STORAGE MICROFLORA AND MYCOTOXINS. A NEW RISK FOR HEALTH.

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Extensive research on mycotoxins has been in isolation, identification, toxicity and analysis of the compounds. A shift in interest from mycotoxins to mycology is taking place. Research is being reported on methods to isolate specific fungi and to determine conditions under which they grow and produce mycotoxins. Concurrent with this is development of methods to prevent fungal growth and to control formation of mytotoxins before harvest and during storage. Research continues to elucidate other aspects of molds and mycotoxins such as the hepatotoxicity of the aflatoxins.

Spoilage of food by fungi is easily recognized by us all. The economic impact of fungal spoilage of food and feed is less easily recognized. Certainly in some areas of the world where the climate is warm and damp the spoilage caused staggering economic loss. Economic loss can also be caused by inadequate handling of crops. It is estimated in the Soviet Union that 20 percentage of the food harvest is lost due to shortages of fuel, storage space, and transportation. The development of knowledge and application of technologies has reduced the amount of fungal spoilage and will continue to do so as better techniques are developed but the facilities for handling harvested crops are essential.

It is estimated that one quarter of the world's food crops are affected by mycotoxins annually (Council for Agricultural Science and Technology, 1989) (CAST). This estimate changes yearly for an individual crop or region because of weather conditions during growing and harvesting. The economic impact of mycotoxins can be from lower yields for crops, from losses to livestock and poultry from death or from less dramatic effects such as reduced growth rates, less feed efficiency, and immune suppression. In addition to these costs add increased prices of the feed as a result of bad crop years, costs to consumers that rise because of restricted production and the associated regulatory costs including monitoring, testing and research (CAST).

Mycotoxins have been suspected for hundreds of years to be related to human disease. In the USSR in 1944-47 Alimentary Toxic Aleukia (ATA) killed thousands of people as a result of eating overwintered grain. It was later shown that the causal agent was the fungal toxin T-2 produced by molds in the genus *Fusarium*. In 1960 the

famous "Turkey X" disease killed some 100,000 turkey poults in Great Britain. Aflatoxin produced by the mold *Aspergillus flavus* was responsible. Since these outbreaks there has been considerable interest in molds that grow on agricultural crops producing mycotoxins either in the field or in storage.

Since the threat of mycotoxins in the food and feed supply was discovered research on new toxins and the molds that are capable of producing them has increased. It is estimated that there are more than 200 known mycotoxins. Of these the most important in terms of food and feed are the toxins produced by *Fusarium* spp. and the aflatoxins. An important part of the mycotoxin story has been surveys of agricultural crops for the presence of mycotoxins.

Evolutionary development has allowed some fungi to grow at moisture levels much lower than any other life form. This ability to grow at low moisture concentrations (low water activity) creates concern for the stored products industry. Water is the single most important factor in fungal growth and in the ability of stored products to resist spoilage (CAST,1989; Magan and Lacey, 1988). Stored products are stable against microbial deterioration if they are maintained at a low enough moisture content. Temperature fluctuations during storage can cause moisture transfer that may be high enough to permit microbial growth. Similarly insect and rodent activity can cause moisture conditions that allow fungal growth in an otherwise stable product. Fungal growth can then cause spoilage or permit mycotoxin formation. Once biological activity starts the associated production of water will provide sufficient water for additional biological activity. If the water activity (a_w) (equivalent to equilibrium relative humidity expressed as a decimal) is below 0.70 the product will be protected from microbial growth for long term storage. Higher a_w storage will allow shorter spoilage free storage time.

This report is an update of stored product protection against microorganisms and their toxins. Recent advances in the ongoing effort of mycology to control spoilage and mycotoxin formation are examined.

MYCOTOXINS

Since the early days of mycotoxin research there has been an increasing interest in solving the agricultural and public health problems presented by these compounds. Figure 1 shows the frequency of citations for the terms Aflatoxin and Mycotoxin from the years 1970-1989 in Biological Abstracts. This dramatically shows the continuing interest in mycotoxins.

Figure 1

The aflatoxins produced by *Aspergillus flavus* and *A. parasiticus* have received the most attention. These two molds produce

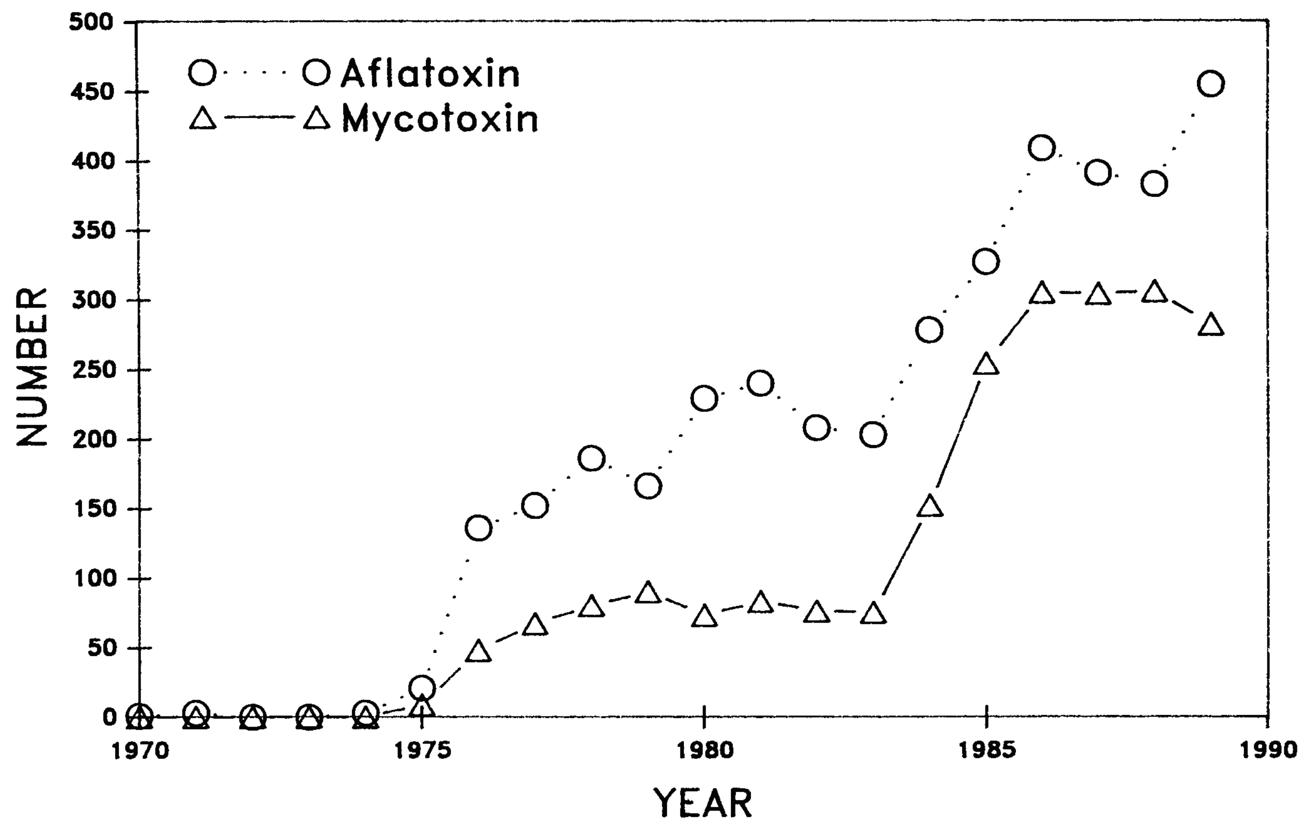

Figure 1. Number of Biological Abstracts citations for aflatoxin and mycotoxin from 1970 through 1989.

aflatoxins B_1, B_2, G_1, G_2 and other mycotoxins. Aflatoxin B_1 is the most toxic of the aflatoxins, is acutely toxic to young animals, especially poultry, and causes hepatic lesions in pigs. The designation of the four aflatoxins is due to their order and fluorescent color (blue or green) on developed TLC plates.

Trichothecenes are produced by the mold genera *Fusarium*, *Trichoderma*, *Myrothecium*, and *Stachybotrys* (Uneo, 1987). Trichothecenes are a major group of at least 148 mycotoxins (Scott, 1990). *Fusarium* mycotoxins--deoxynivalenol (DON), nivalenol, zearalenone and T-2 toxin and others have been detected in a number of grain products (Blaney and Dodman, 1988, Blaney et al., 1987, Jelinek et al.,1989, Scott, 1990), grain sorghum (Hagler et al., 1987) and corn and animal feeds (Abbas et al., 1988). These toxins are of both health and economic concern. They cause mycotoxicosis in animals as well as ATA (Abbas et al., 1988). In addition they cause economic loss because animals eating infected grains exhibit poor feed performance (Gilbert, 1989).

Ochratoxin is produced by several molds. It was first isolated from *Aspergillus ochraceus* and has subsequently been isolated from other aspergilli of the Ochraceus group--*A. sulphureus*, *A. sclerotiorum*, *A. alliaceus*, *A. melleus*, *A. ostianus*, and *A. petrakii* (Krogh, 1987). Ochratoxin is also produced by *Penicillium purpurescens*, *P. commune*, *P. viridicatum*, *P. palatans*, *P. cyclopium*, and *P. variabile* (Krogh, 1978). Ochratoxin causes a kidney disease in pigs now known as mycotoxic porcine nephropathy (Krogh, 1987).

Human diseases. Although numerous other mycotoxins have been isolated and identified only a few are important to food and feed. Clear evidence for casual association of mycotoxins and human disease has been recorded only for aflatoxin, for ATA caused by *Fusarium* toxins, for ergotism caused by fungal alkaloids and possibly for human nephropathy caused by ochratoxin A (CAST, 1989; Krogh, 1989).

Collecting clear evidence for human and animal diseases caused by mycotoxins is a difficult task due to the lack of acute toxicity and the methodological problems in collecting long-term low level exposure data and evaluation of it. Aflatoxin B_1 is acutely toxic to humans and laboratory animals (CAST, 1989) and is highly carcinogenic for selected species of laboratory animals causing hepatocellular carcinoma. Several epidemiological studies have been carried out in Africa and South East Asia to determine the risk of aflatoxin in human liver cancer. The results of these studies on the daily intake of aflatoxin and the incidence rate for liver cancer do not give a clear answer to the relationship of aflatoxin to liver cancer. Other risk factors may be associated with liver cancer beside aflatoxin ingestion--alcohol consumption, smoking or the presence of hepatitis B virus (Autrup et al., 1987; Krogh, 1989). Autrup et al. (1987) report a moderate degree of

correlation between exposure to aflatoxin and liver cancer in a study in Kenya but found no synergetic effect of liver cancer induction with hepatitis B virus exposure. Campbell et al. (1990) did not find a relationship between aflatoxin and liver cancer in moldy grain products in China. They did find a significant relation between infection with hepatitis B virus and liver cancer mortality. They conclude that the role aflatoxins play in the etiology of liver cancer is not proven. These two recent studies illustrate the complexity of the problem of relating mycotoxin exposure and disease.

Mycotoxin occurrence. The natural occurrence of most mycotoxins has not been well documented and the frequency and level of contamination of foods and feeds is not known (CAST, 1989). Mycotoxins cannot entirely be avoided in foods and feeds by current agronomic and processing techniques. They are considered unavoidable contaminants by the U.S. Food and Drug Administration (FDA). To help define the extent of the mycotoxin problem, surveys of products are conducted. Wood (1989) summarized a FDA surveys on aflatoxins in domestic and imported foods and feeds. Commodities sampled were peanuts, corn, tree nuts, cottonseed, milk, spices and various manufactured products. Many products were free of aflatoxin and most that were positive had low concentrations. For instance, 104 samples of peanut butter had an average of 14 µg/kg and a maximum of 27 µg/kg and only four were > 20 µg/kg. Aflatoxin in corn and corn products was correlated with origin in the south eastern states. Similarly,cottonseed containing aflatoxin was correlated with geographic area. Sixty one percent of the cottonseed and 80 percent of the cottonseed meal from Arizona and California were contaminated with aflatoxin. Only pistachio and walnut samples were positive for aflatoxin > 20 µg/kg. Pistachio nuts had a maximum of 252 µg/kg out of 22 samples while walnuts had a maximum of 41 µg/kg with 7 percent of the samples positive. The other 4 nutmeats were negative for aflatoxin. All 206 samples of milk and other products were negative for aflatoxin.

The survey included analyses of a number of imported products. Several nutmeats and nutmeat products had high concentrations of aflatoxin as did some spices, melon seeds and cornmeal. High concentrations of aflatoxin were found in some feeds including sorghum, mixed feeds, and ingredients. Imported products containing greater than 20 µg/kg aflatoxin are denied entry into the United States. A survey of 388 samples of foods and feeds including cereal grains, edible nuts and other products in Spain showed only aflatoxin from mixed feeds, one sample of groundnuts and whole maize flour (Sanchis et al., 1986). Molds including *A. flavus* were routinely isolated from these foods. Figs imported into Switzerland from Turkey were surveyed for aflatoxin and a small number of figs were found to be contaminated. The figs that were contaminated had high levels of aflatoxin in the individual fruits. These could be removed by visual inspection (Steiner et al., 1988). Aflatoxin is typically found in food samples at high

concentration on some individual pieces while other pieces are not contaminated. This makes sampling of the product difficult and requires large samples (Schade et al., 1981)

Jelinek et al. (1989) summarized surveys of foods and feeds for mycotoxins. Corn and corn products, peanuts and peanut products, seeds and some tree nuts continued to have high concentrations of aflatoxins. Rice affected by adverse weather had elevated levels of aflatoxin while raw and paddy rice had none. Refined food oils and other vegetable oils had low levels of aflatoxin but unrefined sunflower, peanut, copra and other oils had elevated levels.

MYCOLOGY

Mycology lagged behind the mycotoxin research because most of the early effort was placed on the determination of structure, toxicity, mode of action, analytical methods etc. for aflatoxin then other mycotoxins. Only later were mycology studies initiated to determine the parameters for fungal growth and mycotoxin production. Along with the increase in mycology research related to mycotoxins, associated research was increased to study spoilage caused by molds.

Mold growth conditions. Molds are often classified for practical reasons into two broad classes, field fungi and storage fungi. The field fungi consist of plant pathogens that invade the growing crop before harvest. They rarely play a significant role in postharvest deterioration (Pitt and Hocking, 1985). However, mycotoxins produced in the field may persist in the stored product such as with aflatoxin from *A. flavus* or *A. parasiticus* in corn or the persistence of T-2 toxin in overwintered Russian wheat. Storage fungi are predominately from the genera *Aspergillus* and *Penicillium*. They have the ability to grow at relatively lower water activity levels than the field fungi (Magan and Lacey, 1988).

Table I lists some foodborne fungi with the growth parameters for temperature and water activity. Optimum temperature for molds is in a fairly narrow temperature range around 20-35°C, with the optimum around 25°C. The total temperature range varies with the individual mold but most have growth limits in a range of about 40°C top to bottom.

Table I also lists the water activity parameters for growth. The optimum water activity for the listed molds is >0.94 except for the unique mold *Xeromyces bisporus*, the most xerotolerant fungus known. This fungus has been isolated from a variety of dried foods including prunes, currants, chocolate sauce and table jelly (Pitt and Hocking, 1985). All the molds with water activity optimum >0.98 are usually regarded as field fungi while those with the lower optimum are called storage fungi. Storage fungi also have a lower minimum water activity. It is this ability to grow at the lower water activity that allows them to damage stored products.

Table I. Temperature and water activity optima for spoilage fungi.

Mold	Temperature °C		Water Activity	
	Optimum	Range	Optimum	Minimum
Aspergillus flavus	25-42	6-45	0.95	0.78
Alternaria alternata	20-25	-5-35	1.00	0.85
Botrytis cinerea	22-25	-2-35	1.00	0.93
Cladosporium cladosporoides	24-25	-5-32	1.00	0.88
Eurotium spp.	25-35	5-43	0.94	0.70
Fusarium spp.	22-28	-2-39	0.98	0.90
Penicillium spp.	20-26	-6-35	0.98	0.78-0.83
Phytophthora infestans	20	4-26	1.00	0.85
Rhizopus stolonifer	26-29	5-37	1.00	0.93
Wallemia sebi	23-25	5-35	0.95	0.69
Xeromyces bisporus	25		0.85	0.61-0.97

(Pitt and Hocking, 1985; Lacey, 1989)

Note that the molds, even though they can grow over a range of water activity, always have the optimum in the high moisture end of the range.

The development of storage fungi is chiefly dependent on water content and temperatures. If the moisture level is high enough then some storage fungi may grow. If the water available is low the fungi will die off (King and Schade, 1986). In a series of papers Magan and Lacey (1984a, 1984b, 1984c) have shown the relationship between water activity, temperature and substrate on growth of fungi. The minimum water activity tolerated by the fungus changes with the temperature. The closer to optimum temperature the lower the tolerated water activity that will permit growth. The interaction between temperature, water activity, carbon dioxide concentration and oxygen concentration was also studied (Magan and Lacey, 1984c). The further from optimum conditions the more retarding these parameters became. Storage fungi exhibit interactions between themselves, some being antagonistic while others are benign Magan and Lacey, (1985). Research to determine which fungi will become dominant will allow development of biological control measures for fungal growth in storage.

Mycological Media

Two basic growth methods are used to evaluate fungal contamination of foods. One method involves removal of the fungal propagules from the food by stomaching or blending the sample in diluent and then plating on an appropriate medium to determine the count per g or ml. The other method is direct plating of the food directly on a medium and determining the percentage contamination. The latter is often used after surface disinfecting with chlorine to remove all the surface contamination leaving only the molds that are established in the product (Andrews, 1986). Development of this kind of data shows that the fungi have many unique niches, especially in food and feed commodities, and require unique media for isolation and counting.

General Purpose Mycological Media. Mycological media have been developed to serve several unique purposes. Previously, it was recommended that a general medium, such as potato dextrose agar acidified to pH 3.5 to inhibit bacterial growth, be used for routine examination of foodstuffs. Mossel et al. (1970) introduced a medium, Oxytetracycline Glucose Yeast Extract Agar (OGY) containing the antibacterial antibiotic oxytetracycline as a general purpose fungal medium that is still in use today. Koburger and Rogers (1978) noted that the use of antibacterial antibiotics would allow use of a higher pH in fungal medium and thereby avoid the inhibitory effect of low pH. Jarvis (1973) developed a medium, Rose Bengal Chloramphenicol Agar (RBC), with rose bengal added to slow the spreading growth of some molds and allow easier counting of colonies. To avoid the problem of rapidly spreading fungi,

especially the Mucorales, King et al. (1979) developed a general purpose medium Dichloran Rose Bengal Chloramphenicol Agar (DRBC). The medium developed from the Jarvis RBC medium contains the fungicide dichloran (2,6-dichloro-4-nitroaniline) at 2 mg/l concentration, rose bengal at 5 mg/l to restrict spreading, and chloramphenicol as an antibacterial. This medium inhibits bacteria, strongly inhibits growth of rapidly spreading fungi but does not prevent their growth entirely. DRBC causes the mold colonies to grow compactly so counting is easier and more accurate. DRBC allows growth of food spoilage fungi yet effectively restricts the rampant growth of *Rhizopus* and *Mucor*. OGY, RBC and DRBC are widely accepted by mycologists for cultivation of molds and yeasts and are in commercial production (King et al., 1986; Pitt, 1989).

Selective Mycological Media. Xerophilic fungi (a fungus which is able to grow at or below a water activity of 0.85 (Pitt and Hocking, 1985)) require less moisture (lower water activity) than other molds. Hocking and Pitt (1980) developed a medium with reduced water activity to successfully grow these molds. The medium,Dichloran 18% Glycerol Agar (DG18), contains glycerol in a nutrient base to lower the water activity to 0.955 and dichloran as well as chloramphenicol. A wide variety of fungi will grow on this medium including most species of *Aspergillus* and *Penicillium* found in foods such as stored grains, nuts and spices. It also supports growth of a range of the less fastidious xerophilic molds and yeasts including *Eurotium* species, *Wallemia sebi* and *Saccharomyces rouxii*. DG18 is available commercially and is a highly satisfactory isolation medium. Fastidious xerophilic fungi can be isolated on medium with lower water activity. No inhibitors are necessary because the low water activity restricts other microorganisms. Media such as Malt Extract Yeast Extract 50% Glucose Agar (MY50G) or a similar medium with less sugar can be used to isolate spoilage mold from dried stored products (Pitt and Hocking, 1985).

Isolation media is needed, especially for the mycotoxigenic fungi. Pitt et al. (1983) developed Aspergillus Flavus and Parasiticus Agar (AFPA) from Aspergillus Differential Medium (Bothast and Fennell, 1974) which lacked selectivity. Ferric ammonium citrate in these media will, in the presence of *A. flavus* and *A. parasiticus* metabolites, produce an intense orange reverse color that is diagnostic. AFPA is recommended for the detection and enumeration of aflatoxigenic fungi. The medium is suitable for enumeration by non-mycologists and can be counted in less than 48 hrs. This medium is available commercially.

Frisvad (1983, 1986) developed Pentachloronitrobenzene Rose Bengal Yeast Extract Agar (PRYES) as a selective screening medium for *P. viridicatum* and *P. verrucosum*, important mycotoxin producers including ochratoxin, from stored cereal products. The reverse violet brown coloration is associated with *P. viridicatum* group II

while *P. viridicatum* group I and *P. aurantiogriseum* has a yellow reverse and obverse color.

Fusaria and dematiaceous hyphomycetes can be isolated from cereals by Dichloran Chloramphenicol Peptone Agar (DCPA) (Andrews and Pitt, 1986). This medium has only peptone as a nutrient which inhibits the growth of *Aspergillus* and *Penicillium* by absence of carbohydrate while dichloran inhibits the mucoraceous fungi. Thus this medium is useful for isolation of *Fusarium* and *Alternaria* species that each produce many mycotoxins (Gilbert, 1989: King and Schade, 1984).

Other recent mycology developments are to detect mold growth by chemical methods rather than to have to rely on growth before analysis (Jarvis et al., 1983; Williams, 1989). ATP (adenosine triphosphate) measurement by luciferin/luceferase photochemical reaction has been proposed for assessment of fungi growth as well as other microbial cells. Impedance measurements are used to measure bacterial cells and if a suitable medium could be developed, would be also for fungi (Williams, 1989).

An enzyme linked immunosorbent assay (ELISA) using antibodies against the heat stable, water soluble polysaccharide antigens produced by molds has been developed by Notermans et al. (1988). They developed a test for *Penicillium* and *Aspergillus*, the major group of molds found in food. The technique is simple once the test reagents are developed and can be highly reliable. Another test that detects extra-cellular polysaccharide (EPS) is the latex agglutination assay (Kamphuis et al., 1989). Latex beads are coated with immunoglobulins sensitized to EPS and when they come in contact with the EPS antigen produced by the mold, the complex that is formed agglutinates. The authors claim this test is faster but not as sensitive as the ELISA test. Both of these tests will detect viable and non-viable mold so they give different results than viable counts.

Williams (1989) reviews the chemical analysis of fungal biomass by detection of either chitin or ergosterol. Both of these components of fungal mycelium are heat stable and will persist through food processing. Analysis of them gives information about the amount of fungal biomass. Both analyses require several chemical steps and are therefore somewhat cumbersome (Williams, 1989). With the chitin test interference from glucosamine in food or from insect exoskeletons are limitations of this test (Cousin, 1986). The chitin test is a hydrolysis of chitin to sugar which is estimated colorimetrically and takes around 5 hours. Ergosterol assay is claimed to be highly sensitive and specific to mold contamination in grains (Seitz et al., 1977). The HPLC assay takes about one hour including extraction, saponification and detection (Williams, 1989). Another rapid method for screening samples for ergosterol uses a TLC detection method. Iodine vapor treatment of the TLC plate makes ergosterol fluoresce greenish-blue under longwave UV

light. Chemical confirmation tests using acid sprays on the plate are also reported (Rao et al., 1989).

CONCLUSIONS

Fungal spoilage and mycotoxin production are both economic and public health problems to the food and feed industry. They must be controlled. Fungal spoilage of stored products is caused by excess moisture and growth of xerotolerant fungi. The control of this kind of spoilage will be aided by the data on growth requirements of fungi and the interaction of fungi. Biological control of fungal spoilage will one day be a reality. Before control of mycotoxins can be achieved, the conditions for mycotoxin production must be fully understood both for field fungi and storage fungi. Defining the conditions of mycotoxin production and for control of fungal growth is needed. New mycological media and chemical tests to detect and to study the fungi are being developed.

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MYCOLOGIE ET MYCOTOXINES DANS LES STOCKS DE DENREES, UNE MISE A JOUR

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RESUME

Les recherches intensives sur les mycotoxines ont consisté à isoler, à identifier, à mesurer la toxicité et à analyser les diverses substances. Un changement d'intérêt se portant des mycotoxines vers la mycologie a lieu actuellement.

On fait état des recherches qui portent sur la création de méthodes visant à isoler des mycoflores spécifiques et à mesurer le conditions dans lesquelles elles se développent et produisent leurs mycotoxines.

Il s'ensuit un développement de méthodes préventives qui visent à empêcher la croissance fongique et à éliminer la formation des mycotoxines avant la récolte et pendant le stockage.

Les recherches sont poursuivies pour trouver des solutions à d'autres aspects de la formation des moisissures et des mycotoxines tels que l'hépatotoxicité des aflatoxines.