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«TRAINING ACTIVITIES ON FOOD CONTAMINATION CONTROL  
AND MONITORING WITH SPECIAL REFERENCE TO MYCOTOXINS»

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TOXIN-FORMING IMPERFECT  
FUNGI AND THEIR CULTIVATION  
IN THE LABORATORY



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Numerous data on the contamination of agricultural food material, food stuffs, and fodders with spores of various microscopic fungi can be found in the literature. Improper storage of these substrates, which most frequently occurs during the harvesting of vast masses of grain with high humidity, may cause the intense development of many species of mould fungi. In a number of cases, this is accompanied not only by changes in the chemical composition of the contaminated substrate and its processing properties, but also by the accumulation of the toxic metabolites (mycotoxins) of a number of fungi.

One of the most well known mycotoxicoses of alimentary origin is ergotism connected with the consumption of bread made of grain affected by ergot, the Claviceps purpurea fungus.

Most of fungi capable of forming mycotoxins belong to the group of the so-called imperfect fungi (Fungi imperfecti, Deuteromycetes). This vast group of fungi, numbering up to 25,000 described species, includes fungal forms with a multicellular mycelium containing no higher sporophores. The imperfect fungi represent at times sterile mycelia without any reproductive organs. More frequently various conidial sporophores are developing on them. Established for individual species of the imperfect fungi is an ascosporic stage which usually has its special name; however, in most fungi no such stage has been revealed and they represent true Fungi imperfecti.

The imperfect fungi exceed all other groups not only in the number of species but also in the number of individuals. They occur both in saprophyte and parasite conditions and are distinguished by the capacity for rapid and copious growth and hardiness in regard to environmental conditions. Representatives of various species of imperfect fungi are capable of growing in a broad range of temperatures, from 0 to 50°C, and at pH 2-9 and beyond. Owing to a rich choice of various hydrolytic enzymes, they are capable of populating most diverse substrates. All these properties of mould fungi has ensured them a broad distribution in nature. In his practical activities, man constantly has to take measures to prevent the development of mould fungi not only on agricultural materials, foods, and fodders but also on many other materials of the organic nature.

Of the greatest importance for the contamination of food and fodder substrates with mould fungi are temperature and relative humidity. The humidity which favours practically no contamination of grain is 14.5-15.5% for wheat, rice, and barley, 12-14% for maize and millet, 10-11% for low-oil sunflower seeds, 6-9% for high - oil sunflower seeds, 8-9% for flax seeds. The humidity being constant, the degree of grain contamination differs with temperature.

Imperfect mould fungi are distinguished for their potential to form numerous different organic compounds, many of which are toxic to man and animals. Among the imperfect fungi known as producers of mycotoxins, one should note first of all the fungi from the Aspergillus and Penicillium genera and also various species of the Fusarium, Alternaria, Trichotecium,

Stachybotrys, Helminthosporium, Trichoderma, Cephalosporium,  
Myrothecium genera.

Though the main habitat of the fungi is the soil, the number of toxin-forming strains in it is usually not very great. The source of mass distribution of toxin-forming fungi causing the outbreaks of diseases in man or agricultural animals, are the substrates attacked by them. Toxin-forming fungi may be found on the surface of plants and will damage them during harvesting and storage under conditions during their development, or attack the grain during vegetation, as for example, is done by the phytopathogenic species of Fusarium, or the Aspergillus flavus and A. parasiticus fungi.

High on the list for the frequency of revealing toxigenic strains and for the hazard to man and animals caused by mycotoxins are the mould fungi of three genera - aspergillus, Penicillium, and Fusarium. These fungi include producers of various mycotoxins, and not infrequently individual strains of one and the same fungal species may form different toxins. Meanwhile, far from all the strains of those fungal species, which are known as producers of definite mycotoxins, possess toxigenic properties. The toxigenic strains of mould fungi occur in nature considerably more seldom than could be expected, and it is important to bear this in mind when drawing up forecasts regarding the relative hazards represented by toxigenic fungi. For the same reason, the screening of toxigenic fungal strains and the study of their prevalence in food and fodder substrates is one of the most important tasks in solving the problem of mycotoxins.

Another no less important task consists in working out ways and means of cultivating toxigenic fungi in the laboratory providing optimum conditions for the formation of mycotoxins by them. Without fulfilment of these tasks, it is impossible to study toxigenic properties of fungal isolates nor to understand the pathways of the biosynthesis of mycotoxins by fungi. Moreover, studies along these lines create the necessary prerequisites for obtaining sufficient quantities of mycotoxins needed for studying their chemical nature and biological mode of action.

In this connection let us examine the microbiological methods of the detection and isolation of mould fungi from food and fodder substrates contaminated by them, and also of the formation of pure cultures which can be cultivated under laboratory conditions with due regard for their specific biological features.

Detection of Fungi and Their Isolation  
From Food and Fodder Substrates (2,3)

When substrates are sent for examination with obvious indications of their contamination with microscopic fungi, i.e. with altered organoleptic properties and signs of moulding, the detection of fungi responsible for their deterioration may be carried out by direct microscopic examination of the substrate. For this purpose a fragment of the mouldy zone scraped off with a scalpel or bits of the product itself are examined in a drop of water at low microscopic magnification, which, in a number of cases, enables the detection of the hyphae and sporophores of the fungus and establish its genetic

and, sometimes, specific identity.

In the presence of surface sporophores and easily washed off fungal spores, one may use the method of spore washing-off. The test material is ground and placed in a flask; water is added to cover the material and the flask is shaken for 20 minutes. A drop of the thus obtained suspension is examined under the microscope. If the suspension is insufficiently concentrated, the fluid is decanted and centrifuged to give a precipitate which is then studied microscopically.

These tentative methods establish the genus and sometimes the species of the fungus affecting the substrate but do not provide information on its toxigenic properties nor do they permit a quantitative evaluation of the degree of the substrate's contamination with the fungi or the isolation of the fungi into a pure culture.

For obtaining a more complete information during mycological analysis of the substrate, various nutrient media are inoculated with the test material; this is followed by isolation of the pure fungal cultures. For isolating and cultivating the fungi in the laboratory, standard artificial media are routinely used, such as the Czapek agar and the Czapek-Dox agar, sometimes with the addition of peptone or yeast extract, malt agar, wort agar, potato agar, potato-dextrose agar, agar with sucrose and yeast extract, and a whole number of others.

The isolation of fungi from agricultural material, foods, and fodders is hampered, as a rule, by bacterial growth. The most efficient means for preventing it is the increase of the acidity of the nutrient medium. Most of fungi need for their

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development pH 4.5 to 6.8, occasionally to 3.5, whereas saprophyte bacteria develop at a pH 7.0 and higher. Mineral acids (hydrochloric, orthophosphoric) and particularly organic acids (citric, lactic, more seldom tartaric) are used for acidification. Also used for suppressing bacterial development are nutrient media containing antibiotics: streptomycin (30/4 g/ml), aureomycin (2  $\mu$  g/ml), tetracycline (30 ppm) and others.

The isolation of Aspergilla and Penicillia from the substrates is frequently hampered by Mucor fungi. For suppressing their growth, L.I.Kursanov (4) recommended to add a 0.1-0.15% solution of copper sulphate to the nutrient medium after sterilization.

There are several different approaches to the mycological analysis of test material, depending on its special features and physical properties.

Sometimes it becomes possible to isolate fungi which had developed on a food or fodder substrate by a direct passaging of the fungal spores from the mouldy specimen to the nutrient agar with a loop. For example, Davis et al. (5) when screening toxigenic fungi in food stuffs, isolated a number of mould fungal cultures by direct inoculation from the foods into an agarized medium with dextrose. Then the fungi were isolated into a monoculture at room temperature (25-30°C) either on a Czapek-Dox Medium or on a potato-dextrose agar, and identified.

The method of direct inoculation was also successfully used when analysing samples of mouldy cheese (6). The spore material was transferred from the test samples with a loop

moistened in a 0.05% solution of Tween-80 onto a potato-dextrose agar containing 30 mg/kg of tetracycline. Methods of this kind may be used, however, only in cases of evidently moulded test material.

When the fungi are being isolated from grain, cereals, legumes, or seeds, the surface microflora (fungal spores and other elements of the mycelium which are present on the surface only) is often determined together with the in-depth contamination.

For detecting the surface microflora, the grain is covered with a definite quantity of water to which a wetting agent (Tween-20 or Tween-80) is added and the mixture is shaken to produce a spore suspension, after which it is transferred onto the surface of a solid nutrient medium or introduced into molten agar cooled to 45°C, then poured into Petri dishes; the agar is set and the dishes are incubated at a definite temperature.

For studying the in-depth contamination, the surface of the grains is first disinfected. For this purpose, the grain are placed for a period from 30 minutes to 2 hours into a 1:300 formalin solution or for 1 minute into a 1% solution of sodium hypochlorite; after that the grains are washed in sterile water. A 1:1000 solution of mercuric chloride or silver nitrate or a 0.5% solution of potassium permanganate can be used for the same purpose. After disinfection, the grains are cut longitudinally with a scalpel and from 5 to 10 grains, depending on their size, are placed into the agar surface in Petri dishes. The total number of grains

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should be at least 50.

For practical purposes, the in-depth and the total mycoflora of the grain is determined. For finding the total mycoflora, the nondisinfected grains are placed on the surface of a nutrient medium. The first to appear will be colonies of fungi present on the surface of the grains and latter those which developed within the grain tissues. Grown fungal colonies are studied under the microscope; rather frequently, not only the generic but also the specific identify of the fungi can be determined already at this stage and their isolation into a pure culture performed. For a quantitative characterization of the fungal contamination, the grains around which colonies of certain fungal species had developed are counted and the percentage of these grains calculated.

Two methods are used for detecting fungi in bulk products. The first one (direct inoculation) consists in that a weighed portion of the test material is placed in small mounds on the surface of the agar - 10 mounds per dish. With this method it is difficult to produce isolated colonies, which hinders the identification of the grain fungi and a quantitative assessment.

The second one (the pouring method) is used for examining cereals and legumes; these products are preliminarily ground to obtain a homogeneous mass. The method is used also for the analysis of liquid material.

A definite amount of the ground substrate (1-10g) is diluted with sterile water or saline containing a wetting agent to obtain a 1:10 dilution. If a high content of fungal spores and mycelium is expected in the material, 1 ml of the suspen-

sion from the first dilution is diluted again to obtain such dilutions as 1:25, 1:50, 1:100, etc. to 1:1000 and 1:10000. Then 1 ml of the suspension of the required dilution is distributed on the surface of the solid nutrient medium with a spatula or introduced into a sterile Petri dish and covered with cooled molten agar so that the material is evenly distributed in the medium.

There are different modifications of these basic techniques. Thus, in Japan, when analysing yellow rice, two approaches were followed (7,8).

In one case a 10-20 g of rice specimen was 20 times washed off in sterile water (total amount of water 1 600 ml); then the washed grains were cultivated, each grain separately, on Czapek-Dox agar slants at 28°C. After cultivating for 7-10 days, the number of fungal colonies was calculated. From 100 to 200 grains were analysed in each sample.

The second approach consisted in that 1 g of the sample was suspended in 99 ml of a sterile 0.1% agar solution with glass beads (5 mm in diameter). Then 1 ml of the thus obtained suspension was mixed with an agar-containing medium, with added peptone, glucose, and chloramphenicol, poured into 9 cm Petri dishes, and cultivated at 25°C. The fungal colonies were calculated 5-7 days later on 4 Petri dishes for each sample.

The fungi from dairy produce and eggs are isolated by direct transfer of pieces of mycelium or fungal spores, when colonies develop on the substrate, or by the pouring method. In the latter case, a weighed portion (not more than 1 g) is thoroughly shaken in a nutrient medium (preliminarily warmed

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up to 25-30°C to obtain a homogeneous emulsion. The degree of dilution is selected in every individual case. The obtained mixture is inoculated into acidified agarized media.

From juicy fruits and root crops, the fungi are isolated by direct transfer of mouldy bits into a nutrient medium. In cases when the fungus appears as a mycelium within the tissues of the natural substrate, it is recommended to carry out obligatory surface disinfection. The use of a mercury chloride or silver nitrate, solution and sometimes flaming, for these purposes produces good results. The surface parts of the test material are removed and small pieces (about 1 mm<sup>3</sup>) are extracted from the deeper layers and placed into test tubes (one in each) or onto the surface of a solid nutrient medium in Petri dishes (5-6 pieces in a dish). Before inoculating the pieces, make sure that they contain fungal mycelium.

For isolating individual groups of fungi from natural substrates, special nutrient media are suggested on which the fungi of the group to be detected either grow better than the fungi of other groups or form a pigment characteristic of them alone.

Thus, for isolating the fungi of the Fusarium genus, the moist chamber method is used (9), whereby sterile circles of filter paper (2-3 pieces) are placed in sterile Petri dishes, moistened with sterile water or a liquid nutrient medium and test material is placed on them after surface disinfection or without it. The dishes are incubated in a thermostat. Fungal growth is monitored on the test substrate and the grown fun-

gal cultures are isolated under a 20-30 X binocular magnifying glass. Acid wort agar is also recommended for isolating these fungal cultures.

In our studies we isolated different species of Fusarium fungi from rye or wheat grains on Czapek's agar with sucrose. The agar was poured into Petri dishes and individual grains were placed on its surface, after surface disinfection or without it, depending on the task. The dishes were placed into a moist chamber and incubated at 20-22°C.

When isolating the Fusarium fungi from barley grains contaminated with different fungi in a number of districts in Japan, certain difficulties were encountered in detecting the fungi of this genus, since the Rhizopus and Mucor fungi rapidly spread over the entire surface of the medium. For this reason, a special nutrient medium was developed on which the Fusarium fungi grew well, while other fungi did not form large colonies and the growth of the Rhizopus and Mucor fungi was completely suppressed (10).

Bothast R.D. and Fennell D.I. (11) proposed a nutrient medium for a rapid identification of fungi of the Aspergillus flavus group. This medium facilitates the separation of fungi of this group from accompanying microorganisms, since A. parasiticus and A. flavus form a stable bright-yellow pigment. The authors inoculated Petri dishes containing this medium with the spores of 55 collection cultures and 10 cultures isolated from grain and flour products. All the strains of A. flavus, A. parasiticus and 6 strains of the A. oryzae group formed a yellow pigment. The identification and counting of the colonies of A. flavus with respect to cultural and morphologi-

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cal traits in agar with yeast extract gave the same results as the detection of the colonies of these fungi by a characteristic yellow pigmentation of the medium.

In all cases pure fungal cultures are isolated from the grown colonies or a monospore culture is obtained. The isolated fungal cultures are preserved on solid media in a refrigerator.

Pure fungal cultures isolated from food substrates are identified relative to their genus and species. The identification of the isolated cultures is carried out by routine methods on standard solid nutrient media, mainly wort agar and Czapek medium, according to the recommendations of well-known manuals (9, 12-15).

Along with the identification of the isolated fungal cultures, their toxigenic properties are studied. For this purpose, the fungal isolates are inoculated into a certain natural nutrient substrate or on an artificial nutrient medium of a definite composition and, after incubation for several days at a selected temperature, the substrate and the mycelium grown on it are examined. The presence of toxic properties is investigated biologically on animals, chicken embryos, tissue cultures, or on other test objects; also, the toxic component is isolated and its identify with known mycotoxins determined by physical and chemical methods. It is crucial at this stage of the analysis to correctly select the pattern for the cultivation of the fungal isolates whose toxic properties are under study, so as to ensure favourable conditions for toxin formation.

Cultivation of Fungal Isolates in the Laboratory

Toxin formation is a complex process. The conditions of the accumulation of toxic substances are insufficiently studied for many fungi. The dependence of toxin formation on various factors has been studied by numerous investigators. Much attention has been paid to physiological properties of toxin-forming fungi as well as to the methods of mycotoxin isolation. In these studies, both surface and in-depth methods of cultivation on various nutrient media (liquid, solid, natural, synthetic) have been employed. It has been found that fungal toxins may be detected in the mycelium, spore-bearing organs, and the nutrient substrate which supports the development of the fungus. Optimum conditions for growth and toxin formation are not the same for different fungi. It has been found that the conditions for maximum synthesis of a number of toxins do not coincide with the conditions providing for the maximum growth of the mycelium. It must be stressed that only pure and monospore fungal cultures are used in all investigations into the toxicity of the fungi.

Apart from the genetic properties of the fungus itself, the basic factors on which the yield of toxin depends, are the nature of the substrate, its humidity, incubation temperature, aeration, duration of incubation. The best toxin formation in various fungal species and the accumulation of toxins take place on those substrates on which they developed under natural conditions: on oats, barley, maize, wheat, peas, to name but a few.

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Spore suspensions from pure cultures grown on test-tube agar media are used for inoculation. Sterile water or saline with wetting agents, usually Tween-20 or Tween-80 (0.02 - 0.05%) added to it are employed for preparing a suspension. The mycelial material which is introduced either as a small piece or in the form of a suspension in sterile water or saline may be used as inoculum as well. The inoculated nutrient media are incubated at temperatures from 20 to 30°C, and for individual fungal species at lower or higher temperatures.

The duration of cultivation depends on the species of the fungus, humidity, temperature conditions, and the composition of the nutrient substrate. Some authors recommend the following durations of incubation: 20-25 days for Fusarium and Trichoderma 10-15 days for Aspergillus and Penicillium, 15-20 days for Mucor, Rhizopus and Alternaria. For further examination following incubation, various extracts are prepared from the mouldy substrate or a fungal culture grown on a nutrient medium is used.

Agar or liquid artificial nutrient media are also taken for obtaining toxic substances from various fungi. Most frequently used for this purpose are (Czapek-Dox media, Czapek medium with 2% sugar, a glucose-peptone medium, a medium with sucrose and yeast extract, and a number of others.

There are two approaches to investigating the toxigenicity of fungal cultures. In the first case, fungal isolates from food are studied for their capacity to produce various metabolites possessing toxic effect towards biological test objects (animals, chicken embryos, tissue cultures, etc.).

These studies are not aimed at detecting a strictly definite group of mycotoxins; they embrace the entire spectrum of toxic substances formed by the fungi concerned. This approach may be exemplified by experiments carried out by B.Gedek (1) who has studied the capacity of 134 strains of 10 Aspergillus species and 13 Penicillium species isolated from grains to form toxic metabolites. The isolated fungal strains were incubated in a sucrose-yeast medium for 7 days at 32°C and for 10 days at 22-24°C. Then the fungal cultures were extracted with chloroform and the extracts were examined by thin-layer chromatography for the presence of known mycotoxins. Besides, the filtrates of the fungal cultures were tested for toxicity on tissue culture and chicken embryos. These experiments have revealed fungal strains forming toxic metabolites and 5 known mycotoxins were identified among them.

The other approach involves the screening of fungal strains forming a strictly definite group of mycotoxins. For carrying out such investigations, one needs to know the conditions on which the formation of a given toxin depends, and also the corresponding techniques and methods of cultivating the producer fungi. In this connection let us take a look at several patterns of cultivating fungi producing the most widespread and most intensely studied mycotoxins.

#### Aflatoxin-forming Fungi (16,17)

So far the best studied are the factors influencing the synthesis of aflatoxins by the Aspergillus flavus and A. parasiticus fungi.

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In the laboratory these fungi were cultivated on various natural and synthetic substrates. It was found that toxin formation proceeds more intensely on natural substrates: sterile moistened grains of rice, rye, wheat, peanuts, oats, soya beans - on which very high toxin yields were produced. The fungi are incubated at 24 - 30°C. Before sterilization, the substrate is moistened with water. Sometimes instead of water a solution of microelements is added.

The maximum yield of aflatoxins was obtained when the producer fungus was grown under stationary conditions, although the growth of the fungi and the formation of aflatoxins by them proceed faster during incubation with stirring.

In synthetic liquid nutrient media, the yield of aflatoxins was considerably lower than on natural substrates. For obtaining a significant yield of toxins into synthetic media, it is necessary to add a maize (up to 8%) or yeast (up to 2-2.5%) extract. Efficient formation of aflatoxins is ensured by such carbon sources as sucrose, glucose, maltose, glycerol, and ethanol; 20-30% is believed to be the optimum concentration of glucose and sucrose.

Among the microelements, zinc is of great importance for the biosynthesis of aflatoxins. The addition of zinc the synthetic medium at a concentration of 1-25  $\mu$  g/ml raises more than 30 times the amount of aflatoxins formed. The optimum pH values for K. flavus and A. parasiticus are 5.0-5.5.

It should be noted that the four main aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub> may be simultaneously produced by one and the same

culture. Most of fungal strains producing aflatoxin G<sub>1</sub> also form aflatoxin B<sub>1</sub>, but not all strains forming aflatoxin B<sub>1</sub> may synthesize aflatoxin G<sub>1</sub> (18).

Aflatoxins belong to the class of secondary metabolites. The onset of aflatoxin synthesis in the fungal culture coincides with the end of the period of active mycelial growth and the beginning of spore formation. Aflatoxins start to be detected in the growing culture usually on the 2nd-3rd day of incubation. During incubation, the amount of aflatoxins in the fungal culture reaches a maximum, after which it diminishes. The time of attaining the maximum amount of aflatoxins and their total quantity largely depend on the quantity of the spore inoculum, as we have demonstrated by the following experiment (19). Definite volumes of spore suspension were placed into flasks containing sterile moistened grain of rye or wheat so that the inoculum would contain 1 spore per gram of grain in one test and 10<sup>4</sup> spores per gram of grain in another. The fungi were incubated at 28°C for 28 days and as they grew, the content of aflatoxin B<sub>1</sub> was determined in the grain by thin-layer chromatography. At the same time we checked microscopically the appearance of newly formed fungal spores. Aflatoxins appeared in the grain with the beginning of spore formation and their quantity increased parallel with the number of spores in the fungal culture. In flasks with a larger dose of the inoculum (10<sup>4</sup> spores per gram of the substrate), the period of maximum accumulation of aflatoxin B<sub>1</sub> coincided with the period of the maximum spore formation, after which a gradual decline in the content of aflatoxin B<sub>1</sub> in grain was

observed. In flasks containing grain inoculated only with 1 spore per gram of grain, the quantity of aflatoxin B continued to rise even after spore formation in the fungal culture had ceased. In this test, the maximum accumulation of aflatoxin B<sub>1</sub> was reached considerably later but its yield was 1.5 times higher than for the massive inoculum (see the Table).

In another series of tests sterile rye grain was infected with different numbers spores of A. flavus and A. parasiticus (from 1 per 10 g of grain to 10<sup>4</sup> per 1 g of grain) and the formation of aflatoxin B<sub>1</sub> was determined in the grain within 14-20 days and within 28-31 days of incubation (20). When the amount of the inoculum was increased to exceed 10<sup>2</sup> spores per 1 g of grain, the yield of aflatoxin diminished in comparison with the grain inoculated with a lower quantity of spores. Concerning those data, it should be noted that usually in studies of toxin formation in mould fungi a rather massive spore inoculum is used containing 10<sup>4</sup> and more spores per 1 g or 1 ml of nutrient substrate. On the one hand, this somewhat shortens the period of incubation of the fungal culture, but, on the other, it apparently prevents the obtaining of a maximum yield of the toxin.

However, it should be said that when screening aflatoxin-synthesizing fungal strains in food and fodder substrates, it is often not so important to obtain a maximum toxin yield as to establish the very fact of toxin formation. Moreover, it is very important to have a rapid high-quality method for telling toxin-forming strains from non-toxic ones. For this purpose rapid methods were suggested revealing the formation of

Table

The Amounts of Spores and Aflatoxin B<sub>1</sub> per gram of Rye and Wheat Grain During Fungal Growth

Inoculum, number of spores per gram of grain	Incuba- tion time, days	Rye		Wheat	
		Number of spores	Amount of aflatoxin, $\mu\text{g/g}$	Number of spores	Amount of aflatoxin, $\mu\text{g/g}$
1	2	0	0	0	0
	2.7	$1.8 \times 10^6$	1.6	-	-
	3	$2.8 \times 10^6$	1.3	$3.2 \times 10^6$	15
	3.7	$1.6 \times 10^7$	27	$9.0 \times 10^6$	59
	4	$3.0 \times 10^7$	28	-	-
	4	$1.0 \times 10^8$	296	-	-
	7	$8.0 \times 10^8$	488	$1.6 \times 10^8$	320
	10	$1.7 \times 10^9$	649	-	-
	14	$1.7 \times 10^9$	694	$1.6 \times 10^{10}$	742
	21	$1.1 \times 10^{10}$	751	-	-
28	$6.6 \times 10^9$	1.171	$9.8 \times 10^9$	1,126	
$10^4$	1	0	0	0	0
	1.7	$1.9 \times 10^7$	18.3	$8.3 \times 10^6$	23
	1.8	$2.0 \times 10^7$	13.6	-	-
	2	$4.9 \times 10^7$	23.0	$1.7 \times 10^7$	37
	2.7	$1.2 \times 10^8$	291	$7.0 \times 10^7$	215
	4	$8.0 \times 10^8$	477	-	-
	5	$4.6 \times 10^9$	721	-	-
	7	$2.0 \times 10^9$	654	$5.6 \times 10^9$	694
	14	$4.0 \times 10^9$	459	$1.8 \times 10^{10}$	749
	28	$1.0 \times 10^{10}$	473	$8.7 \times 10^9$	424

aflatoxins during fungal growth directly in Petri dishes on media of special composition. In these works the property of aflatoxins to fluoresce under ultraviolet rays was advantageously used.

For detecting strains producing aflatoxin, Hesselstine C.W. et al. (21) have suggested a nutrient medium which they designated APA. Isolated strains of fungi are inoculated into the center of glass Petri dishes containing this medium and incubated in darkness for 7-10 days at 28°C. Then the dishes with the culture are examined in a 366 nm ultraviolet light for blue fluorescence of the agar surrounding the colonies. To make sure that the fluorescence around the fungal colonies on their medium is due to the excretion of aflatoxins, the authors extracted the medium with chloroform and identified mycotoxins by thin-layer chromatography. Thus, the suggested rapid method included three steps: (1) the growing of the fungal strain on a special nutrient medium and the detection of the agar fluorescence around the colonies in ultraviolet light; (2) the chloroform extraction of toxins from the fluorescing agar; (3) the identification of aflatoxins by thin-layer chromatography.

Another method was suggested by Torrey E.S. and Marth E.H. (22). These authors grew aflatoxin-producing strains of A. flavus and A. parasiticus on a nutrient medium of a special composition with the addition of silicic acid, which led to the formation of a gel. Already at the next day of incubation, a zone with blue fluorescence in long-wave ultraviolet light appeared around the developed fungal colonies.

The authors hold that although the suggested medium is not optimal for the formation of the maximum quantities of aflatoxins, it facilitates the detection of a fluorescent zone indicating the presence of aflatoxins in the medium at concentrations above 0.04 micrograms.

Sterigmatocystin-Forming Fungi (16,17)

Aspergillus versicolor which is widespread in agricultural products is believed to be the main producer of sterigmatocystin. The metabolism of this fungus is being intensely studied due to the potential hepatocarcinogenicity of its metabolites. Sterigmatocystin is formed by this fungus in great amounts on various nutrient media, both synthetic and natural (on sterile maize, rice, wheat, etc.).

Ochratoxins-Forming Fungi (23,24)

A group of fungi forming very dangerous ochratoxins not infrequently attacks agricultural food and fodder plant substrates, particularly grain. These fungi have been detected also in peanuts and black pepper. The group of ochratoxin producers includes several fungal species from the Aspergillus and Penicillium genera, including P. purpurescens, P. commune, P. viridicatum, P. politans, P. cyclopium, P. variabile, A. sulphureus, A. sclerotiorus, A. alliaceus, A. melleus, A. ochraceus, A. ostianus, A. petrakii, and others. These fungi are very widespread and the hazard of contamination of agricultural foods and fodders is very high indeed.

Though it has been established that ochratoxin A may be

formed by different mould fungi of the Aspergillus and Penicillium genera, its main producer is A. ochraceus and P. viridicatum. The most toxigenic strain of A. ochraceus is known as K-804.

A. ochraceus forms ochratoxin A most intensely in the temperature interval from 20 to 30°C. The maximum formation of this toxin is observed at 30°C and substrate humidity 39%. At lower temperatures (around 15°C), the highest quantity of ochratoxin A is formed at substrate humidity 52% (25,26).

The Penicillium genus includes psychrophilic species. It has been demonstrated that P. viridicatum may form ochratoxin A at 5 - 10°C. Therefore the contamination of agricultural plant products with ochratoxins in regions with a cold climate, such as Canada and Scandinavia, is due mainly to infection with fungi of the Penicillium genus.

In the laboratory, A. ochraceus and P. viridicatum are usually cultivated on natural substrates, such as sterile grain of wheat, maize flour, wheat flakes, soya beans. When cultivated on wheat flakes, the amount of synthesized ochratoxin A was 239 mg per 1 g of substrate after 17-25 days of incubation. Proper humidity was maintained by adding 40-70 ml of water to 100 g of flax. These conditions are regarded to be optimal for obtaining ochratoxin A. A. ochraceus does not grow on ordinary synthetic laboratory media. It forms ochratoxin A on mycological broth with 0.5% of yeast extract and on a medium with 2% of yeast extract and 15% of sucrose (YES). On the latter nutrient medium, 9.0 mg/kg of ochratoxin A were obtained

by cultivating the M-298 strain of A. ochraceus.

As a rule, ochratoxin A and B are formed simultaneously when the fungal growth attains the logarithmic phase, i.e. after 3-4 days of cultivation, and the synthesis is completed within 12-24 hours. In a medium with 4% of sucrose and 2% of yeast extract, the yield of ochratoxin A per 100 ml of medium was 29 mg. If the concentration of sucrose was above 8%, ochratoxin B was synthesized. The best sources of nitrogen for the synthesis of ochratoxins by A. ochraceus are proline and glutamic acid, the best source of carbon is sucrose.

#### Toxigenic Fungi from the Fusarium Genus (9)

The next important group of fungi are those from the Fusarium genus which synthesize (zearealenone (28,29)). The producers of zearealenone are Fusarium graminearum, F. tricinctum, F. oxysporum, F. sporotrichioides, F. moniliforme.

It should be noted that the F. graminearum fungus is known also as the cause of a toxicosis which bears the name of "drunken bread"; the disease was investigated by Russian scientists N.S. Voronin, N.A. Pal'chevsky, N.A. Yachevsky, and others.

Different strains of F. graminearum are distinguished by their capacity to produce zearealenone.

Various nutrient media suitable for the formation of zearealenone and studying its biosynthesis were examined. The nutrient requirements of Fusarium fungi were studied on such balanced nutrient systems as Czapek and Czapek-Dox media with 20% sucrose and some others. However, the maximum yield of the toxin was obtained on solid natural substrates.



The following pattern of cultivating the F. graminearum fungus is recommended for the production of zearalenone. Autoclaved rice or maize in quarter carboys is inoculated with a suspension of fungal spores. Before autoclaving, maize is moistened to a 45% humidity and rice to a 60% humidity. The cultures are incubated at 24-27°C for 1-2 weeks for the formation of a sufficient quantity of fungal biomass and then kept at 12-14°C for 4-6 weeks to ensure a maximum yield of the toxin. It is presumed that low temperatures activate the enzymes involved in the zearalenone synthesis.

It must be noted that the formation of zearalenone on sterile rice grains is enhanced by the addition of 1% peptone.

Some species of the Fusarium genus are capable of synthesizing mycotoxins of still another group - trichothecenes (30). One or several mycotoxins of the trichothecene nature were isolated from the strains of the following Fusarium species: F. lateritium, F. episphae, F. nivale, F. oxysporum, F. rigidiseulum, F. sclani, F. roseum, F. tricinctum (synonym F. sporotrichioides). In addition to Fusarium fungi, trichothecenes are formed also by fungi of the Cephalosporium, Myrothecium, Trichoderma, and Stachybotrys species.

In order to obtain trichothecene mycotoxins by cultivating producer fungi in the laboratory, various modifications of Czapek-Dox medium and complex nutrient mixtures containing corn extract, malt or yeast extract, peptone, mineral salts, and glucose are usually taken.

For the formation of nivalenol and fusarenon, sterile rice is used as a medium and for obtaining diacetoxyscirpenol, glu-

cose and ammonium nitrate are employed. Most of fungi grow in an immersed culture for 2 - 4 days at 25°C with aeration and stirring. However, diacetoxyscirpenol, T-2 toxin, and HT-2 toxin were obtained in a stationary culture. In this case the formation of trichothecenes takes from 2 to 4 weeks.

The optimum temperature required for the biosynthesis of most of trichothecenes lies within a 24- 30°C range. T-2 toxin, however, is an exception. According to a number of authors, the maximum yield of this toxin was obtained at a low temperature about 8°C. At this temperature, the culture of F. sporotrichioides, F. tricinctum (strain 63) on rice grain synthesised within 48 days of incubation 4,500 mg T-2 toxin/kg. At 14° and 25°C, the toxin in the fungal culture on the grains of maize, rice, wheat, and barley appeared earlier than at 8°C, but its content in the grain was considerably lower (31).

We have successfully cultivated various strains of F. sporotrichioides in the laboratory on millet prepared in a special way: before inoculation it was moistened by adding 50 ml of water to 100 g of millet. The culture of the fungus on millet was incubated for 7 days at 20-22°C and then kept for another 14 days at 5-7°C. Depending on the strain, we have obtained a high yield of T-2 toxin which in a number of cases attained 3 mg per kilogram of the substrate. There are quite a number of other mycotoxin-forming fungi with a more limited distribution. The conditions of their formation are less thoroughly studied and the literature lacks exhaustive information on this matter. Of these, we shall briefly look at four groups of fungi.

Patulin-Forming Fungi (32,33)

Patulin has been found in apples, apple juice, rotting pears, and other stone fruits, in mouldy bread. This mycotoxin is formed by various fungal species of the Aspergillus and Penicillium genera, including P. urticae, P. claviforme, P. expansum, A. clavatus, A. giganteus, A. terreus, and also by the Byssochlamys nivea species.

The formation of patulin depends on the strain specificity, substrate and temperature. Thus, P. expansum, strain 550, formed 40  $\mu$ g/ml of patulin after 12 days of incubation at 4°C on a mineral salt medium with sucrose. At 25°C the same strain formed patulin in the amount of only 3  $\mu$ g/ml. The maximum amount of the mycotoxin (170 ppm) was formed by the P. urticae fungus strain 512, cultivated on tomato pulp with pH 3.7 at 10°C. At 0° and 25°C the formation of the toxin was negligible.

Penicillic Acid-Forming Fungi (32)

Penicillic acid is a mycotoxin formed in maize legumes, tobacco. It possess a carcinogenic effect and is very toxic to man and animals. Penicillic acid is synthesized by numerous species of fungi of the Penicillium and Aspergillus genera. They include: P. puberulum, P. stoloniferum, P. cyclopium, P. madriti, A. thomii, P. suaveolens, P. palitang, P. boarnense, P. madriti, A. ochraceus, A. sulphureus, A. guercinus, A. melleus.

Optimum conditions for the formation of penicillic acid by producer fungi appear at 15 - 22°C. This toxin was first

isolated from the culture of *P. puberulum* grown on maize. The cultivation of *A. ochraceus* on a sucrose-salt medium with glutamic acid gave a 0.7 g/litre yield of penicillic acid.

#### Rubratoxins-Forming Fungi (34)

It is at present difficult to evaluate the role of *P. rubrum* in the emergence of mycotoxicoses under natural conditions. However, there is no doubt that the strains of this mould are capable of forming great amounts of toxic metabolites in the laboratory. It has also been established that these fungi, isolated from mouldy feeders, are toxic to animals. The producers of rubratoxins, *P. rubrum* and *P. purpurogenum*, are widespread in nature. They have been isolated from cereals and legumes, rice husks, maize, bran, sunflower seeds.

The growth of *P. rubrum* on simple synthetic media is limited and toxin formation is light. It grows better on complex natural substrates. First the toxin was isolated from the culture of this fungus on a nutrient medium consisting of sterile rice with 1% of sucrose. A good yield of the toxin was obtained on a stirred medium containing glucose and yeast extract. After 4-6 days of incubation, the amount of the toxin was 220 - 600 mg/l. All the toxin synthesized by the fungus was contained in the cultural fluid. The washed and dried mycelium was non-toxic to laboratory animals. The initial pH value of the medium favourable for the growth of the fungus and the synthesis of the toxin was 3.7.

Rubratoxins as such are not very toxic. However, they are capable of synergistic effect when given together with

afatoxin B<sub>1</sub> to mice. This observation is noteworthy, since P. rubrum and A. flavus occupy similar ecological niches and are frequently isolated together from mouldy foods.

Luteoskyrin Producer (35,36)

One of the fungal species that were identified in the 1940's as being responsible for the yellow rice disease, is P. islandicum. Apart from luteoskyrin, this fungus also forms (potentially carcinogenic mycotoxins, such as islandotoxin, rugulosine and cyclochlorotin, as well as a number of other toxic metabolites.

Luteoskyrin is readily formed during the growth of a culture in the laboratory both on synthetic Czapek medium and on sterile grains of rice, wheat, oats, soya beans, and maize. The yield of the toxin obtained on rice reached 430 mg/kg of substrate.

It is more difficult to obtain islandotoxin. The highest yield of this toxin was obtained on spring red wheat to which proline and potassium chloride were added.

With this we shall conclude our examination of the special features of laboratory cultivation of fungal cultures forming the most dangerous and best-studied mycotoxins.

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