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Mycotoxicoses in veterinary medicine: Aspergillosis and penicilliosis

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Abstract

Molds and mycotoxins are contaminants of animal feed causing spoilage and clinical intoxication. Animal exposure to mycotoxins reflects diet composition with major differences occurring between animals kept predominantly of pastures, i.e. ruminants and horses, and those consuming formulated feed like pigs and poultry. Mixed feeds are composed of several ingredients, often sourced from different continents. Subsequently, practitioners may confront endemic diseases and signs of toxin exposure related to toxins imported accidentally with contaminated feed materials from other countries and continents. Mycotoxins comprise more than 300 to 400 different chemicals causing a variety of clinical symptoms. Mycotoxin exposure causes major economic losses due to reduced performance, impaired feed conversion and fertility, and increased susceptibility to environmental stress and infectious diseases. In acute cases, clinical symptoms following mycotoxin ingestion are often non-specific, hindering an immediate diagnosis. Furthermore, most mold species produce more than one toxin, and feed commodities are regularly contaminated with various mold species resulting in complex mixtures of toxins in formulated feeds. The effects of these different toxins may be additive, depending on the level and time of exposure, and the intensity of the clinical symptoms based on age, health, and nutritional status of the exposed animal(s). Threshold levels of toxicity are difficult to define and discrepancies between analytical data and clinical symptoms are common in daily practice. This review aims to provide an overview of *Aspergillus* and *Penicillium* toxins that are frequently found in feed commodities and discusses their effects on animal health and productivity.

Introduction

Fungi are ubiquitous and fulfill an essential role in the recycling of nutrients from decaying matter in soils, vegetation, and water. Fungi may be saprophytic or parasitic as they lack true chlorophyll and might promote or impair plant growth and development. *Fungi imperfecti* are known to produce a variety of secondary metabolites which seem to improve their competitiveness in nature.[1] Primarily, these secondary metabolites are directed against substrate competitors such as bacteria (anti-biotics) or other fungal species (antimycotics), however, they may help protect the plant host from insect damage. Peramine, for example, a secondary metabolite of endophytic fungi, is a strong insect repellent.

As fungal metabolites exert diverse biological effects, any effect-based classification remains arbitrary. The group of penicillin's, fumagillin, and monacoline, exemplify this as these compounds have been originally isolated from fungal cultures and further developed into pharmaceutical entities for therapeutic purposes. Other fungal metabolites exhibit an intrinsic toxicity even at low concentrations, resulting in the collective classification as mycotoxins. Thus, the terms mycotoxins and mycotoxicosis refer to the multiple adverse health effects exerted by fungal metabolites towards mammalian species including man.

Toxinogenic fungi are cosmopolitan and reside all around the world. Their geographic prevalence depends on their tolerance to low water activity (moisture), pH and their temperature and substrate preferences.[2] Since the outbreak of turkey-X-disease in the UK in the early '60s,[3] it has become evident that the international trade in bulk raw materials used in animal diets, facilitates the transfer of mycotoxins from endemic regions to areas of intensive

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animal farming. At present, the contamination of feed commodities by molds and mycotoxins is considered to be one of the most important negative factors in crop production and animal feed quality, as more than 300-400 toxic mold metabolites have been identified.[4] However, their impact on animal health varies considerably and only a few mycotoxins have been related to important animal diseases.

Mycotoxins may exert acute intoxications, however, more importantly, they impair animal productivity by reducing weight gain, feed conversion, and resistance to infectious diseases.[5] Studies with purified toxins have identified the major target organs for toxic effects, and comprehensive reviews summarizing the experimental data have been published for all major toxins, including the aflatoxins.[6] However, specific experimental data often fail to resemble the multiple clinical symptoms of field outbreaks of mycotoxicoses. The latter may be explained by the fact that toxinogenic molds commonly synthesize more than one toxin and that spoiled feed commodities may be contaminated by more than one fungal species.[7] In addition, typical (patho-) physiological features of the target animal species account for the observed interspecies variation in susceptibility and clinical presentations.[8] The following review deals with the *aspergillus* and *penicillium* toxins clinical effects on animal health and productivity.

***Aspergillus* toxins and their effect on health of animals.** *Aspergilli* may invade a broad variety of feed and food commodities, including corn, cereal grains, soybeans, nuts, and oilseeds. Certain *Aspergillus* strains (for example *A. oryzea*) have been used for centuries in food fermentation and preservation all around the globe.[9] Typically, *Aspergilli* are found in tropical or subtropical areas, as optimal fungal growth and particular mycotoxin production requires average temperatures above 24.00 ˚C. The most prominent toxins produced by Aspergilli (*A. flavus, A. parasiticus*) are aflatoxins, comprising aflatoxin B_1, B_2, and G_1, G_2, identified and classified based on their fluorescence (blue or green) in the presence of ultraviolet light. Aflatoxin B_1 (AFB_1), the most toxic aflatoxin, has been identified as the causative agent in the turkey X – mycotoxicosis.[3] AFB_1 exerts its toxicity after metabolic activation by tissue oxygenases (primarily CYP450 enzymes). This results in the formation of reactive epoxides, of which the *exo*-epoxide readily binds to macromolecules such as proteins and DNA, causing (liver) cell toxicity and DNA damage. The reactive epoxides are further metabolized by glutathione S-transferases, resulting in inactivation and excretion.[10] The species- and organ-specific capacity to metabolize, activate and deactivate AFB_1 explains the predilection sites of its toxic effects (hepatotoxicity) and the species variability insensitivity towards aflatoxin exposure.[11] AFM1 and AFM2 as milk AFs are monohydroxylated metabolites of AFB1 and B2, respectively, which are not destroyed under the normal cooking condition and even pasteurization processes. The hepatocarcinogenesity of AFM1 has been documented while the potency of AFM1 due to being a poor substrate for epoxidation is less than AFB1.[12]

Clinical symptoms of acute intoxication comprise hepatocellular necrosis and lipidosis around central veins, bile duct hyperplasia, icterus, hemorrhagic disease or coagulopathy, weight loss, and anorexia.[6] In monogastric species, these symptoms are observed primarily after ingestion of feeds containing levels above 1,000 μg kg^{-1}, however, considerable interspecies variation in AFB_1 tolerance has been observed, depending on the age, sex and condition of the animal.[13]

Chronic exposure to moderate concentrations of AFB_1 results in elevated liver enzymes, impairment of hepatic function including icterus and hypoproteinemia, followed by general symptoms such as reduced growth, reduced feed conversion, and rough hair coat.[14] Non-specific signs of aflatoxin exposure include immunosuppression, resulting in increased susceptibility to infectious diseases such as salmonellosis, candidiasis, coccidiosis, and liver fluke infections.[15] Outbreaks of acute intoxications are presently confined to rural areas of tropical and subtropical countries, as control measures implemented worldwide, prevent animal exposure to high aflatoxin concentrations in formulated feeds.[16] Recently, p53-dependent apoptosis induction and cyclin D_1 down-regulation in AFB_1-exposed rodents has been reported.[17]

Feed commodities invaded by aflatoxigenic fungal species may also contain trace amounts of sterigmatocystin (ST), the natural precursor in the aflatoxin biosynthetic pathway.[18] As ST shares many of the toxicological properties of aflatoxins, it is generally recommended to analyze feed for the total amount of aflatoxins. By adding the amount of any ST to the amount of the four natural aflatoxins (B1, B2, G1, and G2) present, it is possible to estimate the acceptability of a certain feed batch for animal feed production. The genotoxicity of ST in HepG2 cells through oxidative stress and lysosomal leakage has been documented.[19]

Aflatoxinogenic fungi often also produce cyclopiazonic acid (CPA) by a distinct biosynthetic pathway, and the co-occurrence of aflatoxin and CPA may vary from 51.00% in corn samples to 90.00% in peanut samples, in which CPA has been found in concentrations as high as 10.00 mg kg^{-1}.[20] CPA is an indole-tetramic acid that has been primarily classified as a neurotoxic mycotoxin.[21] Studies on the mode of action of CPA have indicated an inhibitory effect on calcium ATPase in the sarcoplasmic reticulum of muscle cells that explain the clinical symptoms such as weakness, incoordination, and lethargy.[22] Poultry seems to be particularly sensitive and CPA has been discussed as a co-toxin in the initial outbreak of turkey X - disease, accounting for the neurological signs and opisthotonos

observed in affected animals.[23] The occurrence of CPA in broiler's diet (0.95 ± 0.35 μg g^{-1}) and CPA-induced hepatic and renal disorders in broilers have been reported.[24] Very recently we showed CPA-induced detrimental effects on the male reproductive system in the rodent model. Exposure to CPA at 0.06 mg kg^{-1}, BW dose level for 28 days resulted in a significant (p < 0.05) reduction in sperm count, sperm viability, sperm motility, chromatin quality of sperm and testosterone level.[25] Although CPA may form residues in edible tissues of animals, the concentrations measured seem to be too low to represent a health hazard to consumers. However, under stress conditions, CPA can impair meat quality and this has been demonstrated in young turkeys.[26]

Other typical *Aspergillus* toxins included patulin, produced by *Aspergillus clavatus* (and also by *Penicillium* species) and gliotoxin formed by *Aspergillus fumigatus*. The intoxication of livestock due to patulin exposure has been reported incidentally as a consequence of feeding malt products (originating from beer breweries) to cattle. Clinical symptoms include incoordination, paralysis, neuronal degeneration of the cerebral cortex, and even death. Whether or not these symptoms can be solely attributed to patulin remains to be elucidated.[27] Moreover, patulin is frequently found as a contaminant in apple juices and tomato products intended for human consumption. There are several lines of evidence to show the molecular mechanism(s) of cytotoxicity of patulin including its high reactivity with sulfhydryl-containing compounds such as cysteine or glutathione, inhibition of Na^{+}-K^{+} ATPase and inhibition of biosynthetic enzymes such as RNA polymerase and aminoacyl-tRNA synthetases. Induction of inflammation and ulceration in the gastrointestinal tract of patulin-exposed animals has also been reported in *in vivo* studies.[28]

Gliotoxin, a piperazine-derivative, is a strong immune-suppressive agent in *in vitro* systems,[29] however, its relevance in clinical toxicology is becoming a matter of increasing concern. *Aspergillus fumigatus*, originating from the animal's environment, is known to invade animals (and human) tissues, such as lungs, gastrointestinal and uro-genital tract. Following the distribution, toxin production occurs directly at the site of infection (endomycotoxicosis), leading to high tissue concentrations,[30] followed by a respiratory syndrome due to immuno-suppressive effects of gliotoxin in poultry and late abortion in cattle.[31] The cytotoxic effects of *Aspergillus fumigatus* toxins on human T lymphocytes (Jurkat cells) are shown in Figure 1.

***Penicillium* toxins and their effects on animal health.** *Penicillium* species are typical storage fungi, invading feed commodities at the post-harvest stage, although exceptions have been reported. For example, ochratoxin A producing *Penicillium* strains have been found on oats and barley before harvest.[32] *Penicillium* species share a preference for protein-rich commodities and low pH substrates. This is indicated by their widespread industrial use in the fermentation of dairy products in soft cheese production (*Penicillium camenberti,* and *Penicillium roqueforti*).[33]

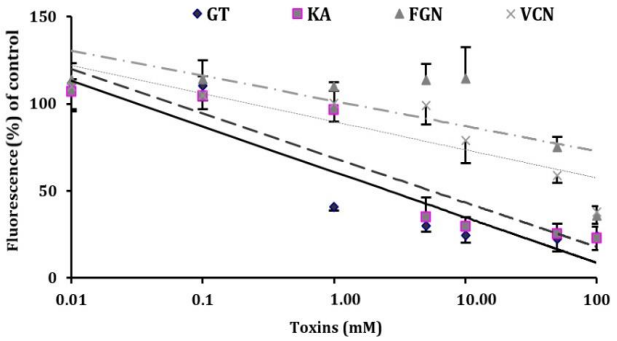

Fig. 1. *Aspergillus fumigatus* toxins cytotoxicity on human T lymphocytes (Jurkat cells) by Alamar blue reduction test; GT: Gliotoxin, KA: Kojic Acid, FGN: Fumigallin, and VCN: Verruculogen.

Penicillium toxins, including CPA, verruculogen, roquefortine, and penitem, originate directly from fungal amino acid metabolisms.[21] They may contain a functional amino acid side chain, for example, ochratoxin A, which consists of an isocoumarin moiety linked to phenylalanine. The biosynthetic origin of *Penicillium* toxins may explain a number of their typical features: The ease with which they bind to proteins explains their affinity to cellular enzymes and their antibacterial activity (penicillin-binding protein in bacteria) and their long biological half-life due to tissue and serum-protein binding in mammalian species.[34]

The most prominent *Penicillium* toxin found in feed commodities is ochratoxin A. Ochratoxin A is the common toxic representative of some structurally related ochra-toxins, which are secondary metabolites of a variety of *Penicillium* species, including *P. aurantiogriseum, P. freii, P. tricolor P.verrucosum, P. viridicatum, and P. polonicum.*[35] Their occurrences in feedstuffs, particularly grains, exhibit a typical geographic pattern.

Ochratoxin A is primarily a nephrotoxin, exerting tubular damage and fibrosis, followed by renal function impairment. Typical intoxications have been described in pigs (porcine nephropathy), however, the kidneys are the major target organ in all monogastric species.[36] Pathological changes of the kidneys develop after prolonged exposure over several weeks following in-feed concentrations > 200 μg kg^{-1}. Clinical symptoms may be mild with polyuria, polydipsia, and reduced growth and feed efficiency; however, can and often remain undetected until the time of slaughter where pale, firm kidneys indicate toxin exposure.[37] Other toxicological effects exerted by ochratoxin A comprise impaired coagulation, leukopenia, immunosuppression, teratogenicity, and induction of tumors in the urinary tract.[38] These toxic effects have been predominantly observed in experimental feeding trials, in which high doses of the toxin were given. Previously its

toxic effects via releasing of calcium from internal stores in human neutrophils have been reported.[39]

Field-outbreaks of ochratoxicosis are known to occur in pigs and poultry, however, ruminants successfully degrade the toxin in the rumen.[40] Ochratoxin is gaining increasing attention as a potential risk factor in the human diet and it can be found in numerous cereals, grains and by-products, and coffee.[41] Due to its long half-life in monogastric animals, residues of ochratoxin A in animal tissues often contribute to risks of human exposure.[42] Endemic diseases such as Balkan Endemic Nephropathy and Urinary Tract Tumours have been attributed to human exposure to ochratoxin A.[43]

A second nephrotoxin produced by *Penicillium* species is citrinin, which shows some structural similarities to ochratoxin A, however, it lacks an amino acid-side chain. Citrinin has not been well studied, however, it has been found to aggravate the effects of ochratoxin A and causing renal impairment in feeding trials in poultry.[44] Recent experimental studies on zebrafish showed that citrinin-induced nephrotoxicity was due to the induction of the expression of the pro-inflammatory genes including COX2, TNF-α, and IL-1β.[45]

Other prominent *Penicillium* toxins such as gliotoxin (discussed already above with the group of *Aspergillus* toxins), and the group of *P. roqueforti*-toxins [46] including patulin, mycophenolic acid, the roquefortins (iso-fumiclavin), PR-toxin and penicillic acid, which are found particularly in silage as *P. roqueforti* is well adapted to low pH conditions.[47] Despite their demonstrated toxicity in experimental studies, the significance of *P. roqueforti*-toxins as feed contaminants is limited and their low bioavailability after oral ingestion seems to prevent clinical intoxication. However, they may exert adverse effects due to antimicrobial activity resulting in dys-bacteriosis. The antibacterial effects of *P. roqueforti* toxins on two important bacteria are shown in Figure 2. The ruminal flora is particularly sensitive and impaired fatty acid synthesis may result in ketosis which in turn can increase the risk of sub-clinical mastitis in dairy cows.[48]

Some of the *P. roqueforti* toxins have been allocated to the group of tremorgenic mycotoxins, a broad group of mycotoxins sharing an indole moiety. They include the penitrems, the verruculogen-fumitremorgen group, and the aflatrems.[49] Experimental investigations in laboratory animals have demonstrated the tremorgenic effects of these mycotoxins particularly after parenteral injection of purified toxins. The potassium and in particular big potassium channel inhibition has been documented as a molecular-based mode of action of indole-diterpene tremorgens.[50] Their neurotoxicity seems to be related to interactions with GABA-receptor gated ion channels and sodium ion channels as demonstrated in some studies with penitrem A by Knaus *et al*.[51] Penitrem A has been discussed previously as the causative agent of field-outbreaks of tremors in sheep and other incidental intoxications.[52] However, the oral bioavailability of these indole derivatives is very limited and their actual role in clinical intoxications needs to be established. The cytotoxic effects of *P.roqueforti* toxins on two different neuronal cell lines are depicted in Figure 3.

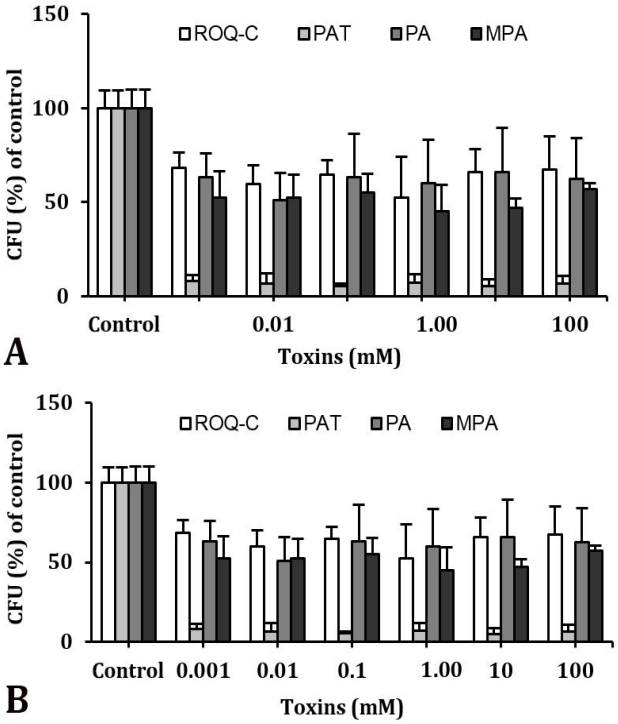

Fig. 2. The antibacterial effect of *Penicillium roqueforti* toxins on **A)** *E. coli*, and **B)** *Staphylococcus aureus*, ROQ-C: Roquefortine C, PAT: Patulin, PA: Penicillic Acid, and MPA: Mycophenolic acid. All four toxins at examined concentrations could exert significant antibacterial effect with the following range: PAT > MPA > PA > ROQ-C.

Methods of prevention. The most effective way of preventing mycotoxicosis is to make feed diets free of mycotoxins or containing negligible amounts of toxin. As this is rarely achievable under practical conditions, numerous alternative approaches have been attempted to degrade, sequester or complex mycotoxins present in feed.[53]

Chemical decontamination through ammonia treatment was the first method to be applied to detoxify products contaminated with aflatoxins, and large-scale technical processes were effective in removing aflatoxins or decreasing to very low residual amounts. However, ammonification reduces the nutritional value of feeds. Moreover, its effect is limited to the removal of aflatoxins, as other mycotoxins are incompletely degraded. In another approach, the treatment of contaminated commodities with electrically generated ozone has been suggested.[54] Other chemical degradation methods have not achieved broad application by feed manufacturers.

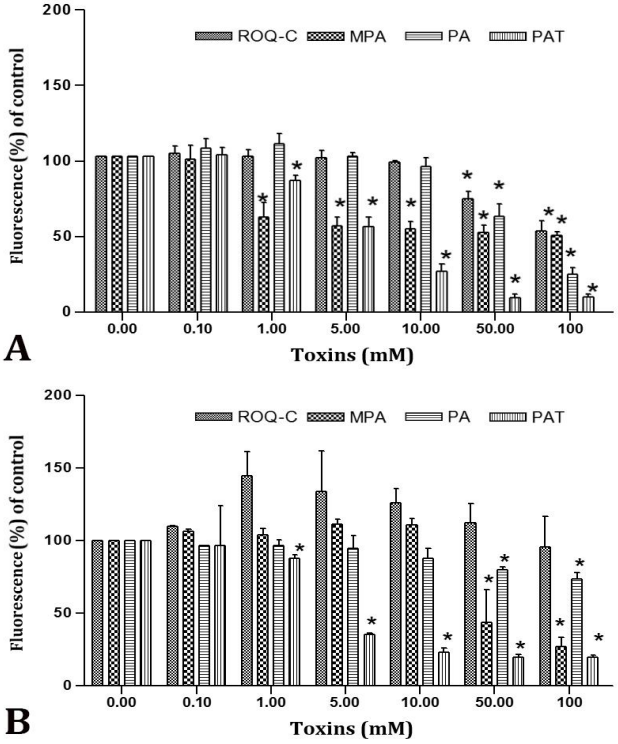

Fig. 3. Cytotoxicity of *Penicillium roqueforti* toxins on **A)** Neuro-2a cells, and **B)** Genetically engineered PC-12 Tet-Off (PTO) cells, ROQ-C: Roquefortine C, PAT: Patulin, PA: Penicillic Acid, and MPA: Mycophenolic acid. Patulin exerts the most potent cytotoxic effects and roquefortine C caused the weakest cytotoxic effects on both cell lines. Asterisks represent significant differences between non-exposed (control) and mycotoxins-exposed cells ($p < 0.05$).

As an alternative to chemical degradation, silica clays may scavenge aflatoxins successfully. The ester bond of the furan-ring has been found to provide a docking site binding the aflatoxin molecule covalently to natural clays. Although this method is successfully applied worldwide, it needs to be emphasized that different clays have different binding capacities, thus, effectiveness has to be verified for every individual product. Furthermore, it is important to stress that optimal results in mycotoxin binding to silicates have been achieved only for aflatoxins.[55] Other mycotoxins, having chemically different structures, such as the trichothecenes, ochratoxins or zearalenone, bind only with low affinity to silica clays and toxin exposure is therefore only partly prevented.[56] Other methods to reduce mycotoxin exposure have also been tested. In another study which was conducted to measure the effectiveness of yeast product (a yeast cell wall from baker's yeast) in adsorption performances for mycotoxins, it has been reported that the aforementioned yeast product was only able to adsorb 29.00% AFB1 and 69.00% OTA, indicating that adsorption capacity mainly depends on yeast composition and mycotoxin concentration. [57]

The most promising results have been obtained with fermentation processes, in which bacterial, yeast, or fungal enzymes facilitate biodegradation and detoxification of multiple toxins at moderate temperatures. Most mycotoxins are degraded successfully by ruminant microflora and by caecal flora of pigs.[58] However, the search for one single microorganism able to detoxify various mycotoxin classes has so far been unsuccessful (perhaps not surprising given the diverse chemical structures of mycotoxins). Future attempts will be directed towards the combined use of different microorganisms, targeted to the major classes of mycotoxins. Such pre-fermentation might not only detoxify mold-contaminated feed but could also improve digestibility and nutritional value, as indicated by early trials with *Lactobacillus* and *Bifidus species*.[59]

Public health aspects. In human food, mycotoxins have gained increasing importance as they have been implicated in the etiology of various diseases, particularly in liver hepatocellular carcinoma (HCC), and Balkan endemic nephropathy. The latest report on human aflatoxicosis has been reported from Kenya that in that special outbreak several hundred deaths due to consumption of aflatoxin-contaminated maize occurred.[60] The question addressed to veterinary public health is the possible carry-over of mycotoxins into edible tissues, milk, and eggs. This risk was first recognized for aflatoxin M_1, aflatoxin metabolites excreted in milk. Furthermore, the long half-life of ochratoxin A in animal tissues has been debated, however, found to be insignificant in comparison with direct exposure to ochratoxin and citrinin from plant-derived foods. At the same time, relatively high OTA concentrations were reported from human milk, which may be a great concern from public health.[60]

Conclusion

Whilst mycotoxins in animal feeds may cause acute intoxication, their greatest impact is their negative effect on animal performance and productivity. Thus, practitioners are confronted with difficult differential diagnoses, as mycotoxin intoxications usually lack specific clinical symptoms. Besides, farm managers require optimal animal production and will question the quality of their feed supplies expecting advice from the veterinary professionals for possible prevention of mycotoxin contamination and treatment for its effects. Treatment currently is limited to symptomatic therapy in cases of acute intoxication, as specific antidotes are not available. There is a need for more effective toxin binders to prevent the absorption of toxins and thus exposure. Other forms of in-feed medication (antioxidants, probiotics) that can reduce the detrimental effects of mycotoxins, deserve consideration. Finally, the modern agricultural practice has to consider the impact of advanced crop production as new techniques may provide more optimal conditions for mold development and toxin production. Equally, genetic engineering may produce plants less susceptible.

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