FEMS Symposium No. 69

# THE GENUS *ASPERGILLUS*

## From Taxonomy and Genetics to Industrial Application

EDITED BY

KEITH A. POWELL
ANNABEL RENWICK
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# The Genus *Aspergillus*

## From Taxonomy and Genetics to Industrial Application

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PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging-in-Publication Data

The Genus Aspergillus : from taxonomy and genetics to industrial application / edited by Keith A. Powell, Annabel Renwick, and John F. Peberdy.
p. cm. -- (FEMS symposium ; no. 69)
"Proceedings of a symposium held under the auspices of the Federation of European Microbiological Societies, April 5-8, 1993, in Canterbury, Kent, United Kingdom"--T.p. verso.
Includes bibliographical references and index.
ISBN 0-306-44701-0
1. Aspergillus--Congresses. 2. Aspergillus--Industrial applications--Congresses. I. Powell, Keith A. II. Renwick, Annabel. III. Peberdy, John F., 1937- . IV. Federation of European Microbiological Societies. V. Series.
QK625.M7G46 1994
589.2'3--dc20 94-15373
CIP

Proceedings of a symposium held under the auspices of the Federation of European Microbiological Societies, April 5-8, 1993, in Canterbury, Kent, United Kingdom

ISBN 0-306-44701-0


A Division of Plenum Publishing Corporation
233 Spring Street, New York, N.Y. 10013



Printed in the United States of America

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## PREFACE

Every sixteen years or so it is appropriate to review progress in the understanding of a genus, its pathogenicity, practical utility, genetics and taxonomy, secondary metabolites and enzyme production. This book is the second attempt at such a work on the genus *Aspergillus*. It is a compilation of papers from a conference organised by the British Mycological Society and sponsored by the Federation of European Microbiological Societies. Additional sponsorship came from Pfizer, SmithKline Beecham and Zeneca Agrochemicals.

The purpose of the conference, held from 5th to 8th April 1993 was to enable a cross-discipline discussion of the properties of the genus. As can be seen from the chapters which follow the conference was broad and gave a wide coverage of topics. We are delighted to be able to produce this book relatively rapidly after the conference and hope that readers will find it both interesting and a useful source of reference as indeed was the first *Aspergillus* book produced by the British Mycological Society in 1977.

This text contains a comprehensive review of the fungal genus *Aspergillus* with contributions from a diverse group of experts including taxonomy, molecular genetics, medical pathology, industrial fermentation, food and agriculture; offering the reader a current overview of the subject.

The aspergilli have been used for many years by the fermentation industry for the production of citric and other organic acids, and have been used for centuries in the preparation of soy sauce. More recently the aspergilli have been exploited for the production of enzymes widely used in industry for the manufacture of a variety of materials.

A wide range of secondary metabolites are produced by these fungi including the potent carcinogens aflatoxins. Different aspects of this important topic are covered in several chapters. Cotty, Baymna, Egel and Elias have produced a comprehensive review of the occurrence of *Aspergillus* and aflatoxins in agriculture. Other chapters cover food spoilage in animal and human feed.

*Aspergillus nidulans* is one of the few filamentous fungi for which molecular genetics tools are being developed. The use of *A. nidulans* for heterologous gene expression is discussed which is of importance both when considering the industrial utility of these fungi and as a model system. The regulation of gene expression is illustrated by proline utilisation and *A. nidulans* is being used as a model for the genetic analysis of secondary metabolism in fungi as illustrated by Turner using penicillin biosynthesis. An area of current interest is the mechanism regulation of cell division. *A. nidulans* is one of the few fungal organisms being studied in detail. The role of two genes *bimB* and *bimD* are discussed especially with regard to their function in mitosis by May *et al.*

*Aspergillus* is one of the few fungal genera well characterised by traditional taxonomic methods based on morphological features. The advent of biochemical and molecular genetics techniques has offered new approaches to the detection and identification of different species of the aspergilli. Bainbridge reviews and compares the suitability of the different molecular approaches to understanding the taxonomy of this genus.

The aspergilli are major fungal pathogens of mammals. Campbell reviews the forms of aspergillosis which can occur in both mammals and birds. The nature of *Aspergillus* pathogenicity is discussed in two papers, Hearn and Latge *et al.* Hearn's paper covers the role of the cell wall in infection and host response, while Latge *et al.* investigate the role of molecules produced by *A. fumigatus* and how these affect host defense mechanisms. Both papers stress the importance of development of a diagnostic technique for the detection of early infection.

It is clear from the papers produced for this book that there will be much more to learn about *Aspergillus* in the future. The next sixteen years will no doubt warrant a future conference on the genus!

K. A. Powell, J. F. Peberdy, A. Renwick

September 1993

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## ACKNOWLEDGEMENTS

The BMS would like to thank the following organisations for their support:

FEMS
SmithKline Beecham
Pfizer
Zeneca Agrochemicals

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# AGRICULTURE, AFLATOXINS AND *ASPERGILLUS*

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## INTRODUCTION

Human activities affect both the size and structure of fungal populations. Construction, war, recreation, and agriculture disrupt large expanses of vegetation and soil; disruption causes redistribution of fungal propagules and makes nutrients available to fungi. Many fungi, including the aspergilli, exploit these human engineered resources. This results in the association of large fungal populations with various human activities, especially agriculture. When crops are grown or animals raised, fungi are also grown. From a human perspective, most fungi associated with cultivation increase inadvertently. Human activity, however, partly dictates which and how many fungi occur and the fungi, both directly and through fungal products, influence human activities, domestic animals, and even humans themselves.

During warm, dry periods, several of the aspergilli increase rapidly in association with crops. These include aspergilli in the *Aspergillus flavus* group. Prior to 1960, interest in the *A. flavus* group resulted both from the use of certain strains in processing of agricultural products in Europe and the Orient (Beuchat, 1978), and from the ability of some strains to parasitize insects. In the early 1960's fungi in the *A. flavus* group were implicated as the producers of aflatoxins ("*Aspergillus flavus* toxins"), the toxins which poisoned thousands of poultry, pigs and trout; in trout these factors were associated with liver cancer (Goldblatt and Stoloff, 1983). It soon became apparent that aflatoxins also occurred in the human diet and that aflatoxins could pass from feed to milk with only slight modification (Goldblatt and Stoloff, 1983). The most common aflatoxin, aflatoxin $B_1$ , was found to be a potent hepatocarcinogen in rats and trout; carcinomas were induced at rates below $1 \mu g kg^{-1}$ body weight (Robens and Richard, 1992). Aflatoxin content of foods and feeds was eventually regulated in many countries (Stoloff *et al.*, 1991). In some products, such as milk or infant foods, aflatoxin levels below $0.02\ \mu g kg^{-1}$ are mandated. Thus, for many, the focus of interest in this diverse and important fungal group became the production of aflatoxins.

There clearly are interactions between agriculture, and both aflatoxins and the fungi in the *A. flavus* group. Some consequences of these interactions are obvious, others are virtually unexplored. The relationship of crop contamination cycles to the life strategies of

*The Genus Aspergillus*, Edited by Keith A. Powell *et al.*,
Plenum Press, New York, 1994

*A. flavus* group fungi is uncertain. The role agriculture plays in structuring *A. flavus* populations and their toxigenic potential is also uncertain. This chapter will address some aspects of the interactions of *A. flavus* with humans and human activities; it includes suggestions on how these interactions may be altered to reduce human exposure to aflatoxins and other detrimental fungal traits.

## INFLUENCES OF THE *ASPERGILLUS FLAVUS* GROUP

### Effects of Aflatoxins on Humans and Domestic Animals

Although aflatoxins are most often noted for ability to induce liver cancer at very low doses, they can cause several problems of economic importance during animal production. The presence of relatively high levels of aflatoxins in feeds can lead to animal death; rabbits, ducks and swine are particularly susceptible ($LD_{50}$= 0.30, 0.35, and 0.62 $mgkg^{-1}$, respectively; Pier, 1992). However, at much lower concentrations, aflatoxins have other effects on domestic animals including immunosuppression and reduced productivity (Pier, 1992; Robens and Richard, 1992). Once consumed, aflatoxins are also readily converted to aflatoxin $M_1$ which occurs in milk and can thus cause both human exposure and sickness in animal offspring (Pier, 1992; Robens and Richard, 1992).

**Incidence of Health Effects due to Contaminated Foods.** In many developed countries, regulations combined with both an enforcement policy and an abundant food supply can prevent exposure of human populations, in most cases, to significant aflatoxin ingestion (Stoloff *et al.*, 1991). However, in countries where either food is insufficient or regulations are not adequately enforced, routine ingestion of aflatoxins may occur (Hendrickse and Maxwell, 1989; Zarba, *et al.*, 1992). In populations with relatively high exposure, a role for aflatoxins as a risk factor for primary liver cancer in humans has repeatedly been suggested, but is still not clear (Robens and Richard, 1992). However, aflatoxins cause a variety of effects on animal development, the immune system and a variety of vital organs. Exposure to aflatoxins, particularly in staples (*i.e.* corn or peanuts) of people dependent upon relatively few nutrient sources, must be considered a serious detriment. The relationship between aflatoxins and kwashiorkor may be only one reflection of this detriment (Hendrickse and Maxwell, 1989).

**Effects of Aflatoxins on Agricultural Enterprise.** Controversies regarding the possible role of aflatoxins in primary liver cancer of humans are moot in the contemporary international marketplace. Brokers and producers of agricultural commodities have found aflatoxins increasingly costly as careful monitoring of aflatoxins limits the use and value of contaminated products (Cappuccio, 1989). Regulations in most developed countries and even many less developed countries restrict the import of contaminated foods and feeds (van Egmond, 1991; Stoloff *et al.*, 1991). Assessing the aflatoxin content of crops is a routine aspect of brokering and often a prerequisite of shipping. Contamination is highly variable and allowable concentrations are at such low levels (some below 1 $\mu gkg^{-1}$), that analysis prior to shipping cannot always ensure acceptable levels upon receipt, even if no increases occur during transit (Horwitz *et al.*, 1993). This increases commodity costs and can decrease competitiveness of imported products. Regulations applied more rigorously to imported than domestic products or set at zero, where the limit of detection determines the enforcement level, can serve as barriers to trade which again increase the cost of products. These increased costs may be the primary effect of aflatoxins felt by most consumers in developed nations.

**Effects of Aflatoxins on Health of Agricultural Workers.** Labourers engaged in production and processing of commodities may be exposed to aflatoxins through inhalation

(Shotwell, 1991). Crops grown under conditions favouring aflatoxin contamination often become covered with large quantities of *A. flavus* propagules. Furthermore, air in areas where contaminated crops are produced may contain thousands of propagules per cubic meter (Lee *et al.*, 1986). These propagules, which are mostly conidia, remain associated with the crops through harvest and processing. Conidia contain large quantities of aflatoxins (over 100 $mgkg^{-1}$ in some strains; Wicklow and Shotwell, 1982). Since most contamination occurs in damaged crop components, fines and dust generated during crop processing have much higher toxin contents than the crop as a whole (Lee *et al.*, 1983). The conidia, fines, and dust, may be inhaled and thus pose an avenue of exposure to aflatoxins; this exposure has been quantified in certain cases (Shotwell, 1991). Recently, occupational exposure to aflatoxins through the handling and processing of contaminated agricultural products has been associated with increased risk of both primary liver cancer and other cancers (Alavanja *et al.*, 1987; Olsen *et al.*, 1988).

## *Aspergillus flavus* group Fungi as Allergens and Animal Pathogens

Several allergic and infective conditions of humans and certain other vertebrates are caused by *Aspergillus* species (Rinaldi, 1983; St. Georgiev, 1992; Wardlaw and Gedes, 1992). These include allergic bronchiopulmonary aspergillosis and invasive pulmonary aspergillosis. The most common cause of most of these conditions is *Aspergillus fumigatus* (Rinaldi, 1983; St. Georgiev, 1992; Wardlaw and Gedes, 1992). However, other aspergilli, including members of the *A. flavus* group, are also often implicated.

**Insect Pathogen.** During epidemics of aflatoxin contamination, high concentrations of *A. flavus* group propagules are associated with most objects resident in fields, including insects; thus insects may serve as vectors (Stephenson and Russell, 1974; Widstrom, 1979). *A. flavus* readily grows and multiplies on insect damaged crops, insect frass and on insects themselves both as dead debris and as parasitized hosts (Sussman, 1951,1952; Stephenson and Russell, 1974; Goto *et al.*, 1988). Many insects typically carry *A. flavus* group isolates internally and many insects are hosts of at least certain strains (Stephenson and Russell, 1974; Widstrom, 1979; Goto *et al.*, 1988). Domesticated insects are included among the hosts of the *A. flavus* group. Domesticated insect diseases include Stonebrood, a rare disease of the honey bee which is of minor importance to bee keepers (Gilliam and Vandenberg, 1990) and koji kabi disease of cultivated silkworm larvae (Ohtomo *et al.*, 1975; Goto *et al.*, 1988).

## Benefits of *Aspergillus flavus* group Fungi

**Industry.** Fungi in this group have had a long history in processing to increase product utility and value. *A. flavus* group strains are used to produce enzymes for food processing and other industrial uses and even to produce therapeutic products such as urate oxidase and lactoferrin (Chavalet *et al.*, 1992; van den Hondel *et al.*, 1992; Ward *et al.*, 1992). A variety of traditional fermented food products have been made with fungi in the *A. flavus* group for centuries (Beuchat, 1978).

**Ecological Benefits**. Although *A. flavus* group fungi are not commonly recognised as beneficial, these ubiquitous organisms become dominant members of the microflora under certain circumstances and exert multiple influences on both biota and environment. These fungi are important degraders of crop debris and may play roles in solubilising and recycling crop and soil nutrients (Ashworth *et al.*, 1969; Griffin and Garren, 1976). *A. flavus* can even degrade lignin (Betts and Dart, 1989). As insect pathogens, these fungi may serve to limit pest populations (Wadhwani and Srivastava, 1985) and have even been considered potential agents to replace chemical insecticides ( Roberts and Yendol, 1971).

## Contamination Cycles

**Contaminated components.** *A. flavus* causes a variety of plant diseases typical of largely saprotrophic "weak" plant pathogens (Widstrom, 1992). These diseases include boll, ear, and pod rots which result in both decreased yield and reduced quality (Shurtleff, 1980; Watkins, 1981). However, crop infection by *A. flavus* takes on a different importance than infections for which concern might focus on yield and quality loss, or increased free fatty acids. Aflatoxins are compounds regulated in parts per billion; yet, these toxins occur in certain infections at concentrations over 100,000 $ugkg^{-1}$. This situation causes high-toxin-containing components to greatly exceed in cost the value of the same components if not contaminated. Variability among components of crops in aflatoxin content is extreme (Figure 1). Most infected components contain low aflatoxin concentrations (below 50 $\mu gkg^{-1}$). However, a small percent contain very high toxin levels, at times exceeding 500,000 $\mu gkg^{-1}$ (Cucullu *et al.*, 1966; Schade *et al.*, 1975; Lee *et al.*, 1990; Steiner *et al.*, 1992). In many cases, elimination of highly contaminated components (over 1,000 $\mu gkg^{-1}$) would result in a commodity with an acceptable average aflatoxin content (Schade *et al.*, 1975; Steiner *et al.*, 1992).

Crop components damaged by wounding or severe stress are colonised and decayed by a variety of fungi. During hot and dry conditions, fungi in the *A. flavus* group out compete many colonising microbes and become the prominent fungi degrading damaged components. In most crops the majority of contamination occurs in damaged plant parts (Wilson et al., 1977; Lee et al., 1983; Cotty and Lee, 1989). Damaged seed can be sorted from high value crops for less profitable use such as production of vegetable oil. However, crushing contaminated seed to produce oil concentrates aflatoxins in the resulting meal which is used for feed. Such toxic meal caused the first recognised aflatoxin problems; peanut meal caused turkey X disease in England and cottonseed meal caused trout hepatocarcinoma in the United States (Goldblatt and Stoloff, 1983). Such meal must either be detoxified (i.e. through ammoniation) or put to non-feed use (Park *et al.*, 1988).

**Geography determines frequency and severity.** Geographic location greatly influences frequency of contamination. Many agricultural areas at low elevation and between the latitudes 35 N and 35 S have perennial risk of contamination. Countries in this zone (which include many countries with insufficient food supply) may view elimination of aflatoxins from the food supply differently than countries whose major agricultural lands lie out of this zone (*i.e.* developed countries in Europe and North America). Producers of contaminated products may base allowable levels of aflatoxins on toxicological data, whereas consumer nations which rarely produce contaminated products may base allowable levels at the lowest level detectable (Stoloff *et al.*, 1991).

Contamination cycles can be considered perennial, sporadic or infrequent based on locale and crop. In all three situations, populations of *A. flavus* are long term residents. However, populations in different areas differ in magnitude (Figure 2) (Griffen and Garren, 1974; Manabe *et al.*, 1978; Shearer *et al.*, 1992) and possibly in the distribution of both qualitative and quantitative traits (Manabe *et al.*, 1978; Cotty, 1992*b*). During periods not conducive to contamination, perennial areas (*i.e.* the desert valleys of Arizona; Lee *et al.*, 1986) support higher *A. flavus* populations than areas with infrequent contamination, *i.e.* midwest corn producing areas (Shearer *et al.*, 1992). Areas with sporadic contamination may have perennial contamination at low levels but, have less regular exposure to important predisposing factors such as hot, dry conditions, *i.e.* contamination of corn in certain areas of the southeastern United States (Widstrom, 1992) or insect pressure, *i.e.* pink bollworm pressure on cotton in western Arizona (Cotty and Lee, 1989). During periods conducive to contamination, a shift in the microflora occurs and aflatoxin producing fungi become dominant colonisers and decayers.

Processes through which crops become contaminated with aflatoxins are varied and complex (Diener *et al.*, 1987). However, certain generalities might be suggested. Contamination cycles may be divided into three phases: Prebloom, Crop Development, and Post Maturation (Figure 2).

**Prebloom.** Contamination does not occur in the field during the period after crop removal and prior to bloom. However, both the microflora and crop may become predisposed to contamination. During this phase: 1. propagules (conidia, sclerotia, colonised organic matter) are dispersed through cultivation, planting, pruning or other activities of animals (including man) or the environment; 2. *A. flavus* populations fluctuate, first decreasing after crop removal and then, if conditions are favourable, increasing on debris from current and

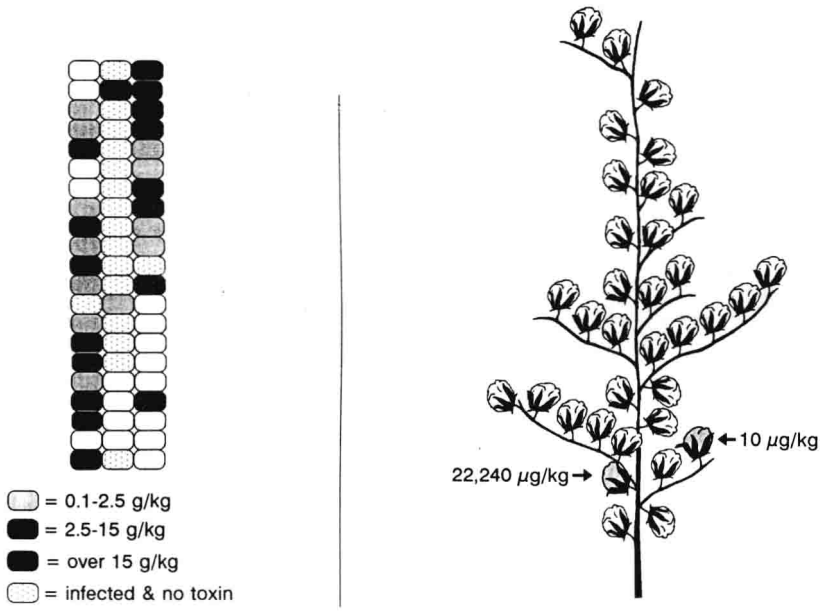


**Figure 1.** Distribution and aflatoxin content of maize kernels on an ear (drawn from data in Lee *et al.*, 1980) and bolls on a plant (Cotty and Lee, 1990). Contamination is highly variable and not all infected seed becomes contaminated.

prior crops (Ashworth *et al.*, 1969; Griffen and Garren, 1974; Lee *et al.*, 1986) 3. The crop may become predisposed by long periods of drought or by luxuriant growth followed by drought (Cole *et al.*, 1982; Deiner *et al.*, 1987; Shearer *et al.*, 1992); 4. Overwintering insects emerge and develop.

**Crop Development.** From flowering to maturation, seeds and fruits are vulnerable to various perturbations. During this phase: 1. If conditions are hot and dry, populations of the *A. flavus* group, in canopy and soil, will outcompete many saprophytic microbes and increase in size. 2. High temperatures and/or drought stress may interfere with crop development and weaken plant defences making the crop more susceptible to infection and contamination (Jones *et al.*, 1981; Cole *et al.*, 1985; Wotton and Strange, 1987; Widstrom,

1992). 3. Wounding of fruits at middle to late stages of development can lead to portions of the crop with very high toxin levels (Lillehoj *et al.*, 1987; Cotty, 1989*b*). In several crops, most aflatoxin is formed during this phase and in certain locations crop predisposal to contamination can be attributed to specific wound types caused by specific insects. Examples are pink bollworm exit holes in cotton in the desert valleys of the western United States (Cotty and Lee, 1989), maize weevil damage in the southern United States (McMillian *et al.*, 1987), navel orange worm damage in nuts in the western United States (Schade *et al.*, 1975; Sommer *et al.*, 1986), and lesser corn stalk borer damage in peanuts in the southern United States (Lynch and Wilson, 1991). In some crops, components prevented from maturing due to stress or early harvest are particularly vulnerable to contamination (Cole *et al.*, 1985; Lynch and Wilson, 1991).

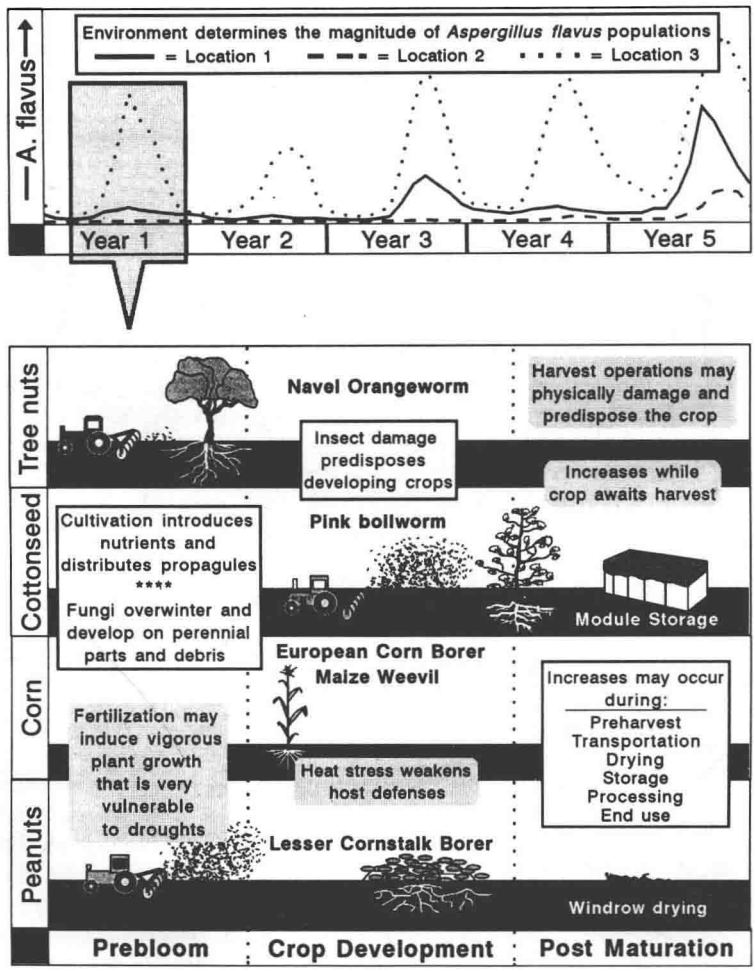


**Figure 2.** Contamination cycles can be divided into three phases. Local conditions determine both the extent of contamination and the magnitude of *A. flavus* populations associated with the crop. Boxed information applies to all crops.

**Post Maturation.** Most crops are susceptible to aflatoxin contamination at maturity and if the crop was grown in an area with perennial contamination or during a period conducive to contamination, the mature crop will be associated with large quantities of *A. flavus* group propagules. These propagules remain associated with the crop as it awaits harvest in the field, during harvest, field storage (i.e. peanuts in windrows, cotton in modules), shipment and processing, and even during storage by the end user. Exposure of the mature crop to periods of wetting and drying under warm conditions may lead to increased contamination. Aflatoxin concentrations are known to be dependent on environmental conditions and competing microflora (see Strain Isolation and Accumulation of Aflatoxins). Mature fruits and seeds are living organisms and factors which compromise seed health, such as wounding or stress, predispose these products to infection and contamination. Harvest operations can simultaneously damage crops and introduce *A. flavus* into wounds (Schroeder and Storey, 1976; Sommer *et al.*, 1986; Siriacha *et al.*, 1989). Insect activity after harvest can disperse aflatoxin-producing fungi and, by increasing host susceptibility, increase aflatoxin levels in a manner similar to insect damage during crop development (Dunkel, 1988). The same insect can affect contamination both prior to and after maturation (ie. the navel orange worm on pistachios).

Post maturation contamination dictates that each handler of the crop be responsible and minimize the potential for aflatoxin increases. Thus dairies which purchase feed with undetectable toxin must still store the feed properly or contaminated milk may occur. With indeterminate crops (*e.g.* cotton) crop development and post maturation phases may occur simultaneously and with all crops the prebloom and post maturation phases occur simultaneously, although at different locations.

Initially, the crop development phase was ignored because all contamination was thought to occur post harvest; recently, most research has been directed at contamination

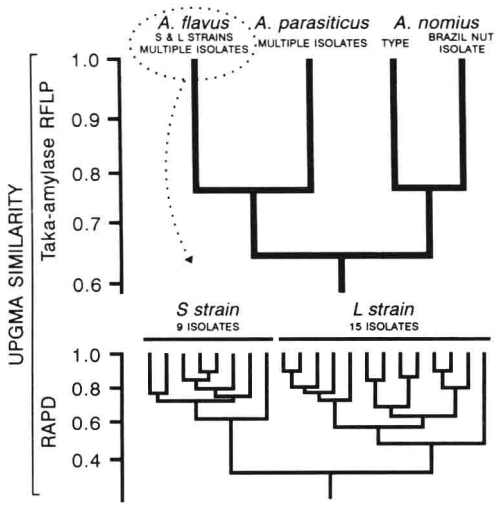


**Figure 3.** Phenograms of *A. flavus* group isolates. Taka-amylase data from Egel and Cotty, (1992); RAPD data from Bayman and Cotty, (1993).

before harvest, sometimes without distinguishing periods of active crop development from periods after maturation (Lillehoj *et al.*, 1976; Goldblatt and Stoloff, 1983). The contamination process can be divided many different ways besides those presented here. However, failure to segregate the contamination process into different phases may result in data that suggests no clear pattern and apparent contradictions. For example, in Arizona, most cottonseed contamination occurs during crop development in cottonbolls damaged by pink bollworms in the absence of rain (Cotty and Lee, 1989). Still, rain on a mature crop awaiting harvest can lead to significant contamination during post maturation, even if developing bolls were not damaged (Cotty, 1991). Similarly, a great fervour occurred about contamination of the midwest U.S. corn crop, in the field, during droughts of 1983 and 1988 (Kilman, 1989; Schmitt and Hurburgh, 1989; Shearer *et al.*, 1992). Yet, in Thailand, contamination typically occurs during the wet season, not during the dry season (Goto *et al.*, 1986). In Thailand's rainy season, contamination occurs during post maturation (Siriacha *et al*, 1989); in the midwestern United States, it typically occurs during crop development (Lillehoj *et al.*, 1976).

## FUNGAL POPULATIONS

### Diversity

**Species of Aflatoxin-Producing Fungi.** There have been a variety of taxonomic schemes used to classify *A. flavus* group strains (Thom and Raper, 1945; Klich and Pitt, 1988; Samson and Frisvad, 1990). Each species represents an assortment of strains which behave as clonal organisms with the exception of occasional parasexuality between members of the same vegetative compatibility group (Papa, 1984, 1986). For the purposes of this discussion we will place all isolates within this group into four species *A. flavus*, *Aspergillus parasiticus*, *A. nomius,* and *Aspergillus tamarii*. Depending on interpretation, these species are supported by clustering algorithms based on DNA polymorphisms (Kurtzman *et al.*, 1987; Moody and Tyler, 1990*a,b*; Egel and Cotty, 1992; Bayman and Cotty, 1993). *A. tamarii* is of minor interest here because no isolates in this species produce aflatoxins. *A. tamarii* isolates apparently have some markedly different adaptations than the remainder of the group and *A. tamarii* is more distantly related to the other three species, than the three are to each other (Kurtzman *et al.*, 1987; Klich and Pitt, 1988). *Aspergillus oryzae* and *Aspergillus sojae* are apparently derived from *A. flavus* and *A. parasiticus*, respectively (Kurtzman *et al.*, 1986) and will be mentioned only in an industrial context. *A. nomius* was named after the genus of alkali bees from which several isolates were obtained (Kurtzman *et al.*, 1987). *A. nomius* comprises a group of strains that are distinct by both physiologic and molecular criteria (Kurtzman *et al.*, 1987; Bayman and Cotty, 1993). The name "*nomius*" may be misleading in associating this species predominantly with the alkali bee when isolates are known from several crops, including wheat (the type isolate) and peanuts (Hesseltine *et al.*, 1970).

**Diversity Within *Aspergillus flavus*.** Within each of the three aflatoxin producing species, there is a great deal of variability among isolates. It may be, that if we sought out all the unusual or atypical isolates within this group and examined them, we would find a continuum as suggested by Thom and Raper (1945). Indeed, based on polymorphisms in the Taka-amylase gene, we have found strains intermediate between *A. flavus* and *A. parasiticus* as well as *A. nomius* isolates almost as different from the *A. nomius* type strain as the *A. parasiticus* type from the *A. flavus* type (Egel and Cotty, 1992; see Brazil nut isolate in Figure 3). Variation among isolates is evident in genetic, physiological and morphological characters. Each of the above species is composed of at least several Vegetative Compatibility Groups (VCGs) and *A. flavus* is composed of many (Papa, 1986; Bayman and Cotty, 1991; P.J. Cotty, unpublished). Physiological and morphological traits are typically much more consistent within a VCG than within the species as a whole (Bayman and Cotty,