSU, 2.2 at 0.2 % (w/v) Rylux BSU, 1.0 at 1 % (w/v) Rylux BSU, 3.1 at 5 % (w/v) Rylux BSU, 3.9 at 10 % (w/v) Rylux BSU, and 4.0 at 15 % (w/v) Rylux BSU. If spore suspensions were treated with Rylux BSU, the UV mutagenization resulted in a significantly higher variability in colony morphology, growth rate, sporulation, pigmentation, surface texture and colonial form.

The observed stimulatory effect of Rylux BSU on germination rate could be useful to improve the sensitivity of detection of pathogenic fungi in clinical specimens and to increase the recovery of mutants in UV induced mutagenesis.

Stress proteins in fungi

Stresové bílkoviny u hub

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Cell and molecular biology studies of the stress response have become a self-contained field with many connections to the basic questions of living systems. On

the other hand, knowledge about stress proteins has opened a new era of their practical exploitation. In this context, fungi play two important roles: 1) as model organisms and 2) as important pathogens for humans, animals and plants.

In this review the roles of stress proteins in individual fungi and their families are discussed. Special interest is focused on the stress proteins of pathogenic fungi, which could be immunodominant antigens, and their potential use in DNA vaccine development.

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Effect of peroxynitrite on resting spores and germlings of Aspergillus fumigatus in vitro

Účinek peroxynitritu na dormantní a klíčící spory Aspergillus fumigatus

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Our previous investigations (Kunert 1995) led to the conclusion that nitric oxide (NO^-) is not a major effector molecule in the killing of *Aspergillus fumigatus* conidia by phagocytes. Recently, peroxynitrite ($ONOO^-$), a strong oxidant arising in the reaction of nitric oxide with superoxide (O_2^-), was shown to be the principal candidacidal molecule of activated macrophages (Vázques-Torres et al. 1996). We tested the killing of resting and swollen conidia of *A. fumigatus* by authentic peroxynitrite and a peroxynitrite donor, SIN-1 (3-morpholinosydnonimine). The decrease in colony-forming units after treating the conidia was evaluated. Inhibition of germination and growth by both effectors was also tested on conidia germinating in nutritive media in microtitration plate wells. Authentic peroxynitrite did not kill any resting or medium-swollen (4 h) conidia in concentrations up to 6.25 mM. Germlings (after 6 and 9 h in the glucose-peptone medium) were only slightly inhibited in further growth when peroxynitrite (5 mM final concentration) had been added. Likewise, SIN-1 (up to 10 mM, 24 h in pH 7 buffer) did not kill any conidia. However, it had an inhibitory effect on germination and growth, depending on the medium. In the glucose – sodium nitrate medium 5 mM SIN-1 slowed down the germination and 10 mM SIN-1 stopped it completely for 20 hours. This effect was, however, only fungistatic and all spores germinated in the fresh medium. In a rich (glucose-peptone) medium the effects were much weaker. In ten other strains of *A. fumigatus* from the Czech Republic, Slovakia, Germany and France the effects of peroxynitrite and SIN-1 were very similar to those in the model strain.

One *Candida albicans* strain used for comparison was much more susceptible to peroxynitrite and its donor than *A. fumigatus*. E.g., 2.5 mM peroxynitrite killed all yeast cells in 2 hours.

The results indicate that peroxynitrite is even less effective against conidia and germlings of *A. fumigatus* than its precursor molecule – nitric oxide. Therefore it does most probably not contribute substantially to the fungicidal activity of phagocytes against the above fungus.

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Conserving of microscopical fungi for collection and demonstration

Uchovávání kultur mikromycetů pro sbírky a demonstrace

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The author describes methods used for establishing collections of microscopical fungi and conserving their typical macroscopical and microscopical traits. This is important for comparison with further isolates as well as for purposes of teaching.

Sabourauds glucose agar enriched with thiamine and histidine and cultivation at 27 °C are recommended for reference cultures. The "macroculture method" includes inoculation of the fungi onto 3 mm thick layer of the medium in plastic Petri dishes. Mature, typical colonies are fixed in formaldehyde vapours and the medium dried to one half of its original thickness. Petri dishes are sealed airtight with acrylate glue and kept at room temperature in dark. In the authors laboratory, prepared colonies are stable for at least ten months. Only about 15% of the cultures had to be discarded because of contamination or insufficient fixation.

For microcultures the fungi are cultivated on thin agar blocks covered with microscopical cover glasses, fixed with methanol and mounted in a gelatin medium. Such cultures can be kept for a long time; in the authors laboratory 15 years old preparations are still being used.

The chitin synthase inhibitor Nikkomycin Z causes disorganized cell wall synthesis in hyphal apices of Basidiobolus ranarum

Inhibitor chitin synthas Nikkomycin Z způsobuje dezorganizaci polarizované syntézy buněčné stěny u hyfových vrcholů Basidiobolus ranarum

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Chitin plays an important role in fungal cell wall rigidity. Stimulation of chitin synthesis was detected in cases of cell wall damage, in mutants with cell wall defects and in response to hypotonic shock. Nikkomycin Z is a tripeptide which inhibits chitin synthases competitively. In the yeast *Saccharomyces cerevisiae* K_i varies for different chitin synthase isozymes between 0.21 and 890 μM. In the filamentous fungi *Mucor plumbeus* and *Geotrichum candidum* 1 μM Nikkomycin Z was described to induce swelling of hyphal apices.

We tested the effect of Nikkomycin Z in the filamentous fungus *Basidiobolus ranarum*. The effect of Nikkomycin Z was concentration-dependent. The highest concentrations of Nikkomycin Z caused immediate swelling of hyphal apices and lysis. Intermediate concentrations of Nikkomycin Z caused formation of large spherical cells with irregular cell wall deposits formed at the inner side of the cell wall after prolonged exposition (24 hours). The lowest effective Nikkomycin Z concentrations caused only minor changes in growth pattern – the hyphae were not as straight as in the controls and dumbbell-like cells were observed. The lowest effective concentration of Nikkomycin Z was determined to be 0.5–1 μM.

Formation of irregular cell wall deposits after prolonged exposition to Nikkomycin Z indicates that the inhibition of chitin synthases causes spatial disorganization of cell wall synthesis and induces cell wall synthesis. As the material of these deposits was stained with Rylux BSU very intensively, it might consist of chitin. However, this has to be proved by chemical analysis.

Position and prospect of Slovak mycology at the turn of the second millennium

Postavenie a výhľady slovenskej mykológie na prelome tisícročia

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Slovak mycology was immediately connected to Czech mycology until the last decade of this century. In the future this historical fact must always fully be respected and correctly interpreted. Today mycology in Slovakia already has

a comparatively rich tradition. Its roots reach down to the past centuries from which the first information on the occurrence of fungi on this territory originate. History shows that the territory of Slovakia, already in the past time, was very interesting in respect of mycology.

The results attained during the last 40 to 50 years document the good level of Slovak mycology which gain respect and appreciation among biological disciplines. In the last years a big advance was made in the field of promotion of macromycetes and prevention against poisoning by poisonous fungi. To the progress of this field largely contributed not only professional mycologists but also amateurs.

At present scientific mycology is developed at the institutes of Slovak Academy of Sciences, universities and some departments of institutes focused on phytopathological mycology in agriculture and forestry. Many research tasks are investigated through scientific projects. Remarkable results were attained by studies of physiology, biochemistry and genetics of fungal pathogens of cereals, sugar beet, grapevine, tobacco, introduced and native trees as well as medicinal plants. It is necessary to inform on the significant results of research in the field of dermatomycoses and mycotoxins. Slovakia attained a prominent international position by significant results of the study in the field of yeasts. Rightly can be spoken of a Slovak school in basic and applied research. Similar results were attained in the study of wood-destroying fungi. In the future attention need to be payed above all to the study of fungal systematics with regard to elaborated mycoflora of Slovakia. The systematics of fungi must be developed on modern basis, i.e. to bring the morphology and anatomy of fungi, close to general mycology, to pay more attention to fungal physiology and ecology.

New possibilities and demands of the research are unceasingly opened in the field mycogeography. It appears that bigger space in research must be provided for micro- and macromycetes because their mycelium forms mycorrhiza with forest trees, shrubs and plants.

For the future an urgent need appears to be the study of theoretical and practical questions of fungal physiology and genetics.

Attention must be paid to the collection of vital fungal cultures and mycosociology.

To fulfil the given theoretical and practical questions it will be necessary to expand special staffs of mycologists, support their further specialisation and coordinate the mycological research according to the demands of the newest scientific knowledge.

Book review

H. L. Barnett & Barry B. Hunter:

Illustrated Genera of Imperfect Fungi. 4^{th} Ed.

APS Press, St. Paul, Minnesota, USA, 1998, 218 p., ISBN 0–89054–192–2.
(The book is deposited in the library of the Society.)

The book "Illustrated Genera of Imperfect Fungi" by H. Barnett was for a long time a very useful manual of a large group of conidial fungi. Its third edition was published in 1972. In the new, fourth edition Barry B. Hunter has included several additions: a general introduction to the imperfect fungi, a simplified key to common fungal genera and 24 photos (mainly scanning electron micrographs).

The book is devided into an introductory section and two parts. The introductory chapter deals with the major group of imperfect fungi and their importance in the biosphere. It is followed by rather short chapters on cytological and morphological features and on factors affecting growth and sporulation.

The first part ("Physiology") is short, too. It covers methods of isolation, culture media, maintenance, nutrition and examples to illustrate some biological principles. The latter chapter is very interesting for student practices.

In the second part ("Taxonomy and identification"), the old Saccardoan system of four form orders is followed: *Moniliales, Sphaeropsidales, Melanconiales* and *Mycelia Sterilia*. Members of the *Mucorales* (*Zygomycetes*) are also included because of their similarity to some imperfect fungi. In this part separate keys of each of the five orders are presented. These keys are followed by the newer Hughes-Tubaki-Baron system of classification completed with an alternate key based on the mode of conidial formation and with scanning electron micrographs. The photos are practical and apposite. Unfortunately, the term "aleuriospore" is treated, for a long time widely used in various senses, but at present considered a confusing term. On the other hand, new terminology connected with this group of fungi (i.e. mitotic fungi, anamorph, teleomorph) is lacking.

The most important chapter of this book is "Description and illustration of genera". This part covers over 400 genera. In contrast to the 3^{rd} edition, some infrequent fungal genera were omitted. Each genus account includes a short morphological description, drawings and references. In this basic chapter, however, some shortcomings are visible. For example, an alphabetical ordering of genera (or another type of arrangement) within the individual orders (or other groups) would have been very useful. Some drawings are not too reliable, e.g. in the picture of *Aureobasidium* hyaline conidia are missing. Although data on teleomorphs are given in some genera (e.g. *Chromelosporium*, *Chalara* and *Sphaceliа*), they are missing in others (*Botryotrichum, Sporothrix, Scopulariopsis, Trichophyton* etc.). Several taxa are listed under old names, although they are not accepted today (e.g. *Cephalosporium* instead of *Acremonium*, *Monilia sitophila* instead of *Chrysonilia sitophila, Fusoma* — doubtful name). Very frustrating are the references. Literature references from 50^{th} to 70^{th} are prevailing. However, in the past twenty years an extensive development has been observed in this area. Many recent and even less recent references were omitted (monographs on *Acremonium* and *Verticillium, Penicillium, Aspergillus, Fusarium* etc.). Also some other errors can be found in the book: in *Penicillium* the same reference is mentioned as in *Aspergillus*, *Nodulisporium* is referred to as *Nodulosporium*, *Papulaspora* as *Papulospora*. The book is completed with a very useful glossary with examples of fungi.

Comparing this book with the similar "Genera of Hyphomycetes" by Carmichael et al., there are apparent differences in the extent of information and drawings, which are more detailed and successful in Genera of *Hyphomycetes*.

Despite the above mentioned criticism, the book is a practical identification guide and will provide significant basic information to all friends of microfungi. The unique combination of micromycetes belonging to different groups (*Zygomycetes*, mitotic forms of *Ascomycetes* and some *Basidiomycetes*, too) as well as listing both saprotrophs and parasites is very useful for users, because they are often specialized in a particular taxonomic or ecological group of fungi.

Alena Kubátová

Book review

S. S. TZEAN, L. S. HSIEH AND W. J. WU

Atlas of Entomopathogenic Fungi from Taiwan

Council of Agriculture, Executive Yuan, Taiwan, R. O. C., 1998. 214 pages.
ISBN 957-97217-6-9. Price 50 USD.
(The book is deposited at the Library of Dep. Bot., Fac. Sci., Charles University, Prague)

This book is another well-produced atlas from Taiwan (following e.g. Tzean et al., 1990, 1994). It is part of the output of the project "Exploration and application of insect microbial pathogens". The book focuses on fungal pathogens belonging to the *Zygomycota*, *Ascomycota* and *Deuteromycota* and fills the gaps in the knowledge of tropical and subtropical entomopathogenic fungi.

The book is divided into several chapters. The introduction deals with occurrence and impact of arthropod-pathogenic fungi, taxonomy, pathogenesis and gives a review of contributions from Taiwan. In this chapter, some members of *Chytridiomycetes* and *Oomycetes* are mentioned, too. As the authors mention in the chapter Material and methods, this work is based on over 830 fungal-infested cadavers of insects, spiders, and mites they collected during the period 1988 to 1996 in various niches of different parts of Taiwan. This chapter is supplemented with a very useful survey of references important for the identification of individual genera of entomopathogenic fungi. It is followed by a chapter on the occurrence of entomopathogenic fungi in Taiwan with a list of herbarium specimens.

The main part of the book is opened with genus descriptions and keys to genera and species. The most important and extensive part contains detailed species descriptions and illustrations of 66 species, 37 of them newly recorded from Taiwan. This part also includes four new species, one new variety and one new combination. The figures include high quality black-and-white micrographs of fungi, colour photographs of insect specimens with fungal cover and very instructive drawings of morphological structures of the microfungi. Each species is clearly described and illustrated on two pages. Many interesting fungi are covered, for example *Neozygites floridana* from mites, *Cordyceps* spp. from ants and other arthropods, *Torubiella* spp. from spiders and other hosts, *Aschersonia* spp. associated with a homopteran scale insect and whitefly, *Gibellula* attacking arachnids and *Beauveria*, *Metarhizium* or *Paecilomyces* spp. known also in our region.

The book is supplemented with an extensive bibliography and a helpful index of athropod hosts and entomopathogens.

The Atlas of Entomopathogenic Fungi from Taiwan is a lavishly illustrated book with 134 figures and its content is well in agreement with the title of the book. This beautiful atlas will be a very useful guide for all mycologists interested in microfungi as well as for entomologists and workers investigating biocontrol.

Alena Kubátová

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Prof. Dr. Zdeněk Urban, DSc passed away

VÁCLAV ŠAŠEK

On January 4, 2000 the Czech mycological community lost a prominent personage, Zdeněk Urban, professor of Botany at the Department of Botany of the Faculty of Science, Charles University in Prague. Prof. Urban was associated with this Department for practically his entire scientific and pedagogical life. After finishing university studies (1945–1949) he joined the Department of Botany as a PhD student (1951–1954), continued his work there as an assistant professor, from 1967 as an associate professor, and in 1984 he was nominated Professor of Botany. In the period 1971–1984 he was Head of the Department of Botany.

Prof. Dr. Zdeněk Urban, DrSc. Sept. 30, 1986
Photo Dr. František Kotlaba

Zdeněk Urban was born on July 11, 1923 at Opatovice nad Labem near Pardubice. Already during his studies at Pardubice Gymnasium he started to be involved in natural science taking part in meetings of the Natural History Club under the Museum in Pardubice. Influenced by Dr. Emil Hadač, he took aim at mycology that became his main field of interest. Prof. Urban dedicated most of his work to rusts and smuths — their distribution, morphology, taxonomy and biology. Most of his publications deal with distribution and ecology of graminicolous rusts that was also the topic of his DSc thesis (1975). However, he studied also other fungal groups. For instance, his PhD thesis (1956) was a revision of stromatic, lignicolous pyrenomycetes in the former Czechoslovakia and their phytopathological significance. Prof. Urban had no hobby, his only interest were fungi. He spent most of his weekends in the countryside collecting graminicolous rusts for his life-work on the distribution of *Puccinia* species in the Czech Republic. After he retired in 1988, he would regularly go to the Department of Botany to continue his mycofloristic research and to take part in several research projects.

Prof. Urban spent a lot of time on organising mycological research. For many years he was vice-president of the Czechoslovak Scientific Mycological Society and editor-in-chief of its journal Česká Mykologie (now Czech Mycology).

I knew Prof. Urban for more than 40 years. I met him first in the late 1950s as a mycology student and from that time our friendship grew. We often discussed a broad spectrum of mycological problems and different questions of

social development in our country, sometimes sitting in a winehouse with a glass of a red wine. The last year of his life I regularly visited him at his home because due to serious malady he was not able to leave his house. It was very mournful to see him gradually pass away. I admired his wife Věra, who, though also ill, took care of her husband until his last day.

With the death of Prof. Zdeněk Urban the science of mycology in the Czech Republic has lost a significant representative and some of us a good friend.

Bibliography of prof. RNDr. Zdeněk Urban, DrSc. since 1973

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