ASPERGILLUS FLAVUS AND THE DETERIORATION OF FARM-STORED BARLEY GRAIN

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SUMMARY

Inadequate farm storage of barley can result in moulding of the grain mass with a corresponding danger of mycotoxin production in that grain and subsequent risks to animal health. Dilution plating techniques utilising progressive washing and surface sterilization have been used in this study to investigate the mycoflora of the husks of 12 samples of farm-stored barley grains, with particular reference to the presence of *Aspergillus flavus*, the producer of the potent mycotoxin, aflatoxin. These techniques allowed differentiation of fungi and fungal numbers on the inner and outer surfaces of the husks to be made and related to the extent of deterioration of the grain.

The dilution plating method used for examining the husks revealed that total viable counts of the outer surface were not a reliable index of the condition of the samples, whereas inner surface counts were consistently related to the degree of mouldiness. A "condition line" could be established at 2.0×10^2 CFU/g grain for such inner surface counts.

The most common *Aspergillus* species isolated by dilution plating were *A. flavus*, *A. glaucus* and *A. fumigatus*. The most common of the other genera were *Alternaria*, *Cladosporium* and *Aureobasidium*. *A. flavus* was the most widely distributed species in both clean and mouldy samples, but was present mainly on the outer surface. The distribution of the various genera on the outer and inner surfaces of the husks was also found to be related to the degree of mouldiness of the sample. In clean samples the field fungi (*Alternaria*, *Cladosporium* and *Aureobasidium*) were dominant, but they were replaced by storage fungi (*Aspergillus* and *Penicillium*) in mouldy samples.

A further technique allowing direct examination of the fungal mycelium within husk tissue using a vital stain was developed. This allowed an assessment to be made, by means of three comparative scales (relative mycelial score, comparative mycelial score and relative viability score), not only of the abundance of such mycelium but also of its viability. Most samples of husk tissue showed abundant mycelium

but estimation of viability obtained by this direct plating technique showed that whilst hyphae in husks from mouldy samples were active, much of the mycelium in clean samples was dead. The most common species of *Aspergillus* in the husk tissue of mouldy samples were the spoilage fungi *A. glaucus*, *A. restrictus* and *A. fumigatus*. Only 2 samples yielded *A. flavus*. Fungal genera isolated mainly from clean samples were *Alternaria*, *Monilia* and *Papulospora*. This technique thus reinforces the findings obtained by dilution plating and emphasises the location of spoilage fungi within the husk tissue of mouldy samples.

Barley isolates of *A. flavus* have been compared to soil isolates for their ability to produce aflatoxin on different media. *A. flavus* isolates from barley were first screened for aflatoxin production on coconut agar. All were negative. Several isolates from soil, however, were found to be toxigenic. Selected barley and soil isolates were examined for their ability to form aflatoxin on various media (semi-synthetic, Weet-bix, pearled barley and barley husk), culture filtrates being analysed by the minicolumn technique and by TLC. Aflatoxin B_1 and traces of B_2 were detected by the TLC method in culture extracts from 7 out of 9 soil isolates of *A. flavus*. No aflatoxin was detected in cultures of barley isolates.

The studies reported suggest that although *A. flavus* is common in stored barley, it is mainly a surface contaminant and present largely as spores. It seemed to play little part in the actual spoilage of the grain, as indicated by its infrequent occurrence as mycelium within the husk tissue. Furthermore, elaboration of aflatoxin does not appear to be a problem in the barley samples examined, as judged by the absence of toxigenic *A. flavus* strains in those samples. However, soil isolates were toxigenic, and it is possible that other samples of stored grain may on occasions become contaminated with these strains, with the concommitant danger of aflatoxin production if the grain is not adequately stored.

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INTRODUCTION

For many years there was a tendency to regard moulds in grains destined for animal consumption as of little significance other than as gross spoilage organisms. Investigations of the possible role of toxic fungi in diseases of grain-fed animals was confined in the main to problems of restricted local significance (Forgacs and Carll, 1962). However, it was the discovery of aflatoxin in 1961 and the widespread concern which it generated, which inspired a vast amount of research into the natural production of mycotoxins and the consequent mycotoxicoses affecting both animal and human health, aspects of which have been fully reviewed in a number of recent publications (Purchase, 1974; Rodricks, 1976; Rodricks *et al.*, 1977; Wyllie and Morehouse, 1978 and Cole and Cox, 1981).

Mycotoxin production can occur anywhere, in the field, or during harvest, processing, storage and shipment, or during the feeding period on the farm. Commodities grown and stored in areas where high levels of insect damage and poor farming and storage practices prevail appear to be the most susceptible (Ciegler, 1978). Nevertheless, natural contamination with a variety of mycotoxins has been reported for most of the major agricultural commodities in the world (Hesseltine, 1974) and those mycotoxins currently of most concern are: aflatoxin, trichothecenes, zearalenone, ochratoxin, citrinin and some tremorgens (Ciegler, 1978). However, there are over 23 known mycotoxins which can be associated with grains and some of those currently less well known may prove to be significant in the future.

The aflatoxins, produced by *Aspergillus flavus* and *A. parasiticus*, have been the most studied of the mycotoxins associated with fungal contamination of foods and feeds in general (Jones, 1977) and almost all food commodities are susceptible to contamination. The production of mycotoxins in agricultural commodities and the ensuing mycotoxicoses represent an extremely complex series of interactions betwe

the causative fungi, the contaminated products, physiochemical environmental factors and the intoxicated host (Ciegler, 1978) and although *A. flavus* has a worldwide distribution, various factors may thus restrict the areas in which natural outbreaks of aflatoxicoses may occur.

A considerable amount of research has been conducted on aflatoxin contamination of corn, wheat, rice, peanuts and other agricultural commodities, but relatively little similar work has been published on barley, a crop which has become a major animal feedstuff in many parts of the world (Chang and Markakis, 1981). Thus there is scope for increasing our understanding of *A. flavus* contamination and aflatoxin production in this crop. It will not be surprising if we record what Hamilton (1978) has noted as being statements made by a respected scientist about aflatoxin in corn in the U.S.A.: 1971: "*A. flavus* does not occur in U.S.A. corn." 1973: "*A. flavus*, but not aflatoxin, occurs in U.S.A. corn." 1975: "Aflatoxin occurs in U.S.A. but only in southern corn." and 1977: "Aflatoxin occurs in U.S.A. corn, even in my laboratory."

1. Barley Production and Uses.

Barley is a hardy and drought-resistant cereal, grown in widely-distributed areas of the globe from semi-tropical regions to the Arctic Circle. Its production during the last decade has expanded more rapidly than that of any of the other principal grain crops (Commonwealth Secretariat, 1973). Although it is a staple human food in many parts of the world, as well as being produced for the brewing and distilling industries (Chang and Markakis, 1981), barley's most significant role is as an animal feed, and it is used extensively by manufacturers of compounded rations for various classes of livestock.

In New Zealand, most of the barley grown is used as animal feed, with a lesser quantity for brewing purposes. Currently, the most commonly grown cultivars are Zephyr and

Triumph (McKinnon, 1983). The 1983 statistics of the N.Z. Ministry of Agriculture and Fisheries (M.A.F.) show that the area cultivated in barley and total yields increased very markedly during the 1982 season in comparison with other cereals, particularly wheat (Table 1). Thus barley has now become the most widely grown of the cash crops (maize production in 1982 was of the order of 200,000 tonnes).

Table 1. Comparative area and yield of barley and wheat grown in New Zealand, 1978-1982.

	Wheat		Barley	
Year	Area grown (000 hectares)	Yield (000 tonnes)	Area grown (000 hectares)	Yield (000 tonnes)
1978	91	329	71	259
1979	87	295	77	264
1980	86	306	66	228
1981	81	326	67	271
1982	76	320	94	407

The sharp increase in barley output in 1982 was due to the increased demand for high quality malting barley, with the opening of a new plant in the North Island, and this trend is expected to continue. Some 65,000 tonnes were used for malting purposes during 1982 (Canterbury Malting Co., personal communication). Approximately 12,600 tonnes of the crop were kept as seed (M.A.F., personal communication) and 62,000 tonnes were exported (McKinnon, 1983). Thus, from available records, it can be calculated that about 267,000 tonnes of the 1982 barley crop was available for use as animal feed.

Such a substantial rise in tonnage has obviously required a rapid expansion of both commercial and on-farm storage facilities and with this has come problems concerning

the avoidence of gross spoilage of the grain, with its consequent risk of contamination of animal feedstuffs with toxic microbial metabolites affecting animal, and indirectly human, health.

2. Grain Storage and Spoilage Problems.

Studies done on storage facilities and conditions for safe storage of whole grains have in general dealt more with wheat and maize than with barley. Balley (1974) has discussed the range of available facilities. The most simple methods include the piling of unprotected grain on the ground, or in underground pits. Various types of containers may also be used, with varying degrees of protection, or the grain may be bagged and stored in piles. More advanced facilities include storage bins and silos of many sizes and shapes and types of construction.

Farm storage facilities in New Zealand tend to range from small wooden enclosures in the barn, through small round steel bins to silo-type facilities, with or without aeration and other devices for controlling storage conditions. Commercial silos tend to offer very large storage space with fast handling facilities prior to storage, eg. for cleaning and drying. Storage conditions are usually controlled well, eg. by aeration and temperature control, often with automatic control devices.

To maintain grain quality, it is necessary to maintain safe storage conditions. This means protecting the grain from weather and attack by microorganisms, rodents, insects and birds. Accidental introduction of moisture and exposure to high temperatures must be avoided, otherwise contamination will occur (Balley, 1974).

It has been stated that the world loses about 20% of its food commodities by deterioration, more than half of which is due to the growth of moulds (Moreau, 1979). In 1960 such losses were calculated by the Food and Agriculture

Organisation of the United Nations at many thousands of millions of dollars (Golumbic, 1965).

Grain that is spoiled or unfit for human or animal consumption can be recognized easily by conspicuous, objectionable features such as heating, caking, discoloration and off-odour, and such features are used as quality factors in routine inspection and grading of various grains including barley. Tests of viability and biochemical tests (fat acidity and non-reducing sugar content) can be useful as measures of the extent of the actual damage which has taken place. During the early stages of deterioration, however, incipient spoilage is usually detectable only by sensitive instruments and techniques.

It has been suggested that other tests may be used to determine the condition of apparently clean cereal grains and to predict their future storage behaviour, including estimations of the fungal population (Christensen and Kaufmann, 1974). Mould counts could be good indices of incipient deterioration and studies of the fungal flora could be expected to provide useful information in attempts to control spoilage. However, because of the complex structure of barley grain, with its tight covering of husk tissue, clearly defined techniques for assessment of fungi present in the various layers would be needed if such information is to be used to gauge incipient deterioration. Studies of the actual genera present within the grain would also be useful when considering the risk of contamination of the grain with mycotoxins.

3. Structure and Composition of Barley Grain.

Barley grain is a dry, indehiscent fruit botanically known as a caryopsis (Bengal and Clemencet, 1962). The base of the caryopsis is occupied by the embryo and the remainder by the starchy endosperm. In hulled barley the structure of the fruit is complicated by the presence of the lemma and palea or "husk". The lemma and palea are

tightly wrapped around the caryopsis but they are not organically fused with it.

3.1 The Caryopsis.

The caryopsis of barley grain is composed of the endosperm, embryo, scutellum and aleurone layer (Figure 1). A transverse section of the mature grain reveals that most of the cells of the endosperm are filled with simple and compound starch grains, with the hemicellulose cell walls and remains of the protoplasm forming an irregular network amongst the grains (Bengal and Clemencet, 1962). The endosperm and embryo develop together during the ripening of the seed. The structure associated with the embryo which secretes enzymes and absorbs the nutrient products of enzyme action is a layer of pallisade cells, the scutellum, which is directly in contact with the endosperm (Harris, 1962). It shares with the aleurone layer the ability to secrete diastatic enzymes which enable the young plant to utilize the starch which is present in the endosperm. The aleurone layer is composed of two to four layers of thick-walled cells (aleurone cells) which are more or less rectangular or polygonal in shape (Warnock and Preece, 1971). Aleurone cells are free of starch but contain aleurone grains and small globules of fatty material in their protoplasm. This aleurone layer is rich in nitrogenous substances.

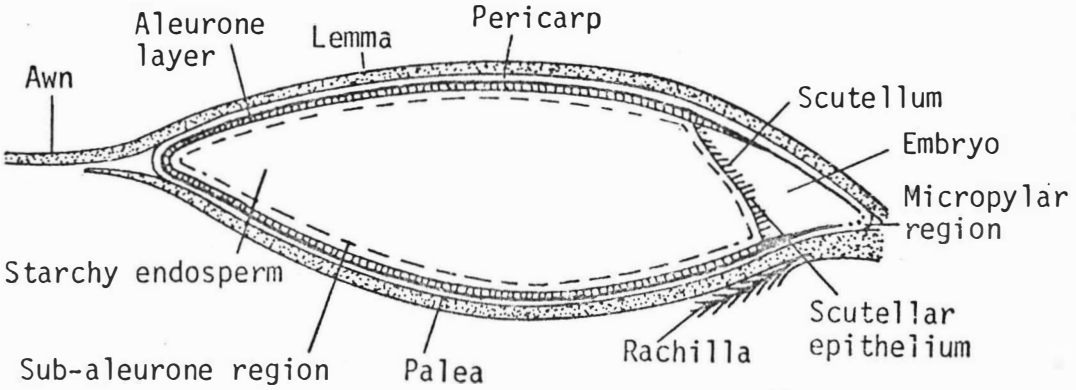
3.2 The Husk.

The mature caryopsis is enclosed within the lemma and palea (the husk). The lemma, comprising 60% of the surface area of the husk, extends some two-thirds of the way around the grain, its margins overlapping those of the palea (Harper and Lynch, 1981). The husk averages 13% of the whole grain (compare endosperm 73%).

Warnock and Preece (1971) described the barley husk as being composed of four layers; an outer epidermis consisting largely of elongated cells; a layer of sclerenchyma fibres;

Figure 1. Schematic longitudinal and transverse sections through a barley grain showing the disposition of the parts.
(From: Malting & Brewing Science, Vol.1, by Briggs *et al.*, 1981.)

Dorsal side

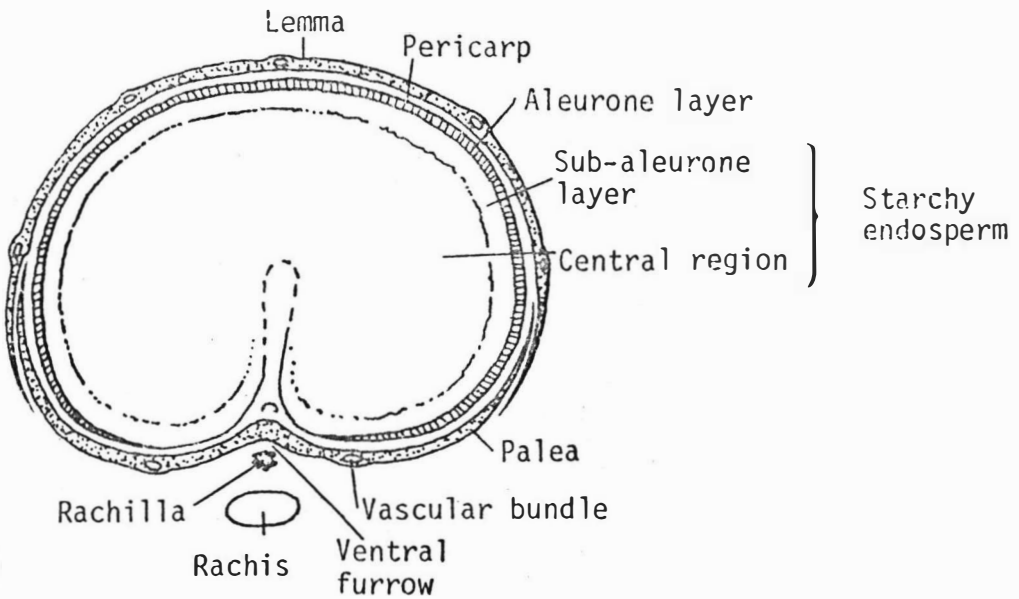


Distal end

Proximal end

Ventral furrow side

Dorsal side



Ventral side

a parenchyma layer, throughout which run the fibro-vascular bundles, and an inner epidermis of more or less polygonal cells, trichomes and stomata.

Another structure associated with the true husk is the pericarp layer, formed from the fusion of the inner epidermis of the husk with the outermost layers of the caryopsis. The pericarp consists of several layers of longitudinally elongated cells which are very compressed in cross-section and can not easily be distinguished. Briggs *et al.* (1981) consider the true husk and pericarp make up 9 - 14% of the dry weight of the whole barley and may be considered together, since they are fused together and appear to serve the same functions.

The major polysaccharide components in the cell walls of barley husk are cellulose, lignin, hemicellulose and gums (Flannigan, 1977). The available information on the relative chemical composition of husks and whole barley is shown in Table 2. Cellulose comprises about 5%, hemicellulose about 8% and gums 2% of the whole grain (Briggs *et al.*, 1981). The main hemicellulose is arabinoxylan (67% xylose and 11% arabinose, with small amounts of 4-O-methyl glucoronic acid, glucose and galactose [Aspinall and Ferrier, 1957]). The husk tissue also contains traces of protein, polyphenols, sugars, amino acids and pectin. Its surface layers are particularly rich in silica.

3.3 Barley Grain as a Substrate for Fungal Growth.

The moisture content (M.C.) of the grain is an important factor in relation to the number of viable fungal propagules likely to be present. Lacey (1971) has noted that grain can be stored for a long time without deterioration only if it contains less than 13.6% water. If conditions become suitable for the growth of fungi present as dormant spores or mycelium, deterioration of the stored grains can involve a decrease in percentage germination of the seed, discoloration, various biochemical changes,

Table 2. Chemical composition of whole barley and barley husk.

Composition	Whole barley	Husk
Moisture (%)	14.5	7.4
Protein (%)	9.5	7.1
Starch (%)	54.0	8.2
Fats (%)	2.5	2.1
Crude fibre (%)	5.0	22.6
Silica (% of total ash)	26.0	71.0
Phosphate (as P_2O_5, % of total ash)	35.0	6.0
Pentosans (%)	9.0	20.0
Non-nitrogenous extract (%)	3.0	22.0
Amino acids (No.)	18	?
Minerals (No.)	14	?
Vitamins (No.)	5	?
Fatty acids (No.)	6	?

From Moll (1979) and Harris and Douglas (1981).

mycotoxin production (which would constitute a health hazard for man and his animals) and also heating and complete spoilage of the grains (Christensen and Kaufmann, 1965).

Fungi growing on such grain must be equipped with the necessary enzymes required for the breakdown of the grain and components such as starch and cellulose, by degrading them to produce the monosaccharides necessary for fungal growth. Some of these enzymes, such as amylases and 1,3-β-glucanases, are constitutive in a wide range of fungi, but others, such as cellulases and xylanases, are adaptive enzymes (Flannigan, 1977). For example, Flannigan and

Sellars (1977) examined a number of fungi for their ability to produce amylase. Only one isolate (*Absidia corymbifera*) did not degrade starch. The most active was *A. fumigatus*. In contrast, Flannigan (1970b) and Flannigan and Sellars (1972), assaying fungal isolates for their ability to degrade arabinoxylan and carboxymethyl cellulose, found most were able to break down arabinoxylan, but fewer apparently possessed the ability to release reducing sugars from cellulose, as judged by degradation of carboxymethyl cellulose. Hesseltine *et al.* (1970) tested 28 strains of the *A. flavus* group for assimilation of carbon supplied from 36 various compounds, and all strains showed positive utilization of hemicellulose and pectin but not cellulose.

The principal monosaccharide released by most fungi from arabinoxylan is xylose, which is regarded as a good carbon source for the growth of many fungi (Cochrane, 1958). Flannigan (1970b) suggested a classification of fungi based on their xylolytic and cellulytic activity:

1. Species which seem to be suited for growth on husks, in being able to degrade both major polysaccharide components (cellulose and arabinoxylan) of the cell walls, eg. *Trichoderma viride*.
2. Species which are less well adapted in that they appear to degrade only one component, the arabinoxylan, eg. *Botrytis cinerea*. (*A. flavus* can be included within this group.)
3. Species which appear poorly adapted in that their activity against the structural polysaccharides is low or absent, eg. *Alternaria repens*.

Thus a range of fungi, including *A. flavus*, have the ability to be involved in spoilage processes of barley grain, but a number of other controlling factors determining the actual species present have emerged as our knowledge of the mycoflora of grains has increased.

4. Mycoflora of Barley Grains.

4.1 Early Investigations.

The association of fungi with cereal grains has been studied for many years. Black and Alsberg (1910) and Alsberg and Black (1913), cited by Christensen and Kaufmann (1974), discussed reports from Italian workers from the early 1900's dealing with corn imported from the U.S.A. and supposedly made toxic by fungi. However, in comparison with investigations on the mycoflora of wheat, corn, rice etc., relatively little specific work has been done on the mycoflora of barley grains.

Early investigations on barley tended to be in comparison with other cereals. In such early work, more attention was paid to the "field fungi" (i.e. *Alternaria*, *Fusarium*, *Helminthosporium* etc.) than to what later became known as the "storage fungi". In 1935 Christensen and Stakman studied the relationship between *Fusarium* and *Helminthosporium* in barley seed to seedling blight and yield. They stated that the feeding value of barley would be considerably reduced if a large percentage of the blighted kernels were affected with *Gibberella saubinettii* (*Fusarium saubinettii*), and the barley was also likely to be toxic to pigs. Gordon (1944) studied the occurrence of *Fusarium* spp. in wheat, barley and oats from 1937 to 1942, isolating *Fusarium* spp. from 39% of wheat samples, 55% of barley samples and 50% of samples of oats.

Mead (1942) concentrated on the relationship of seed-borne disease of barley to the field fungi, particularly *Helminthosporium sativum*; in particular, the relationship of this fungus and its mycelium to various parts of the barley kernel and the developing embryo. He considered the dormant mycelium present within the husk to be capable of remaining viable for two to five years. Machacek *et al.* (1951) performed an intensive study of Canadian barley, when they tested thousands of samples of wheat, barley and

oats over a 7-year period. The mycoflora of dried barley grain showed a resemblance to the mycoflora of other cereal grains and *Alternaria tenuis* was the dominant field fungus present on all the cereals.

Following reports of many cases of vulvovaginitis of pigs in many parts of the world apparently related to mouldy barley, McErlean (1952) isolated *F. graminearum* from the barley and this species caused signs of hyperoestrogenism in swine when fed experimentally. McErlean suspected that this fungus produced a toxin responsible for those symptoms.

Hot spots due to fungal growth attracted the attention of Gilman and Barron (1930) who showed in laboratory tests that *A. flavus*, *A. niger* and *A. fumigatus* raised the temperature of wheat, barley and oats with a M.C. of 18%. In 1948 Christensen and Gordon confirmed and extended these findings and noted that *A. flavus* and *A. candidus* were common on moist stored grains. Semeniuk (1954) and Wallace and Sinha (1962) studied the fungi associated with hot spots in farm-stored grains and also found *A. flavus* and *A. candidus* to be common.

Detailed studies were done by Lutey and Christensen (1963) on the influence of M.C., temperature and period of storage upon various fungi, especially *Fusarium* and *Helminthosporium*. They suggested that viable field fungi die rapidly in grains held at a M.C. in equilibrium with a relative humidity of 70-75%.

Tuite and Christensen (1952, 1955) were the first to study the "storage fungi" in barley in detail, and the growth of *Aspergillus* and *Penicillium* spp. in relation to M.C. Their results indicated that these fungi remain static when barley is stored at M.C. of 10-13%, but at M.C. of 13.8-14.2% *A. restrictus* and some species of the *A. glaucus* group such as *A. repens*, *A. amstelodami* and *A. ruber*, in addition to *A. candidus* and *Penicillium* spp. began to invade

the seeds.

4.2 The Changing Fungal Flora during Storage.

After these early studies, it became apparent that there were distinct groups of fungi associated with grains at various stages of storage, and the fungi associated with cereal grains became a major area of investigation.

Christensen and Kaufmann in 1965 classified the fungi of cereal grains into three types: a) Field fungi, which invade the grain before harvesting and include species of *Alternaria*, *Fusarium*, *Helminthosporium* and *Cladosporium*. b) Storage fungi, which invade the grains after harvesting and mainly include species of *Aspergillus* and *Penicillium*. c) Advanced decay fungi such as *Fusarium graminearum (Gibberella zea)*, *Papulospora*, *Chaetomium* and *Sordaria* spp.

Christensen and Kaufmann considered the storage fungi to be superficially present on seeds at harvest and started to be active in stored grain when the M.C. was in the range of 13.2% to 18%. The *A. glaucus* group, *A. ruber* and *A. restrictus* were active in grain at the lower end of the range 13.2% to 15%. Above 15% the predominant organisms were *A. candidus*, *A. ochraceus*, *A. flavus*, *A. versicolor* and *A. tamarii*. *Penicillium* spp. were often present, especially in lots having a M.C. above 16% and stored at a low temperature.

Christensen and Kaufmann (1969) reported more than 150 species of fungi from cereal grains and confirmed that kernels were not extensively invaded by storage fungi at harvesting. Similarly, Clark *et al.* (1966) found that *Aspergillus* and *Penicillium* spp. were almost entirely absent from freshly-harvested barley.

The sequence of development of the various fungal groups were termed "phases of the mycoflora of the stored grains" by Clark *et al.* (1967). Phase 1 consisted of the

mycoflora before or at harvest, eg. *Alternaria*, *Cladosporium*, *Fusarium*, *Epicoccum* etc. Phase 2 showed a decline or disappearance of these fungi, early in the storage period. The rate of decline was much more rapid at high M.C. Phase 3 was characterized by a fungal flora of very few species, and consisted largely of yeasts (eg. *Candida* spp. and *Hansenula anomala*) and *Penicillium roqueforti*, in cases of high M.C. The fungi in this phase persisted throughout the 9 to 12 months for which storage continued. Phase 4 was the final phase and included the appearance of typical "storage fungi" such as *A. candidus* and several species of the *A. glaucus* group. In the second half of the storage period *Absidia corymbifera*, *A. terreus*, *A. fumigatus* and *Mucor pusillus* appeared.

Lacey (1971) investigated six unsealed farm silos loaded with moist barley grain and his results agreed with the phases of Clark *et al.* Flannigan (1977) studied spontaneous heating of grain and noted growth of thermotolerant and thermophilic organisms such as *A. candidus*, *A. flavus*, *A. nidulans*, *A. fumigatus*, *Absidia* spp. and later *Thermoascus crustaceus* and *Thermomyces langinosus*.

Although there is no completely uniform world pattern for the occurrence of fungi in barley grain, Moreau (1979) listed the following species which contaminate barley as common in most countries: *Alternaria, Fusarium, Cladosporium* and *Mucor* (dominant at harvest time); *Aspergillus, Penicillium* and *Absidia* (more frequent after storage); three months after harvest the most common species are *P. cyclopium, P. roqueforti, Absidia corymbifera, Aspergillus candidus* and *A. terreus*. However, if temperatures reach 37-45°C, thermophilic species such as *A. fumigatus, A. terreus, Absidia corymbifera, Mucor pusillus* and *Doctylomyces crustaceus* may be isolated.

The most intensive work done on storage fungi was by Flannigan and colleagues (1969, 1970a, 1972 and 1978). In 1969 Flannigan reported on his investigations of the

mesophilic and thermophilic or thermotolerant, mycoflora of barley. He used three different media (potato dextrose agar, malt salt agar and tryptone soya agar) at three different incubation temperatures (25, 37, 50°C), to isolate fungi from non-disinfected, surface-disinfected and heat-treated whole barley. The most common species isolated were *Alternaria, Penicillium* spp., *A. glaucus, A. fumigatus, Aureobasidium, Cladosporium, Epicoccum* and *Absidia corymbifera.*

In 1972 Flannigan and Dickie examined all stages of commercial pearling of barley for human consumption, using direct culture and dilution plating methods. The most common fungi on or in pearled barley were found to be *Alternaria tenuis, A. glaucus, Cladosporium, Penicillium* and *Sporobolomyces.* They also noted that the most viable inoculum was present in or on the first 5% of the husk and pericarp removed by abrasion during the pearling process. These husk fragments were dominated by species in the *A. glaucus* group, *Alternaria tenuis, Aureobasidium pullulans, Penicillium* spp; *Thermomyces lanuginosus* , and actinomycetes and other bacteria. These observations add support to the contention that most fungi are present on the outer parts of the husk tissue and suggest that the topographic distribution of fungi can be of significance in later grain deterioration.

4.3 The Topographic Distribution of Fungi within Grains.

4.3.1 General distribution.

The spatial distribution of fungal propagules within barley grains has received some attention. Published work in general indicates that husk tissue is consistently more frequently colonized by fungal mycelia than is the caryopsis. Mulinge and Chesters (1970a) found that fungi were more frequent in husks than in the caryopsis (5% internal grain infection compared with 65-80%

infection in the husk) and Mulinge and Apinis (1969) showed that most storage fungi were confined to the husk tissue. Similarly Flannigan and Dickie (1972) noted over 90% of the viable propagules to be located in and on the husk, and Warnock and Preece (1971) found that fungal mycelium is mostly associated with the husk and pericarp rather than the caryopsis.

The various investigators developed different techniques for isolating fungi from different parts of the whole grain in their attempts to determine which fungi are surface colonists, which invade the husks and which penetrate the interior of the grain. For example, Mulinge and Chesters (1970a) found that whole grains plated on MSA provided the highest viable counts and suggested that this was a reflection of the deposition of fungal spores or mycelium on the grain surface. A second approach by these authors was to plate whole grains on MSA after washing with tap and distilled water. The results of such cultures were considered to represent the fungi which had invaded the husks and grain. A third treatment, in which the grains were sterilized with full-strength sodium hypochlorite, gave the lowest counts. After the grains were washed and dehusked, the husks and caryopsis halves were plated separately and the highest counts were obtained from husks. All the isolates were *Aspergillus* and *Penicillium* spp. (plus some yeasts) and this may be because they used only MSA medium. Basically similar techniques were used by Flannigan (1969, 1970a), as has been discussed in the previous section.

It therefore seems that the husk is the major reservoir of potential spoilage fungi, these being present both as spores and as mycelium.

4.3.2 Hyphal growth within the husk tissue

A number of investigators have attempted to find a suitable technique for distinguishing actual fungal mycelium on and within the husk tissue or epidermis

of various grains. Even within the husk itself it is necessary to determine which fungi are surface colonists and which actually invade the husks. The assessment of the actual amounts of internal mycelium present and whether this component is potentially capable of toxin production is of major importance (Warnock and Preece, 1971).

Several authors have developed direct observation techniques to study fungal hyphae within wheat and barley seeds. Hyde (1950) attempted to estimate the amount of fungal hyphae present in wheat. After a preliminary soaking of the grain in water, the epidermis was removed and stained with aniline blue, before microscopic examination. He noted that mycelium was commonly present, sometimes in abundance, beneath the pericarp, particularly in areas where the epidermis was loose, i.e. at the two ends of the grain or along the sides of the crease. Hyde developed a quantitative method of assessing the amount of the mycelium present based on the product of density and distribution estimates. The identity of the fungi was not established. In another study, Hyde and Galleymore (1951) investigated the identity of the subepidermal mycelium. Wheat grains were washed, surface sterilized, washed again and portions of the epidermis stripped from each grain and cultured. The most common isolate was *Alternaria tenuis* (64.4%).

Mead (1942) studied the mycelium of *Helminthosporium sativum* in barley grain. He used both microtome-sectioning of whole grains and removal of strips of lemma, palea and pericarp after soaking the kernels in formalin-acetic acid-alcohol overnight. These sections or strips were stained and examined microscopically. Mycelium was present in all of the seed coverings, particularly at the proximal end (head) of the kernel. No mycelium was found in the embryo.

Tuite and Christensen (1955) observed abundant fungal mycelium in the parenchyma layer of the husk and less abundant hyphae in the pericarp layer when they sectioned and stained barley grain, but they did not attempt identification

of these fungi.

A more accurate direct observation technique was used by Warnock and Preece (1971), using cryostat sectioning and phenol-acetic acid-aniline blue staining for studying the distribution of the internal fungal mycelia. An assessment of extent of mycelium present was based on the number of observations of "hyphal units" in serial sections. They found that fungal mycelium was only present in the parenchyma layer of the lemma and palea, and in the pericarp layer. Warnock (1971) used the same assessment technique for the entire surface of the husk without sectioning and later (1973a) described the development of internal mycelium and its origin before harvest. He concluded that mycelial growth (particularly of *Cladosporium*) in grains of barley originates both from spores deposited inside the lemma and palea and from spores deposited on the anthers.

Another technique used by Warnock (1971) involved an indirect fluorescent antibody technique to detect and determine the extent of *Penicillium cyclopium* mycelium in barley husk. The amount of mycelium was estimated by counting the number of sampling units of each lemma and palea in which specific fluorescent stained mycelium was present or absent. In this way he found the mean areas in which *P. cyclopium* mycelium was present, eg. 4.0% for the lemma and 4.7% for the palea. In 1973b he presented further evidence of the value of the immunofluorescence method for detection of *Alternaria*, *Aspergillus* and *Penicillium* mycelium and the estimation of the total amount of the mycelium present. Slide cultures of a number of fungal species treated with antiserum of *Alternaria alternata*, *A. flavus* and *P. cyclopium* indicated only a reasonable "genus-specific" nature for each antiserum. *Aspergillus* mycelium was present in most samples examined but could not be identified specifically as *A. flavus*.

The above review indicates that although *A. flavus* has frequently been noted as a fungus associated with heated,

spoiled grain, little is known of its presence in apparently clean samples or as mycelium within or on husk tissue of barley. The latter factor can be of considerable significance in relation to production of aflatoxin within the grain.

5. Aflatoxin Contamination of Barley.

It has been stated that corn is the grain in which aflatoxin contamination is most likely to be encountered (Stoloff, 1977). In the Philippines 97% of samples were positive, in Uganda 41% and in Thailand 40%. A number of surveys done on cereals other than corn suggest the general incidence and level of aflatoxin in such grains and their products, on a world wide basis, are relatively low (Jones, 1977), but little work has been published on barley.

Probably the first report of aflatoxin from barley was from the Tropical Products Institute in London where amounts of aflatoxin at biologically significant levels were detected in samples from around the world (Wogan, 1968). Aflatoxin was also isolated from 17% of barley samples in France (LaFont and LaFont, 1970) and has also been noted in barley in Japan (Kurata and Tanabe, 1973). In the U.K., Hacking and Biggs (1979) found aflatoxin B_1 contamination at levels of 125 μg/Kg in farm-stored mouldy barley. Recently Bryden (1982) in Australia summarized the results of investigations of about 20 different feedstuffs and compound feeds from Australia. Some animal feeds were contaminated with aflatoxin, particularly B_1. Barley samples showed about 14% positive samples with concentrations ranging between 10 - 500 μg/Kg. Thus it seems that although not recognized as being of any particular significance in relation to barley until quite recently, aflatoxin contamination may well prove to be of importance in future years, particularly in view of the current increased production of this cereal and the necessity for expanded farm storage.

5.1 Factors Affecting *A. flavus* Growth and Aflatoxin Production.

Factors influencing growth and toxin formation by fungi in general, do not act singly but in multiples (Hesseltine, 1976) and growth and toxin production by *A. flavus* are no exception. An understanding of these factors is important in relation to both natural contamination of feedstuffs and to production of aflatoxin in culture.

Jarvis (1971) and Hesseltine (1976) considered all the factors which may be involved and divided them into three groups; chemical, physical and biological. All the factors were operative during storage of the commodity but not all were effective in the field or at harvest. Many of the factors are ill-defined, eg. country, time of year, agricultural practices, crop types and varieties, weather, storage and shipping condition (Ciegler, 1978).

Physical factors include moisture content, relative humidity, temperature, time, development of hot spots, rapidity of drying and the rewetting of grain. Of these, moisture content and temperature are usually controlling factors for the growth of the fungus. For stored barley, Chang and Markakis (1981) found that if the M.C. reached 16% or higher at 25°C, aflatoxin contamination may occur. The optimum temperature for aflatoxin production, when other conditions are near optimal, is approximately 27°C; the lower limit is about 12°C and the upper limit about 40°C (Davis and Diener, 1970). The optimum temperature for actual growth of *A. flavus* is around 33°C, with growth limits between 12° and 14°C (Hocking, 1982).

In vitro the time period for maximum toxin production varies according to the strain, the medium used, and the temperature of incubation. Diener and Davis (1966) reported maximum yields after 15 days at 20°C or 11 days at 30°C. Maximum concentration of aflatoxin in the medium is correlated with the exhaustion of fermentable carbohydrate and

the onset of mycelial autolysis (Jarvis, 1971). In the case of shaken cultures the incubation time can decrease to about four to five days optimum and the amount of aflatoxin produced is higher than in stationary cultures (Davis and Diener, 1968; Hesseltine *et al.*, 1966).

Chemical factors include the nature of the substrate, the mineral nutrients available, CO_2, O_2, pH and any chemical treatment to which the commodity was subjected. A higher proportion of toxigenic strains of *A. flavus* have been isolated from oil seeds, especially peanuts and cotton seed, than from rice or sorghum (Ciegler, 1978). For any given strain of *A. flavus* the substrate will also influence the amount of aflatoxin produced (Butler, 1974).

In laboratory studies of toxin production, various carbon sources have been used successfully in synthetic and semisynthetic media for aflatoxin production. Glucose, sucrose or fructose are the preferred carbon sources (Mateles and Adys, 1965; Davis and Diener, 1968). During its growth, the fungus also requires a number of trace elements such as iron and zinc (Davis *et al.*, 1967) and various vitamins and amino acids, eg. thiamin, biotin. Natural substrates such as groundnuts, maize, rice and wheat have also proved suitable and the yield of toxin has frequently been superior to that obtained on synthetic media (Stubblefield *et al.*, 1967; Wildman *et al.*, 1967; Shotwell *et al.*, 1966). The highest amount of aflatoxin produced in culture (8 g/Kg) was reported by Arseculeratne *et al.* (1969) who used coconut flesh as substrate.

Biological factors include plant stress, plant varietal differences, presence of invertebrate vectors, degree of fungus infection, fungal strain differences, spore loads and the general microbiological ecosystem. Probably the fungal strain is the most significant factor because not all strains of *A. flavus* are able to produce aflatoxin and even the toxigenic strains vary in their ability to produce the various aflatoxins (B_1, B_2, G_1 and G_2) (Hesseltine

et al., 1970). Strains of tropical origin seem to be more toxigenic than strains from temperate regions - 46% of the former compared to only 15% of the latter (Jacquet and Boutibonnes, 1970, cited by Moreau, 1979). According to Stoloff (1977), *A. parasiticus* is the more likely species to be encountered in the warmer environments and it is that species which is more likely to produce the greatest variety of aflatoxins.

5.2 Determination of Aflatoxins.

There are several approaches for investigating the natural occurrence of mycotoxins in foods and feeds. A direct, precise but expensive, approach is to examine suspect material by chemical methods to determine whether a specific mycotoxin or mixture of mycotoxins is actually present. An indirect approach is to isolate fungi from the substrate in pure culture, grow on a suitable medium and then test for their ability to produce toxin.

The direct approach is the only definitive way of assessing the significance of mycotoxin-contaminated feedstuffs in natural outbreaks of disease. For example, the growth of *A. flavus* from commodities or their products does not automatically imply the presence of aflatoxin in those commodities. These isolates may be non-toxigenic strains or growth of the fungus may not have actively occurred in the commodity, or occurred to only a minor degree. Conversely, aflatoxin can be detected without it being possible to isolate the mould, perhaps because the fungus grew long ago and produced the toxin, but the fungus itself has since disappeared (Moreau, 1979). However, the direct approach has little value when a commodity is showing only incipient deterioration - it gives no indication of the potential significance of any fungi which may be present, i.e. the actual origin of the mycotoxin, nor can it differentiate the actual location of toxin production within the substrate, as sampling procedures require bulk analysis of the material.

The approach taken in this investigation was first to isolate *A. flavus* strains, subject them to a screening procedure to determine their toxigenicity, and then carry out confirmatory tests to determine the amount and types of aflatoxin which toxigenic strains were able to produce. The significance of this information could then be assessed in relation to the actual topographic origin of the isolates.

5.2.1 Screening procedures for fungal isolates.

A number of screening techniques are available which utilise the production of fluorescence on agar media of various composition, eg. coconut agar (Lin and Dianes, 1976), corn-steep liquor agar (Hara *et al.*, 1974) and peanut extract agar (Connole, pers. comm.). Of these, coconut agar is the most convenient.

Coconut was described by Arseculeratne *et al.* (1969) as an excellent substrate for aflatoxin production. When grown on freshly-grated coconut, a toxigenic *A. flavus* strain (NRRL 2999) produced 8 mg (total) aflatoxin/g coconut when incubated at 24°C for 9 days with agitation. These authors also used coconut agar medium to induce fluorescence within the agar. Later, Lin and Dianes (1976) used this method successfully, using both fresh coconut extract and commercial coconut extract, for screening a number of *A. flavus* isolates. Only aflatoxin-positive isolates showed characteristic blue or blue-green fluorescence surrounding the colony on the agar.

Lin and Dianes noted that the minimum time required to detect fluorescence on this medium was 32 hours with a strong aflatoxin producer (NRRL 2999), and 3 days' incubation was sufficient for detection of even very weak aflatoxin production.

5.2.2 General analytical procedures

Jones (1977) listed the general steps for aflatoxin detection and analysis in a given commodity or fungal culture.

a) Sample preparation for analysis: the sample, usually about 1 to 5 Kg is ground and mixed until the whole sample is homogeneous. A 50 g (usually) sub-sample for the actual analysis is then taken using quartering procedures.

b) Defatting: this step may be necessary if the material to be analysed contains more than about 5% of oil and other fatty materials.

c) Extraction and purification of extract: Suitable solvents and clean-up procedures are then used to remove potentially interfering fluorescent materials etc. Most methods of extraction and purification are based on the solubility of aflatoxins in such organic solvents as chloroform, methanol, ethanol, acetone and benzene, and their insolubility in lipid solvents such as hexane, petroleum ether and diethyl ether (Moreau, 1979).

d) Chromatographic separation and assessment of aflatoxin concentration in the extract: various techniques have been used for separating aflatoxins but the more popular systems, two of which have been used in this study, involve thin-layer chromatography and column chromatography.

TLC provides a reasonably easy method for separation of the individual aflatoxins. The use of plates coated with a layer of silica gel (Kiesel gel G) for separating the four aflatoxins B_1, B_2, G_1 and G_2 was first reported by De Iongh *et al.* (1962, cited by Moreau, 1979). They used a mixture of chloroform and methanol (98:2) as developing solvent and classified the aflatoxin components according to their Rf's and colour. Subsequently, different solvent systems have been used. The Methods of the Association of Official

Analytical Chemists (1980) list 12 such solvents which can be recommended for use in one-way chromatography systems. Many investigators now use 2-dimensional TLC, particularly when working with mixed feedstuffs, as by this method aflatoxin is better resolved from coextractives (Patterson and Roberts, 1980).

A commonly-used method for the quantitative estimation of aflatoxins by TLC involves direct comparison of the intensity of spots on chromatograms of test extracts examined under U.V. light with standards of known concentration of aflatoxin (Nesheim, 1964), although errors of as much as 50% can be involved (Moreau, 1979). Densitometric or spectrophotometric methods are more accurate (Nabney and Nebitt, 1965; Pons and Goldblatt, 1969).

Minicolumns are a refinement of larger scale column chromatographic techniques, and were developed to provide a simple and inexpensive method for use at the plant or field level, to enable acceptance or rejection of lots (Romer *et al.*, 1979). They have mostly been applied to surveys of peanut and corn samples. Holaday (1968) was the first to report the use of minicolumns for aflatoxin detection. The column was prepared by filling a length of 4 mm diameter glass tubing with silica gel to a depth of about 4.5 cm and developed with a chloroform-methanol extract of the sample. Aflatoxin-positive samples showed a blue fluorescent band at the lower end of the column when examined under long-wave U.V. light. Sensitivity was 5 ppb (Holaday, 1968).

Of the various minicolumn methods in use, a combination method, (Holaday-Velasco) for aflatoxin in corn has been adopted by both the Association of Official Analytical Chemists and the American Association of Cereal Chemistry (Shotwell and Holaday, 1981), and also the International Union of Pure and Applied Chemists (Romer *et al.*, 1979).

e) Confirmatory tests: additional chemical

confirmatory tests are needed to differentiate unambiguously between the aflatoxins and other fluorescent compounds which may be present in an extract (Jones, 1977). Derivatization directly on the chromatogram using, for example, a mixture of acetic or formic acid with thionyle chloride, or trifluoroacetic acid, can be used to differentiate aflatoxin from artifacts (Wiley and Waiss, 1968).

6. AIMS OF PROJECT

With particular reference to *Aspergillus flavus*, to:

a. Examine the total fungal load and relative abundance of individual species on both inner and outer surfaces of the husks of farm-stored barley grains, in relation to degree of spoilage.

b. Investigate the hyphae within the husk tissue in relation to their viability and their identity.

and

c. Test the strains of *A. flavus* isolated for their ability to form aflatoxin.

MATERIALS AND METHODS

1. Samples Examined.

1.1 Source of Samples

Samples of barley grains held in store for stock feeding purposes were collected from twelve farm silos in the Manawatu District during the month of August, 1981 (Fig. 2). All grain had been stored in sealed metal silos of varying capacities (Table 3) for four to five months except for one sample (PL) which had been stored for two years. Table 3 also lists the visual assessment of the condition of the samples at the time of collection.

1.2 Sample Collection

Approximately 2 Kg samples were collected from each silo. In most cases, the sample was collected from the upper surface of the grain mass by thoroughly mixing an area of grain approximately 1 m^2 x 30 cm deep before the sample was removed. In two cases (FH and SR) samples were collected from the silo shute. Each sample was placed in a brown multi-wall paper bag, which was then sealed with cello-tape. Samples were held in a cold room (0-5°C) until required.

1.3 Moisture Content

1.3.1 At time of introduction to silo

An approximation of the moisture content of the grain at the time of introduction to the silos was reported by the individual farmers from whose property samples were collected.

Figure 2. Sources of farm-stored barley samples collected in the Manawatu District. Each collection site is coded: MI... etc.

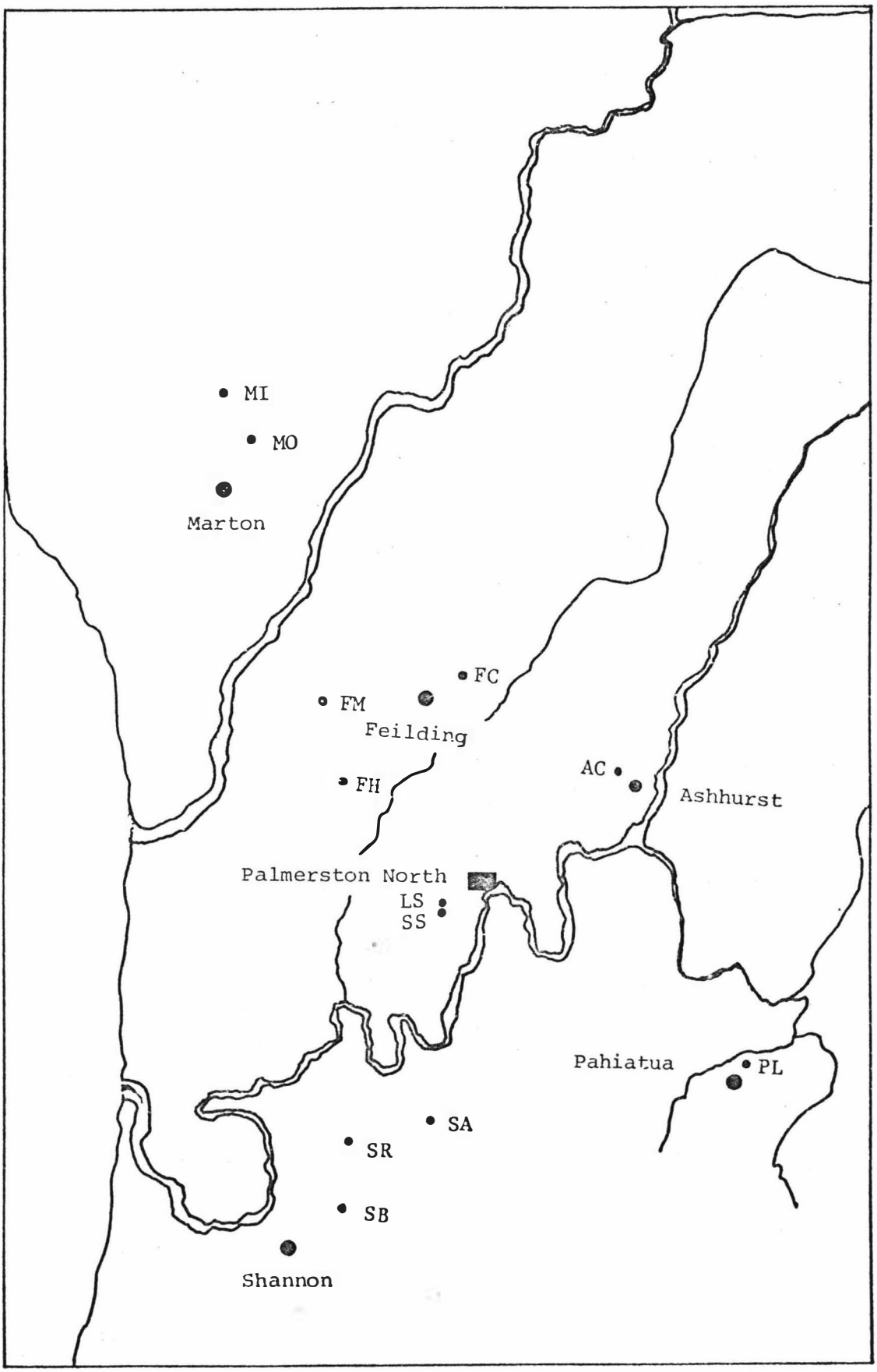

MI
MO
Marton
FC
FM
Feilding
FH
AC
Ashhurst
Palmerston North
LS
SS
Pahiatua
PL
SA
SR
SB
Shannon

Table 3. Details of the silos from which grain was collected and the condition of the grain within each.

Silo code	Capacity of silo (Tonnes)	Aeration system	Storage period (months)	Visual condition of sample
LS	200	+	5	Heated, mouldy, grain mass in upper layer showed grey-coloured surface
PL	100	-	24	Heated, mouldy, mostly damaged grain, caked, white and grey-coloured surface; grain weevils abundant.
FH	75	-	5	Grain had been treated with "Siloguard"*. Some grain was damaged; dead grain weevils present.
SA	80	-	4	Heated, mouldy, grey-coloured surface; grain weevils abundant.
SB	100	-	4	Grain was cone-shaped at bottom of silo; mouldy, caked, some germination.
MO	40	-	5	Slightly mouldy, grey-coloured, mainly at upper door of silo.
AC	34	-	4	Slightly mouldy.
SS	92	+	5	Apparently clean.
MI	50	-	5	Apparently clean.
SR	200	-	5	Apparently clean.
FC	80	-	4	Apparently clean.
FM	530	+	4	Apparently clean; grain had been treated with "Siloguard"*.

* Maldison 2%, Premium grade.

1.3.2 Laboratory determination after sampling

The air oven method was used (Air Oven Method, 14.004, AOAC 1980) for determining the moisture content of each sample within one week of collection.

Procedure

1. Metal dishes with foil covers were dried at 98-100°C for two hours in a vented air oven, then cooled in a dessicator with conc. H_2SO_4 as drying agent, and weighed soon after reaching room temperature.

2. Approximately 2 g of barley removed from the well-mixed sample was accurately weighted into a dish.

3. The sample was heated to 130 ± 3°C in the uncovered dishes for one hour. (The drying period began when the oven temperature was actually 130°C.)

4. The dishes were transferred to the dessicator and weighed soon after reaching room temperature.

1.4 Sub-sampling.

Disposable gloves were worn to thoroughly mix the grain sample and approximately 4 g subsamples were taken randomly and placed in small sterile plastic bags for further processing.

2. Media, Reagents and Apparatus.

2.1 Media.

The following media were used:

a. Potato Dextrose Agar (PDA):

Potato infusion	200.0 g
Dextrose	20.0 g
Agar	15.0 g
Distilled water	1 ℓ

Chloramphenicol (to a final concentration of 0.05 g/ℓ, dissolved in a little 95% alcohol) was added prior to autoclaving for 10 minutes at 15 psi.

b. Malt Salt Agar (MSA):

Maltose (technical)	12.75 g
Glycerol	2.75 g
Bacto-Peptone	0.78 g
Bacto-agar	15.0 g
NaCl	100.0 g
Distilled water	1 ℓ

Autoclaved for 10 minutes at 15 psi.

c. Czapek-Dox Agar (CDA):

Sucrose	30.0 g
Sodium nitrate	3.0 g
Dipotassium phosphate	1.0 g
Magnesium sulphate	0.5 g
Potassium chloride	0.5 g
Ferrous sulphate	0.01 g
Agar	15.0 g
Distilled water	1 ℓ

Autoclaved for 10 minutes at 15 psi.

d. Potato-Carrot Agar (PCA) (Sporulation Medium):

Potato	200.0 g
Carrot	200.0 g
Tap water	1 ℓ

The ingredients were boiled for 20 minutes, strained

and the volume adjusted to 1 ℓ. Then the remaining ingredients were added:

Dipotassium phosphate	2.0	g
Agar	20.0	g

Autoclaved for 15 minutes at 15 psi.

e. Coconut Agar - for primary screening for aflatoxin production by *A. flavus* group isolates.

A 410 cm^3 can of Coconut Cream, containing coconut cream, water, and polysorbate 60 as manufactured by Samoa Tropical Products Ltd. was used as coconut source.

"Coconut Cream"	200	cm^3
Distilled water	600	cm^3
Agar	12.0	g
pH	6.9	

Autoclaved for 15 minutes at 15 psi and plated in plastic petri dishes. The medium was best used within two weeks of preparation.

f. Semisynthetic Liquid Medium (SMKY):
(Diener & Davis, 1966.)

Sucrose - analytical grade	200.0	g
Magnesium sulphate	0.5	g
Potassium nitrate	3.0	g
Yeast extract (Difco)	7.0	g
Distilled water	1	ℓ
pH	5.5	

The medium was dispensed in 50 cm^3 quantities in 500 cm^3 conical flasks. The flasks were plugged with cotton wool wrapped in cheese cloth and the plugged mouth sealed with a foil closure before autoclaving for 10 minutes at 15 psi.

g. Weet-bix Medium:

Malted wheat biscuits as manufactured by Sanitarium Health Food Co., New Zealand, were used.

Crushed Weet-bix	25.0	g
Tap water	12.5	cm^3

Dispensed into a 500 cm^3 conical flask, plugged and sealed as above before being autoclaved for 15 minutes at 15 psi.

h. Pearled Barley Medium:

A quantity of barley grain was pearled mechanically by Manawatu Mill of Farm Products Cooperative Ltd. Husks and other small particles were removed before weighing.

Pearled barley	50.0	g
Tap water	25.0	cm^3

Dispensed into a 500 cm^3 conical flask and plugged and sealed. Left overnight in fridge to soak the grains, then autoclaved for 15 minutes at 15 psi.

i. Barley Husks Medium:

Coarse husks obtained from the pearling process were seived through a 1.6 mm sieve to remove contaminating starch grains and very fine husk particles, and the following medium prepared:

Coarse husks	25.0	g
Tap water	12.5	cm^3

Dispensed into a 500 cm^3 conical flask and plugged and sealed. Soaked overnight in fridge then autoclaved for 15 minutes at 15 psi.

2.2 Reagents.

a. Trypan blue-vital stain:

0.04 g trypan blue* was dissolved in 100 cm^3 1% acetic acid (in sterile distilled water).

b. Mounting fluid - Lactophenol cotton blue:

Phenol crystals	20.0	g
Lactic acid	20.0	g
Glycerol	40.0	g
Distilled water	20.0	cm^3

Dissolved by gentle heat under hot tap and 0.05 g cotton blue (Porrier's blue) was then added.

c. Aflatoxin Standard (B and G):

A standard mixture of aflatoxins was obtained from Sigma Chemical Co., U.S.A. Each cm^3 of benzene-acetonitrile (98:2 v/v) mixture contained 5.0 μg, 1.5 μg, 5.0 μg and 1.5 μg of B_1, B_2, G_1 and G_2 respectively.

d. TLC development solvent:

Chloroform-acetone-water (88:12:1.5) was used as development solvent in unlined unequilibriated glass tanks.

e. Extraction solvent:

An aqueous acetone solution was prepared by mixing 850 cm^3 acetone, 150 cm^3 distilled water and 8 cm^3 glacial acetic acid. The solution was stored in a brown bottle.

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* BDH Chemicals Ltd., Poole, England.

f. Lead acetate solution.

200 g neutral lead acetate was dissolved in distilled water, 3 cm^3 glacial acetic acid added and the solution diluted to 1 ℓ.

2.3 Apparatus.

2.3.1 Minicolumns

a) Local preparation, as described in 26.016 AOAC 1980.

A 19 cm length of glass tubing of 6 mm i.d. was used for each minicolumn. A small plug of glass wool was tamped into one end of the column. The following materials were then added to the heights indicated in the following order and with constant tamping:

Calcium sulphate (anhydrous)	8 - 10 mm
Florisil*	8 - 10 mm
Silica gel	16 - 20 mm
Neutral alumina	8 - 10 mm
Calcium sulphate (anhydrous)	8 - 10 mm

A further small plug of glass wool was tamped on the top of the column (Fig. 3,A) and gentle pressure applied to this glass wool plug with a thin glass rod to assist the compaction of ingredients.

b) Commercially prepared minicolumns were obtained from Tudor Scientific Glass Co., Belverdere, U.S.A. The materials within these columns are shown in Figure 3,B.

All columns were stored in a dessicator containing silica gel as drying agent for at least 5 days before use.

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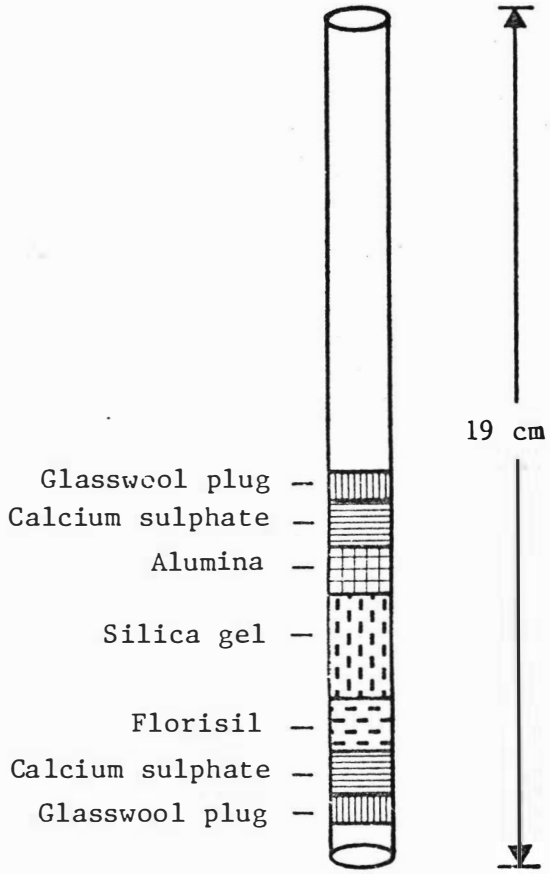
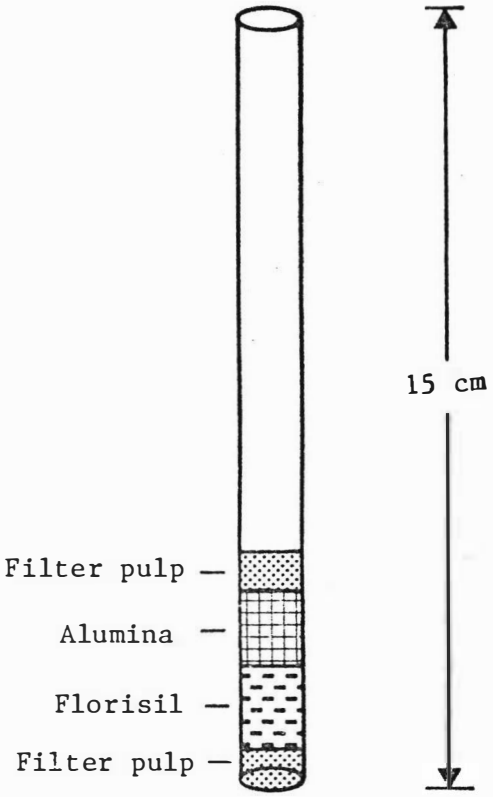
* Florisil - Registered Trade Mark for a brand of activated Magnesium silicate.

Figure 3. Components of the minicolumns used for screening culture extracts for aflatoxin.



A. Locally-prepared.



B. Commercially-prepared.

2.3.2 Thin-layer Chromatography (TLC)

20 cm x 20 cm aluminium sheets precoated with silica gel 60 (without fluorescent indicator) (5553) with a layer thickness of 0.2 mm, were obtained from E. Merek, Darmstadt.

3. Mycoflora of Barley Grain Husk.

3.1 Fungi on outer surface (O.S.)

3.1.1 Sample processing

1 g quantities of each sub-sample (Section 1.4) were accurately weighed into sterile universal bottles (1 oz, screw-cap). 10 sterile glass beads and 10 cm^3 sterile distilled water (s.d.w.) were then added to each. The samples were mechanically shaken for 30 minutes on a reciprocating shaker giving a 1 cm throw, 5 throws per second.

3.1.2 Dilution plating and counting procedures

After the shaking period the supernatant was immediately decanted into another sterile universal bottle. This suspension was shaken vigorously on a mixer* for 20 seconds before transferring 1 cm^3 into 9.0 cm^3 of diluent s.d.w. A series of dilutions (10^{-2} to 10^{-5}) were prepared by standard techniques. Before each transfer, the suspension was shaken vigorously for 10 seconds to prevent sedimentation of suspended material.

Duplicate 1.0 cm^3 quantities of the appropriate dilutions were transferred to sterile plastic petri dishes and 15 cm of molten but cool PDA medium added and mixed thoroughly with the suspension before leaving to set.

All plates were examined after 3, 5 and 7 days of incubation at 25°C. Suitable plates showing between 20 and 200 colonies were used to estimate the total viable count.

* Super-mixer, Lab-Line Instruments, Inc., U.S.A.

Identification procedures are detailed in Section 4.

3.2 Fungi on Inner Surface (I.S.)

3.2.1 Sample processing and dehusking

Sub-samples of 2 g grain were removed from the bulk sample and soaked for 3 hours in 10 cm^3 s.d.w. at 4°C. The water was drained off and the grain was treated with 10 cm^3 of full-strength sodium hypochlorite solution for 1 minute with occasional shaking to disperse air bubbles from the grain surfaces. The sterilant was then decanted off and the grains rinsed in seven changes (20 cm^3 each) of s.d.w. with vigorous hand-shaking for ½ minute each time.

The sample was drained by aseptically placing the grains onto three layers of sterile filter paper contained in sterile glass petri dishes. The grains were dehusked with flamed forceps, starting from the proximal end and peeling to the distal end and from the dorsal side to the ventral furrow, removing both the lemma and the palea.

3.2.2 Dilution plating and counting procedures

All the husks from the 2 g whole grain sample were collected in a sterile universal bottle and mechanically shaken with 20 sterile glass beads in 10 cm^3 s.d.w. on the reciprocating shaker for 30 minutes. The supernatant was then decanted off. From this suspension serial dilutions were made and plated as in Section 3.2.1, for viable counts and identification.

3.3 Fungi within Husk Tissue.

The husks were rewashed in seven changes of s.d.w. (20 cm^3 each) with vigorous hand-shaking for ½ minute at each change.

3.3.1 Microscopic examination of stained husk tissue

Ten husk strips were examined in trypan blue vital stain (Section 2.2). Preliminary experiments had shown that the vital stain at the concentrations used had very little effect on the growth of a range of fungi tested. The strips were soaked in the stain on a slide, with the inner surface of the strip uppermost, for five minutes. A coverslip was applied and the entire surface of the strip was then examined. The presence of fungal hyphae was noted as present (+) or absent (-) and the mycelial density estimated roughly on a scale 1+ to 5+.

3.3.2 Cultural examination

After examining the husk strips in vital stain, five of the strips were plated out onto MSA and five onto PDA, being placed equidistantly on the agar surface. Both plates were incubated at 25°C for two weeks after which the growth was examined and subcultures made for identification if necessary.

4. Identification and Maintenance of Isolates.

4.1 Identification

The isolated fungi were identified according to standard methods of macroscopic and microscopic examination and the criteria described in Barnett and Hunter, 1972; Barron, 1968; Ellis, 1971, 1976; Gilman, 1957; McGinnis, 1980; Raper and Fennel, 1965 and Raper and Thom, 1968.

Non-sporulating colonies were subcultured on MSA and PDA and/or PCA for one month, with periodic examination for spores before discarding.

Isolates of the *A. flavus* group were subcultured on

CDA for three weeks at 25°C to allow further speciation.

4.2 Selection of *A. flavus* strains and source of cultures for aflatoxin assays

4.2.1 Barley isolates

52 isolates of *A. flavus* Link and three isolates which seemed closer to *A. parasiticus* Spear were obtained from barley husks in this study.

4.2.2 Soil isolates

32 isolates from soil samples were supplied by Dr. M. Baxter; 9 were *A. flavus* Link and 23 *A. tamarii* Kita.

4.2.3 Reference strain

A culture of *A. parasiticus* strain NRRL 2999 (a high aflatoxin-producing strain) was kindly supplied by Dr. C. Freke, National Dairy Laboratory, Hamilton.

4.3 Maintenance of isolates.

The agar slant method using PDA in universal bottles was used for the maintenance of cultures, which were held at room temperature with subculturing and checking every six months.

5. Cultural Methods for Aflatoxin Production.

5.1 Coconut Agar

All *A. flavus* group isolates were grown on PDA slants for three days at 25°C, and then subcultured into the centre of coconut agar and incubated at 25°C. The

plates were viewed under long-wave U.V. light (365 nm) in a darkened room after 3, 5 and 10 days of incubation to detect fluorescent material.

5.2 Semisynthetic Liquid Medium.

For this medium, 40 *A. flavus* group isolates were used; 34 from barley, 5 from soil and the *A. parasiticus* strain NRRL 2999. Spore suspensions were prepared from 7-14 day-old cultures on PDA slants, by adding 5 cm^3 s.d.w. and detaching the spores by agitation with a sterile platinum loop. 0.5 cm^3 of spore suspension was inoculated into a flask of SMKY. After shaking to distribute the spores, the flasks were incubated in either a stationary position at 25°C for 7-9 days, or in a shaking incubator* at 28°C for 4-5 days (100 rpm). All flasks were wrapped completely with foil to darken the culture.

5.3 Weet-bix Medium.

Six *A. flavus* isolates (4 from barley and 2 from soil) were cultured on this substrate, and also strain NRRL 2999. 0.5 cm^3 of spore suspension (prepared as in Section 5.2) was inoculated into the medium in 500 cm^3 conical flasks and incubated at 25°C for 10-12 days. To keep a high moisture level, 1 cm^3 sterile distilled water was added daily for five days; thereafter 0.5 cm^3 was added until the time of harvesting (this technique also maintained the O_2 level).

5.4 Pearled Barley and Barley Husks Media.

Inocula of three toxigenic strains and one non-toxigenic strain were prepared as in Section 5.2 and used to inoculate pearled barley and barley husks media. The cultures were incubated in stationary condition at 25°C for

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* Controlled environment incubator shaker, New Brunswick Scientific Co., Canada.

10-12 days.

6. Extraction of Aflatoxin from Cultures.

6.1 Extraction from SMKY Cultures

Chloroform (analytical grade) was used as extraction solvent. 100 cm^3 $CHCl_3$ (2:1 v/v) was added to the culture flask together with approximately 100 g glass beads. The flask was left for one hour with periodic shaking. The contents were then filtered through 16 folds of cheesecloth, the cloth washed with 20 cm^3 $CHCl_3$ and the whole filtrate collected in a 200 cm^3 dispensing bottle. This crude extract was refiltered twice through a folded circle of Whatman No. 4 filter paper, covering the funnel with a glass petri dish to reduce evaporation. The filter paper was washed with 10 cm^3 $CHCl_3$ after each filtration.

All the extract was collected into a 250 cm^3 separatory funnel and shaken vigorously for about one minute, then left for 10 minutes to separate into two layers. The lower ($CHCl_3$) layer was drained into a 250 cm^3 round flask, and this extract concentrated by evaporating the $CHCl_3$ on a rotary evaporator* (avoiding dryness). The extract was re-suspended in 3 cm^3 $CHCl_3$ and stored in a small vial prior to analysis by minicolumn and TLC.

6.2 Extraction from Weet-bix, Pearled Barley and Barley Husks Media

The method used was similar to that described by Pons *et al.*, 1968:

6.2.1 Sample extraction

250 cm^3 extraction solvent (Section 2.2.e) was added to the conical flask containing the fungal culture

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* Buchi Rotavapor-R.

and the whole covered with about 100 g solid glass beads. The culture clump was broken with a stainless-steel spatula into small pieces and the flask then plugged and sealed. The flask was shaken vigorously for 30 minutes on a reciprocating shaker. The extract was filtered through a folded circle of Whatman No. 4 filter paper. About 150 cm^3 of filtrate was collected.

6.2.2 Lead acetate treatment

125 cm^3 of the crude extract was measured into a 250 cm^3 beaker and 20 cm^3 distilled water, and several clean boiling chips (anti-bumping granules) were added. The mixture was stirred and then boiled on a steam bath until the volume was reduced to 125 cm^3. After cooling to room temperature, the contents were transferred to a 250 cm^3 graduated cylinder, washing the beaker with distilled water, and diluted to 200 cm^3. 4 g of filter aid* were added and the whole mixed well before filtering through a folded circle of Whatman No. 4 filter paper. About 170 cm^3 of filtrate were collected.

6.2.3 Chloroform partition

160 cm^3 of the filtrate was measured into a 250 cm^3 graduated cylinder and 50 cm^3 $CHCl_3$ added. The mixture was shaken vigorously for about 1 minute and then transferred to a 250 cm^3 separatory funnel and shaken again for about 1 minute. The $CHCl_3$ (lower) phase was drained through a sodium sulphate drying tube (Pons and Goldblatt, 1965). The filtrate was collected in a clean 250 cm^3 beaker. The extraction was repeated with a second 50 cm^3 quantity of $CHCl_3$. The sodium sulphate tube was washed with 20 cm^3 of $CHCl_3$ and the combined $CHCl_3$ extract (about 100 cm^3) was transferred into a 250 cm^3 round flask to evaporate to near-dryness under vacuum on a rotary evaporator (avoiding dryness of extract). The extract was dissolved in 3 cm^3 $CHCl_3$

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* Celite Hyflo-Supercel filter aid.

and transferred to a small vial prior to aflatoxin assay by both minicolumn and TLC technique.

7. Detection of Aflatoxin.

7.1 Minicolumn Method

7.1.1 Technique

Using a 5 cm^3 disposable syringe with a 15 gauge needle, 2 cm^3 of extract solution was transferred into the minicolumn which was held upright in a support rack. The $CHCl_3$ was allowed to run through the column by gravity. When the flow rate was very slow, a rubber bulb attached to the top of the column was used to provide gentle pressure. When the $CHCl_3$ had completely run through (15-30 minutes) 3 cm^3 of elution solvent ($CHCl_3$ - acetone, 9:1) was drained through. The column could then be examined in a darkened room under long-wave U.V. light to detect any blue fluorescent band at the top of the Florisil layer. Comparison could then be made to a reference minicolumn.

7.1.2 Preparation of reference minicolumn

100 $\mu\ell$ of aflatoxin standard solution (Section 2.2.c) was added to 0.9 cm^3 chloroform in a small vial. The entire solution was run into a minicolumn and 3 cm^3 elution solvent ($CHCl_3$ - acetone, 9:1) was then passed through the column. The solvent was removed by forcing it through the column with nitrogen (pressure at $\leqslant$ 10 cm^3/min.). The column was allowed to dry and then wrapped in foil and stored in a freezer. This reference minicolumn contained 0.5 μg, 0.15 μg, 0.5 μg and 0.15 μg/cm of B_1, B_2, G_1 and G_2 respectively.

7.2 Thin-layer Chromatography (TLC)

7.2.1 Spotting technique

A complete plate (20 x 20) was used for analysing 4 samples simultaneously or a half-plate (10 x 20) for analysing 2 samples. A light pencil line (spotting line) was marked 3 cm from the bottom edge of the plate. Aliquots of 1, 5 and 8 µℓ or 2, 5 and 10 µℓ from the standard aflatoxin and 2, 5 and 10 µℓ or 2, 5 and 12 µℓ from test samples were spotted onto the plates using a 10 µℓ disposable micro-pipette*. The micro-pipette was divided with a wax pencil equally into 4 parts to assist with estimation of aliquot quantity. After application of the samples, the plate was left for 5-10 minutes to dry in subdued light before developing.

7.2.2 TLC development

The plates were developed in subdued light in 100 cm^3 chloroform-acetone-water (88:12:1.5), in an unlined and unequilibriated glass tank, until the solvent front reached a mark 13 cm beyond the spotting line. Then the plate was removed from the solvent and dried in a dark fume cupboard for 20 minutes before being examined under long-wave U.V. light in a darkened room.

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* Lambdas, Drummond Scientific Co., U.S.A.

RESULTS

1. Moisture Content (M.C.)

The laboratory estimations of the M.C. of the samples were lower than the values provided by the farmers and said to be true at the time of storage. On-farm determinations ranged from 13% (2 samples) to 16% (sample FH) (Table 4). Moisture contents determined in the laboratory showed a maximum of 15.7% (sample LS) and a low of 9.5% (sample SS).

Table 4. Moisture content of barley grain samples.

Silo sample code	M.C. before storage *	M.C. at sampling **
LS	14	15.7
PL	13	11.9
FH	16	10.5
SA	14	12.1
SB	14	12.0
MO	14	13.9
AC	14	12.0
SS	14	9.5
MI	14	14.7
SR	14	12.2
FC	14	10.0
FM	13	9.6

* = Moisture content at time of introduction to silos as reported by the individual farmers.

** = Moisture content at time of sampling (lab. determinations).

2. Total Fungal Load of Inner and Outer Surface of Barley Husks.

2.1 Outer surface (O.S.)

In Table 5, the total fungal viable counts are expressed as the average colony forming units (C.F.U.)/g barley grain, as determined by the techniques described in Methods 3.1. Yeast and bacteria were of negligible proportions in all these determinations.

The counts fluctuated between 0.4×10^2 (sample SR) to 2.6×10^6 (sample LS).

2.2 Inner surface (I.S.)

Fungal counts of the inner surface of husks as determined by the techniques described in Methods 3.2 are also given in Table 5.

The inner surface viable counts fluctuated between 0.1×10^2 (sample FM) to 4.1×10^4 (sample LS). Except for samples FH and SS, I.S. counts were lower than those recorded from the O.S. The average mean difference between the two counts for all 12 samples was 2.2×10^5.

3. Fungi Isolated from Inner and Outer Surfaces of Barley Husks.

Only members of the genus *Aspergillus* are considered at species level. All 12 samples examined by the dilution plating technique yielded *Aspergilli* and they were the predominant fungi on both I.S. and O.S. of samples LS to AC. The distribution of isolations of individual *Aspergillus* spp. is shown in Table 6 and summarized in Table 8. A total of nine species was isolated from the samples examined and all samples were positive for at least one species. *A. flavus* was the most frequent (11 of 12 samples positive); 7 samples yielded *A. fumigatus* and 6 *A. glaucus*. The

Table 5. Viable counts of inner and outer surfaces of barley husks.

Sample code	CFU/g grain: Outer surface	Inner surface *	Difference in counts
LS	2.6×10^6	4.1×10^4	2.56×10^6
PL	9.7×10^3	4.2×10^3	5.50×10^3
FH	5.5×10^2	2.5×10^3	-1.95×10^3
SA	9.1×10^3	1.7×10^3	7.40×10^3
SB	7.7×10^3	1.7×10^3	6.00×10^3
MO	2.7×10^4	5.6×10^2	2.64×10^4
AC	4.9×10^3	1.4×10^3	3.50×10^3
SS	0.74×10^2	0.9×10^2	-0.16×10^2
MI	1.4×10^3	0.24×10^2	1.38×10^3
SR	0.4×10^2	0.18×10^2	0.22×10^2
FC	3.1×10^2	0.17×10^2	2.93×10^2
FM	3.1×10^2	0.1×10^2	3.00×10^2
Average mean difference:			2.2×10^3

* CFU of husks obtained from 1 g barley grain.

remaining species were found in between 1 and 3 samples. Most isolations of *A. glaucus*, *A. fumigatus* and *A. restrictus* were made from the I.S. but most *A. flavus* isolates were obtained from the O.S.

The distribution of isolations of genera other than *Aspergillus* spp. from the I.S. and O.S. of the samples examined by the dilution plating method is shown in Table 7. The most common species were *Alternaria* (from 8/12 samples); *Cladosporium* (7/12); and *Aureobasidium* (7/12).

Table 6. Presence of various *Aspergilli* on outer and inner surfaces of husks as determined by dilution plating.

Aspergillus species	Sample code																			
	LS		PL		FH		SA		SB		MO		AC		SS		MI		SR		FC		FM	
	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S
candidus	+	+																						
clavatus	+																							
flavus	+	+	+	*+†	+	+†		+	+	+			+	+†		+		+	+	+		+		+
fumigatus	+	+†	+				+				+				+				+	+	+			
glaucus	+	+	+		+		+	+	+†	+			+											
ornatus									+															
restrictus			+†		+†		+†																	
terreus		+																			+			
versicolor	+	+							+	+														

* = +† the predominant species.

Table 7. Presence of genera other than *Aspergullus* on outer & inner surfaces of husks as determined by dilution plating.

Sample code

	LS		PL		FH		SA		SB		MO		AC		SS		MI		SR		FC		FM	
	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S	I/S	O/S
Alternaria						+					+	+		+	+	+	+	+	* +†	+	+†	+†	+†	+†
Aureo-basidium					+						+†		+†	+	+†	+†		+				+		+
Arthrinium			+																					
Cladosporium						+							+			+	+†	+†		+†		+	+	+
Drechslera																					+			
Fonsecaea							+																	
Fusarium						+								+				+		+		+		
Monilia									+	+														
Papulospora			+	+																				
Penicillium	+	+					+	+†		+	+	+†	+			+	+	+	+	+		+		
Trichothecium											+													
Zygomycetes		+																	+	+				
Non-spor-ulating **		+						+		+				+	+		+	+				+		+
Total genera	2		2		3		2		2		4		5		4		5		5		6		3	

* +† = the predominant genus.

** = not included in total occurrence estimation.

Table 8. Genera isolated from 12 barley samples by the dilution plating method on PDA at 25°C.

Genus	Presence in 12 samples					
	Overall		Inner surface		Outer surface	
	No.	%	No.	%	No.	%
Aspergillus	12	100	10	83	11	92
Penicillium	9	75	6	50	8	67
Alternaria	8	67	6	50	8	67
Aureobasidium	7	58	4	33	5	42
Cladosporium	7	58	3	25	6	50
Fusarium	5	42	0	0	5	42
Zygomycetes	2	17	1	8	2	17
Arthrinium	1	8	1	8	0	0
Drechslera	1	8	1	8	0	0
Fonsecaea	1	8	1	8	0	0
Monilia	1	8	1	8	1	8
Papulospora	1	8	1	8	1	8
Trichothecium	1	8	1	8	0	0
Aspergillus spp:						
A. candidus	1	8	1	8	1	8
A. clavatus	1	8	1	8	0	0
A. flavus	11	92	6	50	11	92
A. fumigatus	7	58	7	58	2	17
A. glaucus	6	50	6	50	3	25
A. ornatus	1	8	1	8	0	0
A. restrictus	3	25	3	25	0	0
A. terreus	2	17	0	0	2	17
A. versicolor	2	17	2	17	2	17

Note: *"Zygomycetes"* were mostly *Mucor* spp.

Fusarium was isolated from 5 samples, but from the O.S. only.

4. Presence, Viability and Identity of Hyphae within Husk Tissue.

4.1 Assessment of fungal mycelium observed within husk tissue

Husk strips from each sample were examined for the presence of hyphae as described in Methods 3.3.2. Trypan blue stains the live mycelium blue or dark blue, husk cells stay yellowish brown, spores and dead mycelium do not stain, and some hyphae appear brown or dark brown and do not take up the stain (Plates 1 and 2). The mycelium frequently extended over the I.S. of the husks, particularly at the two ends and along the sides of the ventral furrow of the grain. In heavily infected samples the mycelium covered the complete husk surface. In most samples, the hyphae were hyaline, but sample PL showed a large amount of brown hyphae (in some strips it was >80% of total hyphae).

The amount of mycelium in each of the 10 strips from each sample was assessed with the hyphal grading scale 1+ to 5+ (Table 9).

4.1.1 Relative Mycelial Score

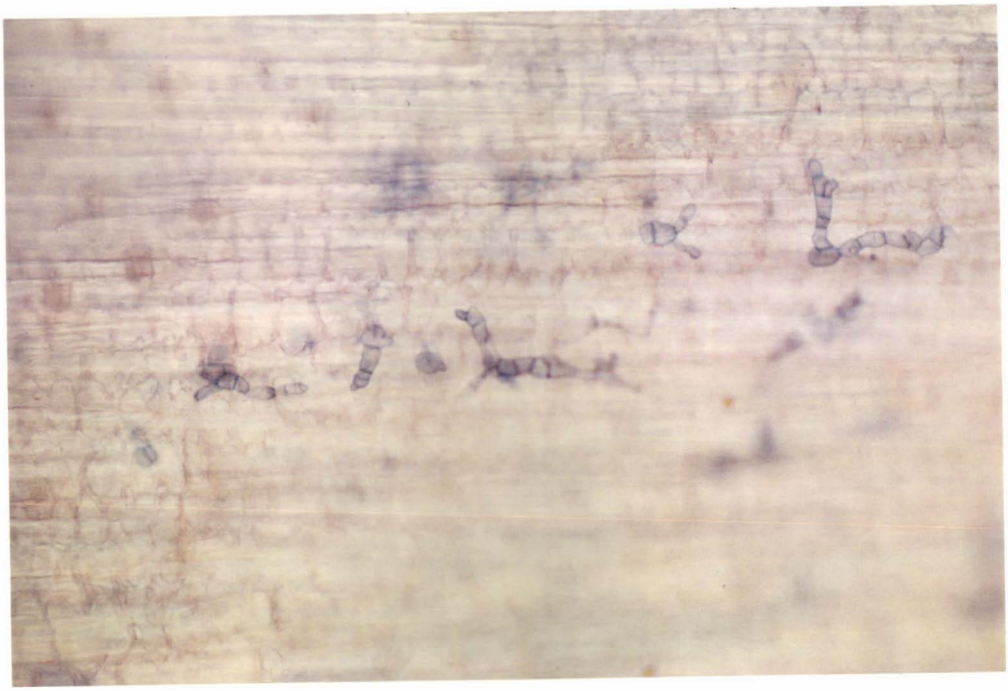
The total number of strips showing hyphae from each sample of 10 strips examined by trypan blue ranged between 7/10 to 10/10, and this ratio constitutes the Relative Mycelial Score (R.M.S.). 5 samples gave a R.M.S. of 10/10, 3 samples 9/10, 3 samples 8/10 and one sample 7/10 (Table 9).

4.1.2 Comparative Mycelial Score

The total amount of assessed mycelium (total pluses) for all 10 husk strips of individual samples is

Plate 1. Fungal hyphae (stained blue by trypan blue), seen on the inner surface of the husk, from Sample FM (clean).
(320 x, using LBT filter.)

Plate 2. Fungal hyphae, heavy invasion of the inner surface of the husk, from Sample LS (mouldy).
(125 x, using yellow filter.)



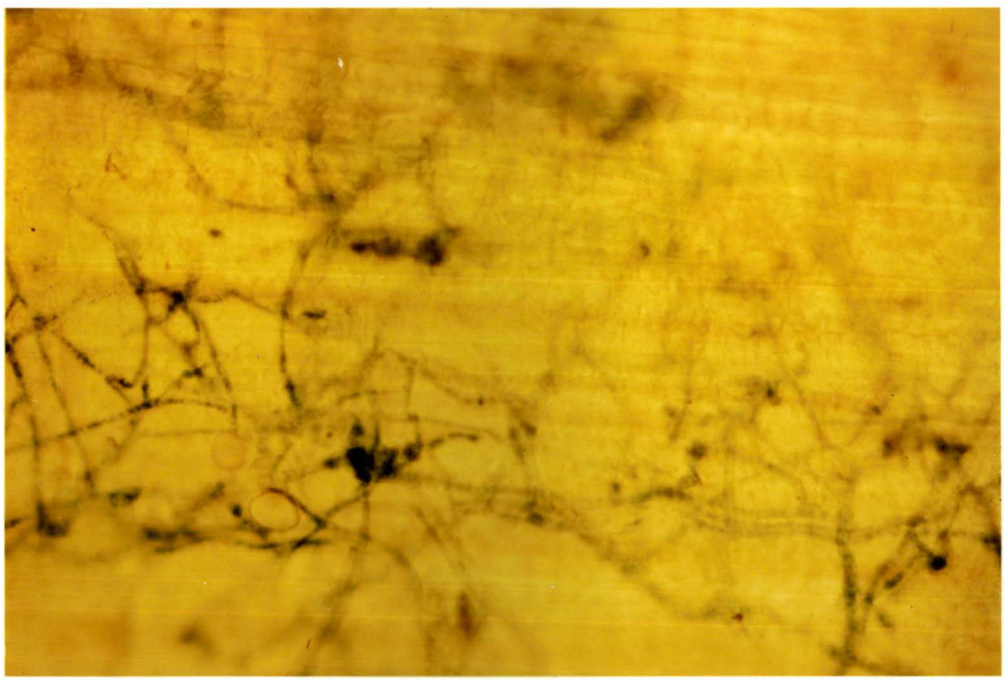


Table 9. Assessment of fungal hyphae observed within ten husk strips/sample and their viability.

	Abundance of hyphae												
Strip	1	2	3	4	5	6	7	8	9	10	R.M.S.*	C.M.S.**	R.V.S.***
Sample code													
LS	4+	2+	2+	4+	1+	4+	2+	2+	4+	1+	10	26	10
PL	3+	5+	4+	3+	5+	2+	3+	4+	3+	2+	10	34	7
FH	3+	1+	3+	0	2+	2+	3+	2+	1+	3+	9	20	10
SA	3+	0	2+	3+	1+	3+	0	3+	2+	0	7	17	3
SB	4+	4+	3+	3+	2+	2+	2+	4+	4+	2+	10	30	10
MO	0	2+	0	3+	2+	1+	2+	3+	1+	3+	8	17	4
AC	1+	1+	2+	3+	1+	2+	3+	1+	2+	1+	10	17	8
SS	3+	0	2+	3+	2+	3+	0	2+	3+	2+	8	20	10
MI	1+	2+	3+	3+	1+	0	1+	3+	2+	2+	9	18	4
SR	1+	1+	0	2+	1+	1+	2+	1+	2+	4+	9	15	1
FC	1+	3+	3+	3+	1+	2+	1+	3+	4+	1+	10	22	0
FM	1+	2+	1+	2+	0	0	3+	1+	1+	1+	8	12	0

* R.M.S. = Relative Mycelial Score (Total No. strips showing hyphae/10); ** C.M.S. = Comparative Mycelial Score (Total pluses/sample); *** R.V.S. = Relative Viability Score (Total No. strips showing growth/10).

expressed as Comparative Mycelial Score (C.M.S.) in Table 9. The scores ranged from 12 (sample FM) to 34 (sample PL).

4.2 Hyphal Viability

4.2.1 Relative Viability Score

Table 9 also compares the occurrence of fungal mycelia within husk strips to their viability as assessed by the techniques described in Methods 3.3.2.

The total number of strips showing hyphae from each sample of 10 strips examined by trypan blue (i.e. R.M.S.) ranged between 7/10 to 10/10. However, when the strips were cultured, 2 samples (FM and FC) showed no growth from any strip, and there was growth from only 1/10 strips from sample SR. The remaining samples gave growth from 3/10 to 10/10 strips. These results are expressed as the Relative Viability Score (R.V.S.) in Table 9.

2 strips from sample SS and one from sample FH had failed to reveal hyphae, but yielded growth on culture.

4.2.2 Species isolated

Aspergillus spp. were the predominant isolates from samples LS to AC, but other genera, especially *Alternaria*, were common in the remaining samples. The detailed results are listed in Table 10, and show that in all but 4 samples a single species was obtained from individual strips. Two samples (FC and FM) had shown hyphae in the strips examined but gave no growth. Two strips of sample SS showed zero hyphal grading but gave *Alternaria* in culture, and one strip of sample FH in which no hyphae had been detected gave *Aspergillus restrictus* when cultured.

A total of 7 *Aspergillus* spp. were isolated (Table 11). Of these *A. glaucus* was the most common (20 isolates from

Table 10. Species isolated from husk strips after examination in vital stain for the presence of mycelium.

		Strip cultured on					
		MSA			PDA		
Sample code.	RVS	Strip no.	Hyphal grading	Isolates	Strip no.	Hyphal grading	Isolates
LS	10	1	4+	*A. candidus*	6	4+	*A. candidus*
		2	2+	*A. candidus*	7	2+	*Penicillium*
		3	2+	*A. terreus*	8	2+	*A. flavus*
		4	4+	*A. terreus*			*A. fumigatus*
				A. candidus	9	4+	*Zygomycetes*
		5	1+	*A. versicolor*	10	1+	*Zygomycetes*
PL	7	1	3+	*A. restrictus*	8	4+	*A. flavus*
		2	5+	" "	9	3+	*Papulospora*
		3	4+	" "			
		4	3+	" "			
		5	5+	" "			
FH	10	1	3+	*A. restrictus*	6	2+	*A. restrictus*
		2	1+	" "	7	3+	" "
		3	2+	" "	8	2+	" "
		4	0	" "	9	1+	" "
		5	2+	" "	10	3+	" "
SA	3	1	3+	*A. fumigatus*	6	3+	*A. glaucus*
		3	2+	*A. glaucus*			
SB	10	1	4+	*A. glaucus*	6	2+	*A. glaucus*
		2	4+	" "	7	2+	*Monilia*
		3	3+	" "	8	4+	*A. glaucus*
		4	3+	" "	9	4+	" "
		5	2+	" "	10	2+	" "

Table 10. (Continued.)

Sample code.	RVS	Strip cultured on MSA: Strip no.	Hyphal grading	Isolates	Strip cultured on PDA: Strip no.	Hyphal grading	Isolates
MO	4	4	3+	*A. glaucus*	7	2+	*Trichothecium*
		5	2+	" "	10	3+	"
AC	8	1	1+	*A. glaucus*	6	2+	*A. glaucus*
				Penicillium	7	3+	" "
		3	2+	*A. glaucus*	8	1+	" "
		4	3+	*A. versicolor*	10	1+	" "
				Trichothecium			
				Penicillium			
		5	1+	*A. glaucus*			
SS	10	1	3+	*Alternaria*	6	3+	*Alternaria*
		2	0	"	7	0	"
		3	2+	"	8	2+	*A. fumigatus*
		4	3+	"	9	3+	*Alternaria*
		5	2+	"	10	2+	"
MI	4	2	2+	*Alternaria*	7	1+	*Alternaria*
		3	3+	"	9	2+	"
SR	1	5	1+	*A. fumigatus*			No growth
FC	NA			No growth			No growth
FM	NA			No growth			No growth

Total isolates:

Aspergillus species : 49

Other genera : 23

72

Table 11. Isolations of *Aspergillus* spp. from 10 husk strips/sample at 25°C (5 on MSA, 5 on PDA).

Sample code.	*Aspergillus spp.* *candidus*		*flavus*		*fumigatus*		*glaucus*		*terreus*		*restrictus*		*versicolor*		Total isolates/ sample.
	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	
LS	3	1		1		1			2				1		9
PL				1							5				6
FH											5	5			10
SA					1		1	1							3
SB							5	4							9
MO							2								2
AC							3	4					1		8
SS						1									1
MI															0
SR					1										1
FC															0
FM															0
Total/ 120 strips	3	1	0	2	2	2	11	9	2	0	10	5	2	0	
	4		2		4		20		2		15		2		49
	8.2%		4%		8.2%		40%		4%		30%		4%		

MSA = Malt Salt Agar (30 isolates); PDA = Potato Dextrose Agar (19 isolates).

4 samples). *Aspergillus* spp. were present in all samples LS to AC, but only 2 of the remaining samples yielded isolates of *Aspergillus* (*A. fumigatus*). One sample (LS) yielded 5 species, 3 samples 2 species, 5 samples yielded 1 species. Samples MI, FC and FM were negative for *Aspergillus* spp. *A. flavus* was isolated from only 2 samples (LS and PL), and grew only on PDA. Overall, MSA gave a greater number of isolations of *Aspergillus* species than did PDA at the same incubation temperature.

Other fungi isolated were mostly field fungi, and were present less frequently than were *Aspergillus* spp. They showed a scattered occurrence among the samples examined. Species of the genera *Alternaria, Penicillium, Trichothecium, Monilia, Papulospora* and also *Zygomycetes* were isolated. *Alternaria* was the most frequent (Table 12).

5. Overall Presence of Fungal Isolates in the Barley Samples.

Table 13 sets out the overall presence of fungal genera isolated from the barley samples examined by the two methods used in these studies, i.e. by dilution plating and by direct culture of husk tissue. Nine *Aspergillus* spp. were isolated, *A. flavus* having the highest frequency from the samples both among the *Aspergilli* and among total fungi isolated (it occurred in 11/12 samples). The next most frequent species of *Aspergillus* was *A. fumigatus*, isolated from 10 samples. *A. candidus*, *A. clavatus* and *A. ornatus* were each isolated from a single sample only.

In addition to *Aspergilli*, 12 other fungal genera were isolated, *Penicillium* being the most common (isolated from 9 samples). *Alternaria*, *Cladosporium* and *Aureobasidium* were each isolated from 7 or 8 samples.

Table 12. Isolations of genera other than *Aspergillus* from 10 husk strips/sample at 25°C (5 on MSA, 5 on PDA).

Genus	*Alternaria*		*Monilia*		*Papulospora*		*Trichothecium*		*Zygomycetes*		*Penicillium*		Total isolates
Sample code	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	
LS										2		1	3
PL						1							1
FH													0
SA													0
SB				1									1
MO								2					2
AC											2		2
SS	5	4											9
MI	2	2											4
SR													0
FC													0
FM													0
Total/120 strips	7	6	0	1	0	1	0	2	0	2	2	1	
	13		1		1		2		2		3		22
% total isolations	59%		4.5%		4.5%		9%		9%		13.6%		

(MSA = 9 isolates; PDA = 13 isolates.)

Table 13. Fungi isolated from barley samples either by the dilution plating method or by direct plating of husks.

Fungal species	Sample code LS	PL	FH	SA	SB	MO	AC	SS	MI	SR	FC	FM
Aspergillus spp.												
A. candidus	+											
A. clavatus	+											
A. flavus	+	+	+	+	+		+	+	+	+	+	+
A. fumigatus	+	+		+		+		+		+	+	
A. glaucus	+	+	+	+	+	+	+					
A. ornatus					+							
A. restrictus		+	+	+								
A. terreus	+										+	
A. versicolor	+				+		+					
Penicillium spp.	+			+	+	+	+	+	+	+	+	
Fusarium spp.			+				+		+	+	+	
Alternaria spp.			+			+	+	+	+	+	+	+
Aureobasidium spp.			+			+	+	+	+		+	+
Monilia spp.					+							
Papulospora ?		+										
Trichothecium spp.						+						
Zygomycetes	+									+		
Arthrinium		+										
Cladosporium			+				+	+	+	+	+	+
Drechslera											+	
Fonsecaea				+								
Non-sporulating & unidentified	+			+	+	+	+		+		+	+

6. Relationship of Sample Condition to the Mycoflora.

6.1 Sample condition, viable counts and the most frequent fungal genera isolated by dilution plating.

A comparison of the apparently 'mouldy' or 'clean' state of the samples in relation to total viable counts and the presence of individual genera can now be made. At the time of collection, the samples' condition had been assessed visually as 'mouldy', 'slightly mouldy' or 'clean', (Table 3; Plates 3 & 4).

6.1.1 Viable counts

Table 5 listed the results of the individual viable counts of both O.S. and I.S. for each sample. These data are summarized in Figure 4. Outer surface counts did not clearly reflect the gross sample condition but counts of the inner surface were consistently higher in those samples which had been classified as mouldy or slightly mouldy. Inner surface counts of definitely mouldy samples LS, PL, FH, SA and SB, and the slightly mouldy samples MO and AC ranged between 1.4×10^3 and 4.1×10^4, and those of the apparently clean samples SS, MI, SR, FC and FM between 0.1×10^2 to 0.9×10^2. A 'condition line' could be established at about 2.0×10^2 CFU/g grain for these I.S. counts. If above this level, the sample could be considered as mouldy. These results agreed with gross visual judgement of the samples' condition but did not differentiate between mouldy and slightly mouldy.

6.1.2 Individual genera

In mouldy samples the predominant genera were either *Aspergillus* or *Penicillium*. In clean samples these genera were isolated only occasionally. Table 14 shows the most frequent genus in each sample (I.S. and O.S.) obtained by dilution plating in relation to the condition of

Plate 3. Gross visual condition of two samples; the one on the left (Sample SS) clean and that on the right (Sample LS) mouldy.

Plate 4. Mouldy, grossly-spoiled grain (Sample PL, mouldy).


a

Figure 4. Viable counts of outer and inner surface of husks in relation to gross visual appearance of sample.

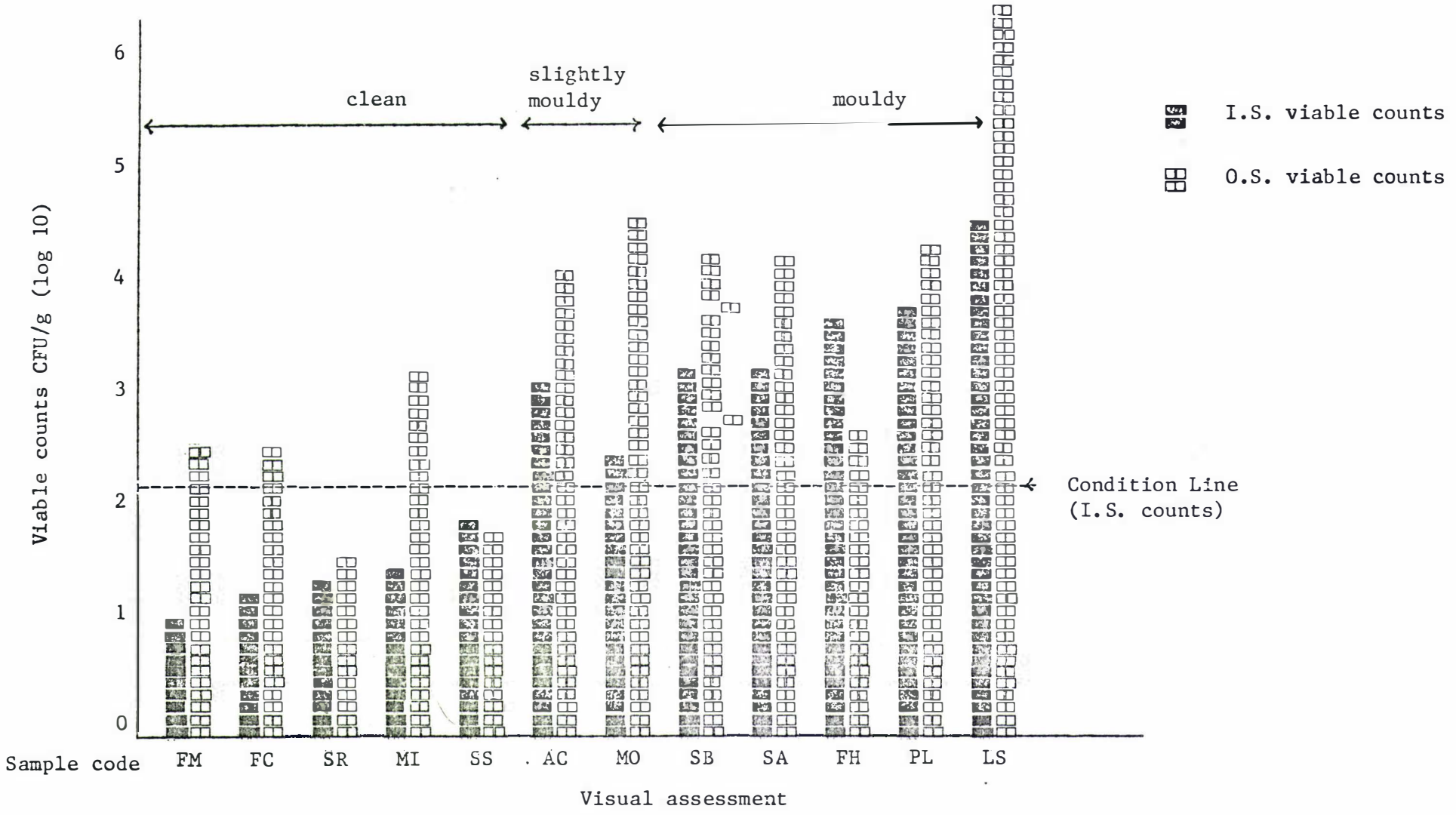


Table 14. Condition of barley samples and the most frequent isolates from outer and inner surfaces of husks (dilution plating method).

Sample code	Condition	Most frequent isolate	
		Inner surface	Outer surface
LS	M	*Aspergillus spp.*	*Aspergillus spp.*
PL	M	*Aspergillus spp.*	*Aspergillus spp.*
FH	M	*Aspergillus spp.*	*Aspergillus spp.*
SA	M	*Aspergillus spp.*	*Penicillium spp.*
SB	M	*Aspergillus spp.*	*Aspergillus spp.*
MO	SM	*Aureobasidium spp.*	*Penicillium spp.*
AC	SM	*Auerobasidium spp.*	*Aspergillus spp.*
SS	C	*Aureobasidium spp.*	*Aureobasidium spp.*
MI	C	*Cladosporium spp.*	*Cladosporium spp.*
SR	C	*Alternaria spp.*	*Cladosporium spp.*
FC	C	*Alternaria spp.*	*Alternaria spp.*
FM	C	*Alternaria spp.*	*Alternaria spp.*

M = mouldy; SM = slightly mouldy; C = clean.

the sample at the time of collection.

Aspergillus spp. were the predominant (i.e. >50% of total isolations) isolates from the I.S. of 5 samples (LS, PL, FH, SA and SB), all mouldy; 3 samples (SR, FL and FM), all clean, had *Alternaria* as the predominant genus; 2 samples slightly mouldy (MO and AC) and 1 clean sample (SS) had *Aureobasidium* as the predominant genus. One sample, MI (clean) showed *Cladosporium*. *Penicillium* was never predominant on the I.S. of any sample.

Similarly on the O.S. *Aspergillus* was the predominant genus in mouldy samples and also slightly mouldy sample AC. Mouldy sample SA and slightly mouldy sample MO had mostly *Penicillium*; 2 clean samples (FL and FM) showed *Alternaria*

as predominant fungus, 2 showed *Cladosporium* and only one sample (SS) had *Aureobasidium* on O.S. Thus a differentiation of mouldy and slightly mouldy samples could be made on the basis of predominant genera on O.S. and I.S.

6.2 Sample Condition and Genera Isolated from Husk Tissues

Table 15 summarizes the culture results from husk strips (10 strips/sample in total), and lists the mean occurrence of fungal genera in the two groups, mouldy and clean. The mean occurrence of *Aspergillus* in the 10 husk strips examined was 6.7 in samples 1 - 7 (mouldy), and only 0.4 in samples 8 - 12 (clean). This result is highly significant ($P < 0.001$). Except for sample SS, which had yielded a high number of *Alternaria* isolates, isolations of genera other than *Aspergillus* were not sufficient to allow a valid comparison to be made.

The relationship between *Aspergilli* and other genera in both groups showed an inverse (negative) correlation which was statistically significant ($r = -0.488$, $P < 0.05$).

6.3 Sample Condition and Comparative Mycelial Score (C.M.S.), Relative Viability Score (R.V.S.) and Relative Mycelial Score (R.M.S.)

R.M.S.'s were not markedly different between samples and bore no relationship to gross mouldy or clean condition (Table 16). The C.M.S. of mouldy samples ranged from 17-34, that of clean samples from 12-22. Samples SS and FC were clean but gave scores of 20 and 22. The highest score of 34 was noted for sample PL, and the lowest of 12 from sample FM.

Overall, the viability of mycelia within the sample strips (R.V.S.) was higher in mouldy samples than in the clean samples. However, clean sample SS showed a high R.V.S. and mouldy sample SA a low score. Table 16 indicates an

Table 15. Sample condition in relation to the total isolations of *Aspergilli* and other genera from 10 husk strips/sample.

Sample code	Isolates/sample				
	Aspergillus spp.		Other genera		Total
	mean			mean	
	mouldy		mouldy		
LS		9	3		12
PL		6	1		7
FH		10	0		10
SA		3	0		3
SB		9	1		10
MO		2	2		4
AC	6.7	8	2	1.3	10
SS		1	9		10
MI		0	4		4
SR		1	0		1
FC		0	0		0
FM	0.4	0	0	2.6	0
	clean		clean		

interesting relationship (also shown in Fig. 5) between the total number of husk strips showing positive growth (R.V.S.) and the comparative mycelial score (C.M.S.) for the two groups, mouldy (7) and clean (5). The differences between the mouldy or clean groups are statistically significant for these criteria, i.e. C.M.S. and R.V.S., ($P < 0.05$). The C.M.S. values for the mouldy samples ranged between 17 to 34 (mean 23), and the R.V.S. values between 3 to 10 (mean 7.4). But clean samples gave lower scores for both criteria (mean C.M.S. 17.4 and mean R.V.S. 3.0).

Figure 5. Relation between the comparative mycelial score and the relative viability score.

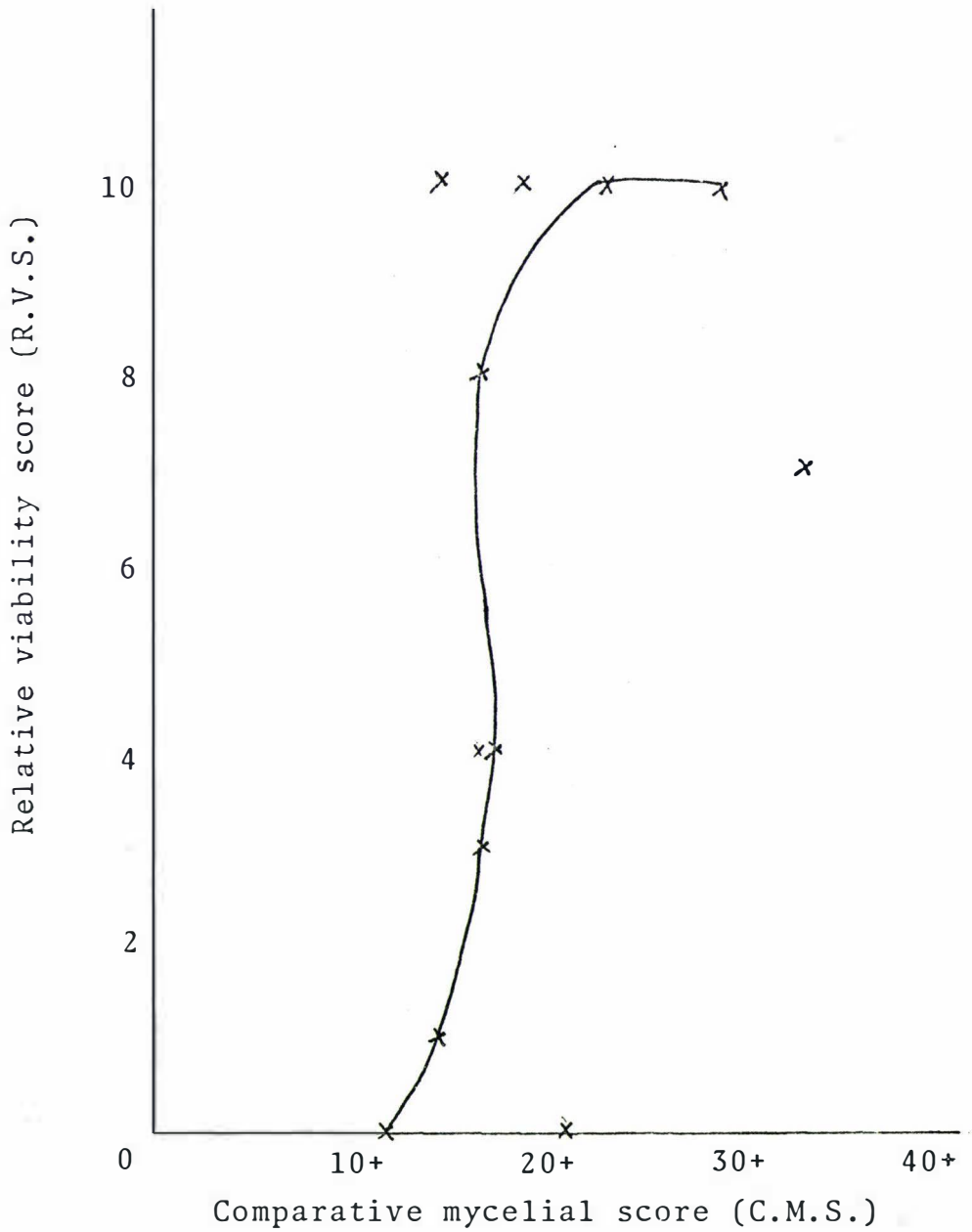


Table 16. Relationship between Comparative Mycelial Score (C.M.S.), Relative Mycelial Score (R.M.S.) and Relative Viability Score (R.V.S.), from 10 husk strips/sample.

Sample code	Sample condition		R.M.S.	C.M.S.	R.V.S.
LS	mouldy*		10	26	10
PL	mouldy		10	34	7
FH	mouldy		9	20	10
SA	mouldy		7	17	3
SB	mouldy		10	30	10
MO	mouldy	**	8	17	4
AC	mouldy		10	17	8
SS	clean		8	20	10
MI	clean		9	18	4
SR	clean		9	15	1
FC	clean		10	22	0
FM	clean		8	12	0

* = Have either *Aspergillus* or *Penicillium* spp. or both as predominant fungi.

** = Mouldy on O.S. only.

7. Aflatoxin Production by *A. flavus* Isolates.

7.1 Screening on Coconut Agar

7.1.1 Husk isolates

55 isolates of *A. flavus*, consisting of 24 isolates from the inner surface and 31 isolates from the outer surface of barley husks, were tested on coconut agar medium. Strain NRRL 2999 (*A. parasiticus*) was included as a known positive control. Culture preparation and examination

under long-wave (365 nm) U.V. light were as described in Methods 5.1. NRRL 2999 gave a clear blue-green fluorescence within 48 hours and the intensity of fluorescence increased up to 5 days. Plate 5 shows this aflatoxin (AT)-positive strain. All cultures of the barley husks isolates were negative at 5 days and continued negative when examined periodically up to 21 days (Table 17). The AT-negative isolate PL4 I.S. is also shown in Plate 5. A few AT-negative isolates produced faint orange-yellow pigmentation on the reverse of cultures on coconut agar and this pigmentation was stronger in the AT-positive strain NRRL 2999.

Table 17. Screening for aflatoxin production on coconut agar medium by isolates of *A. flavus* from I.S. and O.S. of barley husks and from soil.

Source of isolates	No. tested	No. positive	% positive
Barley husks:			
Inner surface	24	0	0
Outer surface	31	0	0
Soil:	32	7 *	22
NRRL 2999:	1	1	

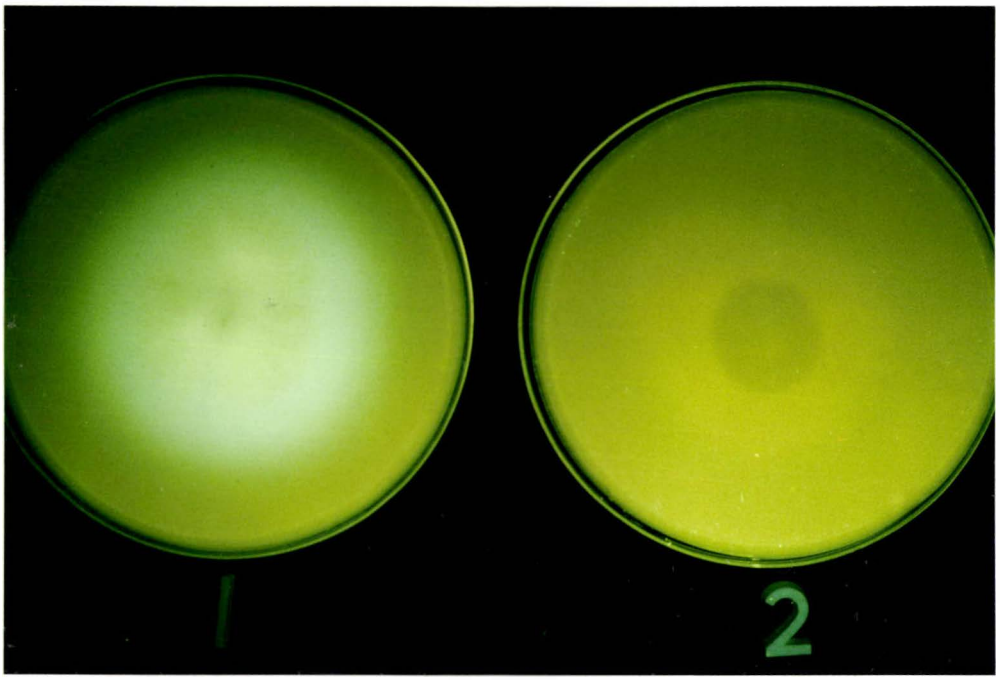
* Strain nos. 6, 7, 8, 18, 22, 29 and 30.

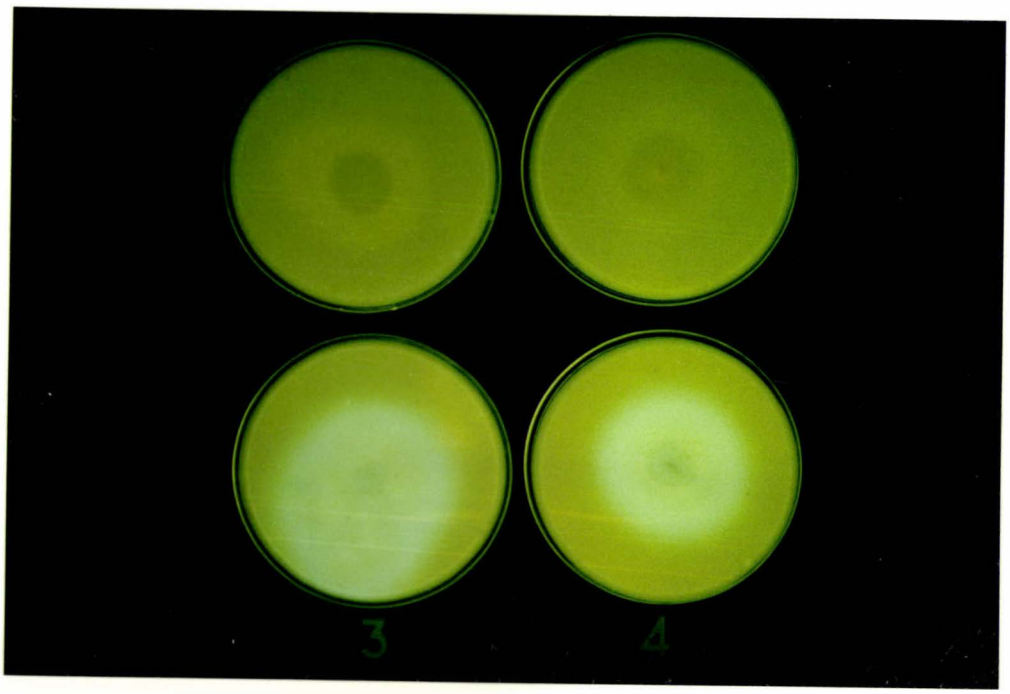
7.1.2 Soil isolates

32 *A. flavus* isolates from soil (Methods 4.2.2) were also tested for their ability to produce AT on coconut agar medium. Fluorescence was evident within 3 days in cultures of 7 (22%) of the isolates, but the intensity of fluorescence was not as great as that with NRRL 2999. All these isolates were *A. flavus* Link. The AT-positive isolates showed increasingly clear blue-green fluorescence up to 10 days of observation. Plate 6 shows 2 AT-negative

Plate 5. Coconut agar plates. No. 1: colony of *A. parasiticus* NRRL 2999 (AT-positive) showing blue-green fluorescence under U.V. light (365 nm). No. 2: colony of *A. flavus* Link isolate PL4 I.S. from barley (AT-negative).

Plate 6. Coconut agar plates. Nos. 1 and 2 (upper): barley isolates FC3 I.S. and SB3 O.S. (AT-negative); Nos. 3 and 4: soil isolates 8 and 18 (AT-positive).


1
2


3
4

and 2 AT-positive cultures. Cultures that did not produce fluorescence within 10 days were considered to be AT-negative.

7.2 Aflatoxin production on Semisynthetic Liquid Medium (SMKY) - Stationary and Shaken Culture.

7.2.1 Aflatoxin detection by locally-prepared minicolumns from SMKY stationary culture extracts

24 I.S. isolates of *A. flavus*, 3 AT-positive soil isolates and the AT-positive strain NRRL 2999 were grown on 50 cm^3 semisynthetic liquid medium (Methods 5.2). Locally-prepared minicolumns (see Fig. 3A) were used for the assay of AT-production in the culture filtrate (Methods 5.2 and 7.1). All the husk isolates were AT-negative by this method (Table 18). The soil isolates and NRRL 2999 gave a clear blue ring at the Florisil layer, which sometimes extended to cover the silica gel layer (particularly NRRL 2999). Some of the husk isolates showed a faint blue-white or orange fluorescent band at the upper calcium sulphate layer, but this was considered as a false positive.

Table 18. Aflatoxin production by *A. flavus* isolates grown on semisynthetic liquid medium (SMKY) in stationary culture at 25°C for 7 days. (Detection by locally-prepared minicolumns.)

Source of isolates	No. tested	No. positive
Husks I/S isolates	24	0
Soil isolates *	3	3
NRRL 2999	1	1

* Strain nos. 6, 8 and 18.

7.2.2 Aflatoxin detection by commercially-prepared minicolumns from SMKY stationary and shaken culture extracts

a) Stationary culture

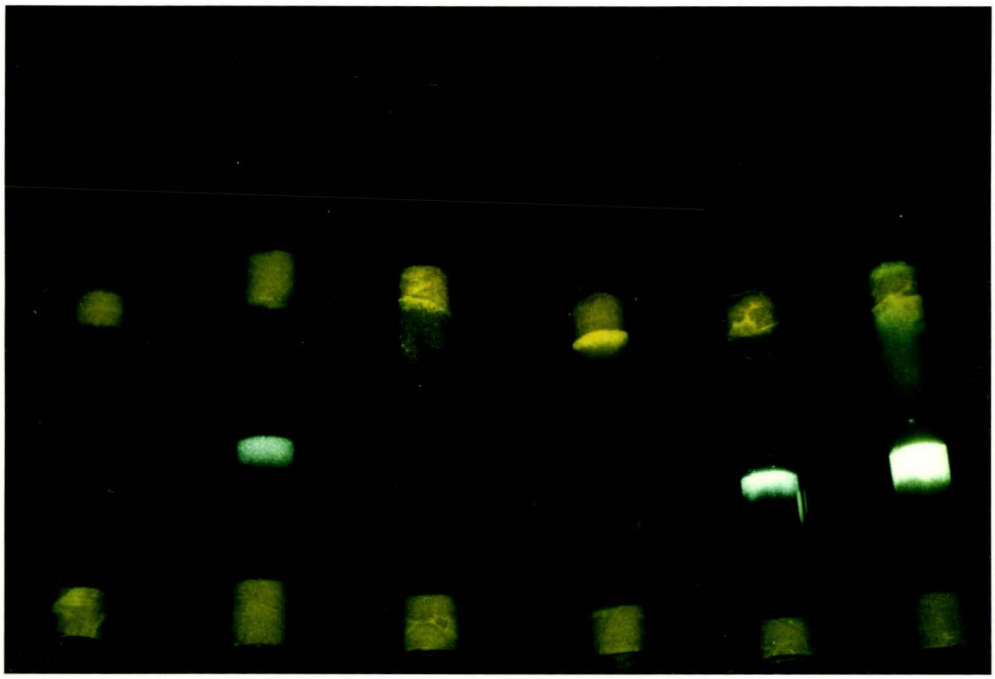
Table 19 shows the results of the screening test of 19 *A. flavus* isolates (13 husks isolates, 5 soil isolates and strain NRRL 2999). The columns containing extracts of these cultures were observed under long-wave U.V. light beside the reference minicolumn (Methods 7.1). A blue fluorescent band was seen in the centre of the column or at the interface of the Florisil and alimina layers. The width and intensity of this fluorescent band could be used to roughly assess the amount of aflatoxin contained in the extract when compared to the reference column. Plate 7 illustrates representative results of these tests. All AT-positive extracts from soil isolates produced less aflatoxin than that produced by NRRL 2999.

Table 19. Aflatoxin production by *A. flavus* isolates on semisynthetic liquid medium (SMKY) in stationary culture at 25°C for 7 days. (Detection by commercial minicolumn.)

Source of isolates	No. tested	No. positive
Husk isolates:		
Inner surface	7	0
Outer surface	6	0
Soil isolates *	5	5
NRRL 2999	1	1

* Strain nos. 18, 7, 8, 30 and 29.

Plate 7. Minicolumns (Holaday-type) used in detection of aflatoxin from SMKY (stationary cultures). From left: unused column, reference, two barley isolates (AC1 I.S. and SB1 I.S.) (AT-negative), soil 18 (AT-positive) and NRRL 2999 (very strong AT-positive).



b) Shake culture

To determine if shake culture conditions were better for AT-production, *A. flavus* strains (2 soil isolates, 5 husks isolates and strain NRRL 2999) were inoculated into 50 cm^3 SMKY medium and incubated in a controlled environment shaker for 5 days. Extracts from husks isolates cultures were negative after this procedure. Soil isolates 30 and 18, and the NRRL 2999 strain produced aflatoxin in greater quantity than that produced on the same medium in stationary cultures (Table 20). Plate 8.1 shows the greater width and intensity of the blue fluorescent band from these AT-positive strains.

Table 20. Aflatoxin production by *A. flavus* isolates on semisynthetic liquid medium (SMKY) in a shaking incubator at 28°C for 5 days. (Detection by commercial minicolumns.)

Isolation reference	Isolates source	Assay result
soil 30	soil	+
PL1 O/S	husk	-
PL2 O/S	"	-
FH3 I/S	"	-
Soil 18	soil	+
SS1 O/S	husk	-
FH6 O/S	"	-
NRRL 2999	reference	+

7.2.3 TLC screening method

The culture extracts used for the minicolumn test were also subjected to TLC analysis (Methods 7.2). TLC was used to confirm the previous results, to avoid any false positives and to attempt to separate the aflatoxin compounds within the extracts. An undiluted aflatoxin standard was run on each test plate.

The development of the TLC plates in the chloroform-acetone-water mixture gave well-separated round fluorescent spots of the individual aflatoxins, B_1, B_2, G_1 and G_2 contained in the standard solution.

A visual comparison of the intensity of fluorescence of the test extract spots with the standard allowed an approximate estimation of the aflatoxin content of each culture extract.

Table 21 shows the results of screening 19 *A. flavus* isolates from both husks and soil, in addition to NRRL 2999 on SMKY medium. 12 extracts were examined from stationary cultures and 7 from shaken cultures. The 4 isolates from soil produced aflatoxin B_1 and a trace amount of B_2. The NRRL 2999 culture produced all 4 aflatoxin compounds B_1, B_2, G_1 and G_2. Plate 8.2 illustrates these results. Aflatoxin G_1 produced by NRRL 2999 on SMKY medium was produced in greater quantity than B_1 (B_1, 2222 $\mu g/100\ cm^3$ but G_1, 2666 $\mu g/100\ cm^3$).

7.3 Aflatoxin Production on Weet-bix Medium

A group of *A. flavus* isolates were tested on 25 g of crushed Weet-bix for their ability to form aflatoxin (Methods 5.3). The cultures were analysed and tested as in Methods 6.2 and 7. The results are summarized in Table 22. All of the 4 husk isolates examined were AT-negative, soil isolate 22 produced only a small amount of aflatoxin B_1, soil isolate 6 produced both B_1 and B_2 (trace) (Plates 9.1

Table 21. Detection of aflatoxin components by TLC method in extracts of semisynthetic liquid culture medium (SMKY) in stationary and shaken culture.

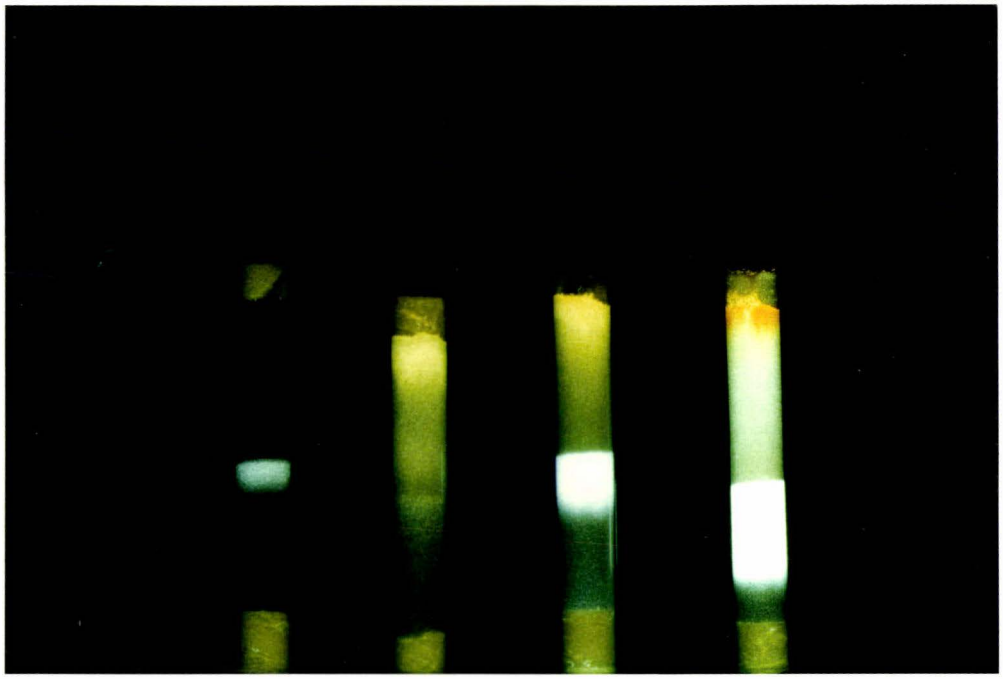
Isolate reference	Isolate source	Culture condition	TLC analysis B_1	B_2	G_1	G_2
FH3 I/S	husk	stationary *	-	-	-	-
PL2 I/S	"	"	-	-	-	-
FH5 I/S	"	"	-	-	-	-
Soil 29	soil	"	+	trace	-	-
LS1 O/S	husk	"	-	-	-	-
LS3 O/S	"	"	-	-	-	-
LS2 I/S	"	"	-	-	-	-
Soil 8	soil	"	+	trace	-	-
SB3 O/S	husk	"	-	-	-	-
AC5 O/S	"	"	-	-	-	-
SR1 I/S	"	"	-	-	-	-
Soil 30	soil	"	+	trace	-	-
PL1 O/S	husk	shaken **	-	-	-	-
PL2 O/S	"	"	-	-	-	-
FH3 I/S	"	"	-	-	-	-
Soil 30	soil	"	+	trace	-	-
SS1 O/S	husk	"	-	-	-	-
FH6 O/S	"	"	-	-	-	-
Soil 18	soil	"	+	trace	-	-
NRRL 2999	reference	"	+	+	+	+
Standard aflatoxin	Sigma		+	+	+	+

* Incubated at 25°C for 7 days.

** Incubated at 28°C for 5 days.

Plate 8.1. Use of minicolumns for detection of aflatoxin from SMKY (shaken cultures). From left: reference column, barley isolate (SS1 O.S.) (AT-negative), soil 18 (AT-positive) and NRRL 2999 (AT-positive). Note increasing width and intensity of fluorescence.

Plate 8.2. TLC analysis of aflatoxin from SMKY (shaken cultures) examined under U.V. light (365 nm). S: aflatoxin standard; 1: barley isolates (SS1 O.S.) (AT-negative); 2: Soil 18 (AT-positive) and 3: NRRL 2999 (AT-positive). S and 3 showed separation of the four aflatoxin components, B_1, B_2, G_1 and G_2 in decreasing order of Rf. No. 2 showed only B_1 and trace amount of B_2.



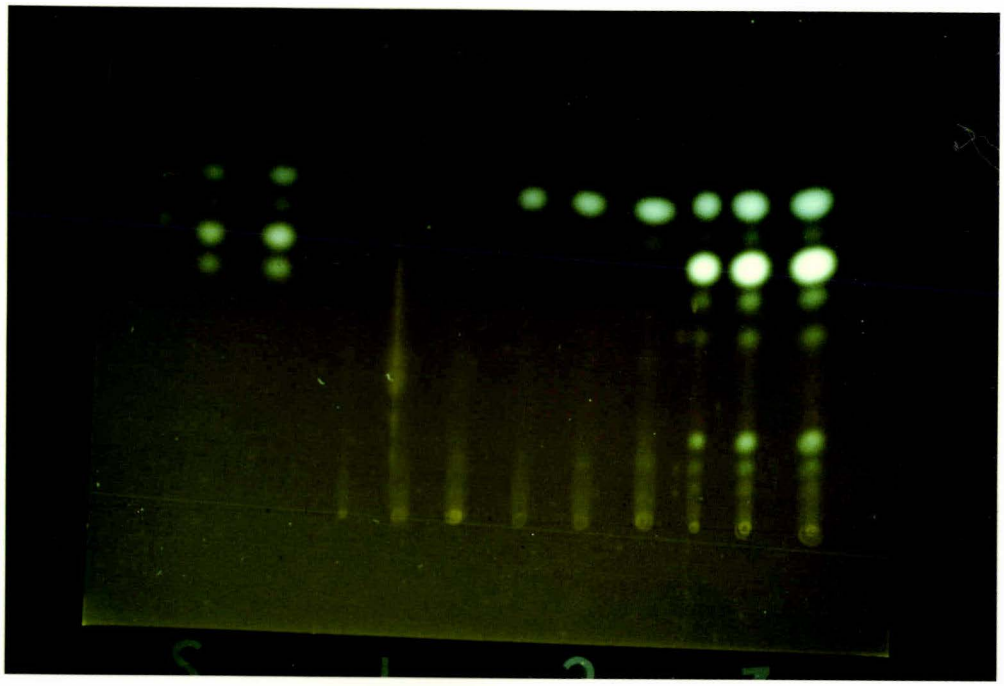


Table 22. Aflatoxin production on Weet-bix medium by 7 *A. flavus* strains.

Isolate reference	Isolate source	Screening methods: Minicolumn	TLC: B_1	TLC: B_2	TLC: G_1	TLC: G_2
Soil 22	soil	+		-	-	-
AC4 O/S	husk	-	-	-	-	-
Soil 6	soil	+	+	trace	-	-
SB2 O/S	husk	-	-	-	-	-
FM1 O/S	husk	-	-	-	-	-
FH4 O/S	husk	-	-	-	-	-
NRRL 2999	reference	+	+	+	+	+

and 9.2) and NRRL 2999 produced all aflatoxin compounds (B_1, B_2, G_1 and G_2). In the last case, G_1 and G_2 were produced in greater amounts than B_1 and B_2 (1200, 7500, 4015 and 1000 µg/25g respectively).

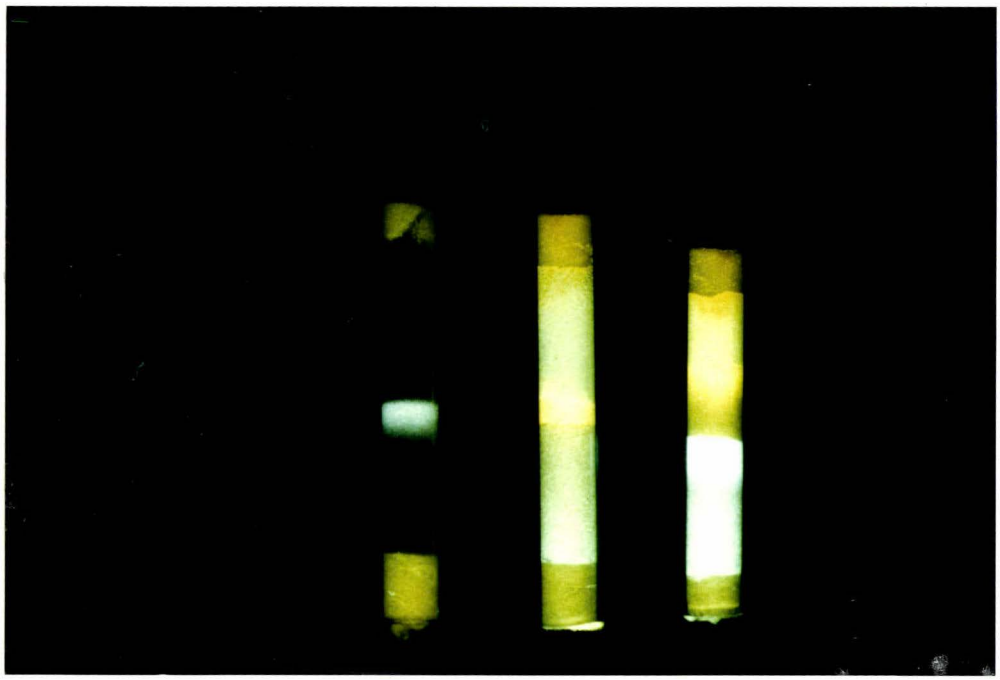
It was noticed that the Weet-bix medium seemed to stimulate the production of other fungal metabolites with blue fluorescence by all the above cultures of *A. flavus*. This pigmentation caused false positive readings in the minicolumn test (Plates 9.1 and 10.1) but the interference was reduced on the TLC method as these blue fluorescent spots showed very low RF values (Plates 9.2 and 10.2). NRRL 2999 was also stimulated to produce a weak orange fluorescent pigment (Plates 10.1 and 10.2).

7.4 Aflatoxin Production on pearled barley and husks

Table 23 presents the results obtained from testing 2 soil isolates (Soil 8 and 30; AT-positive), one husk

Plate 9.1. Minicolumn detection of aflatoxin from Weet-bix medium. From left: reference column, barley isolates (FM1 O.S.) (AT-negative but showing false positive) and Soil 6 (AT-positive.

Plate 9.2. TLC analysis of aflatoxins from Weet-bix medium. S: aflatoxin standards; 1: barley isolate (FM1 O.S.) (AT-negative) and 2: Soil 6 (AT-positive, producing only B_1).



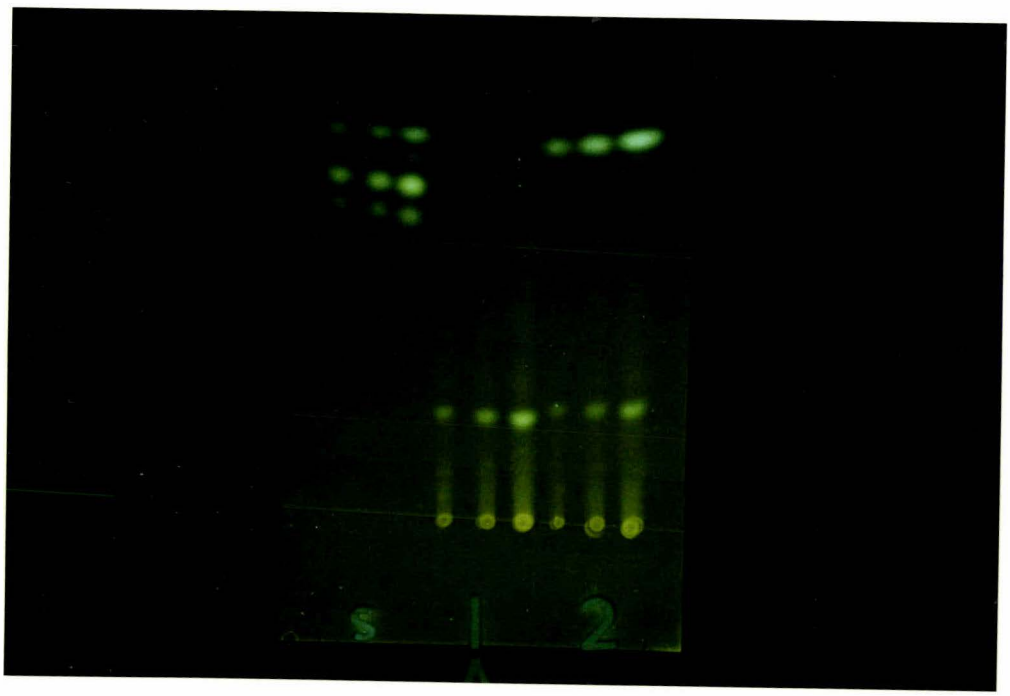
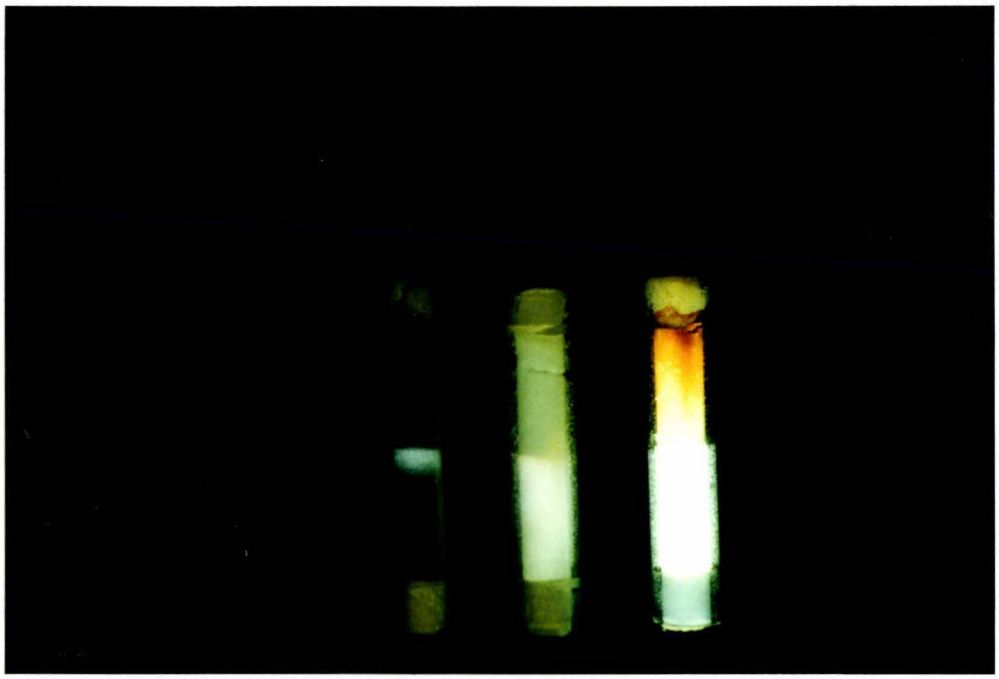
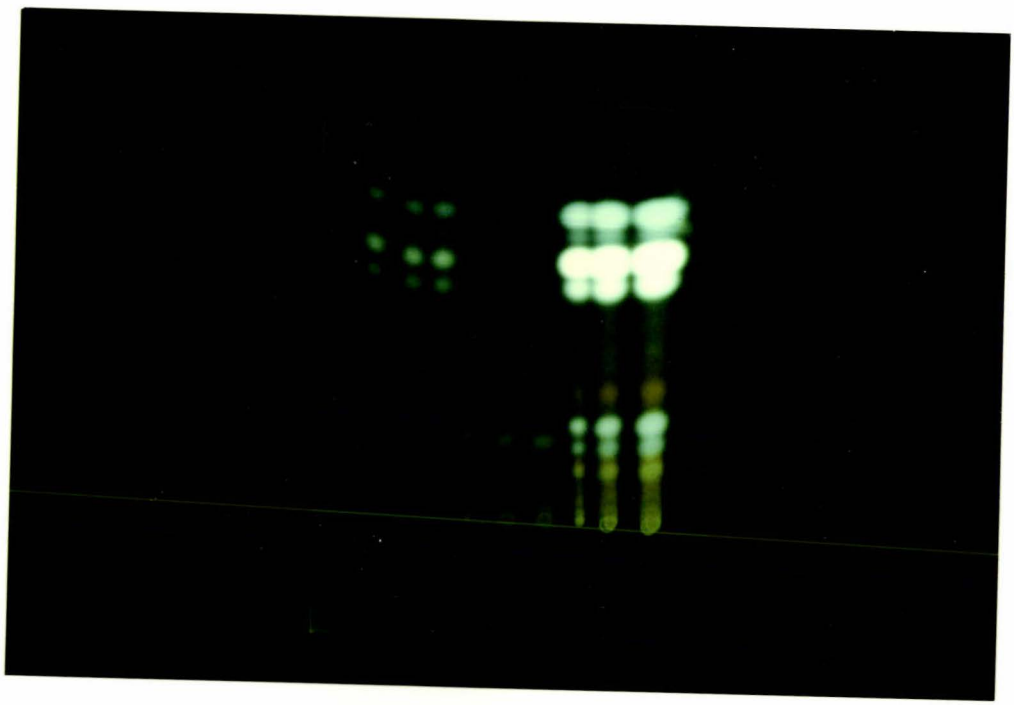

S
2

Plate 10.1. Minicolumn detection of aflatoxin from Weet-bix medium. From left: reference column, barley isolate (FH4 O.S.) (AT-negative but showing false positive) and NRRL 2999 (very strong AT-positive).

Plate 10.2. TLC analysis of aflatoxin from Weet-bix medium; S: aflatoxin standard; 1: barley isolate (FH4 O.S.) (AT-negative) and 2: NRRL 2999 (AT-positive, showing G_1 and G_2 highly fluorescent).





isolate (AC2 O/S; AT-negative) and strain NRRL 2999 on the two substrates, pearled barley and barley husks (Methods 5.4). This experiment was made to investigate the ability of *A. flavus* isolates to utilize the polysaccharide components of the substrates as a carbon source - either starch, the major component in pearled barley, or cellulose and arabinoxylan, the major components in the cell walls of barley husks.

All the culture extracts were tested by the minicolumn method and TLC, and all except the husk isolate (AC2 O/S) showed positive results.

The amount of aflatoxin produced on pearled barley by this method seemed to be greater than that produced on SMKY but less than that on Weet-bix medium, as indicated by the fluorescent band width and the intensity of fluorescence (Plates 11.1 and 12.1). Aflatoxin production on the husks only was the poorest of the various media tested.

TLC analysis showed soil isolates 8 and 30 produced a reasonable amount of B_1 and a very small amount of B_2 (Plate 11.2). Strain NRRL 2999 produced all 4 aflatoxin compounds and again G_1 and G_2 production was higher than that of B_1 and B_2.

A blue fluorescent compound which was not aflatoxin was produced by strain NRRL 2999, particularly on pearled barley, but the production of this was less than that on Weet-bix. NRRL 2999 also produced a considerable amount of yellow-orange fluorescent material (Plate 12.2). A similar material was also produced by other tested isolates, but in smaller amounts.

Table 23. Aflatoxin production on pearled barley and barley husks media by 3 *A. flavus* AT-positive strains and one AT-negative strain.

A. flavus strain	Isolates source	Substrates	Screening methods: Minicolumn	TLC B_1	TLC B_2	TLC G_1	TLC G_2
NRRL 2999	reference	pearled barley	+	+	+	+	+
		husks	+	+	trace	+	trace
Soil 30	soil	pearled barley	+	+	+	–	–
		husks	+	+	–	–	–
Soil 8	soil	pearled barley	+	+	trace	–	–
		husks	+	+	trace	–	–
AC2 O/S	husk	pearled barley	–	–	–	–	–
		husks	–	–	–	–	–

Plate 11.1. Minicolumn detection of aflatoxin from isolate Soil 8 cultured on pearled barley and barley husks media. From left, S: reference column; h: husk medium and p: pearled barley medium.

Plate 11.2. TLC analysis of aflatoxin produced by isolate Soil 8 on pearled barley and barley husks media. From left, S: aflatoxin standard; h: aflatoxin produced on husks medium (B_1) and p: aflatoxins produced on pearled barley (B_1 and trace of B_2).

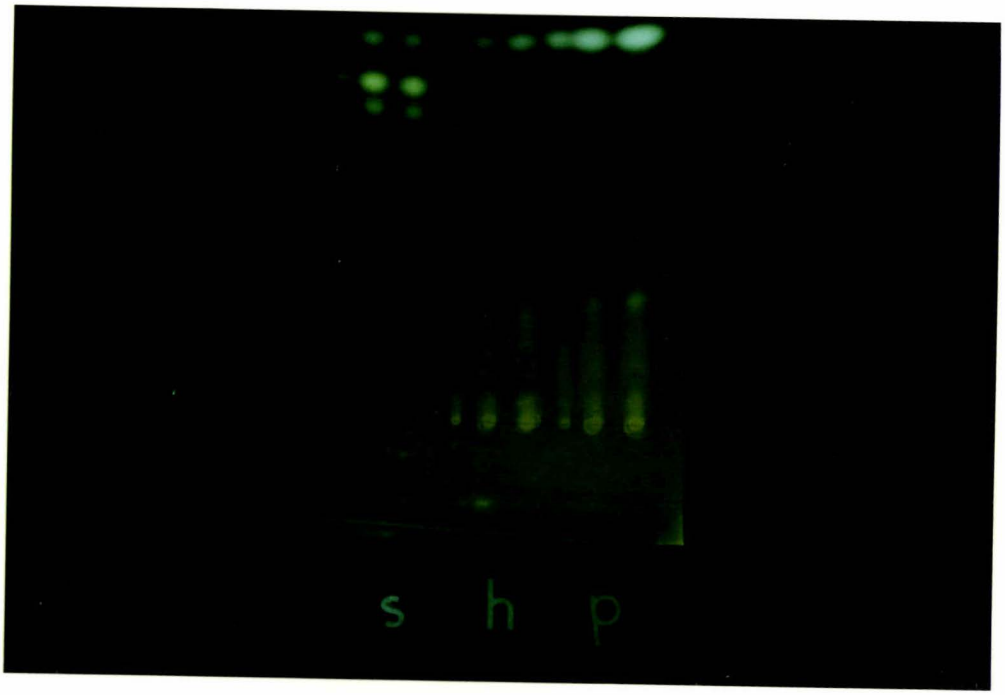
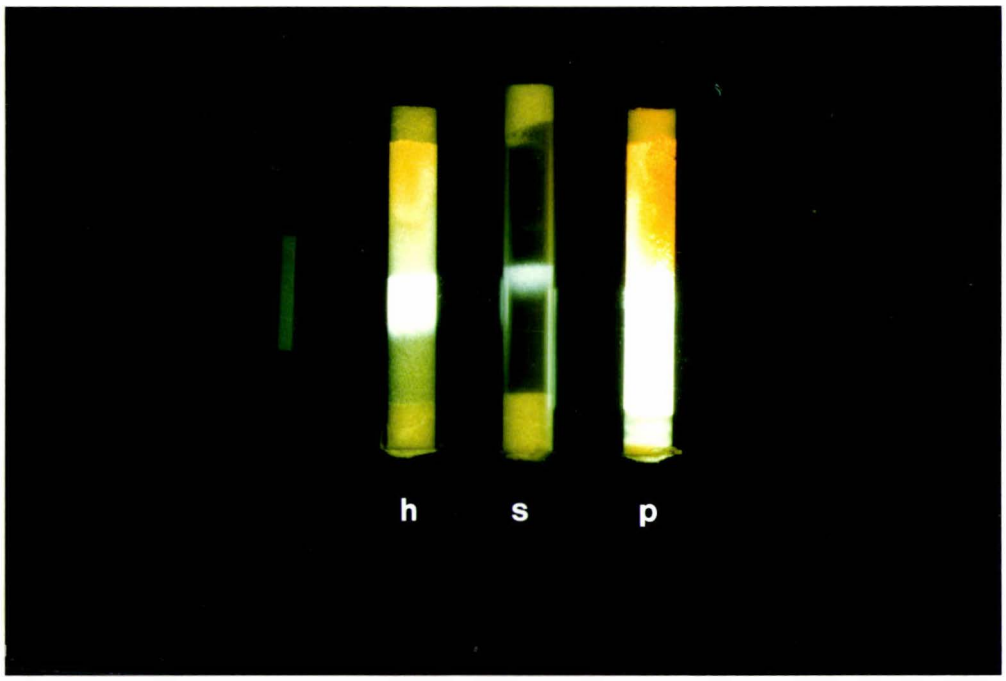
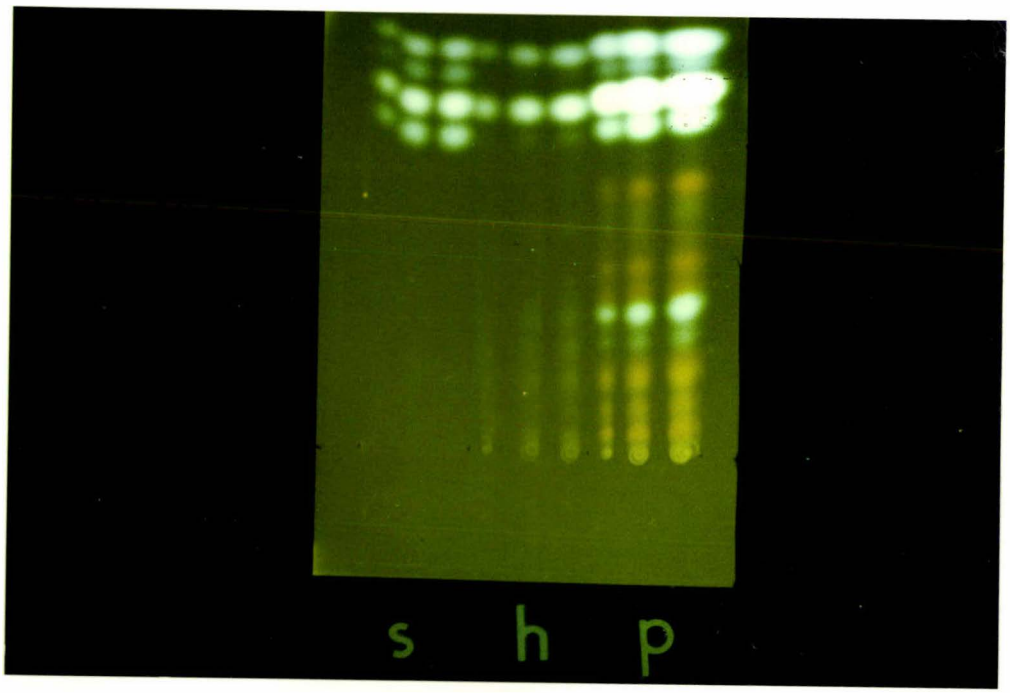

s h p

Plate 12.1. Minicolumn detection of aflatoxin produced by NRRL 2999 cultured on pearled barley and barley husks media. From left, h: aflatoxin produced on husks medium; S: reference column and p: aflatoxin produced on pearled barley medium (highly fluorescent).

Plate 12.2. TLC analysis of aflatoxin produced by NRRL 2999 on pearled barley and barley husks media. From left: S: aflatoxin standard; h: aflatoxin produced on husks medium, and p: aflatoxin produced on pearled barley, which supported G_1 and G_2. Some additional fluorescent spots of unknown identity are shown in p.


h
s
p


s
h
p

DISCUSSION

Ecological studies of the fungal flora of barley and other cereals are necessary because fungi are a major cause of spoilage and toxin production in stored grains. Fungi are said to rank second only to insects as a cause of deterioration and loss in all kinds of stored products throughout the world (Christensen & Kaufmann, 1974). Such studies are particularly important in farm-stored material, as the risk of fungal problems occurring is likely to be higher than when the grain is stored in commercial silos under carefully controlled conditions.

If mycotoxins are produced during any fungal spoilage which may occur, they may well affect animal health if subsequently ingested. When relatively heavy contamination occurs mycotoxins may cause severe illness and even mortality in livestock; when produced in lesser amounts they may result in economically important effects on animal productivity and disease resistance. Other adverse effects include the loss of wholesomeness of animal products destined for human consumption. Animal products can be a source of continuing exposure of human consumers to mycotoxins, in addition to more direct exposure related to plant-derived foods (Herz, 1977).

This study concentrated on the fungal flora associated with barley grain, particularly the barley husk, and on the presence of *Aspergillus flavus* and the possibility of aflatoxin production. The husk was chosen for study because previous studies done on barley have shown that fungal colonization and invasion is predominantly in the husk, with relatively little in the caryopsis (Mulinge & Aspinis, 1969; Mulinge & Chesters, 1970a, 1970b; Flannigan & Dickie, 1972; Warnock & Preece, 1971; Warnock, 1971, 1973a; and Harper & Lynch, 1981).

The choice of techniques was the first problem because no one medium or technique is sufficient to disclose all of

the fungal flora that might be present in a given lot of grain (Wallace & Sinha, 1962). Probably the most widely used technique for enumeration of fungi in cereals is the 'dilution plating technique' (Flannigan, 1977). The number of colonies developing from each sample in the dilution plating technique can be considered to be representative of the fungal spores, hyphae and other propagules which may have been present. However, there are some disadvantages to this technique when it is used alone. Heavily sporulating species tend to be over-estimated while purely mycelial species and weakly sporulating species are underestimated. There is also no recognition or estimation of fungal mycelium which may be present in, for example, husk tissue and not released during the shaking period. Thus the dilution plating technique used on bulk grain samples cannot give an accurate estimation of the occurrence of fungi. Furthermore, it will only give a general idea of fungi present, without indicating which are from the inner surface and which from the outer surface of hulled grains. To overcome these disadvantages the present study used a variety of techniques involving both direct observation and culturing methods, and used surface sterilization at various steps of dehusking to more accurately assess the distribution of the mycoflora. Other problems, particularly with hulled grains such as barley, can occur due to sticky spores adhering to the grain surface, and glass beads were used during the shaking of samples to liberate such slimy and sticky spores.

A number of authors have demonstrated the relationship between moisture content (M.C.) and the growth of fungi on grains (Christensen, 1964) and it is often implied that numbers of fungal propagules are related to M.C. There was, however, no clear relationship between M.C. and the viable counts of the farm-stored samples examined in this study. Only sample LS showed a relatively high viable count and high M.C. One sample (MI) showed a relatively high M.C., thought to be due to leaking seams in the silo, but a low viable count at the time of sampling. Sample PL had been

stored for two years and was grossly mouldy, but its M.C. was only 11.9. Wallace and Sinha (1962) found no relation between 'heated grain' and M.C. of both wheat and oats, and they found variation in the M.C. of the same sample. These variations usually were greater in the vertical than horizontal planes, indicating that moisture tends to move upwards and that there had been concentration near the surface of the stored mass. Perhaps for that reason it was difficult to find a positive relationship in this study between M.C. and viable counts. Christensen & Kaufmann (1974) discussed the differences in the M.C. from place to place within the same bin and found it a common phenomenon. They recommended the determination of the range of M.C. within a given bin or silo by taking samples from different places.

At least in clean samples, the number of fungal propagules present on grain must be a reflection of the numbers of spores etc. deposited onto the grain in the field and during harvesting and storage. The outer surface (O.S.) counts obtained in this study would thus have reflected the high spore population on the O.S. and certain genera, eg. *Fusarium* must have been present only as spores as they were never isolated from husk mycelium. Inner surface (I.S.) counts were lower and are likely to reflect either fungal invasion into the inside of the grain, or spores being deposited in cracked kernels and the loose portion of the two ends, particularly during development of the seed. The results reported agree with those of Christensen and Kaufmann (1969) who found the fungi occurring as external, dormant spores were much more abundant than those present as living, internal mycelium.

In all samples except FH and SS, the O.S. counts were higher than I.S. counts. Some *Aspergillus* species are osmophilic (eg. *A. restrictus*) and sample FH showed *A. restrictus* as the predominant isolate. This sample yielded relatively low viable counts from the O.S. on PDA medium, but I.S. counts on the same medium were higher. A more accurate count may have been obtained if MSA had been used,

as *A. restrictus* is a slow grower on PDA medium. In sample SS the O.S. viable count was slightly lower than that of the I.S. count (difference -0.16 x 10^2). *Aureobasidium* was the predominant fungus on both surfaces. This is not uncommon in clean samples, according to other authors. For example, Lacey (1971) found *A. pullulans* was common in clean barley samples. The lower O.S. count may be due to either this fungus (as a field fungus) starting to disappear from O.S. first, because of the competition with storage fungi, or it may be due to invasion of the inside of the husk and subsequent sporing. Flannigan (1970b) found *A. pullulans* moderately active in degrading hemicellulose, and Pugh (1973) found this species also produced pectinolytic enzymes. In these studies, there was some suggestion that the presence of *Aureobasidium* was a sign of incipient spoilage as it was present as predominant fungus only in sample SS, which showed the highest I.S. counts of the clean samples, and the slightly mouldy samples MO and AC.

Overall, the O.S. counts showed a wide variation between samples, even those apparently 'clean'. These counts did not seem related to the apparent degree of mouldiness of the samples. However, I.S. counts did more consistently reflect the general condition of the grain. The I.S. counts also helped to resolve the standing of 'slightly mouldy' samples. For example, the O.S. count of MO, a 'slightly mouldy' sample, was 2.7 x 10^4, which would place it second to sample LS, within the 'mouldy' category. But its I.S. count was only 5.6 x 10^2, which placed it truly in an intermediate position. The extent of moulding could also be confirmed in relation to the types of fungi which were isolated, and this will be discussed later.

This study concentrated mainly on storage fungi, particularly *Aspergillus* species. *A. flavus* was the most frequently isolated *Aspergillus* species by the dilution plating technique and was most frequent on the O.S. *A. flavus* is said to need a M.C. above 15% for maximum development (Christensen & Kaufmann, 1965) and appears in greatest

numbers only after long periods of storage (Clark *et al.*, 1966). Its high prevalence on O.S. (11/12 samples) in the present study may simply be a reflection of its abundance in the general environment. The *A. glaucus* group also showed frequent isolations but were mostly from I.S. Many factors govern the types of fungi on stored grains, including M.C., amount of fungal inoculum, period of storage, type of fungi contaminating the grain before storage, etc. Flannigan (1978) found exposure of the seeds before harvesting to fungal spores, damage of seeds during and after harvest and the cellulolytic activity of any storage fungi present, played a part in determining whether fungi colonized the I.S. or stayed only on the O.S.

Other species of *Aspergillus*, eg. *A. fumigatus* and *A. restrictus* were also present particularly on the I.S. *A. restrictus* was predominant in mouldy samples only (PL, FH and SA), with no isolates from the O.S. *A. restrictus* is regarded as a most important fungus involved in deterioration of grain stored at moisture content below 15% (Christensen, 1963). *A. glaucus* was also isolated from mouldy samples only and although found on both surfaces, its highest frequency was from the I.S. These results agree well with those of Christensen and Kaufmann (1974) who found that *A. glaucus* and *A. restrictus* were consistently associated with incipient deterioration. They were the only species that could grow on grain when the moisture content was in equilibrium with a relative humidity of about 78% to 80%. These authors used the incidence of *A. glaucus* in grain as a monitor of the grain's condition. If a lot yields *A. glaucus* from 20% to 50% of surface-disinfected kernels, that lot should be regarded as of questionable storability. If *A. glaucus* is isolated from 50% to 100% of the kernels, the lot is deteriorated, even though visible deterioration may not be apparent.

A. fumigatus was a frequent *Aspergillus* species from the I.S., but was equally present in both mouldy and clean samples. This species has a high cellulolytic activity

(Sellars *et al.*, 1976). Warnock (1971) found *A. fumigatus* to be the second most frequent fungus in barley samples, with *A. glaucus* the most common.

Results obtained from dilution plating overall show that the most frequently-occurring genus was *Aspergillus*. As several species of this genus are well known for their ability to form mycotoxins, obviously the risk of mycotoxin contamination could be a possibility. Harrison (1975) investigated moist storage of barley destined for animal feed in the U.K. and found that one of the chief mould contaminants was *A. flavus*, but aflatoxin was not demonstrated in the barley samples. When the strains isolated from barley were grown on other foods, including maize and groundnut, it was shown that they were in fact capable of producing aflatoxin. However, Hacking and Biggs (1979) isolated aflatoxin B_1 from predominantly *A. flavus* contaminated barley samples from farm stored grains in the U.K.

Among the other storage fungi isolated and important as potential mycotoxin producers were *Penicillium* species. *Penicillium* had a relatively high frequency, particularly from the O.S., but was predominant in only two samples (SA and MO) on the O.S. This genus needs a moisture content of about 18.5% and low temperature for optimal development (Christensen & Kaufmann, 1974). *Penicillium* was absent from 2 of the mouldy samples (PL and FH) and was common in clean samples. This may be because this genus frequently contaminates cereals before storage (Flannigan, 1978).

Field fungi recorded from dilution plating showed that *Alternaria* was the most frequent fungus among the clean samples, followed by *Cladosporium* and *Aureobasidium*. *Alternaria* and *Aureobasidium* were usually predominant on the I.S., which may be related to the cellulolytic activity of these fungi as well as other factors which have been mentioned. A few colonies of *Fusarium* species were isolated from the O.S. of 5 samples, mostly clean. *Fusarium* is said to die relatively rapidly in grain stored at M.C. about

12-13% (Christensen & Kaufmann, 1969). Christensen and Kaufmann (1965) obtained *Alternaria* species from 90% or more of wheat kernels of a given lot after surface sterilization, and obtained no storage fungi. They considered this good evidence that the seed was newly harvested or that it had been stored under conditions that do not permit deterioration.

Overall, the frequency of isolation of fungi could be used to monitor the sample's condition. The 12 samples in this study could be classified into three groups: a) mouldy samples (LS, PL, FH, SA and SB) which showed *Aspergillus* and *Penicillium* species as the predominant fungi from both surfaces; b) slightly mouldy samples (MO and AC), showing *Aspergillus* and *Penicillium* to be predominant from the outer surfaces only, and c) clean samples (SS, MI, SR, FC and FM) from which only field fungi (*Alternaria*, *Cladosporium* and *Aureobasidium*) were isolated. This classification was most consistently related to the I.S. viable counts but not to the O.S. viable counts as already mentioned. It was clear that highly sporulating genera (either storage or field fungi) produce a high viable count. The best examples of this were samples SA and MO which showed high viable counts (5.4×10^3 and 1.3×10^4 av. IS + OS) because *Penicillium* species were the predominant fungi, particularly from the O.S., and this genus is well known to be highly sporulating.

Special techniques are required to detect the presence of fungal mycelium in grain, especially in the early stages of decay. It has been said that the presence of mycelium may indicate early stages of deterioration and can be detected long before loss in grade quality occurs (Christensen & Kaufmann, 1974). However, previous reports do not give a complete picture of the mycelium within the grain examined, its biomass, viability and identity. Direct plating techniques have been used by Christensen, (1957), Mulinge and Apinis (1969), Mulinge and Chesters, (1970a) and others for the isolation and estimation of the total fungi present in

and on the grain of barley, but the results of the techniques of such authors were dependent on the selectivity of the media used and temperature and length of incubation. They did not give an assessment of the amount of internal mycelium which was present and could not avoid growth from any remaining spores. No attempt was made to determine the location and amount of the mycelium present within any specific part of the seed. Such mycelium is most important from both the deterioration and mycotoxin-production points of view. To overcome such problems, direct observation techniques were employed by several workers (Oxley & Jones, 1944; Hyde, 1950; Warnock & Preece, 1971), as has been discussed in the Introduction. But these workers concentrated on the assessment and the location of fungal mycelium only, usually making no attempt at identification and/or assessment of viability of such mycelium. Such determinations are necessary to indicate whether deterioration and possible toxin production has occurred. Critical identification of the internal mycelium was not possible in the work of such authors as non-vital stains were used (eg. fuchsin, analine blue etc.). All the above authors agreed, however, that direct observation is a most important technique, although not giving an absolute measurement of the mycelium (Warnock, 1971). To overcome some of these problems, the present investigation has used a vital stain to allow culturing of the observed hyphae. It was found that 0.04% trypan blue in 1% acetic acid produced clear hyphal staining with minimal damage of living hyphae.

This study has attempted to determine the presence, amount, and, in addition, the viability and identity of fungal mycelium within the husks. To avoid contamination by spores, three stages of cleaning the husk were used - surface sterilization, washing several times with mechanical shaking, and using glass beads to liberate any sticky spores. In assessing the amount and viability of mycelium associated with husk tissue, three scales were used: relative mycelial score (RMS), comparative mycelial score (CMS) and relative viability score (RVS). It is appropriate to mention

here that Hyde (1950) and Warnock and Preece (1971) used two different scales. Hyde called his scale, used for assessment of fungal mycelium in the subepidermal layer of wheat, "hyphal score", calculated from rough estimations of fungal density and distribution. Warnock and Preece described an assessment technique based on the number of observations of what they called "hyphal units" present in each sampling unit (the size of sampling unit used was the microscope field of view at x400 magnification). From this they estimated the total amount of hyphae.

RMS was the total number of strips showing hyphae out of 10 strips examined by trypan blue per sample of grain. It does not give a clear indication of the sample's condition because it expresses only the presence or absence of mycelium in each examined strip and takes no account of viability. Thus both mouldy and clean samples can show similar scores, particularly when clean samples are infected with field fungi. The lowest RMS was obtained from a mouldy sample, SA. (This sample also showed the lowest scores among the mouldy samples using the other scales, CMS and RVS.) It is not possible to explain why, in this sample only, fungi did not invade the grain husk and so give a high RMS, when its viable count was so high.

The CMS refers to the total amount of assessed mycelium (total pluses), which is calculated from the grading scale 1+ to 5+. This roughly assesses the relative amount of mycelium seen in individual husk strips, although it, too, expresses the presence of mycelium of both field and storage fungi, without differentiation between live and dead mycelium. CMS was used to compare total mycelium, and seemed more useful in judging the grain's condition. Using this scale, the most mouldy samples showed high CMS. Thus the CMS scale could be of use in determining the degree of spoilage of barley grain. The highest score was obtained from sample PL which was severely spoiled, as noted visually, after having been badly stored for two years. But even this sample showed low viable count compared with sample LS which

was less spoiled visually. Only one clean sample (FC) showed a relatively high CMS, but showed no growth at all (RVS). Possibly this sample was heavily invaded by field fungi before harvest, and these died rapidly during the storage.

The third scale, RVS, indicates the number of husk strips examined by trypan blue showing subsequent fungal growth out of 10 husk strips/sample. It was obtained using husks previously examined microscopically by vital staining, and then cultured on PDA and MSA media. According to this scale mouldy samples showed the highest score, with the most frequent isolates being *Aspergilli*, particularly the *A. glaucus* group, *A. restrictus* and *A. fumigatus*. As has been discussed, *A. glaucus* and *A. restrictus* are regarded as the first storage fungi to invade cereal grains (Christensen & Kaufmann, 1974) and *A. fumigatus* is known to be a highly cellulolytic fungus (Sellars *et al.*, 1976). Among the field fungi, *Alternaria* was the most frequent. The field fungi were mostly isolated from clean samples. These results agree well with those of Christensen and Kaufmann (1969) who found that high-grade lots of wheat show most of the living mycelium beneath the pericarp to be that of *Alternaria* (a fungus not known to cause deterioration of stored seeds). In low-grade lots, most of the living mycelium was that of *Aspergillus* and *Penicillium* species.

Use of 2 media (PDA and MSA) for culturing husk strips allowed a better assessment of the range of species present. Only 2 isolates of *A. flavus* were obtained from the whole husk strips (both on PDA), indicating that it was of little significance as a coloniser of husk tissue. This is perhaps not surprising as although *A. flavus* had been isolated from 11/12 samples by dilution plating it was found to be predominant only on the O.S., and probably present as spores only. Thus it would have been washed off during the preparation of husk strips. It also needs a high M.C. and long storage period to become predominant during spoilage. Highest

numbers of *Aspergillus* species other than *A. flavus* and *A. fumigatus* were obtained from the mouldy samples on MSA medium, indicating that they were osmophilic fungi. *A. restrictus* and *A. glaucus* were the most frequent.

Because of the complex structure of barley husk, it proved difficult to accurately assess the location of fungal mycelium in relation to its inter- and intra-cellular position. However, it seemed that most dead hyphae were present in the head (proximal end) section, as noted by the direct observation technique and confirmed by growth patterns on direct plating of samples. One suggestion for this is that most fungal contamination (infection) started at the proximal end and then extended to cover the other parts of the husk surface. These results are similar to those of Mead (1942), also studying barley grains.

A comparison between dilution plating and direct plating techniques used in this study indicates the value of using multiple techniques for the examination of the fungal population of substrates such as barley. *A. glaucus* and *A. fumigatus* were obtained in reasonable numbers by both methods, but *A. flavus* was isolated most frequently by the dilution plating method. In contrast, most *A. restrictus* isolates were obtained by the direct plating method. Other genera showed similar differences, 11 genera being isolated by dilution plating but only 7 by direct plating. Although *Alternaria* was a frequent fungus obtained by both methods, *Fusarium* and *Cladosporium* isolates were obtained by dilution plating methods only. Dilution plating results largely represent isolates from spores and loosely-attached mycelium which may be present on the surfaces examined, whilst isolates obtained by direct plating of husk tissue should have been from hyphae which had invaded that tissue. Thus both techniques used together give an overall view of the mycoflora of the barley.

This study has demonstrated that barley grains can support a considerable mycoflora and that invasion of husk tissue can be appreciable. Wherever moulds have grown on feedstuffs, there is always the possibility that by-products of their growth will prove toxic to animal consumers. Aflatoxin is considered as one of the most significant mycotoxins affecting animal and human health. Surveys of agricultural commodities, particularly those of major dietary components, are needed to provide a measure of the risk.

In the results of this study, *A. flavus* was the most frequent isolate from the 12 samples examined, and these isolates, together with isolates from soil, were screened for aflatoxin-producing ability. A variety of culture conditions were used to determine the most favourable conditions for toxin production by any toxigenic strains.

Of the total 87 isolates screened on coconut agar, only 7 soil isolates and strain NRRL 2999 showed fluorescent characteristics similar to those noted by Lin and Dianes (1976). Three to five days' incubation period was the best time for looking for the fluorescent zone. It was found that Samoan coconut cream, available in New Zealand, was suitable for the preparation of coconut agar, and this medium was very efficient for screening purposes. As a result of the present investigations, it can be recommended that coconut agar be used as a fast and cheap screening test, so that contamination by toxigenic *A. flavus* strains can be investigated in various environments and commodities.

The coconut agar method has many advantages for screening purposes. The coconut plates have a white background which enhances visualization of the fluorescence and the medium is simple and easy to prepare, and can therefore be used for screening large numbers of isolates. The coconut agar supports only the production of aflatoxin fluorescence and so interference with other fluorescent substances is avoided. Association of aflatoxin production with an orange-yellow pigmentation is claimed to permit the quick

identification of AT-positive isolates without use of U.V. illumination. This pigmentation was noticed by both Arseculeratne *et al.* (1969) and Lin and Dianese (1976) as being produced by only toxigenic isolates, but these observations could not be confirmed in the present study. The identity of the pigment is not known but it was suggested that it may be averufin, an intermediate in aflatoxin biosynthesis (Donkersloot *et al.*, 1972; Lin *et al.*, 1973, and Lin & Dianese, 1976).

After screening on coconut agar, further investigations on other media (semisynthetic [SMKY], Weet-bix, pearled barley and barley husks) of both positive and selected negative isolates were made to determine the influence of nutritional factors and cultural conditions on toxin production. These tests also allowed confirmation of results for positive isolates and determination of which aflatoxin components were produced, using the minicolumn and TLC techniques.

Again only soil isolates and NRRL2999 were found to be positive. Shaken culture conditions were better for toxin production than stationary conditions when using liquid media, and this is in agreement with other investigators (Hesseltine *et al.*, 1966). The results obtained from SMKY confirmed all the barley isolates to be negative and all positive isolates on coconut agar were confirmed to be positive.

Two main disadvantages were noted for locally-prepared minicolumns. Firstly, scattering of Florisil particles along the column wall may contaminate the other chemical layers during packing. Thus fluorescent spots will be seen not only in the discrete Florisil layer but scattered along the column. Secondly, the uneven pressure used for packing the column affected the rate of solvent flow through the column and sometimes air pressure was needed to increase the rate of flow. This may result in misshaping of the chemical layers. However, the commercial minicolumns were

satisfactory and the method could be conveniently used for screening for aflatoxin production.

The TLC technique was used to find which aflatoxin components were produced and to attempt some degree of quantification. However, high errors are possible in quantitative estimations of aflatoxin by visual comparison of intensity of fluorescence with aflatoxin standards. According to Pons (1968) errors inherent in visual aflatoxin estimation can approach ± 20-30% of the amount present on TLC plates. Such possible errors were acceptible in the present work as the main purpose was to screen the isolates for their ability to form the various aflatoxin components.

The TLC assays showed that the positive soil isolates produced mainly aflatoxin B_1, although trace amounts of B_2 were also found. This is a not unexpected result as aflatoxin B_1 is well-known to be a common contaminant of many commodities throughout the world (Bryden, 1982).

Variation of amounts of aflatoxins B and G produced by different isolates have been noticed by several investigators (Davis *et al.*, 1966; Diener & Davis, 1966). Diener and Davis found that more G_1 than B_1 was produced on both SMKY and shredded wheat biscuits. The results reported here support these findings. The amounts of G_1 and G_2 produced by NRRL 2999 were found to be higher than B_1 and B_2 on SMKY, Weet-bix and pearled barley, but not on barley husk. It is possible that B_1 is degraded faster than G_1 or even converted to another aflatoxin metabolite during growth on these media or during analysis.

False fluorescence, white or white-blue and some of a yellow-orange colour, was encountered in the minicolumn screenings. This fluorescence was produced mostly from extracts of Weet-bix and pearled barley. These media also supported blue or blue-green fluorescent spots of very low Rf value on TLC. It is not easy to say if these fluorescent materials were aflatoxin components other than B and G, or

fluorescent materials from the medium itself. The latter is the more likely.

It seemed logical to examine barley husk medium in these studies as fungal mycelium had been found mainly in the husk and pericarp tissue. Also Chang and Markakis (1981) obtained large amounts of aflatoxin (851 μg/Kg) from a hulled barley cultivar compared to a very small amount (36 μg/Kg) on a hull-less barley cultivar under the same cultural conditions. In the present investigations aflatoxin production on pearled barley was reasonable. However, it was found that although barley husks supported production of aflatoxin compounds, particularly B_1, the amount was the lowest among the various media tested.

No information is available on the incidence of AT-producers isolated from soil, but it is well known that *A. flavus* is a constituent of the mycoflora of air and soil and is found in living or dead plants and animals throughout the world (Diener *et al.*, 1976).

The finding of 7/9 soil isolates of *A. flavus* Link to be AT-producers indicates that New Zealand environments can support toxigenic strains and these may pass to agricultural commodities. However, barley grain does not appear to be a significant source of aflatoxigenic isolates, even though most samples were contaminated by *A. flavus*. Even though a variety of methods was used, all barley isolates tested were found to be non-toxigenic. One could not claim that the media and conditions used were not conducive to aflatoxin production as the finding of some positive soil isolates in addition to positive results with NRRL 2999 on the various media gave a good indication that the conditions, i.e. media, temperature, moisture etc. were favourable for aflatoxin production.

The barley samples examined may not, however, be absolutely free from mycotoxin contamination as several of the other storage fungi isolated are known to produce various mycotoxins, but have not been studied in this investigation.

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