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STUDIES OF THE MYCOFLORA OF BARLEY DURING STORAGE

AND ITS RELATION TO

MYCOTOXIN CONTAMINATION

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ABSTRACT

Much of the economic loss occurring during storage of barley grain is that due to fungal deterioration. In addition, the development of mycotoxins during storage may result in contamination of animal feedstuffs.

In this study barley from samples obtained at harvest, from commercial silos after 5 and 9 months' storage, from farm silos after 5 months' storage and from laboratory-stored samples held at 4°C and at ambient temperature were subjected to various fungal isolation techniques. These techniques were designed to provide information on the total viable counts of the whole grain and of the outside and inside surfaces of the husks and caryopses. Isolates of *Aspergillus flavus* were screened for toxigenicity and the barley itself was subjected to multimycotoxin analysis.

To investigate levels of contamination, dilution plating of supernatants from the inner and outer parts of grains were examined. Differential viable counts revealed wide variations between samples at harvest and after storage with most contamination on the outer surface. "Clean" grain showed higher outer than inner counts, but for mouldy grains inside counts were greatly increased. It was concluded that inside counts give a better indication of grain condition.

Commercially-stored grain showed a marked decrease in viable counts over time, as did the laboratory-stored grain although those held at 4°C showed a smaller decrease. In contrast, the farm-stored grain continued to yield high viable counts. These counts could be related to storage conditions.

Amongst the genera isolated from dilution plates, *Alternaria* was the most frequent and persistent, whilst others, including *Cladosporium* and *Fusarium*, showed falling levels over the period of investigation. The genera *Penicillium* and *Aspergillus* showed rising frequencies with storage and were predominant in mouldy samples.

Microscopic examination and culturing of caryopsis sections and husk surfaces revealed the significance and distribution of various

genera. Alternaria was found in all fractions whereas Penicillium was completely absent from grain at harvest, later appearing on the outer husk and finally on the inner husk after prolonged storage at ambient temperature.

Microscopy of stained caryopsis sections showed hyphae only in those from mouldy grain. Microscopy of stained husks revealed hyphae on and in both husk surfaces of all grains, with a greater abundance on the inner surface and in mouldy husks. S.E.M. observations confirmed these findings and established the adherence of spores and hyphae to grain structures and their rough surfaces.

Some hyphal fragments associated with the grain can cause mycotoxin contamination. Loosely-attached hyphae were examined using membrane filtration and micro-manipulation techniques. It was found that whilst total levels of hyphal fragments showed little decrease during storage, their viability dropped considerably. For silo- and laboratory-stored grain, the viability dropped from over 20% to 5%. In contrast, over 45% were viable in a mouldy sample.

Of the fungal species isolated, species of the Aspergillus flavus group were screened for aflatoxin production using coconut agar fluorescence. Positive isolates ranged from 3% for farm-stored grain to 25% of those from grain at harvest. Selected A. flavus isolates were cultured on moist barley which when analysed for aflatoxin gave identical results to those from the coconut agar.

A multimycotoxin technique was used to screen 14 barley samples for aflatoxins, citrinin, ochratoxin, T-2 toxin and zearalenone. Only one obviously-mouldy sample proved positive for aflatoxin, citrinin and ochratoxin. It appears that although A. flavus and other potentially toxigenic fungi can be regularly isolated from barley grains, only in exceptional circumstances are they of significance in relation to mycotoxin contamination of stored grain.

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PREFACE

In spite of advances in technology, millions of dollars worth of cereals are lost each year through spoilage of various sorts. In technologically-advanced countries, (such as New Zealand), where insect and rodent infestations of stored grains can usually be kept under reasonable control, fungi are considered to be the major cause of deterioration and spoilage of newly-harvested and stored grains (Ciegler, 1978). Incipient deterioration in grain quality begins as soon as grain is harvested and under suitable conditions, fungi readily proliferate resulting in gross spoilage.

For many years, there was a tendency to regard fungal growth on food commodities as harmless; a nuisance affecting commodity appearance and perhaps a cause of loss and spoilage. When contamination was severe, commodities deemed unfit for human consumption might perhaps still be used as animal feed. However, ever since the discovery of the aflatoxins and toxigenic Aspergillus flavus species two decades ago, fungal growth in cereal grains was viewed as a potential health hazard.

The genera of fungi most implicated belonged to the "storage fungi" (Ciegler, 1978), but an increasing number of studies have shown the elaboration of toxins by other fungal genera. The range of fungi which can be implicated in toxin formation is now known to be almost limitless. The widespread and frequent occurrence of toxigenic fungi in agricultural commodities indicate a constant potential hazard (Davis, 1981).

The development of specific methods of analysis has led to the discovery of a wide spectrum of mycotoxic compounds at low but significant levels in many samples of food contaminated with fungi. Cereal grains represent the most important commodity contaminated by mycotoxins (Hesseltine, 1974). Contamination is usually the result of faulty harvesting and improper storage, although mycotoxins can be produced before harvest (Scott, 1978; Stoloff, 1979). The presence of fungal toxins is potentially a serious problem as most mycotoxins are stable and even persist in food commodities after the fungi that produced them are killed (Moreau, 1979).

Ingestion of contaminated food can result in acute or chronic symptoms of toxicoses as well as insidious effects in both man and animals (Wyllie & Morehouse, 1978; National Academy of Sciences, 1979; W.H.O., 1979; Pier *et al*, 1980; Pier, 1981). Thus, the economic consequences of crop losses due to fungal attack and concomitant mycotoxin production are of concern when ensuring the production of wholesome food products and livestock feeds for the markets of today.

Barley is widely grown in New Zealand as a feed for livestock, for export and for human consumption and brewing. The studies on grain deterioration and mycotoxin contamination overseas have concentrated mainly on cereal grains other than barley, whilst similar studies are very limited in New Zealand. These factors, together with the increasing production and use of barley particularly in animal feed-stuffs in this country, meant that more intensive studies would be useful in understanding the local situation.

The investigations reported here concern the changing fungal flora of barley grains during storage, the topographic origins of the fungi in relation to grain deterioration and also their potential to produce toxins. Particular attention is paid to *Aspergillus flavus*, as this species is considered to be of considerable importance in causing gross spoilage and many isolates are important mycotoxin producers. Aflatoxins, the formidable mycotoxins produced by many isolates of *A. flavus*, are potent carcinogens, mutagens and hepatoxins (Goldblatt, 1969; Heathcote & Hibbert, 1978). *A. flavus* isolates have been screened for aflatoxin production and selected barley samples analysed for the presence of aflatoxins and other mycotoxins.

CHAPTER 1

THE MYCOFLORA OF BARLEY AND ITS RELATIONSHIP TO SPOILAGE OF STORED GRAINS - A REVIEW

1.1. BARLEY PRODUCTION AND USAGE

Archaeological findings have shown that barley was cultivated by men since neolithic times (Nash, 1978). It was the first cultivated cereal to be used for bread-making and as a feed for livestock. In the days of the ancient Hebrews, Greeks and Romans, barley was the chief bread grain. Bread made from ground barley was also the staple diet of the 15th Century English country peasants until the increasing availability of other cereal grains displaced barley's popularity as a food grain, at least in European societies (Kent, 1983).

Today, barley is grown in many parts of the world, but it is more popular in temperate countries where the cereal is grown mainly as a spring crop. There are two types of barley cultivated - the hulled and the hull-less varieties, with the cultivation of hulled varieties far exceeding that of the hull-less varieties. The world area devoted to barley cultivation increased two-fold from 40 million hectares in 1937-1940 to 80 million hectares in 1981, with world production reaching 158 million tonnes in 1981 (Kent, 1983).

Compared with other cereals, barley now forms a relatively small part of the diet of European and North American communities, but it still ranks amongst the major cereals consumed by people in many parts of Asia, Africa, the Middle East and Latin America. Barley is also used on a relatively small scale in various industrial processes, and as seed.

One of the main uses of barley is in the production of alcoholic beverages such as whisky and beer, but there is no doubt that the principle use of barley, especially in European societies, is as a feed grain for livestock. Hull-less barley rivals wheat as a feed grain as it resembles wheat in composition (Drew _et al._, 1980). Although the presence of hulls renders barley grains rather indigestible except when finely crushed (Kent, 1983), the grain, being essentially an energy-providing carbohydrate (but also with other nutritional qualities) is a valuable feed grain either alone or when used in compounded feeds (F.A.O., 1980). Ground barley is used extensively as supplementary feed for calves, pigs and poultry, and forms the main food for fattening pigs and poultry (McDonald _et al._, 1973).

In addition, brewer's grain, the lesser-known end product after sprouting and malting of barley grain, is also of value when used to feed cattle, sheep and horses (McDonald et al., 1973).

The use of barley in the manufacture of animal feeds, especially compounded feeds, should increase as the demand for monogastric animals increases in Western countries (O.E.C.D., 1981). Its usefulness as animal feed will also increase in developing countries where increasing meat products are consumed in response to increasing population sizes and incomes (Palmer-Jones et al., 1971).

Trends in the manufacture and use of stockfeed in New Zealand also show a continuous increase in the use of cereal grains in feeding livestock (Harris and Douglas, 1981). Expansion in the poultry and pig industries, a change from reliance on milk products and a decrease in the use of wheat as a feed grain, have together contributed to the dramatic increase in the use of barley and corn in feed meals (MacGillivray, 1976). Barley is also the preferred grain because it has 5 times more niacin than corn (Drew et al., 1980).

Of the feed barley used in New Zealand, approximately 46% is used for poultry, 35% for feeding pigs, 10% for dairy cows and the rest for feeding sheep and beef cattle (MacGillivray, 1976). Barley is also often used for supplementary feeding (Drew et al., 1980) and it is especially useful in drought years (MacGillivray, 1976).

There has been an increasing demand for malting barley for export as well as for the domestic market. The Canterbury Malting Company, which is New Zealand's principal user of malting barley, has steadily increased its intake. In the Manawatu region alone, the Company's recently-established plant in Marton was hoping to boost the amount used by 5000 tonnes in 1984, an increase of 17% from 30,000 tonnes in the previous year (Kearney, 1984).

New Zealand has a thriving cereal export industry. The country's agricultural sector was estimating that barley exports would reach a new high of NZ$60 million worth in 1984 and would continue to rise (Paine, 1984). Thus barley is rapidly becoming one of the most important cash crops in the country. The common barley cultivars grown in

the country include Zephyr, Hassan, Mata, Magnum, Manapou, Triumph, Kaniere, Koru, Kym and Goldmarker (Sheridan, personal communication; 1984 Statistics of the New Zealand Ministry of Agriculture and Fisheries). Fleet, Liberty, Goldmarker, Kym, Opiki, Triumph and Koru are more recently used cultivars.

1.2. GRAIN STORAGE

1.2.1. Storage Methods

Prehistoric man realised the necessity to store grain for his future use and developed different methods of storage (Justice & Bass, 1979). Egyptian excavation sites have revealed the existence of small grain-storage pits dating as far back as 4500 B.C. (Nash, 1978). Compared to modern facilities, grain storage in historical times tended to be on a relatively small scale using various underground methods. For example, the keeping of grains in dug pits or buried in earthernware vessels was greatly favoured. Some early above-ground storage methods included storing grains in small enclosures, little huts, in open cribs or in various types of containers.

Except for the less-developed societies of today, underground storage methods are not extensively used. There are numerous alternative methods for storing grain surpluses. Some examples include storing in sacks or bags, in bins, silos and huge grain elevators (Bailey, 1974). With the advent of high technology, building very sophisticated storage facilities, often with controlled atmospheric conditions, is fast becoming the norm in developed countries (Shejbal, 1980).

The seasonal and variable production of grain necessitates its storage to meet continuous demand. A prudent farmer stores surplus grain from a harvest to gain financially from expected higher prices for his produce in between harvesting seasons. In addition, grain storage is essential as a guard against a later poor harvest, to circumvent fluctuating demand and prices, to carry over surpluses into years of natural disasters and for times of acute grain shortages (Anderson, 1973). The primary goal of any merchant or farmer when storing grains is to avoid monetary loss by preventing grain spoilage

and deterioration, and by doing so preserving the original good grain quality and quantity.

1.2.2. Storage systems for Barley in New Zealand

A. On Farm: The majority of New Zealand cereal growers store their grain harvest on-farm for up to 9 months average (Telford, 1978). The grains are bagged and stacked or kept in erect structures which range from wooden bins to well-constructed silo-type bins complete with ventilation. New Zealand farmers are constantly encouraged to provide more storage facilities on their farms. The Marton-based Canterbury Malting Company is one of the recent commercial organisations to introduce an incentive scheme for encouraging farmers to build more satisfactory silos (Kearney, 1984).

B. On Commercial Premises: Commercial grain producers and users build relatively large granaries capable of holding several hundreds of tonnes of grain (Taylor, 1980 - personal communication). These silos and elevators are often equipped with computerised and automated controls to monitor silo atmosphere and conditions, and to assist with loading and unloading of grain. The larger organisations also have grain driers. On-farm driers are not at all common (Telford, 1978).

1.2.3. Storage Failures and Resulting Spoilage Problems

It is the belief of many grain experts that grain deterioration begins as soon as it is put into storage (Bailey, 1974). The F.A.O. reported that the loss of foodgrains attributable to post-harvest storage alone amounted to billions of dollars' worth annually (F.A.O., 1980). The actual magnitude of losses varies from year to year and from country to country, but the losses are more severe in developing countries.

Given clean, adequately-constructed storage facilities, factors such as the nature of the grain, its maturity, the amount of mechanical damage from harvesting, threshing and drying processes, together with the amount of extraneous material present can adversely affect the storability of a given batch of grain. Furthermore, the physico-

chemical variables such as temperature, moisture, oxygen and the biological agents including microorganisms, insects, mites, rodents and even birds are often involved at some stage in the deterioration process. Once deterioration is initiated, these factors acting either individually or simultaneously can bring about severe grain losses in a remarkably short time (Wallace, 1973).

Some of the distinctive features of barley grain deterioration and spoilage are: the production of unpleasant odour, mustiness, caking, the appearance of discoloured and sometimes obviously mouldy kernels. There are other losses in grain quality which might not be detected until the grain is used as seed or is further processed. One example is the decrease in grain germinability when it is used in malting. Undesirable biochemical and nutritive changes in stored grains will only be revealed by conducting laboratory tests on grain samples (Pomeranz et al., 1978). If incipient deterioration which often results in heating and wetness is left unchecked and propitious conditions are maintained for proliferation of the "agents of deterioration", a dramatic decrease in both grain quality and quantity quickly becomes noticeable and very soon complete grain loss will eventuate. Of increasing importance is the formation of deleterious substances, the most well-known being the mycotoxins, during grain storage. The effects of such substances on man and animals is arousing concern throughout the world today as people become more aware of the need for nutritious as well as wholesome food.

1.3. THE MYCOFLORA OF STORED BARLEY GRAIN

Reports published by Duvel (1909) and Shanahan (1910) early this century revealed the presence of fungi in stored grains. At the same time, Black and Alsberg (1910) suggested fungi as the major cause of grain spoilage. Since then meticulous and extensive research has confirmed the important role fungi play in spoilage and deterioration of stored grains.

Other early studies concentrated on the fungal flora of seed barley (Christensen and Stakman, 1935; Mead, 1942; Gordon, 1944; Machacek et al., 1951). In these studies, a wide variety of fungi was isolated and some species of Alternaria, Fusarium and Helminthosporium

were found to cause diseases which plague barley seedlings and plants. The study of pathogenic fungi in seed barley continues to be an important area of plant pathology, but the present discussion will deal principally with the general mycoflora.

1.3.1. The Fungal Flora of Stored Barley

The first detailed study into the fungal flora of stored barley grains was conducted by Tuite and Christensen (1952). They found Alternaria, Cladosporium, Fusarium and Geotrichum species common and frequent in barley seeds. When grains were stored aerated and at moisture content of 12.5 - 17.0%, their numbers decreased or remained constant. On the other hand, Aspergillus glaucus, A. vesicolor and Penicillium species were isolated with increased frequency in grain stored at moisture contents of more than 14%.

Continuing with their studies, Tuite and Christensen (1955) noted the presence of fungi such as Alternaria and Cladosporium in the grains prior to harvest, but they could not find any Penicillium or Aspergillus species in significant numbers at this time. When grains were stored at moisture contents of 10 - 13%, there was no change in the levels of Aspergillus and Penicillium species. On increasing the moisture content to 13.8 - 14.2% a slow and gradual invasion of seeds by Aspergillus restrictus became noticeable. At moisture contents of 15 - 16% stored grains were rapidly invaded by other Aspergillus species such as A. repens, A. amstelodami and A. ruber. However, fungi such as Alternaria, Cladosporium and Fusarium which were originally present in the seeds at harvest disappeared on storage at moisture contents of 15 - 16%.

Similar studies on other major cereal grains have led Christensen and Kaufmann (1965) to arbitrarily divide fungi associated with cereal grains into 3 major categories. The first group comprises the 'field fungi' and includes a multitude of fungal species, the most well-known being Alternaria, Cladosporium, Fusarium and Helminthosporium. These fungi invade grains on standing crops prior to harvest. The next group invades mainly stored grains and is aptly known as 'storage fungi'. Storage fungi include about 15 groups of Aspergillus and several species of Penicillium (Raper and Fennell, 1965). A third

group is the advanced decay fungi of which species of Mucor, Papulospora, Chaetomium and Sordaria are examples.

1.3.2. The Fungal Flora of Freshly-harvested Barley

Freshly-harvested barley samples studied by Clarke et al. (1966) yielded a range of field fungi. Species of Cladosporium, Alternaria alternata, Epicoccum nigrum, Fusarium avenaceum and Aureobasidium pullulans were the most frequent fungi isolated, giving total dilution plating counts of between 1.5×10^4 - 3.4×10^4 colonies per gram of grain. Similarly Lacey (1971) also obtained an average dilution plating count of 0.64×10^6 colonies per gram from freshly-harvested barley grains.

In their examination of freshly-harvested barley, Clarke et al. (1966) also noted the absence of storage fungi. It was considered that species of Penicillium and Aspergillus did not invade barley crops in the field (Tuite and Christensen, 1955; Flannigan, 1969). The extensive studies of cereal grains by Christensen and Kaufmann (1969) confirmed the absence of significant numbers of storage fungi in fresh grains. However there are occasions when high moisture, warm temperatures and insect infestations make conditions conducive to the invasion of standing crops in the field by storage fungi (Martin and Gilman, 1976; Clarke and Hill, 1981). Further, evidence published by Flannigan (1978) showed the presence of low numbers of typical storage fungi in grains at harvest.

1.3.3. The Changing Fungal Flora During Storage

1.3.3.1. Moist Barley Grains

After 4 successive years of investigations, Clarke and his colleagues (1966; 1969) recognised 5 phases in the succession of fungi of moist barley hermetically stored in farm silos. Fungi present at harvest such as Cladosporium, Alternaria and Fusarium dominate Phase I (Clarke et al., 1966). A short time from the start of storage at high moisture content (18.0 - 25.0%), these 'field fungi' begin to decline in numbers and had virtually disappeared after 6 weeks of storage (Phase II). In Phase III, yeasts, in particular Hansenula anomala, quickly replaced these field fungi. The appearance of storage fungi

such as Aspergillus terreus typified the fungal flora of Phase IV. Such storage fungi were quick to develop and very soon raised the temperature in the upper part of the silo to 32°C. A month later, the upper silo temperature had risen to 52°C, heralding the appearance of thermophilic fungi, chiefly Mucor pusillus, Aspergillus fumigatus and Humicola lanuginosa (Phase V).

High moisture levels, slow unloading rates combined with inadequately sealed silos resulted in the heating and moulding of stored moist barley in samples studied by Lacey (1971). A succession of fungi in the stored grain bulk closely resembled the 5 phases of fungal succession suggested by Clarke et al. (1967). Although these 5 phases were distinct, they were not mutually exclusive for in the grain immediately adjacent to the top seal of the silo, a mixture of fungi such as Aspergillus fumigatus, A. flavus, Fusarium species, Trichothecium, Penicillium species and Sporotrichum, representing species from the 5 phases, was present.

Different ecological successions of fungi were reported by Spillane and Pelhate (1982) when moist barley was stored under "safe conditions". Aeration early in storage gradually lowered temperature and moisture content. Prior to the start of aeration, there was a dramatic increase in the numbers of yeasts and bacteria due to a rise in the temperature of the stored grains. Mycological examinations of the ventilated grains showed the existence of both field and storage fungi. The field fungi were present even at the end of the 6-months storage period. The well-controlled storage conditions prevented the proliferation of storage fungi.

Clarke and Hill (1981) found no fungal growth in moist barley stored in well-sealed laboratory silos. In contrast, most farm silos are not completely airtight and this often resulted in the disappearance of field fungi 85 days after storage. Where moisture contents exceeded 19.0%, field fungi were replaced by yeasts (Candida and Hansenula) and Penicillium roqueforti. Heating also occurred in some of these silos. The fungi responsible were the thermophilic Absidia corymbifera, Aspergillus terreus, A. fumigatus, Mucor pusillus and Penicillium variotii.

After 3 months of storage very similar thermophilic fungi were frequently isolated from husked and dehusked grains of moist stored barley (Mulinge and Apinis, 1969; Mulinge and Chesters, 1970a). The mycelium of fungi which were confined to the husk tissue at the start of the storage period proliferated and invaded the inner grain tissues. When moist grain was stored under incompletely-sealed conditions rapid heating of the stored grain occurred.

1.3.3.2. Dried barley grain

When freshly-harvested barley was dried and stored at a moisture content of 12% (wet weight basis) and between 20-30°C, common field fungi such as Helminthosporium and Fusarium disappeared after 53 weeks, whilst Alternaria could still be recovered, but in much reduced numbers (Lutey and Christensen, 1963). Increasing the moisture content by 2% resulted in the death of all 3 genera after only 16 weeks at 30°C, but Alternaria was able to retain its viability for 24 weeks at 20°C. On the other hand, if the grains were kept in the cold, the mycoflora remained at its original level. Thus in storage, common field fungi lose viability at different rates, this effect being most rapid with Fusarium, and slower with Alternaria and Helminthosporium (Christensen, 1963). However, these fungi are known to remain viable in stored barley for up to 10 years provided storage conditions do not become favourable for the proliferation of storage moulds (Machacek and Wallace, 1952).

A large variety of field fungi was isolated from dried barley grains stored at 12 - 15% moisture content within 6 weeks of storage by Flannigan (1969, 1970). The barley kernels also showed significant contamination levels by storage fungi, implying that post-harvest contamination by storage fungi had been rapid as such fungi do not normally invade newly-harvested grains.

Most of the fungi isolated by Flannigan (1969) on 3 different agar media (Potato Dextrose Agar, Malt Salt Agar and Tryptone Soya Agar) at different incubation temperatures (25°C, 37°C and 50°C) were present as surface contaminants, colonising the husks but not penetrating the pericarp layer of barley kernels. This author also found that the barley kernels he examined harboured several

thermophilic fungi (*Absidia corymbifera*, *Aspergillus fumigatus*, *Mucor pusillus*). Besides their ability to cause heating in stored grains, these fungi are also of veterinary and medical importance as potential pathogens.

Hill and Lacey (1983) presented a list of over 80 fungal species isolated from studies of more than 500 samples of stored barley. Of the fungal species identified, more than 15% were *Aspergillus* species and 40% *Penicillium* species. The authors suggested that occurrence and abundance of fungal species are directly related to the storage conditions particularly water activity, temperature, intergranular gas activity, rate of aeration and spontaneous heating. They then went on to investigate the effect of these various factors.

The moisture level surrounding stored grains was recognised as being the single most important factor in detecting the degree of invasion and hence deterioration in stored barley by storage fungi, even if the grains were dried prior to storage.

As early as the 1900's, many researchers have suggested critical moisture levels for the safe storage of barley (Table 1-1).

Table 1-1: Suggested Safe Moisture Contents for Storage of Barley Grains

Moisture Content %	References
13.6 (long-term storage)	Snow *et al*. (1944)
14.8 (short-term storage)	Snow *et al*. (1944)
14.0 - 14.5	Coleman and Fellows (1925)
13.0 (1 year) 11.0 (5 years)	Hall (1957)
14.5	Panasenko (1967)
13.5	Hall (1963)
13.5	Hill and Lacey (1983)

Storage above these critical levels has been found to trigger the proliferation of storage fungi in stored grains (Armolik *et al.*, 1956; Watson and Cameron, 1976).

This is not unexpected for typical storage fungi such as members of the *Aspergillus glaucus* group, *A. amstelodami*, *A. chevalieri*, *A. repens* especially *A. restrictus* and *A. ruber* are xerophilic and can invade grains at moisture contents as low as 13-13.5% (Christensen and Kaufmann, 1965; Justice and Bass, 1979). For this reason, Hill and Lacey (1983) advocated a water content of 13.5% or preferably less for the safe storage of barley.

1.4. OCCURRENCE AND LOCATION OF FUNGI IN STORED BARLEY

Barley grains carry a variety of fungi, the majority in the form of spores, yeast cells and mycelium although sclerotia are sometimes encountered (Tuite and Christensen, 1952).

1.4.1. The Structure of Barley Grains

Before discussing the location of fungi on and in barley grains, it would be appropriate to examine the structure of a barley grain (Figure 1-1). Pomeranz (1972), who made detailed structural determinations of barley grains using scanning electron microscopy, concluded that a normal barley kernel consisted of the husks (which included the lemma and palea), the caryopsis and the rachilla.

The largest part of the barley kernel is the caryopsis, which being the nutritional "store-house" of the grain, is composed of the endosperm, scutellum and aleurone layers, filled with starch, fats and protein materials. Another important component of the caryopsis is the germ or embryo (Briggs *et al.*, 1981).

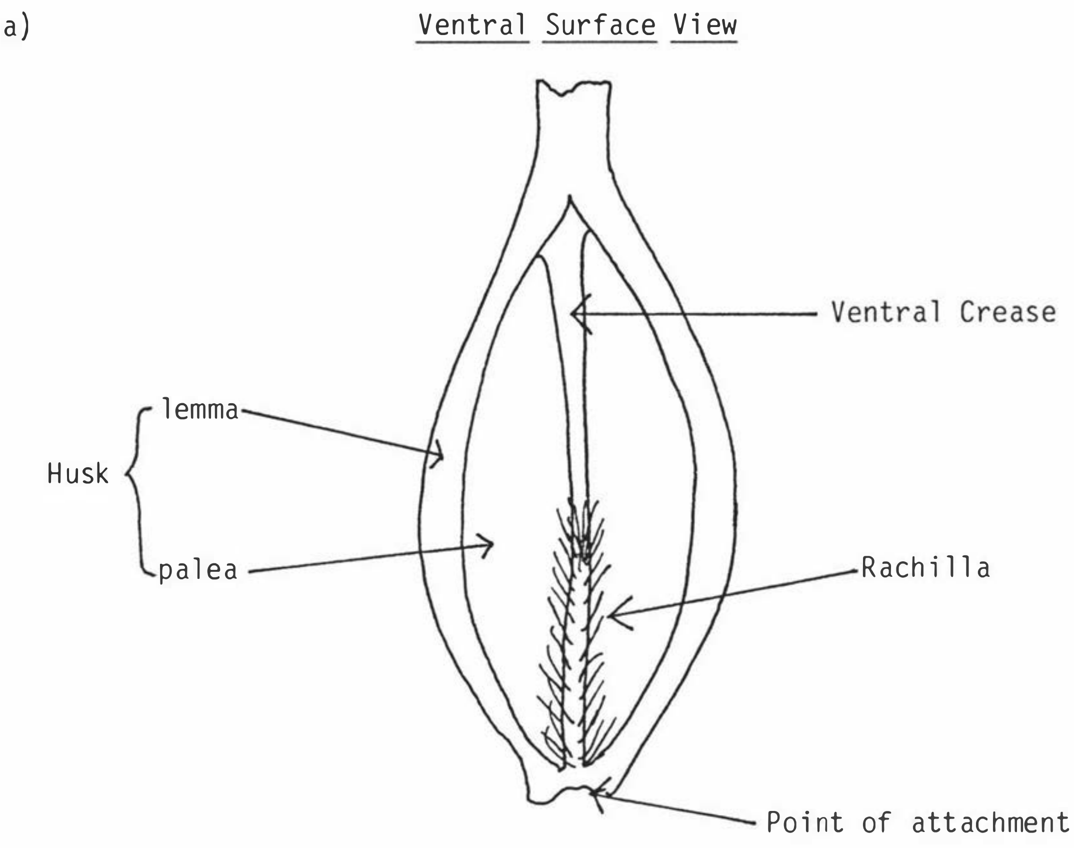
In hulled barley, the mature caryopsis is enveloped by the husks (Pomeranz, 1972), with the lemma stretching two-thirds of the way round the caryopsis to overlap with the palea (Harper and Lynch, 1981). The husk is important in protecting the caryopsis during harvest, drying, storage and handling in marketing as well as in

minimising damage to the germinating grain in the field and in malting processes (Hultin and Milner, 1978).

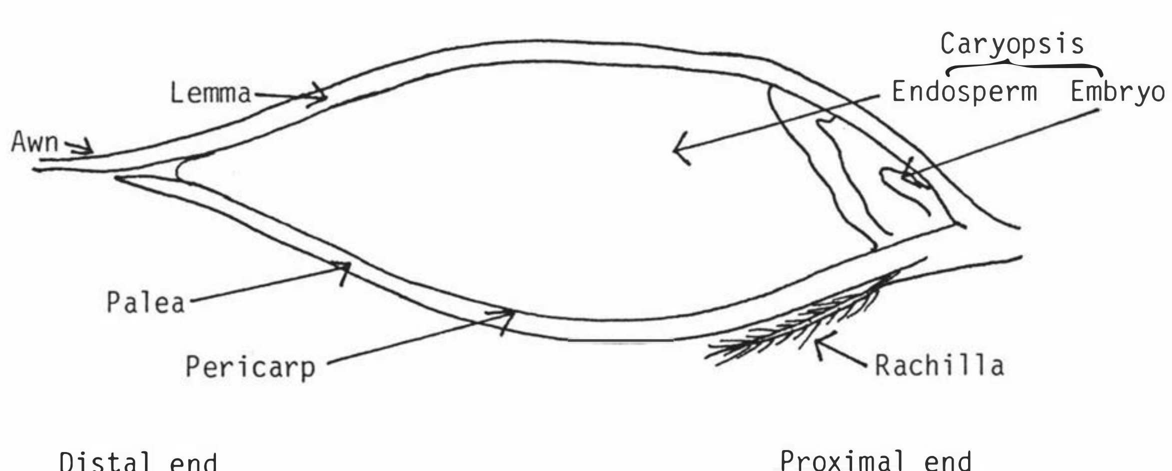
Scanning electron microscopy has shown that the barley husk is rough, being pitted with stomata cells on the outer surface and covered with ridges on both the inner and outer surfaces (Pomeranz, 1972). The lemma surfaces are covered with fine hairs and close to the point where the lemma is drawn out into the awn are found amorphous, silica-like scales and stomata-like cells. The awn itself shows several barbs about 30 μm in diameter and 150 μm in length, numerous ridges and small, blunt protrusions. The palea folds into a v-shaped crease, and on the ventral side of the grain adjacent to it is often found the extremely hairy rachilla.

Figure 1-1: Structure of barley grain
(based on Briggs et al., 1981).

a) Ventral Surface View



b) Median Transverse Section



Distal end

Proximal end

1.4.2. Distribution of Various Fungal Propagules

Many researchers have adopted techniques in their studies of the barley mycoflora which allowed them to isolate, identify and ascertain the locations of the fungi present. For example, Flannigan (1969) plated whole barley grains before and after surface disinfection on different agar media (P.D.A., M.S.A. and T.S.A.)* and incubated them at 25°C, 37°C and 50°C. A dramatic decrease in the species and numbers of fungi after surface disinfection as compared to non-surface disinfected grains, indicated that most of the fungal flora was present as superficial contaminants. However, the persistent presence of some fungi, in particular species of storage fungi, after surface disinfection showed the existence of more deeply-lodged fungi which had probably colonised the husk and caryopsis tissues.

By subjecting the barley samples to a number of different treatments, Mulinge and Chesters (1970b) managed to differentiate fungi which are surface contaminants, those which invade husk tissues and those which penetrate into the grain caryopsis. Plating whole grains on M.S.A. supplemented with Aureomycin allowed the isolation of all spores, mycelium and other fungal bodies on the grain surface.

The second treatment involved washing whole grains under tap water for 15 minutes prior to plating on M.S.A., resulting in the removal of loosely-attached spores, thereby revealing the deeper-seated fungi. Subsequent surface sterilisation of prewashed grains with full-strength sodium hypochlorite enabled the isolation of a relatively low number of fungi which had possibly colonised the grain tissues.

Viable fungal spores or mycelium present within the husk tissue appeared when husks detached from sampled grains were washed and plated on MSA. The final treatment consisted of washing and surface sterilising dehusked grains, aseptically cutting them longitudinally then plating the cut pieces with the cut face down on MSA. This treatment was said to allow the growth of internal fungi without undue competition.

* Refer to p. 12, last paragraph.

Using the procedures outlined above, Mulinge and Chesters located fungi in different parts of the grain and concluded that the husk is the favourable 'abode' of most of the Penicillium, Aspergillus and Absidia species isolated.

The majority of the thermophilic and mesophilic fungi isolated by Mulinge and Apinis (1969) were also found to be largely associated with husk tissue, although some thermophilic fungi were also recorded from dehusked grains. Similarly in their examination of various fractions of mechanically-pearled barley, Flannigan and Dickie (1972) isolated most of the viable fungi from husk fragments. Fungal mycelia in particular are mainly located in the lemma, palea and pericarp layers of not-obviously mouldy barley kernels (Warnock and Preece, 1971).

The above findings led to the conclusion that the grain husk serves as the principal site of fungal inoculum awaiting favourable environmental conditions to proliferate and colonise the rest of the grain.

1.4.3. Fungal Mycelium in Husk Tissue

The early recognition of the existence of fungal hyphae in the grain husks of various grains, particularly wheat, has led to many studies in this area. These studies are important as such mycelium is associated with diseased seeds (Mead, 1942), grain respiration (Oxley and Jones, 1944), grain deterioration and spoilage (Christensen, 1951) and finally there is the likelihood of the involvement of this fungal mycelium in toxin production (Warnock and Preece, 1971).

1.4.3.1. Early studies

In looking for the mycelium of the barley seed pathogen, Helminthosporium sativum, Mead (1942) removed the lemma, palea and pericarp layers after overnight soaking of barley grains in a solution of formalin-acetic acid-alcohol. Mead also prepared stained microtome sections of whole grains. Microscopic examination of these tissues revealed the presence of mycelium in all husk tissues. This mycelium was often concentrated in the proximal end of the grain.

Oxley and Jones (1944) removed pericarps from wheat seeds by making use of the abrasive action of carborundum powder. Grain respiration decreased sharply with the removal of the wheat pericarps. When the pericarps were stained with aniline blue, extensive mycelium was found, especially on the inner epidermis. Unless grains had become obviously mouldy, no hyphae could be found in the outer surface of the epidermis.

The first attempt to quantify mycelium tissue in husks was made by Hyde (1950). Areas of husk tissue showing subepidermal hyphae were allocated 'distribution numbers' which represented the proportion in tenths of the total area covered with mycelium. At the same time, the density of fungal growth was estimated and assigned arbitrary numbers (1-5). The multiplication of the 'distribution numbers' by the appropriate 'density numbers' of a particular sample gave a 'fungal score' which quantified the amount of fungi present. Hyde recorded the universal presence of mycelium in wheat samples from all over the world. The amount of mycelium present varied widely in these samples and he suggested that the atmospheric humidity level present during grain ripening was important in determining the degree of infection.

A year later, Hyde working with Galleymore (1951) made relatively detailed studies of the mycelial position, nature and mode of entry into wheat kernels. Septate hyphae were observed to develop extracellularly in the space between the epidermis (the outermost layer of the wheat husk) and the mesocarp layer of crushed cells (middle layer of husk).

The network of mycelium is believed to be established late in the development of wheat grains but researchers have not been able to obtain concrete evidence on their mode of entry into grain tissue. Furthermore, no attempt was made by any of the workers whose investigations were outlined above, to check the viability and identity of the mycelium they observed.

However in 1951, Christensen successfully induced the growth of viable hyphae in wheat seed husks by culturing pieces of husk in Van Tiegham cells enclosed in petri dishes. He noted that although fungal mycelium was present in both high and low grade lots of wheat, most of

the fungi were dead. On identifying the fungal colonies arising from the growth of dormant mycelium, Christensen found Alternaria to be the most common fungus in high-grade grains, whereas most of the viable hyphae of low-grade lots turned out to be those of Penicillium and various Aspergillus species. Similar storage fungi were also present in high-grade lots albeit at low levels. The presence of dormant fungal mycelium in husk tissue may be of significance in relation to possible spoilage, deterioration and toxin production in stored grains.

Besides the above investigators who studied the mycelium in the husk of wheat grains, Tuite and Christensen (1955) examined the mycelium of freshly-harvested barley grains. The husk tissues were found to abound with fungal mycelium. Examination of aniline blue-stained husk tissues revealed the abundance of this mycelium in the inner surfaces of the lemma and palea tissues and a more moderate quantity within the husk.

1.4.3.2. Recent studies

Research work on fungal mycelium performed in the 1970's concentrated on the development of more accurate assessments of its quality and identity.

Warnock and Preece (1971) used a grading system to determine the extent and location of mycelium present in barley husks. Sections of husks were obtained by cryostat sectioning and stained with phenol-acetic acid-aniline blue. All fungal hyphae or aggregates of hyphae observed were termed 'hyphal units'. By examining up to 50 sections of each barley grain, the 'hyphal units' in each layer of individual grains could be recorded. On subsequent assessment of the total 'hyphal units' for all the layers of a single grain, the authors derived a composite figure representing the overall amount of mycelium in that grain.

Warnock (1971) estimated the lengths of mycelium present in lemmas and paleas of barley grains. He started by detaching the husk layers, staining them in a saturated solution of acid fuschin in lactophenol before mounting in lactophenol for microscopy. The length

of fungal mycelium was estimated by making use of an arbitrary scale. Thus the product of mean mycelial lengths in each assessment class and their respective sampling units (for each lemma and palea) was an approximate length of mycelium present. Lengths of mycelium in the two samples Warnock studied ranged from 19.3 to 177.0 cm.

On applying this method to fluorescent-stained Penicillium cyclopium mycelium in husk tissue, Warnock found that P. cyclopium covered an area of 22.7% and 77.2% of lemma and palea respectively. Warnock considered that such a method could be used to detect and quantify the presence of fungal species such as Aspergillus glaucus, A. fumigatus, Fusarium, Helminthosporium etc. which are important plant pathogens and also significant in causing stored grain deterioration or as toxin producers.

In 1973 (b) Warnock again used immunofluorescence to detect mycelium of Alternaria, Aspergillus and Penicillium in husks of stored barley grains. The mycelium of these fungi were present in all grains but in relatively small amounts. For example, Penicillium mycelium was found to comprise 10-20% of the total amount of mycelium present in two grain samples.

Since the failure of Hyde and Galleymore (1951) and others to establish the mode of entry of fungal mycelium into grain tissue, Warnock (1973a) attempted to closely follow the development of fungi in the lemma and palea of barley kernels on standing crops. From his observations, he concluded that mycelium present in husk tissue is the result of the growth of spores deposited on the inside of lemmas and paleas and on the anthers.

Very recently, Hussein (1983) introduced a relatively simple method of assessing the relative abundance, identity and viability of the mycelium present in husk tissues of farm-stored barley. He examined and cultured strips stained with the vital stain trypan blue on P.D.A. and M.S.A. The amount of mycelium was depicted in numeral scores. The relative mycelial score gave the number of husk strips colonised by fungal hyphae out of 10 strips for each sample examined. The comparative mycelial score was the total amount of assessed mycelium for 10 husk strips of individual grains. This assessment

system was based on an arbitrary scale with the lowest density being awarded a number of 1 and the highest density of hyphae represented by 5. All the samples examined showed the presence of abundant mycelium mainly in the inner surfaces, especially along the crease and the two ends of the husks. When grains became mouldy, the mycelial network enveloped the entire surface of the husks.

The advantage of this method was that the vital stain used did not appear to show any apparent adverse effects on hyphal viability. By culturing the same strips of husk that he had earlier stained and examined, Hussein was able to 'measure' the amount of viable mycelium by counting the number of husk strips which gave rise to colonies. The resultant count per 10 strips of husk examined per sample was then called the Relative Viability Score (R.V.S.). Besides confirming the findings of earlier investigations that mouldy grains contain more viable hyphae than clean grains, Hussein identified *Aspergillus restrictus*, *A. fumigatus*, *A. flavus* colonies emerging from husks of mouldy samples. Fungal genera isolated from clean husks were mainly those of *Aspergillus*, *Monilia* and *Papulospora*.

The development of methods to detect the presence and amount of fungal hyphae in husk tissue in addition to testing their viability and identity is of tremendous value in assessing the significance of fungal mycelium, in particular those of toxigenic fungal species. The presence of viable toxigenic fungal hyphae may serve as a rapid source of inoculum for toxin production as soon as conditions become favourable for their further growth.

CHAPTER 2

MYCOTOXIN CONTAMINATION OF BARLEY - A REVIEW

2.1 MYCOTOXINS AND MYCOTOXICOSES

Mycotoxins are secondary metabolites produced by fungi under favourable conditions of moisture, substrate composition and temperature (Ciegler, 1978). They vary considerably in structure and properties and belong to several different chemical groups (Wilson *et. al.*, 1973). Most mycotoxins are relatively heat stable, non-volatile, of low molecular weight (average 300-600) and all are capable of producing toxic effects in man and animals (Smith, 1981). Steyn (1980) illustrated the various biosynthetic pathways via which a wide range of mycotoxins can be derived from relatively simple compounds such as amino acids, acetate and malate.

Although toxic effects can result from inhalation and by coming into contact with mycotoxins, almost all the mycotoxicoses reported are attributed to the ingestion of contaminated foodstuffs (W.H.O., 1979, Baxter, 1981; Smith, 1981). Every food commodity can support the growth of toxigenic fungi and hence is susceptible to mycotoxin contamination. Humans can develop mycotoxicoses directly by consuming contaminated food or via contaminated animal products (Smith, 1981).

For many centuries, man has known the "poisonings" caused by some Basidiomycetes. But there was a failure to recognise the significance of microfungi in producing toxins despite repeated outbreaks of disease due to the consumption of mouldy foods and feeds. Interest in toxic fungal metabolites began in earnest in the 1960's following the publications on mycotoxicoses and fungal toxins by workers such as Forgacs and Carll (1962), Brook and White (1966) and Wright (1968). At the same time, publicity generated by the outbreaks of aflatoxicoses followed by the successful isolation, identification and characterisation of *Aspergillus flavus* and its toxic and carcinogenic metabolites in contaminated meals served as additional impetus for extensive research into mycotoxins and mycotoxicoses.

Today, close to 100 species of fungi are identified as toxin producers (Pier *et. al.*, 1980). The majority are classified in the classes Hyphomycetes or Ascomycetes, especially in the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Claviceps* (Scott, 1973).

Toxigenic fungi are generally ubiquitous although some are more widespread than others and some are restricted in their distribution (Pier, 1981). In presenting a comprehensive list of fungi associated with toxin production, Moreau (1979) pointed out that some fungi produce more than one toxin whilst some toxins are elaborated by different fungal species. In addition, only some strains of a particular species are toxigenic and these strains will only produce toxins under suitable conditions.

Man and animals differ greatly in their susceptibility to mycotoxins in relation to species, age, sex, the toxin involved etc. (Pier *et. al*., 1980). The ingestion of high levels of toxin often results in acute symptoms of toxicoses and depending on the toxin involved, specific organs or tissues can be affected. For example zearalenone causes oestrogenic effects on the vulva and uterus and aflatoxin damages the liver of affected animals.

Prolonged ingestion of lower concentrations of mycotoxins may bring about carcinogenic, teratogenic, tremorgenic, mutagenic, oestrogenic or immunosuppressive effects (Smith, 1981). Furthermore there are various other insidious effects which result from ingesting low doses of mycotoxins, such as reduction in the rate of growth and decrease in the ability to absorb certain nutrients. Such effects must be of concern especially when their aetiology is often attributed to causes other than mycotoxins.

2.1.1 Mycotoxins Contaminating Cereal Grains

While a combination of favourable moisture, temperature and aeration is imperative to the elaboration of toxins by toxigenic fungi (Pier *et al*., 1980), it is well known that a range of other physical, chemical and geographical factors also plays a role in determining toxin production and yield. The actual factors involved depends on whether mold growth is in the field, at the time of harvest or during storage of the product (Hesseltine, 1976). Furthermore, the fastidious nature of toxigenic fungi in requiring specific conditions for toxin elaboration means that mycotoxins are often found in association with certain substrates and commodities (Ciegler, 1978). The occurrence of mycotoxins on standing crops is often confined to certain

geographical regions, with seasonal variations in their production (Pier, 1981). In contrast, mycotoxin production in stored commodities are not governed to the same extent by geographical and seasonal factors (Pier et al., 1980). Once mycotoxin is produced, the resultant manifestations of mycotoxicosis will depend on complex interactions of environmental factors, the susceptible host and the toxin involved (Tuite, 1979).

The association of cereal grain with mycotoxicoses can be traced back to the days of widespread ergotism in the Middle Ages (Van Rensburg et al., 1974). Table 2-1 lists some of the early mycotoxicoses due to the consumption of mouldy cereal grain and feeds and summarises their effects post-ingestion. The causes of these epidemics were not known then and recurrent outbreaks of disease were common. As a result mortality rates were high and there was great suffering amongst affected man and animals. Of the toxic fungal metabolites that are known today, some are found to occur in significant amounts in cereals (Table 2-2) but there are no reports of epidemics in the proportions seen in the past. There is no doubt that with improved, sensitive analytical methods, even more mycotoxins will be detected.

Cereal grains, being carbohydrate concentrates are highly susceptible to mycotoxin contamination (Hiscocks, 1965). In general, mycotoxin is found in lower grade grain (Shotwell et al., 1977) and more commonly after physical damage, improper storage or pest infestation have predisposed the grain to fungal attack.

Although cereal grains are considered good substrates for mycotoxin production, some appear to support the production of some toxins but not others (Ciegler, 1978). Soybeans, for example are a poor substrate for aflatoxin formation (Hesseltine et al., 1966, Mirocha and Christensen, 1974a), possibly because of the poor growth of toxigenic strains on the substrate (Ciegler, 1978).

Table 2-1: Some early mycotoxicoses associated with cereals and animal feeds

Myco-toxicoses	Period of occurrence	Country/region of outbreak	Cereals associated with disease	Toxic fungi involved	Species affected	Symptoms/effects	References
Ergotism	Since Middle Ages. Epidemics in 18th Century	Europe, France	All kinds, especially rye	Claviceps spp. eg. Cl. purpurea & Cl. paspali	Man and animals	Affects nervous system, causes gangrene, nervous seizures etc.	Van Rensburg et. al. (1974) Groger (1972)
Alimentary Toxic Aleukia	1942	U.S.S.R.	Overwintered grains, eg. millet, wheat and barley	Fusarium poae F. sporotrichioides and Cladosporium spp.	Man	Affects haematopoietic system symptoms synonymous with depressed R.B.C., W.B.C. and platelet formation	Forgacs and Carll (1962) Joffe (1978)
Stachy botryo-toxicosis	1931	U.S.S.R.	Fodder - hay/straw oats	Stachybotrys alternans	Animals, esp. Man & horses	Inflammation, neurosis, hyperthalmia, various haematopoietic syndromes	Forgacs (1972) Rodricks et. al. (1974)
Cardiac "Kakke" Beri-Beri	1600-1800's	East Asia esp. Japan Japan	Rice	Penicillium citreo-viride	Man	Paralysis, convulsions respiratory and cardiac arrest	Ueno (1974)
Red Mold Disease	1900s	Japan	Wheat and Barley	Fusarium spp. (F. nivale F. graminearum)	Man	Fever, chill vomiting	Tsunoda (1970) Saito & Ohtsubo (1974) Yoshizawa (1983)
Aflatoxicosis	1960's	U.K. U.S.A.	Groundnuts & Cottonseed	Aspergillus flavus	Poultry, calves, pigs & trout	Subcutaneous haemorrhages hepatitis, hepatomas, death.	Butler (1974)

Table 2-2: Some mycotoxins found in cereal grains

Mycotoxin	Cereal Grain Contaminated	Country	Levels of Mycotoxins	References
Trichothecenes:				
Deoxynivalenol	corn	Japan	0.16-0.37 ppm	Yoshizawa (1983)
	corn	U.S.A.	0.5-10 ppm	Vesonder *et al.* (1978)
Nivalenol	barley	Japan	0.14 ppm	Yoshizawa *et. al.* (1983)
T-2 Toxin	barley	Canada	25 ppm	Puls *et. al.* (1976)
Zearalenone	corn	U.S.A.	5 ppm	Mirocha and Christensen (1974b)
	corn	U.S.A.	450-750 ppb	Shotwell *et. al.* (1971)
	sorghum	U.S.A.	200-6900 ng g^{-1}	Shotwell *et al.* (1980)
Ochratoxins	wheat	U.S.A.	25-35 ppb	Shotwell *et. al.* (1976)
	wheat	Canada	30-600 ppb	Prior (1976)
	corn	Canada	up to 500 ppb	Prior (1981)
Citrinin	barley and oats	Denmark	160-2000 μg kg^{-1}	Krogh *et al.* (1973)
	wheat	Canada	10-80.0 ppm	Scott *et. al.* (1972)
Aflatoxin	corn	Uganda	133.0 ppb	Ciegler (1978)
	maize	Australia	0.045 mg kg^{-1}	Blaney (1981)
Sterigmatocystin	barley	Britain	3.0 ppm	Shreeve *et.al.* (1975)
	wheat	Canada	0.3 ppm	Scott *et. al.* (1972)

Table 2-3: Some mycotoxins present in barley

Mycotoxin	Country	Levels	References
Trichothecenes:			
T-2	Canada	25.0 ppm	Puls *et. al.* (1976)
Deoxynivalenol	Japan	5.0 ppm	Ueno (1980)
Deoxynivalenol	Japan	7.3 ppm	Morooka *et. al.* (1972)
Nivalenol	Japan	0.1-22.9 ppm	Yoshizawa *et. al.* (1978)
Nivalenol	Japan	not quoted	Morooka *et. al.* (1972)
Vomitoxin	Canada	0.1-6.8 ppm	Nash *et al.* (1981)
Zearalenone:			
1	Ireland	-	Mirocha and Christensen (1974b)
2	England	-	Shreeve (1976)
3	Yugoslavia	-	Minocha and Christensen (1974b)
4	Scotland	500.0-750.0 ug kg^{-1}	Miller *et. al.* (1973)
Citrinin:			
1	Denmark	2.0 ppm	Harwig (1974)
2	Denmark	1.05 ppm	Krogh (1973)
3	Canada	80.0 ppm	Scott *et. al.* (1972)
4	Canada	260.0 ppm	Scott *et. al.* (1972)
Ochratoxin:			
1	Poland	50-200 ug kg^{-1}	Juszkiewicz *et. al.* (1976)
2	Denmark	27.5 ppm	Krogh (1972)
3	U.S.A.	38 ppb	Nesheim (1971)
4	Canada	22 ppm	Scott *et. al.* (1972)
Sterigmatocystin:			
1	U.K.	3.0 ppm	Shreeve *et. al.* (1975)
Viomellein	Denmark	1 mg kg^{-1}	Hald *et. al.* (1983)
Multimycotoxins:			
Ochratoxin A, Zearalenone, Aflatoxin and Sterigmatocystin	U.K.	-	Shreeve (1976)
Zearalenone, Ochratoxin, Citrinin, Patulin and Sterigmatocystin	U.K.	-	Hacking *et al.* (1976)

2.2. MYCOTOXINS DETECTED IN BARLEY

Although many fungi are capable of producing toxins when grown on barley under special conditions in the laboratory, relatively few mycotoxins have been found occurring naturally in barley (Table 2-3).

On occasions, a single barley sample has been shown to harbour more than one mycotoxin (Harwig, 1974; Shreeve, 1976). These mycotoxins may be the product of the same or different species of fungi growing in the barley sample (Hesseltine, 1974).

In comparison with larger grains such as corn, the incidence of mycotoxins in barley not only seems to be lower but the mycotoxins present appear to be less varied (Hesseltine, 1974; Scott, 1978). Nevertheless, some mycotoxins such as zearalenone, some trichothecenes, citrinin and ochratoxins can be of significance on barley especially in areas where the grain is one of the major cultivated crops (Ciegler, 1978, Pier _et al.,_ 1980). This is probably due to the fact that barley is more susceptible to invasion by some fungal species. For example, barley is prone to scab diseases caused by _Fusarium_ infestation (Christensen _et al.,_ 1977). Some of these species will produce potent zearalenone and trichothecenes under favourable conditions of growth (Tuite, 1979).

2.2.1. Early Mycotoxicoses and Consumption of Mouldy Barley

It is not surprising that barley, one of the earliest cereals cultivated, has long been associated with outbreaks of mycotoxicoses. In ancient times, when barley was a staple food grain, man and animals often became ill after ingesting food or feed prepared from barley infected with the ergot fungus, _Claviceps purpurea_. As far back as 460-357 B.C., the Greek physician, Hippocrates was reported to prescribe ground barley for obstetric purposes (Van Rensburg _et. al.,_ 1974). Since barley on its own can have no effect on the childbirth process itself, it is assumed that ergot must have been present in the barley used. The utilisation of ergot alkaloids for medicinal purposes especially in obstetrics was claimed by the Chinese more than 5000 years ago.

2.2.1.1. Ergotism

Epidemics of ergotism spread through many parts of Europe especially France from the 9th to the 18th centuries. Thousands of people succumbed to the disease after consuming bread made from ergot contaminated flour (Pier, 1981). The most feared and distressing symptoms due to the effects of ergot alkaloids such as ergotamine, ergosine, ergocristine and ergokryptine is gangrenous ergotism which begins with lassitude and a prickling sensation in the limbs (Van Rensburg _et. al._, 1974; Yamazaki, 1978). As the disease progresses, there is severe pain, alternating sensations of intense heat and cold as gangrene develops in the afflicted legs and feet, and eventually the limbs may be lost. Ergot alkaloids also affect the central nervous system, producing twitchings, muscular cramps, spasms, convulsions and other nervous disorders. Severely affected patients die and complete recovery of less severely affected patients from convulsive ergotism are rare.

Today, it is widely recognised that all members of the family _Gramineae_ are susceptible to parasitism by various _Claviceps_ species (Van Rensburg _et. al_, 1974). The notorious one is _Claviceps purpurea_ which is the most common species to invade barley crops (Diener _et. al._, 1976). Ergotism no longer occurs in epidemic proportions due to changes in agricultural practices but sporadic outbreaks still occur in many parts of Europe and the U.S.A. (Pier, 1981).

2.2.1.2. Alimentary Toxic Aleukia (A.T.A.)

A.T.A. is another mycotoxicosis which has resulted in dramatic disease and mortality of man (Busby Jr. _et al._, 1979). Outbreaks of A.T.A. have been restricted to the U.S.S.R. and reached epidemic proportions during World War II and in the post war years (Diener _et al._, 1976).

In those times, food was scarce and large populations collected grain that had been covered with snow through the winter. Thus the peak outbreaks occurred in spring and was most severe in several areas where up to 60% mortality rate was common amongst the affected. Consumption of at least 2.0 kg of food prepared from overwintered grain reputedly gave rise to clinical symptoms of the disease. The effects

of toxins on the haematopoietic system produced typical A.T.A. symptoms which included fever, haemorrhagic rash, leukopaenia, agranulocytosis, necrotic angina, sepsis etc (Wyllie and Morehouse, 1978).

A number of fungi were isolated from the overwintered millet, wheat, rye and barley (Wyllie and Morehouse, 1978). *Fusarium sporotrichioides*, *F. poae*, *Cladosporium epiphyllum* and *Cladosporium fagi*, were some of the principal fungi recovered from overwintered grain samples (Bamburg *et al.*, 1969).

2.2.1.3. Red Mould Disease

In the early 1900's, there were a number of intoxications affecting man and animals in Japan attributable to the invasion of edible grain by *Fusarium* species (Miyaki, 1970; Ciegler, 1978). Akakabi-byo (Red Mould disease) causes vomiting and diarrhoea in man and refusal of feed, congestion and haemorrhage in various organs of affected animals (Ueno, 1977). On examination of the incriminated grain, *Fusarium graminearum*, *F. nivale*, *F. poae* and *F. oxysporum* were the most frequent *Fusarium* species present (Saito and Ohtsubo, 1974). Infested barley, wheat and to a lesser extent rice and other food crops were all found to contain the toxins (Tsunoda, 1970). After intensive mycological investigations, Red Mould disease and A.T.A. are believed to be caused by the intake of trichothecenes (Smalley and Strong, 1974; Ueno, 1980).

2.2.1.4 Other spontaneous Diseases attributable to mouldy barley

Reports of disease linked with mouldy barley meals have been sporadic. In contrast to the early mycotoxicoses when major outbreaks involved humans, table 2-4 shows that many of these toxicoses affected mainly domestic animals. One plausible explanation is that barley has changed its role over the years from being a principal human food grain to becoming a prime source of animal feed. Another notable aspect is that almost all the low grade and mouldy grain rejected for human consumption is eventually used as animal feed (W.H.O., 1979).

Table 2-4: Spontaneous disease attributed to mycotoxins in mouldy barley

Mycotoxin Detected	Levels	Country	Species Affected	Syndromes	Fungi Isolated	References
Ochratoxin Zearalenone	Trace	U.K.	Pigs	Death	Aspergillus flavus A. fumigatus, A. niger Penicillum crustosum	Shreeve et al. (1975)
Emetic material Zearalenone	-	-	Pigs	Vomiting and diarrhoea	Fusarium species eg. F. graminearum	Brook et. al. (1966)
Zearalenone	-	Russia	Pigs	Changes in genital organs	-	Kysela (1941)
-	-	-	Sheep and Cattle	Poisoning	Giberella zeae	Christensen and Kernkamp (1936)
Zearalanone	-	Ireland	Pigs	Vulvovaginitis	Fusarium species	McErlean (1952)
Zearalanone	-	Yugoslavia	Pigs	Vulvovaginitis	-	Stamatovic et al. (1963)
Zearalanone	-	Denmark	Pigs	Vulvovaginitis	-	Eriksen (1968)
Zearalanone?	-	Germany Belgium Holland	Pigs Pigs Pigs	Death	Fusarium graminearum	Miessner and Schoop (1929) Beller and Wedemann (1929) Christensen and Kernkamp (1936)
T-2 toxin	-	Canada	Pigs	-	-	Andrews et. al. (1981)
T-2	25 ppm	Canada	Ducks, geese, horses, swine	Upper alimentary diseases, death of geese	Giberella zeae	Greenway and Puls (1975) Puls and Greenway (1976)
Ochratoxin Citrinin	>200 ppb 2 ppm	Denmark	Pigs	Kidney degeneration	Penicillum viridicatum	Krogh (1976)
-	-	Ireland	Pigs	Nephrotoxicity	Aspergillus fumigatus, Absida ramosa Penicillum cyclopium	Buckley (1971)
Cyclopiazonic acid	-	England	Calves	ataxia & muscular tremors	Penicillum cyclopium	Harrison (1971)

Many mycotoxicoses resulting from the consumption of mouldy barley have occurred when the grain has been most susceptible to fungal invasion under prevailing cold and wet conditions (Carlton and Tuite 1977; Mirocha *et al.*, 1974). These mycotoxicoses appear more prevalent in some European countries and Canada (Table 2-4), with most of the cases involving locally grown barley. However, the simultaneous high mortality of numerous pigs in Holland (Christensen and Kernkamp, 1936), Belgium (Beller *et. al.,* 1929) and Germany (Miessner *et al.*, 1929) was traced to blighted barley imported from U.S.A. (Moreau, 1979).

The presence of fungal toxins in barley fed to animals does not invariably produce disease. Shreeve *et al.* (1975) could not detect any apparent clinical syndromes in a herd of dairy cows fed barley containing four mycotoxic compounds. In such cases the mycotoxins may have been present at concentrations below those necessary to elicit acute disease syndromes (Pier *et al.*, 1980).

2.3. AFLATOXINS PRESENT IN BARLEY

Some strains of *Aspergillus flavus* and *Aspergillus parasiticus* growing on suitable substrates under favourable conditions may produce several highly toxic and carcinogenic compounds known as aflatoxins (Pier, 1981). The aflatoxins are named according to their colour of fluorescence when they are viewed under ultra-violet light. For example, aflatoxin B fluoresces blue and aflatoxin G fluoresces green. The family of aflatoxin compounds vary in their degree of toxicity. Aflatoxins B2a and G2a, the hydroxy derivatives of aflatoxins B2 and G2 respectively are much less toxic than aflatoxins B1, B2, G1, G2 and M1. Aflatoxin M1 is a metabolite which appears in urine, milk and tissues after ingestion of food contaminated with B1 aflatoxin. Aflatoxins B1, G1 and M1 are the most commonly encountered aflatoxins in foods and feeds (Ciegler, 1978).

2.3.1. Reports of Contamination

Barley, as the other small grains (wheat, sorghum, millet, rye, oats, rice) is generally considered a "low-aflatoxin risk" material

(Mirocha and Christensen, 1974a). The U.S. Department of Agriculture and The Food and Drug Administration examined 254 commercial barley samples from 1968-1975 for aflatoxins and found none. Nevertheless barley has occasionally been found to contain aflatoxins (Table 2-5).

Working in the U.K., Raymond (1966) and Wogan (1968) were amongst the first researchers to detect aflatoxins in barley samples sent to them from temperate and tropical countries. Lafont and Lafont (1970), in their limited survey of cereals from France, encountered both aflatoxins B1 and G1 in 7% of the barley samples they examined. The other reports of significant levels of aflatoxins in barley (Table 2-5) were the result of biased surveys of either grain associated with suspected cases of mycotoxicoses or grain that showed

Table 2-5: Natural Occurrence of Aflatoxins in Barley

Country	Levels of Aflatoxins	References
U.K.	traces	Raymond (1966)
Asia and Africa	not stated	Wogan (1968)
France	B1<10.0 ppb and G1	Lafont and Lafont (1970)
Britain	B1-0.05 ppm, G1-0.20 ppm	Shreeve *et al.* (1975)
	B1-0.07 ppm, G1-0.20 ppm	Shreeve *et al.* (1975)
Australia	0.1, 0.2 ppm B1	Bryden *et al.* (1975)
Britain	125 $\mu g\ kg^{-1}$ B1	Hacking and Briggs (1979)
Australia	0.01 $mg\ kg^{-1}$ B1	Bryden *et al.* (1980)
Australia	0.051-0.50 $mg\ kg^{-1}$ B1	Connole *et al.* (1981)

obvious signs of fungal invasion, often in conjunction with a history of rewetting. The one exception was the mouldy sample examined by Hacking and Briggs (1979). This particular sample of home-grown barley became mouldy due to inadequate treatment with commercial propionic acid on a British farm.

Thus, it is evident that aflatoxins have contaminated barley, especially barley subjected to improper storage and handling (Stoloff 1977; Busby Jr. and Wogan, 1979). The possibility of aflatoxin contamination will undoubtedly increase as production and usage of barley continues to rise rapidly, creating extra pressure to provide adequate storage facilities.

2.3.2. Aflatoxin Formation in Cereal Grains

2.3.2.1. Aflatoxin producers

Since the outbreaks of turkey X disease in the 1960's, Aspergillus flavus and Aspergillus parasiticus have become well known as producers of aflatoxins. Apart from these two species, several other fungi have in the past been claimed to produce aflatoxins. These include other Aspergilli, some Penicillum species and Rhizopus species (Kulik and Holaday, 1966; Hodges et al., 1964; Parish et al., 1966; Scott et al., 1967; Van Walbeek et al., 1968). However, despite the work of many investigators none of the above claims have been substantiated. The ability to produce aflatoxins seems to be confined to A. flavus and the closely related A. parasiticus, both numbers of the Aspergillus-oryzae group (Raper and Fennel, 1965). Moreover, they are the only aflatoxin producing fungi that are regularly isolated from aflatoxin contaminated commodities and from commodities associated with overt aflatoxicosis (Stoloff, 1979).

Numerous studies including those by Parrish et al. (1966), Wildman et al. (1967) and Diener and Davis (1969) have revealed that toxin producing ability is not a universal characteristic of A. flavus and A. parasiticus species. It is said that only around half the strains of A. parasiticus and A. flavus are aflatoxigenic under optimal environmental conditions (Pier 1981). Perhaps one of the most significant studies was that of Hesseltine et al., (1978) who demonstrated the great variability in isolates of the A. flavus group to

produce aflatoxins. They cultivated 67 strains in vitro on three different natural substrates, under two different incubation temperatures. The fungal strains differed not only in the yield of toxins but also in the proportions of aflatoxin compounds, B, G and M accumulated.

2.3.2.2. Other factors influencing aflatoxin formation

On a given substrate, the growth and subsequent toxin formation of individual strains of A. flavus and A. parasiticus is dependent on the expression of its inherent aflatoxin producing ability under the influence of the environment (Schroeder and Ashworth, 1966). Moisture, temperature and length of incubation are considered the principal factors involved (Christensen et al., 1977),

A. flavus and A. parasiticus are classified as mesophytes, requiring a minimum relative humidity of 80-90% for growth (Diener and Davis, 1969). In starchy grains, the minimum moisture content for growth is therefore about 18.0-18.5% on a wet weight basis (Mirocha et al., 1974). This is also the minimum moisture level required for aflatoxin production in cereal grain (Boller, 1974a). As moisture content increases above this level, the fungus grows more rapidly, producing more aflatoxin. However, when grain is stored, increasing moisture contents activate other fungi present, and due to the increased competition imposed on the A. flavus, the net result is a decrease in the aflatoxin accumulation (Boller, 1974b). More recently Chang and Markakis (1981) published one of the few studies on the effects of moisture levels on aflatoxin production in barley. They reported maximum aflatoxin accummulation in the moisture range of 28-31% for barley stored at 25°C. There was no aflatoxin at moisture levels of 13.5% or lower and only traces of aflatoxin were detected at 16%.

Sorenson et al. (1967), who studied the effect of temperature on aflatoxin production in rice observed the accummulation of the toxin within a temperature range of 11-37°C. The optimum temperature for toxin production was 28-32°C for aflatoxin B and 28°C for aflatoxin G. Virtually no aflatoxin was formed when the temperature increased above 37°C. Temperature was also shown by Davis and Diener (1970) to play a

role in determining the ratios of aflatoxins B and G produced. The ratio of B1 to G1 produced by *A. flavus* is 1 at 30°C; more than 1 at temperatures of incubation greater than 30°C and less than 1 at temperatures lower than 30°C.

The third factor influencing aflatoxin formation is time. Under optimum temperature and moisture levels, some toxin will be detected after 24 hours, with toxin production culminating around 10 days. After this time toxin production may decline (Christensen *et al.*, 1977).

Various other factors, often interacting together in a complex manner, can influence aflatoxin production and yield. These factors include the nature of the substrate, the degree of physical damage, the maturity of the grain, the gaseous effects of oxygen, carbon dioxide and nitrogen, the enhancement effects of trace minerals and finally the complications of microbial interactions. All these are well evaluated in a number of papers and some excellent reviews can be found in Diener and Davis (1969), Moreau (1979) and Detroy *et al.* (1971)

2.3.3. Screening *A. flavus-oryzae* strains for Aflatoxin Producers

In any survey into the mycoflora of commodities, fungi belonging to the *A. flavus* group can usually be isolated on a variety of agar media, possibly after incubation at different temperatures. A knowledge of the toxigenic characteristics of such omnipresent isolates will be useful in assessing the potential significance of these isolates in rendering substrates harmful to humans and animals.

Bothast and Fennell (1974) were the first to describe a selective medium for the rapid detection and enumeration of toxigenic *A. flavus*. The *Aspergillus* Differential Medium (A.D.M.) contained 1.0% yeast extract, 1.5% tryptone and 0.05% ferric citrate. On incubation of inoculated plates at 28°C for 3 days, toxigenic isolates produce a characteristic yellow-orange pigmentation on the reverse of the colonies. More recently Hocking (1982) developed another differential medium, A.F.P.A. (*Aspergillus flavus* and *parasiticus* agar) which is

recommended for isolating aflatoxigenic strains in commodities such as grain, stockfeed, nuts and oilseeds.

There are many methods available for determining the toxigenicity of fungal isolates. Most investigators favour the inoculation of fungal spores on solid or liquid substrates.

2.3.3.1. Liquid Media

An undefined semisynthetic medium will suffice for screening cultures of *A. flavus*, and *A. parasiticus* for toxin production.

Diener and Davis (1966) screened species of the *A. flavus* group from peanuts, feed, corn and other sources, on a semi-synthetic medium, SMKY, containing sucrose, minerals and yeast extract. Later Mehan and Chohan (1973) chose this medium as well as the Yeast extract, sucrose and salt medium (Y.E.S) as described by Davis *et al.* (1966), to test 21 *A. flavus* isolates for their aflatoxin producing abilities. Unfortunately, although confirmation of aflatoxin production is made easier with the relative ease of toxin extraction from liquid culture, some investigators (Maggon *et al.*, 1969) have found that liquid media are in fact rather poor substrates for aflatoxin production. This may result in a reduction in efficiency for detecting weak aflatoxin producers.

2.3.3.2. Natural substrates

Evaluations of substrates for toxin production by members of the *A. flavus* group have shown that aflatoxins are readily produced on a variety of natural substrates under controlled conditions *in vitro* (Detroy *et al.*, 1971). Several investigators, in surveys for aflatoxin producers, have cultured *A. flavus-oryzae* isolates on a range of sterilised cereal grains and oilseeds. Peanuts, rice, wheat, cottonseed, sorghum, corn, rye and oats are some of the substrates used (Ambrecht *et al.*, 1963; Boller and Schroeder, 1966; Taber and Schroeder, 1967; Joffe, 1969; Richard and Cysewski, 1971; Schroeder and Boller, 1973). Some investigators (Diener and Davis, 1966, and Mehan and Chohan, 1973) used a combination of liquid media in addition to natural substrates to identify and verify aflatoxin producers amongst their *A. flavus* isolates.

After a period of incubation (an average of 7-10 days), the fungal cultures in both liquid and solid substrates are checked for the presence of aflatoxins. Any aflatoxin compound present is extracted into organic solvents, the extracts concentrated and the aflatoxins detected by such methods as bioassays, minicolumn or thin layer chromatography, fluorodensitometry, spectrophotometry etc. (to be discussed later).

2.3.3.3. Agar fluorescence

Observations of the fluorescence produced in media on which fungi from toxic groundnuts were grown led to the development of a simple and relatively reliable method for the rapid recognition of aflatoxin-synthesizing fungi (De Vogel, 1965). Agar media specially formulated to detect fluorescent aflatoxins as well as supporting the growth of *Aspergillus* species and subsequent toxin elaboration and diffusion include modified Czapek Dox Agar and peanut extract, (de Jongh *et al.*, 1964 and De Vogel *et al.*, 1965), modified Czapek Dox Agar (CDA) and corn steep liquor (Hara *et al.*, 1974), coconut agar medium (Lin and Dianese, 1976) and a chemically defined medium designed by Torrey and Marth (1976).

The emission of fluorescence when an incubated agar plate is viewed under ultra-violet light is a strong indication of the aflatoxin producing ability of the inoculated fungus. Even weak aflatoxin producers emit fluorescence after 3-4 days of incubation. The distinctive colours of fluorescence can give an idea of the aflatoxin compound produced. For example, aflatoxin B is represented by a bluish fluorescence and the presence of a quantity of aflatoxin G1 changes the fluorescent colour to greenish-blue (Vogel 1965). Furthermore, these aflatoxin compounds can be extracted following the blending of the fluorescent agar media (Hara *et al.*, 1974; Lin and Dianese, 1976).

2.4. ASSAY METHODS FOR AFLATOXINS AND OTHER MYCOTOXINS IN CEREAL GRAIN

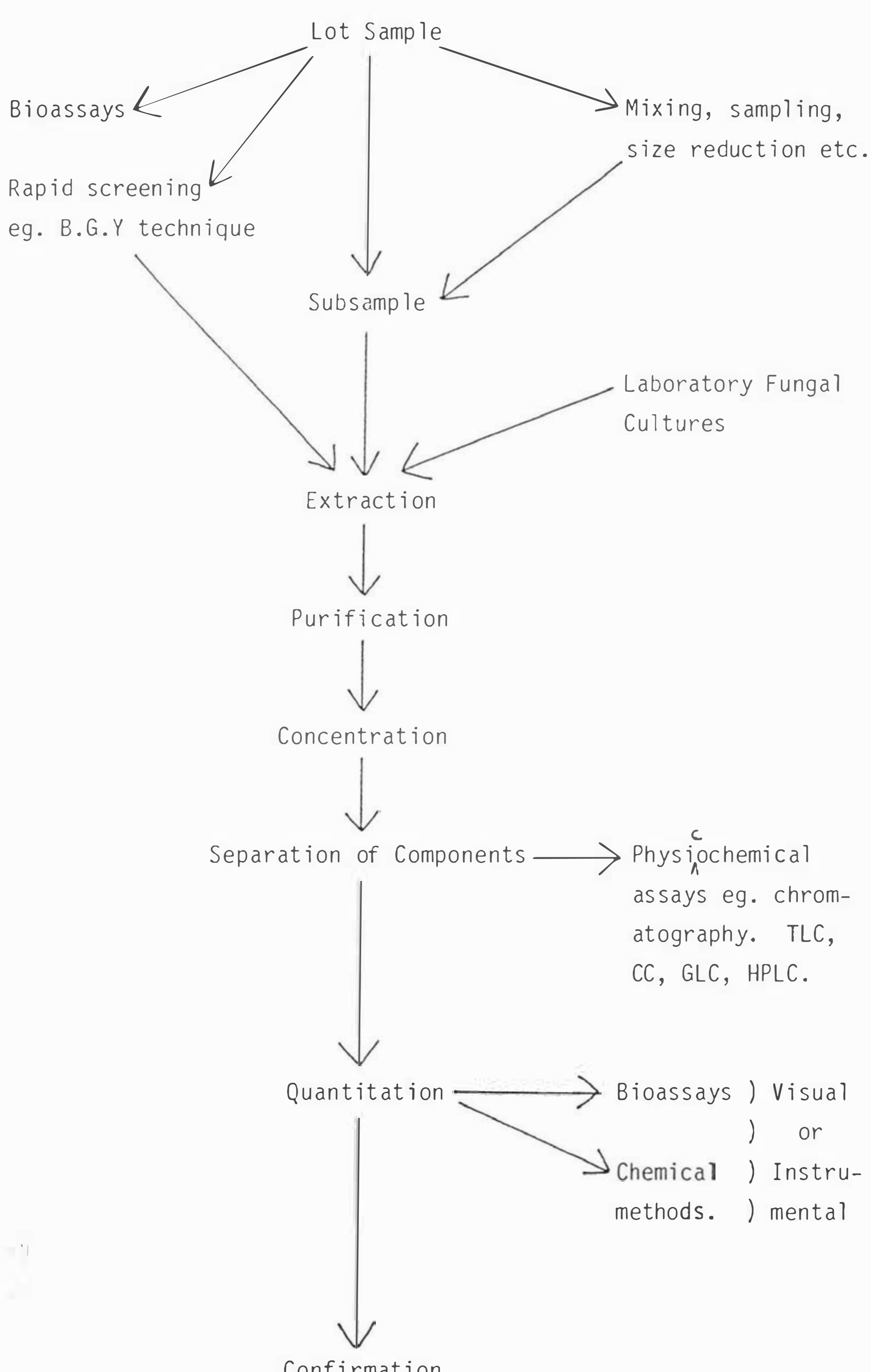
2.4.1. Introduction

Aflatoxins and other mycotoxins are known to occur in a wide variety of agricultural commodities. This together with the fact that they are present amongst a milieu of other compounds and trace elements has led to the development of hundreds of methods for detecting and analyzing them. Even a decade ago, Neshiem (1976) was able to list more than 100 physiochemical and biological methods for the qualitative and quantitative determination of the ochratoxins alone. The diverse physical and chemical properties of the mycotoxins themselves and the possibility of their multiple presence in a given substrate at any one time further contribute to the variability of methods and the complexity of designing mycotoxin assays.

Mycotoxin assays differ in their sensitivity and accuracy, in their rapidity and ease of performance, in their requirements for resources such as equipment, labour, time, materials, and finally in their applicability for extractions of mycotoxins from various materials. Some of these assays have been selected and tested in collaborative studies prior to their adoption as official methods of analysis. A standard book of recommended official methods is that published by the Association of Analytical Chemists (1980). Despite the existence of a large number of proposed methods of analyses, the choice of a method is determined by considerations such as composition, size and quantity of samples to be examined, the availability of resources and the aims of the study.

All mycotoxin assay methods basically comprise the general steps illustrated in figure 2-1. Although the determination of mycotoxins can be carried out using physicochemical or biological methods, chemical methods are preferred for routine analysis and for the quantitation and characterisation of mycotoxins. Biological methods are better employed to complement chemical methods for they are frequently time consuming, expensive and lack sensitivity and precision. Nevertheless, biological assays are essential in demonstrating toxicity and when no reliable chemical methods have been established.

Figure 2.1 Diagramatic Representation of General Assay Methods



2.4.2 Sampling

In any analysis, the total error is the sum of the sampling, subsampling and analytical errors (Whitaker, 1977), with the sampling error generally far exceeding that of the later two errors. This difficulty in sampling arises because of the heterogeneous nature of mycotoxin contamination (Lillehoj, 1979). Amounts of aflatoxins present, for example, may not only be small but these toxins can occur in only a few kernels or grains within the mass (Campbell, 1970). Thus a carefully taken sample is imperative in obtaining a truly representative sample of the lot.

In the recommended protocols for surveys and sampling of cereal grains for mycotoxins, Davis *et al*. (1980) emphasized that samples must be obtained at random from well blended grain lots and the sample sizes must be adequate for producing reliable and useful results. Whenever possible, minimum sample sizes of 5.0 kg per lot should be obtained. Furthermore, the authors advocated stream sampling or the collection and combination of small portions from a moving stream of grain at periodic intervals to give a "pooled" sample as the most effective method. Probe sampling is adequate for recently blended lots and is the next preferred sampling method. Both stream sampling and probe sampling are considered superior to field sampling of ears of grain.

2.4.3 Subsampling

Since it is not practical to analyse the entire sample from a lot for mycotoxins, The sample is often ground, mixed and subsamples taken by coning or quartering (Jones, 1972). Equal care must be taken in subsampling as in sampling from a lot, to ensure a representative sample. Recommended techniques for size reduction, mixing as well as subsampling can be found in the A.O.A.C.'s Manual of Offical Methods of Analysis (1980). The A.O.A.C. also recommends subsample sizes ranging from 20-100 g for different commodities but most subsamples of cereal grains taken for analysis of mycotoxins weigh 50 grams. This quantity is considered sufficient for a representative sample and for solvent economy. The grains should be comminuted to an adequate size so that the efficiency of toxin removal is improved.

2.4.4 General Methods of Analysis for Aflatoxins

When there are many samples to be examined in a survey for aflatoxins, it may be useful to apply a screening test to identify samples for further examination. Several researchers including Shotwell and Hesseltine (1981) and Blaney (1981) utilised the bright greenish yellow fluorescence technique as a rapid, qualitative presumptive test for aflatoxins in corn. Grains contaminated by A. flavus or A parasiticus and aflatoxins emit a B.G.Y fluorescence when viewed under long wave U.V light (365 nm). Unfortunately, the B.G.Y. fluorescence is sometimes due to the presence of kojic acid and not aflatoxins (Tulpule et al., 1977). In addition, Bothast and Hesseltine (1975) found both false positives and false negatives in their evaluation of the method on cereal grains including barley and other agricultural commodities. Nevertheless, it is the most rapid method to date for identifying suspect samples.

2.4.4.1 Toxin extraction

As a preliminary step, some commodities are defatted with diethyl ether, for example, prior to extraction for aflatoxins. This is recommended especially for oilseeds with a fat content greater than 5% (Jones, 1977).

There are two basic extraction techniques:

a) Exhaustive extraction: The most time-consuming; typically requiring 4-6 hours. Nabney and Nesbitt (1965) found that even though all aflatoxin B1 was removed in a 6 hour soxhlet extraction, 1.5-2.0% of aflatoxin was lost per hour extraction. Another disadvantage is the persistence of lipids, pigments and carbohydrates at the end of the extraction despite prior defatting and lipid removal measures.

b) Equilibrium extraction (Pons et al., 1969): A more effective and less time-consuming technique which can be carried out in either one of the following two ways:

i) Shaking the commodity for 10-30 minutes with solvents;

ii) High speed blending of the commodity with solvent in a Waring Blender for 1-3 minutes

Various combinations of organic solvents can be used as extractants. The mixture is allowed to separate after extraction permitting the partitioning of aflatoxins into the separated organic phase. An added advantage of this method is that one can combine a lipid-fat removal step simply by adding a fat-dissolving solvent such as hexane.

Both extraction methods just outlined involved the use of one or a combination of organic solvents (chloroform, methanol, ethanol, acetone, benzene) in which the aflatoxins are soluble (Moreau, 1979). Sometimes an aqueous solution is included in the solvent mixture to facilitate the penetration of hydrophilic tissues in order to extract the toxins.

2.4.4.2 Purification and concentration of toxins

Analysts have a wide choice of purification and clean-up methods to remove interfering lipids, pigments and other impurities extracted along with the aflatoxins. They include: liquid-liquid partitioning of toxins and interfering substances between immiscible liquids, filtration or centrifugation of insoluble precipitates formed by lead acetate or copper carbonate, absorption or physical retention of impurities in processes utilising absorbent materials, dialysis and eluting solvents in column or preparative thin layer chromatography (Jones, 1972).

Depending on the method chosen for initial extraction of toxins, a dilution or concentration step is required in preparing the sample for aflatoxin detection. Concentration of toxins can be achieved by evaporating away extractants in a rotary evaporator or under a stream of nitrogen in a steam or water bath.

2.4.4.3 Separation of components

All known chromatographic methods have been applied with varying degrees of success in separating aflatoxin components in purified

extracts. The early methods favoured paper, column or gas liquid chromatography. In more recent years, high pressure liquid chromatographic methods have gained popularity but undoubtedly thin layer chromatography is the most widely used technique (Moreau, 1979). Thin layer chromatography is a relatively easy method that is also very effective in separating the individual toxin components (Heathcote and Hibbert, 1975). The availability and versatility of commercially prepared precoated plates using high grade absorbent materials has further promoted its usefulness as an analytical tool. Also, where there are problems with impurities in extracts having similar chromatographic and fluorescent properties as aflatoxins, two dimensional thin layer chromatography can be performed to effect separation of toxins (W.H.O., 1979).

A rapid and inexpensive technique which the A.O.A.C. (1980) has adopted in their official procedures, employs minicolumns eluted with solvent mixtures to separate aflatoxins mostly in extracts of corn, cottonseed and peanuts (Romer et al., 1979). Minicolumns as devised by Holaday (1968), Velasco (1972) and others are available (Romer et al., 1979). The minicolumn techniques can detect very low levels of aflatoxins. The combined Holaday - Velasco method for example, can detect aflatoxin levels in the order of 10 $ng\ g^{-1}$ (Shannon et al., 1979). Unfortunately, this commonly used technique does not separate aflatoxin components.

2.4.4.4 Quantitation of toxins

Estimation of the amount of toxin present in a given extract can be achieved by chemical or biological methods with the actual measurement done visually or by instrumental means.

The fluorescence exhibited by aflatoxins is the basis for both visual and instrumental quantitation of aflatoxins following their resolution on T.L.C. plates. Early methods estimated quantities by dilution to extinction of fluorescence (Coomes et al., 1965). Another method introduced in the 1960's and still in use today compares sample fluorescence with fluorescence emitted by spots of known concentrations of aflatoxin standards (Pons and Goldblatt, 1969). Because visual comparison is subjective, accuracy varies according to the

analyst's ability and experience. Errors in estimation are claimed to be as much as 20-50% (Beckwith and Stoloff, 1968; Moreau, 1979). However precision can be vastly improved by the use of fluorodensitometric or spectrodensitometric methods. In cases where aflatoxins are separated by column chromatography, thin layer chromatography or high pressure liquid chromatography, various instruments, some with automatic recording devices are available for sensitive and precise quantitations.

Legator (1969), Detroy et al. (1971) and Moreau (1979) have reviewed the many biological assays which have been used for detecting and quantitating aflatoxins. These assays measure toxic effects of aflatoxins on a range of animal species, microorganisms, and cell and tissue culture systems.

2.4.4.5 Methods of confirmation

In spite of clean-up and separation procedures, the possible presence of extraneous, fluorescent material makes it necessary to perform confirmatory tests that provide conclusive proof of the presence of aflatoxins (W.H.O., 1979).

Aflatoxins can be identified by chromatographing a previously developed T.L.C. plate in an additional solvent system. A further method which has been used for a long time involves the spraying of developed aflatoxin spots on T.L.C. plates with dilute acids thus changing the fluorescence of aflatoxins from blue to green or yellow (Przybylski 1975). The presence of aflatoxins is ruled out should there be no colour change after acid treatment.

The formation of chemical derivatives such as water adducts or acetates by reacting aflatoxin extracts with trifluoroacetic acid (T.F.A.), formic acid plus thionyl chloride or acetic acid-thionyl chloride and concentrated hydrochloric acid and acetic anhydride are recommended as reliable confirmatory methods for aflatoxins by Jones (1972) and A.O.A.C. (1980) The techniques are rapid and simple when derivatization is performed directly on chromatograms (Stack et al., 1975). Moreau (1979) also listed other confirmatory methods utilising the numerous chemical properties of aflatoxins.
Formation of aflatoxin derivatives with trifluoroacetic acid is perhaps the most popular method.

2.4.5 Multimycotoxin Analysis

Surveys for mycotoxins in cereal grains have revealed the concurrent presence of a mixture of mycotoxin compounds (Shotwell *et al.*, 1970; Scott, 1972; Shotwell and Bothast, 1973; Shreeve, 1976 and Andrews *et al.*, 1981). A massive number of assays and techniques based on fundamental toxin extraction procedures and using a variety of solvent extractants and combinations of developing systems are available for the screening and simultaneous extraction of multiple toxins. For example, Gimeno (1979) proposed a T.L.C. method, which is capable of detecting up to 16 mycotoxins from a variety of commodities. Generally, most multimycotoxin methods lack the sensitivity of methods for the detection of individual toxins. However, improvements are continuosly being made on multimycotoxin detection techniques, the majority of which can be found in the journals of the A.O.A.C.

2.5 THE MYCOTOXIN SITUATION IN AUSTRALIA AND NEW ZEALAND

Australia and New Zealand are two countries in the Southern Hemisphere well-known for mycotoxicoses of domestic animals involving toxic standing pasture. Some of the diseases include ergotism, facial eczema, lupinosis, fescue poisoning and ryegrass staggers, of which facial eczema and lupinosis are of considerable economic importance (Culvenor, 1974). For many years, mycotoxin studies in Australia and New Zealand have concentrated on these toxicoses, resulting in a large number of publications which will not be discussed in this review.

Even though the study of mycotoxins associated with agricultural commodities have not been intensive, a few surveys of Australian cereals and other agricultural commodities have demonstrated the presence of aflatoxins, B1, B2, G1 and G2 (Baseden and Aldrick, 1970; Alisauskas, 1974; Bryden *et al.*, 1975; Bryden *et al.*, 1980; Blaney, 1981 and Connole *et al.*, 1981). Livestock feedstuffs and compounded feeds of local origin were the predominant commodities examined in these surveys. Many of these samples were either visibly mouldy or water damaged. The only unbiased survey was conducted by Blaney (1981) who found low aflatoxin incidence in Queensland's 1978 maize

crop. Nevertheless the aflatoxin levels found in these surveys could cause economic losses through insidious effects which reduce animal productivity (Bryden, 1982). As yet no aflatoxins have been found to contaminate Australian animal products (Dulley and Houlihan, 1979).

Sporadic cases of aflatoxicosis occuring in the relatively warmer and more humid regions of Australia have been reported (Table 2-7). These disease outbreaks, involving both housed and grazing animals, were the result of the ingestion of mouldy feed in all cases and in the two cases reported by Baird (1978) and McKenzie _et al_. (1981), the sheep and calves were also under water and food stress in drought conditions. There are other suspect cases of mycotoxicoses where aflatoxins were implicated but not proved to be involved (Bryden _et al_., 1980 and Connole _et al_., 1981). In addition, numerous factors including the complications caused by disease syndromes with features similar to aflatoxicosis make diagnosis of aflatoxicosis difficult (Bryden, 1982).

From the limited data available, it appears that the insidious effects of aflatoxins will be of far greater significance to the Australian livestock industry, but low levels and low incidences of toxin contamination of feedstuffs should reduce the risk of acute disease outbreaks even amongst the poultry and pig industries (Reichman _et al_., 1982; Bryden _et al_., 1982). The extensive grazing industry will only be at risk in conditions of drought or disease.

Table 2.6 Reputed Cases of Aflatoxicosis in Australia

Feedstuffs Implicated	Animals Affected	Aflatoxin levels (Averaged mg kg^{-1}) B1	B2	G1	G2	Australian State	References
Peanut Meal	Broiler Chicken	2.80	-	-	-	W. Australia	Gardiner & Oldroyd (1965)
Sorghum	Turkeys	>2.00	-	-	-	N.S.W.	Hart (1965)
Bread	Dogs	6.70	-	-	-	Queensland	Ketterer *et al.* (1975)
Maize	Ewes	-					Baird (1978)
Peanut Hay	Calves	2.82	0.02	4.25	0.01	Queensland	McKenzie *et al.* (1981)
Bread	Pigs	-				Queensland	
Peanut screenings	Pigs	15.00	0.50	15.00	0.75	Queensland	
Peanut screenings	Pigs	16.67	1.47	10.00	1.07	Queensland	Ketterer *et al.* (1982)
Sorghum	Pigs	3.00	3.95	5.26	0.90	Queensland	
Sorghum	Pigs	0.17	1.02	-	-	Queensland	

A range of toxigenic fungi including species of *Aspergillus, Penicillum, Fusarium* and *Alternaria* have been isolated from Australian agricultural commodities (Connole *et al.*, 1981; Morgan and Hocking, 1984; Sater *et al.*, 1984; Bryden, 1984). But the potential significance of these fungi is not known. The presence of aflatoxins in

Australia is not unexpected as aflatoxigenic strains of Aspergillus flavus are proven contaminants of many commodities, particularly deteriorated grain, in many parts of the country (Baseden and Aldrick, 1970; Bryden, et al., 1975; Connole et al., 1981). The presence of other toxigenic fungi could mean that there is a likelihood of the occurence of mycotoxins other than aflatoxins.

Indeed, Ketterer et al. (1982) reported the presence of 100 μg kg^{-1} ochratoxin A in a sample of sorghum grain used in pig rations, Suter et al. (1984) detected alternariol monomethyl ether and alternariol in a mouldy sorghum lot and Blaney et al., (1984) found zearalenone in 85% of the maize harvested in North Queensland.

There are a few proven cases of mycotoxicoses other than aflatoxicoses in Australia. One example is a case of swine vulvovaginitis in Victoria in 1937 (Pullar and Lerew, 1937). Another is the zearalenone intoxication of pigs reported by Blaney et al. (1984). There is no doubt that in the future increased attention will be paid to the occurrence and significance of various mycotoxins in Australia, in particular the Fusarium toxins, zearalenone and trichothecenes.

The mycotoxicoses which have caused problems to man and animals overseas have not been reported in New Zealand. Even comparable outbreaks of aflatoxicosis as reported in Australia, a neighbouring country with very similar agricultural practices as in New Zealand, is not known to occur in this country. There are a number of possible explanations; research into mycotoxin contamination of local agricultural commodities is scarce, the country is favoured by mild cool climatic conditions thus reducing the risk of mycotoxin formation, the New Zealand agriculture industry maintains high hygiene standards and New Zealand's livestock industry is predominantly pastoral. Feed intensive industries such as pig and poultry production are relatively small. Nevertheless, mycotoxins and mycotoxicosis can be of economic importance especially in relation to animal health in view of expected expansion of these industries. Already the existence of aflatoxigenic fungi has been reported in a survey by Freke and Richardson (unpublished) and from soil samples (Hussein 1983).

CHAPTER 3

THE FUNGAL FLORA OF STORED BARLEY

3.1. INTRODUCTION

Over the years, many studies have investigated various aspects of fungi associated with barley. Most of these investigations have used either direct plating onto agar plates of surface or non-surface disinfected grains, or dilution plating of comminuted grains as methods of isolation (Tuite and Christensen, 1955; Armolik *et al.*, 1956; Christensen, 1963; Sutey and Christensen, 1963; Clarke, Hill and Niles, 1966, 1969; Flannigan, 1969, 1970, 1978; Jorgensen, 1969, 1974; Harrison and Perry, 1976; Watson and Cameron, 1976; Abdel-Kader *et al.*, 1979; Abdel-Hafez and Abdel-Kader, 1980; Clarke and Hill, 1981; El-Kady *et al.*, 1982; and Abdel-Hafez, 1984).

Unfortunately, these two methods give only limited information as to the composition of the barley fungal flora with no indication of the locations of the fungi. As it is well known that various fungal propagules occurring on and within the grain could be responsible for its deterioration, a comprehensive assessment of the numbers and types of fungi occurring as surface or internal flora can be a very important means of determining the condition of the grain and whether deterioration is about to occur.

The studies reported here were concerned with the mycoflora of locally-grown barley, with emphasis on the changing levels of fungal contamination which occur during storage of the grain in commercial silos, in the laboratory and on farms.

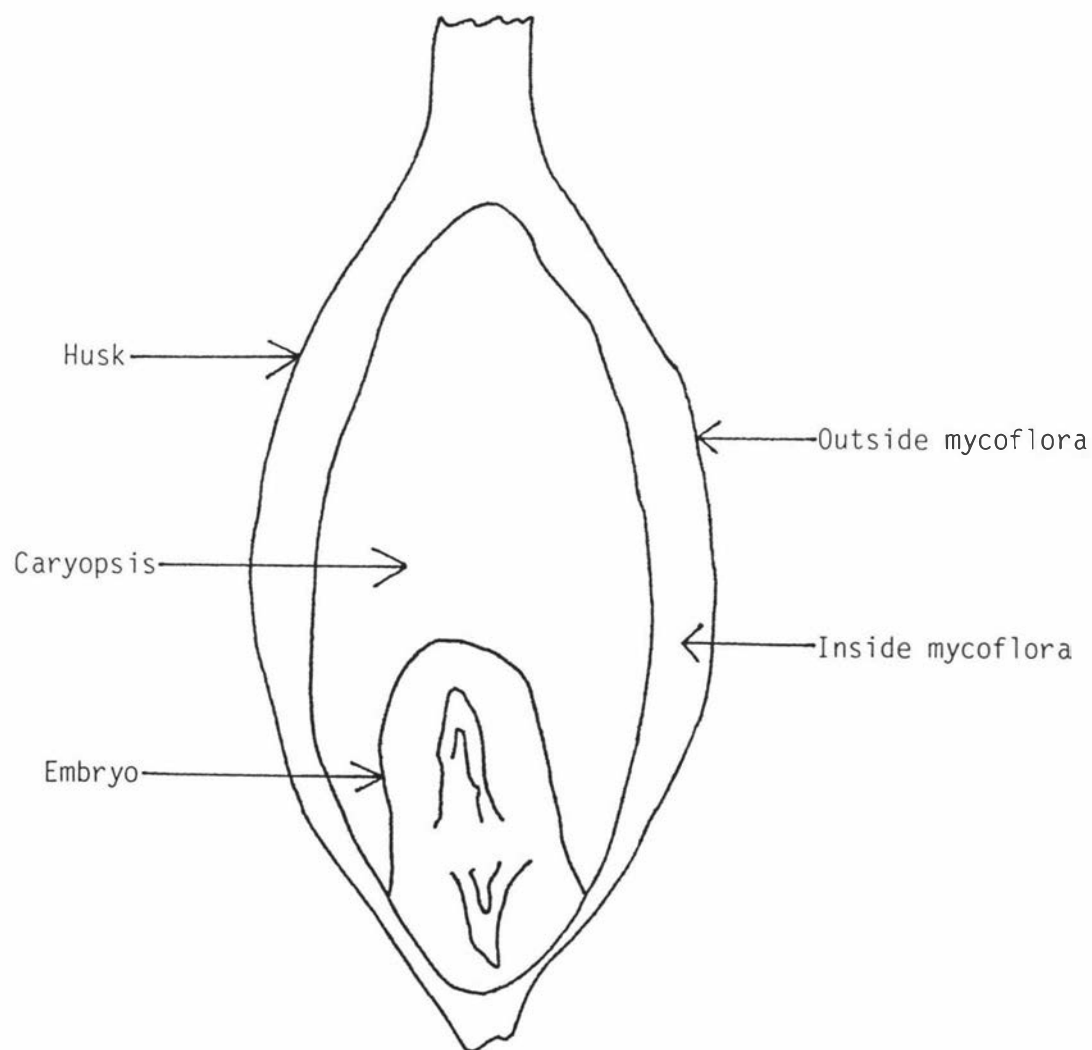
The choice of methods to be used for the isolation of fungi from barley requires a close look at the structure of a grain (Chapter 1, Figure 1-1). This kernel structure is of great significance in the production, storing, marketing and processing of the grain (Pomeranz *et al.*, 1978) and from an experimental point of view, its structure would affect the effectiveness of the selected fungal isolation techniques.

As has been described in Section 1.4.1 the rough and ridged structure of the lemma, the covering of fine hairs on the outer surface and, indeed, the barbed awn and hairy rachilla are significant

in providing excellent sheltered "harbours" for various fungal propagules.

The local barley grains used were characterised by the presence of a fairly 'tight-fitting' husk surrounding the actual caryopsis. A narrow space is thereby created between husk and caryopsis (Figure 3-1). In studying the fungal flora of such grains, it is, therefore, useful to differentiate between fungi found on the outer surface of the grain, which may represent only transient surface contaminants, and those on the 'inside' of the grain, where favourable conditions might lead to extensive development of mycelium which could be related to grain deterioration and later to the production of mycotoxin within the grain. The fungi present in the space between husk and caryopsis (including fungi on the inside surface of the husk) constitute what is differentiated in this study as fungi 'inside' the grain.

Figure 3-1: Simplified structure of a barley kernel showing location of the distinct areas of mycological interest



3.2. MATERIALS AND METHODS

3.2.1. The Barley Samples

3.2.1.1. Sources of samples

A. Grain at Harvest: The barley grain sampled at harvest was from the February-March 1981 crop, from farms located mainly in the Manawatu region of the North Island of New Zealand. Actual sampling (technique and quantity to be discussed later) was carried out on the premises of two major feed mills to which the harvested grain had been transported immediately after harvest. Upon arrival at the mills, the grain was checked for moisture content, and dried if necessary before storage. Forty percent malathion [5-(1,2-di-(ethoxycarbaryl)ethyl) dimethyl phosphorothiolothionate] was sprayed on the grain at the rate of about 1 litre per tonne of grain as it was being loaded into the silos. The sources and codes applied to the samples are tabulated in Table 3-1 (pp. 70-71).

B. Grain Stored in Commercial Silos: Mill A stored barley in 3 metal silos each of 350 tonnes capacity. The temperature of storage was between 11-14°C. Silo 1 was not aerated whilst silos 2 and 3 were aerated with an air extractor attached to the bases of the silos.

Mill B stored grain in sealed concrete silos of 1,050 tonnes capacity. The silos were linked to computerised and automated controls for monitoring silo conditions. The silos were all aerated and although the temperature of the grain bulk varied along the length of the silos, an average temperature of no more than 15.5^{o}C was maintained. Moisture content of the grain was maintained at 12-13%. The stored barley was sampled from both mills after 4-5 months of storage and again at 9-10 months of storage. Unfortunately, it was not possible to directly relate samples collected after storage to any particular sample which had been obtained at harvest time.

C. Laboratory-stored Grain: Samples obtained 'at harvest' were stored in sealed polythene bags in the laboratory at ambient temperature (12.5 - 20°C) for a period of 9 months.

A similar quantity of sample in sealed, double-layered brown bags, was kept in a cold room at a constant temperature of 4°C.

D. Farm-stored Grain: Barley, also from the late summer/early autumn (February-March) harvest but stored on-farm for 5-6 months, was also sampled. The farm-stored grain was either home-grown crop or had been bought from the mills. The grain was stored in sealed metal silos varying in capacity from 50-200 tonnes. Few of the farm silos had aeration systems (Table 3-1[d]).

3.2.1.2. Sampling of grain

A. Samples at Harvest: Grain collected at harvest from Mill A was obtained using a probe to sample the bulk load in trucks arriving at the mill. Grain from Mill B was 'stream' sampled as the grain was emptied from trucks into storage holds from where it was pumped into silos. Samples weighing between 1.0 - 5.0 kg were sealed in either polythene bags or double-walled brown paper bags for transfer to the laboratory.

In the laboratory, the grain was immediately subsampled for examination (see below) and the remainder divided for laboratory storage studies as follows:

i) If the bulk sample weighed about 1.0 kg it was divided into 2 equal portions and each was placed in a polythene bag and sealed. One bag was stored in the cold and the other at room temperature.

ii) Where a bulk sample weighed more than 2.0 kg it was split into 2 equal portions and one portion was halved yet again. One half obtained from the second step was sealed in a polythene bag for storage at room temperature. The other half was combined with the first (halved) portion, sealed in brown paper bags and stored in the cold.

B. Silo-stored Grain: Samples of silo-stored grain at both mills were obtained as the grain was removed for processing. Stream sampling at periodic intervals from shutes carrying the grain out of the silos was used to obtain 'pooled' samples of 2.0 - 5.0 kg each. The grain was sealed in bags for transfer to the laboratory.

C. Farm Samples: Two kg samples were obtained from the upper grain mass of each farm silo. Before each sampling, the grain contained in

an area approximately 1.0 m^2 by 30 cm deep was thoroughly mixed. In one case (Sample No. F8, Table 3-1), grain was collected from the shute. All the collected samples were then individually bagged and sealed.

3.2.1.3. Sub-sampling in laboratory

Sub-sampling of each sample in the laboratory began with thorough mixing of the grains in large glass flasks. Grain samples weighing about 1.0 kg were sub-sampled as a whole. Those weighing in excess of 1.0 kg were halved and halved again, if necessary, until a portion weighing about 1.0 kg was obtained for sub-sampling.

Trays with holding capacities of 1.0 kg and 0.6 kg were used for sub-sampling according to the weight of the grain to be sub-sampled (Figure 3-2a).

The grain from each sample was poured into the selected tray until it was full. The grain was levelled off and 5 flat-bottomed tubes (volume = 53 cm^3 each) were pushed into the tray of grain as illustrated in Figure 3-2a. The loose grain was poured out of the tray carefully in such a manner that the tubes and contents were retained.

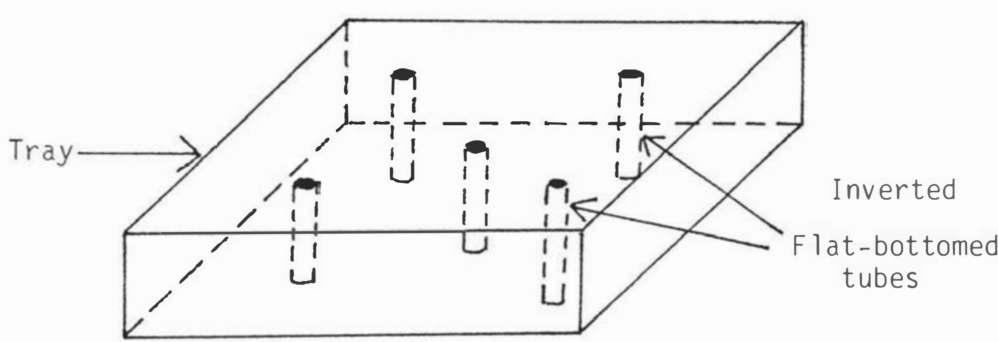
Grain held in these 5 tubes was then poured into a small sterile steel dish, levelled and another similar tube pushed into the centre of the grain mass (Figure 3-2b). The loose grain in the dish was emptied out and the grain trapped in the tube was transferred to a sterile glass bottle with a tight-fitting screw cap. The procedure just described was repeated twice more, using the grain emptied out of the dish each time. In this manner a composite sample weighing about 250 g was drawn from the original sample.

This composite sample was immediately mixed and 2 lots of 5.0 g and 3 lots of 1.0 g were accurately weighed out into sterile 1 oz universal screw-capped bottles for actual experimental purposes. Grain remaining after this last random sampling step was stored in the tightly-capped bottle in the cold.

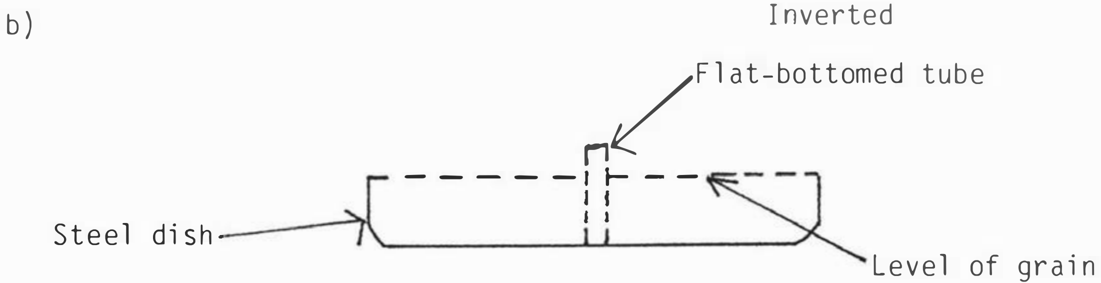
All the containers and other apparatus used were thoroughly cleaned and sterilised by autoclaving or by swabbing the surfaces with 95% ethanol between sub-sampling each barley sample.

Figure 3-2: Apparatus for sub-sampling in the laboratory

a)



b)



3.2.1.4. Moisture content of samples

A. Moisture Content Recorded Prior to Storage: Tables 3-1 (a), (b), (c), (d) show the moisture content of grain measured by mill personnel and by individual farmers before the grain was loaded into the silos.

B. Laboratory Determinations: Following sub-sampling of the grain samples in the laboratory, their moisture content was determined using a method adapted from the American Association of Cereal Chemists, methods 44-15A and 44-19, (1976).

Replicate sub-samples of each barley sample, weighing 5 g each, were used for moisture determinations. The method used determines moisture content as loss in weight of a sample when heated under specific conditions.

i) Apparatus used:

1. Beam balances with accuracies up to 4 decimal places.
2. Oven (gravity convection) equipped with a thermometer capable of reading up to 300°C. The thermometer was kept just above the drying dishes and the temperature could be read through the oven's glass window. The temperature of the oven varied by not more than 7°C from the set temperature.
3. Moisture dishes made of aluminium, covered by pieces of tinfoil. Each dish and foil was labelled with the same number.
4. Air-tight glass desiccators containing concentrated H_2SO_4 as drying agent.

ii) Procedure:

1. Dishes with their corresponding tinfoil covers were dried overnight at 130°C. They were then transferred to desiccators, allowed to cool to room temperature (about 1 hour) and the weights of dishes and foils were individually recorded with a beam balance.
2. Each 5 g grain sample was placed in a separate dish. The uncovered dishes were lined up with their respective covers in a preheated oven at 135°C. Drying of the grain

took 2 hours from the time the oven temperature returned to and settled at about 135°C.

3. At the end of the 2 hours, the covers were put back on their respective dishes, the covered dishes transferred to a desiccator and allowed to cool to room temperature.
4. Individual weights of covered dishes and grain were obtained and the loss in weight calculated.

   The moisture content of each 5 g of grain was calculated applying the following formula:-

$$\% \text{ moisture} = \frac{\text{loss in weight (= loss in moisture)}}{\text{weight of sample}} \times 100$$

5. All the dishes were placed back in the oven to dry at a steady temperature of 135°C for a further 1 hour. The dishes were again cooled in a desiccator to room temperature and moisture content calculated from the losses in weight as set out in the previous step.

   The moisture content listed for each sample in Tables 3-1 (a), (b), (c) and (d) were the averaged moisture contents of duplicate samples obtained after 3 hours of drying.

Figure 3-3: Summary of Total Counting Technique for non-surface sterilised grain (n.s.s. total counts) and surface-sterilised grain (s.s. "inside" counts) - see text.

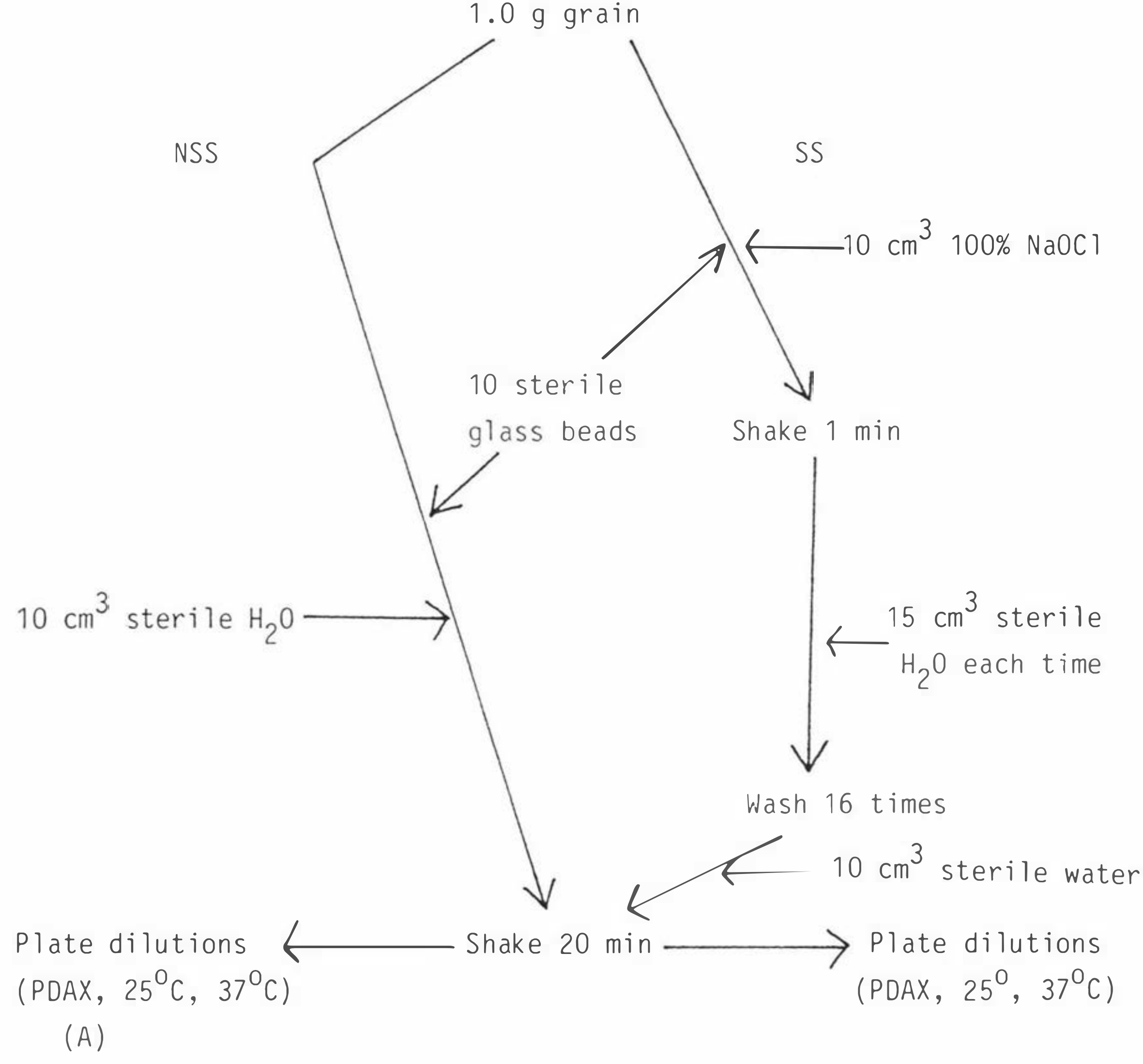
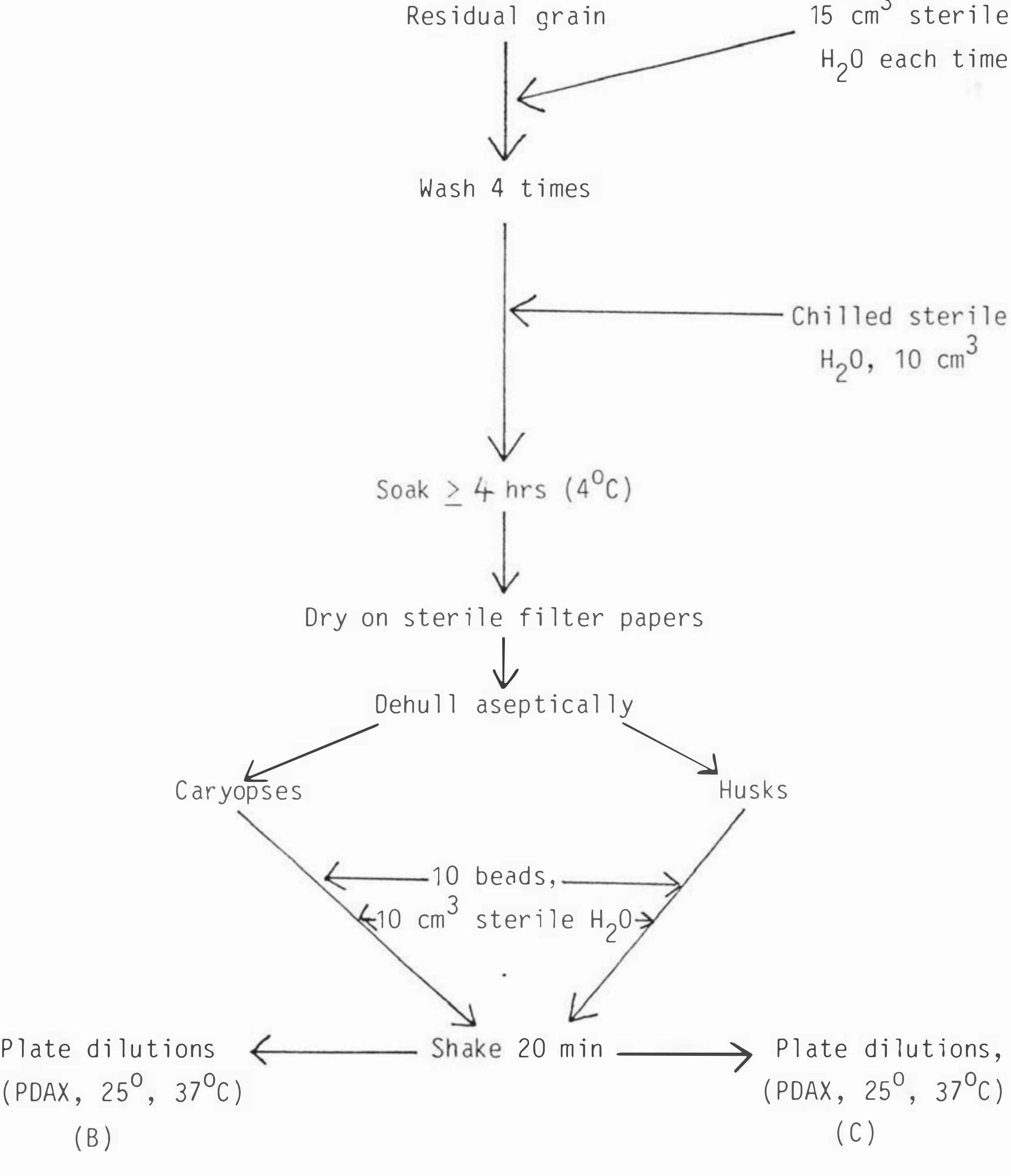


Figure 3-3 (continued)



Total count = A + B + C

Inside count = B + C

Outside count = Total - (B + C)

3.2.2. Mycoflora of Barley Grains

The mycoflora of each barley sample was examined by individually analysing triplicate lots of 1.0 g grains following the scheme described in Figure 3-3.

3.2.2.1. Total viable counts

A. Whole Grain: The total counting technique used in these studies (Figure 3-3) involved shaking 1.0 g grain in 10 cm^3 sterile distilled water with 10 sterile glass beads for 20 minutes on a reciprocating shaker with a 1 cm throw, 5 throws a second.

i. Dilution Plating and Counting: The preparation was shaken vigorously on a "Super-mixer"* for 15 seconds, after which 1.0 cm^3 of the supernatant was instantly and aseptically transferred to 9.0 cm^3 sterile distilled water. Depending on the condition of the grain, a series of dilutions ranging from 10^{-1} to 10^{-5} was similarly prepared. With the exception of mouldy samples, dilutions of up to 10^{-2} of supernatant were sufficient to give satisfactory total counts. Immediately before each transfer of suspension in the diluting process, the suspension was briskly shaken for 15 seconds to thoroughly mix suspended materials and to ensure no sedimentation.

Triplicate 1.0 cm^3 quantities of neat supernatant and dilutions were aseptically plated out for each sample following vigorous mixing. The dilutions were placed in sterile petri dishes, quickly followed by 15-18 cm^3 of molten, warm potato dextrose agar + 0.05 mg/cm^3 chloramphenicol (PDAX). The contents were carefully mixed to uniformly distribute suspended materials by gentle swirling and left to set at room temperature.

One plate of neat supernatant was incubated at 37°C and all the other plates were incubated at 25°C, in the dark. They were first examined on the second day of incubation, and counts of plates with 20 to 200 colonies were recorded on

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

the 3rd, 4th and 6th days of incubation. Suitable colonies were selected from all the plates and subcultured and maintained on slopes and plates of PDAX for identification as set out in Section 3.2.2.5. Fungal colonies were transferred to fresh PDAX plates for identification.

B. Caryopsis and Husks: After plating the supernatant from the first 20 minutes of shaking, the supernatant was discarded and the whole grains washed 4 times with 15.0 cm^3 of sterile distilled water each time. The grains were then soaked for a minimum of 4 hours at 4^oC, before they were dehusked aseptically with sterilised forceps in a cabinet previously sterilised by ultra-violet light irradiation for 1 hour.

i. Dilution plating and counting: The husks and caryopses were put into separate sterile 1 oz screw-capped universal bottles. When all grains in the 1.0 g sample were dehusked, 10 sterile glass beads and 10 cm^3 of sterile distilled water were aseptically added to each universal bottle. The husk and caryopsis fractions were separately shaken and the supernatants plated, and subsequent colony counts made as outlined in Section 3.2.2.1A.

This method can thus give the total viable count per gram of grain by simple addition of the averaged counts for each fraction of grain.

3.2.2.2. Viable counts inside grain

By repeating the method with a similar quantity of grain, but this time subjecting it to surface sterilisation before the first shaking step (as shown on the right side of Figure 3-3), counts of the inside mycoflora can be made by adding the averaged dilution plating results of husks and caryopses.

The additional surface sterilisation step mentioned in this section involved shaking the grain with 10 cm^3, 100% sodium hypochlorite solution in the presence of 10 sterile glass beads, for 1 minute. The NaOCl solution was immediately discarded and the grains

washed 16 times, using 15.0 cm^3 sterile water and gentle agitation each time.

3.2.2.3. Viable counts outside grain

Thus, with the knowledge of the total viable count and the total inside count, a complete count for the outer surface only can be computed from the difference between the two counts just mentioned.

3.2.2.4. Fungi of barley fractions

Supernatants left after dilution plating of husks and caryopses from both surface sterilised (s.s.) and non-surface sterilised (n.s.s.) grains were discarded. The barley tissues from each sample were separately washed 4 times with 15 cm^3 sterile distilled water each time in preparation for culturing and microscopy.

A. Culturing of Tissues: Husks - Ten pieces of husk tissue were picked up with sterile forceps, dried on sterile filter paper and plated on PDAX. Five pieces were placed with their upper surfaces in contact with the agar and the remaining 5 pieces were plated with their inner surfaces down on the agar surface.

Caryopses - Five whole fractions were carefully dried on sterile filter paper and plated on PDAX agar.

A further 2 whole caryopses were similarly dried and then sectioned longitudinally. Thin transverse sections were also made from another 2 whole caryopses. All sectioning was done by hand using a sterilised scalpel for each cut. The longitudinal sections were plated with their cut surfaces on the PDAX plates whilst the transverse sections were carefully laid on similar agar plates.

All the plates of husks and caryopses were incubated at 25°C and examined at the same time as the dilution plates. Any fungal growth from these tissues was transferred to fresh PDAX plates to await identification.

B. Microscopy of Tissues: Pieces of washed lemma and palea were stained in aniline blue, or lactophenol cotton blue prior to microscopy of their inner and outer surfaces.

Microscopy of stained sections of caryopses were carried out only after softening of the tissues with 15% potassium hydroxide.

3.2.2.5. Identification and maintenance of cultures

Fungi subcultured from dilution plates were identified to genus and where possible to species according to their cultural, macroscopic and microscopic characteristics with the aid of identification criteria set out in a number of books including: Gilman (1957), Carmichael (1962), Raper and Fennell (1965), Barron (1968), Raper and Thom (1968),Tousson and Nelson (1968), Smith (1969), Booth (1971), Ellis (1971, 1976), Barnett and Hunter (1972), Pitt (1979) and McGinnis (1980).

Prolonged incubation on PDA, culturing on Malt Salt agar, Czapek Dox agar (CDA), Sabouraud Dextrose agar (SDA), Hay Infusion agar at 25°C and 37°C were some of the techniques used to induce spore formation by non-sporulating colonies in order to facilitate their identification.

Fungal cultures isolated onto PDA from dilution plates and barley fractions were maintained on PDA, CDA and SDA and these cultures were stored in the cold and at room temperature. They were subcultured onto fresh media every six months.

3.3. RESULTS

3.3.1. Moisture Contents

Table 3-1(a) shows that for the eight freshly-harvested barley samples generally the moisture contents (M.C.) reported by the mills were in close agreement with those obtained by laboratory determination. In commercial silos, the M.C. of the grain was about the same after 5 months' storage (average M.C. of 12.68% compared with 12.81% at harvest), but M.C. decreased to an average of 11.92% after a further 4 months in storage (Table 3-1[b]). The M.C. of grain kept in the cold for 9 months was approximately the same as that of the grain at harvest (Table 3-1[c]), but those samples kept at ambient temperature displayed a decrease in average moisture level (averaged M.C. = 12.03%) similar to that observed with samples stored in commercial silos for the same period (Table 3-1[c]).

On the farms, the reported M.C.'s were generally higher than those estimated in the laboratory but in the case of Sample F1, which showed obvious signs of spoilage, the M.C. was higher after storage than at the time of harvest. Also, grain tended to be harvested and stored at higher moisture levels on the farms than those kept in the mills.

Table 3-1: Moisture content and conditions of barley samples

a) Grain at harvest

Sample Source	Code	Moisture Content (MC) A (%)	Moisture Content (MC)# B (%)	Condition of sample
Mill A	A1		13.10	Good*
	A2	About 12.00	12.79	"
	A3		12.63	"
	A4		12.53	"
Mill B	B1	14.10	13.96	"
	B2	12.00	12.07	"
	B3	13.10	12.47	"
	B4	12.10	12.91	"

Average MC = 12.81

* Good condition = grains were clean, good quality, no obvious infestations.

b) Grain stored in commercial silos for 5 months

Source	Storage 5 mths Code	Storage 5 mths MC (B) %	Storage 9 mths Code	Storage 9 mths MC (B) %	Condition of sample
Mill A	A5*	13.44	A8	13.34	Slight weevil infestation, heated
	A6	12.14	A9	11.76	Good
	A7	11.64			
Mill B	B5	13.37	B9	11.71	Good
	B6	12.67	B10	11.78	"
	B7	12.28	B11	11.75	"
	B8	13.22	B12	11.75	"
	Average = 12.68		Average = 11.92		*Silo 1 - not aerated

M.C.: A and B, refer to bottom of p. 71.

c) Grain stored 9 months in cold and at ambient temperature in the laboratory

Storage at ambient temp.			Storage in cold		
Source	Code	MC (B) %	Code	MC (B) %	Condition of sample
Mill A	A1	12.18	A1	12.47	Good
	A2	11.58	A2	12.97	"
	A3	11.75	A3	11.36	"
	A4	12.08	A4	11.45	"
Mill B	B1	12.76	B1	12.15	Good
	B2	11.53	B2	12.24	"
	B3	11.63	B3	12.05	"
	B4	12.69	B4	12.53	"
	Average =	12.03	Average =	12.40	

d) Grain stored on farms 5-6 months

Source code	MC (A) %	MC (B) %	Conditions of storage	Condition of samples at sampling time
F1	14.0	16.42	Silo, aerated	Heated, mouldy, caked & germinated grains
F2	13.0	14.36	Silo, non-aerated	Heated, mouldy, caked heavy weevil infestation
F3	14.0	15.35	Silo, non-aerated	Heated, mouldy, weevil infestation
F4	14.0	12.80	Silo, non-aerated	Mouldy, caked, germinated grains
F5	14.0	13.77	Silo, non-aerated	Slightly mouldy
F6	14.0	12.57	Silo, aerated	Good
F7	14.0	13.69	Silo, non-aerated	Good
F8	16.0	13.63	Silo, non-aerated	Slightly damaged

F8 - Treated with Siloguard, Maldison 2%, Premium grade

MC: A - Moisture content prior to storage as reported by farmers/mill

B - Moisture content determined after sub-sampling in the laboratory

3.3.2. Viable Counts of Barley Fungi

As an aid to assessing the significance of grain contamination it was useful to differentiate levels of surface and internal fungal contaminants, as mentioned in Section 3.1.

All counts are the averaged results of 3 determinations using 1 g lots of grain by the method outlined in Figure 3-3 and the counts are expressed as colony-forming units per gram of grain. No counts were made of the yeast and bacterial populations, thus the viable counts reported here are those of filamentous fungi only.

Due to the variability of the counts obtained and difficulty of establishing a sound base for statistical analysis, these results are simply presented as actual counts and in summarised form in Figures 3-4 to 3-10.

3.3.2.1. Samples obtained at harvest (Table 3-2[a])

Of the eight grain samples obtained at harvest, there was a wide variation in total count (T.C.) ranging from 1.84×10^3 for Sample A1 to 51.9×10^3 C.F.U. g^{-1} for Sample B1. Samples A1 and A3, both originating from Mill A, showed relatively low total viable fungal counts.

Table 3-2: Average viable counts (Colony-forming units g^{-1}) of grain at 25°C

[a] At harvest

Sample	Viable counts C.F.U. g^{-1} ($x10^3$)		
	Outside	Inside	Total Counts
A1	0.95	0.89	1.84
A2	14.28	6.44	20.72
A3	2.17	0.11	2.28
A4	30.17	4.08	34.25
B1	40.52	11.40	51.92
B2	12.48	3.37	15.85
B3	22.00	2.66	24.66
B4	23.92	6.19	30.11

Range = $1.84 \times 10^3 - 51.92 \times 10^3$ C.F.U. g^{-1}

[b] Commercial silos (5 months' storage)

Sample	Viable counts C.F.U. g^{-1} ($x10^3$)		
	Outside	Inside	Total
A5	13.08	1.20	14.28
A6	3.03	0.25	3.28
A7	6.28	0.13	6.41
B5	23.59	2.76	26.35
B6	18.26	1.35	19.61
B7	27.09	1.06	28.15
B8	18.41	0.92	19.33

Range = $3.28 \times 10^3 - 28.15 \times 10^3$ C.F.U. g^{-1}

[c] Commercial silos (9 months' storage)

Sample	Viable counts C.F.U. g^{-1} ($x10^3$) Outside	Inside	Total
A8	919.12	38.23	957.35
A9	3.59	0.43	4.02
B9	7.45	0.63	8.08
B10	2.74	0.18	2.92
B11	5.44	1.20	6.64
B12	4.03	0.15	4.18

Range = 2.92×10^3 - $9\,57.35 \times 10^3$ C.F.U. g^{-1}

[d] Ambient temperature, sealed (9 months' storage)

Sample	Viable counts C.F.U. g^{-1} ($x10^3$) Outside	Inside	Total
A1	0.03	0.01	0.04
A2	0.82	0.10	0.92
A3	0.02	0.00	0.02
A4	1.29	0.12	1.41
B1	0.13	0.02	0.15
B2	0.33	0.07	0.40
B3	0.10	0.03	0.13
B4	0.41	0.14	0.55

Range = 0.02×10^3 - 1.41×10^3 C.F.U. g^{-1}

[e] Cold, $4^{o}C$, sealed (9 months' storage)

Sample	Viable counts C.F.U. g^{-1} ($x10^3$) Outside	Inside	Total
A1	5.90	0.07	5.97
A2	14.95	13.03	27.98
A3	0.50	0.03	0.53
A4	24.07	0.60	24.67
B1	30.58	0.08	30.66
B2	8.17	1.38	9.55
B3	3.98	0.26	4.24
B4	13.94	1.08	15.02

Range = $0.53 \times 10^3 - 30.66 \times 10^3$ C.F.U. g^{-1}

[f] Farm silos (5-6 months' storage)

Sample	Viable counts C.F.U. g^{-1} ($x10^3$) Outside	Inside	Total
F1	2260.00	5073.33	7333.33
F2	634.05	34.29	668.34
F3	586.39	58.39	644.78
F4	283.34	254.50	537.84
F5	224.22	2.45	226.67
F6	11.38	0.12	11.50
F7	16.30	14.80	31.10
F8	255.28	0.62	255.90

Range = $11.50 \times 10^3 - 7333.33 \times 10^3$ C.F.U. g^{-1}

Key to Figures 3-4 to 3-9

▬ Total viable counts g^{-1} of grain

ᴅᴅᴅ Total inside counts g^{-1} of grain

////// Total outside counts g^{-1} of grain

Figure 3-4: Total, inner and outer surface counts of grains at harvest (CFU g^{-1} x 10^3) at 25^{0}C.

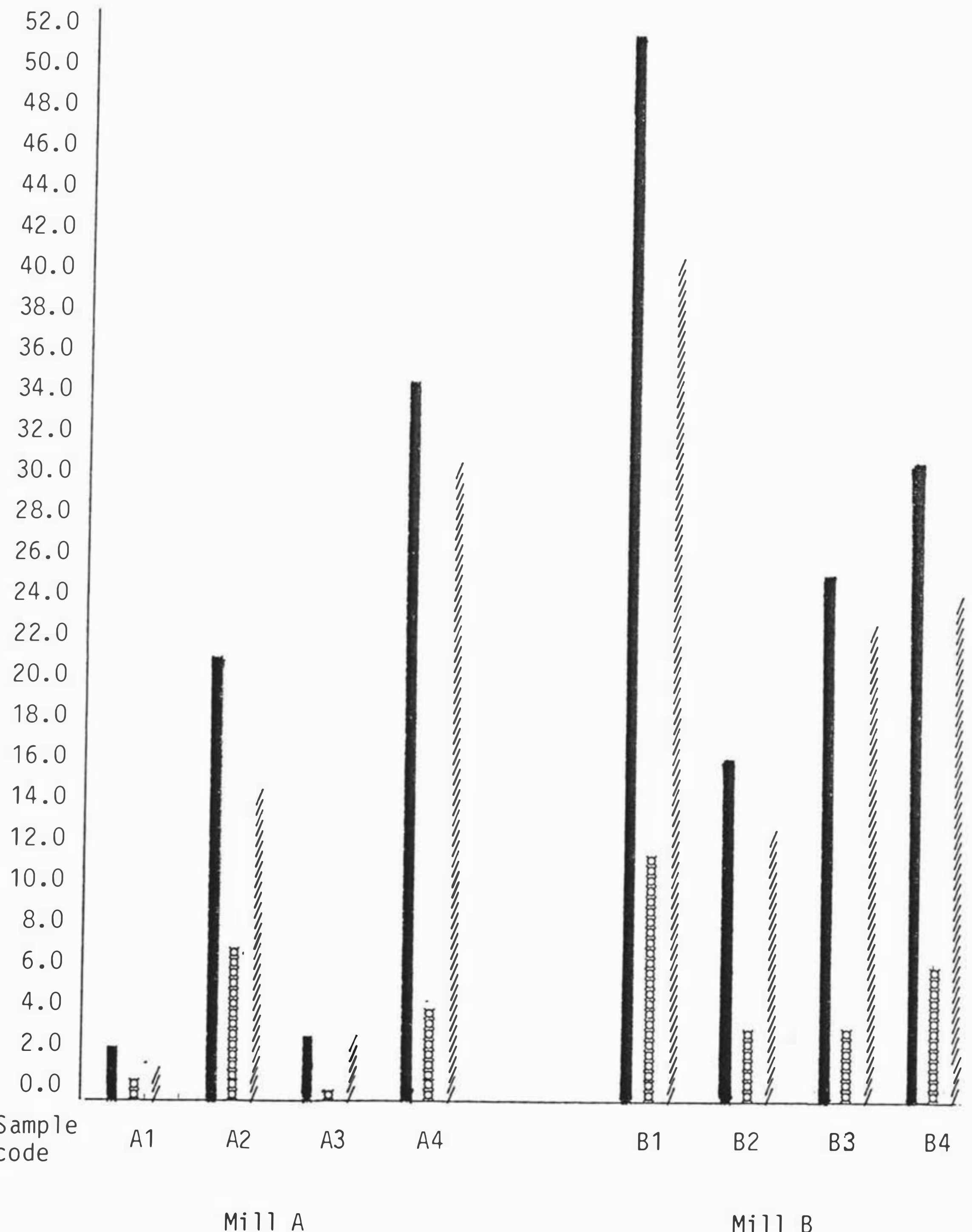


Figure 3-5: Total, inner and outer surface counts of grains stored in commercial silos for 5 months (CFU g^{-1} x 10^3) at 25^oC.

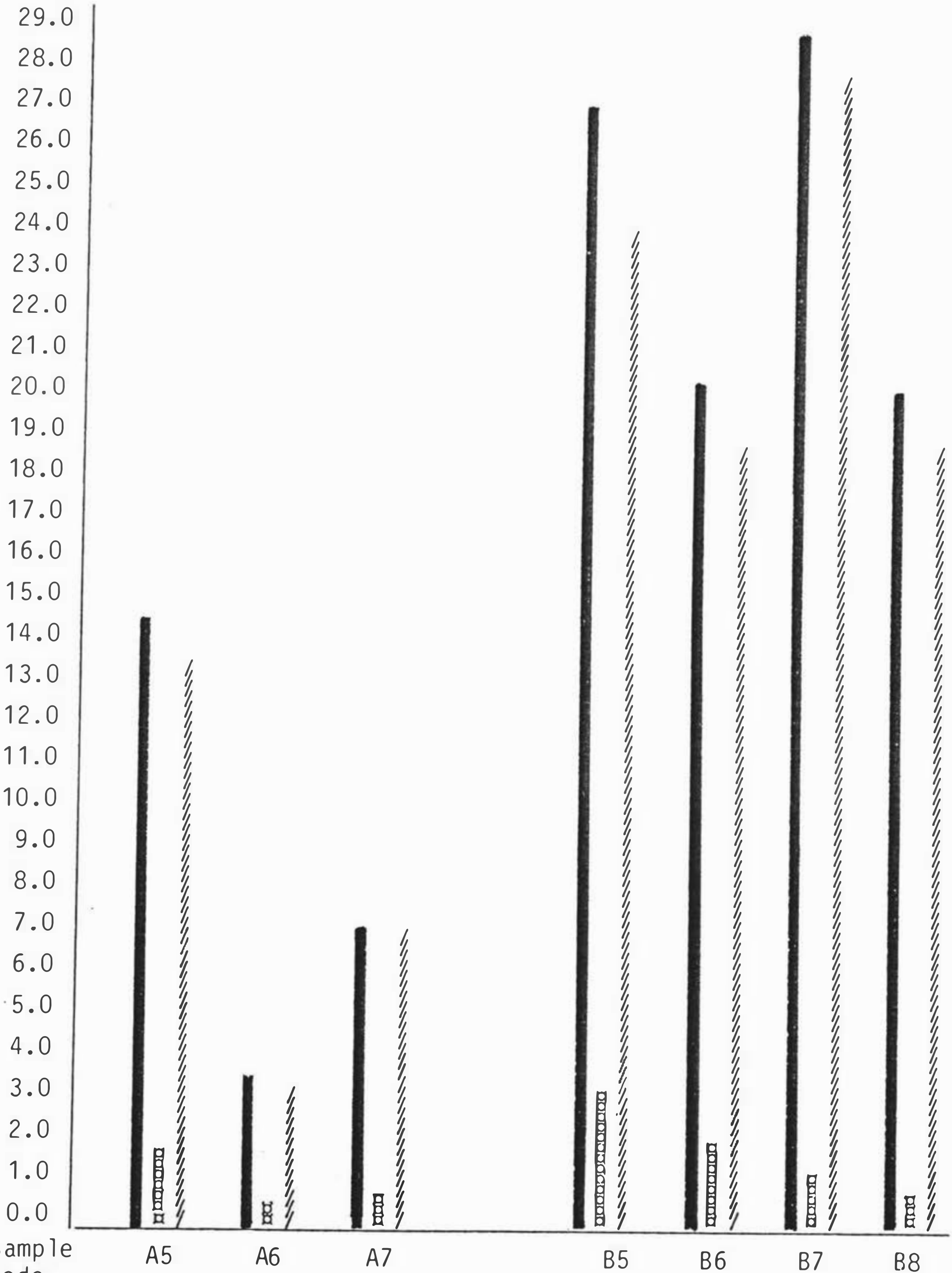


Figure 3-6: Total, inner and outer surface counts of grains stored in commercial silos for 9 months (CFU g^{-1} x 10^3) at 25^oC.

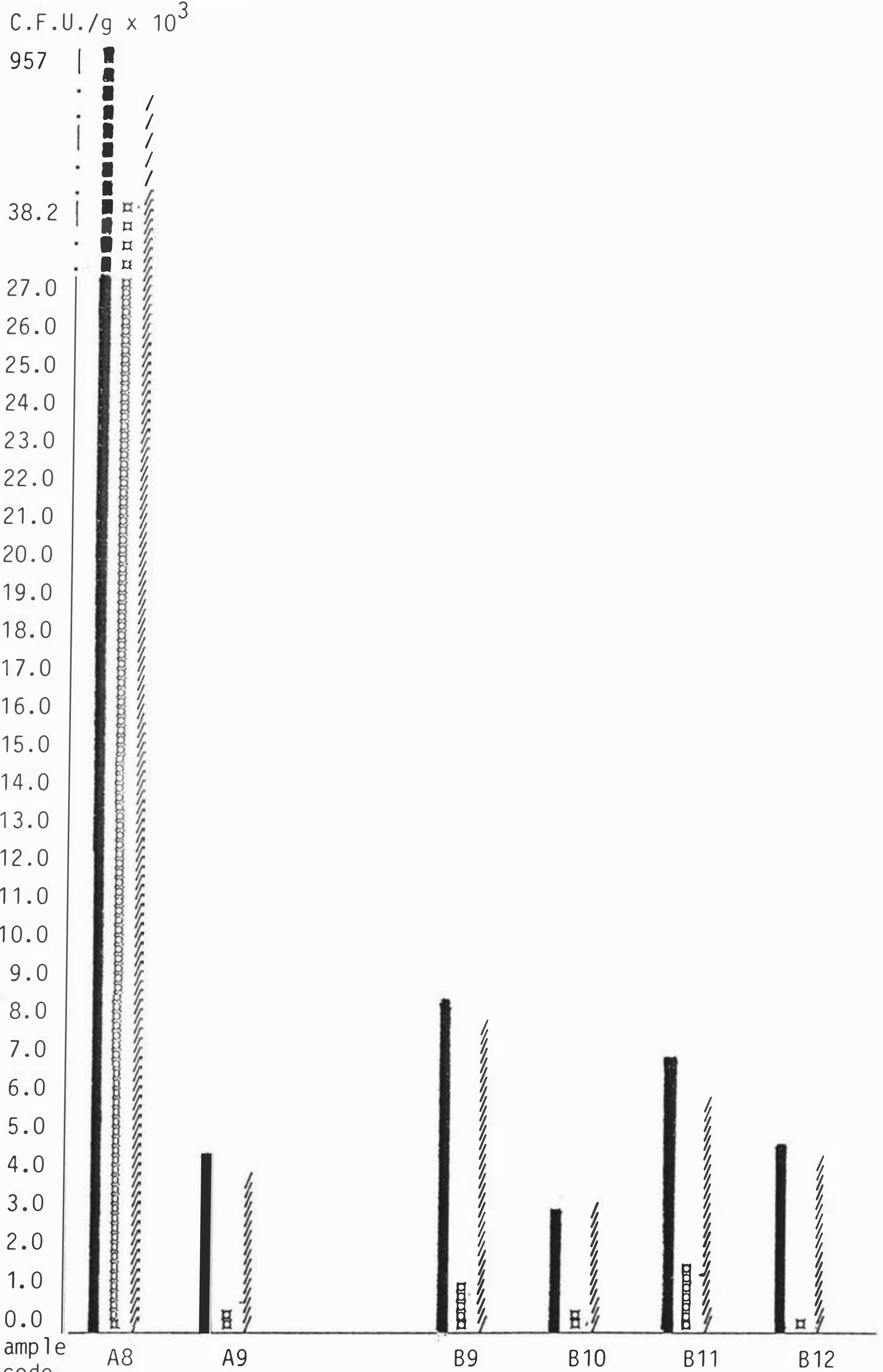


Figure 3-7: Total, inner and outer surface counts of grains stored at ambient temperature for 9 months (CFU g^{-1} x 10^3) at 25°C.

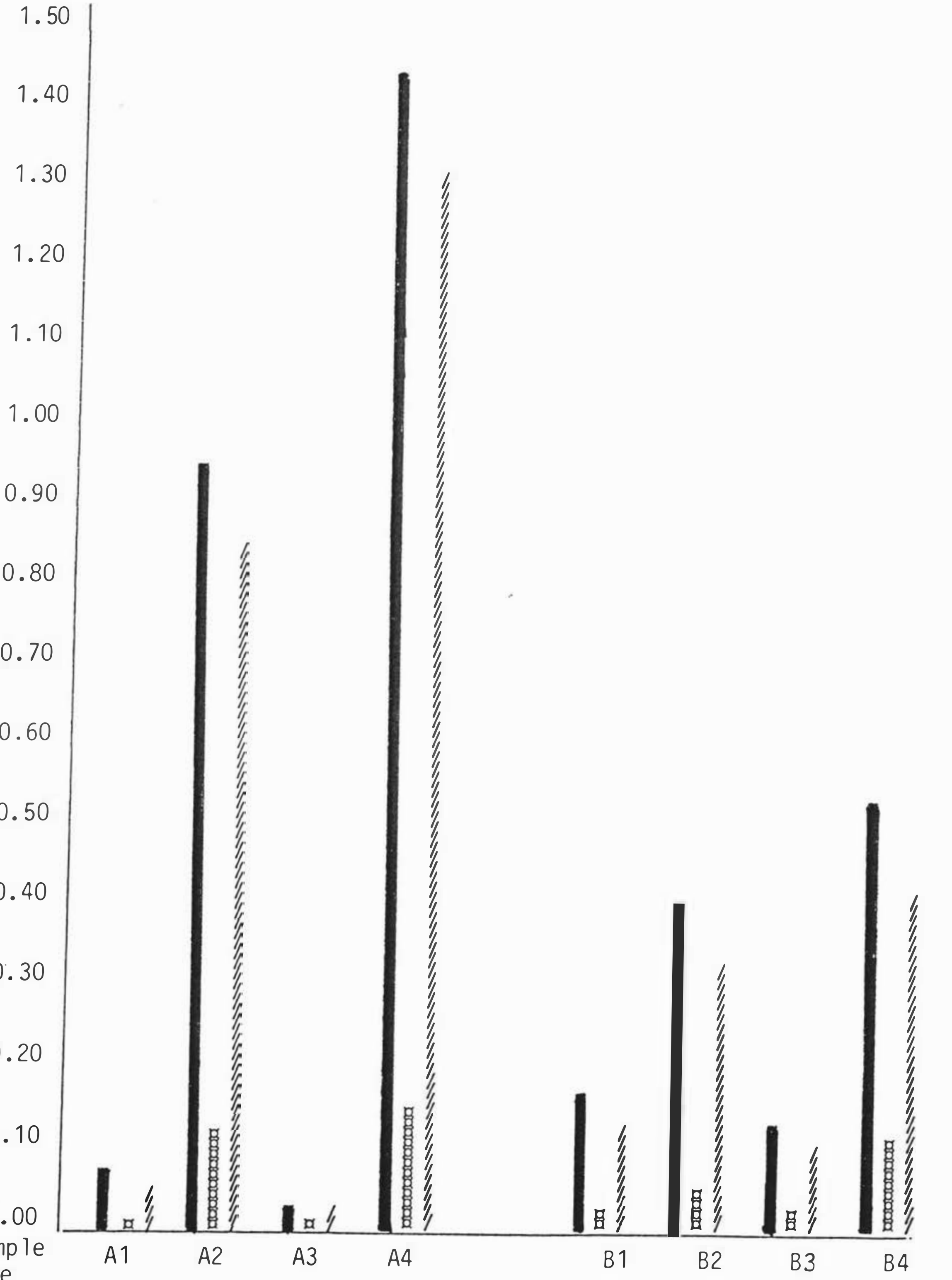


Figure 3-8: Total, inner and outer surface counts of grains stored in cold (4 ^{o}C) for 9 months (CFU g^{-1} x 10^3) at 25^oC.

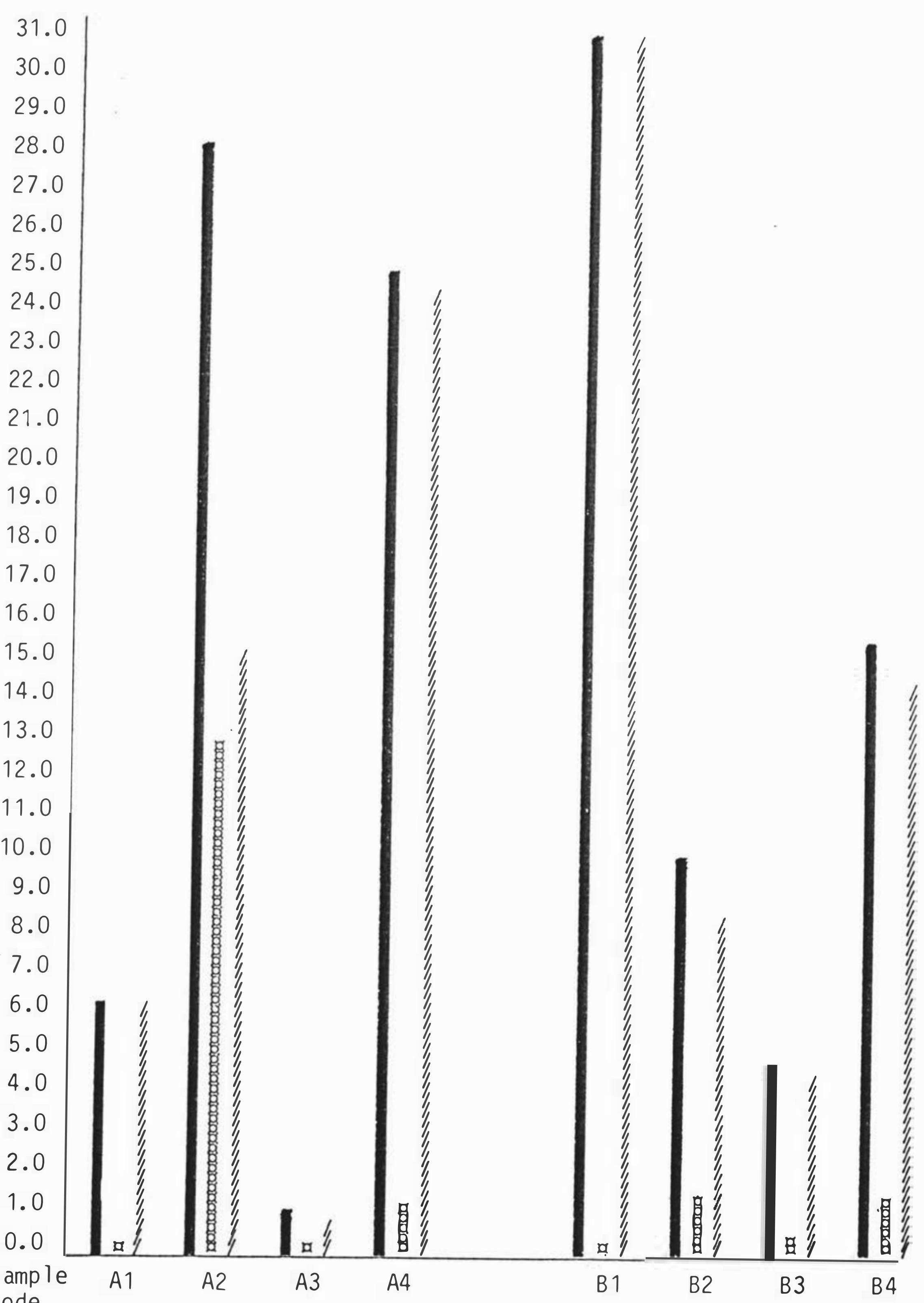
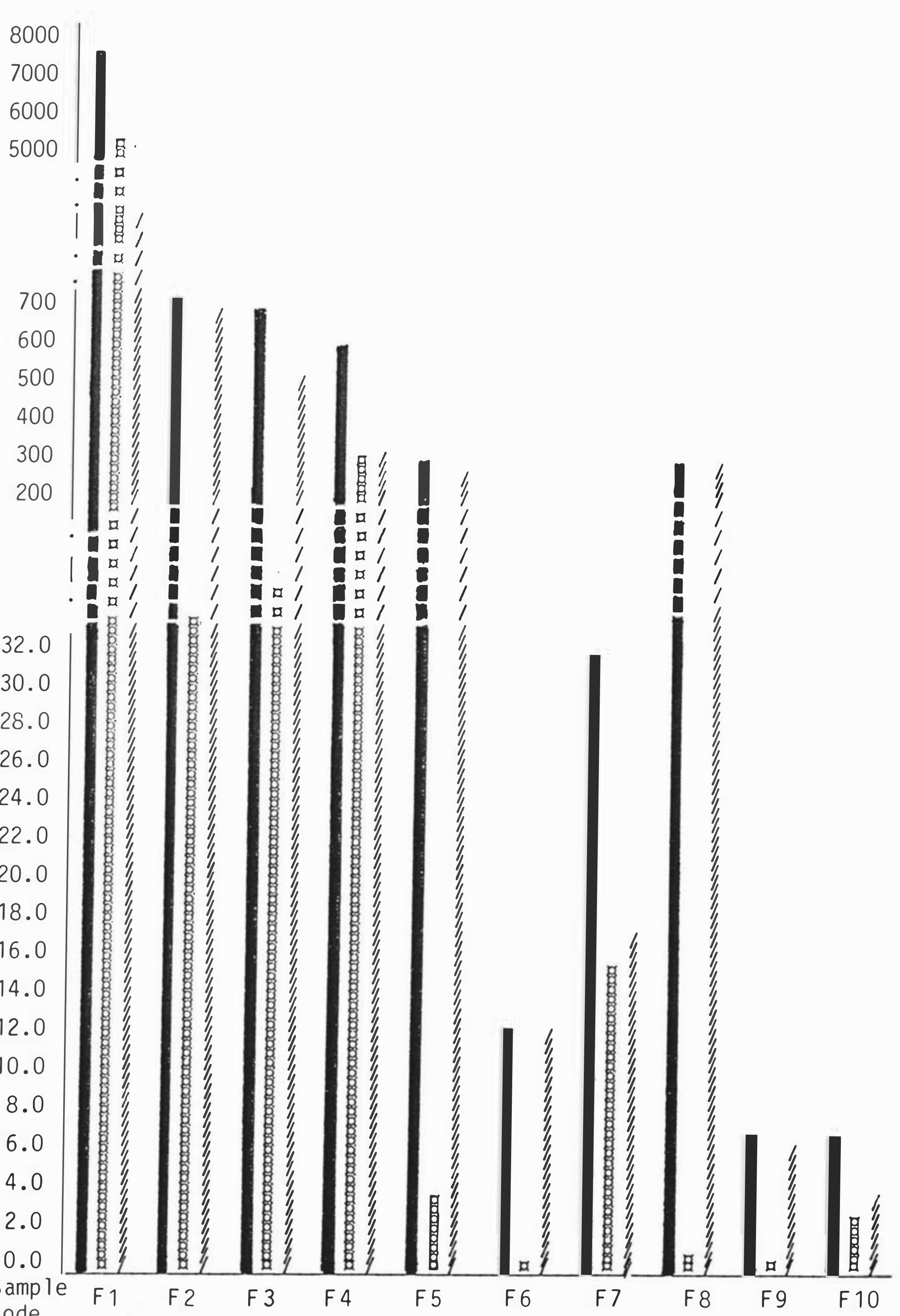


Figure 3-9: Total, inner and outer surface counts of grains stored on farms (CFU g^{-1} x 10^3) at 25°C.



Contamination was highest on the outer surface whilst the inside counts (I.C.) contributed between 1 x 10^3 to 12 x 10^3 to the total. Furthermore, inside counts showed less variation than outside counts (O.C.).

3.3.2.2. Samples from commercial silos - 5 and 9 months' storage

With the exception of the total counts of Sample A8 after 9 months' storage, all the samples stored for 5 and 9 months recorded lower C.F.U. g^{-1} of grain for T.C., I.C. and O.C. compared with those obtained at harvest (Table 3-2[b,c])

At 5 months' storage, grain from Mill B had higher T.C. compared with those at Mill A. When the storage period was extended to 9 months, a dramatic increase in T.C., up to 957 x 10^3, was obtained for Sample A8 stored at Mill A.

Samples stored in commercial silos showed highest contamination on the outer surface, and much lower inside counts were obtained for all the samples in the total period of storage.

3.3.2.3. Laboratory storage samples - ambient and cold temperatures (9 months' storage)

Low O.C.'s and even lower I.C.'s resulted in the overall low T.C.'s of all 8 samples kept at ambient temperature in sealed bags. The lowest value was 0.02 x 10^3 C.F.U. g^{-1} for Sample A3 (Table 3-2[d]).

On the other hand, grain stored in the cold showed a range of viable counts, the counts being much higher than those of the same samples kept at room temperature (Table 3-2 [d, e], and only slightly lower than those at harvest (Table 3-2 [a]). Again Samples A1 and A3 recorded the lowest counts for both laboratory storage conditions, as they had at harvest.

3.3.3. Farm-stored grain (Table 3-2 [f])

In farm-stored grain, there was a vast difference between the lowest T.C. obtained, 11.5 x 10^3 for Sample F6, and the highest T.C. recorded, 7333 x 10^3 for Sample F1. In contrast to the counts for grain stored in the laboratory and in commercial silos, many of the total viable counts obtained here were very high. Samples F1, F2, F3 and F4 showed substantial I.C.'s, while Samples F5 and F6, for example, displayed a similar pattern to the other samples, i.e. having I.C. $<$ O.C. Samples F4 and F7 had their I.C. almost equal to their O.C.. The I.C. of Sample F1 was more than double its O.C.

3.3.4. Fungi Isolated by Dilution Plating

A wide range of fungal species was isolated on PDAX during these studies of the fungal flora of barley (Appendix 3-1). Both storage and field fungi were isolated with varying frequencies from grain at harvest and after storage. The non-surface sterilised grain, however, contributed the larger variety of fungal species.

Some fungi such as *Alternaria*, *Cladosporium* and *Aureobasidium* were invariably found in the supernatants of both surface-sterilised and non-surface sterilised grain. Others such as *Fusarium*, *Pithomyces* and *Stemphylium* were associated with non-surface sterilised grain at harvest and that stored in the cold. *Aspergillus* and *Penicillium* were found in many samples but were primarily associated with the non-surface sterilised stored grain at ambient temperature and their occurrence was significant on surface-sterilised grain stored for 9 months in commercial silos and on grain stored on farms.

The principal fungal genera isolated from plates incubated at 25°C by all techniques are presented in Table 3-3. All the samples from the 6 sources showed *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cephalosporium*, *Cladosporium*, *Drechslera*, *Fusarium*, *Penicillium* and *Rhizopus*, while other genera such as *Pithomyces*, *Trichoderma*, *Paecilomyces* and *Verticillium* were only infrequently encountered. Nevertheless, the majority of the genera which occurred in more than 50% of the samples were field fungi. These fungi were most prominent in freshly-harvested grain samples and those stored in the cold, and

Table 3-3: Principal fungal genera isolated from grain samples on PDAX at 25°C

	At harvest	Commercial silos		Laboratory		Farm silos
		5 months	9 months	Cold	Ambient	
	No. +ve	No. +ve	No. +ve	No. +ve	No. +ve	No. +ve
No.of samples examined	8	7	6	8	8	8
Acremoniella					1	
Alternaria	8	7	6	8	8	6
Arthrinium	1	1	1			
Ascochyta	2			2	1	3
Aspergillus	3	7	6	3	7	8
Aureobasidium	8	7	5	7	7	8
Botrytis	3		1			
Cephalosporium	4	3	3	3	1	3
Chaetomium	1		1	1		1
Chrysosporium	1	2	1			1
Cladosporium	8	6	5	8	5	4
Curvularia	5	1	1			
Drechslera	7	6	5	6	6	3
Epicoccum	4		1	4	1	1
Fusarium	8	5	2	5	1	1
Geotrichum	1			1	5	1
Helminthosporium	1	4	4	4	5	1
Humicola	2			1		
Monilia		1				3
Mucor	2	1	2	1		2
Nigrospora	1					
Paecilomyces		1				2
Papulospora	1					
Penicillium	5	7	6	6	8	8
Phoma	2	1			1	1
Phycomyces		1				2
Pithomyces	1	1		1		
Rhizopus	3	2	4	3	1	2
Scopulariopsis			1	2		
Sordaria	1				1	
Stemphylium	3	1	1	1	1	
Trichoderma			1	1		
Verticillium	1			2		

the proportion of those samples yielding the well-known storage fungi, *Aspergillus* and *Penicillium* was much lower than that recorded for stored grain. Although these storage fungi were present in virtually all the grain stored at ambient temperature, fairly high proportions of the predominant field fungi were also isolated from these samples.

Incubating plates at 37°C reduced the number and thus the variety of fungal genera isolated and identified (Table 3-4). Although *Alternaria* was still recovered from all 6 sources of barley samples, most of the other field fungi did not occur significantly at 37°C. Species of field fungi found at 25°C were displaced by the storage genera *Aspergillus* and *Penicillium*. Also, incubating plates at 37°C revealed the presence of *Absidia* and *Sporotrichum* and increased the isolation of fungi such as *Scopulariopsis*, *Cephalosporium* and *Monilia*.

Amongst the fungi isolated were a number of species of *Aspergillus* (Table 3-5). Selected species of the *A. flavus-oryzae* group were later screened for toxin production (Chapter 4). Members of the *Aspergillus fumigatus*, *A. flavus-oryzae* and *A. glaucus* groups were isolated from both grain at harvest and from stored grain. Other species such as *Aspergillus candidus*, *A. versicolor* and *A. nidulans* only appeared during storage.

Table 3-4: Principal fungal genera isolated from grain samples on PDAX at $37^{o}C$

	At harvest	Commercial silos		Laboratory		Farm silos
		5 months	9 months	Cold	Ambient	
	No. +ve	No. +ve	No. +ve	No. +ve	No. +ve	No. +ve
No.of samples examined	8	7	6	8	8	8
Absidia		1	1			2
Alternaria	4	2	2	6	4	1
Aspergillus	5	7	5	4	5	8
Cephalosporium	4	1	1	4		1
Chrysosporium			1			1
Curvularia	1					
Geotrichum	2		3	1	2	
Monilia	1	3	1	1		3
Mucor					1	1
Paecilomyces						1
Penicillium	3	6	4	3	4	5
Phycomyces						1
Rhizopus	3	1	1	2		1
Scopulariopsis			4	1	2	1
Sporothricum	1			1		
Verticillium	1		1	1	1	

Table 3-5: Principal *Aspergilli* isolated from 6 sources

Aspergilli (groups)	At harvest	Commercial silos		Laboratory storage		Farm silos
		5 months	9 months	Cold	Ambient	
	No. +ve	No. +ve	No. +ve	No. +ve	No. +ve	No. +ve
A. fumigatus	++	++	++	++	++	++
A. flavus-oryzae	+	++	++	++	++	++
A. glaucus	+	+	++	+	++	++
A. candidus		+	++	+	+	++
A. terreus		++	+		++	++
A. versicolor		+	++		+	++
A nidulans			++		+	++
A. restrictus						++

3.3.5. Fungi from Barley Tissues

3.3.5.1. Microscopic observations

Signs of fungal invasion into the caryopsis were absent from all the samples at harvest and after storage in commercial silos and in the laboratory. Only samples from farm silos, i.e. of mouldy and damaged grain, showed such invasion. Such grains often showed discoloration and were difficult to section due to deterioration and destruction of tissue structures, but microscopic examination of squashed caryopsis tissues revealed the presence of mycelium stained blue with lactophenol cotton blue.

However, in the case of husk tissue, spores, sclerotia, fruiting bodies, groups of yeast cells and fungal mycelia were noted on grain from many samples. The fungal structures stained blue-violet with aniline blue and blue with lactophenol cotton blue against a background of brown non-staining husk cells.

Mycelium was present on both the inner and outer surfaces of the husk and also penetration of some hyphae into the husk cell layers was noted. This mycelium was detected in all the "clean" grains examined

Table 3-6: Principal fungal genera obtained on culturing grain tissues on PDAX at 25°C.

	No. samples positive									
	Whole		Caryopses				Husks			
			Longitudinal sections		Transverse sections		Inside surface		Outside surface	
	n.s.s.[+]	s.s.[*]	n.s.s.	s.s.	n.s.s.	s.s.	n.s.s.	s.s.	n.s.s.	s.s.
At harvest										
No of samples	8		8		8		8		8	
Alternaria	8	8	8	8	8	8	8	8	8	8
Aureobasidium	3	2			3	1			1	1
Cladosporium	5	2	2						3	1
Drechslera	3	1	2	1			4		4	
Epicoccum			2							
Fusarium	6	3	5				1		4	1
Penicillium			1							
Rhizopus										1
Commercial silos										
5 months										
No of samples	7		7		7		7		7	
Alternaria	7	7	7	7	7	7	7	6	7	7
Aspergillus		1	1			1	1		3	
Aureobasidium	3		1		2	1		1	2	1
Cladosporium		1	2		2		1			
Drechslera	4	4		3	2		2		5	
Epicoccum									1	
Fusarium	4						1		3	
Monilia		1								
Penicillium	4				1	2			2	1

[+] n.s.s. columns represent fungi from grain tissues of non-surface sterilised grains

[*] s.s. columns represent fungi isolated from grain tissues after whole grains have been subjected to surface sterilisation.

TAble 3-6
(continued)

	No. of Samples Positive									
	Whole		Caryopses				Husks			
			Longitudinal sections		Transverse sections		Inside surface		Outside surface	
	n.s.s.	s.s.	n.s.s.	s.s.	n.s.s.	s.s.	n.s.s.	s.s.	n.s.s.	s.s.
Commercial silos 9 months										
No. of samples	6		6		6		6		6	
Alternaria	5	6	5	4	5	4	6	5	6	6
Aspergillus	6	5	3		2	1	6	4	6	6
Aureobasidium			3	2	3		2	1	5	
Chrysosporium							1		2	
Cladosporium			4		4			1		
Drechslera	4	3	4	3	3	2	2		3	2
Epicoccum			1							
Fusarium	3								2	
Mucor							1			
Penicillium	6	6	6	4	5	2	6	5	6	5
Rhizopus	2			1						
Laboratory 9 months $4^{o}C$										
No. of samples	8		8		8		8		8	
Alternaria	8	8	8	6	4	4	8	8	8	8
Aureobasidium		2	1				3		6	4
Chaetomium				1						
Cladosporium	6			2					7	
Drechslera	7	6	5	5	6		6		7	6
Epicoccum	2									
Fusarium	7	7			2			2	6	4

Table 3-6
(continued)

	No. samples positive									
	Whole		Caryopses				Husks			
			Longitudinal sections		Transverse sections		Inside surface		Outside surface	
	n.s.s.	s.s.	n.s.s.	s.s.	n.s.s.	s.s.	n.s.s.	s.s.	n.s.s.	s.s.
Laboratory 9 months										
Ambient										
No. of samples	8		8		8		8		8	
Alternaria	8	7	8	5	7	5	6	5	8	7
Aureobasidium	8	7	3	2	1		3		4	
Cladosporium	8		4						7	
Drechslera	7	3	7	4		3			8	
Fusarium									4	
Penicillium			2						3	
Farm silos										
9 months										
No. of samples	8		8		8		8		8	
Alternaria	8	8	8	7	7	5	7	5	7	8
Aspergillus	8	8	8	6	7	6	8	7	8	8
Aureobasidium		5	5	2	3		7	4	6	6
Chaetomium									2	
Cladosporium						2			6	
Drechslera	6		5							
Epicoccum									3	
Fusarium	5		4						7	
Monilia			2						1	1
Penicillium	8	8	8	8	8	8	8	7	8	8
Phycomyces	5	1	2		1				3	

as well as in the mouldy grain. Generally, mycelium was often distributed all over the surfaces of the husks, but was most abundant on the inner surfaces, especially at the two ends of the grain and where hairy and barbed structures were located. In heavily infected husk strips from mouldy samples, the mycelium completely covered both the husk surfaces.

Two types of fungal hyphae, brown and hyaline, were noted in the husk strips. The mycelium present in most husk samples was brown hyphae but many of those from farm-stored barley consisted of both types. Husks from obviously mouldy grain had a higher proportion of hyaline hyphae.

3.3.5.2. Barley tissues cultured on PDAX

A smaller variety of fungi was isolated from cultured husk tissue than from dilution plating. It is apparent from Table 3-6 that field fungi, namely Alternaria, Drechslera, Cladosporium and Aureobasidium were once again the predominant fungi present. These fungi were more frequently found in cultures of non-surface sterilised grain. Aspergillus and Penicillium were not always isolated from every barley fraction examined but were encountered from barley fractions of both non-surface sterilised and surface-sterilised stored grain.

Fungi were isolated from all the whole caryopses and longitudinal sections cultured on PDAX. Examination and observations of caryopsis fractions soon after the commencement of incubation showed that no fungi grew from the cut surface of "clean" grain, instead the fungi represented in the table often emerged from the circumference of the whole caryopsis and sections. On the other hand, sections of caryopsis of mouldy grain invariably yielded fungi from all over the caryopsis. It was also noted that fungi tended to grow from the transverse sections of the ends of the caryopsis and from the area of the ventral crease, but transverse sections from the middle areas of, particularly, surface-sterilised grain mostly appeared to be free of viable fungi.

Overall, no significant results were obtained from the pattern of fungal occurrence of the whole caryopses and the longitudinal section

of non-surface sterilised grains. Fungi isolated resembled those of the outer husk surfaces (Table 3-6), and yet again the field fungi were predominant whilst the principal storage fungi exhibited sporadic occurrence except for the farm-stored grain and that kept in commercial silos for 9 months.

Most of the fungi isolated from husk strips were from the outer surfaces, with some commonly-occurring genera also isolated from the inner surfaces. Alternaria was present in all husk tissue examined. Species of Fusarium, Cladosporium and Aureobasidium appeared to colonise the outer husk surfaces.

Penicillium was not isolated from husks of freshly-harvested grain nor from such grain after being kept in the cold. It was isolated from the outer husk surfaces of grain stored in the laboratory at ambient temperature and in silos for 5 months. Grain from farm silos and after prolonged storage in commercial silos yielded Penicillium species from both inner and outer husk surfaces.

In comparison with the occurrence and distribution of Penicillium in husk tissues, Aspergillus was not isolated from husk tissues of samples at harvest nor those stored in the cold or stored in the laboratory at ambient temperature. Species of Aspergilli were present on the outer surfaces of grain which had been in silos for 5 months (they were isolated on the outer and inner surfaces of non-surface sterilised grains), and were consistently isolated from both the outer and inner surfaces of husk strips of both non-surface sterilised and surface-sterilised grain stored for 9 months and on farms.

3.3.6. Changing Levels of Fungal Contamination with Storage

3.3.6.1. Viable counts (Figure 3-4)

Examination of grain held in silos for 5 months showed a decline in total counts from those at harvest, and a much larger drop in counts was found when the grain had been stored for a further 4 months. Both inside and outside counts showed steady declines over this period.

Counts for grain kept at room temperatures of between 12.5-20.0°C for 9 months recorded a 100-fold drop in viable counts compared with those obtained from grain at harvest. This significant drop in total count, however, was not followed by grain kept in the cold (i.e. at 4°C) for the same period. In these, there was less loss of fungal viability for the outer surface counts but the inside counts dropped to a similar level to those of grain stored in silos at ambient temperature.

Although counts of freshly-harvested samples of the farm-stored grains were not available for analysis, a comparison of the counts of these grains with those counts of samples harvested in the same local area and stored in commercial silos revealed high levels of fungal contamination of the farm-stored grain as compared with those from commercial silos, showing dramatic increases in total C.F.U. from counts at harvest. Outside counts and inside counts of farm-stored grains were also comparatively higher and inside counts rose to a similar level as outside counts in storage, a feature not shown by the other grain samples.

3.3.6.2. Fungal genera

The overall frequencies of various genera obtained from all PDAX cultures incubated at 25°C are tabulated in Table 3-7. The frequencies of genera isolated covered the range from 12.5%-100%.

Amongst the genera isolated, Alternaria was the most frequent and maintained its level throughout the 9 months' storage period at ambient temperature. Other genera such as Aureobasidium and Cladosporium showed somewhat decreasing levels and this trend was especially noticeable for Fusarium and Curvularia. In contrast, Penicillium and Aspergillus, the two well-known storage fungi, were isolated at higher frequency in the stored grain. Thus, although genera of field fungi showed decreasing frequencies with storage, they were not actually replaced by storage fungi.

Table 3-7: Overall incidence of selected fungal genera in the barley samples examined based on isolations from all total count determinations at 25°C (%)

	% Samples Positive					
		Commercial silos		Laboratory		Farm silos
	At harvest	5 months	9 months	Cold	Ambient	5-6 months
No. of samples	8	7	6	8	8	8
Alternaria	100.0	100.0	100.0	100.0	100.0	75.0
Arthrinium	12.5	14.3	16.7			
Ascochyta	25.0			25.0	12.5	37.5
Aspergillus	37.5	100.0	85.3	37.5	87.5	100.0
Aureobasidium	100.0	100.0	83.3	87.5	87.5	100.0
Botrytis	37.5		16.7			
Cephalosporium	50.0	42.9	50.0	37.5	25.0	37.5
Chaetomium	12.5		16.7	12.5		12.5
Chrysosporium	12.5	28.6	16.7			12.5
Cladosporium	100.0	85.7	83.3	100.0	62.5	50.0
Curvularia	62.5	14.3	16.7			
Drechslera	87.5	85.7	83.3	75.0	75.0	37.5
Epicoccum	50.0		16.7	50.0	12.5	12.5
Fusarium	100.0	71.4	33.3	62.5	12.5	12.5
Geotrichum	12.5			12.5	62.5	12.5
Helminthosporium	12.5	57.1	66.7	50.0	62.5	12.5
Monilia		14.3				37.5
Mucor	25.0	14.3	33.3	12.5		25.0
Paecilomyces		14.3				25.0
Penicillium	62.5	100.0	100.0	75.0	100.0	100.0
Phycomyces		14.3				25.0
Pithomyces	12.5	14.3		12.5		
Rhizopus	37.5	28.6	66.7	37.5	12.5	25.0
Scopulariopsis			33.3	25.0		
Stemphylium	37.5	14.3	16.7	12.5	12.5	
Verticillium	12.5			25.0		

3.4. DISCUSSION

The barley studied in these investigations was mainly harvested and stored at moisture content of about 13%, which has been considered safe for this grain type (Hill and Lacey, 1983). During storage, the moisture content of the grain stabilised to a low level and dropped with time under well-controlled conditions. The drop in moisture content was more obvious in grain kept on commercial premises where aeration was used to reduce temperatures and in doing so brought about some drying of the grain. The ability to maintain moisture at low levels ensured the grain was "properly" stored and hence most of the grain dealt with in these studies was considered as good quality, "clean" grain.

Local farms from which samples were obtained store their grain at higher moisture content (average M.C. = 14.0%). This places the grain under continual threat of spoilage for even a small change in M.C. can lead to deterioration of the grain during storage (Christensen and Linko, 1963). Thus it is not surprising that many of the farm samples consisted of heated grain, grain infested with mites, insects and fungi. The poor condition of this grain was further exacerbated by the lack of devices to monitor silo conditions resulting in gross spoilage as occurred in two farm silos (Plate 3-1).

The influence of moisture content on fungal growth in cereal grains has been established by many investigators (Christensen and Kaufmann, 1974) and this relationship was also demonstrated in the results of the present study. "Clean" grain at harvest and on being stored at low moisture content showed no signs of mould infection and gave low viable counts compared with grain stored at high M.C. and which had deteriorated. Increased moisture content accompanied by mouldiness was well illustrated in the first two samples of farm-stored grain.

The total counting technique used in this investigation has enabled the total viable counts, outside surface counts and inside counts to be successfully estimated. Substantial differences were obtained between total outside counts and inside counts. Omitting the obviously mouldy grain, almost all the other barley samples examined

Plate 3-1. Mould-infested grain from a farm silo.

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possessed much lower internal counts, essentially the total counts obtained after surface sterilisation (s.s.) of the grain. This presumably indicates that most of the viable fungal propagules were present on non-surface sterilised (n.s.s.) barley grains as superficial contaminants. The regime of surface sterilisation and rather vigorous washing can be assumed to have killed and dislodged these superficial fungi, leaving behind the well-attached and "protected" fungi (Flannigan, 1969, 1970; Flannigan and Dickie, 1972). The numbers and types of surface contaminants fluctuate under the influence of factors such as the spore population, the sources of inoculum, the time of harvest, the period of storage as well as environmental determinants of biological, physical and chemical nature resulting in the relatively large variations in viable counts of samples. The fact that most of the inside counts consisted of fungi either well enclosed in the space covered by the husk, or well established on and within the inner husk surface and the surface of the caryopsis, must account for the comparatively constant internal viable count.

The total viable counts of the freshly-harvested barley samples were varied and generally high, but they are not as high as those recorded by Clarke *et al.* (1965) who dilution-plated comminuted grains. Their freshly-harvested grain gave counts ranging from 3.2×10^4 to 1.5×10^5 per gram of grain, in contrast with a maximum count of 5.2×10^4 C.F.U. g^{-1} and a minimum of 2.0×10^3 C.F.U. g^{-1} obtained by the total counting technique chosen for the present study. These fungal contaminants associated with barley could be introduced in the field from the soil, the air or the vegetative parts of the plant (Flannigan, 1969, 1978). Contamination can also occur during harvesting and subsequent handling of the grain with items such as harvesters, trucks and other equipment (Flannigan, 1978; Tuite and Christensen, 1957).

Two barley samples (A1 and A3) delivered to Mill A had unusually low viable counts. These 2 samples continued to give very low counts after storage in the cold and in sealed bags kept in the laboratory. Subsequent discussions with the mill manager raised a number of possibilities, one of which was that the farmers concerned had

probably sprayed their barley crops with chemical substances which had fungicidal or fungistatic effects.

As part of an intensive survey of the mycoflora of barley grain in Egypt, total counts of fungi by dilution plating of comminuted grains acquired from 40 samples collected by Abdel-Kader et al. (1979) from commercial silos gave a count of 580.9 x 10^3 per gram of grain. This was a considerably larger estimate of fungal colonies on commercially-stored grain than those of grain obtained from New Zealand commercial silos. It was probable that the grain of apparently low moisture content examined by Abdel-Kader et al. was undergoing fungal invasion in storage resulting in high viable counts. A sample giving unusually high viable counts of 957.4 x 10^3 C.F.U. per gram after 9 months' commercial storage in the present study, showed signs of weevil infestation and slight heating. Given these facts, it was likely that incipient fungal invasion had begun in this sample and such a process can take place with no external signs (Watson and Cameron, 1976). Although little information was given by the authors on the condition of the Egyptian grain and its condition of storage, the isolation of Aspergillus at 100% and Penicillium at 82.5% of the total samples further lends support to the inference that the grain was being invaded by storage fungi.

More recently, El-Kady et al. (1982) reported counts of between 53.2 to 2799.9 per gram of low moisture Egyptian-grown barley from 25 1 kg commercial grain samples. Their survey results showed that barley grain was the richest in viable fungal counts relative to those of maize, sorghum and wheat. The counts of viable fungi obtained by El-Kady et al. were in agreement with that of grain stored for 9 months in the New Zealand commercial silos covered in the present study.

Grain sealed in bags and kept in the laboratory at ambient temperature contained very low numbers of viable fungi compared to those obtained for grain stored under other conditions. Absence of fungal growth has also been observed by Clarke and Hill (1981) in their high-moisture barley stored in hermetically-sealed laboratory silos. In their investigations, the decline in fungal viability was related to grain moisture. Field fungi present in the grain at harvest were completely eliminated in samples stored at moisture content

greater than 20% for 100 days. Meiering et al. (1966), (cited by Clarke and Hill, 1981) suggest that the rapid depletion of O_2 and the concentration of CO_2 in the intergranular atmosphere of the high-moisture barley were responsible for the decreased fungal viability associated with such grain. At about the same time, Berck (1966), (cited by Clarke and Hill, 1981) proposed that gaseous compounds other than those of CO_2 and O_2 could also be responsible for the loss of fungal viability. Changes in such gaseous components in the immediate grain atmosphere could also produce similar effects on fungal viability in the low-moisture grain stored sealed in the laboratory at ambient temperature.

Dilution plating of grain stored, sealed, in the cold has revealed relatively high counts of fungi in the present study, second only to those recorded for "clean" barley at harvest. Clarke and his colleagues (1968) obtained similar results from the high-moisture grain they examined after cold storage. Because the overall counts were lower than when the grain was at harvest, it could be concluded that no active fungal proliferation had taken place and the relatively small drop in counts of most samples must point to greater survival amongst fungal species under cold conditions. However, if the barley grain had been stored in the cold for longer, fungal proliferation might have occurred as discovered by Hill and Niles (1969). The time it takes for this to happen was demonstrated by Moubasher et al. (1980) to be inversely proportional to the moisture level of the grain being stored.

Although the total counts of grain stored on local farms differed from sample to sample, an interesting relationship can be traced between the counts and grain condition. Grain described as "good" and apparently healthy at the time of sampling carried low fungal populations. Comparatively higher viable counts were associated with slightly mouldy or slightly damaged grain, and finally, very high levels of viable counts were obtained in grain that showed overt signs of mouldiness.

When the grain was in good condition after storage, the outside counts were relatively low but were still higher than the inside counts. This resembled the pattern of such counts for healthy, clean

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grain studied in other barley samples, thus implying that there had not been much, if any, fungal invasion during storage. Samples F2 and F3 are the exception to this. On closer examination of their counts, it can be seen that the outside counts are very high, indicating probable fungal growth on the outside of the grain; the corresponding lower inside counts which were still comparatively higher than those obtained from healthy grain could signify fungal invasion to the inside of the grain. It is also possible that there was a failure to achieve complete surface sterilisation when grain tissues were heavily invaded by fungi. The same explanation could also apply to the substantial inside counts of two other mouldy samples, F1 and F4.

Thus it can be concluded from these studies of viable counts that a) low outside counts combined with low inside counts, thus low total counts, reflect absence of mouldiness; b) high O.C. indicates fungal invasion on the outer grain surfaces, and c) I.C. $\geq$ O.C. indicates fungal invasion mainly on inner surfaces and hence gross spoilage.

The isolation of a diverse fungal flora from the barley grain in the present study (Appendix 3-1) was not unexpected as a wide spectrum of fungi has been recorded in barley grain from various sources; (at or soon after harvest - Flannigan, 1970; Clarke and Hill, 1981; from commercial silos - Flannigan, 1969; Abdel-Kader *et al.*, 1979; El-Kady *et al.*, 1982; from markets - Abdel Hafez and Abdel-Kader, 1980; Abdel Hafez, 1984; and from farm storage of moist grain - Hill and Clarke, 1980; Lacey, 1981). The barley microflora represented the broadest spectrum of genera and species recorded amongst four cereal grains (wheat, barley, sorghum and maize) in Saudi Arabia (Abdel-Hafez, 1984).

Of the fungi isolated from locally-grown barley at harvest, most were identified as field fungi. *Alternaria*, especially *A. alternata*, *Cladosporium*, *Aureobasidium* and *Fusarium* were present in 100% of the grain samples. Mulinge and Chesters (1970) and Clarke and Hill (1981) have also reported the dominance of field fungi in freshly-harvested barley grain grown in the U.K. These investigators together with Flannigan (1969, 1970) who studied the mycoflora of dried barley grain soon after harvest, also in the U.K., found the fungal propagules of *Alternaria tenuis* (*A. alternata*), *Cladosporium cladosporioides*,

Cladosporium herbarum, Aureobasidium pullulans and Epicoccum to be amongst the most abundant constituents of the barley mycoflora. However, Tuite and Christensen (1955) found that Alternaria, Cladosporium, Fusarium and Geotrichum were often present in large numbers but Aureobasidium and Epicoccum were not commonly found on barley, especially that from the drier regions of the U.S.A.

Several investigators including Christensen and his colleagues (Tuite and Christensen, 1955; Kotheimer and Christensen, 1961, cited by Flannigan, 1969; Lutey and Christensen, 1963; Clarke et al., 1966; and Flannigan, 1974, 1978) were unsuccessful in isolating storage fungi from barley at harvest. However, these fungi were reported to exhibit a sporadic presence in low numbers amongst the newly-harvested barley examined by Clarke and Hill (1981). In contrast, in the grain examined at harvest in the present study the two well-known storage fungi Aspergillus and Penicillium were found to occur in 40% and 60% respectively of the samples examined.

Further evidence has been provided in several studies to confirm the observations that some storage fungi can contaminate crops both in the field and during harvest. One example was the work of Flannigan (1978), who encountered a high incidence of Penicillium species and Aspergillus species (predominantly A. glaucus) by 'plating' whole grains of hand-harvested and combine-harvested barley in Scotland. This author's results indicated that vegetative plant parts were distinct sources of primary contamination of barley by storage fungi. The fairly regular presence of the Penicillia in the samples examined in the present investigation was not surprising as some investigators (Mislevec and Tuite, 1970) have found from many years of isolating Penicillia from corn that Penicillium species could be classified into three categories:

1. Field Penicillium : P. oxalicum, P. funiculosum, P. cyclopium, P. variable, P. citrinum

2. Storage Penicillium: P. cyclopium, P. brevi-compactum, P. viridicatum, P. palitans

3. Unassigned *Penicillium* (ones which are isolated too infrequently in corn to be recognised as belonging to either one of the above categories): *P. frequentans, P. chrysogenum, P. purpurogenum, P. expansum, P. urticae, P. puberulum, P. digitatum, P. steckii, P. janthinellum, P. charlesii, P. implicatum, P. luteum, P. granulatum, P. multicolor*.

The present isolation of numerous storage fungi (species of (*Penicillium, Aspergillus, Mucor, Arthrinium, Geotrichum* and *Scopulariopsis*) from stored dried grain agrees with the findings of other authors that a number of such fungi with varying frequencies can be obtained from dried barley grain stored under different conditions (Flannigan, 1969, 1970; El-Kady *et al*., 1982; Abdel-Hafez, 1984; Abdel-Kader *et al*., 1979). These authors have also recovered field fungi from their stored grain, albeit at lower frequencies.

Storage fungi become conspicuous in isolation from stored grain due to:

a) increasing numbers as a result of incipient and overt growth under the various conditions of storage;
b) declines in the previously large numbers of active field fungi, which tend to proliferate quickly on isolation media.

The various groups of the Aspergilli have their own defined lower limit of moisture content below which no growth occurs, and the groups isolated were noted to occur with differing frequencies from barley samples at harvest and from storage samples. *A. fumigatus, A. flavus* and *A. glaucus* were isolated from all the samples studied in this investigation. Of the 3 species, *A. fumigatus* had the highest occurrence in these samples. Abdel-Hafez and Abdel-Kader (1980) also found it to be the highest-occurring cellulose-decomposing fungus contaminating barley grain. Warnock (1971) and El-Kady (1982) found *A. fumigatus* to be extremely frequent in barley grain and in addition Hussein (1983) found high frequencies in mouldy farm barley.

The *A. flavus* group was found to rank second in order of frequency in the barley grain examined, as it did in the studies of El-Kady (1982), but Hussein (1983) found it to be the most frequent

Aspergillus from dilution plating of farm-stored grain. Flannigan (1969) isolated *A. flavus* at low frequency from dried grain stored for one month. Although the *A. flavus* group of fungi has been isolated from other cereal grain, in particular decayed grain or grain infected by insects at harvest or in the field (Lillehoj *et al.*, 1975, 1976, 1977; Hesseltine *et al.*, 1981), the members of this group require a minimum moisture content of 17.5% to grow in cereal grain during storage (Christensen and Kaufmann, 1974). Their isolation from both n.s.s. and s.s. grain at harvest was an indication of the high levels in the field environment and this would later on determine their occurrence upon subsequent storage.

Even though *A. glaucus* was isolated from grain at harvest and after storage in the cold, in this study it was not found from s.s. grain from these samples. This indicated that *A. glaucus* was likely to be a superficial contaminant in freshly-harvested grain and had not penetrated the inner grain tissues even after cold storage. However, it was frequently found in grain stored for 9 months at ambient temperature and in farm silos, and in these samples, *A. glaucus* was isolated from the inside surfaces of the grains. This particular group together with the *A. restrictus* group are notorious agents of incipient deterioration and spoilage in stored grain (Christensen and Kaufmann, 1974).

Other Aspergilli associated with the grain stored under the conditions described in this chapter included the *A. vesicolor*, *A. nidulans*, *A. candidus* and *A. restrictus* groups. Flannigan (1969, 1970) isolated only low numbers or none of these fungi on PDA plates incubated at 25°C containing n.s.s. and s.s. dried barley kernels. The failure to isolate these Aspergilli was due to the short period (one month) the grain spent in storage. In the present study, *A. vesicolor* just appeared after 5 months in commercial storage and the other two groups of Aspergilli only appeared after a further 4 months in storage silos. When storage conditions were conducive to fungal growth, as found in some farm silos, those fungi could be isolated from the grain after 6 months in storage.

The higher temperature (37°C) at which some of the plates in these studies were incubated appeared to favour the isolation of

storage fungi and thermotolerant fungi from the barley grain (Table 3-4) which might otherwise have been inhibited by large numbers of competing mesophilic fungi which would have grown at 25°C. The absence of low numbers of Absidia, some Aspergillus species, Scopulariopsis and Paecilomyces from grain at harvest perhaps demonstrated this effect. Many of the fungi isolated at 37°C in the present studies were found amongst the lists of thermophilic fungi isolated from stored barley by Mulinge and Apinis (1969), Flannigan (1969) and Mulinge and Chesters (1970a).

3.4.1. Location and Identification of Fungal Propagules on Grain

Spores, mycelium, fruiting bodies, sclerotia and yeast cells were found on both surfaces of non-surface sterilised grains and particularly on the inner surfaces of the surface-sterilised grains.

Microscopy of stained barley husks of s.s. and n.s.s. grains has confirmed the presence and location of mycelium on and in these structures. Such mycelium has been reported by other investigators working on barley fractions of before-harvest samples (Mulinge and Apinis, 1969), and storage samples (Tuite and Christensen, 1955; Mulinge and Chesters, 1970b; and Hussein, 1983). The mycelium was generally more abundant on the inner surfaces of lemma and palea (Tuite and Christensen, 1955), and in the case of n.s.s. "clean" grains, it was most concentrated at the two ends of the grain (Hussein, 1983); in the present study it also abounded where prominent structures existed to serve as "anchors" for the mycelial strands. At harvest or in the field, spores deposited on the inner and outer surfaces of the lemma and palea may germinate but normally do not penetrate deeply into the grain tissues and the caryopses (Flannigan, 1970; Warnock, 1973b). The relative humidity or moisture level during grain development and harvest is the principal factor that checks fungal invasion of the caryopses (Martin and Gilman, 1976).

Similar circumstances could apply in the case of grain stored under the various conditions studied here. Grain properly stored did not show internal fungal invasion as no mycelium was detected in their caryopses, but grain exposed to adverse storage conditions and especially to higher moisture levels displayed mycelial penetration

deep into the tissues. Also fungal mycelium would spread and eventually envelope the whole grain.

It would not be considered unusual for a smaller range of fungi to be isolated from culturing washed barley tissues (Table 3-6) than from dilution platings of the supernatants from shaking barley fractions in a liquid medium (Table 3-3). The fungal species isolated from cultured husks and caryopses must mostly represent the fungi which have managed to lodge themselves on these barley fractions and subsequently colonise them, whilst dilution plating of supernatants will disclose mainly the loosely attached, viable propagules.

Another reason which could contribute to the smaller fungal variety after the plating of barley tissue itself is due to the limitations of the plating method. Fungi which do not need much coaxing to grow and are rapid spreaders soon suppress the appearance of the slow and late starters, obviously leading to the decreased isolations of the later fungi. One possible way to overcome this is to use different methods of isolation, including incubation at different temperatures on different media.

Echoing the results earlier obtained from dilution plating, the present attempts in culturing washed barley fractions also produced predominantly field fungi, especially from those of "clean" grain. All the samples showed the presence of *Alternaria* on both inner and outer surfaces of husks and also the caryopses, and others, such as *Drechslera*, *Cladosporium* and *Aureobasidium* were also fairly abundant on these tissues. These observations support those of Christensen (1951, 1965) and Christensen and Kaufmann (1969) who identified the fungi from dormant mycelium in dried wheat husks and ranked them in the following order of frequency: *Alternaria*, *Aureobasidium*, *Cladosporium*, *Helminthosporium* and *Fusarium*. Similar observations were also reported by Mulinge and Chesters (1970a) who obtained high frequencies of *Alternaria*, *Cladosporium* and *Fusarium* from husks and caryopses of grain at harvest and somewhat decreased frequencies of the same genera following storage.

The present studies also supported the findings of Mulinge and Chesters (1970a) and Tuite and Christensen (1955) that storage fungi

infrequently colonise grain husks or caryopses of grain at harvest, but a noticeable increase in frequency of isolation from barley grain stored at ambient temperature was common. These fungi were also found by Mazen et al. (1984) in the lemma and palea of their stored wheat grain.

Penicillium species were lodged or established only on the superficial surfaces of the grain fractions examined in these studies and only after prolonged storage at ambient temperature. Conditions probably allowed fungal growth to appear on cultures of inner husk surfaces. The hyaline hyphae observed especially in husks of mouldy grain were likely to be those of Penicillium and Aspergillus.

Aspergillus species, on the other hand, were only surface contaminants of the grain collected after 5 months' storage, but penetrated into the inner husk surfaces in grain stored at ambient temperature for 6 months or more. This pattern of occurrence displayed by Aspergillus and Penicillium species has also been observed by Mulinge and Apinis (1969) to be mostly confined to husk tissues at the outset of storage of healthy grain. Invasion of the grain tissues only occurred after prolonged storage, especially when self-heating had occurred or when there was active fungal growth (Hussein, 1983).

It was not unexpected to obtain fungal colonies from all the caryopsis sections as dilution plating counts have shown the existence of fungi inside the husk. Some of these fungal propagules must have attached themselves to the surface of the caryopsis, especially in the excellent sites provided by depressions, crevices and other uneven surfaces on the caryopsis. This has been supported by the observations of fungal growth from the ventral crease areas of the T.S., especially those from n.s.s. grain. Also, barley husks are fairly firmly attached to the caryopsis (Pomeranz et al., 1978) which could make the complete removal of all husk tissues difficult, because husk tissue is well supplied with fungal inoculum, fungal colonies arising from caryopsis sections bearing such tissue would not be difficult to imagine.

Thus the fungi isolated from caryopsis sections will closely resemble those of husk tissues. None of them actually penetrated into the caryopsis tissues except in the case of mouldy grains as evident from microscopic observations of caryopsis tissue from different sources.

3.4.2. Changing Levels of Fungal Flora During Storage

Decreasing colony forming units following commercial storage of dried barley at Mill B, one of the sources of the grain samples used in the present investigations, could be due to two reasons. Firstly, the effectiveness of the compound used in preventing grain damage from mite and insect infestations and subsequent fungal invasion; secondly, the well-monitored silo conditions under which the grain was held decreased the opportunities of increasing moisture levels in pockets of the grain and consequent heating of the grain and fungal spoilage.

The absence or collapse of such preventative measures, even for a short period, would lead to high levels of viable fungal units from resulting fungal activities as occurred in the case of Sample A6 after 9 months' storage in an unaerated silo at Mill A.

The decrease in recorded viable fungal units of stored grain from the time of harvest may principally be attributed to the significant decline in viability of the large populations of field fungi found on both the inside and outside of grain at harvest. Mulinge and Chesters (1970a) suggested that the storage survival of these fungi was dependent on the moisture content of the infected material. Species of Fusarium and Helminthosporium, for example, have been known to die rapidly during storage of dried barley at 12% moisture content and in temperatures not exceeding 30°C (Lutey and Christensen, 1963). The rate of loss of viability by Fusarium and Curvularia was also found in the present study to increase with the length of storage.

Another factor which indirectly contributed to the resultant drop in C.F.U. during storage was the absence of active proliferation of the low numbers of storage fungal propagules already present in the samples at harvest. The infection percentage of moist barley grain studied by Jorgensen (1974) showed a tendency to decrease following 10

weeks of storage and then increase after 40 weeks of storage. In contrast, in barley containing low moisture contents of 13.9 to 15.9%, infection was found to slightly increase after 40 weeks of storage, but no infection by *Penicillium* was recorded for grain with moisture content equal to or less than 16.4%.

A study of the mycoflora of grain kept in the cold at harvest time could provide the clues to the small decrease in the counts observed in grain held continuously in the cold for 9 months. Colonies from dilution plates and cultured barley tissues have shown a strong resemblance between the kinds of fungi isolated from cold-stored grain and from the same grain samples at harvest. From these observations it could be deduced that the dominating field fungi associated with freshly-harvested grain were able to survive for longer periods in the cold (Lutey and Christensen, 1963; Clarke *et al*., 1968) than at ambient temperature. Also, the grain examined was kept in well-sealed bags which allowed the retention of moisture content close to the level at harvest. These higher moisture content levels encouraged the survival of the existing fungal species.

The drop in the inside viable counts to similar levels as grain kept at ambient temperature could be caused by the adverse gaseous changes due to restricted air movements within the relatively small space enclosed by the husks and caryopses. The sealing of bags in which the grain was kept probably did not facilitate the removal of gases such as CO_2, so allowing them to reach harmful concentrations. Nor did the bags allow a continued supply of O_2 necessary for fungal tissue longevity.

Although many fungal species isolated at harvest could still be isolated from grain kept sealed at room temperature, the viable counts recorded for this grain after 9 months' storage were extremely low compared with the same samples stored under other conditions. The low moisture content in this laboratory-stored grain perhaps hindered the survival ability of many field fungi and prevented the growth of the storage fungi, but this possibility alone was not sufficient to produce the drastic drop in numbers. One other plausible explanation is that normal rates of respiration and other metabolic activities could still be pursued by both grain and fungal propagules at room

temperature. This could rapidly lead to undesirable gaseous composition in the intergranular atmosphere of such grain when kept sealed for prolonged periods, which eventually would lead to decreased fungal viability. Yet another possibility is the adverse effects on fungal viability from the accumulation in the enclosed atmosphere of vaporised fumigants and other chemicals used on the grain before or at harvest.

Considerably higher viable counts of farm-stored grain relative to the total viable counts from the other grain samples indicated that the fungal propagules present on this grain must have increased during the storage period. This was evident from the large increase in the inside counts representing fungal growth and invasion of the deeper grain tissues. Additional evidence was also provided by the microscopic observations of abundant mycelium inside husk and caryopsis tissues in some samples.

Hill and Lacey (1983) considered that at moisture contents of 12.5-14.4% and at ambient temperatures, stored barley is slowly invaded by _A. restrictus_. Above 14.4% _A. glaucus_ begins to grow and the process of deterioration quickly gathers momentum, culminating in the appearance of thermotolerant and thermophilic fungi at moisture contents exceeding 17.2%. Thus the invasion of farm-stored grain by the growth of the existing population of storage fungi was not unexpected, especially as the grain was loaded into unaerated silos at moisture content of about 14%. During the course of storage, this grain was left vulnerable to environmental changes.

In the present study, a sharp demarcation between field fungi and storage fungi cannot be distinguished, for many so-called field fungi such as _Alternaria_, _Cladosporium_ and _Aureobasidium_ were isolated at high frequencies from stored samples. Also, typical storage fungi such as _Penicillium_ and _Aspergillus_ were present in grain at harvest although they later became prominent as storage progressed.

Patterns of fungal succession in the dried stored barley analysed in the present study did not closely follow the defined phases of microflora development in moist barley as described by Clarke _et al._ (1966, 1969). They were similar to the fungal successions observed by

Spillane and Pelhate (1982) in moist barley stored under well-controlled conditions.

Initially, field fungi dominated the fungal flora of grain at harvest. During storage, these fungi declined in viability leading to low viable counts in barley examined after 5 months' storage. A rapid increase in the loss of viability following more than 5 months of continuous storage resulted in even lower viable counts in grain examined after 9 months.

On the other hand, storage fungi which were present at low levels in grain at harvest were encountered at increasing frequencies in commercially-stored grain, with respect to their length of storage, but mycological culture and microscopic examinations of the grain showed that the storage fungi existed mainly as spores and did not invade deep grain tissues. Only in the case of farm-stored grain which was not kept in well-controlled storage conditions, did the storage fungi proliferate and cause gross spoilage.

Thus the phases of fungal succession in the stored barley just described were not distinct, nor were they mutually exclusive as suggested by Clarke et al. (1966, 1969). Both mesophilic and thermotolerant species were amongst the fungi isolated from all the grain sampled from the 6 different sources. While the storage fungi were isolated with increasing frequencies from dried stored grain, it seemed that the declining viability of field fungi never actually led to their complete disappearance even after 9 months of storage at ambient temperature, nor were they completely substituted by storage fungi in damaged grain.

Thus assessments of the numbers and kinds of viable fungi contaminating both inside and outside surfaces of barley grains can enable the conditions of the stored grain to be determined and provide useful information on their storage conditions. Such information could help in preventing fungal spoilage and possible toxin formation by any of the potentially toxic fungal species present.

Appendix 3-1. Fungi associated with barley

	At harvest										Farm silos	
			5 months		9 months		Cold		Ambient			
	nss[+]	ss[*]	nss	ss	nss	ss	nss	ss	nss	ss	nss	ss
Acremoniella atra									+			
Absidia spp.			+		+						+	+
Alternaria spp	+	+	+	+	+	+	+	+	+	+	+	+
A. alternata	+	+	+	+	+	+	+	+	+	+	+	+
A. citri							+			+		
Arthrinium spp.	+											
A. state of Apiospora montagnei		+	+		+							
Ascochyta sp.	+	+					+	+	+		+	
Aureobasidium pullulans	+	+	+	+	+	+	+	+	+	+	+	+
Botrytis cinerea	+				+							
Cephalosporium spp.	+	+	+	+		+	+	+		+	+	+
Chaetomium spirale	+				+		+				+	
Chaetomium sp.	+											
Chrysosporium spp.	+		+		+						+	+
Cladosporium spp.	+	+	+	+	+		+	+	+	+	+	+
C. cladosporioides	+	+	+	+	+	+	+	+	+	+	+	+
C. herbarum	+	+	+		+		+	+		+		
C. sphaerospermum		+									+	
Curvularia spp.	+	+	+									
C. prasadii	+				+							
Drechslera spp.	+	+	+	+	+	+	+	+	+	+	+	
D. coicis						+	+		+			
D. poae	+		+									
D. state of Cochliobolus sativus			+									
Epicoccum nigrum	+	+			+		+	+	+		+	
Fusarium spp.	+	+	+	+	+	+	+	+	+	+	+	
F. acuminatum	+											
F. avenaceum	+	+	+	+	+		+	+	+		+	
F. culmorum	+	+	+				+	+	+		+	

	At harvest		Storage								Farm silos	
			5 months		9 months		Cold		Ambient			
	nss[+]	ss[*]	nss	ss	nss	ss	nss	ss	nss	ss	nss	ss
F. equiseti	+						+					
F. graminearum	+						+		+			
F. moniliforme	+							+	+			
F. nivale	+											
F. oxysporum	+	+		+			+	+	+			
F. poae			+									
F. sambucinum	+		+				+					
F. semitectum	+	+	+				+					
F. sporotrichioides				+			+					
Geotrichum spp.	+						+	+	+			+
Helminthosporium spp.				+	+	+	+	+	+		+	+
H. velutinum	+	+	+									
Humicola grisea	+						+					
Monilia spp.	+		+	+		+	+				+	+
Mucor spp.	+				+		+				+	+
M. hiemalis	+		+		+		+					
Nigrospora sp.	+											
Papulospora sp.	+											
Phoma spp.	+		+						+		+	
Phycomyces spp.			+								+	
Pithomyces spp.	+	+	+				+					
Rhizopus nigricans	+	+	+	+	+		+	+	+		+	
Scopulariopsis brevicaulis					+	+	+		+		+	
Sordaria sp.	+								+			
Sporotricum sp.	+								+			
Stemphylium spp.	+						+	+				
S. pleospora herbarum	+	+		+	+		+		+			
S. bothyosum	+	+										
Trichoderma viride					+		+					
Verticillium spp.	+						+					

	At harvest		Commercial silos				Laboratory				Farm silos	
			5 months		9 months		Cold		Ambient			
	nss[+]	ss[*]	nss	ss	nss	ss	nss	ss	nss	ss	nss	ss
Aspergillus spp.			+		+	+				+	+	+
A. candidus			+		+	+	+		+		+	+
A. clavatus												+
A. fumigatus	+	+	+	+	+	+	+	+	+		+	
A. glaucus	+		+	+	+	+	+		+	+	+	+
A. nidulans					+	+			+		+	+
A. niger	+	+									+	
A. restrictus											+	+
A. terreus			+	+	+				+	+	+	+
A. vesicolor			+		+	+			+		+	+
A. flavus - oryzae	+	+	+	+	+	+	+	+	+		+	+
Penicillium spp.	+		+	+	+	+	+	+	+	+	+	+
P. adametzi											+	
P. brevi-compactum					+		+				+	+
P. canescens							+					
P. chrysogenum					+							
P. citreo-viride									+			
P. citrinum	+	+					+					
P. commune					+	+	+		+			
P. cyclopium	+	+	+	+	+	+	+		+	+	+	+
P. decumbens	+							+				
P. digitatum											+	
P. frequentans					+	+	+		+		+	
P. herquei		+	+				+		+		+	
P. janthinellum	+				+							
P. lilacinum	+	+					+					
P. luteum		+		+	+	+	+		+		+	+
P. nigricans					+							
P. notatum	+											
P.oxalicum	+	+					+		+			
P.paxilli					+							
P. puberulum					+	+					+	
P. purpurogenum	+		+	+			+		+		+	+
P. raistrickii									+			

	At harvest		Commercial silos				Laboratory				Farm silos	
			5 months		9 months		Cold		Ambient			
	nss[+]	ss[*]	nss	ss	nss	ss	nss	ss	nss	ss	nss	ss
Penicillium spp.												
P. ramigena									+			
P. restrictum					+							
P. roqueforti		+	+									
P. rubrum			+									+
P. rugulosum					+				+		+	+
P. thomii				+	+	+						+
P. variable			+		+		+	+	+		+	+
P. viridicatum			+		+	+	+		+		+	+
Unknown species	+	+	+	+	+	+	+	+	+		+	+

nss = Fungi isolated from non-surface sterilised grains

ss = fungi from surface-sterilised grains

CHAPTER 4

FUNGAL MYCELIUM ASSOCIATED WITH BARLEY GRAIN

4.1. INTRODUCTION

It is well known that fungal hyphae are present in most of the popular hulled cereal grains grown in the world. del Prado and Christensen (1952) and Takahashi et al. (1984) found fungal mycelium in the husks and kernels of rice. Hyde (1950) found sub-epidermal mycelium in nearly 100% of "clean" wheat grain collected from the world's major wheat-growing areas. Tuite and Christensen (1955), Warnock and Preece (1971) and Hussein (1983) located and noted the extent of mycelium in the husks of barley grown in the U.S., Britain and New Zealand respectively.

Fungal mycelium was not only found in grain after storage (Warnock, 1976; Hussein, 1983) but was also found in grain at harvest Hyde, 1950; Christensen, 1951) and even before harvest (Tuite and Christensen, 1955). In the present studies of fungi contaminating barley, it was therefore considered useful to examine grain for the presence of mycelia. Such mycelium might be lying latent on or in the grain tissues, awaiting favourable conditions for proliferation, so leading to spoilage and possible toxin production. It could contribute substantially to the total viable counts reported in Chapter 3.

Scanning electron microscopy was used to give an overall and in-depth view of the extent and location of mycelium and spores of both normal and mouldy grain. A second technique developed enabled a gross examination of the more loosely attached hyphae to be made. This technique involved shaking a quantity of grain with sterile water, followed by a membrane filtration step. The hyphal fragments retained on the membrane filter could thus be examined and counted.

If a significant proportion of these hyphal fragments was viable, then they could make a considerable contribution to the total count and could also be important to the later rapid invasion of the grain tissue should conditions become favourable. An attempt was therefore made to find out if these hyphal fragments were in fact viable. Using a micro-manipulation technique, selected hyphal fragments were picked for culture on PDAX at 25°C.

4.2. MATERIALS AND METHODS

4.2.1. Scanning Electron Microscopy (SEM)

Quarter segments of barley grains or husk were prepared for SEM as outlined below:-

1. The segments and husks were fixed in 3% gluteraldehyde, 2% formaldehyde in 0.1M phosphate buffer, pH 7.2, for 4 hours at room temperature.
2. They were then washed 3 times with 0.1M phosphate buffer, pH 7.2, for 20 minutes each time.
3. The samples were subjected to secondary fixation in 1% OsO_4 in 0.1M phosphate buffer, pH 7.2, for 2 hours at room temperature.
4. This was followed by 3 buffer washes, again using 0.1M phosphate buffer, pH 7.2, for 20 minutes each time.
5. Samples were then dehydrated in a graded ethanol series - 25%, 50%, 75%, 95%, 100%, 100% for 20 minutes at each concentration but for a minimum of 1 hour at the second 100% ethanol.
6. Finally the samples were critical-point dried using liquid CO_2 as the critical-point fluid.
7. The now dried samples were glued to aluminium stubs using conductive silver paint and sputter-coated with 10-20 nm of gold.

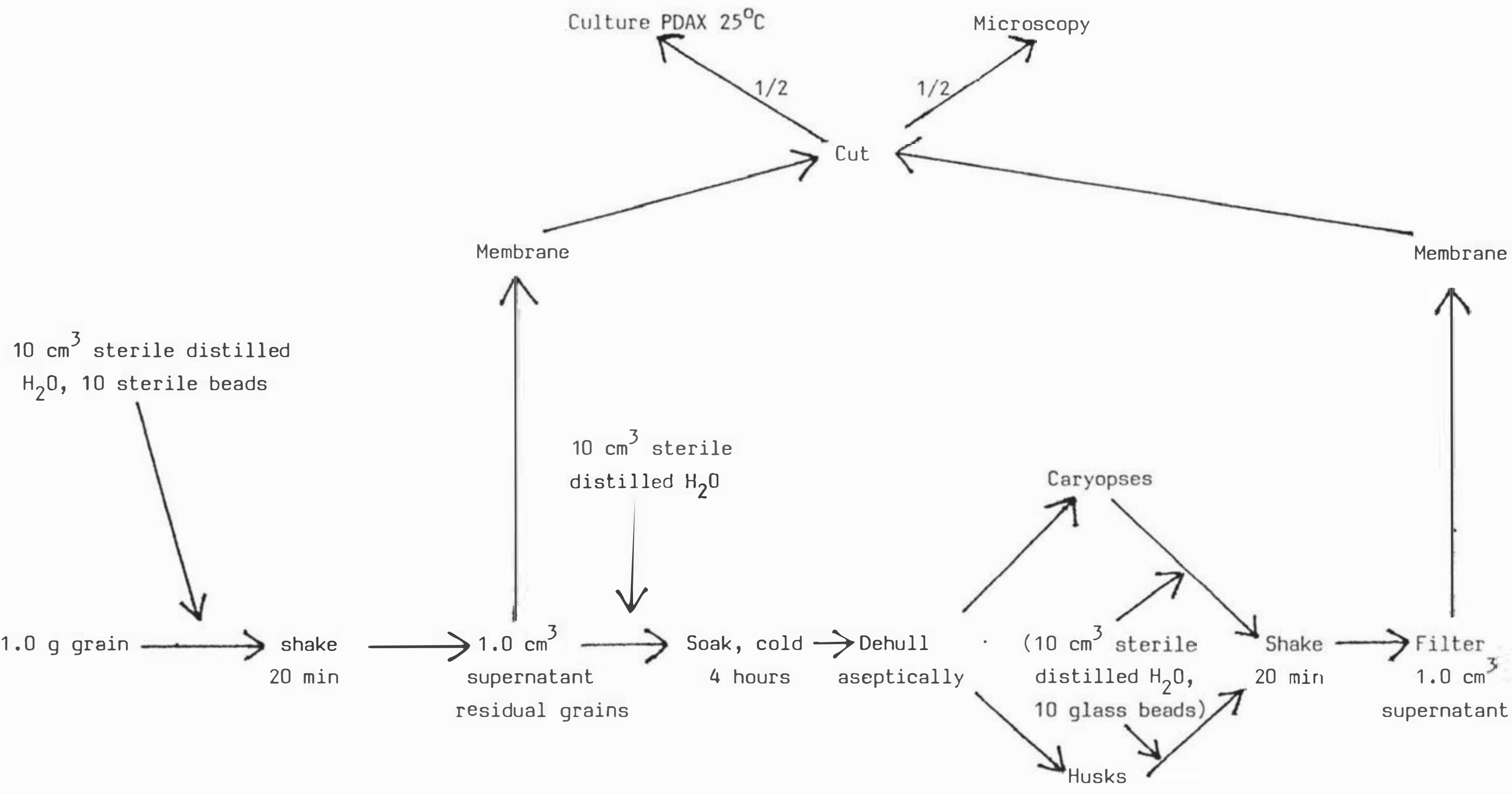
The prepared specimens were examined and photographed in a Cwikscan 100 field emission scanning electron microscope.

4.2.2. Obtaining Hyphal Fragments on Membrane Filters (Figure 4-1)

4.2.2.1. Whole grains

Three x 1.0 g lots of barley grain of each sample from the 6 sources, as obtained in Chapter 3, Section 3.2.1.3 were used. To each 1.0 g grain in a sterile glass universal bottle, 10 cm^3 sterile distilled water and 10 sterile glass beads were added, and the bottle shaken for 20 minutes on a reciprocating shaker. The resulting supernatant was vigorously shaken on a "super-mixer" for 10 seconds and then 1.0 cm^3 was immediately removed and filtered under suction through a millipore membrane filtration system fitted with 0.45 µm

Figure 4-1: Technique for obtaining hyphal fragments on membrane filters



millipore membrane filters. Sterile distilled water (5.0 cm^3) was slowly introduced to wash the sides of the funnel. After completion of filtration, the membrane filter was aseptically removed and treated as outlined in Section 4.2.2.3. The millipore membrane filters, graduated funnels, membrane filter holders and clamps were sterilised each time before use.

4.2.2.2. Husk and caryopsis

The remaining supernatant from the shaking process of whole grains was discarded and the grains gently washed 4 times with 15 cm^3 sterile distilled water before soaking for 4 hours in the cold, in another 10 cm^3 of cold sterile distilled water (Figure 4-1).

At the end of the soaking period, the grains were aseptically dehulled. The husks and caryopses were separately shaken for 20 minutes in 10 cm^3 distilled water with 10 sterile glass beads.

1.0 cm^3 of each resultant supernatant was filtered onto separate membrane filters and the filters were further processed as in the following section.

4.2.2.3. Microscopy and culture of membrane filters

Each membrane filter was aseptically cut into half; one half was used for culture by further cutting that particular half into 8 equal-sized sections and plating the cut sections onto PDAX plates for incubation at 25°C. Any growth arising from these cultured membranes was subcultured onto fresh PDAX for identification.

The other half of the membrane filter was mounted on a slide and covered with a drop of immersion oil for clarification before examination under the microscope. All hyphal fragments retained on the filter were systematically counted, section by section, and simultaneously counts were also noted of hyphal fragments measuring at least 20 um. Single or branched hyphal fragments were considered as "hyphal units" (H.U.).

4.2.3. Separation of Hyphae by Micro-manipulation

4.2.3.1. Source of supernatants

1.0 g subsamples of grain were obtained as described in Section 3.2.1.3 (see Appendix 4-1 for actual number of subsamples). To each 1.0 g grain were added 10 sterile glass beads and 10 cm^3 sterile distilled water before shaking on a reciprocating shaker for 20 minutes.

4.2.3.2. Preparation of agar slabs

Coverslips measuring 22 x 50 mm were placed on a cooled, sterilised area of a large glass plate. The coverslips were sterilised with a bunsen flame and immediately covered with a sterile glass dish. 1.0 cm^3 of sterile molten PDAX was then spread on each cooled coverslip so as to make a thin agar slab. Once the agar had set, another coverslip was flame-sterilised and this was used to cut off a thin strip around the perimeter of the slab. This strip was then discarded.

4.2.3.3. Micro-manipulation technique (Figure 4-2)

A loopful or two of supernatant prepared in Section 4.2.3.1 was spread on one side of a prepared agar slab. The coverslip was then inverted and mounted onto the stage of a platform microscope. Looking under the microscope, hyphal fragments were picked up with the aid of a very fine glass needle and transferred to the opposite side of the agar slab. After this was repeated a few times, the coverslip holding the agar slab was removed from the microscope. With a fresh sterilised coverslip, the agar slab was cut as shown in Figure 4-2, the side containing the hyphal fragments was picked up with a sterilised palette knife and cultured on a thin plate of PDAX. The PDAX plate, which provided a moist environment and nutrients for growth, was incubated at 25°C. In this way, 50 hyphal fragments were picked up from each sample and cultured on PDAX.

4.2.3.4. Observation and isolation of fungi

Over the next 5-7 days, the agar slabs were checked daily under a microscope for signs of growth. The number of fragments yielding colonies was recorded and a small piece of each colony was subcultured onto fresh PDAX plates for identification.

Figure 4-2: Micro-manipulation technique in separating hyphal fragments

Sterile distilled water

Shake

Spread supernatant

Agar slab

Transfer hyphal filaments

Discard

Cut

Culture 25°C, PDAX

4.3. RESULTS

4.3.1. Location of Mycelium and Spores Determined by Scanning Electron Microscopy

Hyphae, spores and other fungal propagules were found sporadically on most surfaces of normal grain specimens viewed by scanning electron microscopy, but as is evident from Plates 4-1 to 4-5, there were areas on the grain where most fungal propagules were concentrated.

The ventral crease formed from folding of the palea and the grooves along the sides of this crease provided excellent sites for the deposition of spores and the attachment of mycelial strands and hyphal aggregates (Plates 4-1 and 4-2). The relatively dense network of rather thick mycelium extended with decreasing density from the groove onto the adjacent area of blunt protrusions and beyond. Many of the mycelial strands present on the grain surface were seen to penetrate the outer epidermal husk layer. The most obvious mode of entry was through stomata as seen in Plate 4-3.

Plate 4-4 shows another preferred site of spores and mycelium. Large concentrations of these fungal elements were observed on the rough surfaces of the rachilla and on the rachilla hairs. There could be seen, under increased magnification of a selected area, mycelial strands wrapped round rachilla hairs and spores lodged in notches especially around the sites of attachment of these hairs (Plate 4-5).

Plates 4-6 and 4-7 show the surfaces and structures of mouldy grains similar to those structures in plates of normal grains (1, 2 and 5), but this time they were covered with abundant mycelium and spores. Even a very small area of mouldy grain, as shown in Plate 4-9, carries a large spore load and dense network of mycelium. Plate 4-8 shows that the fungal mycelium mat, which completely enveloped the grain surface, was made up of relatively slender strands of hyphae intertwined with chains of conidia, chiefly those of *Penicillium* and *Aspergillus*. Of interest were the commonly-occurring large-diameter hyphae which appeared to have collapsed against the grain surface, as shown in Plate 4-9.

Scanning electron microscopy of the outer surfaces of normal husks located relatively low densities of mycelium and spores over large areas (Plate 4-10). Clumps of spores were few and occurred mainly in those areas which allowed their easy attachment, that is areas of depressions, troughs and raised structures (Plate 4-11).

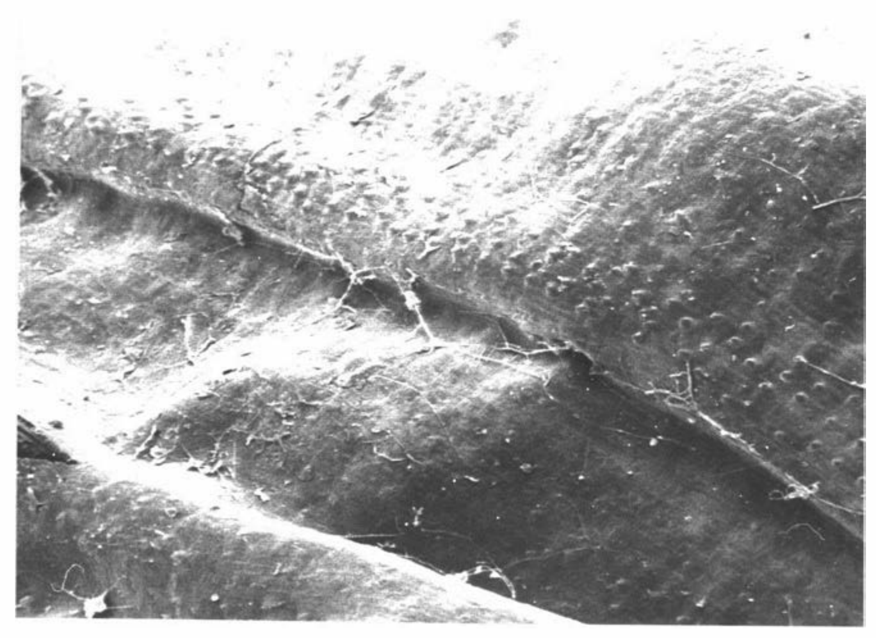
The inner surfaces of normal husk strips (Plate 4-12) were similarly found to contain relatively few spores but the mycelium was in greater abundance than on the outer surfaces. Exceptions were found at the ends and the ventral crease, where mycelium was abundant on the outer surface also.

Plates 4-13 and 4-14, however, showed heavy fungal invasion of inner husk tissues of mouldy grains. Abundant mycelium and spores covered all available surfaces.

Scanning electron micrographs of normal barley grains.

Plate 4-1. Mycelium and spores on ventral surface of whole barley grain (80X) (Commercial silos, 5 months).

Plate 4-2. Interwoven mycelial strands and spores attached to the groove along one side of the ventral crease, with mycelium extending into the adjacent area of blunt protrusions (400X) (Commercial silos, 5 months).



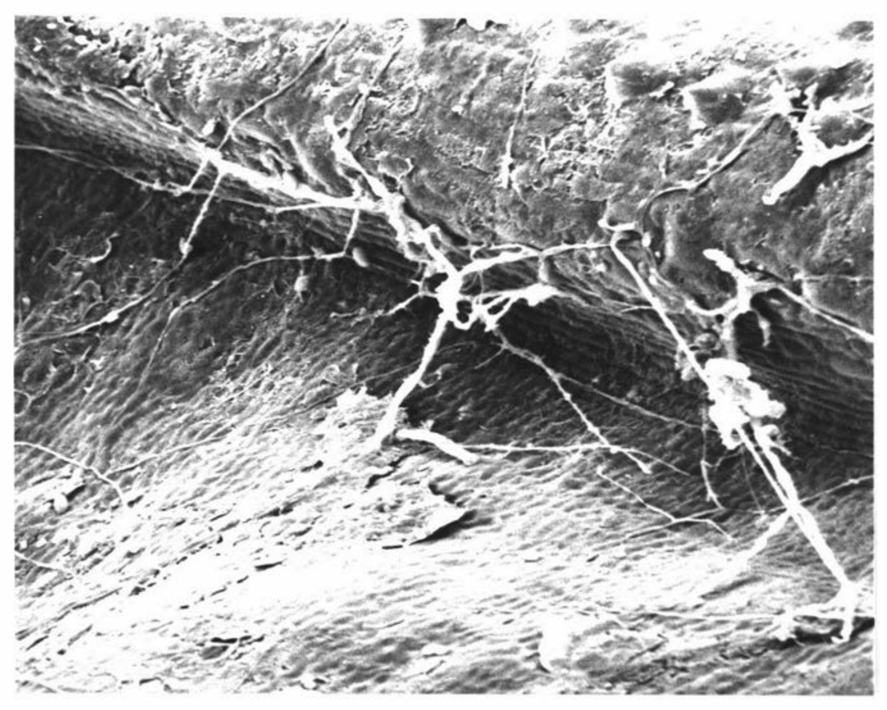


Plate 4-3. Mycelial strands penetrating into stomata cells located just above the groove (400X). Note the silica-like covering of the grain surface especially on the blunt protrusions (Commercial silos, 5 months).

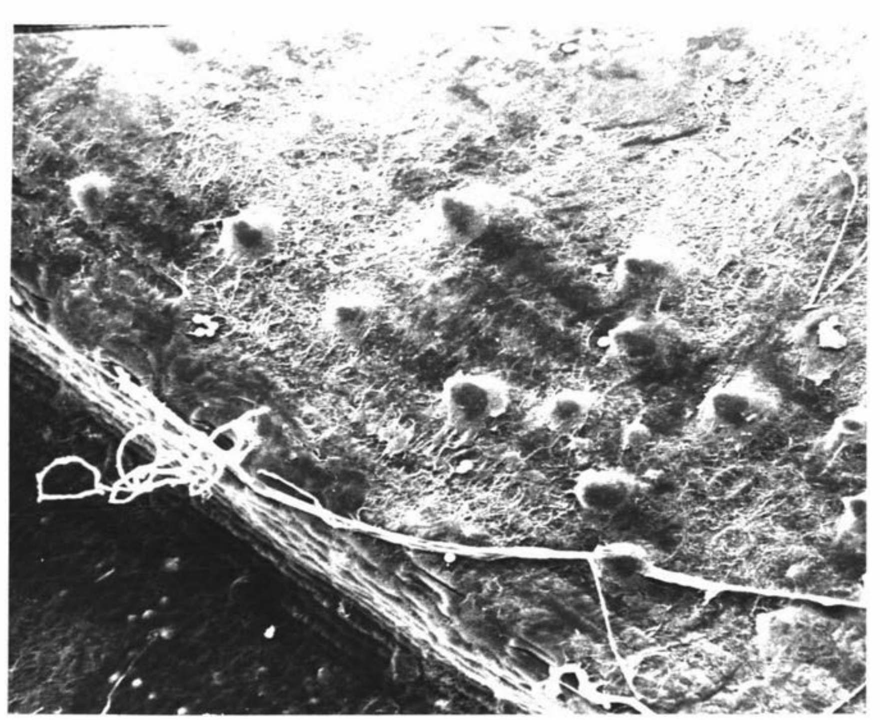
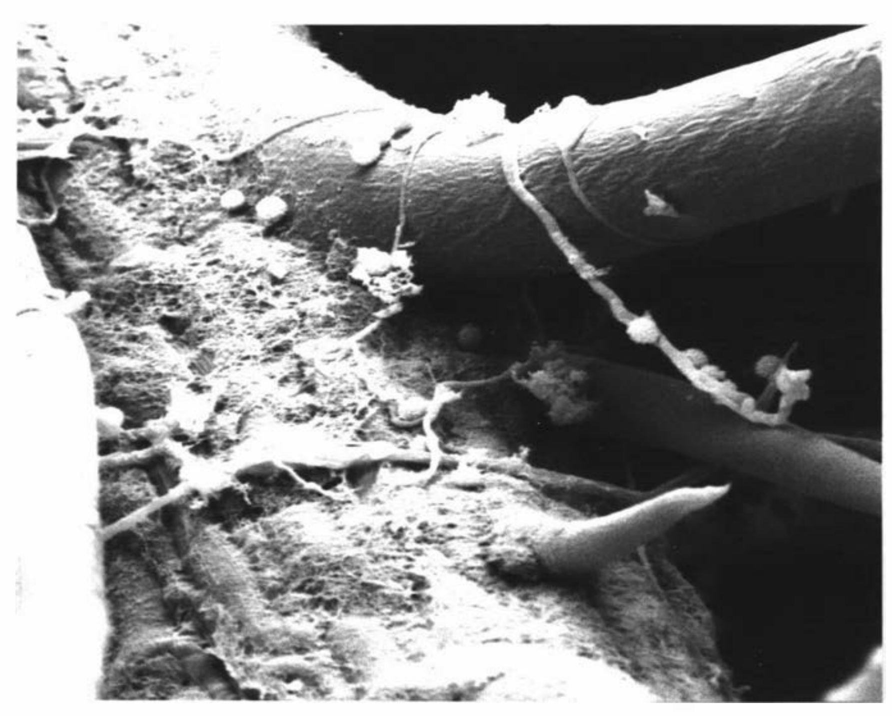


Plate 4-4. Spores "sitting" in indentation and on bottoms of ridges on rachilla surface. Mycelium scattered over the rachilla surface and rachilla hairs (400X) (Commercial silos, 5 months).

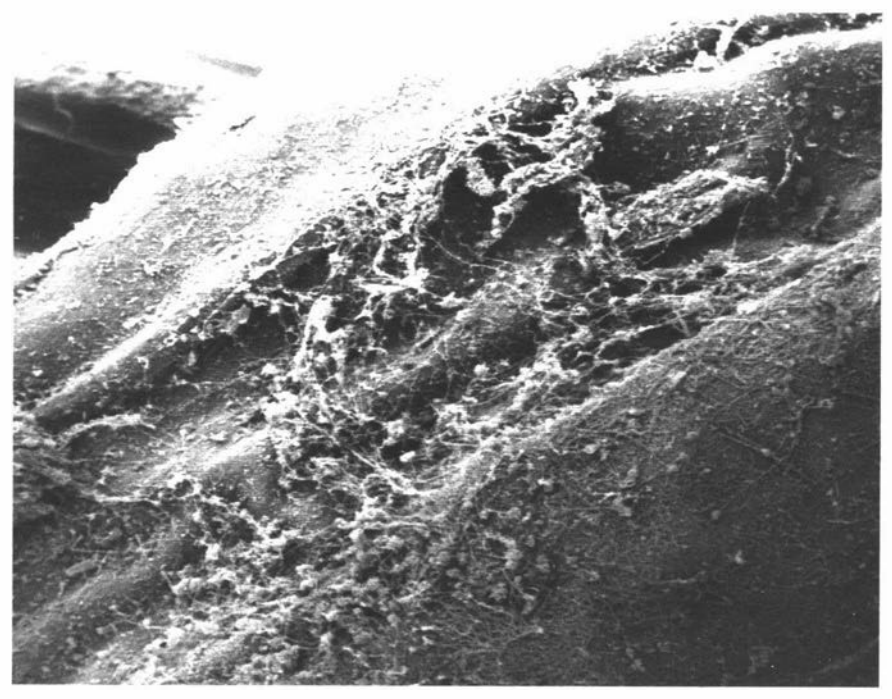
Plate 4-5. "Close-up" view of rachilla surface and rachilla hairs, showing mycelial strands wrapping round rachilla hairs and spores lodged in notches underneath the hairs (1000X) (Commercial silos, 5 months).



Scanning electron micrographs of mouldy grain.

Plate 4-6. Massive fungal contamination of mouldy grain surface, mycelium covering ventral crease (40X) (Farm-stored).

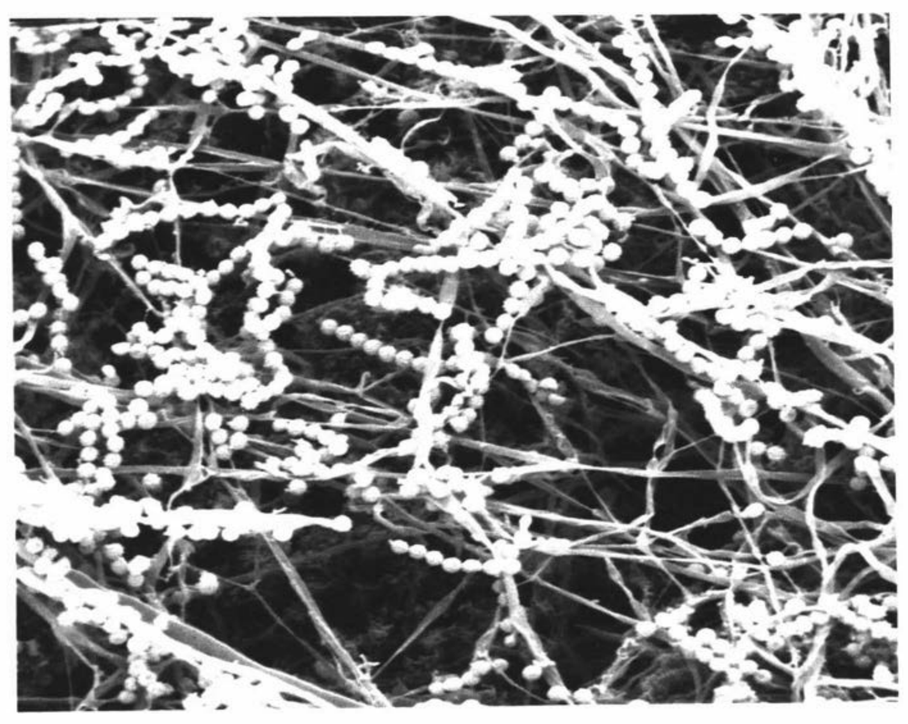
Plate 4-7. Mycelium and spores well established at distal end of ventral crease and on the attached rachilla (50X) (Farm-stored).

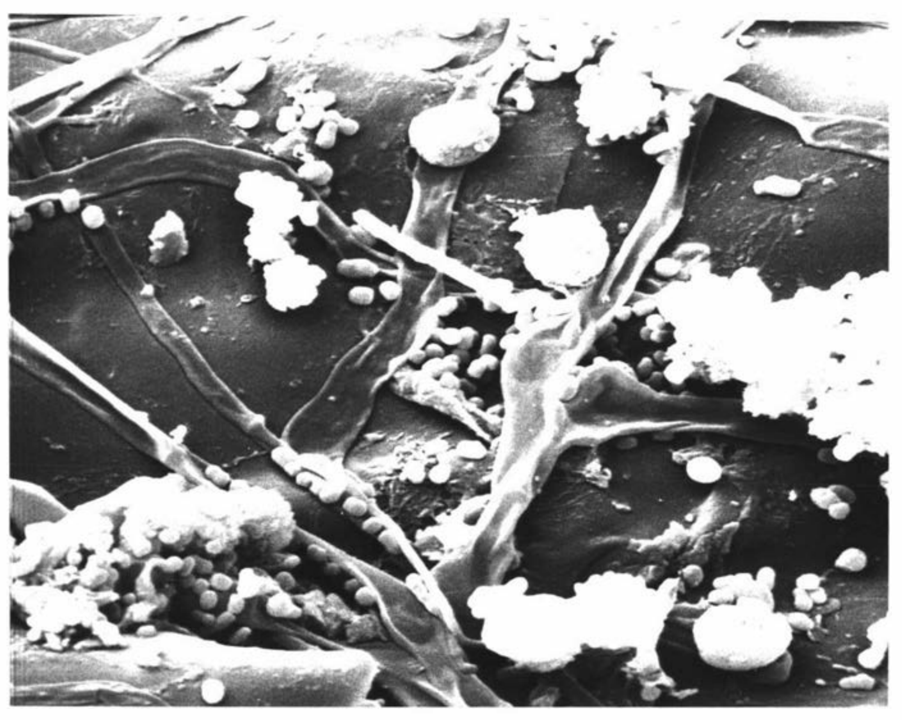


Scanning electron micrographs of mouldy grain.

Plate 4-8. Mycelial mat and chains of conidia completely covering surfaces of mouldy grains (800X) (Farm-stored).

Plate 4-9. Groups of spores amongst interwoven mycelial strands on small area of mouldy grain surface. Numerous bacteria are also present (400X). Note the collapsed, thick hyphae. (Farm-stored).

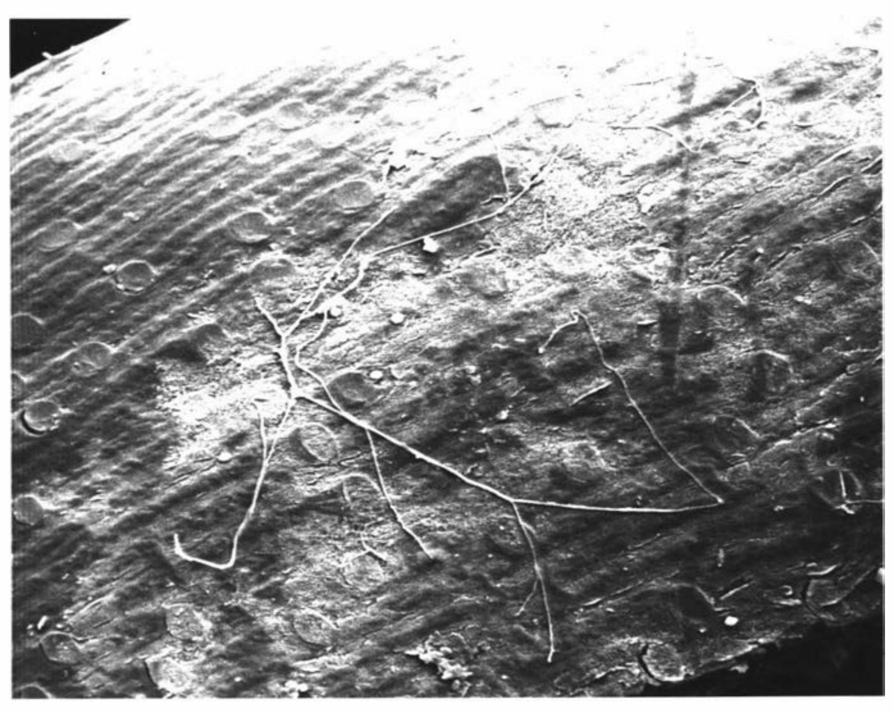


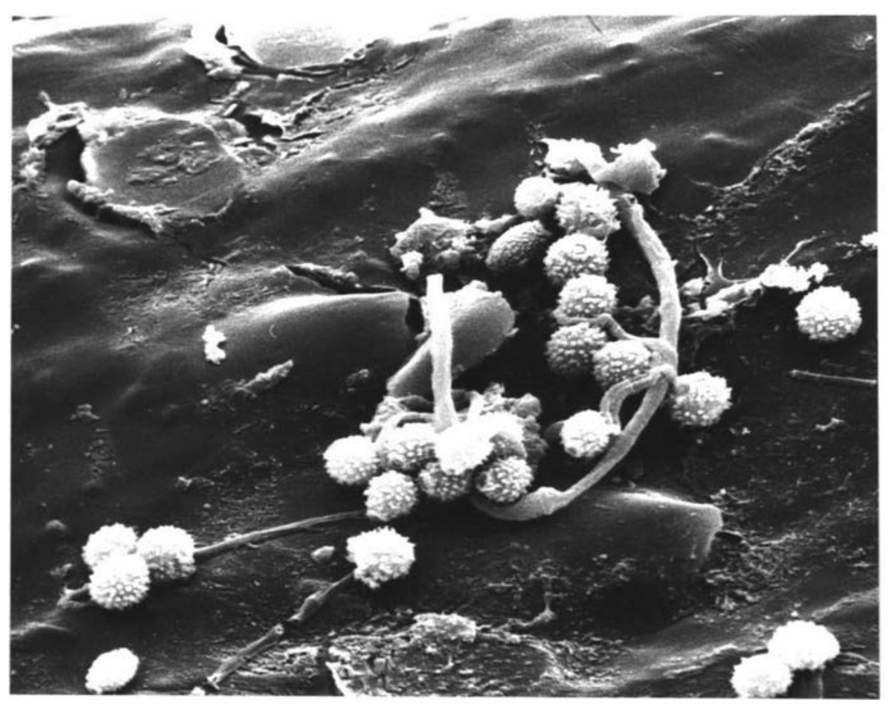


Scanning electron micrographs of normal husk surfaces.

Plate 4-10. Relatively few strands of branching mycelium and sparse spore numbers on outer surface of husk (400X) (At harvest).

Plate 4-11. A clump of spores and hyphae in the depression between 2 blunt protrusions on outer husk surface (2000X) (Farm-stored).





Scanning electron micrographs of inner husk surfaces

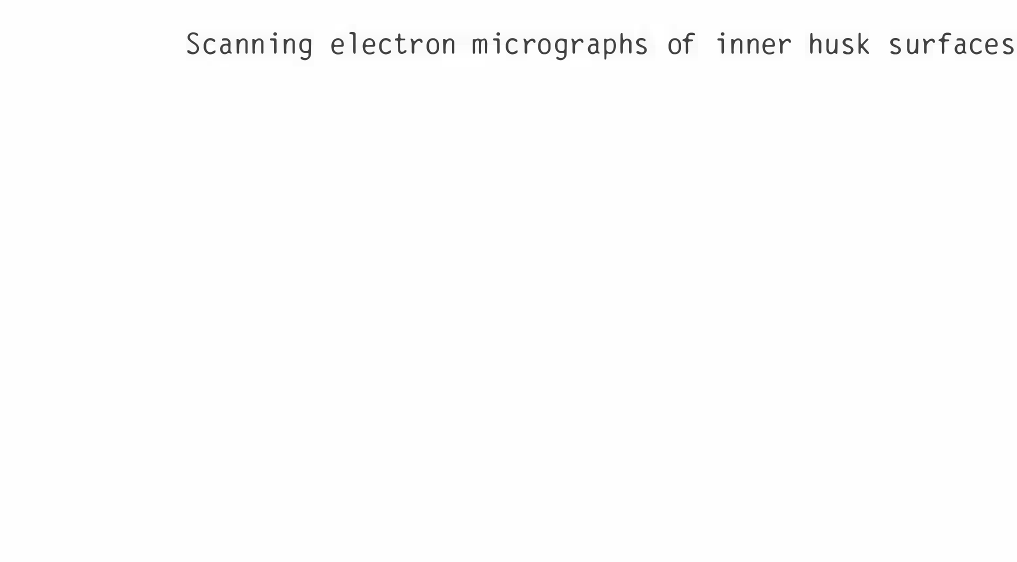
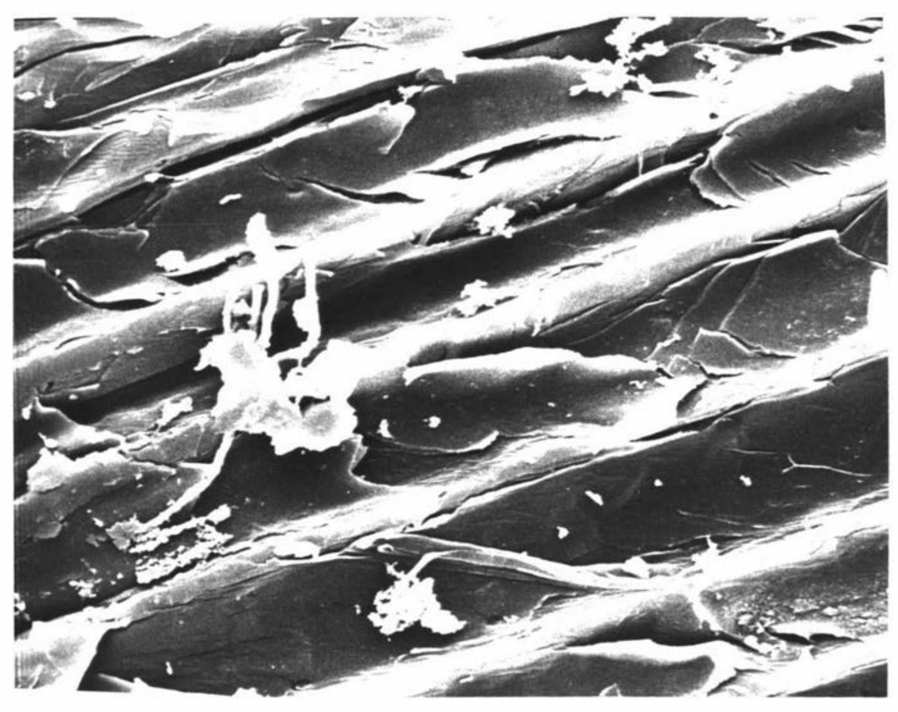


Plate 4-12. A few hyphae and spores populating ridges on inner surface of normal grain (1400X) (Commercial silos, 5 months).



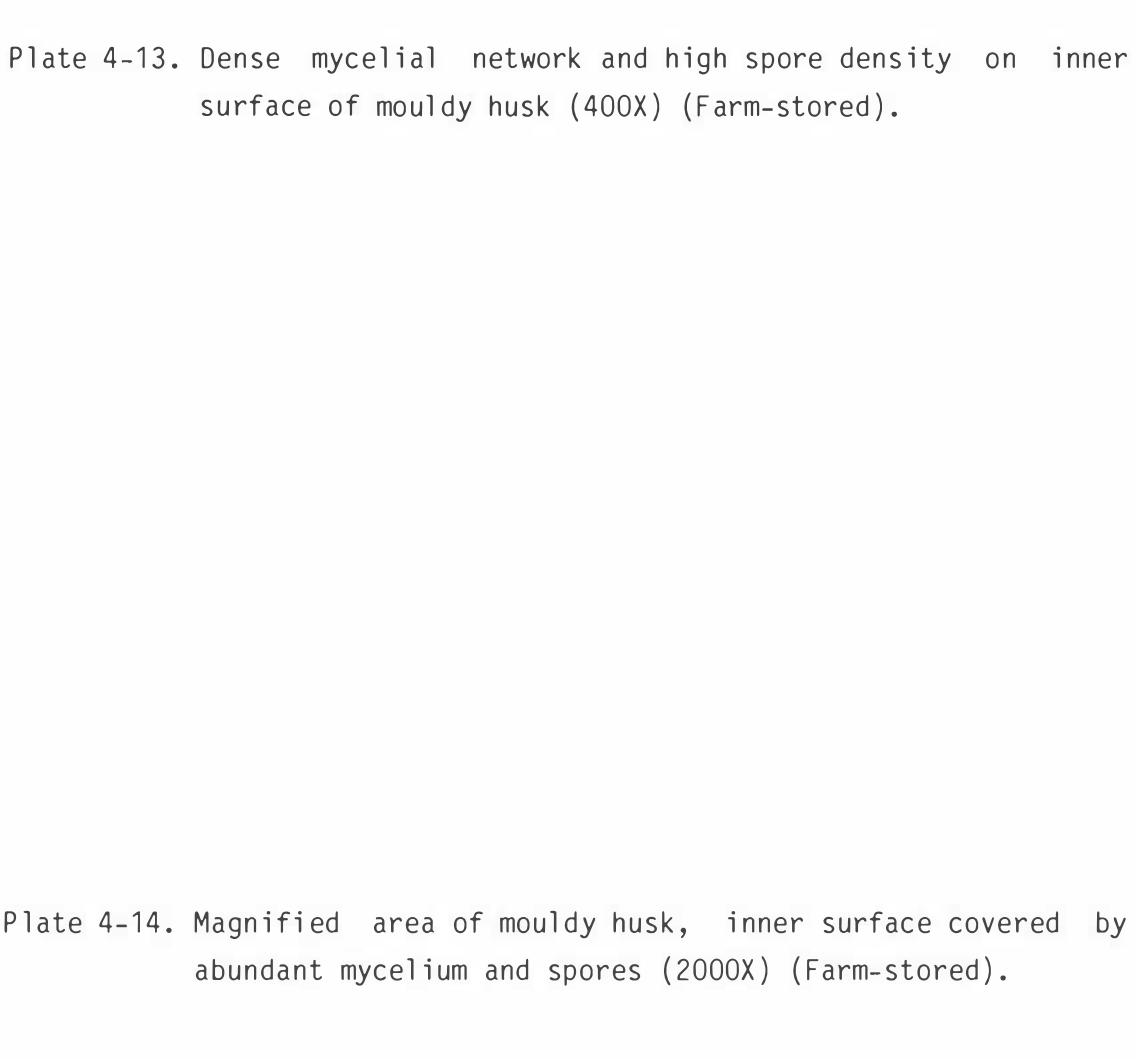
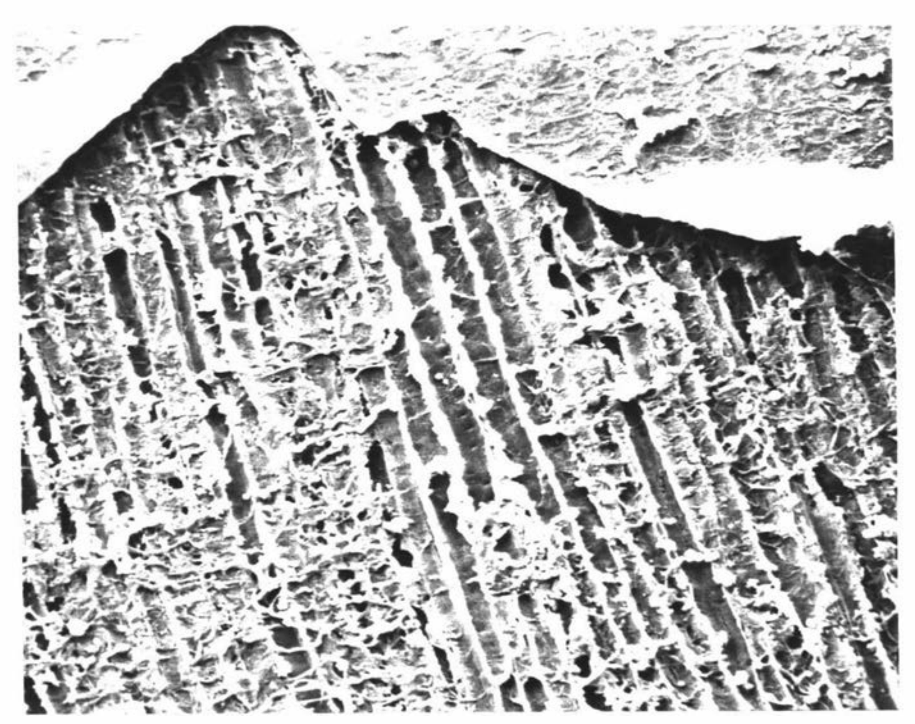


Plate 4-13. Dense mycelial network and high spore density on inner surface of mouldy husk (400X) (Farm-stored).

Plate 4-14. Magnified area of mouldy husk, inner surface covered by abundant mycelium and spores (2000X) (Farm-stored).



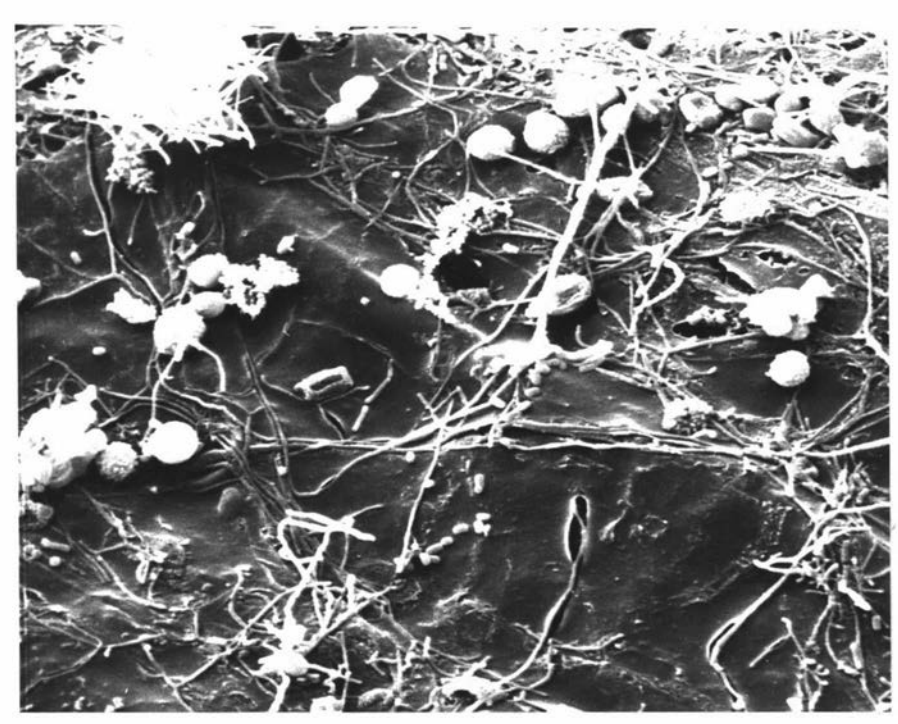


Table 4-1: Counts of hyphal units on membrane filters of barley grains from 6 sources

	At harvest $\times 10^3$			Farm silos 6 months $\times 10^3$	
Sample	Total H.U.*	H.U. >20 μm	Sample	Total H.U.	H.U. >20 μm
A1	2.290	0.900	F1	38.880	15.935
A2	9.260	2.010	F2	16.650	4.100
A3	1.360	0.400	F3	3.124	0.764
A4	11.930	3.520	F4	2.240	0.715
B1	7.940	2.500	F5	5.430	1.780
B2	3.770	0.990	F6	1.417	0.785
B3	4.400	0.950	F7	4.945	1.375
F4	5.110	1.110	F8	2.680	0.660
Mean	5.758	1.548		9.421	3.264

	Commercial silos 5 months $\times 10^3$			Commercial silos 9 months $\times 10^3$	
Sample	Total H.U.	H.U. >20 μm	Sample	Total H.U.	H.U. >20 μm
A5	3.000	0.625	A8	2.575	0.720
A6	4.685	0.920	A9	3.475	1.230
A7	2.415	0.645	B9	4.485	1.455
B5	3.520	0.740	B10	2.585	0.750
B6	3.970	1.190	B11	3.585	1.100
B7	3.120	0.845	B12	1.570	0.545
B8	1.235	0.430			
Mean	3.135	0.771		3.046	0.976

* H.U. = hyphal units

Table 4-1 (continued)

Laboratory x 10^3

Cold			Ambient temperature		
Sample	Total H.U.	H.U. > 20 μm	Sample	Total H.U.	H.U. >20 μm
A1	1.645	0.335	A1	1.620	0.685
A2	6.275	1.935	A2	5.615	2.150
A3	1.265	0.370	A3	0.805	0.410
A4	5.450	1.885	A4	4.535	2.010
B1	6.510	2.410	B1	2.850	1.435
B2	3.240	1.145	B2	1.810	1.030
B3	2.300	0.595	B3	0.835	0.465
B4	2.800	0.830	B4	1.060	0.635
Mean	3.685	1.188		2.390	1.103

4.3.2. Total Counts of Hyphal Fragments

The count of total hyphal fragments obtained by averaging the result from triplicate samples of each grain sample are tabulated in Table 4-1. With the exception of 2 samples of farm-stored grain, there was little difference in the total hyphal unit counts (T.H.U.) for individual grain samples from all 6 sources. This is evident from the mean T.H.U. as shown graphically in Figure 4-3. This figure shows that there was little variation in the mean counts of grain which had been held in cold storage to that held in storage for 5 or 9 months at ambient temperature. Farm-stored grain, however, gave high T.H.U.

The mean counts of H.U. measuring more than 20 μm also showed little variation (Figure 4-3), ranging from 25-35% of mean T.H.U. The one exception was obtained for laboratory-stored grain, kept at ambient temperature. The averaged H.U. counts of fragments more than 20 μm consisted of 46% of the mean T.H.U. for those samples. However, this apparently high frequency was due to the lower mean T.H.U. of these samples.

Figure 4-3: Comparative levels of hyphal units obtained from supernatants of grain samples

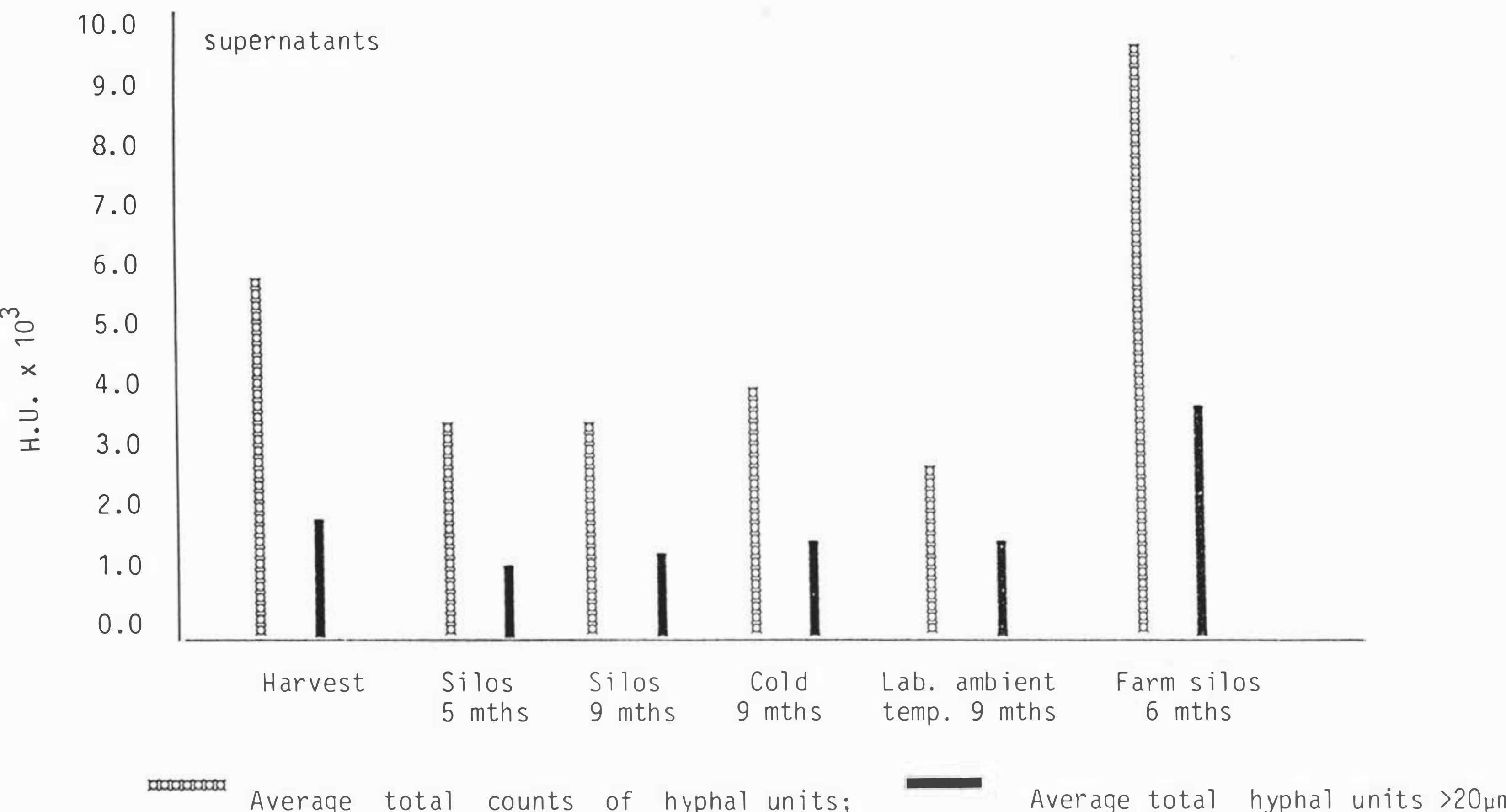
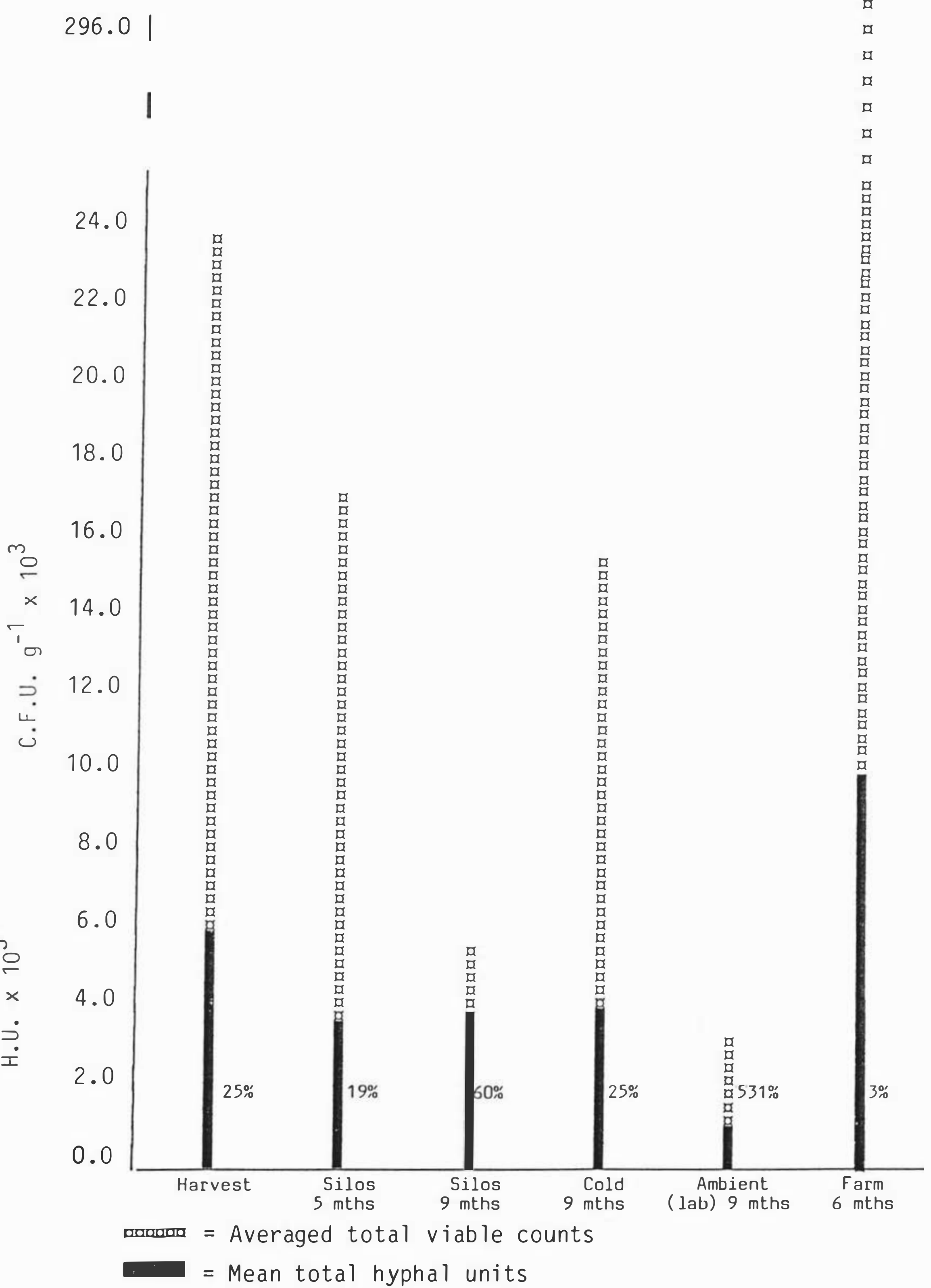


Figure 4-4: Comparison of viable counts as obtained by the dilution plating technique and the counts of hyphal units obtained by the membrane filter technique from 6 grain samples



4.3.2.1. Comparisons of total viable counts and total hyphal units (T.H.U.)

When mean T.H.U. was compared with the total viable count (T.V.C., Chapter 3) for the same grain samples (Figure 4-4), the proportion of T.H.U. to T.V.C. was approximately the same for grain samples at harvest, after commercial storage for 5 months, and after cold storage (19-25%). But the proportion of T.H.U. to T.V.C. rose considerably in the grain which had been stored in commercial silos for 9 months, and a very large increase of more than 500% was recorded in grain stored at ambient temperature in the laboratory. In contrast, the T.H.U. made up only 3% of the T.V.C. in farm-stored grain.

4.3.2.2. Fungi isolated from cultured membrane filters

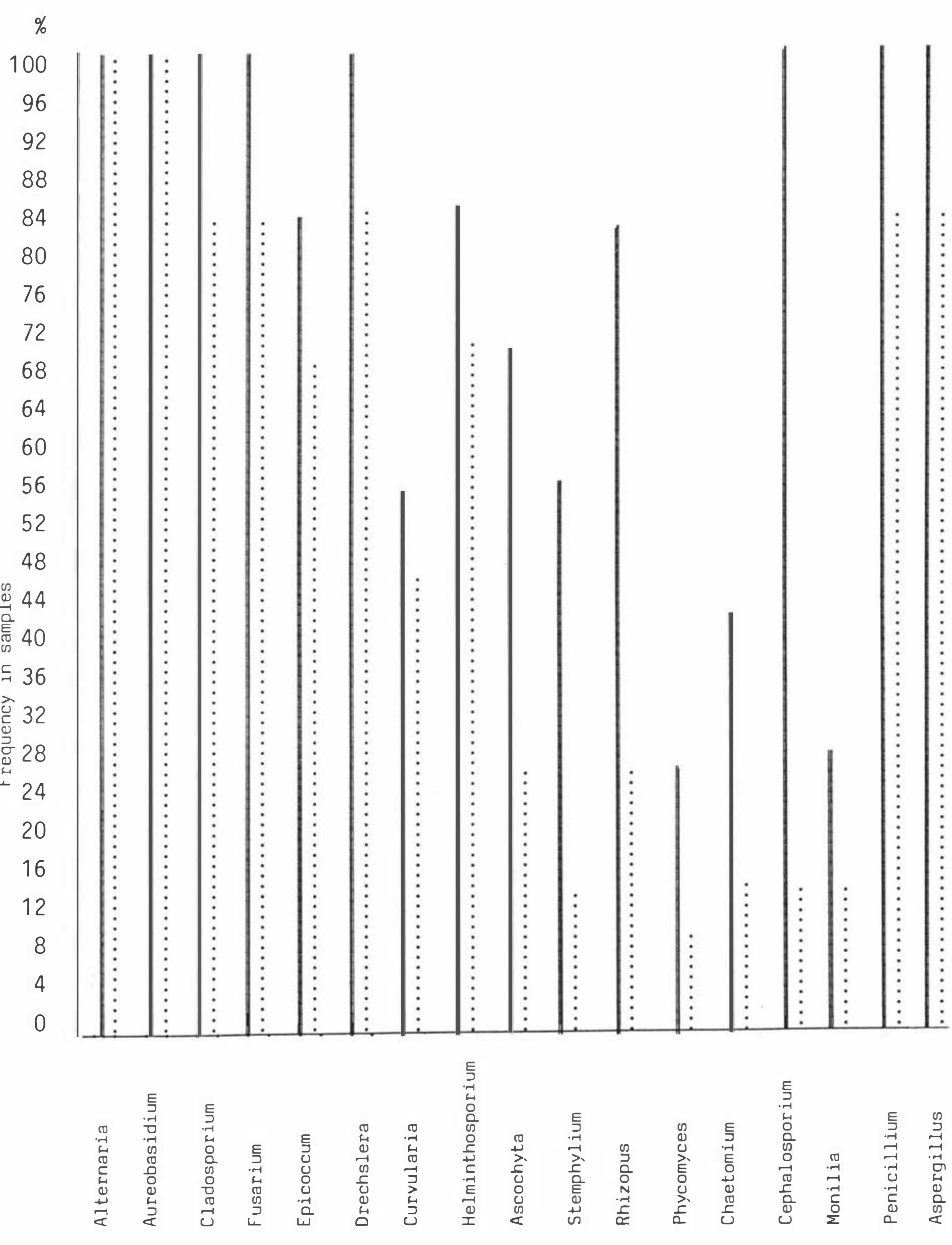
Figure 4-5 illustrates the major fungal genera isolated from PDAX cultures of membrane filter preparations from all the grain samples. Overall, many of these genera were isolated at similar frequencies from dilution plates prepared for total viable counts. Both methods of culture showed the occurrence of *Alternaria, Aureobasidium, Cladosporium, Fusarium, Drechslera, Penicillium* and *Aspergillus* in more than 80% of all the grain samples examined.

4.3.2.3. Viability of hyphal fragments (Figure 4-6)

The viability of the isolated fragments obtained by the micro-manipulation technique showed a decline from 22% viable in fresh samples (compared to total hyphal fragments separated), to 12.5% viable after 5 months' storage to 5% viable after 9 months. There was a small difference in viability loss between silo-stored samples for 5 months (12.5%) and cold-stored grain (18.4%). Viability of hyphal fragments from grain stored in the laboratory at ambient temperatures dropped to 3.5%, a level similar to that of grain stored in commercial silos, for the same period and also at ambient temperature.

In comparison, over 45% of hyphal fragments recovered from an obviously mouldy farm-stored sample were viable. This particular sample had been stored for 6 months.

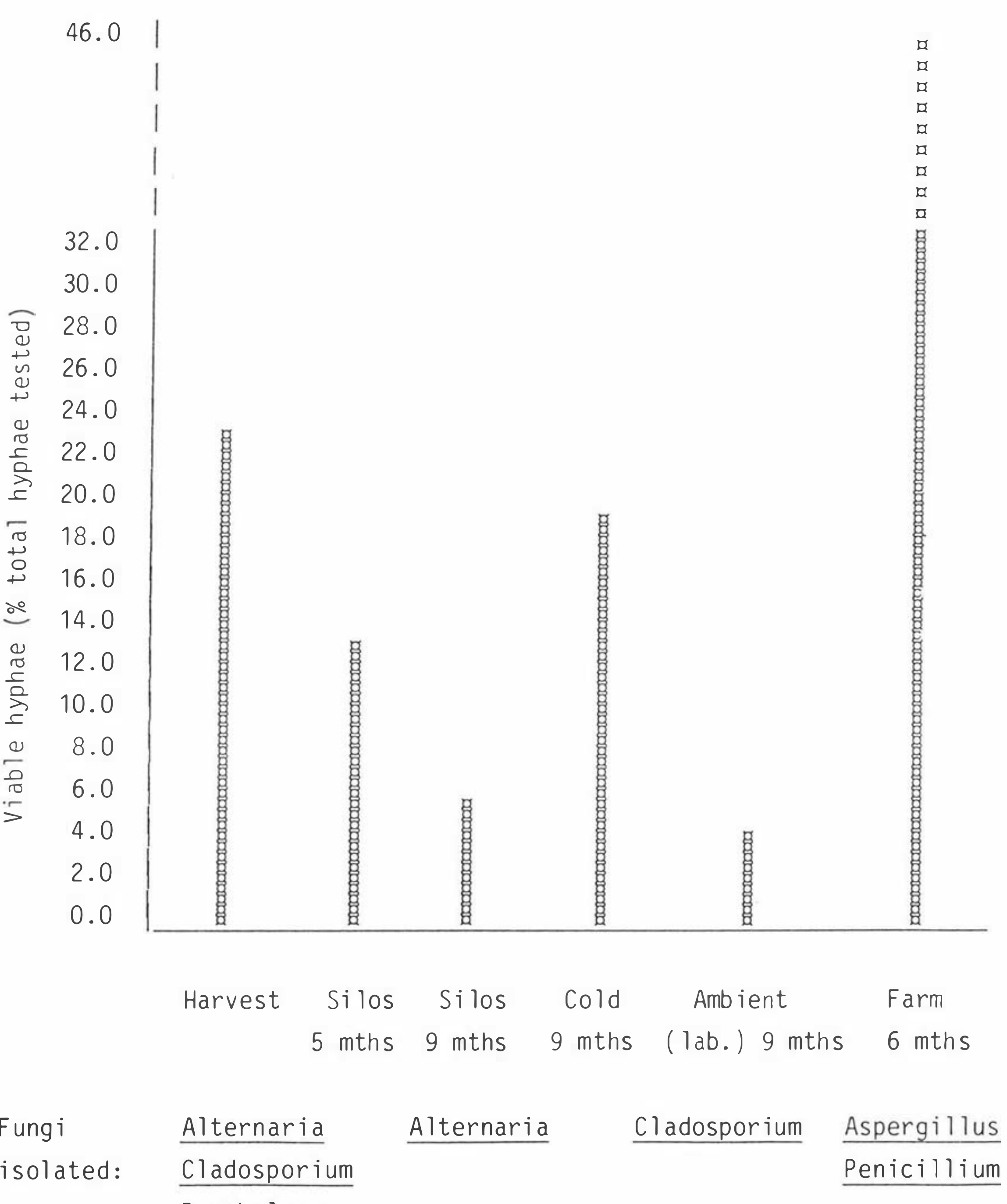
Figure 4-5: Histogram of combined frequencies in grain samples of various genera obtained with total counts and on membrane filters at 25^{0}C



——— Frequency in dilution plate samples (total viable counts)

...... Frequency in membrane filter samples.

Figure 4-6: Viable hyphal fragments in supernatants of grain samples from 6 different sources



Fungi isolated:	Alternaria	Alternaria	Cladosporium	Aspergillus
	Cladosporium			Penicillium
	Drechslera			
	Fusarium			

4.3.2.4. Fungi from hyphal fragments

Colonies which grew from the isolated hyphal fragments obtained from freshly-harvested and stored grain were identified as Alternaria, Drechslera, Fusarium and Cladosporium, with Alternaria by far the predominant fungus, growing from >80% of the hyphae cultured (Figure 4-6). Penicillium and Aspergillus species were obtained only from hyphal fragments obtained from mouldy grain.

The Alternaria and Cladosporium fungi grew from thick brown hyphal fragments, whilst Penicillium and Aspergillus grew from more slender, hyaline hyphae. Some thick brown hyphae isolated from mouldy grain did not grow in culture.

4.4. DISCUSSION

Scanning electron microscopy of barley grains has revealed that all grain surfaces bear not only spores but also a loose network of mycelium, in contrast to the claims of Oxley and Jones (1944) that unless grains were mouldy no hyphae could be found on the outer surface of the epidermis.

However, grains not heavily infected showed relatively sparse mycelium over the outer surface, with the exception of concentrations in highly contoured areas. This finding was in agreement with the direct microscopy examinations of mycelium-containing barley husks described in Chapter 3. Hyde (1950) and Hussein (1983) also observed that the distribution of mycelium in husks was biased towards the two ends and along the sides of the ventral crease. Hussein (1983) and Mead (1942) further suggested that the proximal end of the grain was the most favoured site for the establishment of hyphae in husks. These observations support the work of Warnock (1971) who showed that the area of palea (which folds to form the ventral crease), in which mycelium was present varied from 44.2-92.8%, in contrast to that of the lemma which varied from 19.7-87.7%.

In the present study, scanning electron microscopy has confirmed the findings of light microscopic observations (Chapter 3) that

mycelium and spores occur in great abundance on the rachilla, a structure always found attached to the barley grains examined. The hyphae were seen to wind themselves round the rachilla and its hairs.

So fungal hyphae i) interweave, ii) weave between husk cells and cell layers, and iii) wind around any available structures to form a complex network of mycelium covering the grain. The resultant lattice of fungal mycelium serves as a spore trap in its own right as well as enhancing the effectiveness of other spore-retaining sites such as crevices, ridges and depressions on the rough husk surfaces. Thus it was not unexpected to find that spore distribution was related to abundance of mycelium.

Colonisation of the "internal" barley surface was studied by removing the husk for S.E.M. The internal fungal mycelium of apparently healthy grain was more abundant than that on the outer surface, being especially concentrated within the parenchyma layer as described by Warnock and Preece (1971). The parenchyma layer is the third of the 4 layers of cells which together make up the lemma and palea. Sclerenchyma cells make up the second layer, and these 2 layers are "sandwiched" by epidermal cells.

Warnock (1973a) believed that the hyphae on the inner surfaces were derived from spores deposited on the inner lemma and palea surfaces and from spores deposited on the anthers which remain lodged between the lemma in matured grain. The spread of mycelium could be checked by desiccation beneath the epidermis during drying-out of the ripening grain in the field (Hyde and Galleymore, 1951).

S.E.M. of heavily infected barley grain has not been reported in the literature but many direct light microscopy observations of stained mouldy husks have agreed that mycelium development was normally very extensive and often covered the whole grain including husks and caryopses. It has been found that interspersed amongst the mycelial strands of mouldy grains were clumps and chains of spores in large numbers. These spores were recognised as being chiefly those of Aspergillus as fruiting bodies of Aspergillus were numerous in the S.E.M. of such grains and husks. Further verification of their

identity was provided by Brown and Brotzen (1979) in their book illustrating S.E.M. of phytopathogenic fungi.

The mycelium of mouldy grain and husk consisted mainly of relatively slender hyphae whilst the majority of hyphae associated with normal grain tissues were of thick diameter. Many of these thick hyphae were seen to have collapsed in the scanning electron photomicrographs. These collapsed hyphae were likely to be dead hyphae that earlier had been established during the formation and maturation of the grain.

The method of obtaining supernatants from which hyphal fragments were retrieved was important in the examination of the loosely-attached hyphae of barley. The number and duration of shakings and the number of washings were some of the physical factors contributing to the counts of hyphal fragments obtained from the grains. Provided that the grains were all subjected to identical treatment, comparisons of hyphal fragments from the various grain samples could be made.

It can be deduced from the small variation in counts obtained of H.U. from grain at harvest and after commercial and laboratory storage that there was no active fungal growth during storage of these grain samples. This was supported in the present study by the larger total H.U. count obtained from farm-stored grain, much of which became mouldy during storage. The T.H.U. count for the farm silo grain was almost twice that recorded for grain at harvest and these counts were increased through fungal proliferation. When fungal growth occurs on or in the husk, more hyphae are produced, many growing out from husk tissues. These outgrowths to the existing aerial hyphae form an expanding, loose network of mycelium, fragments of which become easily detached from the grain upon vigorous shaking.

Special note was taken of the quantity of hyphal fragments measuring at least 20 µm in the counts of H.U. from the grain samples because these fragments were likely to have originated from relatively young fungal hyphae. This factor combined with their size gave them a good chance of proliferation should suitable conditions be available. Thus they could contribute to the total viable counts in dilution platings of barley washings and to fungal growth in the grain. Aged

and dead hyphae, on the other hand, tended to break easily into smaller fragments during the shaking process and were less likely to give rise to fungal colonies.

With the exception of the farm-stored grain, the counts of H.U. of at least 20 μm showed little variation, again indicating no fungal proliferation in the grains examined. The counts of H.U. of at least 20 μm of farm-stored grain were again twice those of the grain at harvest, indicating probable fungal growth.

So from the H.U. counts of fragments of at least 20 μm, between 25-35% of the mean total H.U. counts could contribute to the total viable counts provided the hyphae were viable. The high proportion of H.U. of at least 20 μm (46%) for laboratory-stored grain was due to the low T.H.U. counts rather than an actual increase in counts of the fragments. However, this could mean that H.U. measuring at least 20 μm could make significant contributions to total viable counts of this grain. For the reasons given in Chapter 3, the low T.H.U. counts showed that fungi which had infected these grain samples at harvest had disappeared rapidly in sealed storage at room temperature.

T.H.U. counts obtained in the present studies did not appear to have any significant correlation with their respective T.V.C. An increase in T.H.U. could be expected from fungal development during storage. But if storage conditions did not allow any fungal growth, the grains would retain the amount of mycelium they had acquired prior to harvest with the exception of the loosely-attached hyphae lost during handling. Undoubtedly some of the hyphae would also shrivel and disappear when exposed to dry conditions after the harvest and during storage (Milner et al., 1947). This was illustrated in Figure 4-4. The T.H.U. for the grain samples stored in commercial silos and in the laboratory were about the same, but they were lower than for the T.H.U. from grain at harvest.

In addition, the total viable counts were obviously determined by the number of viable units, the majority of which were spores. The factors which determine the number of spores and the viability of spores and hyphae would be different from the determinants of mycelial quantity.

Although no direct relationship could be shown between T.H.U. and T.V.C., on superimposing the T.H.U. of the grain samples onto their respective T.V.C. (Figure 4-4), it became apparent that the proportion of T.H.U. to T.V.C. increased dramatically for grain stored in commercial silos or kept sealed in the laboratory at ambient temperature for 9 months. A comparatively large T.H.U. count could mean that a larger proportion of T.V.C. could consist of colonies arising from the growth of hyphal fragments rather than from spores. This could be due to the initiation of mycelial growth being shorter than the time taken for most spores to break their dormancy, and also to the absence of an overwhelming spore population, meaning less competition for nutrients and other food requirements (Smith, 1975). This situation could be found on PDAX plates, but when it occurred on grains, particularly grains with reduced spore populations, the fungal hyphae could be important sources of rapid fungal invasion of the grain tissues under suitable conditions. When fungal invasion was present as in some farm-stored grain, the fungal hyphae would offer a comparatively less significant contribution than spores to T.V.C. This was not only due to the much larger spore population present but was also partly due to a reduction in the hyphal fragments broken off from the relatively younger mycelia.

The T.H.U. and T.V.C. obtained from laboratory-stored grain kept at ambient temperature revealed an interesting fact: that is, not all hyphal fragments present in the grain supernatants were viable, for if they were viable then the T.V.C. would at least approximate to the T.H.U. The viability of hyphal fragments will be discussed later.

Unfortunately the culture of membrane filters permitted the growth of fungi from spores as well as from hyphal fragments retained on the filters, so it was not unusual for the pattern of fungi obtained from the membrane filter cultures to resemble those obtained from dilution plates. It would be impossible to differentiate the fungal propagules from which fungi such as Alternaria, Cladosporium and Fusarium colonies were subcultured.

The presence of abundant mycelium within cereal grains is not in itself evidence that fungi significant in disease or in spoilage have invaded the seed. Instead the viability and identity of the fungal

hyphae present would be the two most important factors in determining the significance of such hyphae colonising barley tissues.

Whilst the methods used by several investigators such as Hyde (1950), Warnock (1971, 1973b) and Warnock and Preece (1971) were useful in assessing the amount, extent and location of hyphae present in barley grains, no attempts were made to check their viability. The fluorescence methods of Warnock (1971, 1973) were very successful in detecting specific fungal hyphae such as those of *Alternaria* and *Penicillium cyclopium* in barley husk, but did not give any indications of their ability to grow when provided with suitable conditions.

Hussein (1983) was successful in isolating fungi which grew from barley husks cultured on Malt-salts agar and PDA, but it cannot be known with complete certainty that all the fungi isolated grew from the fungal mycelium present in the husks. Although Hussein surface-sterilised the husks with NaOCl and gave them several washes with shaking and with sterile glass beads, not all spore contaminants could be removed. Complete surface sterilisation may not be achieved due to the nature of the barley husks and the surface characteristics of some types of spore. Furthermore, vigorous shaking and washing processes would remove much of the hyphae present on the husk which could be of significance, and it has been suggested that surface sterilisation with NaOCl could result in the death of the mycelium of some more deep-seated fungal species (Milner *et al.*, 1947; Mulinge and Chesters, 1970b).

In comparison, the micro-manipulation technique used in the present studies allowed the isolation and identification of viable hyphae as well as the determination of the frequency of viable hyphal fragments in the supernatants from grain samples without the problems encountered in the methods just discussed.

The 22% viability of hyphal fragments isolated from freshly-harvested grain indicated that most of the loosely-attached hyphae were not living. Drying of the husk tissues during ripening of the grain has been known to arrest the spread of mycelium (Martin and Gilman, 1976) and this drying effect could adversely affect the viability of established mycelium. Moreover, the use of fumigants and

chemical sprays on the grain prior to harvest could cause the demise of a large proportion of these fungal hyphae.

Figure 4-6 showed the continued decline in the viability of fungal hyphae during storage of grain at ambient temperature in commercial silos and in the laboratory. Of all the storage conditions, the moisture content at which the grain was stored had the greatest impact on the viability of the associated fungal hyphae. The field fungi cultured from hyphae detached from freshly-harvested grain were identified as *Alternaria*, *Cladosporium* and *Drechslera*. Such fungi have been known to be susceptible to desiccation. Lutey and Christensen (1963) provided experimental evidence to show that the storage of barley at low moisture content in temperatures of 20°C, resulted in the death of field fungi such as *Helminthosporium* and *Fusarium*, and caused a large reduction in the survival of *Alternaria*. So the loss in fungal hyphal viability in the grain stored at ambient temperature around 15°C and at low moisture levels of 12% could be attributed to the demise of the fungal hyphae of field fungi.

Storage in the cold appeared to preserve the viability of fungal hyphae in the barley grain - compare the small decline in viability of these hyphae with that of hyphae from grain stored at ambient temperature.

Thus although hyphal fragments were numerous in stored grain, relatively few were actually viable. In comparison, over 45% of the hyphal fragments from a mouldy sample examined were viable. This particular sample was the only sample from which *Penicillium* and *Aspergillus* species were obtained from hyphal fragments. Field fungi were likely to be present in this mouldy sample, as evident from the brown hyphae picked up from the supernatant by the micro-manipulation technique, but these hyphae did not grow. One obvious explanation for the failure to grow is that the hyphae were no longer viable. Another plausible explanation, however, is that the hyphal fragments were inhibited by the presence of *Aspergillus* and *Penicillium*. Warnock (1973b) has demonstrated that there is a definite inhibition of *A. alternata* mycelium in the presence of metabolic products by *P. cyclopium*. However, this might not be a factor with the present technique as the fragments had been separated by micro-manipulation.

The colonies which grew from the isolated fragments from freshly-harvested and stored grain were identified as being *Alternaria*, *Cladosporium*, *Drechslera* and *Fusarium*. These results are in agreement with the findings of Christensen (1951) and Elekes (1983) using wheat. Christensen found that most of the living mycelia from high-grade wheat grain were those of *Alternaria* whilst most of the living mycelium from low-grade grain belonged to *Penicillium* or *Aspergillus* species. More recently Elekes (1983) observed that *Alternaria* and some *Helminthosporium* mycelia were common in the red and white wheat grain varieties he collected from Europe, North America and Australia. In addition, he also noted the presence of *Fusarium* hyphae in the wheat husk tissues and particularly high infections were present in old red-grained wheat.

Thus fungal mycelium is not only plentiful in mouldy barley grain but is also commonly found in normal grain. The mycelium exists as a fairly loose network from which fragments can be easily detached. Although most of these hyphae are not living, a small percentage are viable in all normal grain samples and an even higher proportion are viable in mouldy grain. So in some situations these viable hyphal fragments may make a significant contribution to the total viable counts. These hyphae may also serve to give the fungus a good inoculum for later spoilage of the grain if conditions become favourable for their growth, and could also be responsible for mycotoxin production within the grain.

Appendix 4-1. Table showing barley sources, samples and subsamples

Barley sources:	At harvest	Commercial silos 5 months	Commercial silos 9 months	Laboratory 9 months Cold	Laboratory 9 months Ambient	Farm silos 6 months
No. of samples	8	7	6	8	8	8
Experiments						
Moisture determinations	8x2 @ 5g	7x2 @ 5g	6x2 @ 5g	8x2 @ 5g	8x2 @ 5g	8x2 @ 5g
Total counting technique	8x3 @ 1g	7x3 @ 1g	6x3 @ 1g	8x3 @ 1g	8x3 @ 1g	8x3 @ 1g
Membrane filtration technique	8x3 @ 1g	7x3 @ 1g	6x3 @ 1g	8x3 @ 1g	8x3 @ 1g	8x3 @ 1g
Micro-manipulation technique	* 3x1 @ 1g	* 3x1 @ 1g	* 3x1 @ 1g	* 3x1 @ 1g	* 3x1 @ 1g	* 3x1 @ 1g
Multi-mycotoxin analyses	** 2x1 @ 25g	* 2x1 @ 25g	* 2x1 @ 25g	* 2x1 @ 25g	* 2x1 @ 25g	* 4x1 @ 25g

Key for subsampling:

1st figure represents number of samples used
2nd figure represents number of subsamples
3rd figure represents number of grams

* the number of samples actually used

CHAPTER 5

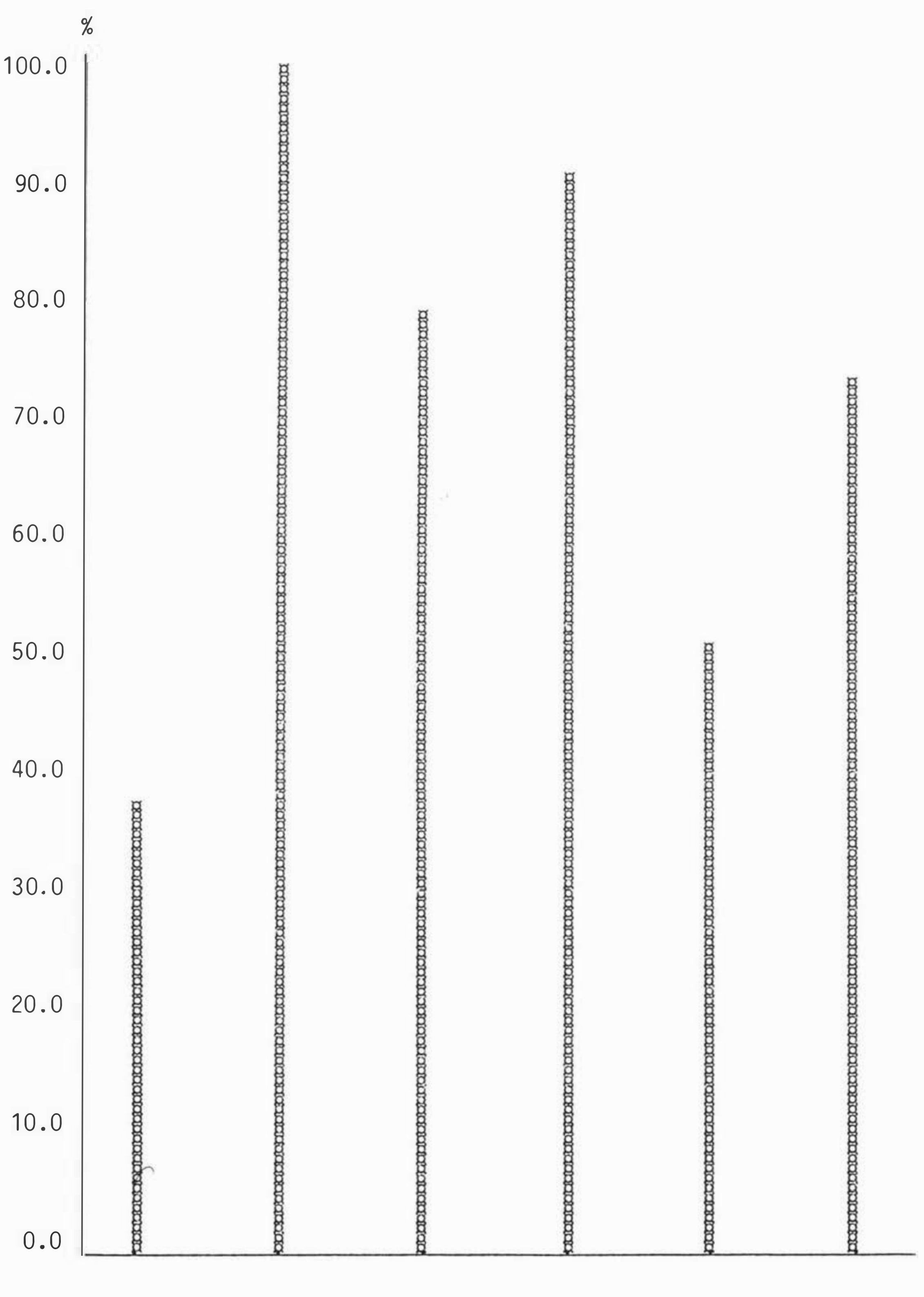
AFLATOXIN-PRODUCING POTENTIAL OF *Aspergillus flavus* ISOLATES

5.1. INTRODUCTION

Actively-growing *Aspergillus flavus* produces various secondary metabolites, some of which are used in flavouring various foods, condiments and drinks especially in the Far East, but others produce adverse effects in man and animals. Some of these biologically-active compounds include aflatoxins, kojic acid, B-nitropropionic acid, aspergillic acid, aspertoxin, flavutoxin, cyclopiazonic acid, aflatrem and dihydroxyaflavinine (Davis, 1981; Wicklow and Cole, 1982). Of these, the aflatoxins are the most significant for their high toxicity and carcinogenicity.

In earlier studies of the mycoflora of barley at harvest and during storage under various conditions, a number of species of *Aspergillus* were amongst the fungi isolated. Members of the *A. flavus* group were isolated both from grains at harvest and from stored grains. The numbers of samples yielding *A. flavus* showed an increase from 37% of those examined at harvest to 70-100% of samples which had been stored at ambient temperature (Figure 5-1).

Figure 5-1: Proportion of barley samples from the 6 sources yielding <u>A. flavus</u>



Sources: At harvest Commercial silos Farm silos Laboratory storage

Storage time: 5 mths 9 mths 6 mths 9 mths cold 9 mths ambient

Thus *A. flavus* strains were present in abundance in most of the barley samples studied, but it was then necessary to determine the presence of aflatoxigenic strains among the total isolates. This was assessed by both screening on coconut agar and by culturing on a natural substrate from which any aflatoxins produced could be "harvested".

Since Vogel *et al.* (1965) first described an agar fluorescence test for detecting aflatoxins elaborated into a Czapek-Dox and groundnut extract agar medium, other agar fluorescent techniques have been used for rapid screening of *A. flavus* populations isolated from foodstuffs, feeds etc. In this study, the coconut agar medium formulated by Lin and Dianese (1976) was chosen in preference to other media (Hara *et al.*, 1974; Torrey *et al.*, 1976 and others) for its simplicity and ease of preparation. Toxigenic strains could be identified by the rapid production of ultra-violet (U.V.) light-induced fluorescence in the agar surrounding the colony of a positive culture. It is also stated by Lin and Dianese (1976) that a specific orange-yellow pigmentation is developed in the reverse of positive colonies.

Cereal grains have been found to be good natural substrates for the laboratory production of aflatoxins by several investigators (Wildman *et al.*, 1967). This is probably due to the large total surface area exposed to air compared to that of liquid cultures, and the fact that moist grains have a high ratio of nutrients to moisture.

It was felt necessary to culture *A. flavus* isolates on a natural substrate to confirm that the fluorescent material produced by some isolates of *A. flavus* in coconut agar is indeed aflatoxin. Some *A. flavus* strains are known to produce other chemical compounds which have the same fluorescent characteristics as aflatoxins (Goldblatt, 1969). The procedure was chosen as barley was the substrate of origin of the strains tested. Aflatoxin production on such a natural substrate could be better related to the situation in nature than would be possible using liquid or agar media.

5.2. MATERIALS AND METHODS

5.2.1. Aspergillus strains

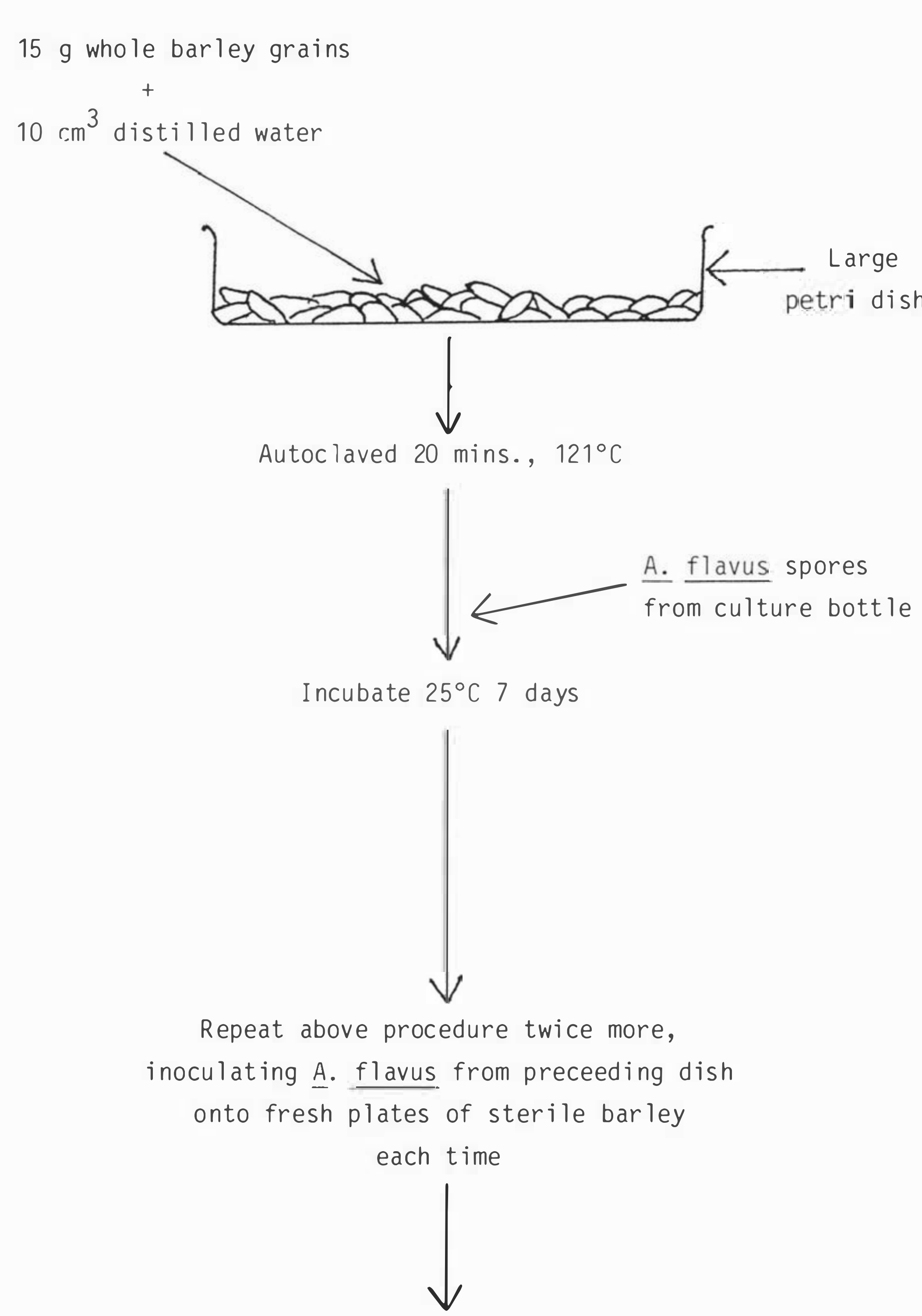
A total of 81 randomly-selected isolates of *A. flavus* was obtained from barley grains at harvest and after storage (see Chapter 3). These isolates were transferred to Potato-Dextrose Agar containing 0.05 mg/cm^3 chloramphenicol and maintained on slants at room temperature until required for testing. Prior to the inoculation of freshly-prepared coconut agar plates the isolates were passaged at regular intervals on a bed of sterilised barley grains as depicted in Figure 5-2. In addition, a known toxigenic strain of *Aspergillus parasiticus* (NRRL 2999) supplied by Dr. Rex Gallagher of the Ruakura Animal Research Centre, Hamilton, was used as a positive control.

5.2.2. Screening method

a) Preparation of coconut agar plates:
Commercial "Coconut Cream" as manufactured by Samoa Tropical Products Limited was used as the substrate. A can of Coconut Cream with a net weight of 420 g contains 430 cm^3 of coconut cream, water and polysorbate 60. To 200 cm^3 of coconut cream was added 600 cm^3 of distilled water and 1.5% agar. The pH of the resulting medium was approximately 6.9. After autoclaving at 121°C for 20 minutes, the medium was dispensed aseptically into sterile petri dishes.

b) Inoculation of plates and observation of fluorescence:
Strains of *A. flavus* isolates were inoculated onto the centre of the coconut agar plates and the plates incubated in the dark at 25°C. From the 3rd through to the 21st day of incubation, the plates were inverted and observed for fluorescence around the colonies under long-wave (365 nm) ultra-violet light from a hand-held lamp or in a Chromato-Vue cabinet.

Figure 5-2: Serial transfers of *A. flavus* species on barley grains



5.2.3. Selection of *A. flavus* isolates for screening

Some of the *A. flavus* isolates previously screened on coconut agar were randomly selected for culture on sterile whole barley grains to investigate their ability to form aflatoxin.

All 81 isolates were grouped into 6 sampling units in relation to their source (Table 5-2). The identification code of every isolate in each unit was written on a piece of paper. The total pieces of paper belonging to one unit were shaken in a bag before being individually drawn out at random. In this way, a "frame" for each sampling unit, made up of identification codes listed in the order in which they were drawn from the bag, was constructed.

The 4 *A. flavus* isolates from grains at harvest were all screened for aflatoxin production, together with 5 isolates from each of the remaining sampling units. Depending on the length of the frame in each sampling unit, one individual was chosen and the other 4 individuals were taken at regular intervals. In cases where an isolate positive on coconut agar (Table 5-4) was not selected, it was included as the 6th isolate to be screened for toxigenicity.

5.2.3.1. Culturing *A. flavus* for toxins

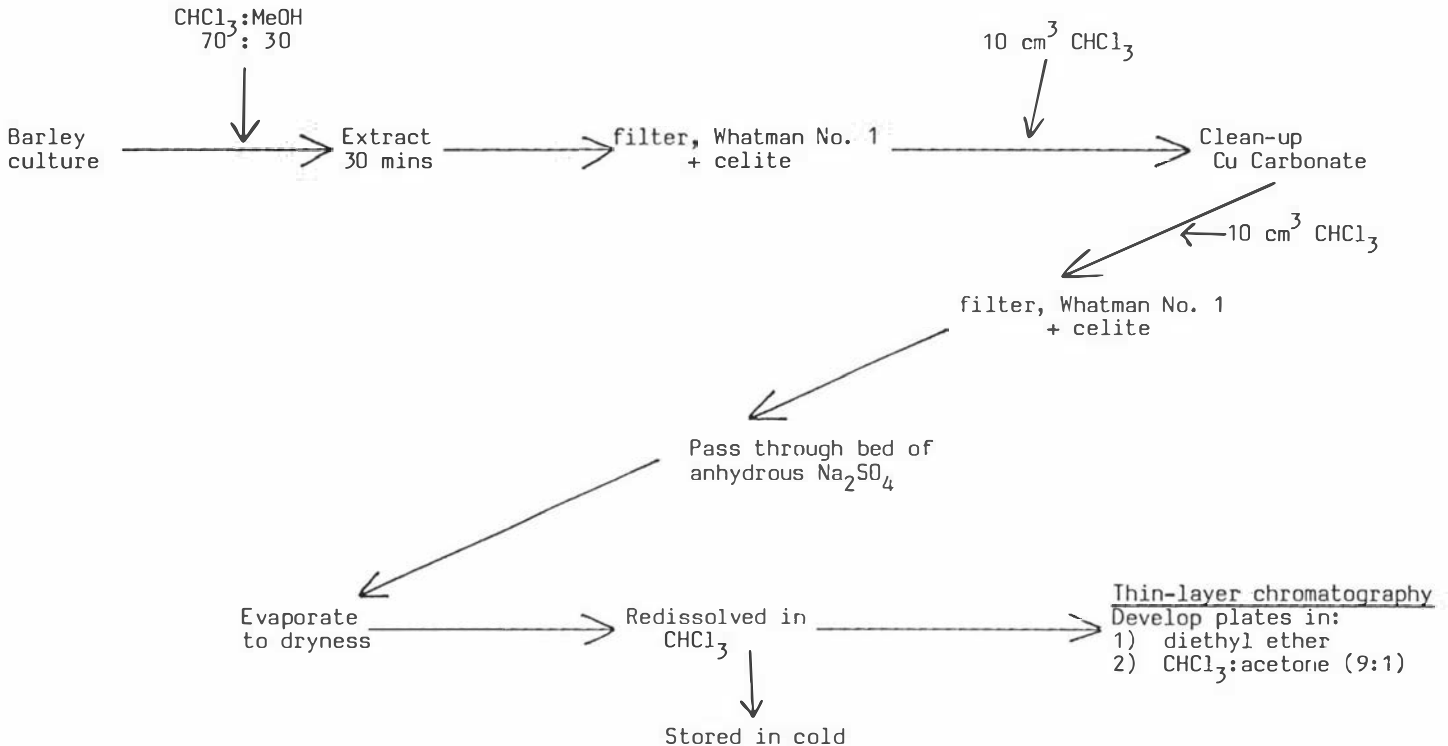
The selected *A. flavus* isolates from coconut agar plates were inoculated onto sterile, moist barley in glass plates, and incubated in the dark for 7 days at 25°C. These cultures were used as the source of inoculum for duplicate test cultures on 15 g of sterile, moist barley contained in wide-mouthed, screw-capped glass bottles. Incubation was in the dark at 25°C. One bottle of each isolate was extracted for toxins on the 7th day and the other on the 18th day of incubation. Analytical grade solvents and chemicals* were used throughout.

5.2.3.2. Recovery of aflatoxins from barley cultures

a) Toxin extraction:
   Using a method recommended by the Tropical Products Institute (Jones, 1972) (Figure 5-3), aflatoxins were extracted with

* B.D.H. Chemicals, Poole, England.

Figure 5-3 Recovery of aflatoxins from barley cultures



chloroform:methanol ($CHCl_3$:MeOH, 70:30). The barley culture was broken up and ground with a pestle after the addition of 15 cm^3 of chloroform. Sufficient chloroform-methanol mixture was added to give a final ratio of chloroform-methanol of 70:30. The aflatoxins were extracted into the solvent mixture by vigorous shaking on a reciprocating shaker for 30 minutes.

b) Clean-up of extract:

After the solid residues were given a few minutes to settle, the solvent mixture containing aflatoxins was filtered through Whatman No. 1 filter paper and Celite Hyflo-Supercel filter aid. Chloroform (10 cm^3) was used to wash the residues. The extract was "cleaned" by swirling with basic copper carbonate. When the copper carbonate had been allowed to settle, the extract was again passed through filter paper and Celite filter aid. The copper carbonate residue was washed with 10 cm^3 $CHCl_3$ and the $CHCl_3$ filtered through the previously-used filter paper and filter aid into the pool of treated extract. Following this, the pooled extract was drained through a bed of anhydrous sodium sulphate (Na_2SO_4). The bed of sodium sulphate was then washed with a further 10 cm^3 chloroform.

c) Concentration of extract:

The pooled extract was evaporated to near dryness in a clean, round-bottomed glass vessel, under vacuum on a Buchi rotary evaporator. The remaining extract was dissolved in 2 cm^3 $CHCl_3$ and transferred to a small glass vial for storage until required for assaying.

5.2.3.3. Detection of Aflatoxins present in extracts

a) Thin layer chromatography:

i) Spotting of extracts:

20 x 20 cm aluminium-backed thin layer chromatographic (TLC) plates* (Merck) were used to examine both test extracts and standards. Before spotting, the plates were activated for 60 minutes at 110°C. The plates were then allowed to cool to room temperature before spotting on a pre-marked line. Disposable

* T.L.C. plates - precoated with 0.2 mm silica gel 60 F_{254} (without fluorescent indicator) E. Merck, Darmstadt, F.R. Germany.

micro-pipettes* were used to spot 5.0 μℓ, 10.0 μℓ, 20.0 μℓ of sample extracts and aflatoxins standards#.

ii) Development of TLC plates:

Spotted TLC plates were first developed in a lined, equilibrated tank of analytical grade diethyl ether, under subdued light to a mark 15.0 cm from the line of spotted extracts. The plates were allowed to air dry in a dark fume cupboard before they were redeveloped in a mixture of chloroform-acetone (9:1) contained in a lined, equilibrated tank to a mark 12.0 cm from the original line of spotted extracts. Developed plates were then air dried in a fume cupboard and finally viewed in a Chromato-vue cupboard under ultra-violet light.

iii) Chemical confirmation of aflatoxins:

Extracts which produced fluorescent spots after developing in chloroform acetone (9:1) at Rfs similar to the standards used were chemically confirmed for the presence of aflatoxins.

iv) Derivative formation with Trifluoroacetic acid:

The method described by Przybylski (1975) was used for derivative formation. Freshly-prepared trifluoroacetic acid (TFA) diluted 1:1 with chloroform, was used throughout. TLC plates were activated and spotted with 5.0 μℓ and 10.0 μℓ of suspected aflatoxin extracts and aflatoxin standards. TFA (10.0 μℓ) was then superimposed on these spots. The spots were allowed to react for 5 minutes in a dark fume cupboard. They were then dried with the aid of an air blower, but care was taken to ensure that the air temperature above the spots did not exceed 40°C. The prepared TLC plates were developed as outlined in Section 5.2.3.3.

v) Treatment with 25% suphuric acid:

Activated TLC plates were spotted with 5.0 μℓ of suspect extracts and aflatoxin standards. The spots were air dried in a fume cupboard before the TLC plates were developed as in 5.2.3.3. After drying the developed plates were viewed in a Chromato-vue cabinet and the fluorescent spots were lightly outlined by pencil. Sulphuric acid was then sprayed onto the

* Capillary micro-pipettes were obtained from Drummond Scientific Co., U.S.A. # See p. 181 for concentrations and source.

TLC plates concentrating on those areas where the outlined spots were located. The treated TLC plates were allowed to air dry in the dark before viewing under ultra-violet light.

5.3. RESULTS

5.3.1. Screening of A. flavus Isolates on Coconut Agar

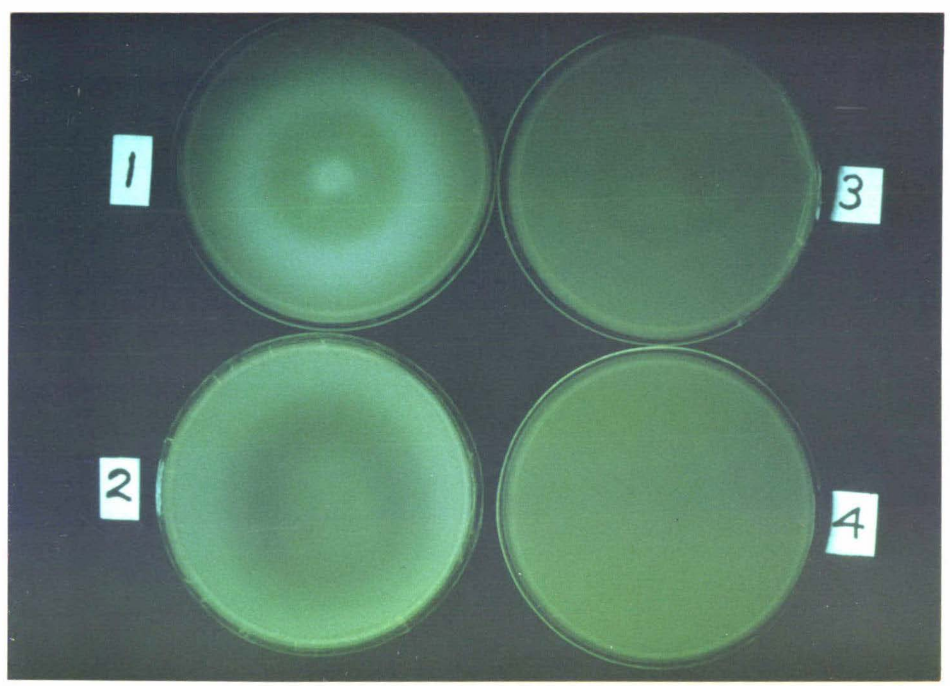
Uninoculated coconut agar plates and plates inoculated with non-toxin-forming isolates appear dull grey under U.V. light. All the aflatoxin-positive (AT) A. flavus isolates tested were observed to produce a characteristic bluish fluorescence similar to that produced by the control strain, A. parasiticus NRRL 2999 (Plate 5-1). The bluish fluorescence of AT-positive isolates became distinct on the 4th day of incubation at 25°C (Table 5-1), and was still perceptible at the end of the 21-day incubation period.

Table 5-1: Production of fluorescence by toxigenic A. flavus isolates (5 isolates from 4 sources)

Source of strain	Appearance of fluorescence No. of days 3	4	5	6	7	10	12	14	17	21	Presence of pigmentation of colony reverse
Harvest samples	-	±	±	+	+	+	+	+	+	+	Yellow-brown
Commercial silos 9 months	±	+	+	+	+	+	+	+	+	+	Light yellow
Farm silos 6 months	+	+	+	+	+	+	+	+	+	+	Yellow
Laboratory storage Cold. 9 months	±	+	+	+	+	+	+	+	+	+	Light yellow
	±	+	+	+	+	+	+	+	+	+	Yellow

Plate 5-1. Fluorescence in Coconut agar under U.V. light.

1. Plate inoculated with known aflatoxin-positive *A. parasiticus*, NRRL 2999.
2. Plate inoculated with test *A. flavus* isolate (Positive)
3. Plate inoculated with aflatoxin-negative *A. flavus* isolate
4. Uninoculated coconut agar plate.


1
3
2
4

Yellow to yellow-brown pigmentation was present on the reverse of all the fluorescent colonies but the presence of pigmentation was not an exclusive feature of toxin-positive isolates. The reverse of colonies of some AT-negative isolates were also yellow to yellow-brown. Isolates which did not produce any fluorescence by the 4th or 5th day of incubation failed to do so even after 21 days of incubation.

Table 5-2 shows the number of toxigenic A. flavus isolates from various samples in relation to the total number of isolates. The percentage of positive aflatoxin producers tested on coconut agar varied from 25.0% of isolates from grains at harvest to 3.2% of those kept in farm silos. But the actual number of positive strains present showed little change from harvest to storage even though the total numbers of isolates may have increased dramatically. Overall, about 6% of the 81 A. flavus isolates screened were capable of toxin production.

Table 5-2: Fluorescence of A. flavus isolates on Coconut Agar

Sources		A. flavus isolates No. tested	No. showing fluorescence	% positive
At harvest		4	1	25.0
Commercial silos	5 months	15	0	0
	9 months	11	1	9.1
Farm silos	6 months	31	1	3.2
Laboratory 9 months	Cold	9	2	22.2
	Ambient	11	0	0
Total		81	5	6.2

Table 5-3 summarises the results of fluorescence testing together with pigment production on coconut agar of all *A. flavus* isolates examined.

Table 5-3: Production of fluorescence and pigmentation on coconut agar medium by A. flavus isolates

Samples	No. of A. flavus Isolates				Pigmentation colour
	Total	Fluorescence +ve	Pigmentation +ve	Pigmentation -ve	
At harvest	4	1	1	3	Yellow-brown
Commercial silos					
5 months	15	0	2	13	Light yellow
9 months	11	1	8	3	Light yellow to yellow-brown
Laboratory					
9 months cold	9	2	4	5	Light yellow to yellow
ambient	11	0	4	7	Light yellow to yellow-brown
Farm silos					
6 months	31	1	15	16	Light yellow to yellow-brown
A. parasiticus					
NRRL 2999	1	1	1	-	Yellow-brown

5.3.2. Screening of Selected *A. flavus* Isolates on Barley

The initial development of TLC plates in diethyl ether helped remove to the solvent front fats and impurities present in the extract. On subsequent development in chloroform-acetone, a chromatogram showing bright fluorescent spots of aflatoxins such as in Plate 5-2 can be obtained. By comparing the Rf values of the fluorescent spots of *A. flavus* extracts and aflatoxin standards, the aflatoxin produced by all the toxigenic isolates were identified to be aflatoxin B1 (Table 5-4).

In no case could aflatoxins B2, G1 or G2 be detected. The table also shows that all the *A. flavus* isolates which produced aflatoxins after 7 days' incubation also produced detectable amounts of the same aflatoxins after 18 days' incubation.

By acid-catalysed hydration, aflatoxin B1 hemiacetals are formed in reacting aflatoxin B1 with trifluoroacetic acid in the presence of water. These show up as bluish fluorescent spots on TLC plates developed in appropriate solvent systems, for example chloroform-acetone (9:1) as in Plate 5-3. The formation of these fluorescent spots of aflatoxin B1 derivative at the same Rf values as corresponding standards confirmed the presence of aflatoxin B1.

Table 5-4: Aflatoxin production by selected A. flavus isolates on sterile barley

Samples		Total no. tested	Days of incubation 7		18		Identity of Aflatoxins
			No. +ve	No. -ve	No. +ve	No. -ve	
At harvest		4	1	3	1	3	B1
Commercial silos	5 months	5	0	5	0	5	-
	9 months	5	1	4	1	4	B1
Farm silos	6 months	6	1	5	1	5	B1
Laboratory 9 months	Cold	6	2	4	2	4	B1
	Ambient	5	0	5	0	5	-

Plate 5-2. Thin layer chromatography plate developed in diethyl-ether followed by chloroform-acetone.

Showing blue fluorescent spot(s) of:

7: aflatoxin standards, B1, B2, G1, G2

6: aflatoxins from *A. parasiticus*, NRRL 2999

3: aflatoxin B1 from positive *A. flavus* isolate.

Nos. 1, 2, 4 and 5 are spots of negative extracts.

Spots and streaks at the top of the plate are impurities in the extracts.

* T.L.C. plate previously spotted with 10 μℓ aflatoxin extracts and standards.

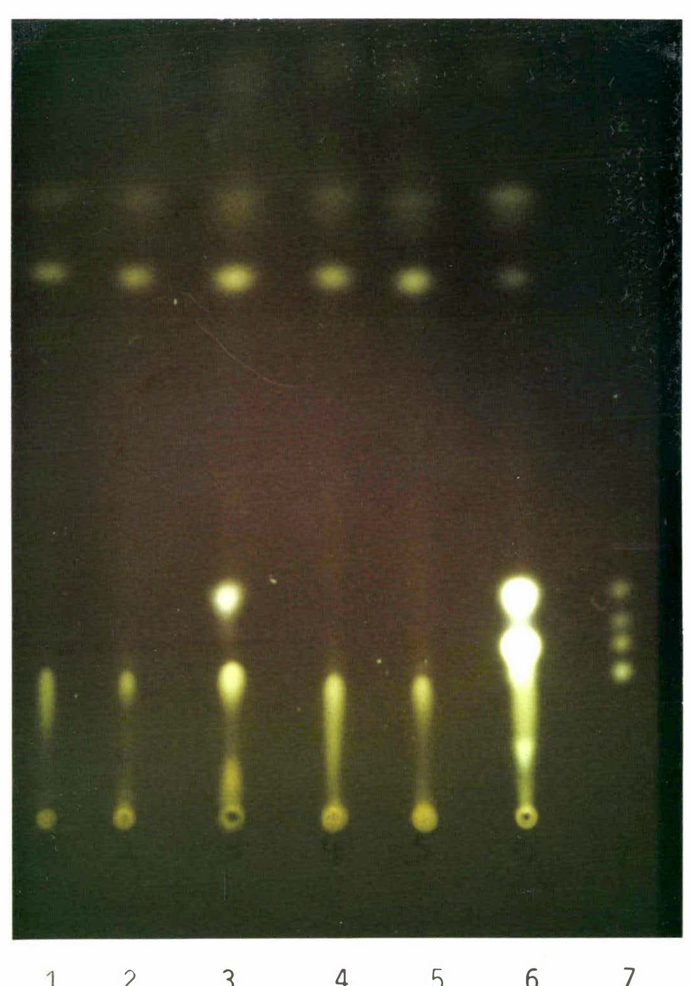

1 2 3 4 5 6 7

Plate 5-3. TLC plate of aflatoxin spots treated with TFA.

A, D, G : Blue fluorescent spots of aflatoxin B1 from positive extracts 10µl

B, E, H : Blue fluorescent spots of hemiacetals from positive extracts + T.F.A. 10µl

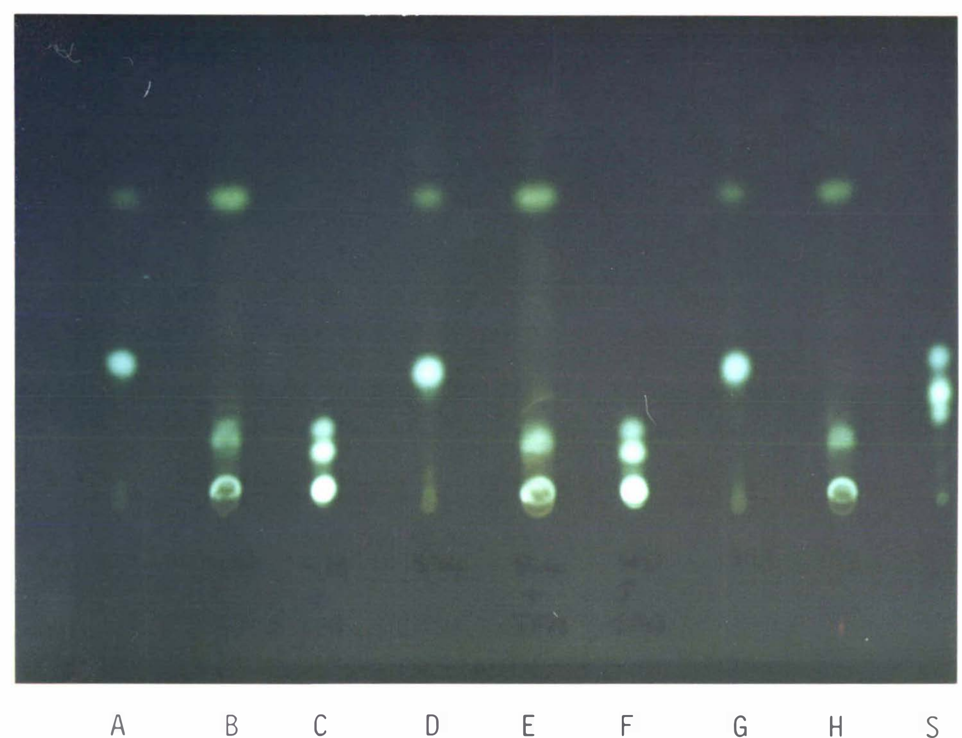
C, F : Blue fluorescent spots of hemiacetals of aflatoxin standards + T.F.A. 10µl

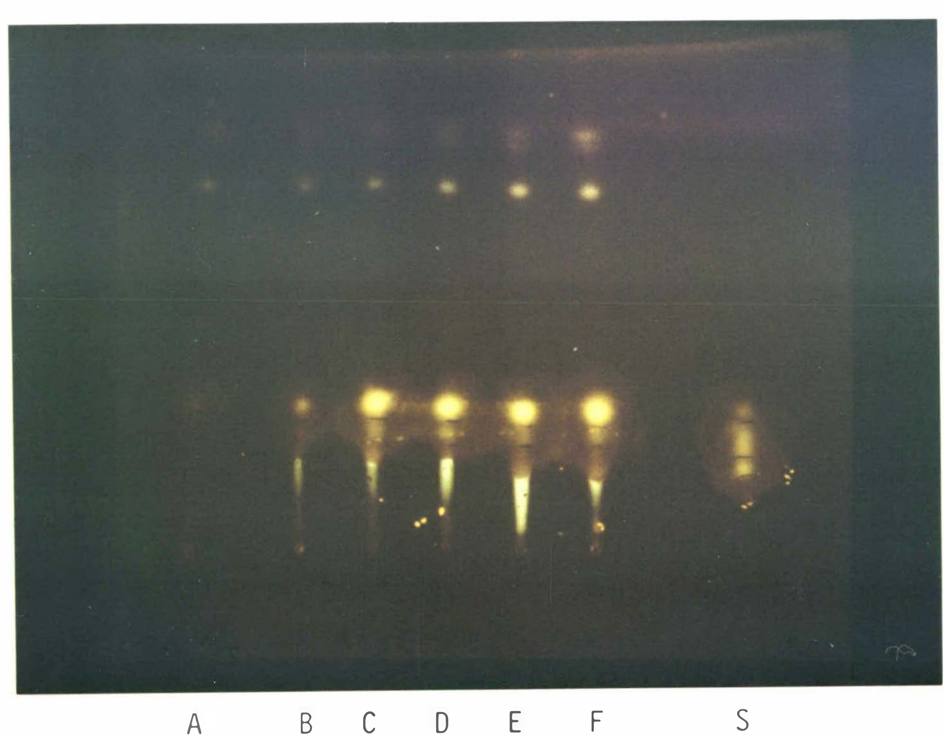
S : Fluorescent spots of aflatoxin standards. (10µl)

Plate 5-4. TLC plate sprayed with 25% sulphuric acid.

A, B, C, D, E, F : Yellow spots of aflatoxins from positive isolates on reacting with sulphuric acid

S : Yellow spots of aflatoxin standards + sulphuric acid


A B C D E F G H S


A B C D E F S

Further confirmation of the presence of aflatoxins in the samples was obtained by spraying with 25% sulphuric acid, which changed the fluorescent spots of _A. flavus_ extracts from blue to yellow (Plate 5-4). The bluish fluorescence of the standard aflatoxin B1 derivative also changed to yellow on being sprayed with sulphuric acid.

Without exception, all the _A. flavus_ isolates which produced bluish fluorescence in coconut agar also produced aflatoxins when cultured in sterile barley. None of the coconut agar negative isolates produced any aflatoxins (Table 5-5).

Table 5-5: Aflatoxin production on coconut agar and in sterile barley culture by selected A. flavus isolates

Source of isolate (Grain samples)	Fluorescence on coconut agar	Aflatoxin production on barley: Days of incubation 7	18	Identity of aflatoxins
At harvest				
1.	+	+	+	B1
2.	-	-	-	-
3.	-	-	-	-
4.	-	-	-	-
Commercial silos 5 months				
1.	-	-	-	-
2.	-	-	-	-
3.	-	-	-	-
4.	-	-	-	-
5.	-	-	-	-
Commercial silos 9 months				
1.	-	-	-	-
2.	+	+	+	B1
3.	-	-	-	-
4.	-	-	-	-
5.	-	-	-	-
Farm silos 6 months				
1.	-	-	-	-
2.	-	-	-	-
3.	-	-	-	-
4.	-	-	-	-
5.	-	-	-	-
6.	+	+	+	B1
Laboratory storage cold, 9 months				
1.	-	-	-	-
2.	-	-	-	-
3.	-	-	-	-
4.	+	+	+	B1
5.	-	-	-	-
6.	+	+	+	B1
Laboratory storage ambient temperature 9 months				
1.	-	-	-	-
2.	-	-	-	-
3.	-	-	-	-
4.	-	-	-	-
5.	-	-	-	-

5.4. DISCUSSION

A. flavus has been reported to grow and produce aflatoxins on coconut and coconut products in nature (Diener and Davis, 1969; Scott, 1978). In the laboratory, Arseculeratne *et al* (1969) found coconut to be far superior to many natural substrates for aflatoxin production. In fact they recorded massive yields of 8 mg of total aflatoxins per g of substrate. It was suspected that high yields of aflatoxins were possible because their production on coconut was enhanced by coconut fats and carbohydrates. Coconut also has a relatively low content of unsaturated fatty acids which are responsible for the formation of lower quantities of peroxidized esters that have been demonstrated to degrade aflatoxins (Ciegler, 1966).

In addition, the use of coconut as the major constituent in a medium described by Lin and Dianese (1976) had many other advantages. The coconut medium not only provides an opaque, white background against which any fluorescence produced can be easily distinguished, the fluorescence produced in the medium by aflatoxigenic isolates is stable. Within 3-5 days, all the aflatoxigenic isolates of the 81 *A. flavus* isolates tested produced characteristic bluish fluorescence which did not fade throughout the 21 days of incubation. This mignt be due to little aflatoxin degradation in the medium combined with continuous aflatoxin production as *A. flavus* enzymes maintained lipolysis of coconut fatty acids.

None of the AT-negative isolates produced any kind of fluorescence in coconut agar. Even AT-positive isolates produced only characteristic fluorescence indicating that the medium is very likely to be specific for aflatoxin-induced fluorescence.

Lin and Dianese (1976) reported the production of orange-yellow pigments on the reverse of aflatoxigenic colonies cultured on coconut medium. This pigment is likely to be that of averufin, an intermediate compound in the biosynthesis of aflatoxins (Donkersloot *et al*., 1972; Lin *et al*., 1973; Lin and Dianese, 1976). Wildman *et al*. (1967) also noted the association of yellow pigments with toxigenic *A. flavus* strains and Arseculeratne *et al*. (1969) observed that toxigenic strains of *A. flavus* produce abundant yellow pigments on grated

coconut after one day's incubation. These pigments turned light brown by the 3rd day of incubation. In the present study, when 81 *A. flavus* isolates were screened on coconut agar medium, pigment production was not only confined to the 5 toxic isolates. This observation was in agreement with those reported by Hussein (1983) who also could not substantiate the observation that pigment production indicates the presence of toxins. Thus the appearance of pigments in coconut medium is probably not reliable as a sole criterion for identifying toxigenic *A. flavus* isolates as proposed by Diener and Davis (1976).

The coconut agar screening technique may have limited usefulness for detecting weak AT-producers. Diener and Davis (1976) observed fluorescence produced by a weak aflatoxigenic *A. flavus* strain after 5 days' incubation. Mylvaganam (1984) who used coconut agar to screen *A. flavus* isolates from poultry feeds, poultry litter and the air of poultry sheds found that *A. flavus* isolates that produced weak fluorescence on coconut agar after 12 days' incubation did not produce any detectable aflatoxins on subsequent culture on semisynthetic liquid medium (SMKY; see Chapter 1). It was suggested that either too low levels of aflatoxin were produced in the liquid medium to be detectable on chromatograms, or the coconut agar test was falsely positive.

When a total of 31 selected *A. flavus* isolates, which included fluorescent positive and fluorescent negative producers were cultured on sterile barley in this study, it was found that all the isolates that produced fluorescence on coconut agar also produced aflatoxins on barley. Similarly Hussein (1983) found that all the *A. flavus* isolates that produced fluorescence on coconut agar also produced aflatoxins when cultured on a variety of natural substrates and a semisynthetic liquid medium (SMKY). It can be inferred from these studies that false positives from coconut agar tests, especially where strong characteristic fluorescence was exhibited, were unlikely to occur. Furthermore, *A. flavus* isolates which did not produce any fluorescence on coconut agar were found to be non-toxin producers on barley, hence ruling out the possibility of false negatives. So, when coconut agar medium is chosen for routine screening, it would be necessary only to apply another test to confirm cases where fluorescence production was doubtful or to seek weak aflatoxin producers amongst those that produced no fluorescence.

Amongst the populations of *A. flavus* isolates screened for toxigenicity, the frequencies of toxic strains varied from 25% of isolates from grains at harvest to 3% of isolates from grains stored under different conditions. These variations in frequencies can be attributed to the considerably larger number of *A. flavus* isolates obtained from stored grains compared with grains at harvest. The actual number of toxigenic isolates remained fairly constant indicating that conditions under which the grains were stored did not encourage the active growth of such fungi.

Several investigators have shown significant variations in the prevalence of toxigenic *A. flavus* isolates obtained from a variety of natural substrates in Australia - New Zealand (Table 5-6) and from other parts of the world (Table 5-7). Whilst a proportion ranging from 2.7% in India to 95.7% in U.S.A. of *A. flavus* isolates tested *in vitro* was capable of aflatoxin production, the overall 6.2% aflatoxigenic isolates selected from barley in this investigation was comparatively low. Also, Hussein (1983) did not succeed in obtaining any aflatoxigenic strains of *A. flavus* isolated from barley stored on New Zealand farms, although he tried screening *A. flavus* strains on a variety of semisynthetic and natural substrates.

It has been known for quite some time that not all randomly-selected *A. flavus* isolates are capable of synthesizing aflatoxins (Boller and Schroeder, 1966; Wildman *et al*, 1967; Wogan, 1969;

Table 5-6: Toxigenic *A. flavus* isolates from natural substrates in Australia and New Zealand

Origin of isolates	Country	Culture media	No. *A. flavus* surveyed	% positive	Aflatoxin compounds produced	References
Soil	New Zealand	SMKY, 'weetbix', pearled barley & barley husks	9	77.8	B1 trace B2	Hussein (1983)
Poultry litter poultry feed	New Zealand	Coconut agar, SMKY	>300	20.0	All 4	Mylvaganam (1984)
Feedstuffs	Australia	Sorghum & peanut	20	55.0	B1 trace B2	Baseden and Aldrick (1970)
Sorghum	Australia	Glucose ammonium nitrate & Czapek Dox agar + peanut extract	29	3.5	B1	Connole and Hill (1970)
Feedstuffs	Australia	Wheat	49	81.6	All 4, mainly B1	Bryden *et al.* (1975)
Feedstuffs	Australia	Maize	200	49.0	B1, G1	Connole *et al.* (1981)

Table 5-7: Isolates of the *A. flavus* group producing aflatoxins in various parts of the world

Origin of isolates	Country	Culture Media	No. *A. flavus* surveyed	% positive	Aflatoxin compounds produced	References
Peanuts & peanut products	India	Peanut	150	2.7	B1	Sreenivasamurthy *et al*. (1965)
Rice	U.S.A.	Peanuts, rice	284	94.4	All 4	Boller and Schroeder (1966)
Peanuts	U.S.A.	Rice, peanut	213	50.2	B1, B2	Taber and Schroeder (1967)
Peanuts, soil	Israel	Wheat	1626	89.6	All 4, mainly B1 & B2	Joffe (1969)
Soil	India	Liquid media, eg. YES, YES + salt, G.A.N.	9	77.8	B1 & B2	Maggon *et al* (1969)
Corn	U.S.A.	SMKY & corn	32	31.3	B1 & B2	Trenk and Hartman (1970)
Corn	U.S.A.	Rice	15	46.7	B1 & B2	Richard and Cysewski (1971)
Peanuts, cottonseed, rice,	U.S.A.	Peanuts, rice cottonseed sorghum	416 165 571	95.7 79.0 35.3	All 4	Schroeder and Boller (1973)
Sorghum			110	49.0		
Cotton, maize wheat	India	YES + salt, SMKY	21	76.2	B1	Mehan and Chohan (1973)
Peanuts & soil	Senegal	Liquid media	-	53.0	-	Lafont and Lafont (1977)
Corn	Argentina U.S.A.	YES	48 21	49.0 53.9	-	Sanchis *et al*. (1982)

Alisauskaus, 1973; Moreau, 1979). Their variable aflatoxin-producing ability under specific conditions are a reflection of their genetic composition. Thus the low frequency of toxin-producing strains amongst barley isolates indicates that the majority of A. flavus isolates contaminating New Zealand grown barley are likely atoxic genotypes.

The expression of toxigenicity in vitro is influenced by the methods of culture and by the laboratory conditions provided, eg. the age of the inoculum, the length of the incubation period and the moisture content and temperature of incubation. In the 1960's, De Vogel (1965), Diener and Davis (1966) and others noted that successive subculture on synthetic and semisynthetic media could result in a progressive decrease in the yield of aflatoxin produced by proven toxigenic A. flavus strains. Torres et al. (1980) provided further evidence to show that the gradual loss of aflatoxin-synthesizing ability may eventually be complete and the loss following repeated subcultures could be fairly rapid. However, not all cultures were equally affected. Since the A. flavus isolates used in this study were mainly maintained in the laboratory on synthetic media before they were tested for toxin production, serial transfers at regular intervals on the substrate of their origin were carried out to stimulate and restore their aflatoxin-producing ability. This phenomenon has previously been observed by several investigators (Ambrecht et al., 1963; De Vogel, 1965; Kulik and Holaday, 1966; Murakami et al., 1968a, b and 1970).

Aside from the inherent toxigenicity which is fundamental to aflatoxin production, the frequency of toxic A. flavus also appears to be dependent on the substrate from which A. flavus has been isolated and their geographical origin. In the U.S.A., Schroeder and Boller (1973) reported that of the A. flavus they screened for aflatoxin production, 96% of isolates from peanuts, 79% of isolates from cottonseed, 35% of isolates from rice and 49% of isolates from sorghum were toxigenic. Although the sources of toxigenic strains do not appear to affect aflatoxin production and level (Schroeder and Hein, 1967; Joffe, 1969; Mehan and Chohan, 1973 and Moreau, 1979), some substrates such as peanuts and cottonseed are notably associated with a higher incidence of toxigenic strains than those of rice or sorghum.

Of the *A. flavus* isolates from peanuts in 9 different geographical areas in Texas, U.S.A., Taber and Schroeder (1967) found 50% of the population toxigenic whereas Borut and Joffe (1966) cited by Moreau (1979) found 71% of their *A. flavus* isolates from peanuts grown in Israel to be toxigenic. Sreenivasamurthy (1965) obtained only 2.7% toxigenic *A. flavus* amongst the isolates from Indian peanuts and peanut products. Also, Joffe (1969) obtained varying percentages of toxigenic *A. flavus* from peanuts during 5 years of study. This was said to indicate that ecological factors could play a role in affecting the incidence of toxigenic strains in a specific crop in any one year. It is thus possible that the mild, temperate climate of New Zealand and other environmental factors including those encountered during storage of the barley grains studied combined with the intrinsic characteristics of barley and the microenvironment provided by the barley grains, do not appear to encourage the selection of toxigenic *A. flavus* species.

It is interesting to note that thin layer chromatographic resolution of extracts produced by toxic strains of *A. flavus* isolated in this study showed the formation of only Aflatoxin B1. The results of many other investigators also showed that *A. flavus* isolated from natural substrates produced primarily Aflatoxin B1 (Table 5-8). Most of the naturally-occurring *A. flavus* strains reported in the literature produced only B and no G aflatoxins (Maggon *et al*., 1969; Taber and Schroeder, 1967; Schroeder, 1969; Wogan, 1969, Trenk and Hartman, 1970 and Bryden *et al*., 1975).

If any of the *A. flavus* strains screened for toxin production in sterile barley in the present study were capable of producing aflatoxins G_1 or G_2, then they were not produced in detectable quantities. The composition of aflatoxins produced can be attributed to the interactions of mould strain, substrate and environmental conditions.

Table 5-8: Some laboratory screenings of naturally-occurring *A. flavus* isolates which produce only Aflatoxin B1

Sources	References
Groundnuts	Rao *et al.* (1965)
Peanuts	Sreenivasamurthy *et al.* (1965)
Various agricultural commodities, eg. peanuts, corn, feed, soybeans etc.	Diener and Davis (1966)
Spanish peanuts	Taber and Schroeder (1967)
Pecans	Lillard *et al.* (1970)
Sorghum	Connole and Hill (1970)
Hams	Sutic *et al.* (1972)
Ground black pepper	Schroeder and Carlton (1973)
Cotton, maize and wheat	Mehan and Chohan (1973)
Agricultural Commodities, feeds, animal organs, chicken fluff, insects etc.	Connole *et al.* (1981)

In 1970, Hesseltine and his colleagues reported an intensive investigation into the aflatoxin-producing abilities of 67 strains of *A. flavus* on 3 different natural substrates under 2 fermentation conditions. Their studies illustrated that the amount and type of aflatoxins produced were strain dependent. The *A. flavus* group of fungi could be divided into 5 distinct groups. Group 1 consisted mainly of *A. parasiticus* isolates which consistently produced all aflatoxins B, G and M. Group 2 represented strains of *A. flavus* which

produced large sclerotia on Czapek Dox agar and also produced all aflatoxins B, G and M. The 3rd and largest group was made up of *A. flavus* strains which produced only aflatoxins B1 and M. Strains put into the 4th group did not produce any aflatoxins, and group 5 consisted of 1 unusual strain which produced high levels of B1 but no G1.

Furthermore Maggon *et al*. (1969) suggested that the production of aflatoxins B and G may be controlled by different haploid nuclei, the presence and absence of which can affect fungal metabolism and hence the final ratios of aflatoxins formed. Further proof that the composition of aflatoxin produced is dependent on genetic capacity was provided by Papa (1977) who produced a mutant of *A. flavus* by genetic treatment with nitroguanidine. The mutant gave larger proportions of B2 than B1.

Substrates have been shown to interact with aflatoxin-producing strains to influence the formation of aflatoxin compounds, B1, B2, G1 and G2. In general, rice is considered the best substrate for the production of aflatoxin B1 and wheat is best for aflatoxin G1 production (Hesseltine *et al*., 1970).

Other environmental conditions are also known to influence the ratios of aflatoxins produced. One of the most important is the effect of temperature. Diener and Davis (1966) showed that although toxigenic strains are able to produce both aflatoxins B1 and G1 over a range of temperatures, the proportion of aflatoxins B1 to G1 decreases with increasing temperature.

The experimental data in this investigation showed that aflatoxin-producing *A. flavus* strains are present on barley grains at harvest and on stored grains. These *A. flavus* isolates have been demonstrated to be capable of producing aflatoxins on the substrate of their origin. Aflatoxin B1 appeared to be the only aflatoxin produced by all the toxigenic strains tested. Although the percentage of contamination by toxigenic strains may not be high, conducive conditions especially during storage can lead to an explosive increase in the number of toxigenic isolates and even if a small proportion of the grain is infected, toxin levels considered toxic and dangerous to man and animals may be reached.

CHAPTER 6

MULTIMYCOTOXIN SCREENING OF SELECTED BARLEY SAMPLES

6.1. INTRODUCTION

Barley, as other cereal grains, carries a large number and variety of fungal spores of natural origin. Numerous studies have shown that amongst these fungi are many potentially toxigenic species. Under favourable conditions, it is possible for several of these fungi to proliferate and produce toxins and hence the barley can become contaminated not just by one but by several toxins.

As described in the preceding chapter (5), toxigenic *A. flavus* species have been isolated from locally-grown barley at harvest and after storage. However, it is not sufficient to assume from this that aflatoxin or indeed other toxins formed by potentially toxigenic fungi present will occur in stored barley samples. It is necessary to screen the barley samples themselves for the presence of naturally-occurring toxins.

A multimycotoxin method based on the extraction techniques used regularly by Roberts and Patterson (1975) and Patterson and Roberts (1979) and many others in the United Kingdom, was chosen to simultaneously screen locally-grown barley for aflatoxins, citrinin, ochratoxin A, T-2 toxin and zearalenone. These mycotoxins of most concern in barley (Table 2-3) have also been found in significant amounts in other cereal grains (Hesseltine, 1974) and they have been implicated in natural cases of mycotoxicity (Mirocha and Christensen, 1974; Pier, 1981). Aflatoxins are notorious hepatoxins, carcinogens and mutagens (Goldblatt, 1969), citrinin and ochratoxin are both nephrotoxins, zearalenone is an oestrogenic toxin and T-2 is a potent dermal toxin (Romer, 1984).

6.2. MATERIALS AND METHODS

6.2.1. Selection of Barley Samples

A total of 14 barley samples, collected at harvest and after storage under different conditions, including samples which yielded aflatoxigenic *A. flavus*, was analysed for the presence of aflatoxins and other mycotoxins.

6.2.2. Sampling

Individual barley samples weighing between 1-5 kg were thoroughly mixed in separate sterile glass vessels and then halved. One half of the mixed grain was left aside and the other half of the grain was mixed and halved yet again. This procedure was repeated until between 50-100 g of grain remained. This final grain sample was finely ground, mixed thoroughly and 25 g were removed for toxin extraction. Where clumps of mouldy grain occurred, the clumps were broken up before actual sampling.

6.2.3. Multimycotoxin Assay

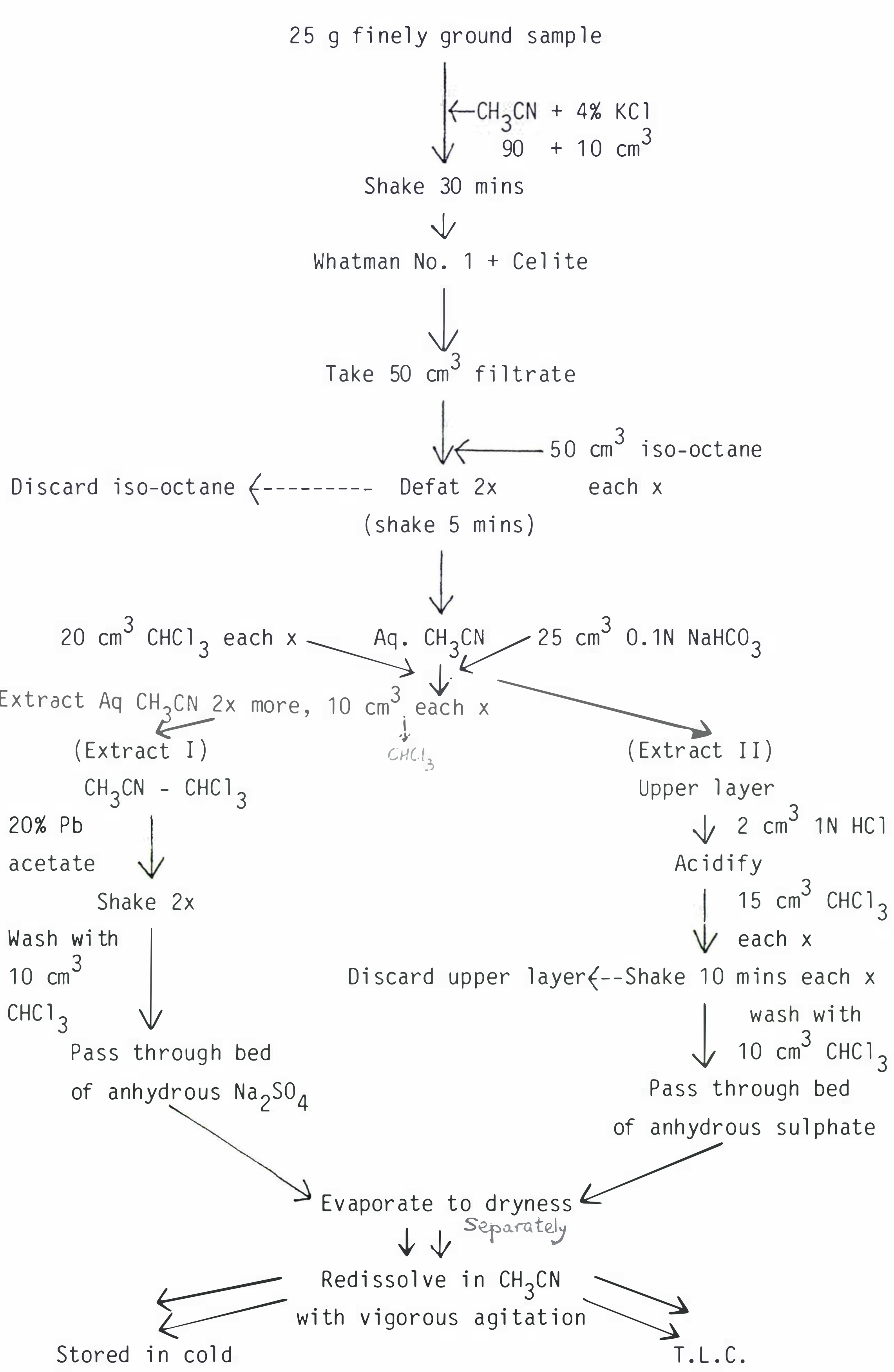
The method illustrated in Figure 5-1 is a modification of those described by Roberts and Patterson (1975) and Patterson and Roberts (1979) for the detection of several mycotoxins, namely aflatoxin, citrinin, ochratoxin A, patulin, penitrem A, penicillic acid, sterigmatocystin, zearalenone and some trichothecenes from various straight and compounded feedstuffs, including barley. Preliminary trials using solvents listed in Figure 6-1 with spiked samples found this method to be satisfactory.

6.2.4. Extraction and cleanup

A. Ground Barley: A 25 g ground barley sample was placed in a wide-mouthed glass bottle with a screw cap and 100 cm^3 of analytical grade acetonitrile - 4% potassium chloride (9:1) mixture was added. Toxins present were extracted by shaking for 30 minutes on a reciprocating shaker.

The solid residues were allowed to settle and the extract was filtered through Whatman No. 1 filter paper and celite Hyflo-Supercel filter aid. 50 cm^3 of the resulting filtrate was defatted with an equal volume of iso-octane (2,2,4,trimethylpentane) by shaking for 5 minutes in a separatory funnel. When the two layers had separated, the upper layer was discarded and the lower layer was defatted with another 50 cm^3 iso-octane in the manner just described, again discarding the upper layer of iso-octane.

Figure 6-1: Method for the extraction of aflatoxins, ochratoxin, zearalenone, sterigmatocystin, T-2 toxin and citrinin.



To the lower layer of aqueous acetonitrile (CH_3CN) was added 25 cm^3 0.1N sodium bicarbonate ($NaHCO_3$). This was followed by toxin extraction, adding 20 cm^3 chloroform (CH_3Cl) and shaking for 10 minutes. The mixture was allowed to settle to give 2 separate layers. The lower layer of aqueous $CHCl_3$-CH_3CN was drained into a clean glass vessel. The top layer was extracted twice more with 10 cm^3 $CHCl_3$ each time. The combined $CHCl_3$-CH_3CN extracts and the upper aqueous layer were separately analysed for toxins.

B. <u>$CHCl_3$-CH_3CN Extracts</u>: Impurities in the $CHCl_3$-CH_3CN extracts were removed by treating with 20% lead acetate solution twice, using 50 cm^3 freshly-prepared lead acetate solution each time. The aqueous $CHCl_3$-CH_3CN extract was then passed through a bed of anhydrous sodium sulphate (Na_2SO_4) contained in a cone of filter paper. The anhydrous Na_2SO_4 was then washed with 10 cm^3 $CHCl_3$. The combined "cleaned" $CHCl_3$-CH_3CN extracts were termed Extract I.

C. <u>Aqueous Layer</u>: The aqueous layer remaining in the separatory funnel was acidified with 2 cm^3 1N HCl. Toxins were extracted twice, by shaking for 10 minutes in the presence of 15 cm^3 $CHCl_3$ each time. The lower layers of $CHCl_3$ were filtered through a bed of anhydrous Na_2SO_4 on Whatman filter paper No. 1, and finally the Na_2SO_4 was washed with 10 cm^3 $CHCl_3$. The combined $CHCl_2$ extracts constituted Extract II.

6.2.4.1. Concentration of extracts

Extracts I and II were evaporated to near dryness in a round-bottomed glass vessel under vacuum in a Buchi rotary evaporator. The extracts were redissolved with vigorous agitation in 1 cm^3 CH_3CN, transferred to a glass vial for storage in the cold and later used for thin layer chromatography (T.L.C.) assays.

6.2.5. Detection of Mycotoxins in Extracts

6.2.5.1. Thin layer chromatography (T.L.C.)

A. <u>Spotting of Extracts</u>: 20.0 cm by 20.0 cm aluminium-backed Merck non-fluorescent T.L.C. plates* were activated at 110^oC for 60 minutes

* See bottom of p 156.

and cooled prior to spotting with extracts and toxin standards. Disposable micropipettes delivering 10 µl aliquots were used to spot 0, 20 µℓ of extracts as well as toxin standards. The toxin standards* used were aflatoxins mixture (5.0 µg/cm^3 B1, 1.5 µg/cm^3 B2, 5.0 µg/cm^3 G1, 1.5 µg/cm^3 G2 in benzene-acetonitrile [98:2]), ochratoxin A (100 µg/cm^3 acetonitrile), citrinin (100 µg/cm^3 acetonitrile), zearalenone (100 µg/cm^3 acetonitrile), and T-2 toxin (50 µg/cm^3 acetonitrile).

B. Development of T.L.C. Plates: Spotted T.L.C. plates were developed in lined, equilibriated tanks, under subdued light in either chloroform acetone (9:1) or toluene-ethyl acetate - 90% formic acid, T.E.F. (60:30:10) solvent systems to a mark 12.0 cm from the original line of spotted extracts. The plates were air-dried in a dark fume cupboard before they were viewed in a chromato-vue cupboard under both long- and short-wave ultraviolet light.

6.2.5.2. Analysis for T-2 toxin

To detect T-2 toxin, T.L.C. plates developed in T.E.F. were allowed to dry and then sprayed with 20% H_2SO_4 in methanol and heated at 120^oC for 10 minutes. The treated spots were examined for charred spots and viewed under long-wave ultraviolet light for blue fluorescent halos similar to those produced by spots of standards (Plates 6-1 and 6-2).

6.2.6. Additional and Confirmatory Tests

In addition to just developing spotted plates in T.E.F. and observing fluorescence under ultraviolet light as in 6.2.5.1B, some T.E.F. developed plates spotted with extracts and standards were sprayed with a saturated solution of $AlCl_3$ in 95% ethanol. The plates were subsequently heated at 105^oC for 10 minutes, allowed to cool and examined for characteristic fluorescent spots with R_f values similar to those of the standards. Expected results of this procedure and that described in Section 6.2.5 are summarised in Table 6-1.

For samples showing positive aflatoxin fluorescent spots on developed T.L.C. plates, the extracts were confirmed for the presence of aflatoxins by derivative formation with trifluoroacetic acid and by

* Analytical grade mycotoxin standards from Sigma Chemical Co., U.S.A.

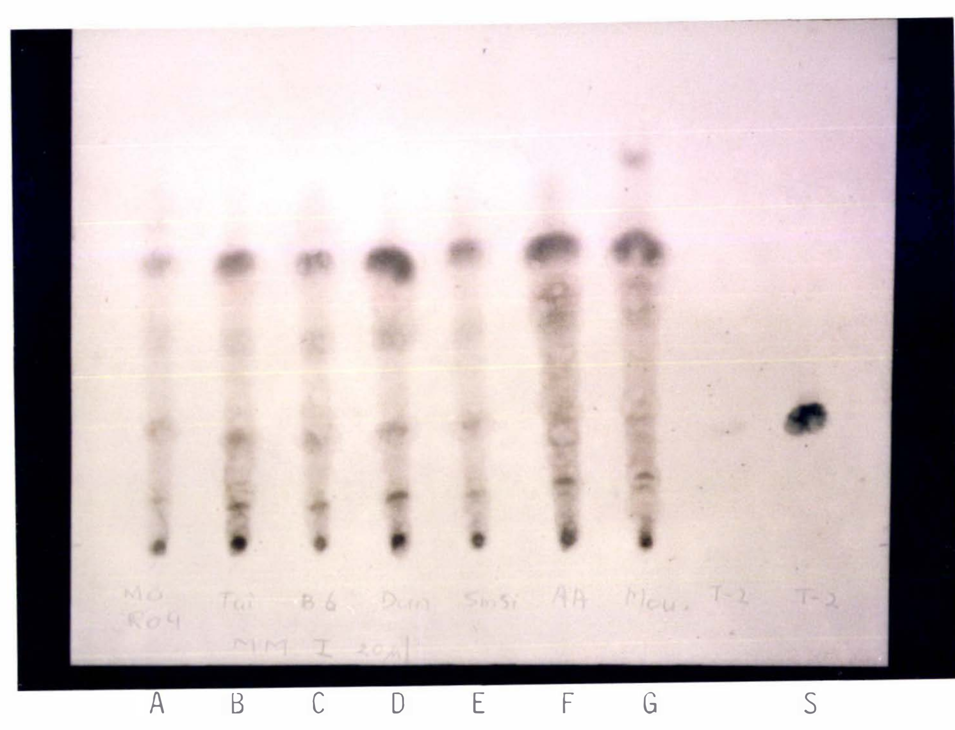
Plate 6-1. T.E.F. (60:30:10)-developed plate sprayed with 20% H_2SO_4 in methanol.

S - Charred spot of T-2 standard
A-G - Absence of charred spots in samples.

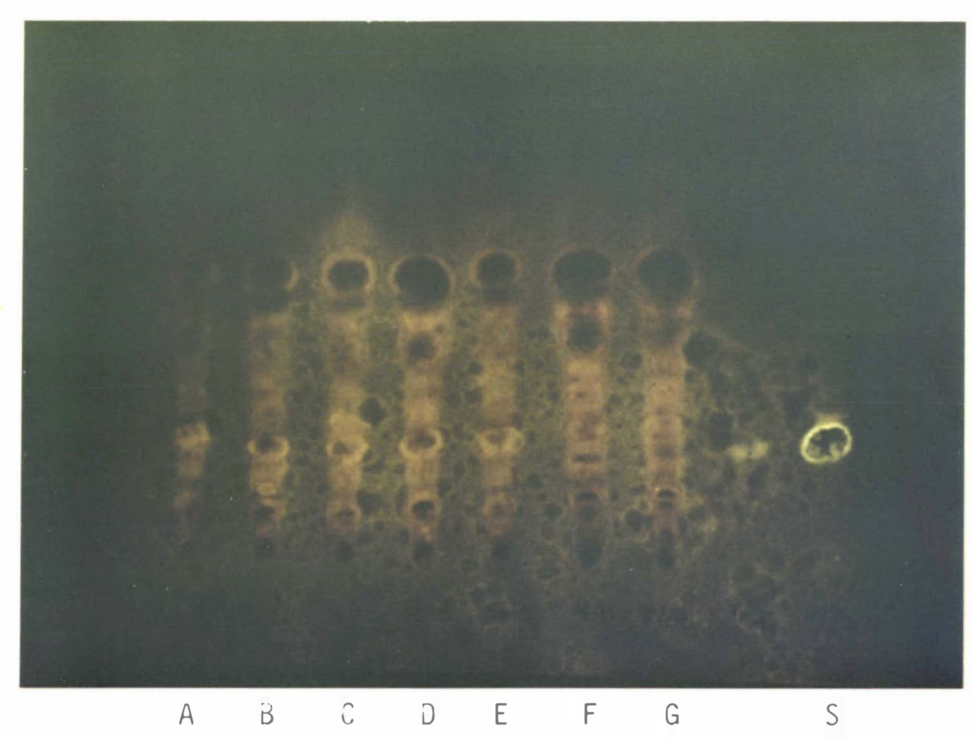
Plate 6-2. Developed T.L.C. plate viewed under U.V. light for T-2 toxin.

A-G - Absence of fluorescent spots of T-2 toxin in samples
S - Fluorescent halo around charred spot of T-2 standard.

* T.L.C. plates previously spotted with 10 $\mu\ell$ aliquots of sample extracts and T-2 standards.



A B C D E F G S



A B C D E F G S

spraying plates developed in $CHCl_3$-acetone (9:1) with 25% H_2SO_4 as in Section 5.2.3.3 of the previous chapter.

Table 6-1: Fluorescent colours of toxins under both short- and long-wave U.V. light after two different treatments

	T.L.C. developed in TEF		T.L.C. developed in TEF and sprayed with $AlCl_3$	
	Colour of fluorescence under U.V. light			
Toxins	Long λ	Short λ	Long λ	Short λ
Extract I				
Aflatoxins	B - blue G - greenish-blue	B - blue greenish-blue	blue greenish-blue	blue greenish-blue
Sterigmatocystin	Dull red		Bright yellow	
Zearalenone	Blue	Bright blue	Bright blue	Bright blue
T-2 toxin	Blue	Blue		
Extract II				
Citrinin	Yellow	Yellow	Yellow	Yellow
Ochratoxin A	Blue-green	Blue-green	Blue-violet	Blue-violet
Overnight air-dried or exposed to NH_3 vapour	Intense blue-violet	Intense blue-violet	Intense blue-violet	Intense blue-violet

6.3. RESULTS

Patterson and Roberts (1979) reported that the addition of $NaHCO_3$ facilitates the extraction of Ochratoxin A and citrinin, which if present can be detected in Extract II. Most of the other mycotoxins are recovered in Extract I. Of the 14 barley samples examined for mycotoxins in the present study, only one was found to be positive for aflatoxin B1, ochratoxin A and citrinin (Table 6-2). The remaining 13 samples were negative for all the 5 mycotoxins tested.

A fluorescent spot of aflatoxin B1, in the positive sample, can be seen in Plate 6-3. This spot was confirmed to be that of aflatoxin B1 by the formation of bright blue fluorescent spots of aflatoxin B1 hemiacetals on reaction with T.F.A. on T.L.C. plates, identical to that formed by aflatoxin B1 standards. Further proof that aflatoxin was present in the extract was obtained when chloroform -acetone (9:1) -developed T.L.C. plates of spotted extracts changed fluorescent colour from blue to yellow after being sprayed with 25% H_2SO_4.

Plate 6-4 shows a T.L.C. plate of the positive and a negative sample Extract II developed in T.E.F. (60:30:10) and viewed under long wavelength U.V. light. The presence of a bright yellow streak of citrinin and a blue spot of ochratoxin A is obvious in the positive sample, labelled F. The following plate, 6-5, shows the same positive sample extract on a T.E.F. developed T.L.C. plate subsequently sprayed with $AlCl_3$ in ethanol.

Table 6-2: Multimycotoxin screening of 14 selected barley samples

Sample			Extract I				Extract II	
			Aflatoxins	Sterigmato-cystin	T-2 toxin	Zearalenone	Citrinin	Ochratoxin
At harvest		1	-	-	-	-	-	-
		2	-	-	-	-	-	-
Commercial silos	5 mths	3	-	-	-	-	-	-
		4	-	-	-	-	-	-
	9 mths	5	-	-	-	-	-	-
		6	-	-	-	-	-	-
Laboratory	Ambient	7	-	-	-	-	-	-
		8	-	-	-	-	-	-
	Cold	9	-	-	-	-	-	-
		10	-	-	-	-	-	-
Farm		11	-	-	-	-	-	-
		12*	-	-	-	-	-	-
		13*	-	-	-	-	-	-
		14*	+ (B1)	-	-	-	+	+

* Mouldy farm samples.

Plate 6-3: T.E.F. (60:30:10) developed T.L.C. plate spotted with 10 μls sample extracts (I) and standards

I - Fluorescent spots of aflatoxin standards B1, B2, G1 and G2

H - Blue fluorescent spot of zearalenone standard

F - Blue fluorescent spot of aflatoxin B1 in extract of positive sample

A-E and G - Extracts of negative samples.

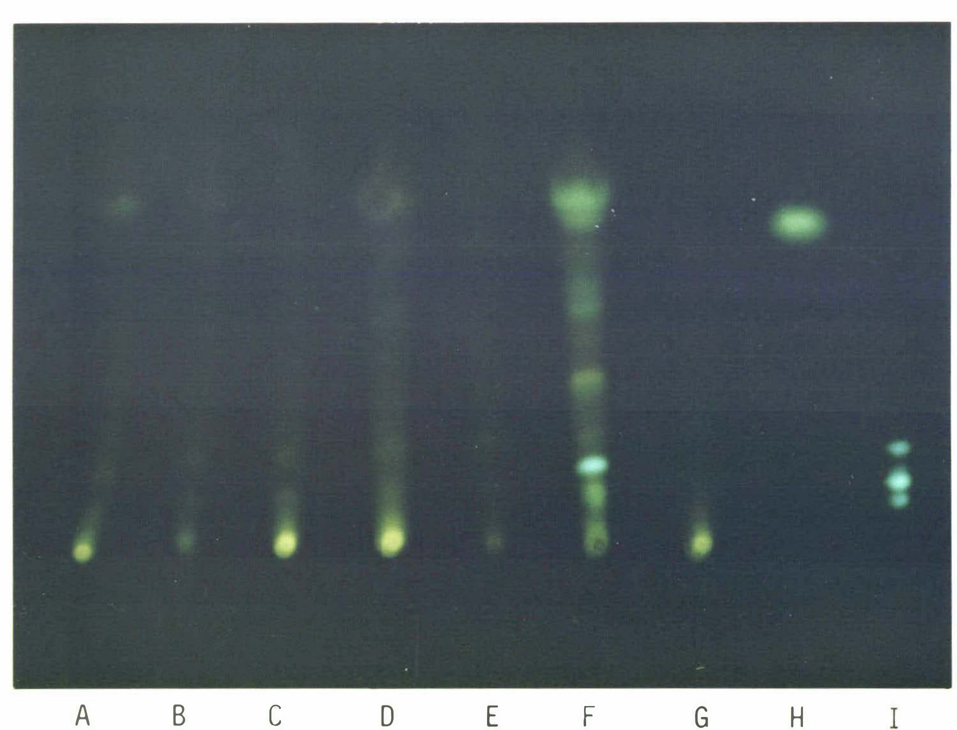

A
B
C
D
E
F
G
H
I

Plate 6-4: T.E.F. (60:30:10) developed T.L.C. plate of sample extracts (II) and standards

Spots of:

I - Ochratoxin A standard

H - Streak of citrinin standard

F - Sample positive for both ochratoxin A and citrinin

A-D, E and G - Negative extracts

Plate 6-5: T.E.F. (60:30:10) developed T.L.C. plates followed by spraying with $AlCl_3$ in ethanol of sample extracts (II) and standards

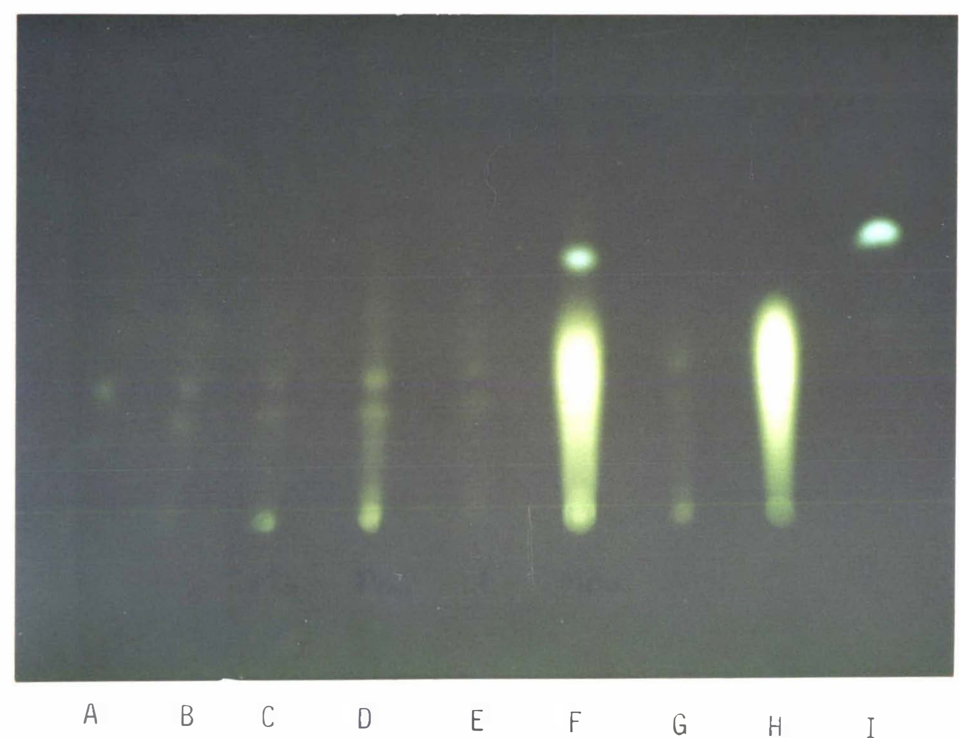
E - Sample positive for ochratoxin A and citrinin

B - Sample negative for both toxins

I - Spot of ochratoxin A standard

H - Streak of citrinin standard

* T.L.C. plates spotted with 10 $\mu\ell$ of sample extracts and standards.


A
B
C
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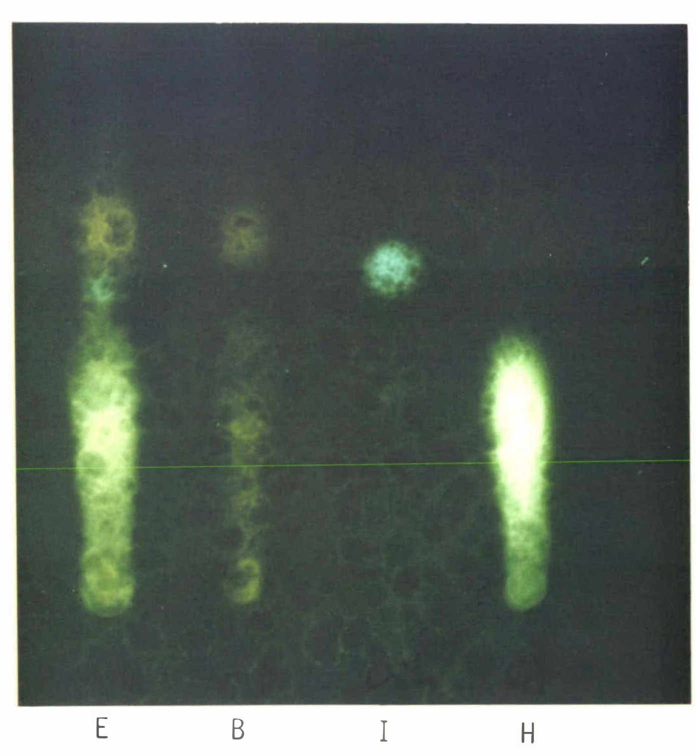
G
H
I


E
B
I
H

6.4. DISCUSSION

The only sample which yielded aflatoxin B1, citrinin and ochratoxin A was a visibly mouldy sample from a farm silo. According to Stoloff (1979), there are at least four possible ways in which multimycotoxin contamination could occur: a) by the mixing of products contaminated with different mycotoxins, b) by the growth of a mixed population of toxigenic moulds, c) by sequential growth of different toxigenic moulds on the same substrate, and d) by multimycotoxin production by a single mould species.

Contamination by possibility a) in this case was least likely because other than the mixing of barley grains harvested from different fields or at different times, no other substances or products were added to the barley prior to and during storage. The sample concerned was in a healthy condition when it was put into storage, thus the likelihood of aflatoxin contamination of the crop in the field or at harvest is not likely, although it cannot be ruled out completely. The formation of citrinin and ochratoxin A in the field have never been reported (Haggblom and Ghosh, 1985). With the knowledge that the barley sample became mouldy during storage, and that there was no distinct demarcation between the initiation of growth of one fungal species and others, almost any permutation of the remaining three possible ways of contamination could account for the concomitant presence of the three mycotoxins in the mouldy sample concerned.

On the basis of the extraction and subsequent confirmation of aflatoxin B1, citrinin and ochratoxin A, it can be concluded that fungi of the genera Aspergillus and Penicillium (Table 6-2), the presumptive producers of these toxins, must have proliferated in the barley to produce these toxic secondary metabolic products. As grain spoilage progressed, moisture and temperature probably increased to levels which could support the development of the mycotoxic fungi. Fungi responsible for the production of aflatoxin, citrinin and ochratoxin A are capable of growth and toxin formation at moisture levels greater than 14% and over a range of temperatures (Ciegler, 1978), and suitable combinations of these two factors would likely occur in pockets of the grain bulk.

The influence of other organisms and fungal species as encountered in a natural situation and in particular during spoilage is of importance in determining the ability of toxigenic strains to produce toxins. Christensen *et al.* (1977) provided evidence to show that aflatoxin formation was severely depressed or inhibited in the presence of a mixture of naturally-present fungi even if moist grain was inoculated with a known aflatoxin-producing fungus and incubated under favourable conditions. Similarly, Boller and Schroeder (1973) demonstrated that *A. chevalieri* could inhibit aflatoxin production by *A. parasiticus* and, *A. niger* and *Trichoderma viride* were found to suppress aflatoxin formation in corn by toxigenic *A. flavus* by Wicklow *et al.* (1980). Weckbach and Marth (1977) grew *A. parasiticus* NRRL 2999 in the presence of various microorganisms and found that *Rhizopus nigricans* and *Saccharomyces cerevisiae* inhibited aflatoxin production whilst *Brevibacterium linens* caused a slight inhibition.

Although the above evidence does not apparently support the possibility of multimycotoxins as a result of simultaneous growth and toxin formation of multiple moulds, it must be noted that mycotoxins such as aflatoxins are still formed, albeit at much reduced levels in comparison with their formation in pure culture (Ashworth, 1965). Besides, the presence of some organisms such as *Acetobacter aceti*, *Penicillium rubrum* and *Lactobacillus plantarum* have been observed to stimulate *A. parasiticus* growth and aflatoxin production (Weckbach and Marth, 1977; Alderman *et al.*, 1973, cited by Moss and Badii, 1982).

The production of toxins is also affected by the sequential growth of individual populations of fungi and other microorganisms in response to changes in temperature and moisture during spoilage. Experimental evidence provided by Ashworth (1965) showed that, if toxigenic *A. flavus* was inoculated a few days following the growth of another mould, little aflatoxin was produced. Similar results were found by El-Gendy and Marth (1981), using *Lactobacillus casei* and *A. parasiticus*. Aflatoxin production was reduced when *L. casei* was allowed to grow 3 days before the subsequent growth of *A. parasiticus*. Wiseman and Marth (1981) cited by Northolt *et al.* (1982) reported that another bacterium, *Streptococcus lactis*, inhibited aflatoxin production by *A. parasiticus* only if *S. lactis* was allowed to grow before inoculating with *A. parasiticus* conidia or if both were allowed to

grow simultaneously. But if A. parasiticus were to grow first, the subsequent addition of S. lactis did not inhibit aflatoxin production.

Despite the complex interactions between both toxic and non-toxic fungi and other microorganisms, the multimycotoxin contamination of a given lot of grain by the growth of successive populations of toxigenic fungi could be envisaged. As the moisture requirements of fungal species vary (Table 6-3), given favourable temperatures and other environmental conditions, individual fungal species would be sequentially activated from their dormant state in response to the increasing moisture levels which invariably accompany spoilage. Depending on the fungal species involved, once a toxigenic species has an opportunity to elaborate toxins, the appearance of other toxigenic dominant moulds in the fungal population may not signal the advent of adverse effects on the toxin-producing process of the established mould nor on its toxic metabolites. Moreover, many authors have found that toxin previously formed could remain stable and persistent even if the producing organism did not survive drying-out or the presence of fungal succession in storage (Moreau, 1979; Mills, 1982). Of special interest is the discovery of the investigators Moss and Badii (1982), who recorded a pronounced increase in the production of aflatoxins, especially of aflatoxin G1, by A. parasiticus species in a chemically-defined medium, in the presence of a low concentration of 10 $\mu g/cm^3$ of rubratoxin B, a toxic product of Penicillium rubrum. More recently Fabbri and his colleagues (1984) showed that T-2 toxin, a metabolite of certain Fusarium species, enhanced the production of aflatoxin by Aspergillus parasiticus.

Table 6-3: Moisture content required for growth of selected mycotoxin-producing fungi

Moisture content (%)	Fungi
10.0 - 17.0	Aspergillus ochraceus,
15.6 - 21.0	Penicillium citrinum, P. cyclopium, P. oxalicum, P. palitans, A. versicolor, P. viridacatum
18.0 - 20.0	A. flavus, A. parasicitus
22.0 - 33.0	Alternaria species, Fusarium moniliforme, F. roseum, F. tricinctum

Source: Ciegler (1978)

The final possible source of multiple toxins in a substrate is attributable to the ability of some mould species to synthesize more than one toxin in nature. Strains of Fusarium graminearum, for example, can elaborate zearalenone and vomitoxin (Seaman, 1982) whilst isolates of P. islandicum have been associated with the formation of at least five hepatotoxins, namely luteoskyrin, cyclochlorotine, islanditoxin, erythroskyrine and rugulosin (Mislivec, 1981). In the case of the mouldy farm samples analysed for mycotoxins, toxigenic strains of P. viridicatum or P. palitans could be individually responsible for the presence of ochratoxin A and citrinin (Table 6-3). The capacity to produce multiple toxins is not solely dependent upon the genetic potential endowed upon the fungal strain concerned but also on the environmental factors which play a role in deciding the final synthesis system activated and hence the toxin formed. Furthermore, there is evidence to show that a strain of A. ochraceus, a producer of two toxins, forms penicillic acid at low temperatures of 15-22°C and low moisture content, and high temperatures and high moisture content favour the formation of ochratoxin B (Bacon et al., 1973).

Table 6-4: Aflatoxin, citrinin and ochratoxin-producing fungi

Fungal species	Aflatoxin [a]	Citrinin [b]	Ochratoxin [b]
Aspergillus	*A. flavus*, *A. parasiticus*	*A. candidus*, *A. niveus*, *A. terreus*	*A. alliaceus*, *A. ostianus*, *A. melleus*, *A. petrakii*, *A. ochraceus*, *A. sclerotiorum*, *A. sulphureus*
Penicillium		*P. canescens*, *P. citreo-viride*, *P. claviforme*, *P. expansum*, *P. fellutanum*, *P. implicatum*, *P. jenseni*, *P. lividum*, *P. palitans*, *P. steckii*, *P. velutinum*, *P. viridacatum*	*P. commune*, *P. cyclopium*, *P. palitans*, *P. purpurascens*, *P. variable*, *P. viridacatum*

Sources: Krogh (1976) [b]; Diener and Davis (1969) [a].

Other than the one positive sample, the majority of the barley samples analysed did not yield any mycotoxins. A large number of reasons could account for the absence of mycotoxins.

Mydotoxins could be present in pockets of the grain bulk but were not collected for analysis. The chances of this occurring in the present investigation were diminished by the care taken in sampling bulk grain (Chapter 3) and in reducing sample sizes for actual toxin analysis. Toxins present in samples examined could be at levels too low to be detected by the method of analysis used. The original method of Patterson and Roberts (1979) employing dialysis tubings for membrane clean-up of extracts and 2-dimensional T.L.C. in various solvent systems to improve sensitivity could detect mycotoxins in barley at minimum levels of 0.2 µg/kg for aflatoxin B1, 5 $\mu g/kg^{-1}$ for ochratoxin A, 50 $\mu g/kg^{-1}$ for zearalenone and 200 $\mu g/kg^{-1}$ for T-2 toxin. Although the actual minimum concentrations of toxins which the modified method used in the present studies could detect were not estimated, the method was tested with samples spiked with various toxin concentrations to ensure its efficiency.

Despite the likely presence of toxigenic fungal strains amidst the natural fungal flora of the barley grains studied, the lack of opportunity for these fungi to thrive and elaborate toxins must be the principal reason for the absence of toxins in all the negative samples. Other than the obvious lack of conducive environmental conditions for toxin formation, these grains were dry and some were treated with chemical substances such as Malathion and Maldison prior to storage. These substances could also have an inhibitory effect on fungal growth and toxin formation. Depending on the fungal species and the insecticide used, decreased yields of aflatoxin and ochratoxin A were obtained by Vandergraft (1973). Similarly, Draughton and Ayres (1981) reported that aflatoxin production was inhibited by 100 ppm of the following insecticides: Dichlorovos, Diazinon, Landrin, Malathion, Pyrethrum and Sevin, but no account of such inhibition could be taken in the present investigations.

The samples analysed in the present study included two obviously mouldy ones which were negative for mycotoxins. There are other

reported instances when mould infestations were not synonymous with toxin production in cereal grains (Christensen and Mirocha, 1977; Shotwell, 1977; and Niles *et al*., 1985). This apparent discrepancy can be explained by recognising that not all moulds are toxin-producers and not all conditions are suitable for toxin production (Shotwell, 1977).

In the event of spoilage when fungi of various types abound, any toxigenic species must survive and have the competitive advantage to proliferate before they can produce toxins. Studies cited earlier in this discussion have illustrated the restricting effects other colonists have on fungal growth and toxin production. The general mycoflora present can inhibit toxin production in a number of ways. Other fungi could be ardent competitors for nutrients, especially in the advanced decay stage. Because the nutritional status of toxigenic strains is important in determining toxin-producing ability (Moss and Badii, 1982) any deficiency in availability of readily-metabolizable substrates could check enzymes responsible for toxin production (Abdollahi and Buchanan, 1981). In localised areas of the grain bulk, the activities of any competing mycoflora can quickly alter the micro-environment to an extent which depresses toxin formation. Moisture availability could be severely reduced to a level too low for toxin formation. Growth of thermophilic species, for example, can raise temperatures, thereby decreasing toxin yields and temperatures can be elevated to such a level that no toxin formation occurs (Mislivec *et al*., 1977; cited by Stoloff, 1979).

Fungi such as *Penicillium citrinum* and *P. expansum* inhibit aflatoxin production by *A. flavus* with the production of a filterable and heat-stable substance (Mislevec *et al*., 1977; cited by Mislivec, 1979). Still others compete by breaking down the toxic metabolites or changing them to less toxic compounds as soon as they are formed (Ciegler *et al*., 1966; Christensen *et al*., 1977; Doyle *et al*., 1980). The role of *Aspergillus niger*, *Fusarium roseum*, *Rhizoctonia solani* and *Macrophomina phaseoli* in reducing aflatoxin accumulation by *A. flavus* in a liquid medium and in peanuts was studied by Ashworth *et al*. (1965). The amount of toxin broken down was dependent upon the initial concentration. Doyle and Marth (1978) showed that biological degradation of aflatoxins was also carried out by mycelia of the

aflatoxin-producing fungus *A. parasiticus* NRRL 2999. The degradation process was greatly enhanced by fragmented mycelia and the mechanism of degradation was traced to intracellular degradation constituents.

Although the number of samples analysed for toxins in this study was small, the trend obtained for the natural ocurrence of mycotoxins was in agreement with the incidence of toxins in small grains examined overseas. Normal and good grade grain samples recorded none or very low levels of aflatoxins, zearalenone, T-2 toxins, ochratoxin and citrinin (Shotwell *et al.*, 1970, 1971, 1976, 1977, 1980; Nesheim, 1977; Krogh *et al.*, 1973 cited by Harwig and Munro, 1975; Stahr, 1981). On the other hand, thousands of samples of grains which had been overwintered in the field or harvested and stored at high moisture levels, or allowed to become heated or rewetted during storage, frequently became invaded by fungi and contaminated with mycotoxins.

The 1972 corn crop in the northern Corn Belt of the U.S.A., which was exposed to high moisture in the field and after harvest, was later found to be contaminated with zearalenone, aflatoxin and T-2 toxin (Eppley *et al.*, 1974). Of the 57 obviously damaged corn samples collected by Stoloff *et al.* (1976) from 116 different farms and country elevators located in the U.S.A., 30% were positive for aflatoxin and 7% were positive for zearalenone. On examination of 29 farm samples of heated grain from Saskatchewan, Canada, ochratoxin A was detected at concentrations of 0.03-27 ppm, citrinin at 0.07-80 ppm and a sample was also contaminated with sterigmatocystin (Scott *et al.*, 1972). A survey of the mycotoxin literature published will yield many more similar examples.

Hence the chances of mycotoxins occurring naturally in sound, freshly-harvested and properly-stored grains are remote, whilst grains that have become mouldy can be a source of toxins. The isolation of multiple toxins from a single mouldy sample, as in this case, indicated that more than one fungal species was involved in the spoilage process. Such joint ventures by fungal species in forming toxins in an individual substrate is of concern, for the health risks involved in consuming multimycotoxin-contaminated feed can be amplified by the probable synergistic effects of the toxins present.

CHAPTER 7

GENERAL DISCUSSION

Fungi are usually present in grains. Their kind and abundance depend on a number of factors which include the grain variety, the environment during grain formation, the harvesting process and the conditions of storage. Once grains are harvested and stored, it is principally the fungi and their interactions with other biological, physical and chemical factors that bring about deterioration and spoilage.

In addition, the proliferation of, particularly, the so-called storage fungi, under favourable conditions of moisture and temperature, can result in the production of metabolites which are toxic both to animals and to man. These mycotoxins can be ingested with feedstuffs prepared from the contaminated grains.

In the case of barley-containing feeds, mycotoxin contamination is often a result of earlier fungal growth on and within the grain itself, and this is most likely to occur during storage of the grain. Therefore it is important to examine the mycoflora associated with the grain and to study the changing levels of fungal contamination which occur during storage of barley.

In the present studies, reasonable effort was taken to ensure that all the samples and subsamples selected for examination and analysis were truly representative of the populations being studied. So, valid unbiased inferences could be made from the information obtained.

The sampling of granular material particularly for mycotoxins is difficult because of factors such as the sheer bulk of the material to be sampled and the uneven distribution of fungi and toxins (Davis _et al._, 1980). To get adequate, random samples of barley grain, samples were taken after a lot of barley had been blended by harvesting, loading and unloading or by manually mixing. Two effective and highly recommended methods of sampling, namely stream and probe sampling, were used (Davies _et al._, 1980).

In the laboratory, all samples were well mixed, systematically reduced, and in the case of multimycotoxin analysis, comminuted to

ensure uniformity of the material before subsampling and subsequent examination and analysis.

The choice of examination technique used to determine the barley mycoflora was also important since such factors as the choice and strength of the disinfectant solution used to surface-sterilise grains, the time of immersion in the solution and the type of agar medium used influence the results (Semeniuk, 1954; Pepin, 1976). Nevertheless, no one technique or medium will divulge all the fungi associated with a given lot of grain (Wallace and Sinha, 1962). Of the two widely-used methods in the study of barley grains, the grain plate method and the dilution plate method, the latter was the preferred choice in these investigations as it was more suitable for estimating the total number of fungal propagules contaminating the grain.

Potato dextrose agar, a medium routinely used for the isolation and identification of numerous fungi (Ogawa _et al._, 1978) was used to enumerate and isolate viable fungi in the dilution platings of supernatants resulting from the shaking of barley grains in sterile water. Preliminary experiments had shown that PDA was the best medium to give a broad spectrum of both the fungi themselves and their numbers, compared with more selective media such as tryptone soya agar, malt salt agar and Czapek Dox agar. Also, 0.05 mg/cm^3 chloramphenicol was incorporated in the agar to suppress the growth of bacteria.

However, the dilution plate method, too, has its limitations. Most investigators follow the method of Christensen (1957) by comminuting grain in a blender before dilution plating. This tends to grossly over-estimate total fungal inoculum by subdividing existing 'viable fungal structures', thus increasing the number of colonies found during plating. Even without comminuting the grains, the method still tends to favour the enumeration of the heavily-sporulating species. Whilst the method gives a general picture of the fungal flora present, it does not distinguish those which grow from spores and those which grow from hyphae. Neither does it distinguish between fungi existing on the surface from those within the grain.

Thus, a number of different techniques is required to give accurate assessments of the number and kinds of fungi, especially with respect to the levels of both internal and external contamination in stored grains, which was an important aspect of the present investigations.

The total counting technique used to obtain viable fungi from barley grains had certain advantages over other techniques. Viable counts of fungal propagules obtained could be differentiated into total viable counts, total outside and total inside counts (see Chapter 3). Total viable counts, which consisted of mainly superficial contaminants, were obtained by a twofold shaking process. First, whole grains, and then after dehulling, their husks and caryopses, were separately shaken in sterile distilled water. In order to enumerate viable fungi inside the grain, that is fungal propagules enclosed between husks and caryopses, the grains were first subjected to surface sterilisation with sodium hypochlorite. They were then dehulled and the resultant grain fractions were shaken in sterile distilled water. To improve the efficiency of surface cleansing both for surface sterilisation and collection of exterior particles, sterilised beads, as suggested by Mulinge and Chesters (1970b) were added prior to the shaking process. These beads assist in releasing slimy, sticky spores trapped in cracks, crevices and other rough structures on the grain surfaces.

The types of fungi cultured from whole grains and grain fractions could be distinguished in a similar manner to the fungal genera from the dilution plates i.e. genera originating from inside the grain could be differentiated from those originating from the outside surfaces. All the fungi that grew on plates of supernatants from surface-sterilised grains, in addition to those colonies from cultured grain fractions, were presumed to be fungi occurring inside the grain.

The culturing of fungi that grew mainly from fungal hyphae which had penetrated or colonised the husk was another important aspect of the present studies and was achieved as follows. Surface-sterilised grains were washed 16 times with sterile distilled water, dehusked, and then the husks were washed in sterile distilled water 4 times and cultured. The fungi that grew from these 'husk' cultures could thus

be distinguished from those which occurred mainly as superficial contaminants in the form of spores.

The culturing of caryopses and caryopsis sections enabled the isolation of fungi which existed inside the grain and to a limited extent those which had penetrated caryopsis tissues. However, to ensure that any existing internal fungi of caryopses would have a good opportunity of being isolated and not be exposed to undue competition, the caryopses should have been surface-sterilised before they were cultured as a whole or as cut sections. This latter step should be included in order to improve the results obtained in Section 3.3.5.2 which implied that the fungi isolated from the cultured caryopsis tissues were mainly those which occurred on the surface of the caryopses.

The evaluation and determination of the location of fungal mycelium occurring in the barley grains examined involved not only the culture of husk strips and caryopses but also a combination of other methods. Caryopsis sections and strips of husk were stained in lactophenol cotton blue or aniline blue for direct microscopy. Both inner and outer surfaces of husks were examined for the types of fungal hyphae present and for their locations. The results obtained by light microscopy were further supplemented by scanning electron microscopic observations of whole grains and husks.

In addition, hyphal fragments which were detached during shaking of the grains were examined and quantified using the membrane filtration technique. This method was quick and simple to use, but it had the disadvantage of not differentiating the viable from the non-viable hyphal fragments retained on the membrane filters. As a result, a micro-manipulation technique was used to pick up the detached hyphal fragments and deposit them on PDA for culturing, to identify any viable hyphae.

The micro-manipulation technique was also found to be simple to use, and although somewhat time-consuming, it was an effective way of determining viability of hyphal fragments. It avoided both the uncertainties introduced by incomplete surface sterilisation of spores contaminating husks cultured for viable mycelium (Mulinge and

Chesters, 1970b) and the probable harmful effects on fungal mycelium of sodium hypochlorite (Milner _et al_., 1947), a surface sterilant commonly used by many investigators.

In the present investigations the method was only used to check the viability of loosely-attached hyphae, but it could be readily adapted for other uses, for example, to determine the viability of internal hyphae, provided the husk tissues were first softened and gently macerated in such a way as to loosen fragments from the internal network of mycelium.

To investigate the levels of grain contamination by fungi the following counts from dilution platings of supernatants from whole grains, husks and caryopses were taken: total viable counts, outside counts and inside counts. It was found that for all the "clean" grains examined the outside counts constituted the major proportion of the total viable counts. The outside counts obtained showed considerable variations but this was not unexpected since they consist of fungal propagules which happen to lodge on the outer grain surface.

The total inside counts recorded for "clean" grains were low and showed little variation. The inside counts of such grains represented the total contaminants introduced from the environment into the space between the lemma, palea and caryopsis during grain maturation. However, once the grains went mouldy, the internal counts rose dramatically both as a result of the growth of the internal contaminants and as a result of husk penetration by outside contaminants. This was illustrated by the high internal counts obtained for mouldy farm-stored grains. During storage any increase in the internal count would tend to indicate that incipient growth was taking place and therefore these counts could prove helpful in determining grain condition before visible mould appeared.

The genera associated with each grain source were isolated from dilution plates incubated at 25°C and 37°C. These genera isolated were therefore mainly mesophilic fungi with some thermotolerant species appearing at the higher temperature. A comparison of the fungal flora of dried barley isolated in these studies with those

found by several other investigators overseas revealed a great similarity in both genera and their frequencies of occurrence.

In a New Zealand study of the mycoflora of barley seeds grown in the Wairarapa district, Chong and Sheridan (1982) isolated a variety of fungi from 32 seed samples using 4 different methods. Many of the genera isolated were also found in the present studies and with relatively similar frequencies. For example, Alternaria, Cladosporium, Fusarium were present with frequencies over 90% in both studies whilst Aspergillus, Mucor and Trichoderma were present at relatively low frequencies.

Amongst the fungi found in the barley samples examined, there were some which are of economic importance as plant pathogens, spoilage organisms and toxin producers. Several of the field fungi encountered have been cited as important seed-borne pathogens (Machacek et al., 1951; and Sheridan, 1976). Fungi such as Epicoccum purpurascens, Nigrospora oryzae, Alternaria alternata were found to be pathogenic to rice seedlings (Lau and Sheridan, 1974). Many of the Drechslera species associated with barley are important plant pathogens (Hampton and Matthews, 1978). D. teres, for example, is the causal agent of net blotch, an important disease of barley crops in New Zealand (Arnst et al., 1978).

Under favourable conditions grain deterioration and spoilage can occur by the growth of fungi present in the grain. In stored barley, spoilage is often attributed to the growth of storage fungi, especially strains of Aspergillus and Penicillium, many of which were present in the barley samples analysed (see Appendix 3-1). Moreover, the damage caused by the growth of these groups can be enhanced should certain combinations of species be present. For example, A. flavus and A. candidus growing together appear to cause much more germ damage than the simultaneous growth of some members of the A. glaucus group (Qasem and Christensen, 1958). Certain fungi isolated in the present studies not only cause grain spoilage but also produce a number of mycotoxins given appropriate conditions. Both field fungi like Alternaria and Fusarium, as well as storage fungi including several species of Penicillium and Aspergillus fall into this category (Ciegler, 1978; Mills, 1982; and Bruce, 1984).

With the exception of mouldy grains, fungal contamination in the form of spores and hyphae as detected by dilution platings, from direct platings of husk fractions and by direct microscopy, occurred mainly on the outer surfaces. This is in agreement with the findings of several other investigators. Mulinge and Chesters (1970a) found 5% of the caryopses they cultured were infected, compared with 65-80% infection of husks. Flannigan and Dickie (1972) detected most viable fungal propagules in the first 5% of the husk and pericarp removed during pearling of barley. Mycelium, too, was found to be located in the husk rather than the caryopsis by Warnock and Preece (1971). The absence of high internal contamination would serve to indicate that grains have been properly stored and no significant fungal invasion has occurred.

In the present investigations, field fungi were predominant in husk and caryopsis fractions of freshly-harvested grains. After prolonged storage at ambient temperatures, Penicillium and Aspergillus species penetrated the inner husk surface. The presence of these internal fungi could prove to be important as they are in a position to rapidly invade the grain and could also produce mycotoxins within the grain.

Whilst hyphae were only observed in the caryopsis tissues from mouldy grains, hyphae were present in both the inside and outside of all husks. The abundance of mycelium associated with "clean" grain is closely related to environmental factors, in particular that of relative humidity prevailing during ripening of the grain (Hyde, 1950). It was subsequently suggested by Hyde and Galleymore (1951) that the spread of mycelium in the husk was restricted by desiccation within the husk.

S.E.M. was a useful technique for confirming the findings of observations by light microscopy of mycelium distribution on and within the grain husk. The mycelium associated with clean grains was mainly confined to the ends of the grain, the ventral crease and grain surface structures, whilst in mouldy grains the mycelium forms a loose network covering the entire grain.

In the study of mycelium associated with barley grains, loosely attached hyphae were obtained, their numbers estimated as hyphal units and their viability checked by membrane filtration and micro-manipulation techniques as detailed in Chapter 3. There was little variation between hyphal unit counts for freshly-harvested grain, samples from commercial silos and laboratory-stored samples, indicating that no fungal growth had occurred during storage. This was supported by the decreasing levels of viability which were obtained for hyphal fragments from well-stored grains. For mouldy samples, however, high hyphal unit counts and high percentage viability of hyphal fragments were recorded.

Most of the hyphal fragments associated with the barley grains were dead. Only 22-25% were viable in freshly-harvested and well-stored grains. This increased to 45% of the hyphae isolated from mouldy grains. This confirms the findings of other investigators (Christensen, 1951; Hussein, 1983) who also noted that most of the mycelium associated with high-quality, "clean" grains were dormant or dead whilst a larger proportion associated with low-grade, mouldy grains were viable.

Although no clear relationship could be demonstrated between total viable counts and total hyphal units in the grain samples examined, the hyphal units could make a significant contribution to total viable counts and hence the inoculum potential of the grain fungi in some situations. Such situations occur where comparatively large quantities of hyphal units composed of larger and therefore more likely viable fragments are coupled with low total viable counts. In the present studies, examples of this were encountered in the commercial silo-stored and laboratory-stored grains at ambient temperatures. In both these cases total viable counts had decreased to low levels during storage but the abundance and viability of hyphal fragments remained relatively constant.

In agreement with other investigators (Christensen, 1951; Hussein, 1983; and Elekes, 1983) the viable fragments cultured yielded field fungi, mainly species of _Alternaria_ as well as _Drechslera_ and _Fusarium_ from all the clean freshly-harvested and well-stored samples.

Mouldy samples yielded only Aspergillus and Penicillium from cultured hyphae.

The presence of the normally invisible yet physiologically active hyphae dispels any earlier myths that "spoilage produces mould" (Christensen and Kaufmann, 1974). Moreover, their presence in the grain samples examined could be of concern should the grains become exposed to conditions which would permit their proliferation leading to grain spoilage and toxin contamination.

In connection with toxin contamination, recently Morita et al. (1984) and Takahashi et al. (1984) have separately reported the use of fluorescence microscopy in detecting mycotoxins associated with fungal mycelium. Morita et al. detected zearalenone in the mycelium of Fusarium graminearum whilst Takahashi et al. found very good correlations between the distribution of fungal mycelium and sterigmatocystin, an endo-type toxin (Purchase and van der Watt, 1970) in brown rice kernels naturally infected by Aspergillus versicolor.

With the knowledge that any fungal growth is likely to contribute both to the existing network of mycelium and its viability, such examination of hyphal fragments for numbers and viability could provide clues as to grain condition. This could be especially useful for checking grain storability given that hyphal counts and viability decreased under well-controlled storage conditions.

In order to study the changing levels of fungi during storage, it was first necessary to know the fungal genera, their counts and their distribution in grains prior to storage. The mycoflora present in freshly-harvested grain is known to show wide variations from year to year depending on grain variety, seasonal conditions, crop rotations and harvesting methods (Jorgensen, 1969; Flannigan, 1978; Cantone, 1983).

Storage fungi are a major factor in determining the storability of grain. Even if the grains are only lightly invaded and hence damaged, at harvest, these fungi would continue to develop at a lower moisture content and lower temperature than those required for the invasion of sound grains (Christensen, 1973). Hence the presence of

species of Aspergillus and Penicillium in grains at harvest may render the grains more susceptible to invasion during storage.

The vast majority of freshly-harvested grains examined in the present studies showed low fungal contamination (Table 3-2) which existed mainly as spores, as evidence from comparisons of total viable counts and hyphal unit counts. The hyphal unit counts represented considerably less than one-half of the total viable counts at harvest.

Amongst this contamination, storage fungi were present in relatively low frequencies and, it seemed, exclusively as spores. This can be concluded by the absence of hyaline mycelium in the husks and the absence of these fungi from cultured viable hyphal fragments. At this stage, these fungi were only cultured from surfaces of husks and it was only after prolonged storage that they were detected in inner husk tissues. Based on the above observations, the freshly-harvested grains stored in the present studies were considered "clean".

When barley grains were stored after harvest with moisture contents of 12-13% and kept under well-controlled conditions, the quantities of viable fungal propagules decreased with the length of storage. This drop in fungal contaminants occurred on both the inside and outside of the grain as revealed by a reduction in the inside and outside counts.

This also proved to be the case for grains kept sealed in the laboratory at ambient temperatures, but the cold-stored grains showed a markedly smaller drop in fungal viability. It would appear that cold storage tends to promote the longevity of fungi and therefore grains stored in the cold should be checked regularly.

In most cases when grains were initially stored at slightly higher moisture contents of 14-16% in an unregulated environment (i.e. farm-stored samples), the moisture content rose with storage and the grains often became heated and infected by weevils. This was accompanied by mould infestation as evidenced by high total viable counts and for larger internal counts as compared with the well-stored grains.

As storage progressed, so the pattern of the fungal flora changed. Generally the field fungi which were predominant at harvest gradually decreased until they were present at low frequencies whilst the opposite trend was displayed by the storage fungi.

The patterns of fungal change during storage thus depended mainly on the moisture content, the temperature, the length of storage and the genera present. When the grains were initially stored at low moisture contents and kept in a controlled environment then species of Cladosporium and Aureobasidium showed gradually falling levels whilst the levels of Fusarium and Curvularia dropped sharply. Alternaria, which was the most frequent genus at harvest, maintained its levels throughout the 9 months' storage period and the storage fungi, Aspergillus and Penicillium, were isolated from stored grains at increasing frequencies with the length of storage.

However, when grains were stored at higher moisture contents and were subjected to changes of temperature and moisture, a different pattern emerged. Within 6 months most of the field fungi had dropped to low levels and the storage fungi, especially Penicillium and Aspergillus, had become prominent.

The monitoring of the numbers and species of fungi in stored grains can therefore be used to determine the grain condition and also to give an indication of the storage conditions.

One aspect in the present studies of stored barley was concerned with the aflatoxin-producing potential of isolates. The general medium, PDA, was used in the isolation of these fungi in preference to A. flavus selective media such as Aspergillus differential medium and Aspergillus flavus and parasiticus agar (Bothast and Fennell, 1974; Hocking, 1982). This simple medium was chosen to facilitate the isolation of a wide and representative range of fungi with consideration to the scope of the studies. It has also been suggested that selective media are likely to give a distorted view of the spectrum of fungal species compared with fungi naturally present (Bell et al., 1967).

The *A. flavus* strains isolated were screened for toxin production on coconut agar which is a proven, easy-to-use and successful medium for the identification of toxigenic strains (Lin and Dianese, 1976). The next stage was an attempt to culture all the coconut agar-positive strains on sterilised barley along with a selection of the negative strains. In this way it could be determined whether all toxigenic strains in coconut agar were also toxigenic on barley and whether any of the non-toxigenic isolates on coconut agar might be positive on barley. It was shown that the results on barley were identical to those on coconut agar (see Chapter 5) and we can therefore conclude that toxigenic *A. flavus* isolates were amongst the fungi isolated from barley, and barley could be a potential substrate for aflatoxin production under suitable conditions. Coconut agar's ease of use as compared with sterilised barley and the identical results obtained justifies its use for such screening tests.

The results in Table 5-2 show that 3% to 25% of the isolates of *A. flavus* were toxigenic in freshly-harvested and in stored grain. Although low, these results are of concern and aflatoxin contamination cannot be ruled out as a problem in New Zealand.

Even low numbers of toxigenic strains in stored grain could lead to severe contamination should storage conditions become conducive to *A. flavus* growth and aflatoxin production. Studies have shown that *A. flavus* requires a minimum temperature of 15°C and an average water activity (A_w) of 0.95 before growth occurs on barley (Niles *et al.*, 1985), whilst Chang and Markakis (1981, 1982) found that a temperature higher than 13.5^{o}C and a minimum moisture content of 16.5% are necessary for aflatoxin production on barley. *A. flavus* is a typical member of the Aspergilli in that even early mycelial growth produces a large number of spores which could be easily dispersed (Moreau, 1979). With the onset of growth of any toxigenic strain in a localised area of the grain bulk, levels of moisture and temperature invariably rise and these rises coupled with the large spore numbers lead to accelerated growth which results in increased aflatoxin production (Chang and Markakis, 1982, cited by Niles *et al.*, 1985).

Within New Zealand toxigenic *A. flavus* are present in environmental sources to which grains are exposed, as shown by their

isolation from New Zealand soils by Hussein (1983) and by their presence in grain at harvest in the present studies. Under favourable ecological and climatic conditions, there is therefore the possibility of contamination by toxigenic *A. flavus* in the field, which may serve as an inoculum for aflatoxin formation during storage. These conditions could also produce quantities of toxigenic *A. flavus* propagules particularly in the form of sclerotia which could remain in the environment and contaminate future crops (Wicklow *et al.*, 1982, 1984).

Given the wide variety of fungi associated with barley (see Chapter 3), it is not difficult to envisage that some toxigenic strains were present in the samples examined. If the grain was subjected to unfavourable conditions even for a short period either in the field, during harvesting or during storage, these toxigenic strains would have the opportunity to grow and synthesize toxins in the substrate.

It should be noted, however, that only one of the samples analysed contained toxins. This particular sample became mouldy during storage in a farm silo (personal communication with the farmer), which suggests that mycotoxin contamination is most likely to occur as a consequence of fungal proliferation during storage of the grain. Two other mouldy samples were analysed (see Table 5-2) and as they were found to be toxin-free, it can be concluded that the obvious presence of mould is not in itself an indication that toxins will be present. This finding is in agreement with the observations of several other investigators (Jackson and Ciegler, 1978; Prior, 1981, and Abramson *et al.*, 1983).

Three different toxins, namely aflatoxin B1, ochratoxin A and citrinin, were found in the sample that proved positive, and this indicated that several toxigenic strains of *Aspergillus* and *Penicillium* were responsible. The most likely conditions of storage that this particular sample was subjected to were deduced by consideration of the growing conditions required by the presumptive producers of these toxins and the conditions that would have prevailed for the production of the toxins. Hence at some stage, the grain was exposed to temperatures of at least 13.5^{o}C and minimum moisture con-

tent of 18.0% for the toxin producers to grow and synthesize the three mycotoxins present in the sample (Ciegler, 1978; Chang and Markakis, 1982).

The concomitance of three different toxins in one grain lot would be of concern should the grain be consumed by animals or man. The consumption of mixed toxins can give rise to multiple toxic effects or aggravated disease symptoms from any synergistic effects of these toxins.

T-2 toxin and zearalenone, two other mycotoxins tested for in the present studies, were not encountered in any of the samples, but this does not mean that they will never occur in New Zealand barley or other cereal grains. The toxigenic Fusaria, such as species of Fusarium graminearum, F. culmorum and F. sporotrichioides responsible for the production of these two toxins require very high moisture content for growth to occur (Mills, 1982; Seaman, 1982). This is more likely if the barley crop is overwintered in the field, but can also arise if rewetting of grain occurs during storage. In the former case these toxins would be present in freshly-harvested grain and would probably persist throughout storage.

When assessing the importance of grain contamination by fungi in stored barley, the studies reported here provide useful techniques for determining the levels of both internal and external contamination. The presence of spores and mycelium, their identity, location and distribution were also determined and their relevance to grain condition and spoilage was evaluated. Whilst A. flavus strains capable of producing aflatoxins were isolated and indirect evidence of other toxigenic species found, only in exceptional cases when gross spoilage occurred did the barley become contaminated with mycotoxins. The present studies have shown that by examining the fungal flora of stored grains by the described techniques at regular intervals, the condition of the grains and the likelihood of spoilage and mycotoxin production can be evaluated.

To date most of the studies on the mycoflora of barley have taken place overseas and have concentrated upon moist barley. In New Zealand, however, most of the barley is stored at low moisture content

in silos. Thus these studies have made an important contribution to the knowledge of the changing fungal flora of stored dried barley and its relevance to spoilage and mycotoxin contamination.

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