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

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MYCOLOGICAL STUDIES INTO MYCOTOXIN PRODUCERS

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A Brief Description of Fungi

Fungi make up a vast group of organisms comprising some 100,000 species among which we find both the well-known edible and poisonous mushrooms and also the microscopic species which constitute the majority.

According to the traditional classification of living organisms into the animal and vegetable kingdoms, fungi used to be placed with the plants. Gaining currency at present, however, is the view of fungi as an independent kingdom of organisms fundamentally differing both from plants and animals (L.L.Velikanov et al., 1981).

Characteristic features of the fungi are the absence of the ability to effect photosynthesis and a primarily heterotrophic type of nutrition by absorption, the presence of a well-pronounced cell wall mostly containing chitin, the formation of glycogen in the cells as a reserve product, reproduction by spores, absence of mobility in the vegetative state, and unlimited growth. It is presumed that fungi emerged at a period when the division of organisms into the animal and plant kingdoms was taking place.

With respect to the method of nutrition, fungi are divided into saprophytes and parasites. Parasites feed on the living tissues of plants and animals. The source of nutrition for saprophytes are the dead remains of plants and animals. It is not always easy to draw a clear-cut demarkation line between saprophytes and parasites. A number of parasitic fungi may develop

on vegetable remains, while some saprophyte fungi under certain conditions adapt to the parasitic mode of life on animal and plant organisms.

The vegetative body of most of fungi is a mycelium consisting of branching filaments which are called hyphae, with apical growth and lateral ramification. The mycelium penetrates into the nutritive substrate and absorbs nutrients through its entire surface. It may also rise above the substrate as a superficial or aerial mycelium on which the reproductive organs usually form.

By structure all fungi are divided into lower and higher ones. Typical of the lower fungi is the noncellular nonceptate mycelium seeming to appear as a giant single ramified cell with a great number of nuclei. The higher fungi have a multicellular mycelium divided by septa into individual cells. The vegetative body of some fungi, for example yeasts, is represented by single budding or dividing cells.

The mycelium of many fungi is transformed into mycelian strands sclerotia consisting of densely intertwinned mycelian hyphae.

Fungal reproduction may be vegetative, asexual, and sexual. In vegetative reproduction, non-specialized parts, such as oidia, chlamydospores, arthrospores detach from the mucelium and give rise to a new mycelium.

Asexual reproduction in the lower fungi is carried out by zoospores (mobile unicellular spores with flagella) or sporangiospores formed endogenously in the zoosporangia and sporangia, respectively.

In the higher fungi, asexual reproduction is performed by means of exogenous spores or conidia developed on conidiophores which represent the mycelium branches. The conidiophores reveal great structural variety.

The product of sexual reproduction in the lower fungi is the gamete, the oospore, the zygote and, in the higher fungi--the ascospore and the basidiospore.

Fungal systematics is based on a complex of characters, the main being the structure of the sexual and asexual reproductive organs, and the composition of polysaccharides within the cell walls.

On the basis of contemporary concepts of the origin and development of fungi, they are subdivided into seven classes (J.A.Von Arx, 1968; H.Kreisel, 1968; D.K.Zerov, 1972, and others). The first four of them are the lower fungi:

The class of Chytridiomycetes. The mycelium is weakly developed or the vegetative body appears as a single, occasionally nonseptate cell. Asexually reproduced by means of zoospores with a single posterior flagellum. The sexual process is gametogamy of various types. The cell walls contain chitin and glucuns.

The class of Hyphochytridiomycetes. The vegetative body is represented by single cells, sometimes naked; often a rhizomycelium is formed. Asexually reproduced zoospores with one plumat anterior flagellum. The sexual process is gamogamy. The cell walls contain chitin and cellulose.

The class of Oomycetes. The mycelium is well developed, nonseptate. Asexually reproduced by means of zoospores with two

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flagella, filiform and plumat. The sexual process is oogamy. The cell walls contain cellulose and glucans.

The class of Zygomycetes. The mycelium is well developed, nonseptate in most cases. Asexual reproduction occurs predominantly through sporangiospores. The sexual process is zygogamy. Cell walls of the mycelium contain chitin and chitosan.

Higher fungi are represented by three classes:

The class of Ascomycetes. The mycelium is well developed, cellular. Asexually reproduced by means of conidia. The spores of sexual reproduction are formed endogenously in an ascus. The cell walls contain chitin and glucans. Two species of this class cause myxotoxicoses; these are Claviceps purpurea and Cl.paspali.

The class of Basidiomycetes. The mycelium is well developed and is cellular. Asexually reproduced by conidia. Spores for sexual reproduction are formed endogenously on a basidium. The cell walls contain chitin and glucans. Belonging to this class are poisonous mushrooms, smut and yellow rust of cereals.

The class of Deuteromycetes or imperfect fungi (Fungi imperfecti). A well-developed cellular mycelium. Asexually reproduced by conidia. The sexual process is absent. Known in the fungi of this class is the parasexual cucle whereby the mycelian nuclei merge and recombination takes place during the mitotic division. The overwhelming majority of mycotoxin producers belong to this class.

Microscopic Mould Fungi are widespread in nature. They occur in great amounts in the soil, on various vegetable substrates, food products, in water, and in the air.

When products, particularly of vegetable origin, are stored under unfabourable conditions (elevated relative humidity of air and high temperature), the fungi start vigorously developing on them. Some fungal species form toxic metabolites. At present, mycotoxins have been found in a great number of microscopic saprophyte fungi.

Mycological studies consist of a complex of methods used for establishing or confirming the role of gungi in the etiology of mycotoxicoses. Studies are started after the preliminary exclusion of an infectious pathogen, poisonous plants, and chemicals as causes of a disease.

Correct sampling is of importance for a successful study of fungal flora. A specimen weighing about 200 g is taken for mycological analysis from an average sample composed by a generally accepted method. Additionally studied is the fungal flora of fodder or good products taken from darkened, mouldy areas or foci.

Carried out in the laboratory are the organoleptic analysis of the specimen, the determination of the toxicity of the fodder, its mycological examination, and the determination of the toxicity of isolated fungal cultures.

Special cultivation methods and techniques are used in every individual case for the differentiation of the species and varieties of fungi.

Fungal contamination of grain may be in-depth and superficial. For revealing the in-depth affection, the grain is preliminarily disinfected with a 3% formalin solution or 70% ethyl alchohol and washed with sterile water. The grains are ar-

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ranged on the surface of the culture medium in Petri dishes (direct inoculation method).

The isolation of gungi from flour, bran, mixed fodder, grist, and other similar products is carried out by the method of pouring.

When fungal growth is noticed, the cultures are transferred from the Petri dishes into test-tubes with an appopriate culture medium.

When cultivation is completed, the colonies are studied and the fungus is examined microscopically. Its specific identity is established on the basis of cultural-morphological characteristics.

An indispensable stage of the work is the toxico-biological examination of food stuffs and fungal cultures.

The main method for determining the toxicity of grain, coarse and mixed fodder, and silage is the skin test.

The toxicity of fodder is determined also by the oral administration of an extract to laboratory animals.

The toxicity of many kinds of vegetable foods may be established by feeding them to test animals and young foul.

When unknown toxigenic fungi are isolated from the test samples, the feeding of the obtained samples to animals is the primary and basic method of toxico-biological analysis in establishing their role in the etiology of the poisoning.

The toxicity of fungal cultures isolated from the samples should be determined when the suspected fodder causes illness or death of test animals, and also in case of a positive skin test.

The toxicity of gungal cultures is determined by different methods, including the skin test on rabbits, feeding the animals with fungal culture on grain and hay, watering the animals with extracts, the intravenous, intraperitoneal, and subcutaneous administration of extracts from the fungal cultures to the animals, testing the extracts on chicken embryos and tissue cultures, chemical and chromatographic analyses, etc.

The fungal film removed from the surface of the culture medium (preferably liquid), from which extracts are prepared, the culture medium (fluid nutrietn medium on which the fungus was grown), a culture of the fungus grown on grain, hay or other vegetable substrate are used for determining toxicity.

In all cases it should be borne in mind that the isolation from the test substrates only of single cultures known as producers of mycotoxins is not sufficient for the diagnosis of mycotoxicoses. One should always take into account the general epidemiological and epizootological background, the clinical manifestations of the disease, the post-mortem investigation, observations of the terms of the outbreak of the disease after a new batch of the fodder (food) products were included in the ration. An erroneously made diagnosis of mycotoxicosis inevitably leads to most undesirable actions, such as the discarding and loss of large quantities of fodder for which there is still great demand in many regions of the world.

Toxin-producing fungi occupy various positions in the systematics and belong to different classes (A.Kh.Sarkisov, 1954; A.Kh.Sarkisov, et al., 1971).

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Among fungi developing on a vegetable substrate and forming mycotoxins the following species are best studied: Fusarium sporotrichiella, F.graminearum, Stachybotrys alternans, Dendrodochium toxicum, Aspergillus flavus, A.fumigatus, and also Claviceps purpurea, Cl.paspali, and Pithomyces chartarum which parasitize on plants.

Let us examine the most dangerous species of toxin-producing fungi.

The Claviceps purpurea fungus belongs to the class of Ascomycetes; it is a strict parasite on a number of cultured cereal plants. The mostly affected cereal is rye; more seldom affected by the ergot fungus are wheat, oats, barley, rice, The development of ergot on various wild-growing cereals is not well studied. It has been reported that ergot may parasitize on approximately 170 varieties of cereals (both cultured plant and wild-growing weeds).

The development and the toxicity of ergot is influenced by soil and climatic factors. Ergot is widespread in Europe, Asia, and the Americas, on the islands of the South Seas (Jawa, New Zealand). It is frequently found in lowlands, in moist localities, along rivers, in valleys on the fringes of forests and sown areas.

This fungus develops in three stages: sclerotial (resting), ascosporic, and conidial. It is the resting state of the fungus-the sclerotium (ergot spurs) - that is toxic to animals.

Ergot sclerotia are elongated, usually somewhat curved, in shape. They are blackish-purple in colour, whitish-purple inside. The number of ergot spurs on an affected ear of rye may vary

from one to several dorens. The weight of ergots collected from 1 kg of rye may vary from 10 to 500 mg. The weight of ergots collected from wild-growing cereals is considerably less. Dimensions vary from 0.5 to 4.5 cm in length and from 3 to 5 mm in diameter.

The quantity of alkaloids in the sclerotia is not constant and depends on many factors, important among which are climatic conditions, characteristics of the host plant, the size of the sclerotia. During long storage after harvesting, the toxicity of the sclerotia decreases under the effect of light, temperature and increased humidity, and steeply drops after 91-12 months.

The Claviceps paspali fungus is a strict parasite of various kinds of herbs of the Paspalum genus. It belongs to the Ascomycetes class. The poisoning of animals is observed mostly during the grazing period, in autumn, when grasses are eaten together with the sclerotia at the time when the fungal sclerotia ripen. The fungus is widespread in a number of states of South America, Africa, and Oceania. It occurs along the coast of the Black and Caspian seas.

The sclerotia of Cl.paspali are roundish, greyish-yellow--brown with a rough-rugose surface, 1.5 to 3.5 mm in diameter. From 10 to 50 and even more sclerotia are formed on a single ear.

The sclerotia are very resistant to low winter temperature, high humidity, prolonged aeration, insolation, and other environmental factors.

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The Stachybotrys alternans fungus belongs to the Deutero-

mycetes class and is a cellulolytic saprophyte. Under natural conditions, it grows and develops well on substrates rich in cellulose: straw, hay, various weeds, and vegetable remains. It attacks cotton, flax articles, wood, sackcloth, paper. On straw and grain it forms a black powdery, easily removable coating.

A velvety black colony with a white border develops on nutrient media (Czapeks agar, wort agar, filter paper). A dark brown pigment is infused into the medium. The conidiophores are septate, sympodial-ramified, pale olive-green, with a bundle of sterigmata on their tips where black elliptic conidia are formed (3-0-7.7 x 6.9-14.0 m).

An important part in the development of the fungus and the formation of toxin is played by temperature and humidity. The optimum temperature is 20-25°C and the best humidity of the fodder is 60-75%. To determine the toxigenicity of the isolated strain of S.alternans, a sterile substrate (straw, oats, and other cultures) is inoculated.

Toxin formation is a stable physiological characteristic of this fungus. The toxins are contained mostly in the conidia and excreted into the culture medium, most actively on sterilized substrate with a high content of cellulose (straw).

The period of toxin formation coincides with the beginning of sporulation and ends with the completion of fungal growth by the 10th-25th day.

The toxins are stable in storage and are resistant to the effect of temperature, mineral and organic acids, light, and ultraviolet rays. Stachybotryotoxins are destroyed in strong alaklis. Laboratory methods for isolating them into a pure culture

are well developed.

The Dendrodochium toxicum fungus belongs to the class of Deuteromycetes; it is a cellulolytic saprophyte well developing in fodders rich in cellulose, such as straw and hay.

The fungus grows well on artificial nutrient media such as Czapek's agar, wort agar, and potato agar on which at first a dense network of snow-white mycelium develops and then a mass of green sporodochia appears, getting black with age. The sporodochia are roundish, up to 1 mm in diameter, consist of conidiophores. The olive-green conidia are eliptical, with tapering tips (2.7-3.5 x 6.5-8.0 μm).

The optimum temperature for the fungal growth and toxin formation is 20-25°, humidity is 50-60%. The appearance of the straw and hay attacked by the fungus does not differ from that of the healthy material. The toxins form both when natural substrates are attacked and during development on various nutrient media; they have been found in the mycelium, conidiophores, conidia, and culture media.

For determining the toxigenicity of the isolated strain of D.toxicum, the culture is grown on a sterile cellulose-containing substrate.

The fungi of the Fusarium genus, classe Deuteromycetes, attack cereal crops, cereal fodder, straw, hay, bran, the remnants of cereals on the fields, the grasses of winter pasture lands. They develop well on various nutrient substrates and sterile grains of cereals.

The greatest danger among the numerous Fusarium species comes from the Fusarium sporotrichiella species.

Organoleptically, the toxic fodders are frequently indistinguishable from healthy ones. In cases when smallish rugose, light grey or pinkish-red grains are found in the samples, one may suspect that the grains are attacked by Fusarium. Upon placing Fusarium affected grains on Czapek's agar in Petri dishes, flufy white or pink colonies develop.

The colonies of F.sporotrichiella on potato agar are fluffy or arachnoid, frequently powdery, white or slightly pinkish, with the mass formation of pseudopionnotes or sporodochia. The nutrient medium sometimes gets stained into purple shades. Micro conidia of two types are formed --spherical, lemon- or pear-shaped and elongated spindle-elliptical. The sporodochia and pionnotes consist of conidiophores on which sickle-shaped macroconidia form. There are numerous chains of chlamidospores.

A specific feature of the F.sporotrichiella fungus is that it may develop at low temperatures (below 2°C) with optimum growth at 18-20°C. A temperature of -20°C does not kill the fungus. It accumulates toxic substances at low temperatures. The optimum temperature of toxin formation lies in the range from +4 to +12° at the substrate's humidity above 50%.

Toxic substances are formed in the mycelium, conidia, and culture fluid. They are resistant to the effect of high temperatures and insolation.

The main producer of zearalenone (F-2 toxin) is F.graminearum (G.M.Birone et al., 1972; A.N.Leonov, 1972, and others). The formation of this toxin has been also discovered in a number of other Fusarium species (F.culmorum, F.moniliforme, F.semitectum, and others). Zearalenone is usually formed on cereal crops

and coarse fodder in storage, when moderate and cool periods alternate.

Among aflatoxin-producing fungi, the greatest importance is attached to the Aspergillus flavus, class Deuteromycetes. This fungus is widespread on various types of fodder. A.flavus most frequently can be isolated during the analysis of peanuts; besides, it readily develops and forms toxins on many other varieties of fodder (soyabeans, wheat, maize, sorghum oats, rice, rye) and also on coconuts, almond, citrus fruit rind, and cotton seeds.

The colonies of A.flavus on Czapek's agar are yellowish-green and granular with roundish black sclerotia up to 1 mm in diameter appearing along the margin of the colony. The conidiophores have roundish terminal tips with one or two layers of sterigmata; the conidia are round and spinous.

The optimum growth temperature for A.flavus is 36-38°C and that of toxin formation is around 27°C. The fungus is capable of developing and forming aflatoxins at temperatures from 11° to 37°C.

The best nutrient medium for the formation of aflatoxin is the potato-dextrose medium (Hesseltine C.W.et al., 1966). Peak toxin production during the development of the fungus on artificial nutrient media falls on the 5th-6th day. This period corresponds to the most intense spore formation in this culture.

Among the fungi of the Aspergillus genus, high toxicity is also notable in the A.fumigatus species, class Deuteromycetes. This fungus affects fodder flour, fodder products, and other substrates.

The colonies of A.fumigatus on nutrient media (Czapek's agar, wort agar) are flat, velvety, dark-green.

The fungus grows well at temperatures from +25°C to +37°C. The optimum toxin formation temperature is +25°C and lower. Toxic substances are excreted into the nutrient medium.

The toxic substances of A.fumigatus are unstable at high temperatures. The temperature required for the inactivation of the toxin excreted into the nutrient medium is within 70 to 90°C at 30 minutes' exposure, whereas the endotoxin contained in the mycelium and spores of the fungus is destroyed at 60°C within 15 minutes. Fungal cultures grown on grain lose their toxicity after autoclaving at 120°C for one hour.

Fodders affected by toxic strains of A.fumigatus may be de contaminated in fodder steaming units at 100°C and after cooling may be fed to cattle withou restriction. At the same time, the toxins of this producer are resistant to the effect of low temperatures (including temperatures below 0°C).

The sporidesmin producer in Pithomyces chartarum, class Deuteromycetes, causing facial eczema of sheep, is found in dead parts of clover, rye grass, and other herbs. The area of this micromycete is limited to Australia and New Zeland.

The fungus develops well on nutrient media. Colonies on potato-dextrose agar are flat, felt-like, olive-black. Single conidiophores are straight, darkly coloured at the apex. Single conidia are formed at the aspices of the conidiophores; they are oval in shape, with longitudinal and transverse septa, olive-black in colour.

Sporidesm in is formed during the development of the fingus

on natural substrates and in culture on nutrient media. The toxin is found mainly in the spores.

Spore formation and rapid growth of the fungus require a 100% humidity of the substrate and a temperature not below 13°C. The optimum toxin formation temperature is +24°C.

Smut species of Ustilago Tilletia (class Basidiomycetes) and rust fungi (Puccinia, Uromyces) are parasites which have adapted themselves to development on certain varieties of cultivated and wild-growing cereals and other plants. Smut fungi most frequently affect the ovary and inflorescences of the ears, sometimes the leaves and stems, while rust fungi develop on the leaves and stems.

Smut fungi cause smut of maize, loose and stinking smut of barley, loose smut of oats. By the time of ear formation and ripening of cereals, the smut mycelium in the ear falls apart into separate cells covered by a thickened coating, viz.chlamydospores. At this time a black, sooty, dusty mass consisting of the fungal spores appears in the ear instead of normal grains and the ear seems to be burned.

Rust fungi(Puccinia genus) cause crown rust of oats and stem rust of wheat. The affected parts of the plants have a red or rusty mealy coating consisting of the fungal teleutospores.

According to some data, particularly those published many years ago, the consumption of plants affected by smut and rust may cause the poisoning of the animals manifesting itself in the disturbances of the central nervous system, the respiratory and alimentary organs. However, the early reports have not been substantiated.

Pronounced toxicity among fungi belonging to the Basidiomycetes class is found in Amanita phalloides. Numerous cases of human poisoning upon eating this mushroom occur also at present and have been described in a number of foreign countries.

Group of probable but poorly studied mycotoxin producers

Ever new species of fungi causing mycotoxicoses in animals have been described in recent years.

Many reports refer to the toxic effect of the A.ochraceus fungus. Ochratoxins, the toxic metabolites of the fungus, were discovered in wheat, barley, oats, maize flour, rye, sorghum, buckwheat, soya beans, peanuts, mixed fodder, alfalfa hay. The colonies of this fungus in culture on nutrient media resemble the colonies of A.flavus. But the coloration of the colonies is always bright yellow without green tints, the conidial heads are larger than in A.flavus. The conditions of the synthesis of ochratoxin were studied. The highest yield of ochratoxin was obtained in a medium containing 4% of sucrose and 2% of yeast extract. The optimum temperature for the formation of ochratoxin is 26°C.

Ochratoxins are also formed by other species of fungi belonging to the Aspergillus genus: A.sulphureus, A.alliareus, A.melleus, A.ostianus, A.petrakii and species of the Penicillium genus: P.viridicatum, P.commune, P.cyclopium, P.purpurescens.

The ability to form toxins has been established in many Penicillium species.

Rubratoxins have been isolated from P.rubrum and P.purpuro-

genum. Toxic substances from these fungi are contained only in the culture fluids of the nutrient media and in the substrate on which they debelop. The fungi are isolated from different kinds of cereals.

From the P.citrinum fungus a hephrotoxic compound citrinin has been isolated.

P.islandicum forms luteoskyrin and excretes toxic metabolites into the substrate.

P.patulum forms patulin. This fungus contaminates many fodders.

From P.citreo-viridi a toxin citreoviridin has been isolated. The fungus has been isolated from mouldy rice which had been associated with diseases among humans.

Toxic substances have been also isolated from some other species of Penicillium.P.palitans, P.cyclopium, and other species form metabolites which produce a tremorgenic effect on the animal organism. Laboratory methods for isolating pure cultures of the above enumerated producers of mycotoxins are the same as for A.flavus.

The toxic metabolite slaframin is formed by the Rhizoctonia leguminicola fungus, class Deuteromycetes, which affects clover. The eating of contaminated clover causes strong salivation in animals.

This is far from being a complete list of fungi whose toxic properties have been described.

Thus, there are many potential producers of mycotoxins in nature. However, the toxicity of many of them has been established so far only experimentally, and mostly on laboratory animals.

Further studies of the effect of new mycotoxin producers on the organism of farm animals and man are required.

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