A study of some of the inter-relationships between maize and the seed storage fungi as typified by *Aspergillus flavus* var. *columnaris*

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Preface

The experimental work described in this thesis was carried out in the Department of Biology, University of Natal, Durban, under the supervision of Professor Berjak.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

Dedicated to my parents,
sorry you never got to see it Dad.

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Abstract

The seed storage fungi (xerotolerant) species of the genera Aspergillus and Penicillium) are renowned for their devastating effects on stored grain and grain products. In view of the fact that most of these fungi liberate toxins which can be harmful to both man and his livestock this problem is becoming increasingly relevant, particularly in developing countries. The seed storage fungi are said to be saprophytes and opportunistic invaders of dead or naturally dried organic matter, and as such no direct host-pathogen relationship has been ascribed to them. This dissertation reports aspects of an investigation into the modes/pathways utilised by these fungi in their infection of maize caryopses (seeds) and plants. The work involved studies on: the effects of protracted storage on maize seeds; the morphology of storage fungi; extra-cellular enzymes of storage fungi; the pathways utilised by the storage fungi in invasion of seed tissues; and the effects of the storage fungi on the seeds. Correlations have been made on a species basis between the extent of seed deterioration and fungal aggressiveness. The results of these investigations indicated that apart from affecting seed vigour and viability, these fungi can also affect plant vigour. This latter aspect was further investigated to determine whether a seed storage fungus could infect germinating maize seeds, and remain an internal contaminant of the tissues during plant growth and development. These latter studies revealed that Aspergillus flavus var. columnaris is capable of systemic transmission from one seed generation to the next. This hitherto unrecognised phenomenon apart from indicating that the fungal species is in fact a biotroph as well as a saprophyte, also has implications in control measures.

Contents

Chapter 1

Introduction

1 Introduction

The development of man and his ancestors is a scenario put together from fossil evidence collected throughout the world. By 12 000 years ago man had become a dominant species but was still a hunter gatherer; he remained very much part of the biosphere, in that he had to compete with other animals for food. The development of agriculture by humans about 10 000 years before the present time released man from this competition as he was capable of producing sufficient food for his own survival (Brouk, 1975). The advent of agriculture was also the beginning of man's greatest impact on the ecosystems of the earth. Cultivating humans have modified vegetation to suit their needs and these alterations, which are still occurring, have introduced plant species that cannot survive without human care (Berrie, 1977).

Today the plants utilised by man are divided into a number of categories:

a Plants consumed by humans
b Industrial plants
c Animal fodder plants
d Medicinal plants
e Plants for shelter
f Ornamental plants

The group of plants that are consumed by man are divided, in colloquial and commercial language, into

a cereals
b vegetables
c fruits
d nuts

It is important to note that these divisions do not necessarily follow botanical principles (Berrie, 1977).

The term "cereal" is derived from the Latin cerealia munera; the gifts of the goddess Ceres (Berrie, 1977). All the cereals are native to the Old World except maize which originated in central America. They are the most important group of plants consumed by man and botanically are grasses which produce dry fruits of high

nutritional value. The group comprises barley, maize, oats, rice and wheat, amongst others. Nutritionally there is little difference between the various species, the most nutritious form being the entire caryopsis. Unfortunately the removal of the husk and outer layers of endosperm by modern milling results in the loss of important vitamins (Vickery and Vickery, 1979).

There is ample archeological evidence that man has been storing cereal grain (in particular maize) for future supply since the pre-history era. Through the ages he has learned that the two important aspects of storage, that is preservation of quality and prevention of losses can be achieved by storage under the correct conditions (Neergaard, 1977). Cereal grains and their products if stored incorrectly are potential substrates for the growth of micro-organisms. The variety of micro-organisms which is present depends on the moisture content and the temperature at which the grain is stored. The predominant pathogens are fungi and these are usually divided into two categories, viz field fungi which infect grain prior to harvesting and storage fungi which invade stored grains (Christensen and Kaufmann, 1969, 1974; Roberts, 1972; Christensen and Sauer, 1982).

The field fungi comprise members of the genera Alternaria, Cladosporium, Curvularia, Epicoccum, Fusarium and Verticillium. These fungi are responsible for seed discolouration and reduced germinability. Their activity is usually halted under storage conditions as they require high relative humidities (in excess of 95%) and seed moisture contents above 25% for growth (Christensen and Kaufmann, 1969, 1974; Roberts, 1972; Christensen and Sauer, 1982).

The storage fungi are an ecological group composed of xerotolerant members of the genera Aspergillus and Penicillium. Members of this group can be metabolically active in stored grains with moisture contents as low 12%. Most storage fungi degrade the germ layers and the embryo, initially causing discolouration but ultimately total decay (Christensen and Kaufmann, 1969, 1974; Roberts, 1972; Hudson, 1986).

The problem of deterioration of stored grains has been with man since the advent of agriculture and even despite modern technology large quantities of grain are still lost to invading pests. The problem is particularly prevalent in under-developed countries where cereal grains are grown by the farmer as a subsistence crop. In these countries the availability and maintenance of the correct storage conditions are not economically possible for the average farmer (Neergaard, 1977). The problem is further intensified by the fact that many of the storage fungi produce mycotoxins which are harmful to both man and stock animals (Mislivec, 1981).

In this dissertation some aspects of the interactions of some the seed storage fungi, and in particular Aspergillus flavus Link var. columnaris with both stored maize caryopses and the maize plant have been examined. By way of an introduction to this topic, various aspects of the antagonist/pathogen (Aspergillus flavus var. columnaris) and the technology involved in both the production and storage of the host (maize) are discussed.

1:1 The pathogen

Prior to the development and perfection of the light microscope, moulds were classified on the basis of their colour and texture. Once microscopy became a useful technique, it was possible to distinguish differing and characteristic structures within the moulds (Raper and Fennell, 1965, 1977). However, already in 1729 Micheli noted that a number of fungi were distinguishable by their rough heads and strings of spores, he gave the moulds having these features the name aspergilli, the word meaning rough head (Raper and Fennell, 1965, 1977).

Today there is extensive literature pertaining to the aspergilli. The Aspergillus genus is classified in the Fungi Imperfecti or the Deuteromycota. The following is the accepted classification of the aspergilli as proposed by Ainsworth (1973) and adopted by Webster (1980).

Kingdom	Plantae
Division	Eumycota
Sub Division	Deuteromycotina
Class	Hyphomycetes
Genus	*Aspergillus*

Raper and Fennell (1965) recognised 132 species of *Aspergillus* with 18 varieties, these being distributed amongst 18 distinct groups. By 1979, a further 90 species had been added to this genus (Samson, 1979).

Link is believed to have first described *Aspergillus flavus*, having isolated it from an herbarium specimen. He distinguished it from other species by its yellow-green hue, thus justifying its name (Raper and Fennell, 1965). Thom and Raper (1945) provided a basic description for members of what they termed the *Aspergillus flavus - oryzae* group. Those authors prepared species descriptions based on cultures from the koji moulds of the orient. These moulds are used in the east in the manufacture of sake and soya sauce. Raper and Fennell (1965), having studied numerous cultures and after extensive literature surveys, grouped a number of species into the *Aspergillus flavus* group, each species being described using a key strain.

Members of the *A. flavus* group, which comprises nine species and two varieties, are cosmopolitan, occurring in most parts of the world, from desert sands to peat bogs to polar lakes (Raper and Fennell, 1977; Christensen, 1981). They are common in cereals and in fact are found associated with most foods consumed by man and livestock (Rodricks, Hesseltine and Mehlman, 1977; Moreau, 1979; Kozakiewicz, 1989). If conditions are favourable, members of this group can grow profusely. Olafson (1969) reported that in a silo holding grain with an elevated moisture content (15 - 17%) 50 - 100% of the spores identified were those of *A. flavus* group members, and under such conditions the fungal species had grown on the surface of the grain to form a mat up to 600 mm in depth.

The secondary metabolites produced by some fungi can be beneficial, for example antibiotics and anti-tumour agents. However, other microbial secondary metabolites are harmful to both man and stock animals: these substances being termed mycotoxins. Humans can be exposed to mycotoxins by direct contact and by consumption of contaminated foods (Moreau, 1979) and most mycotoxins can pass along the food chain thereby concentrating their effects (Goldblatt, 1969; Allcroft, Carnagan, Sargeant and O'Kelly, 1961; Rodricks et al., 1977). Shortages of food in many parts of the world (particularly the third world countries) increase the mycotoxin hazard, since more microbially-damaged and mycotoxin-containing foods are consumed rather than being discarded (Neergaard, 1977).

The discovery in 1961 of the elaboration of aflatoxins by A. flavus and A. parasiticus and moreover the subsequent realisation of its universal nature as a potentially lethal contaminant has led to world-wide imposition of acceptable levels of mycotoxins in food (Allcroft, et al., 1961; Goldblatt, 1969; Rodricks et al., 1977). In America the acceptable level of aflatoxin is 20µg per kilogram of foodstuff whereas in South Africa, where cereals form the staple diet for the majority of the population, the restrictions are greater with only 5µg aflatoxin B_1 permitted per kilogram of foodstuff (Marasas, 1989).

The aflatoxins comprise a very complex group of molecules with malonyl CoA being a precursor. At least six different aflatoxins have been described and are classified as aflatoxin B_1, B_2, G_1, G_2, M_1 and M_2. All differ slightly in their chemical structure and this is correlated with significant differences in their toxicities. Aflatoxins B_1 and G_1 are said to be potentially the most dangerous hepato-carcinogens known, with the former inhibiting both replication and transcription of DNA (Goldblatt, 1969; Steyn, 1980).

Aflatoxin contamination of maize and maize products is a very serious problem in the U.S.A., particularly in the south east, the major maize producing region of that country (Nichols, 1983).

That author estimated that approximately US $238 million was lost by both the public and private sector in 1980 through the action of A. flavus and its production of aflatoxin. In Africa, maize, groundnuts and millet form the staple diet of the majority of the population: all are suitable substrates for the growth of A. flavus and A. parasiticus. However, the positive diagnosis of mycotoxins and mycotoxic diseases requires a highly trained and multidisciplinary approach, and this is not always available. Aflatoxicosis outbreaks have been reported in Kenya, Mozambique, Swaziland and South Africa (Marasas, 1989). These reports however, must be considered to represent only a small percentage of the actual problem in Africa.

Long before the discovery of the production of aflatoxins by A. flavus and A. parasiticus, it was known that the members of this group produced a number of secondary metabolites, some dangerous and some potentially useful.

Aspergillic Acid:- this secondary metabolite was first isolated by White and Hill in 1943. It is an antibiotic which has remarkable inhibitory effects on gram-positive and gram-negative micro-organisms.

Kojic Acid:- It has been known since 1907 that A. flavus produces kojic acid. When this toxin is in high concentrations it acts as a neurological poison causing uncoordinated body movement, exophthalmus and convulsive seizures (Raper and Fennell, 1977; Steyn, 1980).

B-Nitroproprionic Acid:- This secondary metabolite is unique amongst the mycotoxins in that it possesses a nitro group. When in high concentrations it has a vaso-dilatory action on peripheral blood vessels. The toxin also reacts with haemoglobin and impairs oxygen-carrying capacity (Raper and Fennell, 1977; Steyn, 1980).

Oxalic Acid:- All members of the A. flavus group produce oxalic acid. This substance produces gastric irritation along with rapid depletion of calcium in the serum. The oxalic acid combines with

calcium to form calcium oxalate which precipitates out of solution and can cause kidney stones. In high concentrations, this metabolite has neurological effects and can cause coma followed by death (Raper and Fennell, 1977; Steyn, 1980).

Tremorgenic Substances:- Except for aflatrem which has been characterised, the composition of this group of secondary metabolites has not been fully elucidated, but when these substances are injected into mice they cause uncontrolled shaking which may continue for up to three days (Raper and Fennell, 1965).

Four members of the A. flavus group viz A. flavus Link; A. flavus Link var. columnaris Raper and Fennell; A. oryzae Alhb (Cohn) and A. parasiticus Speare have been classified as seed storage fungi. All four species have been isolated in this laboratory from maize seed grown in South Africa. Individual species of the seed storage fungi are being characterised with the ultimate aim of producing a dossier of the distinctive features of this group (Berjak, 1984). As part of a research programme in co-operation with the International Seed Testing Association (ISTA) studying the effects of the seed storage fungi on stored grain, a multifaceted approach has been adopted in this characterisation programme. The four members of the A. flavus group mentioned above are accordingly described in Chapter 2.

1:2 The host

The place of origin of Zea mays L. is still under debate although most people believe southern Mexico to be the most probable possibility. Pollen grains of this species have been collected from geological cores taken from old lake beds near Mexico City, and have been dated at 80 000 years (Langenheim and Thiman, 1982). Man is believed to have spread maize south from Mexico to the Andes, east along the northern coast of South America, then by island-hopping from one Caribbean Island to another and into North America (Berrie, 1977). At the time of the

discovery of the Americas in the 15th century the plant was already in a state of cultivation. Primitive cultivation was simple, either in slash and burn forest clearings or on seasonally moist lands. Planting was apparently achieved by making holes in the soil with a stick then inserting a seed. Some cultures practised primitive fertilizer application by burying a piece of fish under the seed (Langenheim and Thiman, 1982).

The closest relative to maize is a plant which grows wild in central America, Zea mexicana (commonly called teosinte). Zea mays is not a direct descendant of teosinte: rather it is thought that both are derived from a common ancestor. Modern cultivars of maize resemble the primordial form only remotely. Small cobs measuring 20 mm in length and dating from 7 000 years ago have been found in Tehuacan in south Mexico, whilst cobs dating 3 500 before the present are much larger, reaching 100 mm (Langenheim and Thiman, 1982). This indicates that there has been considerable deliberate human selection of maize to produce higher-yielding types. Since the discovery of the Americas the species has spread world-wide and further selection has occurred. However, man's greatest impact on this species has occurred only in this century with the introduction of hybrid lines in 1920 (Langenheim and Thiman, 1982; Jones and Clifford, 1983).

Modern maize is a tall, vigorously growing annual with flowers occurring on separate male and female inflorescences. The male flowers are borne on terminal inflorescences called the tassel. The tassel comprises a panicle on which the spikelets are two-flowered, one of which is sessile and the other pedicellate. In the female inflorescences, which are located half way down the plant, only one of the flowers in each spikelet is fertile. The paired spikelets are sessile and are located on a spadix known as the cob. It is this monoecious nature of the plant that allows for its regulated or controlled crossing. The cob develops as the swollen tip of a branch with extremely short internodes. Overlapping modified leaves develop from these compressed nodes and form the husk which sheaths the cob. The species is characterised by monocotyledonous endospermic caryopses, each consisting of an axis

which is differentiated into a plumule at one end and a radicle at the other, and one cotyledon. Additionally, there is a mass of food-storing tissue, the endosperm. The axis, the cotyledon and endosperm are enclosed by a testa or seed coat which is fused with the fruit wall, constituting the pericarp. This is perforated at only one point, the micropyle. The micropyle is the point of attachment of the caryopsis to the cob. It is important to note that whether a cob has 40 or 1 000 caryopses, each grain can be genetically quite different (Johann, 1935; Vines and Rees, 1972; Berrie, 1977; Agarwal and Sinclair, 1987;).

There are many categories of maize grain, but most fall into five main types.

Flint maize: this has hard starch, and was the type being grown by the Indians when the pilgrims arrived in America.

Dent maize: the external side of each grain is externally indented. This type usually gives the highest yields and has a hard starch core in most of the caryopsis, but is capped by soft starch.

Flour maize: this is characterised by soft starch.

Sweet corn: in which most of the starch has been converted into sugars.

Popcorn: in this type the starch expands on heating and the grains explode. It is possibly the most primitive type of maize (Langenheim and Thiman, 1982).

Maize is currently the world's third most important grain crop, with over 350 million metric tons produced annually (Berrie, 1977). In Africa, particularly tropical Africa, the crop forms a major part of the population's staple diet, whereas in the U.S.A. 90% of the yield is used for livestock feed (Nichols, 1983). Whether used for human or stock animal consumption wherever maize is stored it has to be protected from pests, in particular insects and micro-organisms. As a consequence the practice of saving seed for use at a future time has led to the development of a very specialised multidisciplinary technology.

1.3 Seed Technology

Ewart, in 1908, reviewed a wide spectrum of seeds in terms of their longevity in storage. He found that some seeds cannot be dried to a moisture content in equilibrium with ambient relative humidity without loss of viability: such seeds have since been termed recalcitrant (Roberts, 1973) or homoiohydrous (Berjak, Farrant, Mycock and Pammenter, 1990). Storage life of such seeds ranges from a few weeks to a few months. At the other extreme, certain orthodox hard-seeded species, for example some of the legumes, can survive many years and even centuries, without loss of viability. Most seeds, however, fall into an intermediate category and can be stored for 1 - 7 years, such seeds having been termed orthodox (Roberts, 1972, 1983).

Seed technology involves all the steps necessary to ensure the production and availability of high quality seed. As are all the other cereal crops, maize is characteristically an orthodox seed species. Since cereals provide the basic diet for most of the world's population the technology involved in their production and storage is well developed.

For an orthodox species, seed technology involves specialised methodology in:

1 Production
2 Harvest
3 Cleaning and separation
4 Drying
5 Processing into storage
6 Storage
7 Germination and plant growth

(Justice and Bass, 1978)

At any point in this cycle, the seed is open to attack by pests and micro-organisms, and, in the following consideration emphasis is placed on the factors which can influence microbial activity during storage.

1:3:1 Seed production

Maturing grain can be damaged on the parent plant by insects, birds, rodents and environmental adversities such as hail (Neergaard, 1977). Such damage can allow for the subsequent activity of both field and storage fungi (Christensen and Kaufmann, 1969, 1974; Christensen and Sauer, 1982).

1:3:2 Seed harvest

The harvesting of seed crops includes cutting the plant (reaping) and then separating the seed (threshing). Combines can reap, thresh and partially clean seed in one operation. There are, however, many crops which cannot be harvested with a combine as their seeds shatter easily, or the seed is produced so gradually that mature seed, flowers and buds can be on the same plant at the same time. These types have to be harvested by hand. Reaping or threshing whether by combine or hand can cause physical injury to the seed thereby rendering it susceptible to microbial invasion (Qasem and Christensen, 1960; Christensen and Kaufmann, 1974; Neergaard, 1977).

1:3:3 Seed cleaning and separation

A great number of specialised machines have been developed to separate and clean seed, with most making use of differences in the physical characteristics of the seed. Damage can often be incurred during these processes and the extent of injury is often influenced by the moisture content of the seed (Neergaard, 1977; Christensen, 1982). In maize, injury to the pericarp increases with seed moisture content above 12% (Christensen, 1982).

1:3:4 Drying

At harvest an orthodox seed is either in equilibrium with the relative humidity (RH) of the ambient atmosphere and hence requires no further drying, or it is moist and must be dried. In

the latter case the seed could be moist for a number of reasons: (a) it is in a moist fruit such as a tomato; (b) the growing season is too short to allow for natural drying, as is the case for maize in some temperate regions; (c) the seed is harvested during the rainy season. Whatever the case, the seed must be dried as quickly as possible to a seed moisture content in equilibrium with 70% RH or lower so as to retain germinability and prevent the action of microbes (Neergaard, 1977; Christensen, 1982).

Drying can be achieved in number of ways: (a) Seeds may be dried in the sun. This method is cheap and is often used for maize in African countries when the crop was harvested during the rainy season. Such a method is not successful where the ambient RH is high. (b) Ambient air may be blown through the storage bin. Such drying is relatively inexpensive but again can only be effective if the ambient air is dry. (c) The most usual drying operation is by passing heated air over the seed. However, too rapid drying by this method can cause cracking of the cotyledons and endosperm. Commercially 45 °C is considered a safe maximum drying temperature for cereals and 35 °C for vegetable seed (Justice and Bass, 1978; Foster, 1982). However, use of such elevated temperatures has been correlated with the development of subcellular abnormalities, and lowered vigour and viability of maize seed (Berjak, pers. comm.).

1:3:5 Processing into storage

On receipt, the cleaned and dried seed is weighed at the silo. The weighed grain is fed into a dump pit from where it flows under the influence of gravity into a leg. The leg is a conveyor belt which elevates the grain to the top of the storage and thence into the bin. The movement of the grain during processing into storage can and does cause damage, this is particularly true of the flow into the leg and the drop into the bin (Pomeranz, 1976; Neergaard, 1977).

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1:3:6 Seed Storage

The two important aspects of seed storage are the preservation of quality and the prevention of losses. Any orthodox seed placed into storage will age naturally. Additionally, micro-organisms, in particular the storage fungi, are responsible for the spoilage of stored grain. The two environmental factors which influence the rate at which a seed will age, and which also control the activity of micro-organisms are temperature and relative humidity (Neergaard, 1977; Roberts, 1983; Agarwal and Sinclair, 1987).

Seeds are hygroscopic hence their moisture content comes to equilibrium with their surrounding atmosphere (Roberts, 1979). Relative humidity is a measure of water vapour in the air relative to the amount the air can hold at saturation, and this is temperature-dependent. Consequently these two environmental parameters are linked and should be considered together. However, for simplicity, the two are reviewed separately.

1:3:6:1 Relative humidity

For air-dry seeds, the higher the seed moisture content the more rapid the decline in germination capacity with increasing storage time, due to the accelerated accumulation of biochemical and ultrastructural abnormalities (accelerated ageing) and microbial activity (Neergaard, 1977). Since seeds are hygroscopic, storage at low relative humidities will increase storage life. However, different seed constituents have different hygroscopic equilibria (Justice and Bass, 1978; Lehninger, 1979). Proteins are the most hygroscopic compounds in seeds, with cellulose and starch less so and lipids are essential hydrophobic. Thus at the same relative humidity a seed high in protein and low in oils will have a much higher moisture content than one with a high oil content (Lehninger, 1979).

When it was first discovered that storing seeds very dry increased their survival in storage it was postulated that the lower the moisture content the better (Ewart, 1908). However, too low a moisture content can be detrimental to the seed, as the intracellular bound water could be removed (Aksenow,

Askochenskaya and Golovina, 1977). The bound water is believed to act as a protectant against oxidative processes. A seed contains natural anti-oxidants, the tocopherols, but these are believed to be utilised by the seed during storage without further production. If this is so, and if storage at very low moisture contents is prolonged, the seeds will die (Agarwal and Sinclair, 1987). According to those authors, it is necessary to store seed at a moisture content that is not too low to allow oxidation and not too high to allow the invasion of micro-organisms. However, despite the presence of tocopherols, drying to too lower moisture contents may perturb membrane lipids thereby bringing about irreversible damage (Berjak pers. comm.).

1:3:6:2 Temperature

The other main environmental factor affecting seed longevity is temperature. The cooler the temperature the slower the loss of viability (Neergaard, 1977). This applies to temperatures below zero as long as the seed moisture content is in equilibrium with 70% RH or less so as to prevent ice crystal formation. At high temperatures, not only is subcellular deterioration accelerated but insect and micro-organism activity is increased. At 5 °C and below, although insects become inactive, some micro-organisms can continue to degrade the seed (Neergaard, 1977; Cazalet and Berjak 1983).

The amount of water vapour air can hold increases with increasing temperatures: with each 10 °C rise in temperature the amount of water held is approximately doubled. For example, at 20 °C 1 kg of dry air can hold 14.8 g of water vapour but at 30 °C the same volume can hold 26.4 g of water vapour. Conversely, as temperature drops the amount of water vapour that can be held declines. If air at 20 °C has a relative humidity of 50%, there is 7.4 g of water vapour in each kilogram. As the temperature drops, the relative humidity will increase because the air can hold less and less water vapour. If the temperature is dropped to a point where the air is saturated, water will condense as liquid: this is the dew point. This fact must be taken into

consideration when seeds are placed into storage (Neergaard, 1977; Christensen, 1982). In a large silo where air is passed through the seed, the seed moisture content comes to equilibrium with the relative humidity of the moving air (Neergaard, 1977). However, if seeds are sealed in a can (as they often are) and stored at a low temperature it is possible that the dew point will be reached within the container, and water will condense on the surface of the seeds (Neergaard, 1977). As a consequence the micro-environment of the seed may become conducive to insect and fungal activity. This activity can further increase the moisture content, as well as the temperature in isolated areas (Berjak, 1987). These 'hot spots' as they are termed, represent areas of intensive decay. Hot spots can also develop in silos through the action of insects and micro-organisms. Most modern silos, however, are equipped with electrical resistance probes which detect areas of deterioration using electrical impulses (Waterer, Muir and Sinha, 1985) thus allowing for immediate remedial action.

1:3:6:3 The Storage Bin

In a good seed store, apart from regulation of temperature and relative humidity, there must also be protection against theft action of rodents, birds and insects.

Most seed stores have only one entrance, since this tends to minimise theft (Neergaard, 1977). Pests, however, still manage to destroy vast amounts of grain. In India, for example, rodents are responsible for destroying 9% of the grain each year (Neergaard, 1977). Rodent activity can be minimised by building the store from brick, concrete or metal (rodents tend to eat wood) and by having the entrance at least 1 metre above ground level with a removable gangway (Neergaard, 1977; Harris and Bauer, 1982).

Storage insects are far more damaging than rodents and are also more difficult to control. There are some 43 species of storage insect that are responsible for seed damage, loss of viability and for the introduction of storage fungi (Cotton and Wilbur, 1982; Mills, 1983). Weevils, for example, which have both larval and

pupal stages that develop within seed, not only destroy the seed they are inhabiting, but are also capable of carrying large numbers of fungal spores into the storage environment (McMillan, Widstrom and Wilson, 1981). The metabolic activity of the insects can also result in condensation of water thereby bringing about a localised increase in seed moisture and allowing invasion by storage fungi (Neergaard, 1977; Christensen,1982). Weevils, for instance have been known to elevate the moisture content of wheat from 14% to 23% (Cotton and Wilbur, 1982).

Insects are usually introduced into the storage bin along with the seed and this makes fumigation an effective way of reducing their presence (Mills, 1983). Fumigants such as carbon disulphide, methyl bromide and ethylene oxide are often used (Neergaard, 1977; Agarwal and Sinclair, 1987). However, if the seed moisture content is high these fumigants can be deleterious to seed vigour. Thus storage at lowered temperatures is a superior means of insect control (Hyde and Burrell, 1982).

Storage becomes increasingly expensive as storage temperature and relative humidity are reduced. These parameters are usually controlled through the use of ventilation, dehumidification and refrigeration. Proper ventilation (the most commonly used method) requires consideration of both temperature and relative humidity of the ventilating air. In areas where the ambient air has a high relative humidity, dehumidification is necessary, as is temperature control. These procedures are associated perforce with elevation of costs. Refrigeration of air-dry seeds is the most effective, but costly form of storage (Neergaard, 1977; Hyde and Burrell, 1982).

1:3:6:4 Types of Seed Storage

It is necessary to note that the underlying reason for seed storage will vary with the needs of the population utilising the seed. In the USA for instance, 90% of the annual maize yield is used as livestock feed (Nichols, 1983), whereas in developing

countries a far larger proportion of the annual harvest is for human consumption. Consequently storage types will vary from country to country. In first world countries , where well developed storage programmes are in operation, there are four types of seed storage.

i) Commercial Seed Storage: About 80% of the need of first world countries for seed storage is for conservation of commercial seeds from harvest to the next planting season. For most species, maize included, the requirements of such storage are relatively simple, since the storage time is the same as the length of time the seed is naturally quiescent. Such seed stores range from woven sacks, to barns, to silos. These stores are protected against rain and theft and are usually fumigated.

ii) Carryover Seed: This seed is stored through the first growing season after harvest until the time for the second planting, i.e. it is in storage for 12 - 18 months. Such stores are ventilated, fumigated and protected against rain and theft.

iii) Foundation Stock: This group of seeds may need to be kept for several years. Since genetic drift is minimised by reproduction, foundation stock is replanted periodically. These seed stores have stringent control of all parameters and therefore are expensive.

iv) Germplasm: These seeds are kept for many years. Their storage is usually below 0 °C and is in equilibrium with relative humidities between 20 and 25%. Such storage is extremely expensive (Justice and Bass, 1978).

However, the quantity of seeds stored for both foundation stock and germplasm, is small, and the costs are borne by agricultural agencies and not individual farmers or producers.

1:3:6:5 Consequences of Seed Storage

The storage of air-dry seeds for longer than the period between their production and harvest and the start of the next growing season is an artificial situation. In nature a seed will reach maturity and be shed at a moisture content in equilibrium with the relative humidity of the atmosphere. Under dry winter conditions such a seed will remain desiccated and quiescent, but when favourable conditions prevail it will become hydrated and initiate germination, this usually being the following spring. A non-dormant dry seed is therefore geared to survive in that state for a period of months only (Berjak, 1987). Seed storage for human related-purposes often imposes the requirement of seed survival over long periods, and usually storage is under conditions which are far from optimal. Consequently seeds in storage deteriorate, but far more quickly under poor storage conditions.

1:3:6:5:1 Natural Ageing:

Any seed placed into storage will naturally age and this process is influenced by the temperature and relative humidity of the store. Harrington (1963) has given two rules-of-thumb pertaining to seed ageing.

a) For each 1% increase in seed moisture content, the life of the seed is halved. This applies when seed moisture content is between 5 and 14%, when oxidation is the major deteriorative process involved. Above 14%, storage fungi enhance degradation & rapidly destroy the seed.

b) For each 5 °C increase in seed temperature the life of the seed is halved. This applies over the range 0 - 50 °C.

These two rules apply independently: For example, a seed at 10% moisture content which is stored at 20 °C will probably survive the same length of time as a seed at 8% moisture content held at 30 °C (Neergaard, 1977).

The ageing process can be divided into three periods the length of each period being species-specific. In the first stage, germination is slower, there is an inability to germinate at the extremes of the environmental range and a greater susceptibility to attack by micro-organisms. During this stage the cells of the meristematic region of the embryonic axis of the maize caryopses retain an apparently high degree of cytoplasmic organisation although there is some nuclear lobing, and membrane damage to mitochondria and plastids. The observed delay in germination during this stage is ascribed to repair mechanisms becoming operational before germination can be initiated. In the second stage, germinability is reduced over the entire environmental range. At the ultrastructural level, nuclear lobing is extreme and there is extensive damage to organelles. Repair mechanisms can operate in some cases only. In the final stage of ageing, no visible development growth occurs and in most cases cell lysis occurs upon imbibition. At the ultrastructural level the nuclei are deeply lobed and the chromatin is clumped. There is some deranged metabolism but technically the cells are dead (Berjak and Villiers, 1972a, b, c).

1:3:6:5:2 Genetic Changes:

Over a long period in storage a seed population will become genetically different from the original lot. This genetic drift can be ascribed to two causes:

i) During storage many seeds die and it can be assumed that those that survive do not have the same genetic composition as the majority of the original batch;

ii) An accumulation of mutations as storage time increases (Christensen and Kaufmann, 1969, 1974; Neergaard, 1977; Agarwal and Sinclair, 1987).

1:3:6:5:3 Microbial Activity:

At harvest there is a mixture of both field and storage fungi associated with seed. In the storage environment activity of field fungi is reduced and they are no longer able to perpetuate. Once these fungi no longer dominate the micro-

environment of the stored seed a succession of first Aspergillus and then Penicillium species can establish. The activity of the storage fungi reduces germinability, causes discolouration and can effect total decay (Christensen and Kaufmann, 1969, 1974; Christensen and Sauer, 1982). This transition from one dominant group to the other, and the effects of medium-length storage are the topics of Chapter 3.

According to Christensen and Kaufmann (1974), the first Aspergillus species to appear are the slow growing A. restrictus and A. glaucus group members. These fungi are reputed to invade seeds at moisture contents of 13 -14% and, through their metabolic activity, elevate the seed moisture content. As a consequence, the less xerotolerant species such as A. versicolor and A. ochraceus can invade at moisture contents of 14.5 - 15.5% and 15 - 15.5%, respectively. They, in turn, alter the micro-environment and allow the least xerotolerant species to invade, such as A. candidus (m/c 15%-16%) and A. flavus (m/c 17%). Members of the genus Penicillium then take over the decay process.

Aspergillus shares with Penicillium the role of the "most widespread and destructive agents of decay on earth" (Pitt, 1981). The F.A.O. has estimated that some 5% of cereal grains are rendered inedible by seed storage fungi and this figure may be as high as 30% in some countries (Neergaard, 1977). If one considers maize alone, of the 350 million metric tonnes produced annually, between 17.5 and 105 million are lost through the action of these fungi. The realisation that the storage fungi represent only one sector of the agents of decay others being bacteria, insects, and rodents, makes it obvious that the problem of seed deterioration deserves intensive investigation. The problem is further confounded by the production of mycotoxins by the storage fungi.

However, for such effects to occur the fungi have to gain access to the seed tissues. The aspergilli are considered saprophytes and opportunistic invaders of naturally dried or dead organic matter and, as such, no direct host-pathogen relationship has been ascribed to them (Raper and Fennell, 1965, 1977). For

the saprophytic fungi in general, there are a number of accepted mechanisms of infection and although each can be considered separately they are all probably involved in a natural infection (Hudson, 1986).

Any physical damage to the seed readily allows for invasion of the underlying tissues. Seed can be damaged at any point in its growth, harvest, cleaning, drying and processing into storage. Once in storage, the grain can be further affected by the action of insects and mites (See earlier) (Qasem and Christensen, 1960).

The pericarp, which covers an entire maize grain, is naturally discontinuous at only one point, the micropyle. This is a pore transversing the testa and is subtended by loose tissue, the peduncle, which is the remains of the structure that attached the caryopsis to the cob. The micropyle and peduncle are common to many seeds, and numerous fungi have been reported to make use of this route of infection (Koehler, 1938; Agarwal and Sinclair, 1987). As storage fungi are encountered within caryopses which are not obviously externally damaged, one or more species may gain access to the seed tissues via the peduncle and micropyle. This is the topic of Chapters 4 and 6.

Most fungal pathogens are capable of releasing cell-wall-and cell-membrane-degrading enzymes into their immediate environment (Agarwal and Sinclair, 1987). The widespread occurrence of the enzymes suggests an essential rôle for them in the infective process (Dean and Timberlake, 1989). Additionally, it is now becoming clear that pathogens sequentially release enzymes capable of degrading the various other components of the seed (Raper and Fennell, 1977; Agarwal and Sinclair, 1981). This however, does not mean that each pathogen secretes an entire spectrum of enzymes, most appear to liberate enzymes specific to certain components. Chapters 5 and 6 discuss the ability of a number of Aspergillus species to produce extracellular enzymes capable of degrading the various seed tissues.

1:3:7 Seed Germination and Plant Growth

Soil-moisture levels range from insufficient for seed germination to waterlogged in which oxygen deficits may occur (Etherington, 1978). Temperatures in the soil vary from below zero to above 40 °C and pH, although it may be extreme, generally ranges from 3 to 8. (Etherington, 1978). Throughout this range of conditions there are numerous insects and approximately 1 500 types of micro-organism and virus which can survive and adversely affect germinating seeds (Agarwal and Sinclair, 1987).

Oospores, sporangia, chlamydospores, sclerotia and conidia are the dormant micro-organism structures found in soil, and they are dormant because of the soil environment (Hudson, 1986). The actual infection of a germinating seed by a soil-located pathogen is influenced by a number of parameters, some of which also control seed germination.

According to Agarwal and Sinclair (1987), atmospheric and soil moisture levels are important in controlling micro-organism propagule germination as well as seed germination. Excessive moisture reduces oxygen and this generally inhibits germination, but there are some pathogens which are adapted to this condition and can germinate, invade and adversely affect germinating seeds and the resultant plant. Like moisture, temperature affects spore germination in the soil, the infection process and the subsequent disease development, and again there are pathogen species adapted to a wide range of temperatures. The amount of inoculum, as well as its type, aggressiveness and location in the rhizosphere relative to the seed, are also important factors. Additionally, during seed germination there is leakage of carbohydrates and amino acids from the seed tissues and these can stimulate spore germination and aid the infective process (Agarwal and Sinclair, 1987).

The actual infection process by an externally located pathogen can be very complex, involving the use of specialised enzymes and structures, or can be a simple opportunistic invasion of damaged

areas of the seed (Jones and Clifford, 1983). Alternatively, there is the possibility that pathogens may be seedborne - that is, they are internal to the seed at germination. Such pathogens must be specialised in their ability to survive the various stages of plant and seed development and subsequent seed storage in order to be transmitted successfully to the next plant generation. The ability of A. flavus var. columnaris to infect germinating maize seeds is the topic of Chapter 7, whilst its capability of survival in the developing plant and seed is discussed in Chapter 8.

Therefore, the intentions of this dissertation were an examination of the modes of entry employed by the seed storage fungi in the invasion of maize caryopses. To this end all the routes ascribed to an opportunistic pathogen were investigated, and although particular attention was placed on the interactions of A. flavus var. columnaris with maize, several other seed storage fungi were also examined. A. flavus var. columnaris was chosen as the test organism because of its relative rarity in South African maize. Hence confusion between the occurrence of the test organism and any chance infection, would be minimal. The results of those investigations indicated that A. flavus var. columnaris can act as a more complex pathogen and consequently the possibility of seed to seed transmission of this fungus in maize was also investigated.

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CHAPTER 2

Characterisation of four members of the *Aspergillus flavus* Group

Publication

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Mycock, D.J., McLean, M. and Berjak, P. (1984). Seed storage fungus characterisation: The Aspergillus flavus Group. Proceedings of the Electron Microscopy Society of Southern Africa, **14**, 63 - 64.

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Conference Presentation

Mycock, D.J., Lloyd, H.L. and Berjak, P. (1987). Characterisation of four members of the Aspergillus flavus Group. Proceedings of the South African Association of Botanists.

2:1 Introduction

The genus Aspergillus comprises 132 species and 18 varieties classified into 18 groups (Samson, 1979). Fifteen species from nine of the groups are distinct in terms of their ability to grow in the desiccated tissues of air-dry seeds, and are denoted as seed-storage fungi. It is becoming increasingly accepted that this ecological group can cause a great deal of damage to stored seeds with considerable economic losses resulting from their activity. Even if their growth is kept minimal, many of the species involved liberate mycotoxins which can be harmful to both man and his livestock (Smith and Moss, 1985).

In our laboratory, species of fungus within the seed storage group are being individually characterised with the ultimate aim of producing a dossier of the distinctive features of this group. A multifaceted approach, using macroscopic features, and light and electron microscopy, has been adopted in this venture (Berjak, 1984). To date, 13 species isolated from maize seed of local origin and to a lesser extent from New Zealand, have been described from colonies grown on potato dextrose agar containing 6% NaCl (PDA). The rationale for using this medium was that the storage fungi are xerotolerant, thus the elevated salt content would apply a water stress on the fungus as would occur in an air-dry seed. Raper and Fennell (1977) are the authorities most widely consulted in the identification of members of the aspergilli. Those authors describe colonies grown on malt extract agar (MEA) and on Czapek-Dox agar (CDA). In order to make our observations and measurements comparable with those given by those authors, the 13 species so far described are being re-described from colonies grown on CDA. It must, however, be noted that the observations of Raper and Fennell (1977) were limited by the resolution of the light microscope. Electron microscopy, particularly scanning electron microscopy (SEM), should facilitate not only more detailed descriptions of the members of this ecological group, but perhaps also resolve fine distinctions among individuals presently classed as group species.

Reproduction in the A. flavus group is by asexual means. Individual cells of the mycelium become differentiated into foot cells, which may be submerged in the agar, or above the surface the medium. Generally half way along the length of the foot cell, a perpendicular branch forms, which develops into a conidiophore (Fig. 2.1). The length, width and surface characteristics of the conidiophore are species-specific. When mature, the apex of the conidiophore gives rise to the conidial head, initiated when swelling occurs to form a vesicle. Aspergillus species may produce sporogenous heads which are either uniseriate or biseriate. A vesicle which is destined to be uniseriate produces a single rank of cells called phialides over part or all of its surface. One which is destined to be biseriate produces two ranks of cells, the first layer comprising the metulae, and each metula producing up to three phialides. In either case the phialides are the spore- or conidium-forming cells (Fig. 2.1) . Spores are produced in a typically blastic formation achieved by sequential nuclear division and cell wall formation (Vines and Rees, 1972; Cole and Kendrick, 1981; Hudson, 1986).

This chapter details the asexual reproductive structures of Aspergillus flavus Link, Aspergillus flavus Link var. columnaris Raper and Fennell, Aspergillus oryzae (Ahlb.) Cohn and Aspergillus parasiticus Speare. The descriptions and size ranges are for strains isolated from local maize caryopses. Additionally, the key strain WB 4818 of Aspergillus flavus var. columnaris obtained from the International Mycological Research Institute, Kew, UK, has been characterised in parallel allowing for comparisons between strains of this variety.

Fig. 2.1 A schematic representation of the asexual reproductive structures produced by members of the aspergilli.

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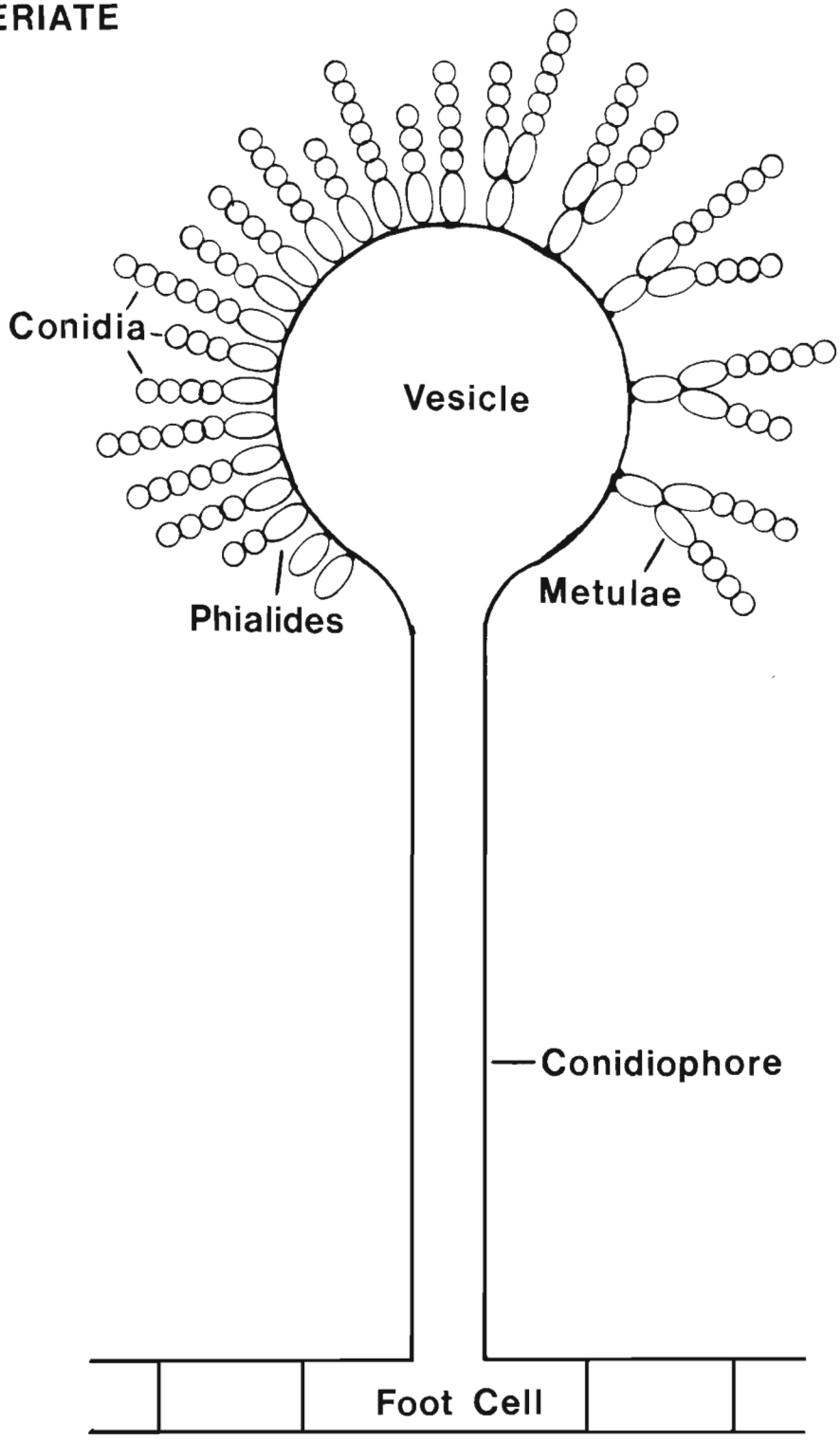


2:2 Materials and Methods

2:2:1 Media

Potato Dextrose Agar (PDA)

Potato Dextrose agar	12.5 g
Bactoagar	7.5 g
NaCl	30.0 g
Distilled water	500 ml

The components were mixed in water, then autoclaved at 121 °C (1.05 kg/cm^2) for 20 minutes. Once the solution had cooled to ± 60 °C it was poured aseptically into sterile plastic Petri dishes and allowed to set. The solidified plates were stored at 4 °C ± 2 °C in sealed plastic bags. Prior to use the plates were equilibrated to ambient temperature.

Czapek-Dox Agar (CDA)

Bactoagar	15.0 g
$NaNO_3$	3.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4.7H_2O$	0.01 g
Sucrose	30.0 g
Distilled water	1000 ml

The agar was dissolved in 500 ml hot distilled water, and the other components dissolved in the remaining 500 ml distilled water, the two solutions mixed and then autoclaved. The plates were poured and stored as described for PDA.

Malt Extract Agar (MEA)

Agar	20.0 g
Malt extract	20.0 g
Peptone	1.0 g
Dextrose	20.0 g
Distilled water	1000 ml

The plates were prepared, poured and stored as described for PDA

2:2:2 Isolation of Local Fungal Strains

Locally obtained maize seeds were:

i. Rinsed in sterile distilled water.

ii. Surface sterilised for 20 min in a 2% solution of sodium hypochlorite containing a few drops of a wetting agent, usually teepol.

iii. Rinsed three times in sterile distilled water.

iv. Aseptically halved through the long axis of the embryo.

v. Plated cut-side down on PDA.

vi. Each plate was sealed with Parafilm and incubated at 25 °C for up to 10 days. Any fungal growth was isolated and identified.

vii. All identifications were verified by the Mycological Research Institute, Pretoria.

2:2:3 Subculturing

Using a sterile inoculation needle, a clump of spores was picked up from an individual culture and placed in the centre of an agar plate. The plate was sealed with Parafilm (American Can Company), and incubated at 25 °C. Long-term maintenance of cultures was achieved on inoculated agar slopes, in McCartney bottles or test tubes. The slopes were incubated at 25 °C for five days then stored at 4 °C. These were sub-cultured every 10 - 12 weeks.

2:2:4 Colony Description

Colony Measurement: Colony diameter in millimetres was measured from the reverse side after 3, 5, 7, 10 and 15 days of growth. Two measurements, at right angles, were taken per colony, with 20 replicates.

Colony Colour: This was assessed in daylight using the Methuen Handbook of Colour (1978). Immature and mature colony colour was noted.

Exudate Droplets: Where present, exudate colour was recorded (as above) and whether it was produced sparingly or copiously.

Pigment Production: The production (or lack thereof) and colour of pigments in the medium were recorded.

Reverse Colouration: Exudate and pigment production can cause the reverse colony colouration to be a species characteristic, therefore presence or lack of such changes were recorded.

Colour Texture: Colony morphology in terms of the appearance of the mycelium was recorded.

2:2:5 Scanning Electron Microscopy (SEM)

Four-day-old colonies were utilised, and all were processed as plugs in the following manner:

i. 10-mm-diameter discs from the edge of a colony were removed using a leaf punch and forceps.
ii. Excess agar was trimmed away.
iii. The plug was placed in a pill vial and flooded with a freshly prepared 1:1 mixture of 4% glutaraldehyde and 1% osmium tetroxide made up in 0.1 M sodium cacodylate buffer, pH 7.2.
iv. The material was fixed overnight at 4 °C.

v. The samples were gently washed with ten changes of 0.1 M sodium cacodylate buffer and then five changes of sterile distilled water.

vi. The material was dehydrated through a graded ethanol series (25%, 50%, 75% and 100%). Each stage was allowed 20 minutes with one change.

vii. The samples were then critical-point dried using liquid CO_2 in an Hitachi C.P.D.1.

viii. The dried preparations were mounted on stubs and coated with a mixture of gold/palladium in a Polaron sputter coater.

ix. Viewing and photography was carried out using an Hitachi SEM 520.

x. Ten measurements per fungal structure from ten different colonies were taken from the microscope screen or from electron micrographs.

2:3 Results and Discussion

2:3:1 Aspergillus flavus Link (local strain)

On MEA the sulcate colonies were uniformly green attaining an average diameter of 67 mm in 10 days (Fig. 2.2). Colonies on PDA were more floccose and usually some shade of yellow-green, becoming browner with age, and attaining a diameter of 55 mm in 10 days. On CDA, growth was more adpressed and restricted (48 mm in 10 days) the colonies being green, and producing a yellow, diffusible pigment. A sparse hyaline exudate was also produced on the latter. Sclerotia, which were white when immature, became black with age and were more abundantly produced on CDA and MEA than on PDA (Fig. 2.3).

The conidiophores of this species develop from foot cells which may be submerged in the agar or aerial and uniformly decorated along their length (Fig. 2.4). A feature of A. flavus seldom observed in the other species was the bifurcation of the conidiophore (Fig. 2.5). The conidiophores tended to be shorter and narrower when the fungus was cultured on PDA than on CDA, and in both cases shorter than those reported by Raper and Fennell (1977) [Table 2.1]. As there were no significant discrepancies between the LM and SEM measurements, shrinkage during SEM preparation was excluded [Table 2.1] and it is probable that the shorter conidiophores are a characteristic of this strain. The apical tip of the conidiophore (Fig. 2.6) developed into a vesicle, agar type having no effect on the shape of these structures which were globose to sub-globose (Figs 2.7 and 2.8) The mature vesicles were distinctly ornamented (Fig. 2.8).

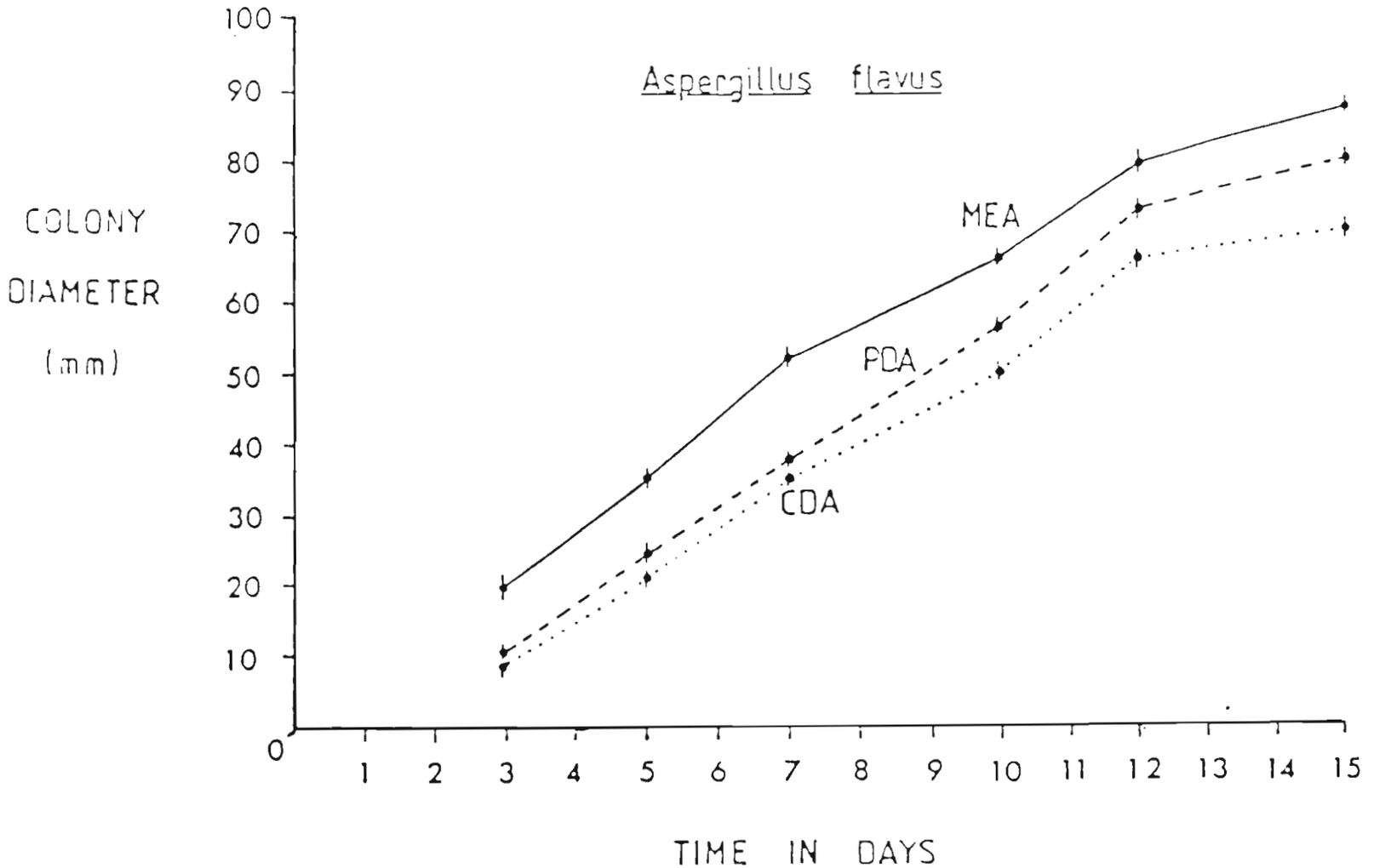
The strain was predominantly uniseriate on PDA and typically biseriate on CDA. In the uniseriate condition, phialides developed synchronously over three quarters of the surface of the vesicle, to which they were perpendicular (Figs 2.9 and 2.10). When mature, these structures were smooth and ampulliform (Fig. 2.10), and tended to be smaller from PDA colonies [Table 2.1].

The range of lengths of the phialides given by Raper and Fennell (1977) is smaller than that obtained presently [Table 2.1], for material cultured on CDA.

In the biseriate condition the metulae were smooth and barrel-shaped and gave rise to two or three phialides (Fig. 2.11). The size ranges obtained by SEM agree favourably with those given by Raper and Fennell (1977). The phialides produced in the biseriate condition were similar in length, width and shape to those formed by the uniseriate conidial heads.

In both the uni- and biseriate state, conidia were produced from the tip of each phialide in a blastic-type formation. The spores became barrel-shaped to globose as they matured (Fig. 2.12), and the verrucose nature developed with increasing age (Fig. 2.13). There was close agreement between the conidium dimension ranges given by Raper and Fennell (1977) and that obtained from colonies grown on CDA. As with the other fungal structures, the spores from PDA colonies were smaller than those from CDA colonies, but there was no difference in the pattern of surface ornamentation or shape.

Fig. 2.2 Growth curves of A. flavus on the three media. Bars indicate standard deviation.


Aspergillus flavus
COLONY DIAMETER (mm)
100
90
80
70
60
50
40
30
20
10
0
MEA
PDA
CDA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
TIME IN DAYS

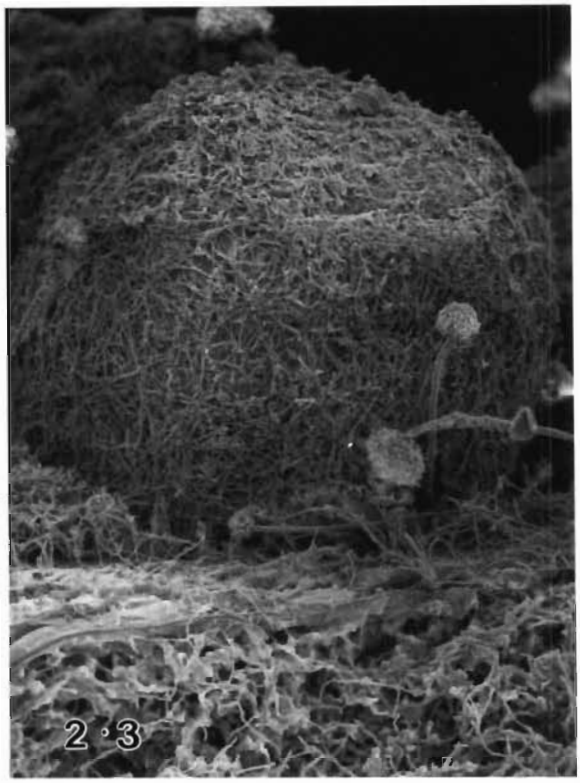
Figures 2.3 - 2.13 are all scanning electron micrographs of *A. flavus*.

Fig. 2.3 (x 200) A typical sclerotium on CDA. These structures were developed more abundantly on CDA and MEA than PDA.

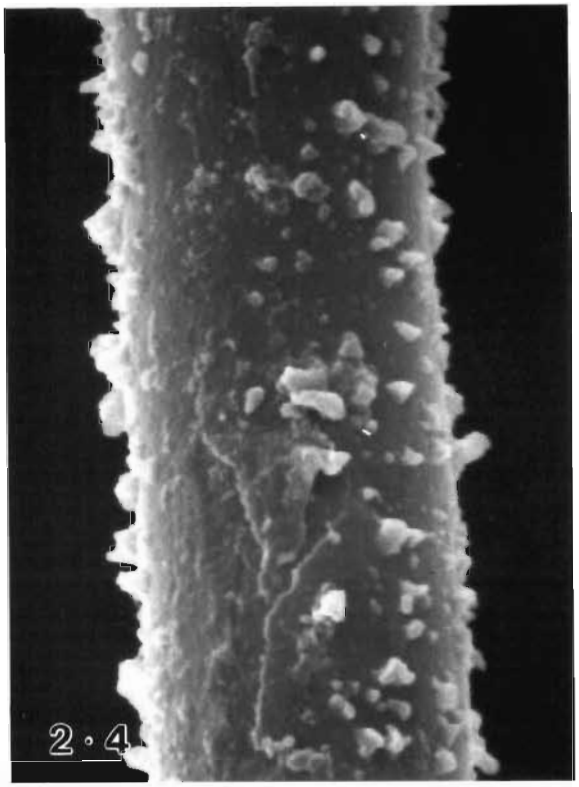
Fig. 2.4 (x 10 000) Verrucose decoration of the conidiophores of *A. flavus* was uniformly distributed along their length (CDA).

Fig. 2.5 (x 800) Bifurcation of the conidiophore allows for the development of two conidiogenous head (CDA).

Fig. 2.6 (x 6 250) Apical tip of the conidiophore (CDA).

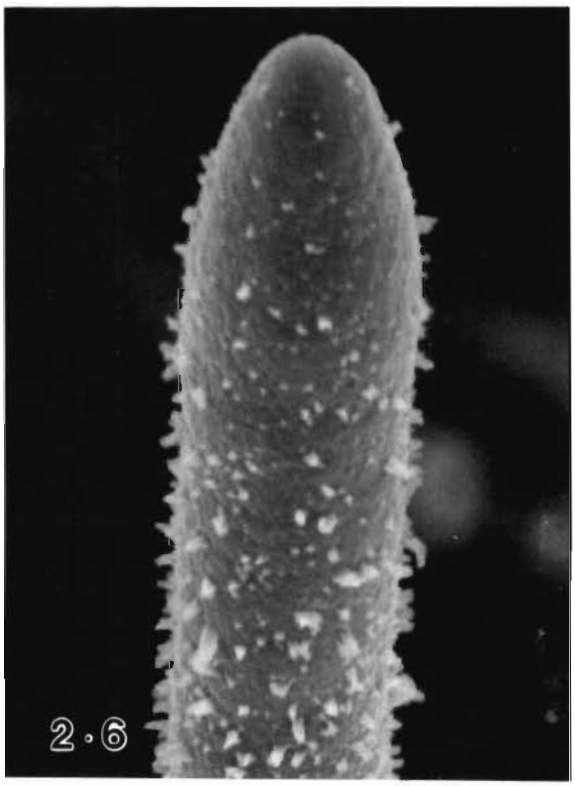


2·3



2·4

2·5



2·6

Figs 2.7 to 2.10 taken from CDA-derived colonies.

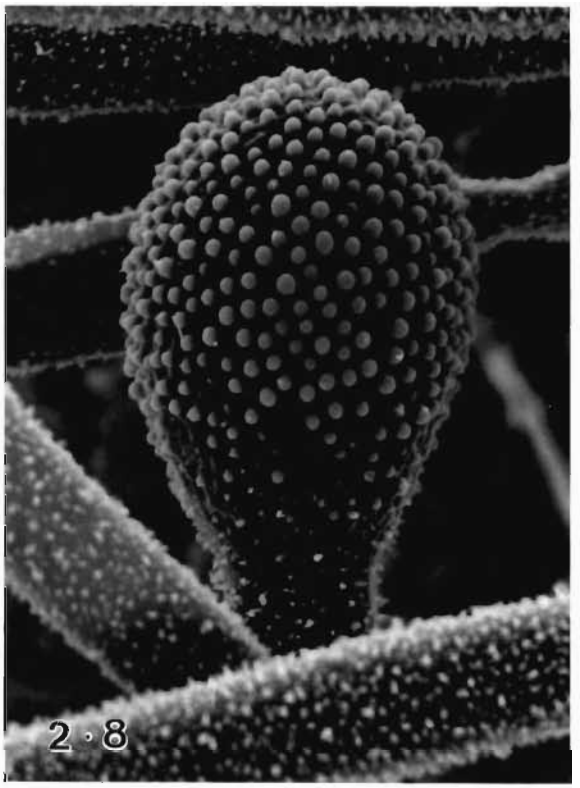
Fig. 2.7 (x 3 000) Sub-globose vesicle

Fig. 2.8 (x 3 000) Globose vesicle. Note the ornamentation of the vesicle surface.

Fig. 2.9 (x 2 600) Development of phialides was perpendicular to and over three quarters of the surface of the vesicle.

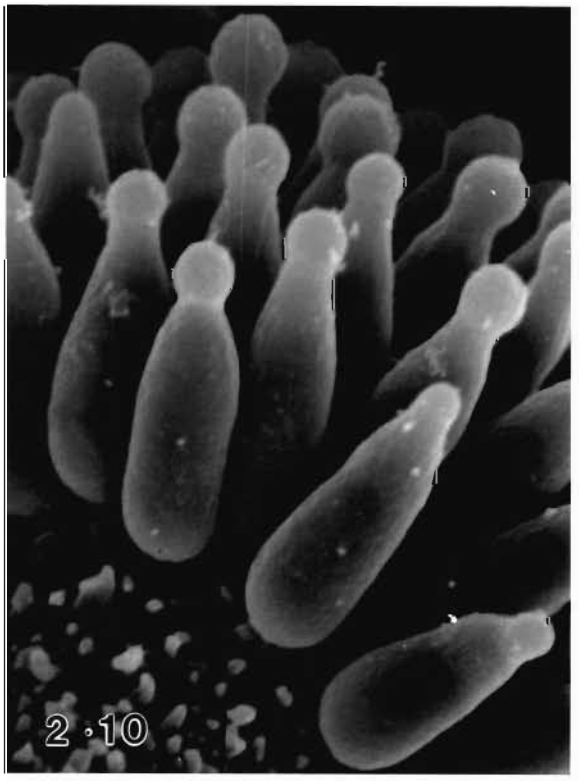
Fig. 2.10 (x 10 000) Smooth ampulliform phialides developed on the uniseriate conidiogenous heads.

2・7



2・8

2・9



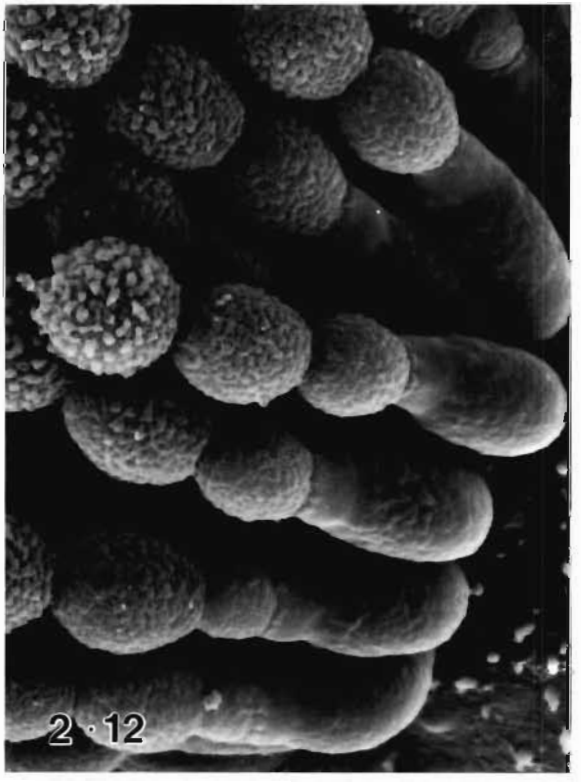
2・10

Fig. 2.11 (× 4 000) In the biseriate condition up to three phialides could form from each metula (CDA).

Fig. 2.12 (× 10 000) Blastic type formation of conidia from the phialides (uniseriate head) [CDA].

Fig. 2.13 (× 10 000) Mature seceded conidia. Note the variation in size (CDA).


2·11


2·12

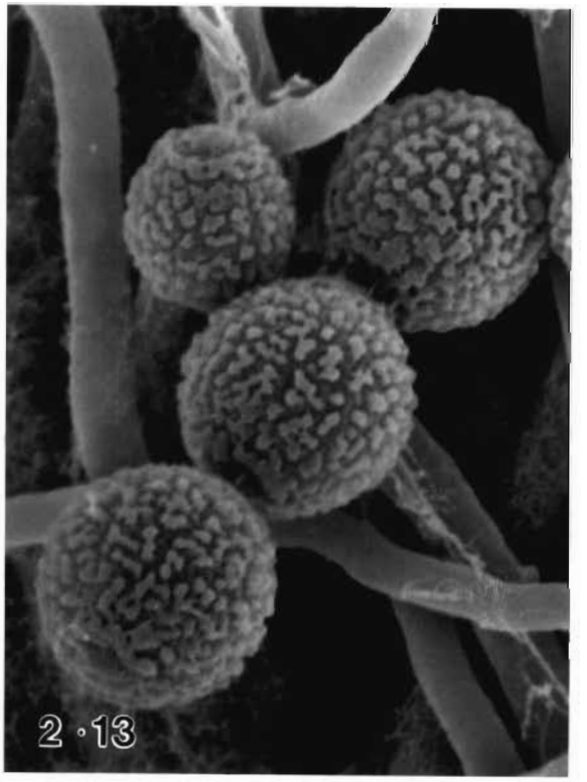

2·13

Table 2.1 Dimension ranges of the reproductive structures of A. flavus

Measurements in µm	Conidiophore Length	Conidiophore Width	Vesicle Width	Metula Length	Metula Width	Phialide Length	Phialide Width	Conidium Length	Conidium Width
PDA (SEM)	300-700	4-15.5	12-40	N.F.	N.F.	3-5.5	1.5-3.5	3.5-4.5	2.5-4.5
CDA (SEM) local	400-850	5-20	15-40	5-10	4-6	3-9	2-3.5	3.5-5.5	3.5-5
CDA (LM) local	400-850	5-20	18-40	5-10	4-6	3-10	2-4	3-5	3-5
CDA (LM) R+F	400-1000	5-15	25-45	6-10	4-5.5	6.5-10	3-5	4.5-5.5	3.5-4.5

R+F* Raper and Fennell (1977)

N.F. Not formed.

2:3:2 Aspergillus flavus Link var. columnaris Raper and Fennell (local strain and WB 4818)

Colonies of the local strain when grown on PDA (46 mm in 10 days [Fig. 2.14]) were a shade of green and remained so until very old, when they turned brown. There was a sparse production of a colourless, odourless exudate. The sulcate colonies were velutinous to intermediate. On CDA, growth was more restricted (38 mm in 10 days [Fig. 2.14]), and the green floccose colonies produced a copious colourless exudate. A yellow diffusible pigment was also produced. On MEA (60 mm in 10 days [Fig. 2.14]) an important feature of the green colonies was that every conidial head was distinctly columnar, whereas on PDA and CDA some radiate heads were encountered.

Colonies of WB 4818 grew more slowly on all three media (Fig. 2.15). As with colonies of the local strain, colonies of WB 4818 were sulcate, velutinous and green when grown on PDA. On CDA all the characteristics exhibited by the local strain were evident, except that colony colour was a darker green. The columnar nature of the sporogenous heads (the characteristic which gives the variety its name) was clearly evident when the colony was established on MEA (Fig. 2.16). As there were no differences in the shape of the structures developed by the two strains on the various media, the following description is for the variety.

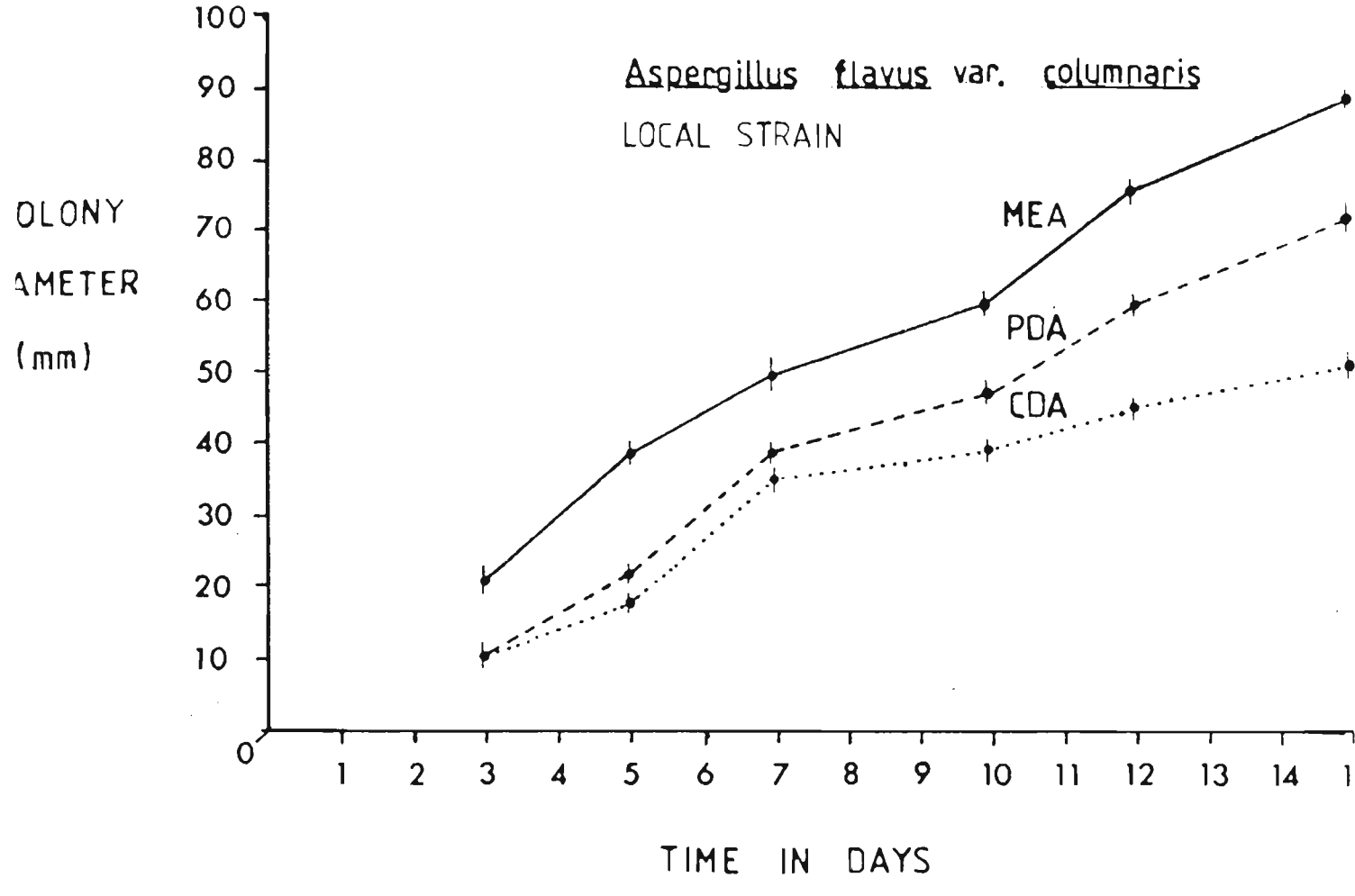
The conidiophores were increasingly ornamented away from the foot cell (Fig. 2.17), the latter being submerged in the agar or aerial. The tip of the conidiophore developed (Fig. 2.18) into a sub-globose vesicle (Fig. 2.19). The species was characteristically uniseriate with ampulliform to ovoid phialides developing over more than three quarters of the vesicle surface (Figs 2.20 and 2.21). As the conidia matured they became sub-globose to globose and ornamentation increased (Fig. 2.22). The mature heads were columnar (Figs 2.23 and 2.16).

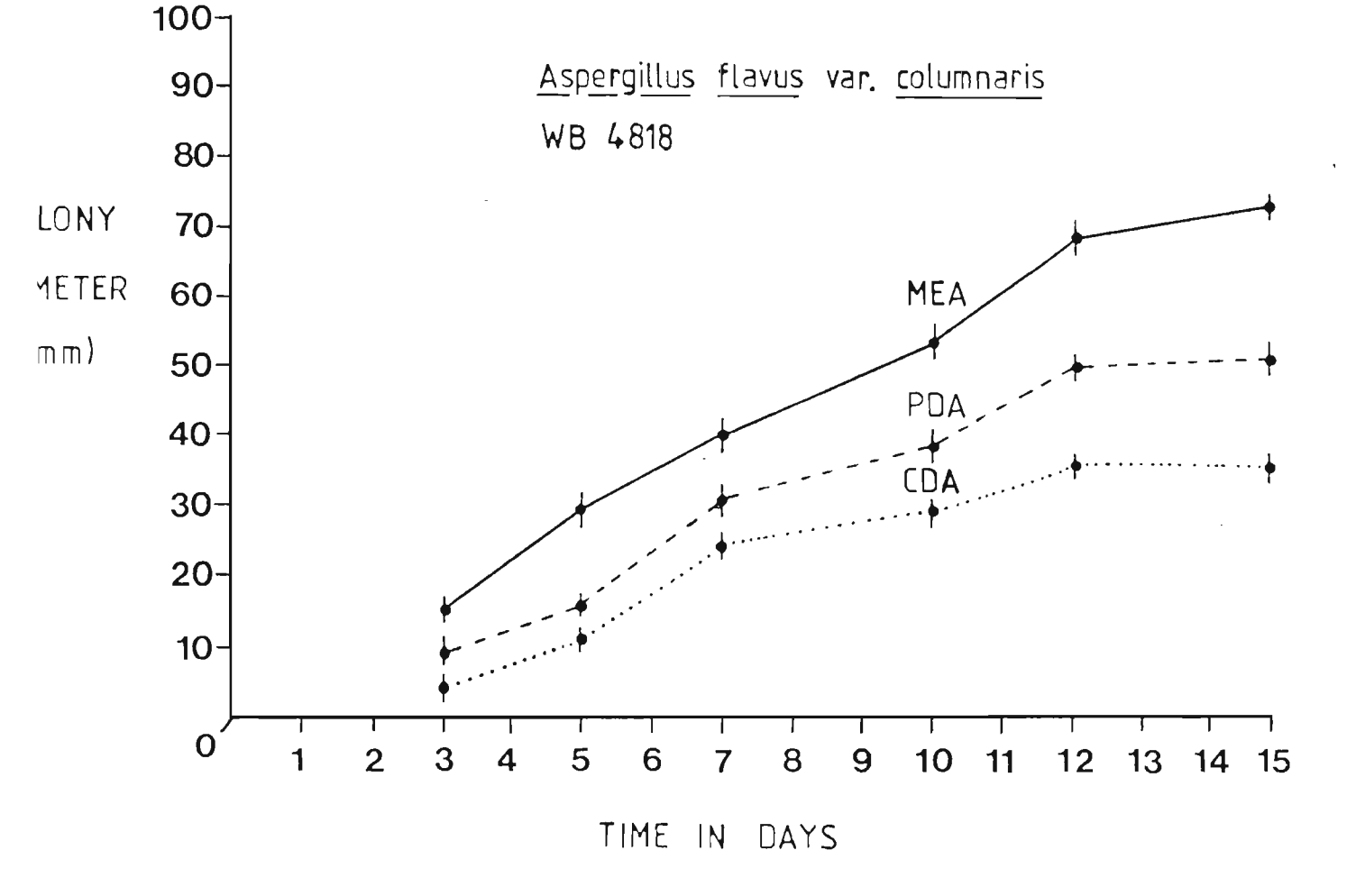
Structures developed in colonies grown on PDA were consistently smaller than those grown on CDA (Table 2.2). Of the colonies grown on CDA there was parity between all structures

except the length of the phialides, with the local strain having shorter phialides than the strain originally described by Raper and Fennell (1977). Scanning electron microscopy indicated that A. flavus var. columnaris WB 4818 has larger condia than previously described (Table 2.2). This discrepancy is probably a result of the limitations of light microscopy. When a three-dimensional structure on a slide is viewed with a light microscope haziness often occurs at the periphery of the structure and this is particularly true for small objects such as spores. This is because only part of the structure can be in focus in any one plane (so-called "Airey disc" by Meek, 1970). Such an effect must compromise the accuracy of measurements.

Fig. 2.14 Growth curves of the local strain of A. flavus var. columnaris.

Fig. 2.15 Growth curves of the key strain of A. flavus var. columnaris (WB 4818).
Bars indicate standard deviation.


Aspergillus flavus var. columnaris
LOCAL STRAIN
MEA
PDA
CDA
OLONY
AMETER
(mm)
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
TIME IN DAYS

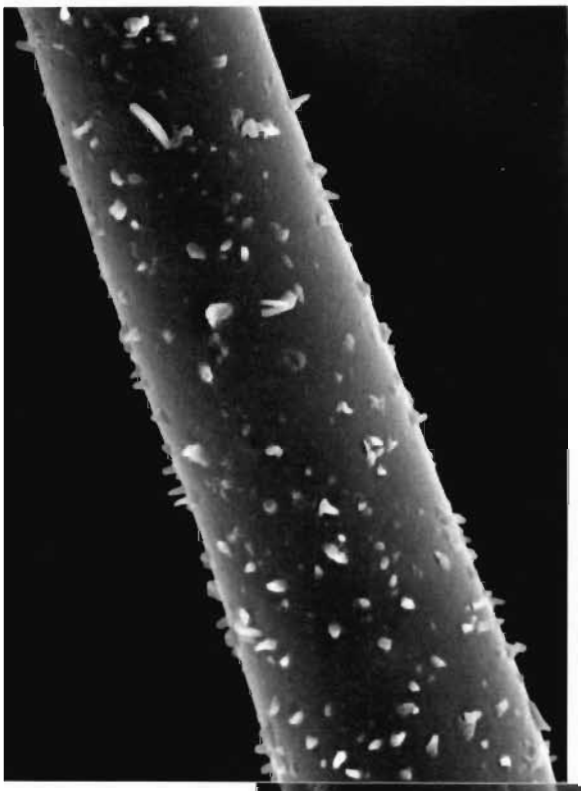

Aspergillus flavus var. columnaris
WB 4818
MEA
PDA
CDA
LONY
METER
mm)
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
TIME IN DAYS

Figures 2.16 - 2.23 are scanning electron micrographs of A. flavus var. columnaris.

Fig. 2.16 (× 1 000) The columnar nature of the conidiogenous heads give this variety of A. flavus its name.

Fig. 2.17 (× 6 000) Decoration increases along the length of the conidiophores of this variety.


2·16



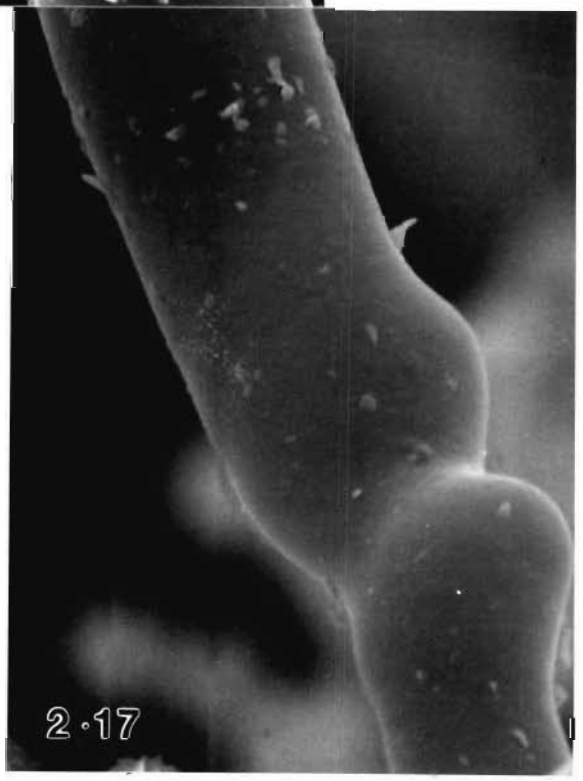

2·17

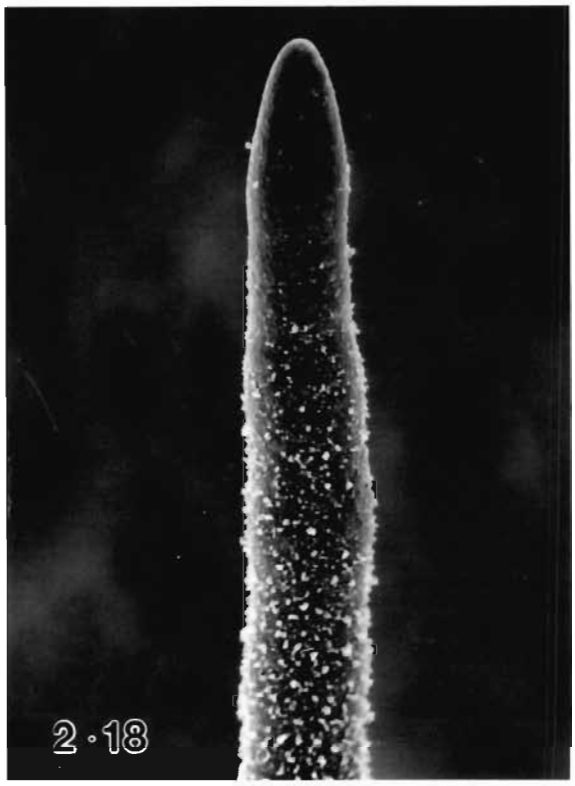
Fig. 2.18 to Fig. 2.21 taken from CDA colonies.

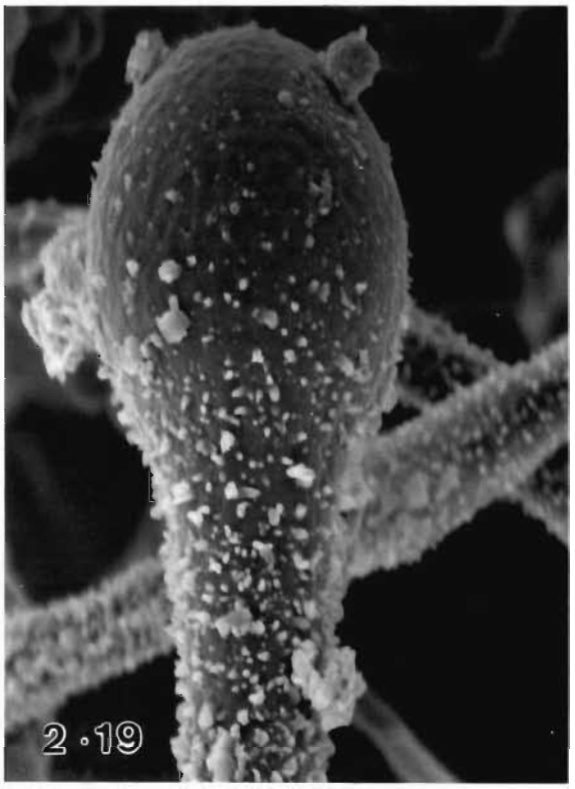
Fig. 2.18 (× 2 400) Tip of the conidiophore.

Fig. 2.19 (× 4 000) Sub-globose vesicle characteristic of this variety.

Fig. 2.20 (× 5 000) Phialide development from the vesicle surface.

Fig. 2.21 (× 8 000) Phialides when mature are smooth and ampulliform to ovoid.


2·18

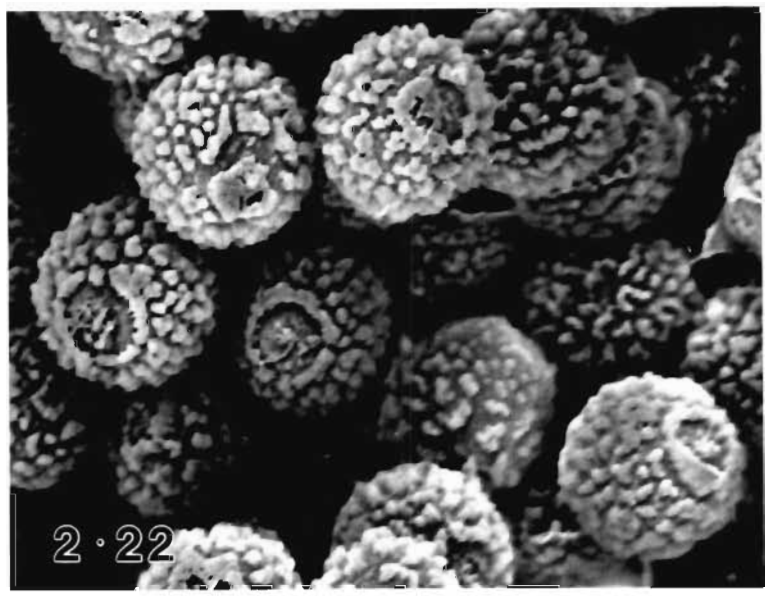

2·19


2·20


2·21

Fig. 2.22 (× 10 000) Mature globose conidia (CDA).

Fig. 2.23 (× 4 000) The columnar nature of the mature heads is achieved by the angling of the phialides (CDA).


2·22

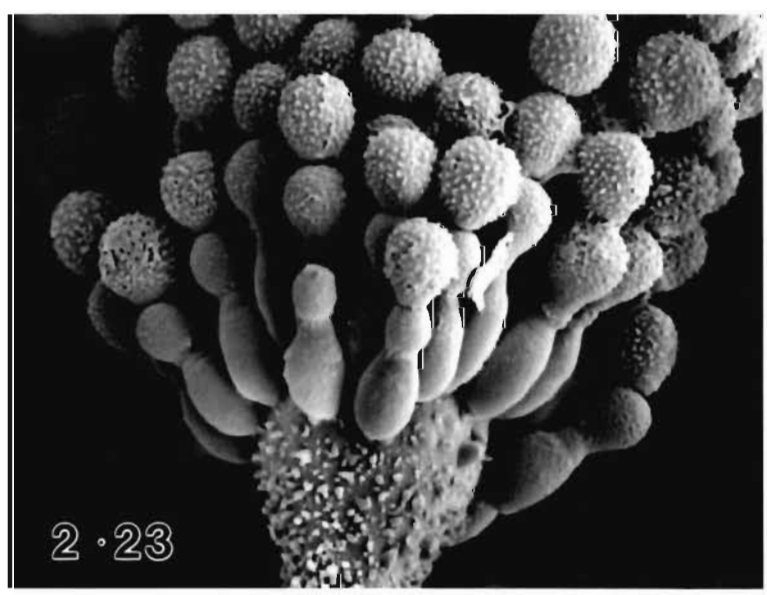

2·23

Table 2.2 Dimension ranges of the reproductive structures of the two isolates of *A. flavus* var. *columnaris*

Measurements in µm	Conidiophores Length	Conidiophores Width	Vesicle Width	Phialide Length	Phialide Width	Conidium Length	Conidium Width
PDA (SEM) local	250-600	4-5.5	11-30	2.5-6.3	1.5-3.7	2-5.3	1.5-4.2
CDA (SEM) local	250-600	5-12	10-35	3.5-8	3-4.5	3-6.5	2.5-5.5
CDA (SEM) WB 4818	140-600	5-12.5	11-35	4-12	2.5-4	4-6.5	3-6
CDA WB 4818 R+F*	500	not given	15-40	7-12	3.3-4.4	4.5-5.5	3.5-4.5

R+F* Raper and Fennell (1977)

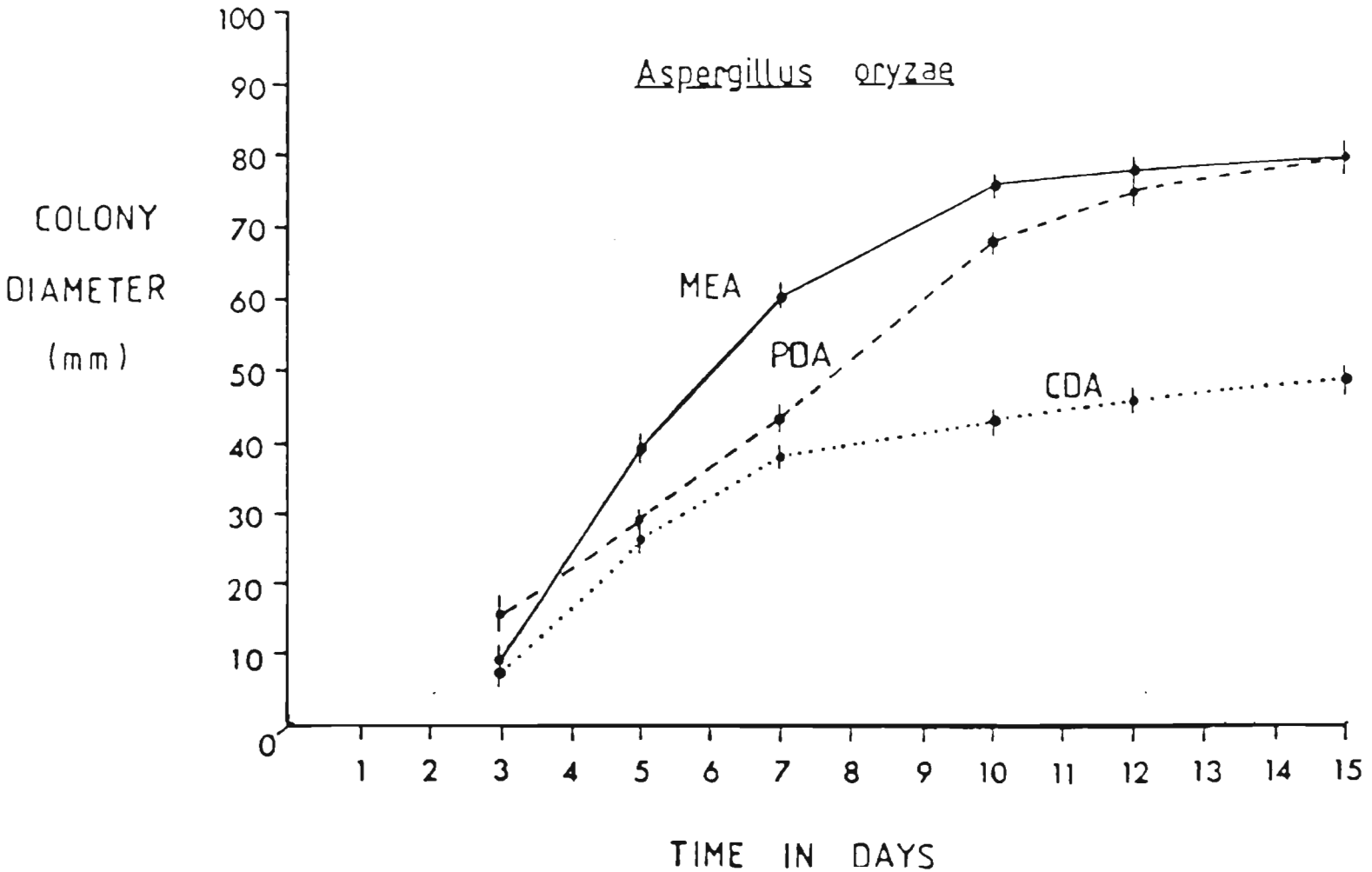
2:3:3 Aspergillus oryzae (Ahlb.) Cohn

Growth on CDA was restricted (Fig. 2.24) and adpressed (42 mm in 10 days), whereas the floccose colonies characteristic of PDA and MEA growth tended to be sulcate. On all three media colonies were white to Naples Yellow at first, becoming olive to deep green with maturation of the conidia, while very old colonies were brown. A copious yellow exudate was produced at all stages, irrespective of the medium.

The hyphae of A. oryzae occurred both aerially and submerged in the substrate. While conidiophores developed predominantly from foot cells which were submerged in the agar, some also developed from aerial foot cells (Fig. 2.25). The long conidiophores (Table 2.3) were increasingly ornamented along their length and the vesicles, when mature, varied in shape, being clavate, sub-globose or globose (Figs 2.26, 2.27 and 2.28) irrespective of the medium. The species formed both uniseriate and biseriate sporogenous heads on all the media, and occasionally both arrangements of conidiation developed on the same vesicle. The smooth, ovoid phialides of a uniseriate head developed over two-thirds to three-quarters of the vesicle surface (Fig. 2.29), while metulae in the biseriate condition were also smooth, but cylindrical. Two phialides developed asynchronously per metula (Figs 2.30 and 2.31). The conidia which developed from the phialides of both conidial-head types were initially smooth and eliptical (Fig. 2.29), becoming sub-globose to globose and ornamented with maturity (Fig. 2.32).

Size ranges of the reproductive structures of A. oryzae are given in Table 2.3. As with the other species, the structures from colonies grown on PDA were generally smaller than those from CDA colonies. In general, there was parity between the SEM-derived measurements from the CDA colonies and the dimension ranges given by Raper and Fennell (1977).

Fig. 2.24 Growth curves of A. oryzae on the three media. Bars indicate standard deviation.


Aspergillus oryzae
COLONY DIAMETER (mm)
100
90
80
70
60
50
40
30
20
10
0
MEA
PDA
CDA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
TIME IN DAYS

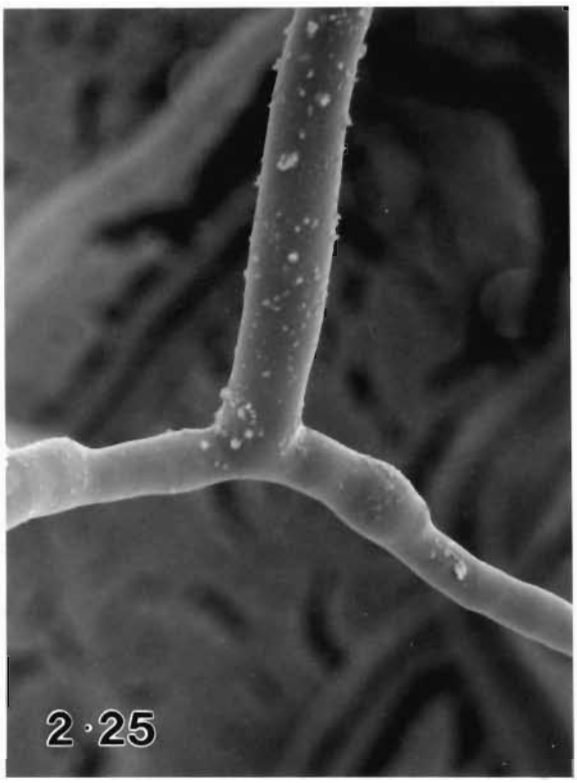
Figs 2.26 - 2.32 are of material of A. oryzae cultured on CDA

Fig. 2.25 (x 3 000) Aerial foot cells were commonly observed, when this species was grown on either PDA or MEA.

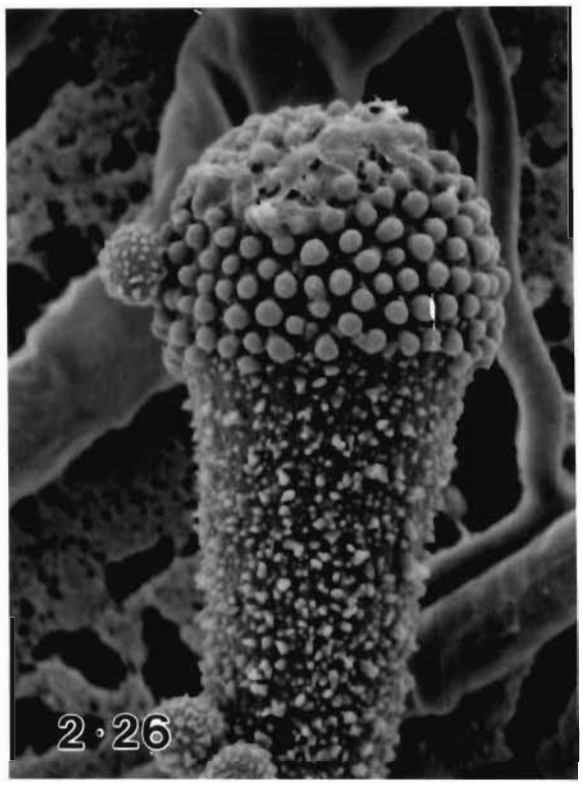
Fig. 2.26 (x 6 000) Clavate vesicle.

Fig. 2.27 (x 5 000) Sub-globose vesicle.

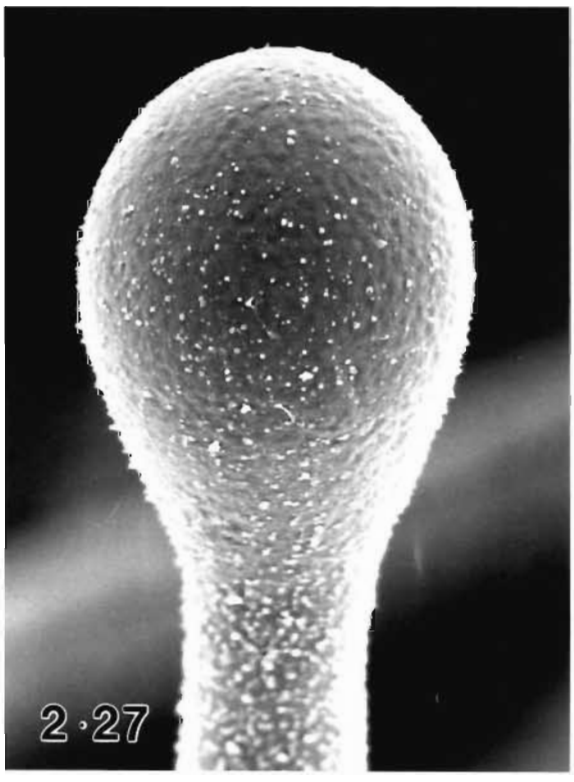
Fig. 2.28 (x 3 500) Globose vesicle.



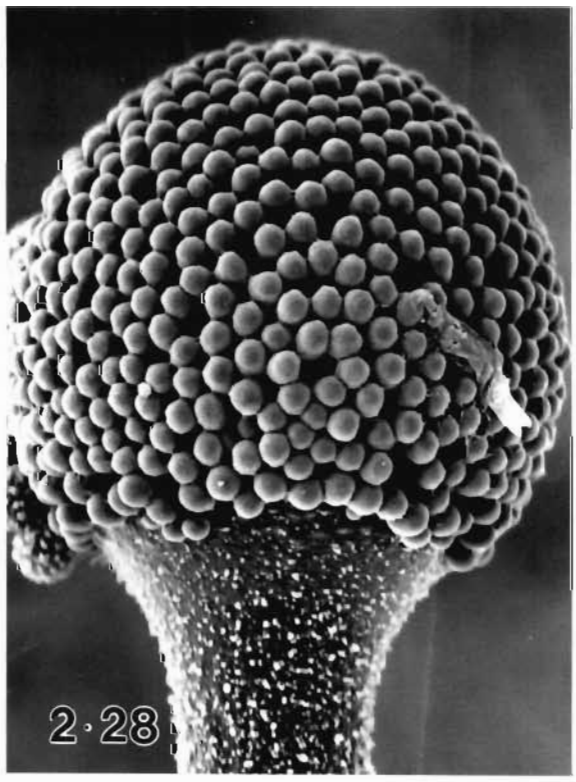
2·25



2·26



2·27



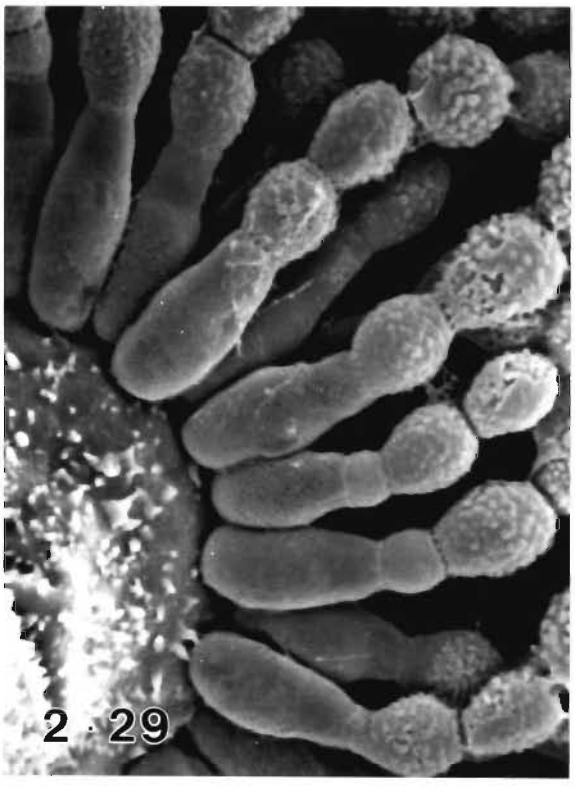
2·28

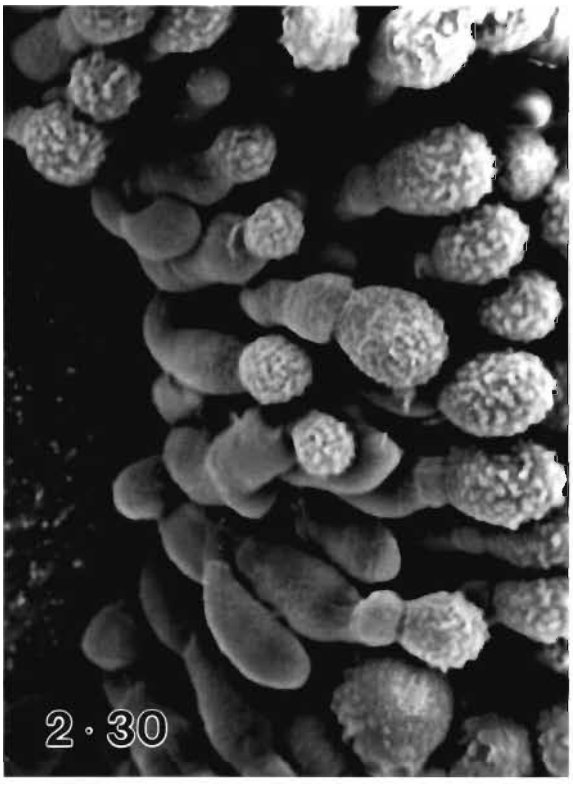
Fig. 2.29 (× 8 000) Ovoid phialides producing conidia.

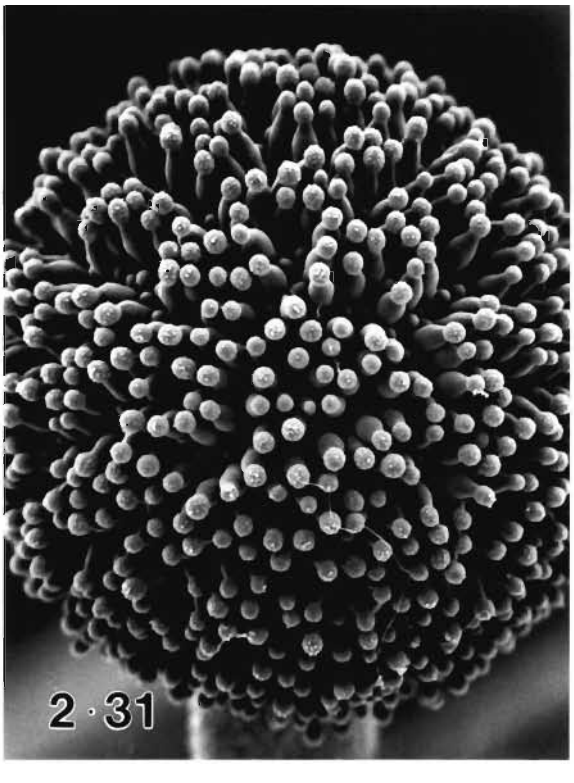
Fig. 2.30 (× 7 000) Phialide development from the metulae was asynchronous.

Fig. 2.31 (× 2 250) The primary phialides produced conidia before the secondary phialides were fully formed.

Fig. 2.32 (× 6 000) Mature globose conidia.


2 · 29


2 · 30


2 · 31


2 · 32

Table 2.3 Dimension ranges of the reproductive structures of *A. oryzae*

Measurements in µm	Conidiophore Length	Conidiophore Width	Vesicle Width	Metula Length	Metula Width	Phialide Length	Phialide Width	Conidium Length	Conidium Width
PDA (SEM) local	up to 2mm	8-24	17-35	-	-	4-7	2-4	3.5-6	3-5
CDA (SEM) local	up to 2.5mm	10-26	24-44	6-10	3-5	5-8	2-4	4-7	3-5
CDA (LM) R+F*	up to 2.5mm	12-25	40-50	8-12	4-5	8-10	3-3.5	4.5-7	not given

R+F* Raper and Fennell (1977).

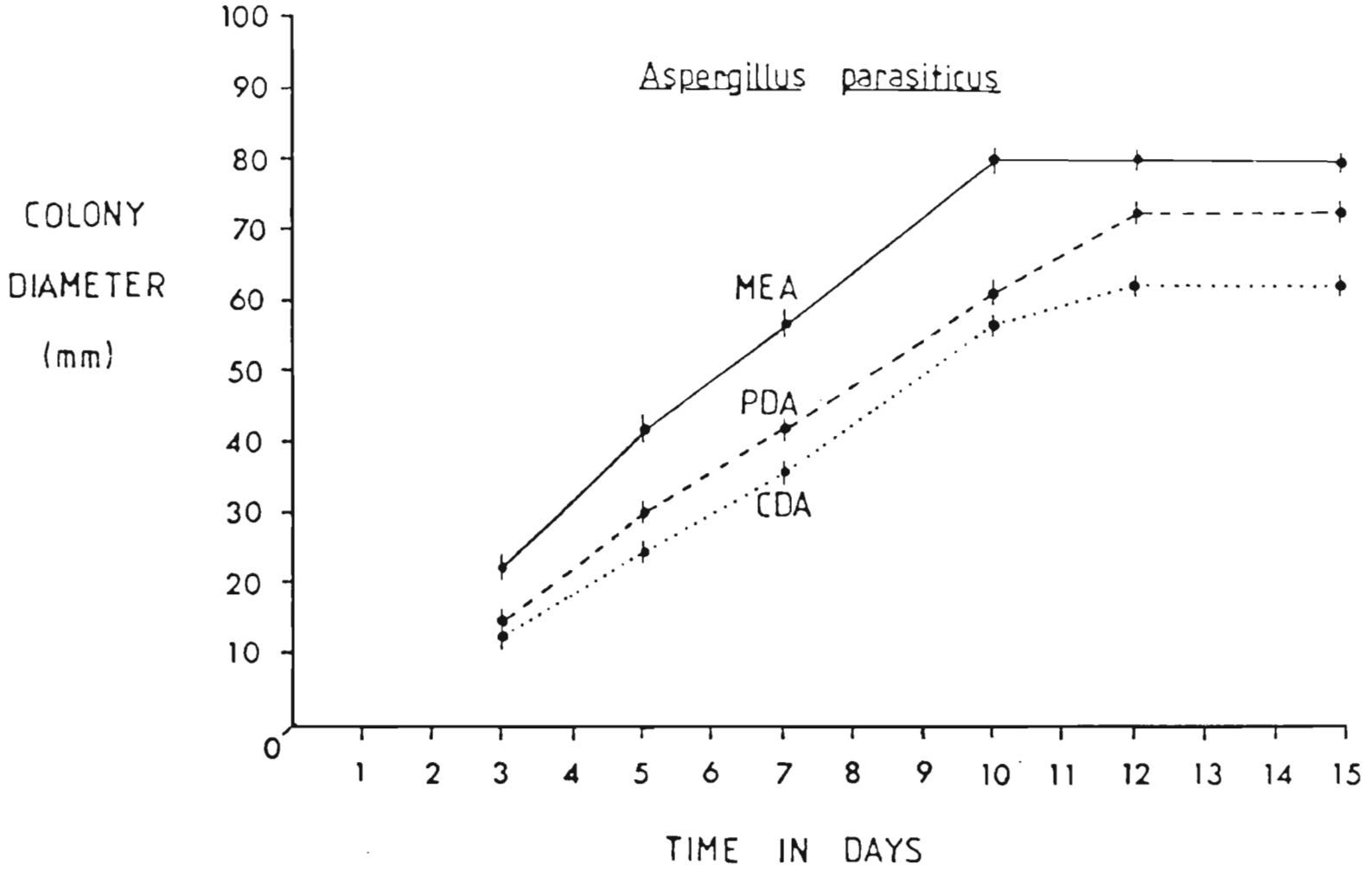
2:3:4 Aspergillus parasiticus Speare

Growth on MEA was rapid (80 mm in 10 days [Fig. 2.33]) with the mycelium being submerged. A copious, colourless, odourless exudate was produced. The velutinous, dark-green colonies turned brown with age (28 days). On CDA, growth was less adpressed but more restricted (56 mm in 10 days [Fig. 2.33]). As the conidia of the radiate conidial heads matured they passed from white through yellow to Cedar green. The floccose growth (60 mm in 10 days [Fig. 2.33]) characteristic of PDA colonies exhibited a similar colour transition.

The conidiophores which were uniformly decorated along their entire length, developed from foot cells that were either submerged in the agar or were aerial. The species was characteristically uniseriate, with the sub-globose to globose vesicles (Fig. 2.34) developing phialides over three-quarters of the surface (Fig. 2.35). The phialides were smooth and ovoid to ampulliform (Fig. 2.36). The verrucose conidia changed from ellipsoidal to sub-globose as they matured (Figs 2.37 and 2.38).

With the exception of vesicle width, the discrepancies between the size ranges of structures from PDA and CDA colonies were not as marked in A. parasiticus (Table 2.4) as for the other species. There was an excellent agreement between the size ranges obtained presently from CDA colonies and those given by Raper and Fennell (1977).

Fig. 2.33 Growth curves of A. parasiticus grown on CDA, MEA and PDA.
Bars indicate standard deviation.

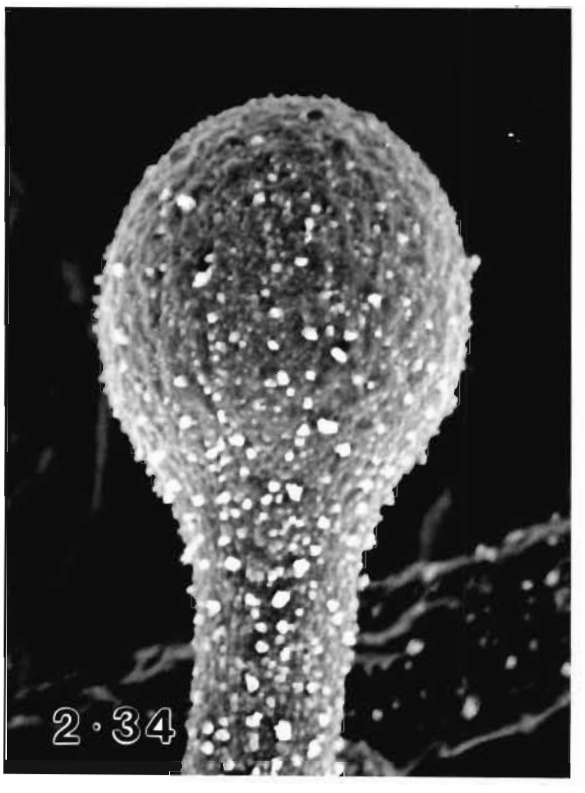

Aspergillus parasiticus
COLONY
DIAMETER
(mm)
100
90
80
70
60
50
40
30
20
10
0
MEA
PDA
CDA
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
TIME IN DAYS

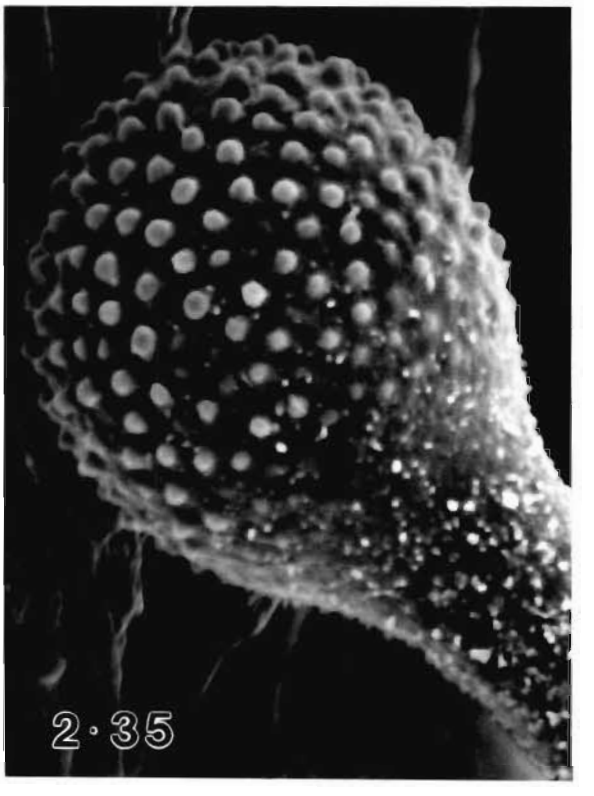
Figs 2.34 - 2.38 from colonies of A. parasiticus grown on CDA.

Fig. 2.34 (x 6 000) Mature vesicles of A. parasiticus were sub-globose.

Fig. 2.35 (x 6 000) Phialide initials developing over three quarters of the surface of the vesicle.

Fig. 2.36 (x 6 000) Mature ovoid phialides

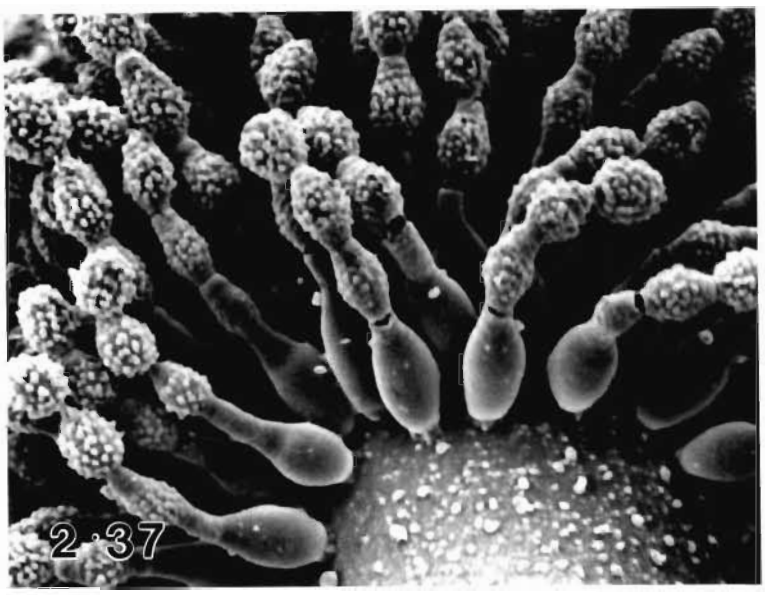

2·34


2·35

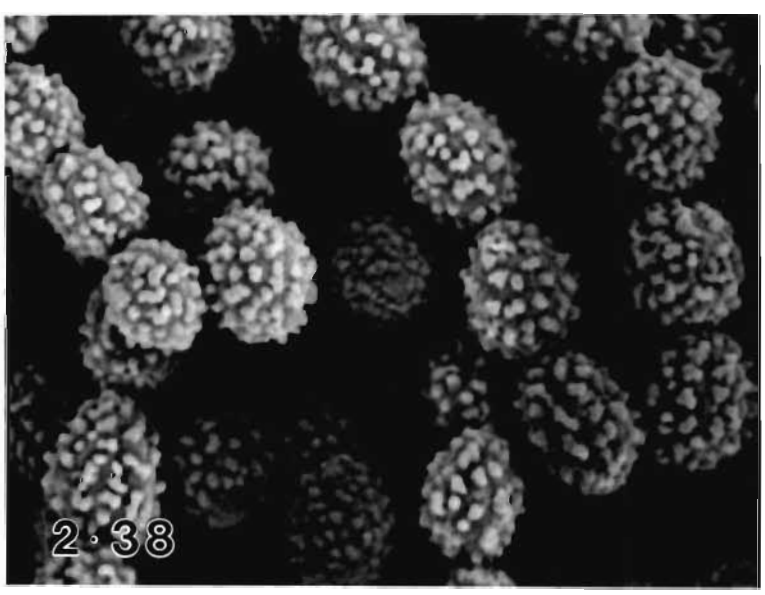

2·36

Fig. 2.37 (x 4 000) Conidial chains, formed by sequential cell wall formation.

Fig. 2.38 (x 8 000) Mature conidia.



2.37



2.38

Table 2.4 Dimension ranges of the reproductive structures of *A. parasiticus*

Measurements in µm	Conidiophore Length	Conidiophore Width	Vesicle Width	Phialide Length	Phialide Width	Conidium Length	Conidium Width
PDA (SEM) local	120-500	8-15	14-27	5-8	2.5-4	3.5-6	3.5-5
CDA (SEM) local	200-850	8-15	14-35	6-9	2.5-4	3-6	3-6
CDA (LM) R+F*	300-1000	10-12	20-35	7-9	3-4	not given	3.5-5.5

R+F* Raper and Fennell (1977).

The application of SEM technology to the description of fungal species has been both advocated and criticised (Smith, Anderson, Deans and Davis, 1977; Kozakiewics, 1982, 1989; King and Brown, 1983). The major criticism stems from the possibility that the methods used (particularly critical point drying) in the preparation of material for SEM can cause shrinkage and distortion of the fungal structures (King and Brown , 1983; Beckett, Read and Porter, 1984; Allan-Wojtas and Yang, 1987). However, Beckett and Read (1986) did make the point that the drying procedure for SEM can be modified to minimise undesirable changes. The present investigation has found that if fixation is complete, dehydration thorough and if the samples are not exposed to air after fixation, little or no shrinkage and distortion occurs. In regard to the latter point, a specimen holder designed by Evers, Robinson and Maistry (1983) was utilised. The construction of the holder allowed the simultaneous removal and addition of the consecutively used fluids, thus minimising exposure to air. The close agreement between the LM-derived size ranges of the various structures of A. flavus (Table 2.1) and those obtained using SEM, indicated that if: a) sample size is statistically valid (a minimum of 100 measurements), and b) precautions are taken in preparation for SEM, the criticism of use of critical point drying of fungi are not valid.

In contrast to such criticism, Kozakiewics (1982) working with A. parasiticus, proposed that SEM would be the most powerful tool available for differentiating between two morphologically similar fungi. That author, utilising SEM technology, has since produced a monograph on the aspergilli (Kozakiewics, 1989). However, the limitation of that work is that the SEM descriptions are based on air-dried conidia. The excellent descriptions of conidiogenesis in some of the aspergilli by Tokunaga, Tokunaga and Harada (1973) also emphasize the usefulness of SEM in fungal taxonomy. In the present study, the differences in conidial surface characteristics are clearly evident between the four A. flavus species (compare Figs 2.13, 2.22, 2.32 and 2.38). Such distinctions could not have been so unequivocally resolved at the LM level. Similarly, the species-specific characteristics of conidiophore

ornamentation are decisive using this microscopical mode. Christensen (1981) reported that this ornamentation could not be visualised using LM in two of the A. flavus group members. Since this feature is important in the identification of A. flavus, the advantage of the use of SEM is obvious.

However, despite the greater accuracy of fungal descriptions obtained using SEM, the usefulness of LM must not be ignored particularly in terms of its availability, cost, ease and rapidity with which observations can be made.

In summary therefore:

i. Although medium composition affected size of fungal structures it did not alter form.
ii. Fungal structures developed on CDA were generally larger than those on PDA colonies, the elevated salt content probably being the causal factor.
iii. SEM-derived measurements are more accurate and the resolution of this microscopical mode allows descriptions to be far more precise.
iv. For any one species there are no differences between size ranges obtained using LM and or SEM, thereby negating the criticism of shrinkage during SEM preparation.

2:4 References

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Chapter 3

Medium term storage of maize caryopses

3 Medium term storage of maize caryopses

3.1 Introduction

The length of time seed is in storage, and the type of facility used, depends on the requirements of the utilising society. In some countries tremendous effort and expense are invested in the conservation of plant diversity, and this can take the form of foundation seed stocks and germplasm banks (Roberts, 1972; Neergaard, 1977; Agarwal and Sinclair, 1987). In those seed stores the risk of loss through the action of pests (for example, insects) or micro-organisms is kept minimal by the application of insecticides and fungicides, and most effectively by storage at low relative humidities (RH) and temperatures (Neergaard, 1977; Justice and Bass, 1978). In shorter term stores, such as commercial and carryover seed stores, where some protection against rodents and insects is afforded, the RH and temperature are not as strictly controlled and, as a consequence, seeds in such stores are more vulnerable to infection by micro-organisms (Neergaard, 1977). The most susceptible seed store is that of the subsistence farmer who can provide little or no protection against the influence of the environment, rodents, insects and micro-organisms.

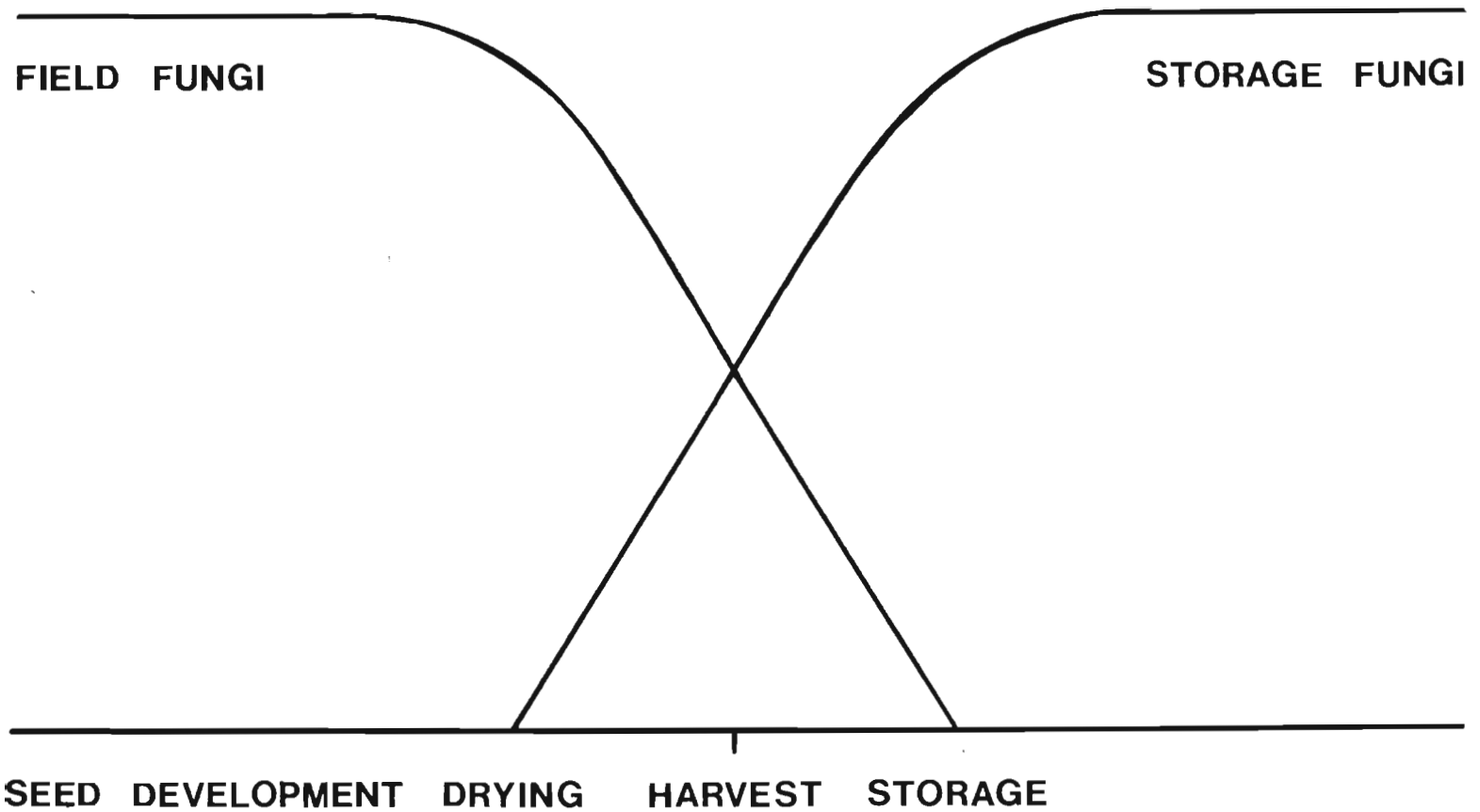
Even if the storage conditions are good, stored seeds will naturally age (Harrington, 1963; Roberts, 1972; Neergaard, 1977, Roberts, 1983). This process manifests itself as a narrowing of the environmental range over which the seed can germinate, reduction in vigour, and ultimately as loss of viability (Berjak and Villiers, 1972; Roberts, 1983). Additionally, genetic drift and the accumulation of mutations can cause the seed population to become different from that which was originally placed into storage (Christensen and Kaufmann, 1974). The time taken for any particular seed type to undergo these inevitable deteriorative changes is species-specific. On the other hand, if the storage conditions are, or become, conducive to the proliferation of micro-organisms, the natural consequences of storage become over-shadowed, and the ensuing microbial deterioration can be devastating.

At harvest there is a mixture of both field fungi (Alternaria, Curvularia, Epicoccum and Verticillium spp. amongst others) and storage fungi (xerotolerant spp. of Aspergillus and Penicillium) associated with the seed (Christensen and Kaufmann, 1969, 1974; Roberts, 1972; Agarwal and Sinclair, 1987). Pelhate (1979, 1981) has suggested that depending on the thermal gradient and moisture regime, an intermediate group may also exist, and proposed that this group comprises members of the genera Cladosporium, Rhizopus, Verticillium, Mucor and Trichoderma which Christensen and Kaufmann (1969, 1974) would group as field fungi.

Concomitant with the decline in seed moisture content after harvest, and with increasing time in storage, there is a decline in the incidence of the field fungi and intermediate fungi and an increase in that of the storage fungi (Christensen and Kaufmann, 1969, 1974; Pelhate, 1979, 1981).

Thus there is an overlap between the groups (Fig. 3.1). Christensen and Kaufmann (1969, 1974) proposed that during the first few months of storage the activity of the field fungi can continue, but ultimately, due to the lowered moisture content and to the storage conditions, they are unable to perpetuate and eventually die out (Fig. 3.1). On the other hand, the ubiquitous spores of the storage fungi are able to germinate and establish on the surfaces of the naturally drying seed whilst it is still on the parent plant. Their activity, however, is kept minimal by the vigorous field fungi, and it is only when the seed is in storage that they come to dominate the micro-environment of the seed (Fig. 3.1). Once this transition has occurred, and if storage conditions become suitable for their growth, a succession of first Aspergillus and then Penicillium species can spoil the grain. The temperature and relative humidity required by each species of the seed storage fungi is specific, although the general range is 70 - 90% RH and temperatures in excess of 25°C (Christensen and Kaufmann, 1969, 1974; Neergaard, 1977; Roberts, 1983; Agarwal and Sinclair, 1987). The first storage fungi to appear are those of the

Fig. 3.1 A schematic diagram of the transition from field to storage fungi during seed development, maturation drying and processing into storage, based on the proposals of Christensen and Kaufmann (1974).


FIELD FUNGI
STORAGE FUNGI
SEED DEVELOPMENT
DRYING
HARVEST
STORAGE

Aspergillus restrictus and A. glaucus groups (the former have never been isolated in South Africa [Roux pers. comm.[1]] The A. glaucus species
invade seed at a moisture content (mc) between 14 and 14.5% (Christensen and Kaufmann, 1974) and cause mustiness of the grain and discolouration of the germ layers. These species are slow growing and generally do not cause localised increases in seed temperature, consequently their presence usually goes undetected. However, they are capable of elevating the seed moisture content so that the next Aspergillus species in the succession, the A. versicolor and A. ochraceus species, can invade. The latter species infect seeds with moisture contents of 15.0 to 15.5%, and can rapidly discolour the germ layers and reduce viability (Christensen and Kaufmann, 1974). Their rapid growth can produce localised increases in temperature and these 'hot spots', as they are termed, can be detected using electrical probes, thus allowing for remedial action (Waterer, Muir and Sinha, 1985). As with the A. glaucus species, the A. versicolor and A. ochraceus species elevate the seed moisture content so that the A. candidus group members can infest the seed tissues 15.0 - 15.5% mc. These fungi grow extremely rapidly and can reduce seed viability substantially. The A. candidus species are out-competed by the least xerotolerant aspergilli the A. flavus group species, which can dominate seeds with moisture contents in excess of 18%. The activity of this group of seed storage fungi can be devastating, and has been known to elevate grain temperature to 130 °F (C 55 °C) (Christensen and Kaufmann, 1974). Members of the penicillia then take over the degradative process.

This chapter details some of the changes, in viability, moisture content and fungal status in two batches of maize seed (one white and one yellow) over a period of years.

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3:2 Materials and Methods.

3:2:1 Seeds and Seed Storage

Caryopses (seeds) of Zea mays L. var Hickory King [a white grain] were obtained in September 1984 from the Pioneer Seed Company, Greytown, Natal, South Africa. These seeds had been in 4 °C storage for one year prior to receipt. The yellow-grain maize (of unknown variety) was received immediately after harvest from the same company in May 1986. The seeds were stored in sterile, hermetically sealed glass jars, at the moisture content at which they were received (10.5% and 9.6% respectively) and at 4 °C ± 2 °C.

3:2:2 Moisture Content

Immediately after receipt and at yearly intervals for Hickory King and at six-monthly intervals for the yellow maize, moisture content (mc) of 25 seeds was determined on a wet mass basis, as follows

i. Wet mass determined (WM)

ii. The seed dried in an oven at 80 °C until a constant mass was achieved.

iii. Dry mass determined (DM)

iv. Moisture content calculated using the following equation

$$mc = \frac{WM - DM}{WM} \times 100$$

3:2:3 Germination Studies

At each sampling, 100 seeds were set to germinate under sterile conditions. Radicle length (divided into two classes: less and greater than 5 mm) and total germination were determined every 24 h for 96 h. The germination index (Czabator, 1962) was calculated as follows

$$GI = MDG \times PV$$

where

GI = Germination index

MDG = Mean daily germination (final percent germination divided by the length of the test period)

PV = Peak value (percent germination on a particular day divided by the number of days taken to achieve that percentage)

The seeds, whether germinated or not, were then planted in sterile vermiculite. The dry mass of the aerial portions of the plants was determined after 14 days.

3:2:4 Infection Studies

3:2:4:1 Surface-sterilisation

At each sampling, 100 seeds were surface-sterilised in a 2% solution of sodium hypochlorite and 1% sodium dodecyl sulphate for 20 min, then rinsed three times with sterile distilled water and tested for internal infection.

3:2:4:2 Internal infection

Surface-sterilised seeds were halved longitudinally through the embryo and aseptically plated onto potato dextrose agar containing 6% NaCl. The plates were incubated at 25 °C, and any fungal growth identified and quantified on a species basis.

3:3 Results and Discussion

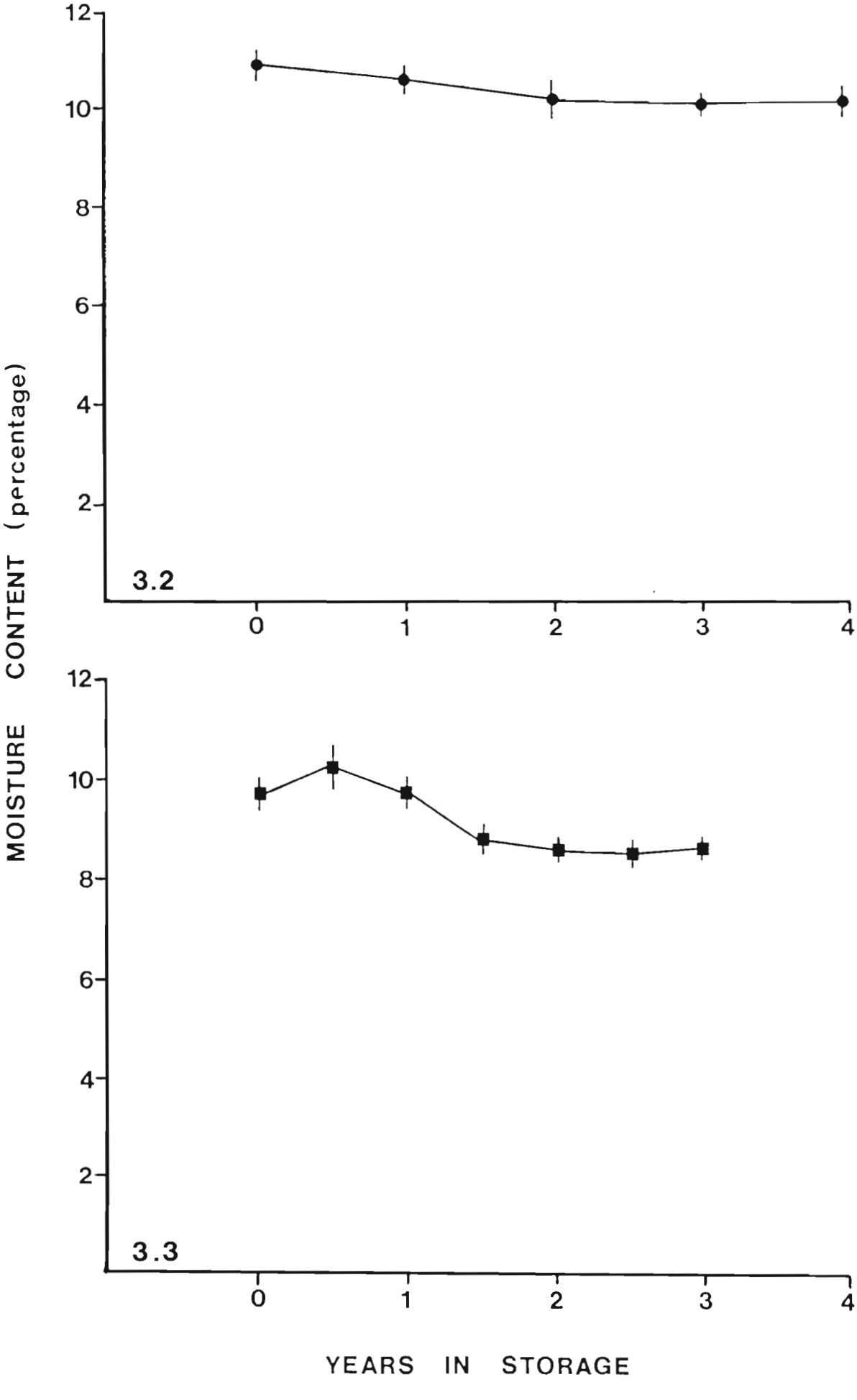
The storage of a non-dormant seed for any period longer than that for which it is naturally geared is an artificial situation. Since most human related seed storage is over protracted periods, seeds deteriorate in storage. The two environmental factors which influence the rate at which a seed will age and which control the activity of micro-organisms, are temperature and relative humidity (Neergaard, 1977; Roberts, 1983; Agarwal and Sinclair, 1987).

The moisture content of each of the batches of seed taken from hermetic storage was representative of an equilibrium reached between the seeds and the storage environment. In both seed batches the initial moisture content was below 11% and this declined with increasing time in storage (Figs 3.2 and 3.3). As the seeds were also stored at 4 °C, the storage conditions were taken as good and presumed to preclude any active fungal growth and to minimise natural ageing (Neergaard, 1977; Roberts, 1983; Agarwal and Sinclair, 1987).

Accelerated and artificial ageing is a process used by seed technologists to predict the longevity of a seed batch (Justice and Bass, 1978). Berjak, Dini and Gevers (1986) have proposed that the changes associated with both natural and artificial ageing (at moderate seed mc and storage temperature) are the same and are initiated by free radicals. At the ultrastructural level the free radicals are proposed to damage most sub-cellular structures, but particularly the nucleus, intracellular membranes and the mitochondria. Those authors proposed that because of their complexity, these particular organelles are capable of tenaciously binding more water than other cellular components. Consequently, because of their elevated moisture content relative to the other sub-cellular structures, they are more susceptible to free radical damage.

Fig. 3.2 Seed moisture content (average of 25 seeds) of the white maize caryopses over the five year experimental period. Bars indicate standard deviation.

Fig. 3.3 Seed moisture content (average of 25 seeds) of the yellow maize caryopses over the three year experimental period. Bars indicate standard deviation.


MOISTURE CONTENT (percentage)
12
10
8
6
4
2
3.2
0
1
2
3
4
12
10
8
6
4
2
3.3
0
1
2
3
4
YEARS IN STORAGE

Whether the cellular damage is brought about by free radical action on one or all of these subcellular constituents is still contentious (Berjak et al., 1986). It may be that the primary site of damage is the membranes, and in particular, the lipid components of these structures. One of the ultrastructurally visible effects of ageing is the coalescence of lipid droplets (Berjak et al., 1986; Samuel, Berjak and Lamb, 1986; Smith, 1989) and many believe that, by following the changes in isolated lipids, a measure of the extent of damage can be determined (Pommeranz, 1974; Harman and Mattick, 1976; Priestley and Leopold, 1979, 1983; Powell and Mathews, 1981). Hailstones and Smith (1989) have correlated the liberation of volatile aldehydes, thought to be generated by lipid peroxidation, with the loss of seed viability in soybean.

The relative activity of mitochondrial enzymes, such as succinic dehydrogenase and cytochrome oxidase have also been suggested as being indicators of ageing (Berjak et al., 1986; Samuel, Lamb and Berjak, 1986).

Considerable information exists on the effects of long term storage and of artificial ageing on the nuclear material. Damage of this sort can not only affect seed germination but can also reduce the vigour of the developing plant (Cheah and Osborne, 1978; Piech and Supryn, 1979; Osborne, 1980; Banerjee, Choudhuri and Ghosh, 1981).

Regardless of the form of damage, and despite the presence of certain natural anti-oxidants (e.g. the tocopherols) which can reduce the level of injury on imbibition, an aged seed seemingly must undergo a period of repair before germination can commence (Berjak and Villiers, 1972; Priestley, McBride and Leopold, 1980). If, however, the accumulated damage is too great, vigour and viability are affected and in extreme cases lost.

Even though the storage conditions in the present experiments were good, the consequences of protracted storage were apparent in both seed batches. The decline in the germination index of the

var. Hickory King (Fig. 3.4a) was indicative of a drop in both seed vigour and germination totality. The effects of storage on both these parameters were corroborated by the increase in the time required for the onset of germination (decline in rate) and by the decline in the total number of seeds germinating (Figs 3.4b and c). The vigour of the plants produced from these stored seeds (as expressed in terms of dry mass) also declined (Fig. 3.5).

The drop in germination index of the yellow maize was not as marked as that of the white maize (Fig. 3.6a), although comparisons of Figs 3.6b and c revealed that there was an increase in the time required for the onset of germination (that is a decline in rate) and a reduction in germination totality. However, the effects of storage on this seed batch were most apparent in the plants they produced (Fig. 3.7), there being a decline in plant dry mass with increasing storage time. This reduction in plant dry mass indicated that storage of this seed batch had more effect on vigour than viability.

With increasing storage time there was a lag in the onset of germination in both batches, due to the necessity for repair mechanisms to become operational during imbibition. The drop in the total number of seeds germinating can be ascribed, at least partly, to the inability of some of the seeds to repair extensive damage. Further, the decline in the dry mass of the plants developed from these naturally aged seeds suggested that processes additional to those involved in germination might also have been affected during storage. While a persistently debilitated condition of the resultant plants would have suggested damage at the intracellular control (genome) level, this cannot be proposed as unequivocal, as the seedlings were harvested only 18 days after the start of imbibition.

Despite the low seed moisture content and the low storage temperature there was a change in the composition of the mycoflora associated with both batches of seed during the experimental period (Figs 3.8 and 3.9). In accordance with the observations of Christensen and Kaufmann (1969, 1974), there was a decline in the

Fig. 3.4 Germination index (a), germination rate [PV] (b), and germination totality (c) of the var. Hickory King as a function of storage time.

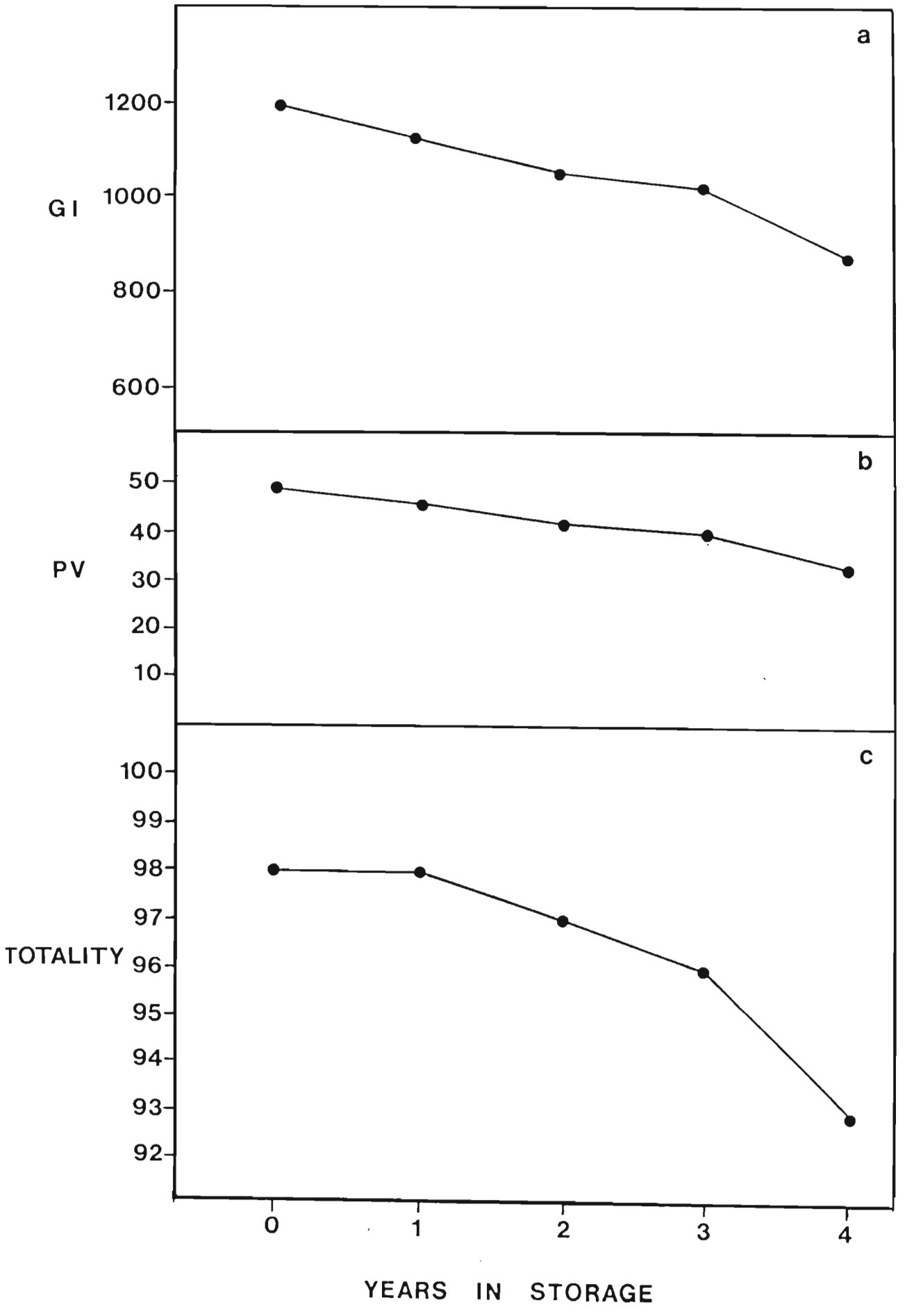

a
1200
1000
800
600
GI
b
50
40
30
20
10
PV
c
100
99
98
97
96
95
94
93
92
TOTALITY
0
1
2
3
4
YEARS IN STORAGE

Fig. 3.5 Average dry mass of the aerial portions of plants produced from the stored white maize seeds. Bars indicate standard deviation.

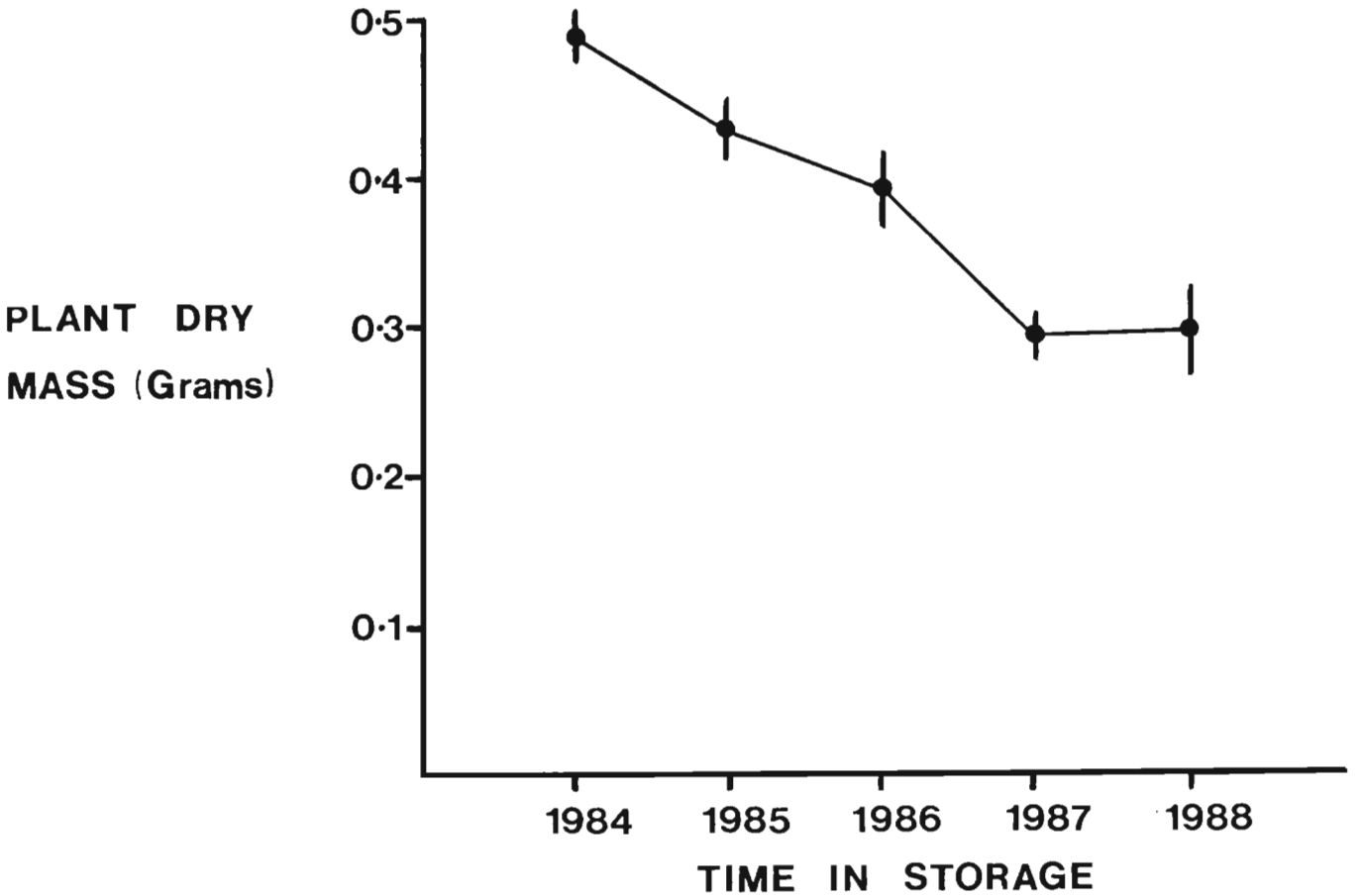

PLANT DRY
MASS (Grams)
0·5
0·4
0·3
0·2
0·1
1984
1985
1986
1987
1988
TIME IN STORAGE

Fig. 3.6 Germination index (a), germination rate [PV] (b), and germination totality (c), of the yellow maize caryopses as a function of storage time.

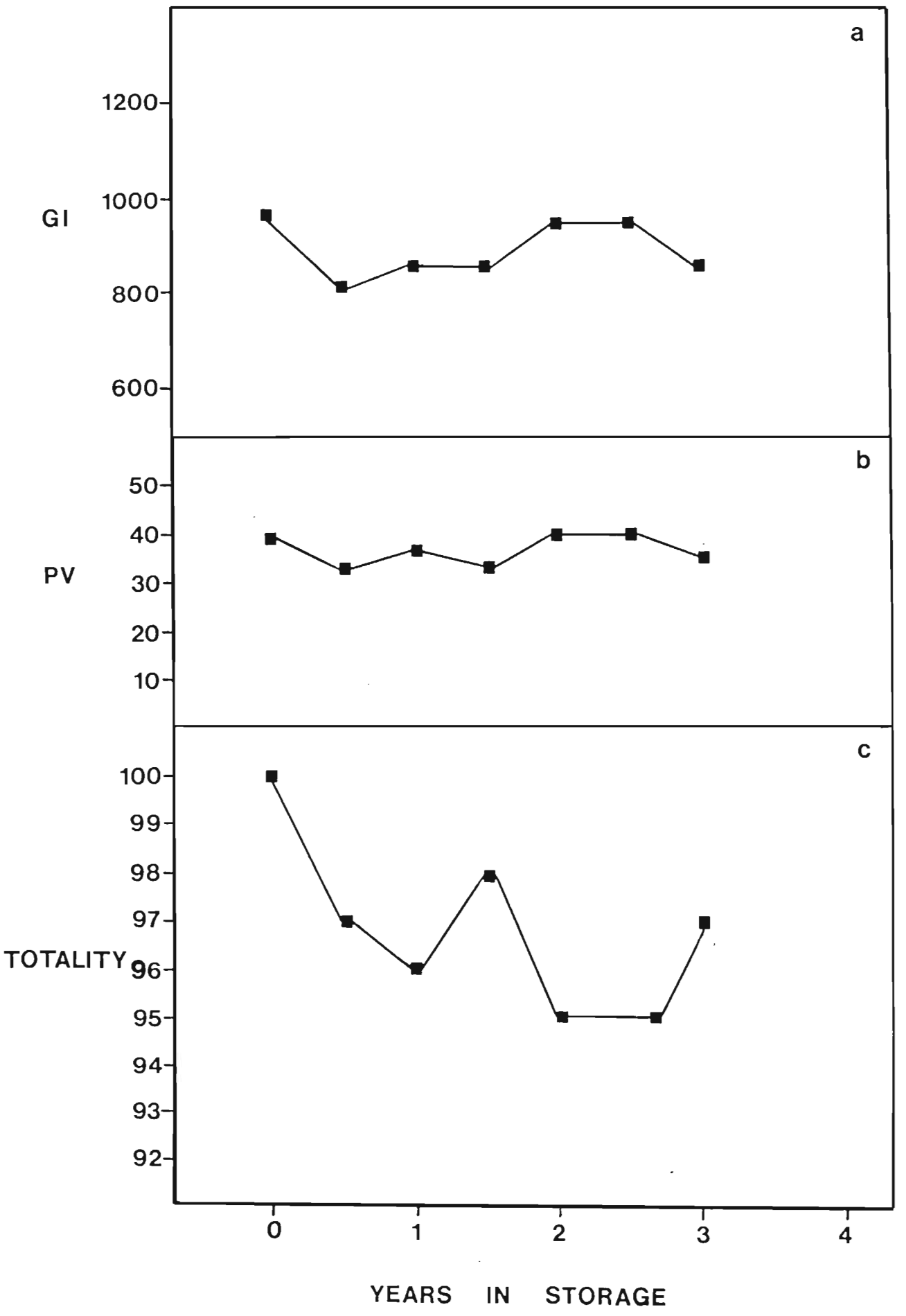
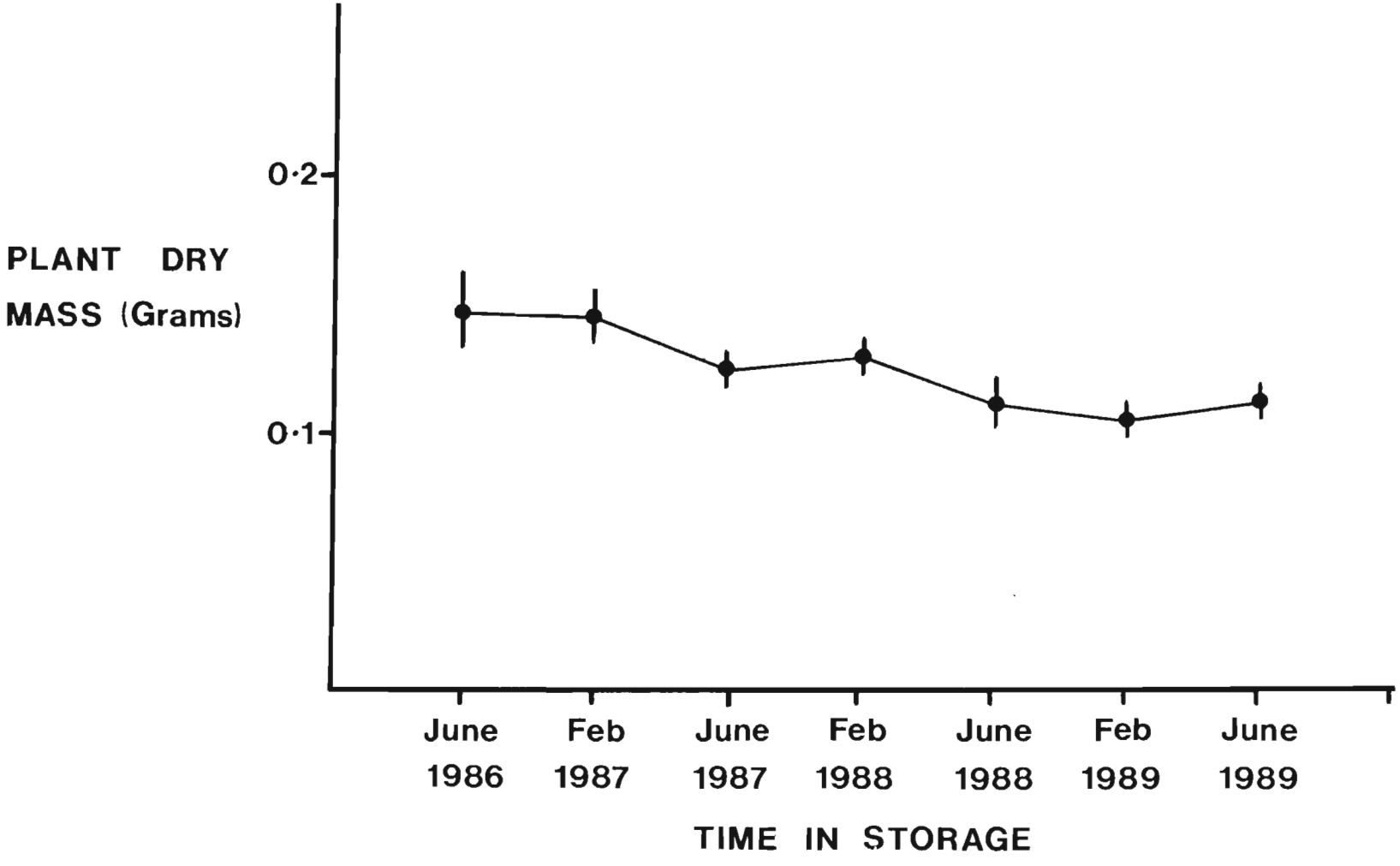

a
1200
1000
GI
800
600
b
50
40
PV
30
20
10
c
100
99
98
97
TOTALITY
96
95
94
93
92
0
1
2
3
4
YEARS IN STORAGE

Fig. 3.7 Average dry mass of the aerial portions of plants produced from the stored yellow maize seeds. Bars indicate standard deviation.


PLANT DRY MASS (Grams)
0·2
0·1
June 1986
Feb 1987
June 1987
Feb 1988
June 1988
Feb 1989
June 1989
TIME IN STORAGE

incidence of the field fungi as typified by Fusarium spp. associated with the yellow maize and an increase in that of the storage fungi (Fig. 3.9). In the white maize (Fig. 3.8) the decline of the field fungi was not evident although there was some increase in the levels of Aspergillus species. This may reflect differences in the longevity of the Fusarium propagules associated with the seeds, or differences in the resistances of the seed. Maize varieties are known to differ in their resistance to fungal pathogens (Agarwal and Sinclair, 1987; Tucker, Trevathan, King and Scott, 1986).

The storage conditions under which the seeds were stored presumably precluded the active growth of fungi (low temperature, and RH in equilibrium with the seed moisture content) and storage was under sterile conditions. It was therefore assumed that the fungal propagules were associated with the tissues when the seeds were placed into storage and were probably in a dormant form. Therefore they were detectable only by plating the surface-sterilised bisected seeds, onto a nutrient medium. Even though the type nor the form of these propagules was known, the results from the yellow maize suggested that those of the storage fungi were better suited to survive the particular storage conditions than those of the field fungi, the latter declining with increasing time. Even in the case of the white maize, the increasing incidence of isolation of the aspergilli with time, suggested a decline in the aggressiveness (vigour) of the fusaria.

The apparent increase in the isolation of the Aspergillus species implied their proliferation, but under the storage conditions used this was unlikely. At the beginning of the experiment, the Fusarium propagules, associated with the yellow maize, were still vigorous. It may be proposed that on plating the half seeds onto nutrient medium, their germination in some way suppressed that of the Aspergillus propagules. As storage time increased and the Fusarium propagules progressively lost vigour and viability, development of the Aspergillus propagules was no longer suppressed and consequently these species could come to predominate. In the yellow maize this transition from one

dominant fungal group to another took two and a half years, whilst in the white maize the transition is suggested already to have occurred when the seeds were received (this seed batch had been in storage for one year prior to receipt). This proposal assumes that both types of propagules were associated with the internal seed tissues at the beginning of the storage period and presumably at harvest. McLean and Berjak (1987) have found that both field and storage fungi can be associated with maize seed tissues during seed development on the parent plant, and these can survive into storage. Those authors showed that, although fusaria predominated at harvest, these declined as species of Aspergillus and Penicillium were isolated with increasing frequency during storage.

The ability of Fusarium spp. to survive for up to six years in storage is of interest. Previously the field fungi were believed to survive only a few months under acceptable seed storage conditions (Christensen and Kaufmann, 1969, 1974). The present findings indicate otherwise, thus further contributing to the confusion referred to by McLean and Berjak (1987), that exists in the separation of the seed-associated fungi into clear-cut field or storage categories. Additionally, the persistence of the fusaria could be of significance to both commercial and carry over seed stores, where seeds are in storage for a maximum of two years (Neergaard, 1977). These seeds are used for re-planting, therefore during seed germination, the associated Fusarium propagules may also germinate and have deleterious effects on seedling establishment and subsequent plant growth, especially as fusaria are implicated in a variety of plant pathological conditions (Agarwal and Sinclair, 1987).

Therefore even under good storage conditions, as time increases:

i. germination rate and totality decrease
ii. plant vigour declines
iii. the incidence of storage fungi increases
iv. the incidence of field fungi declines but they do not die out, at least in the relative short-term

incidence of the field fungi as typified by Fusarium spp. associated with the yellow maize and an increase in that of the storage fungi (Fig. 3.9). In the white maize (Fig. 3.8) the decline of the field fungi was not evident although there was some increase in the levels of Aspergillus species. This may reflect differences in the longevity of the Fusarium propagules associated with the seeds, or differences in the resistances of the seed. Maize varieties are known to differ in their resistance to fungal pathogens (Agarwal and Sinclair, 1987; Tucker, Trevathan, King and Scott, 1986).

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Therefore even under good storage conditions, as time increases:

i. germination rate and totality decrease
ii. plant vigour declines
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iv. the incidence of field fungi declines but they do not die out, at least in the relative short-term

While quality of germination and the resultant plants will decline with increasing age of seeds with increasing cold-storage time, most seeds apparently harbour fungal propagules at harvest (McLean and Berjak, 1987). Thus the increasingly debilitated seeds afford the fungi increasingly optimal conditions in which to develop, once water becomes available. This would, in turn, decrease vigour, germination potential and the ultimate production of viable seedlings.

3:4 References

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Chapter 4

Micropylar infection of *Zea mays* by *Aspergillus flavus* var. *columnaris*

4 Micropylar infection of *Zea mays* by *Aspergillus flavus* var. *columnaris*

Published Article

Mycock, D.J., Lloyd, H.L. and Berjak, P. (1988). Micropylar infection of post-harvest caryopses of Zea mays by Aspergillus flavus var. columnaris var. nov. Seed Science and Technology, 16, 647 - 653.

Conference Proceedings

Mycock, D.J., Lloyd, H.L. and Berjak, P. (1985). Micropylar infection of the caryopses of Zea mays by Aspergillus flavus var. columnnaris. Proceedings of the Electron Microscopy Society of Southern Africa, 14, 107 - 108.

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Mycock, D.J., Lloyd, H.L. and Berjak, P. (1986). Micropylar infection of Zea mays by Aspergillus flavus var. columnaris. Proceedings of the International Seed Testing Association. Brisbane. Australia.

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4:1 Introduction

Despite modern technology, large quantities of stored grains are lost to invading pests, deterioration by fungal pathogens being particularly prevalent in the tropics and sub-tropics. In under-developed countries the availability and maintenance of suitable storage conditions are economically precluded to the average farmer (Neergaard, 1977). The problem is further compounded because many seed storage fungi produce mycotoxins, which are harmful to both man and livestock (Moreau, 1979; Steyn, 1980).

Members of the storage fungi are present at harvest, and are said to become dominant only in grain stored at relative humidities of 70% to 90%, representing seed moisture contents of above 15% (Christensen, 1967). Most of these fungi sooner or later attack the embryo and storage tissues of the seeds, causing discolouration, reduced germinability and vigour, and ultimately total decay (Christensen and Kaufmann, 1969, 1974). For such degradation to occur, the fungi must gain access to these internal tissues. There are a number of documented modes of entry and, although each can be considered separately, more than one might be involved in a natural infection (Agarwal and Sinclair, 1987).

A storage fungus must initially gain access to the seed tissues through some portal of entry, this generally being provided by injury to the pericarp/testa. Maturing grain can be damaged on the parent plant by insects or birds, and fungi can readily infect the damaged tissues. However, such fungi are held to be field fungi which are not generally considered to survive in the seed tissues during air-dry storage. Mature grain is vulnerable to injury at a variety of stages during its harvest and processing (Christensen and Kaufmann, 1974), while insects and mites may cause significant [further] damage to grain in the storage bin (Neergaard, 1977; Mills, 1983; Agarwal and Sinclair, 1987). Damage of any sort at any point in its history, renders the seed vulnerable to fungal attack.

The pericarp, which covers an entire maize grain is naturally discontinuous at only one point, the micropyle (Johann, 1935). This is a pore in the testa surrounded by loose tissue, the peduncle (which is the remains of the tissue attaching the caryopsis to the cob). As storage fungi are regularly encountered in/on caryopses which are not obviously externally damaged, one or more species may gain access to the seed tissue via the micropyle and peduncle.

The present chapter details events occurring over a six-week period, during which caryopses were inoculated with spores of Aspergillus flavus var. columnaris at the start of storage. The aim of this study was to determine whether intact caryopses could be invaded by this storage fungus and if so, what route of entry was utilised.

4:2 Materials and Methods

4:2:1 Seeds

Caryopses (seeds) of Zea mays (var. Hickory King; 9.5% moisture content) were obained one month after harvest from the Pioneer Seed Company, Greytown, Natal, South Africa. Prior to storage, the seeds were treated with hot water and surface-sterilised to eliminate (reduce) as far as possible the inherent infection.

4:2:2 Hot Water Treatment (Daniels, 1983)

Seeds were soaked in sterile, distilled water for four hours, after which they were transferred to vials containing sterile distilled water and maintained at 60 °C for five min. Seed temperature was dropped by continuous washing in cool, sterile, distilled water. The seeds were then surface-sterilised in a 2% solution of sodium hypochlorite and 1% sodium dodecyl sulphate for 5 min, then rinsed with sterile distilled water and tested for internal infection.

4:2:3 Internal Infection

Surface-sterilised seed was halved longitudinally through the embryo and aseptically plated onto potato dextrose agar containing 6% NaCl. The plates were incubated at 25 °C. Any fungal growth after five days was isolated and identified. Five hundred seeds were tested both prior to and after the heat treatment.

4:2:4 Storage

After hot-water treatment and surface-sterilisation, excess water was blotted off with sterile filter paper, and the seed then dried to a moisture content of 11.5% in a stream of air. Experimental seeds were infected by dusting with the spores of A. flavus var. columnaris prior to storage, the controls not being inoculated. The fungal isolate was obtained from maize seed of local origin and identified by the Mycological Research Unit of the Plant Protection Research Institute, Pretoria. A sufficient quantity of seed was stored at 90 and 95% RH (Thewlis, Glass, Hughes and Meetham, 1961) and 25 °C, to allow weekly sampling for six weeks.

4:2:5 Microscopy

Twenty seeds were removed from the storage bins at weekly intervals, each seed being then halved longitudinally through the embryo. Ten half seeds were prepared for scanning electron microscopy (as described previously 2:2:5), while the peduncle region was excised from another ten and processed for light microscopy (Berjak, 1984). The remaining twenty half seeds were plated on PDA (potato dextrose agar) plus 6% NaCl.

4:2:6 Germination Studies

At each sampling, an additional 30 seeds were set to germinate under sterile conditions. Percentage germination was scored after 72 hours. Those seeds, whether germinated or not, were then planted in sterile vermiculite. Seedling mass was determined after 14 d.

4:3 Results

4:3:1 Internal Infection

Prior to hot water treatment the seed showed an infection level of 84%, comprising Aspergillus (34%), Penicillium (34%), bacteria (8%), Fusarium (6%) and Cladosporium (2%). Hot water treatment reduced this internal infection to 18%, comprising Penicillium (9%), bacteria (5%), Aspergillus (2%) and Fusarium (2%). Heat treatment had no effect on the total germination, which was maintained at 98%.

Despite the inherent infection remaining associated with 18% of the hot water treated seed samples, there was no detectable fungal growth on the control seeds at any stage, nor was Aspergillus flavus var. columnaris isolated from any of this material. However, this fungus was isolated from all inoculated seed.

4:3:2 Microscopy

The trends described below were similar in material maintained at both 90 and 95% RH. However, the degree of microscopically visible damage was greater for seeds maintained at the higher RH, for which the microscopical results are described.

After one week in storage, some of the spores with which the experimental material had been inoculated had germinated and hyphae were seen ramifying over the surface of the seed (Fig. 4.1). Similarly, hyphae were seen on the surface of the peduncle (Fig. 4.2). A further seven days in storage allowed for intense fungal growth, with hyphal penetration of the loose peduncle tissue (Fig. 4.3). Fungal asexual reproductive structures, viz. conidiogenous heads, were also observed on the surface of the seed (Fig. 4.4). After three weeks in storage, hyphae were present within the vascular tissue of the peduncle (Fig. 4.5) and traversing the space between the micropylar region and the scutellum (Fig. 4.6). After one month in storage, the hyphae had penetrated into the hollow between the scutellum and the coleorhiza

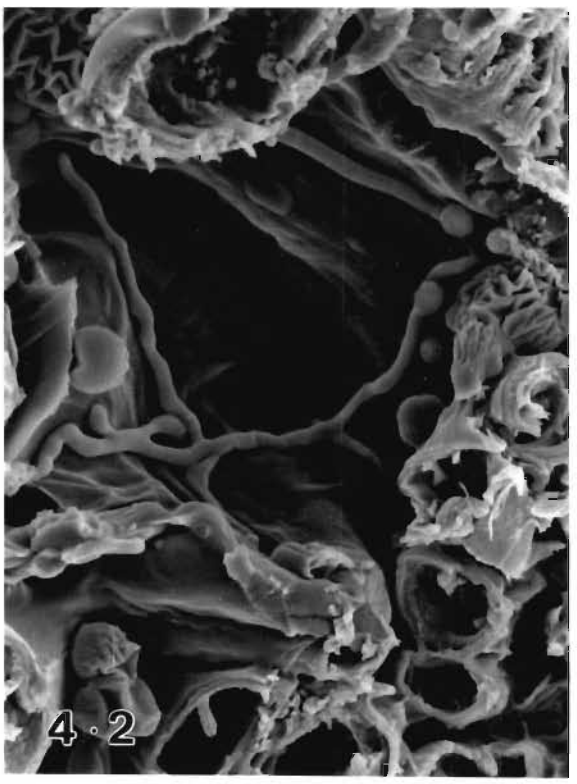
Fig. 4.1 After seven days in storage the fungal spores had germinated and hyphae were ramifying over the surface of the seed (× 2 000).

Fig. 4.2 Hyphae growing on the surface of the peduncle (× 2 400).

Fig. 4.3 After 14 days in storage the fungal mycelia had penetrated into the loose peduncle tissue (× 2 500).

Fig. 4.4 Conidiogenous heads on the surface of the infected seeds(× 60).

4 · 1



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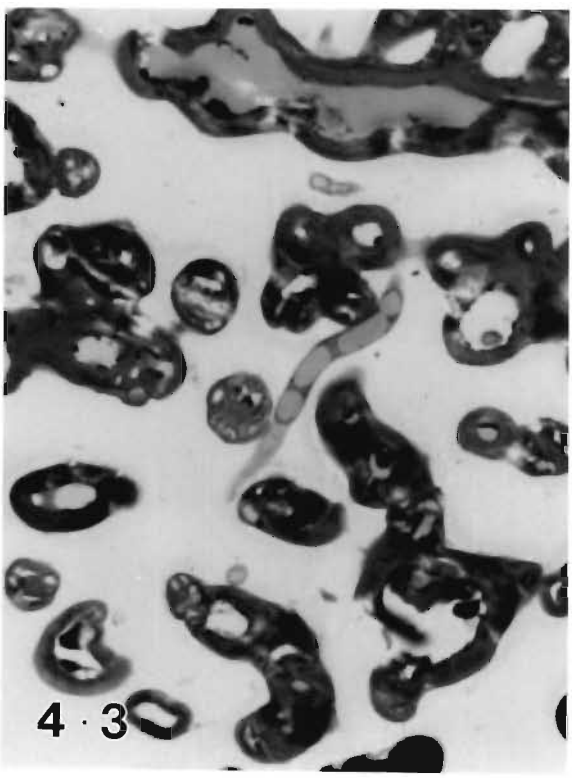

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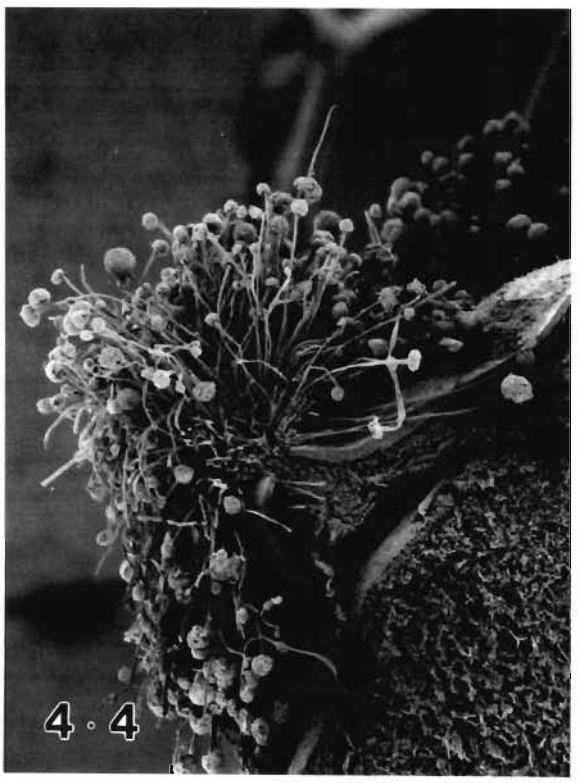


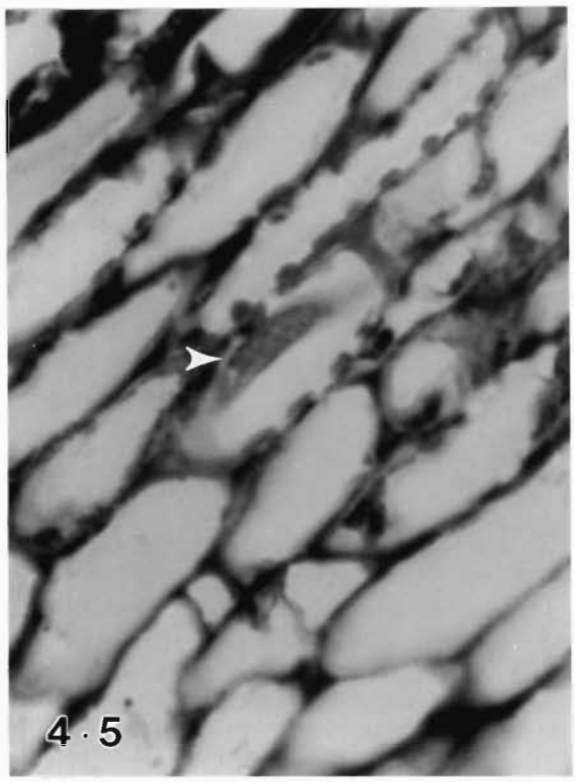
4 · 4

Fig. 4.5 Hyphae within the vascular tissue of the peduncle. (Three weeks in storage) (x 3 000).

Fig. 4.6 Hyphae traversing the space between the micropylar region (m) and the scutellum (s). (Three weeks in storage) (x 300).

Fig. 4.7 The control material was not visibly infected by fungi (x 60).

Fig. 4.8 Hyphae colonising the space between the scutellum and the coleorhiza (x 60). [Four weeks in storage].



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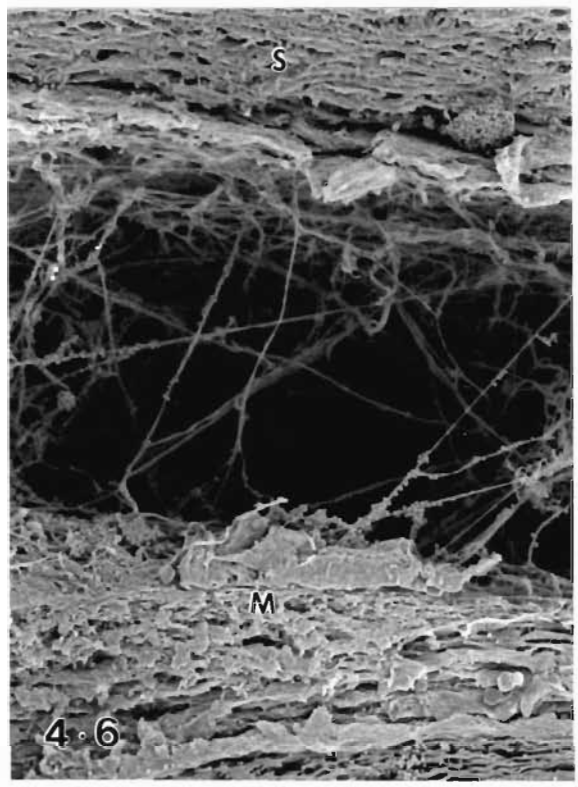

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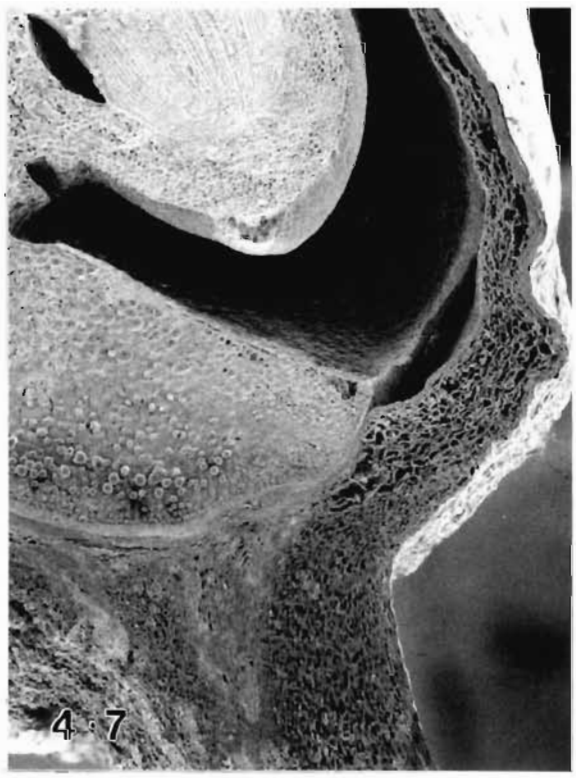
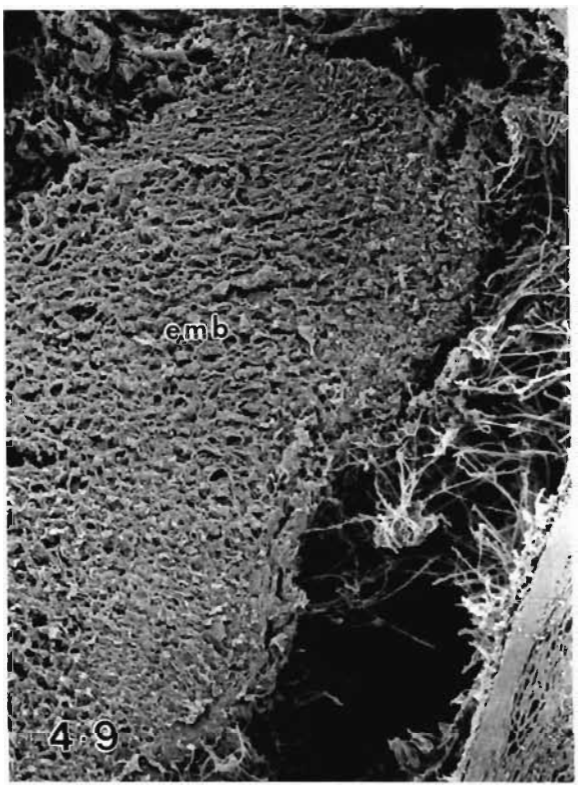


4·7

4·8

Fig. 4.9 After six weeks in storage the embryonic axes (emb) were degraded (x 150).

Fig. 4.10 Sclerotia developed on the peduncle surface after six weeks in storage (x 100).



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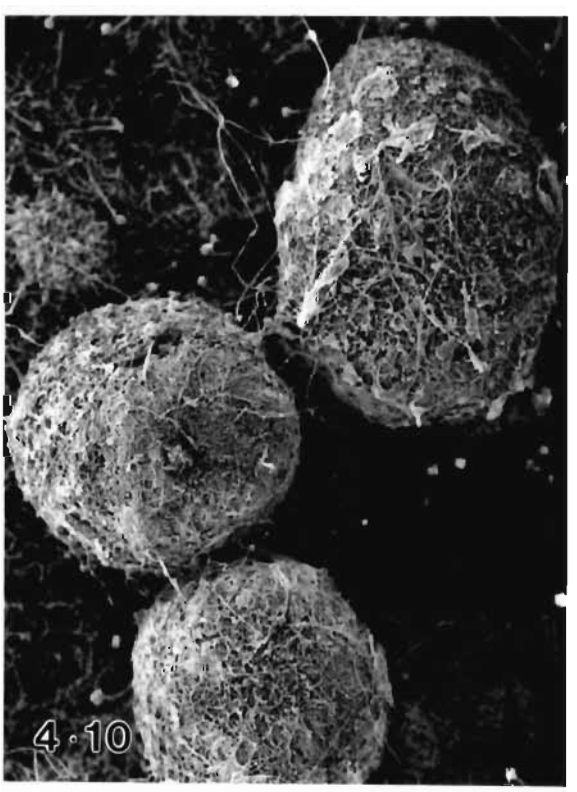


4·10

(compare Fig. 4.7 [control] and 4.8). The embryonic axes were heavily invaded after six weeks in storage (Fig. 4.9), and sclerotia were observed on the peduncle surface (Fig. 4.10).

4:3:3 Germination

There was no effect on the viability/germinability of control seeds over the six week period, at either relative humidity (Figs 4.11 and 4.12). Similarly, in the first month of storage, inoculated seed exhibited maximum germination. After this time, however, germinability of the experimental material was reduced, this reduction being correlated with fungal invasion of the peri-embryonic area and then the axis itself (Figs 4.8 and 4.9 respectively).

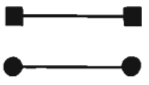
4:3:4 Establishment

Storage of uninoculated seed at 90% RH had no effect on seedling establishment (Fig. 4.13), although it was slightly reduced in the control material after one month in storage at 95% RH (Fig. 4.14). The establishment of seedlings from inoculated seed, however, was substantially affected after only one week of storage at 95% RH and after 3 weeks storage at 90% RH: The reduction was greater at the higher RH value (c.f. Figs. 4.13 and 4.14).

4:3:5 Dry Mass

Seedlings established from both control conditions had a greater average dry mass than those established from inoculated seed (Figs. 4.15 and 4.16). There was a decline in seedling dry mass with increasing seed storage time, this being reflected in both experimental and control material. As was found for seedling establishment, the decline in dry mass was greater in the seed material stored under the higher RH conditions.

Fig. 4.11 Total germination of the uninoculated control and the experimental seeds stored at 90% RH. All seed material was hot water treated prior to the experiment.



■——■ Control

●——● Infected with A. flavus var. columnaris

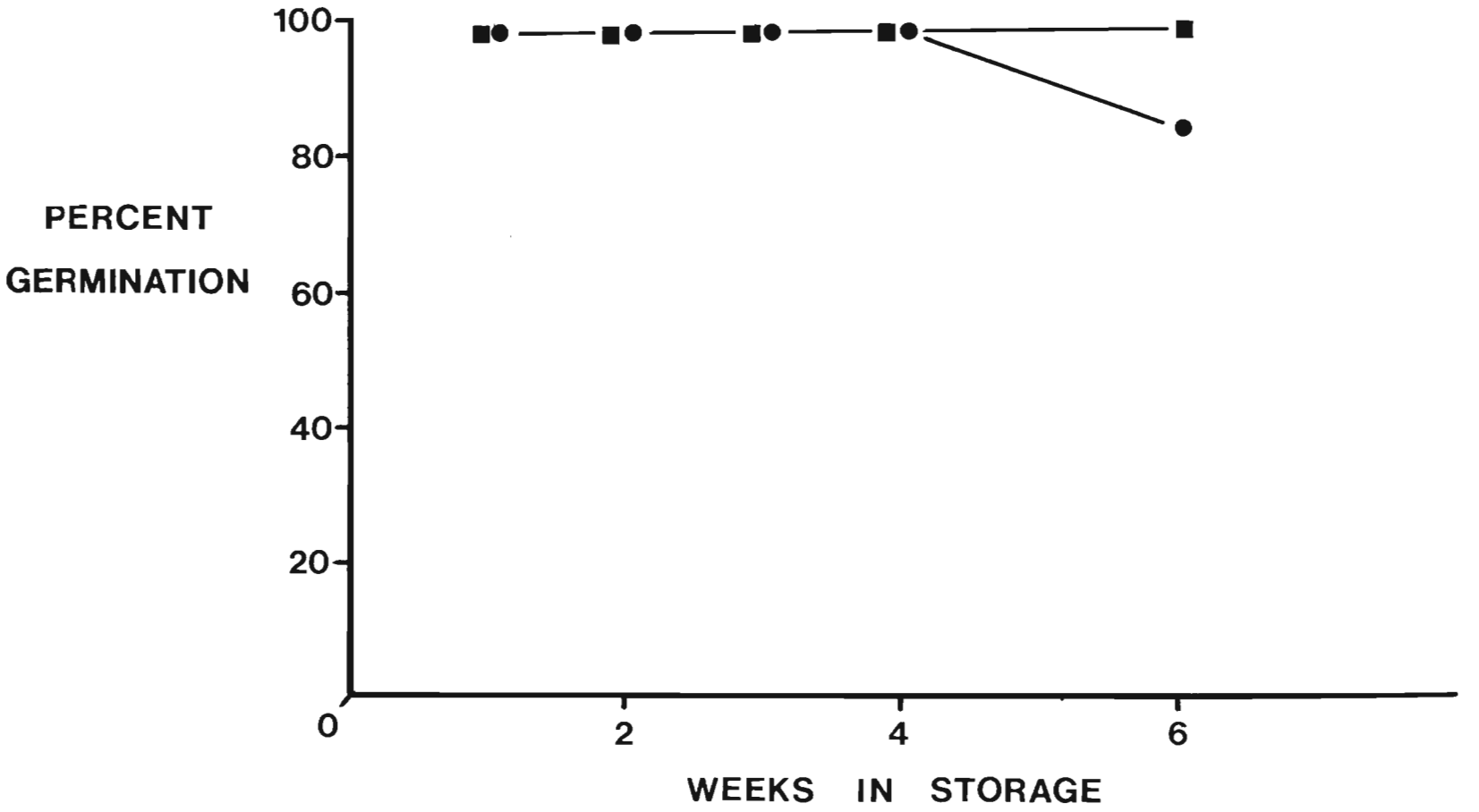

100
80
60
40
20
0
PERCENT
GERMINATION
2
4
6
WEEKS IN STORAGE

Fig. 4.12 Total germination of the control and experimental seeds stored at 95% RH. All seed material was hot water treated prior to the experiment.

Control

Infected with A. flavus var. columnaris

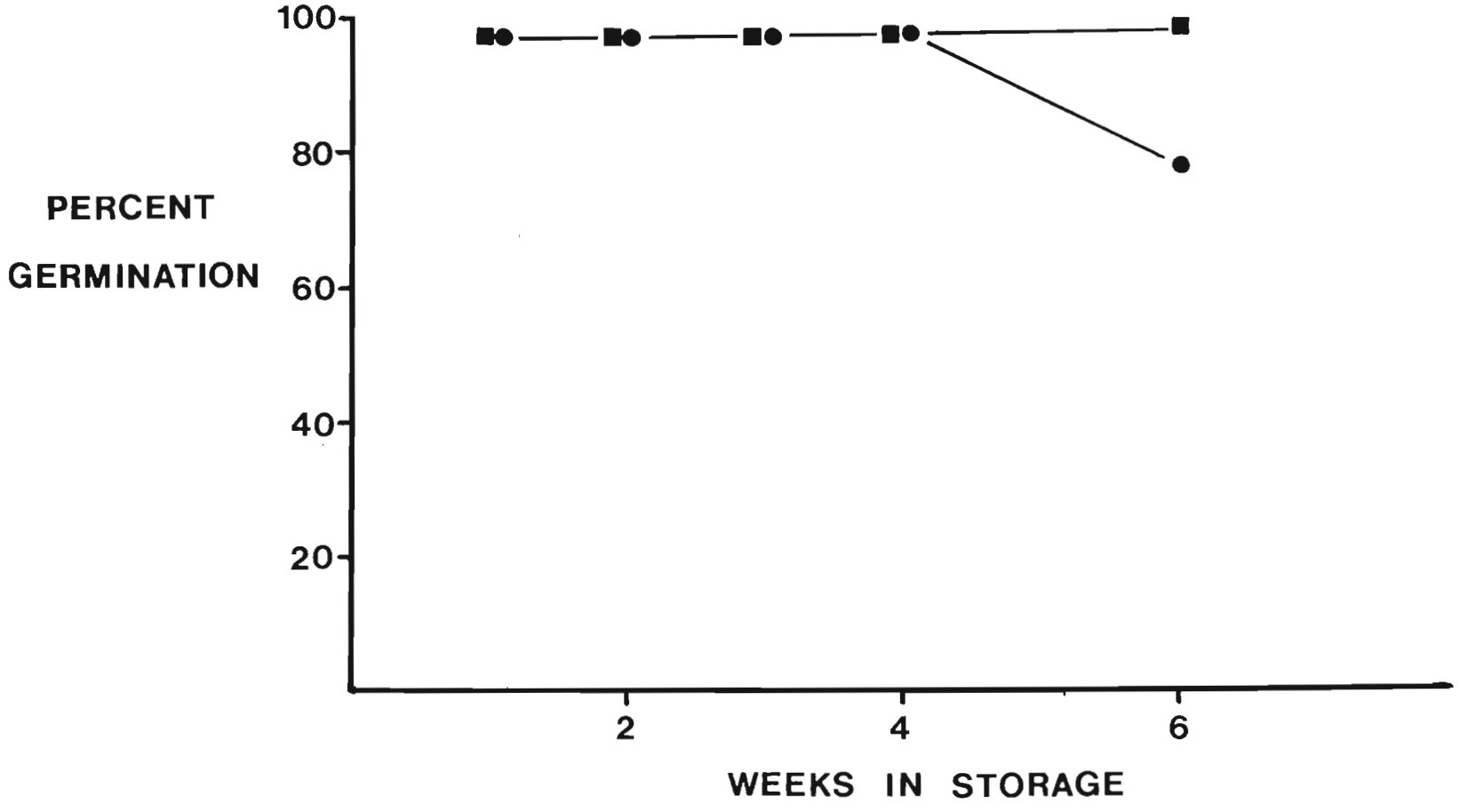

100
80
60
40
20
PERCENT
GERMINATION
2
4
6
WEEKS IN STORAGE

Fig. 4.13 Percent establishment of the plants developed from the uninoculated control and experimental seeds that had been stored at 90% RH.

■——■ Control

●——● Infected with A. flavus var. columnaris

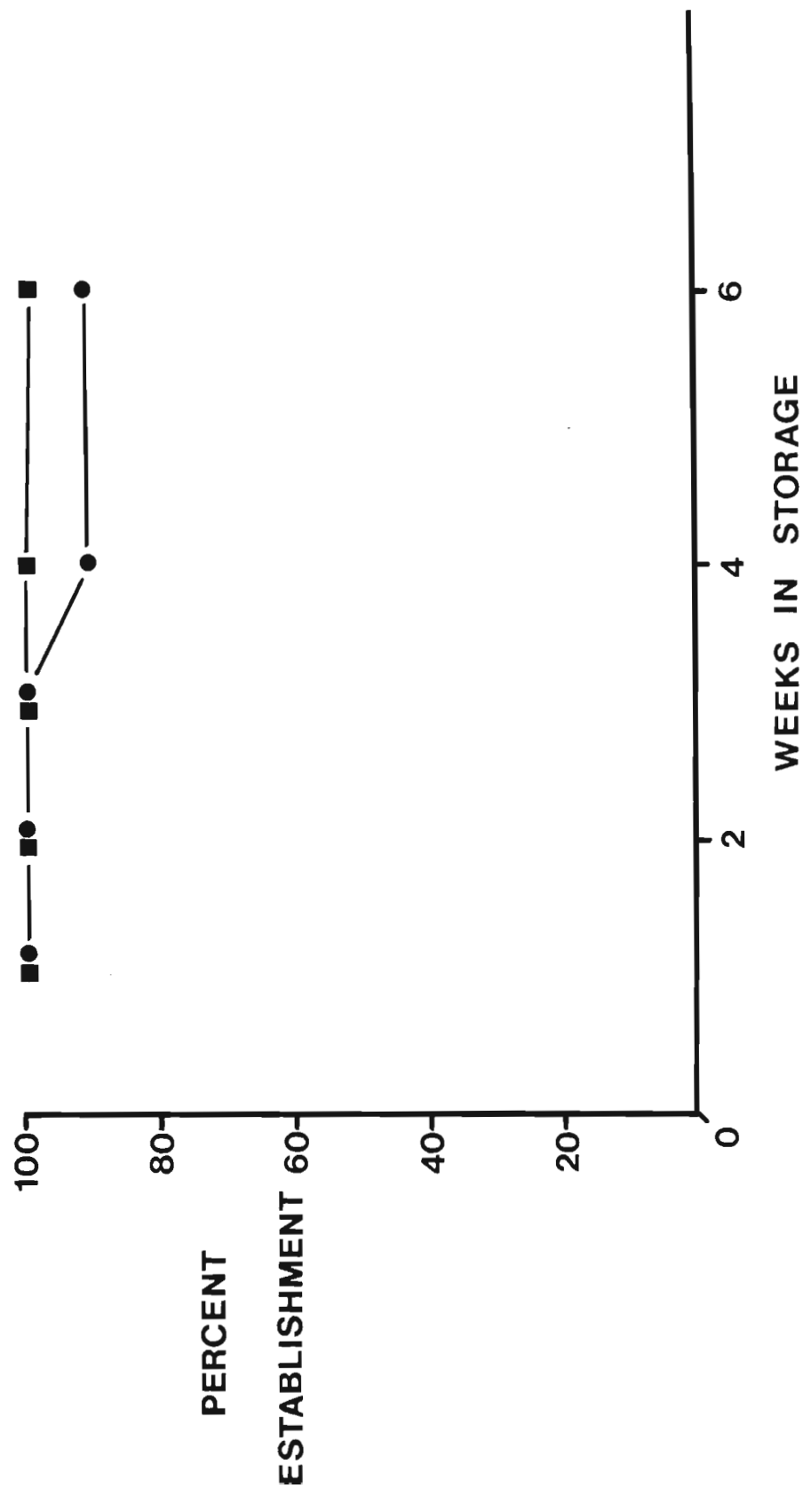
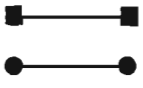

PERCENT
ESTABLISHMENT
100
80
60
40
20
0
2
4
6
WEEKS IN STORAGE

Fig. 4.14 Percent establishment of plants developed from the control and experimental seeds that had been stored at 95% RH.



Control

Infected with A. flavus var. columnaris

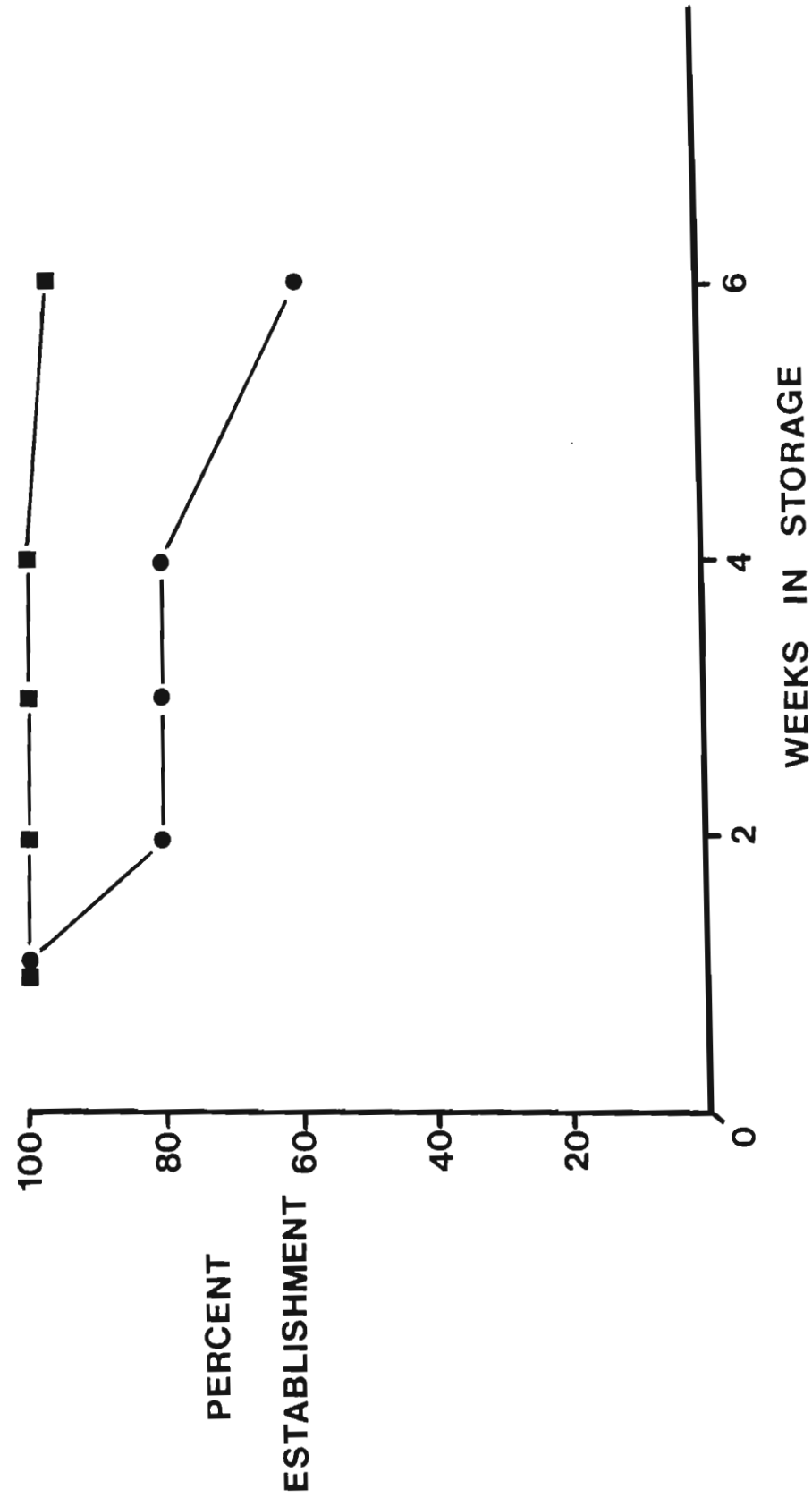
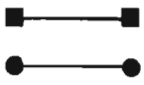

PERCENT
ESTABLISHMENT
100
80
60
40
20
0
2
4
6
WEEKS IN STORAGE

Fig. 4.15 Dry mass of the plants that had developed from seeds stored at 90% RH. Bars indicate standard deviation.



Control

Infected with A. flavus var. columnaris

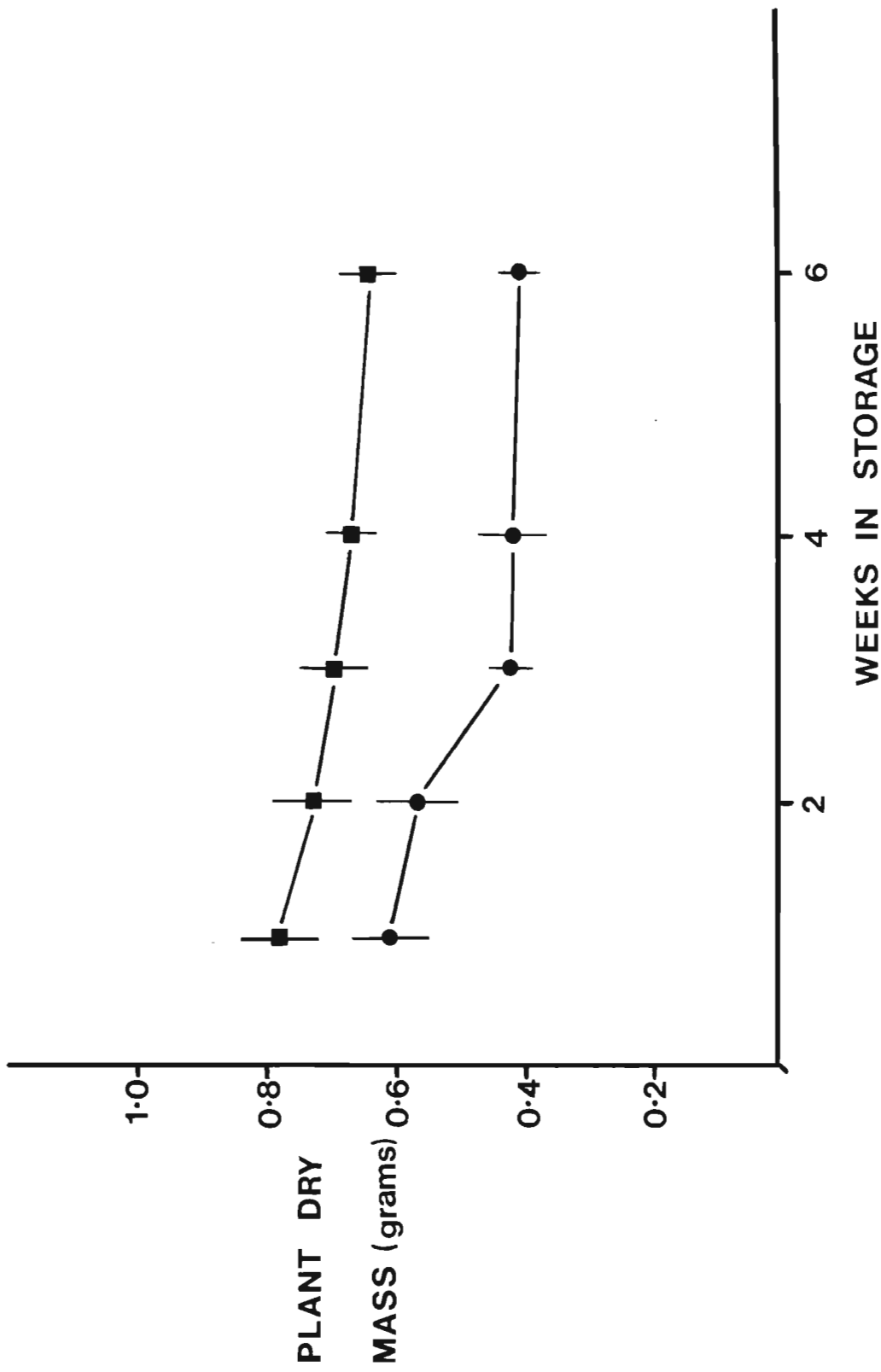
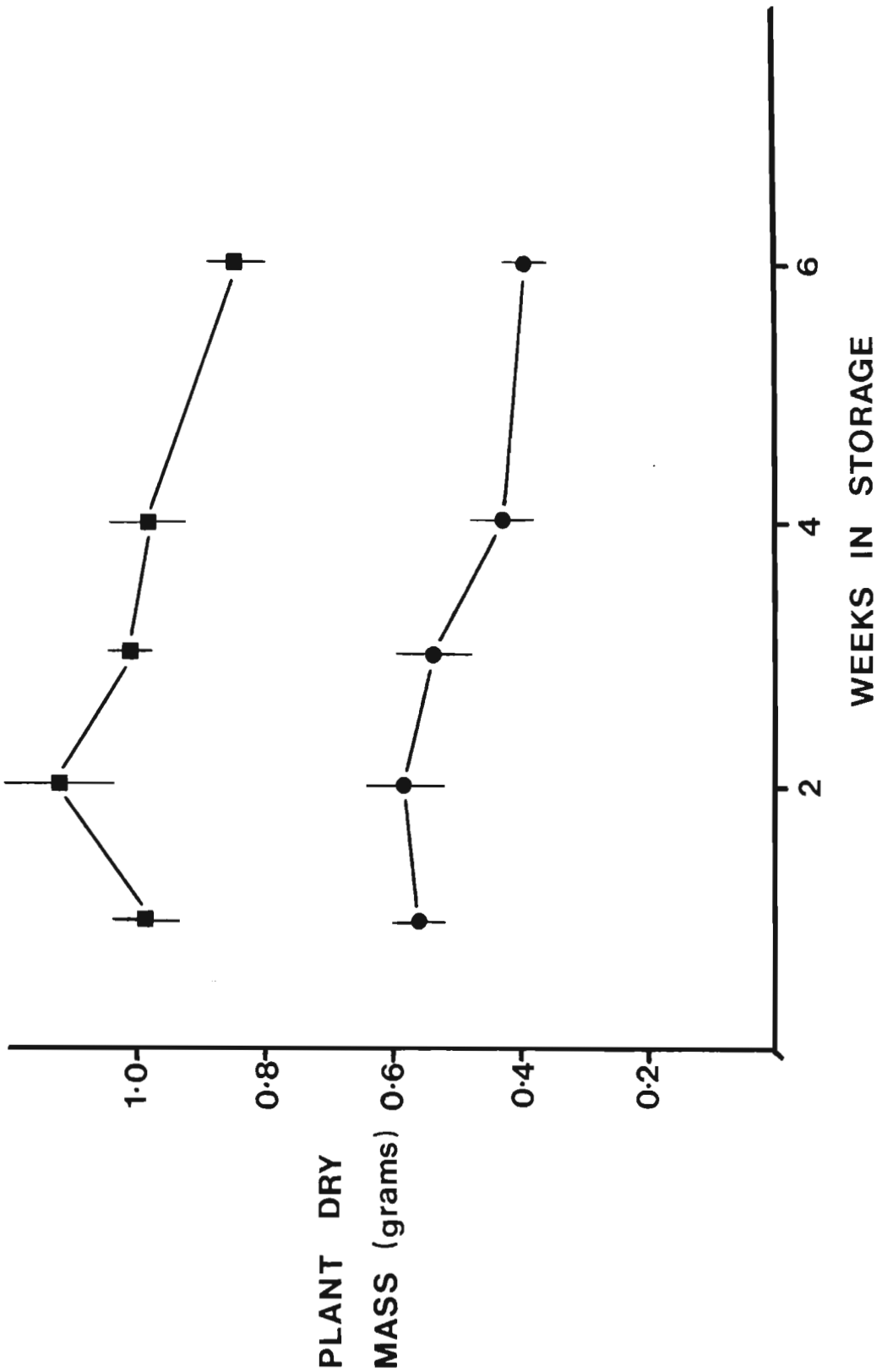

PLANT DRY
MASS (grams)
1·0
0·8
0·6
0·4
0·2
2
4
6
WEEKS IN STORAGE

Fig. 4.16 Dry mass of the plants that had developed from seeds stored at 95% RH. Bars indicate standard deviation.

■——■ Control

●——● Infected with A. flavus var. columnaris


PLANT DRY MASS (grams)
1·0
0·8
0·6
0·4
0·2
2
4
6
WEEKS IN STORAGE

4:4 Discussion

As it has been impossible to obtain uninfected maize seed, the hot water treatment was used to curtail as far as possible the inherent infection at the start of the experiment. A comparison of the control with inoculated seeds throughout the experimental periods, bears out the success of this approach under the circumstances of the present investigation. However, our unpublished results indicate that if the seeds are to be conventionally stored after hot-water treatments, immersion periods of 30 - 45 min are more effective.

Storage of the uninoculated hot-water-treated seeds at 90% RH had no effect on germination and subsequent seedling establishment. The maintenance of similar control seed at 95% RH for one month, although not apparently affecting germinability, did reduce establishment. The effects of storage at 95% RH were apparent only from the establishment studies, and not from the assessment of germinability; however, it is possible that the rate of germination itself was reduced, but this parameter was not measured.

The effect of increasing seed storage time is also reflected in the declining dry mass of seedlings established from both control conditions. The reductions may be due to the initiation of deterioration of the seed ascribable to accelerated ageing under these conditions. Although the temperatures generally used for accelerated ageing over a short period are more extreme than those used here, the elevated moisture content of the seeds could have adversely affected their vigour (Berjak and Villiers, 1972).

The slightly decreased establishment of the control material is, however, most likely to have been brought about by the inherent 18% infection. This effect was particularly noticeable in the material stored at 95% RH. Such conditions, particularly at the favourable temperature used (25 °C), are conducive to ongoing activity of seed storage fungi (Christensen, 1967). The greater dry mass of the control material stored at 95% RH (compared with that at 90% RH) is ascribed to the mass of the fungal mycelium.

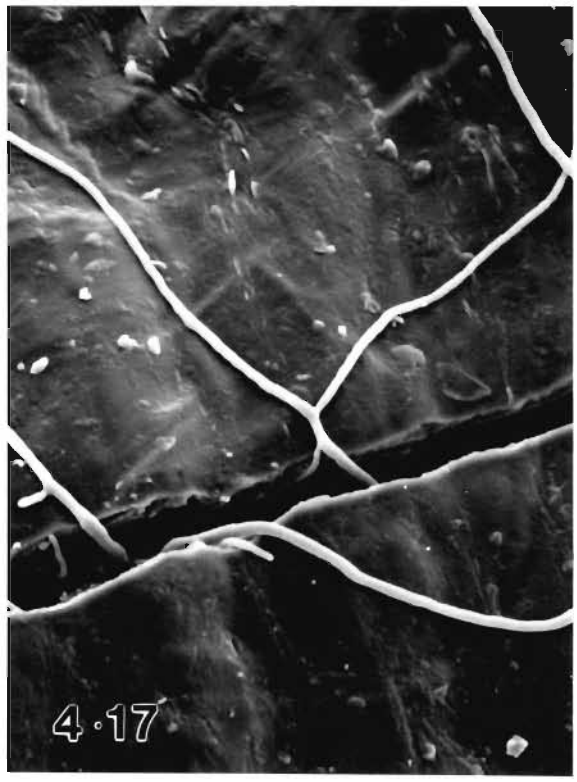
Inoculation of the heat-treated seed at the start of the experiments was correlated with a reduction in germination after one month in storage at both 90 and 95% RH. Similarly, there was a reduction in the establishment of the seedlings from these seeds. This was manifested after a shorter storage period than was the decline in germination, and was more marked in material from the 95% RH condition. The deleterious effects of the fungus were clearly manifested by the substantial reduction in dry mass of the plants, (compared with the control) grown from the inoculated seeds. It is this reduction in germination, seedling establishment and dry mass of the inoculated as opposed to the control material, that can be considered the outcome of fungal activity.

A decline in percentage germination of the inoculated material was first manifested after only one month in storage. Microscopy revealed that although hyphae had penetrated the peduncle tissue within two weeks of storage, it was only after four weeks that the infection process had progressed to invasion of the embryo. Once hyphae become established in the tissues of the embryo, the latter would not only have been physically and physiologically damaged, but also would presumably have been begun to be utilised as a source of nutrient for the fungus. Fungal infection, however, affected the establishment and the growth of the seedlings before its effects were manifested at the level of seed germination.

Microscopical examination showed that in the first two to three weeks of storage, fungal growth was confined to the seed surface and peduncle tissue. It is possible, however, that this mycelium serves as a source of infection of the elongating radicle (and perhaps also the shoot) as germination proceeds (Chapter 7).

Aspergillus flavus var. columnaris appears to gain access to the tissues of an intact maize caryopsis via the peduncle and micropyle. However, hyphae of this species have also been observed penetrating cracks in the surface of damaged grain (Fig. 4.17) [see also Chapter 9]. Peduncular infection is gradual, commencing with growth on the surface of the seed, particularly at

Fig. 4.17 Hyphae of A. flavus var. columnaris penetrating cracks in the surface of damaged grain (× 2 000).


4·17

the micropylar end. The peduncle is composed of scar tissue comprising loose parenchyma (Fig. 4.18). This would presumably facilitate hyphal penetration, compared with the pericarp tissues, the latter being made up of tightly-packed cell layers. Two or three weeks after inoculation, hyphal elements were observed in the vascular tissue of the peduncle. Hyphal advance through this tissue might well be facilitated by the natural perforations of the cell walls.

Penetration through the peduncular scar tissue allows fungal access initially to the underlying scutellum and then directly to the embryonic axis. In the dry maize grain, there is a clearly demarcated space between the scutellum and the radicle tip, the latter being ensheathed by the coleorhiza (Fig. 4.19). Vigorous establishment of the mycelium was particularly marked within this space, which would greatly facilitate growth of the hyphae into the embryonic axis. The production of fungal reproductive structures supports the fact that the seed is being used as a food source, the fungus probably utilising the embryo and cotyledon as a nutrient supply. A. flavus var. columnaris produces highly active proteases and lipases in its extracellular exudate, and these probably facilitate breakdown and utilisation of cell components of the embryo and scutellum (McLean , Mycock and Berjak, 1985), [see also Chapter 5].

The peduncular/micropylar route of infection appears thus to favour particularly establishment of A. flavus var. columnaris in the embryonic tissues. However, invasion of the endosperm cannot be ruled out, although this was not observed in the present SEM study. Tsurata, Gohara and Saito (1981) have reported that other members of the storage fungi (A. candidus, A. chevalieri and A. restrictus) also utilise the tip cap in their infection of stored maize. Similarly Hill and West (1982) found that stored soybean is infected via the hilum.

In summary, microscopical investigations reveal that Aspergillus flavus var. columnaris gains access to the interior of intact maize grains stored under suitable conditions by the

Fig. 4.18 A section through the peduncle (p) of a maize caryopsis (x 300). Note the loose parenchyma. c, cotyledon.

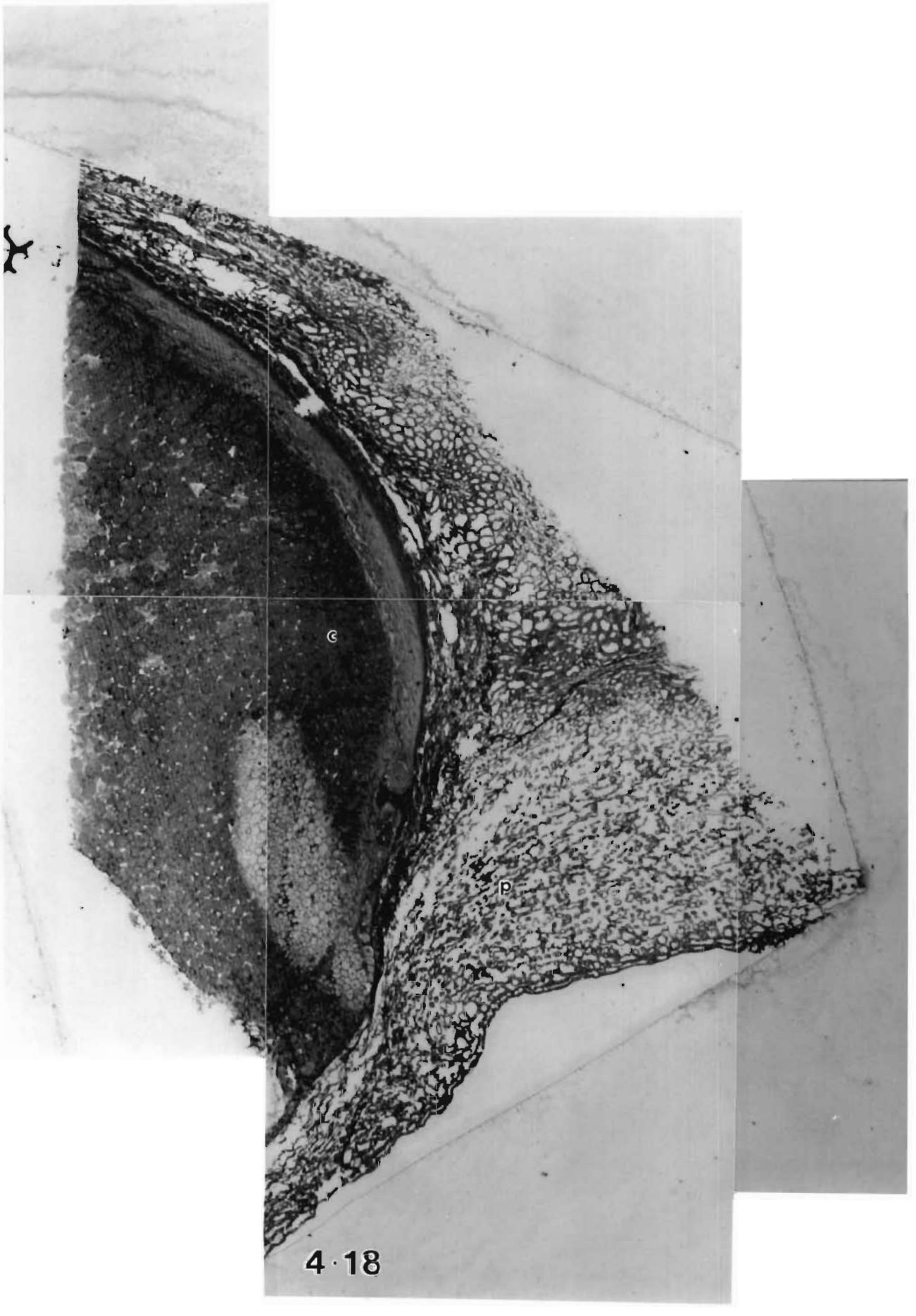

c
p
4·18

Fig. 4.19 A low power scanning electron micrograph of a longitudinally halved maize caryopsis (× 20). r, radicle tip; c, coleorhiza

4 ·19

peduncle/micropyle route. This portal of entry appears also to be favoured above that provided by microscopic pericarp lesions. These observations support the generally accepted view that the aspergilli are opportunistic invaders and saprophytes. Deleterious fungal effects are initially manifested by reduction in seedling establishment and dry mass, indicating, from the peduncular location of the mycelium, that the elongating embryonic axis might remain vulnerable to invasion. However, when the storage period exceeds four weeks, the embryonic tissues have become infected in the air-dry seed and germinability, *per se*, declines.

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Chapter 5

Extracellular enzyme production by some *Aspergillus* species

5 Extracellular enzyme production by some Aspergillus species

The work reported in this chapter was carried out and equally shared with Miss M. McLean, who also utilised the data in her M.Sc. Her contribution is gratefully acknowledged.

Published Article

McLean, M., Mycock, D.J. and Berjak, P. (1985). A preliminary investigation of extracellular enzyme production by some species of Aspergillus. South African Journal of Botany, 51, 425 - 431.

Conference Proceedings

Mycock, D.J., McLean, M., Berjak, P. and Lloyd, H.L. (1985). Extracellular enzyme production by two species of Aspergillus Proceedings of the South African Society for Plant Pathology, Phytophylactica, 17, 53.

5:1 Introduction

Most fungal pathogens are capable of releasing cell-wall- and membrane- degrading enzymes into their immediate environment (Agarwal and Sinclair, 1987). The widespread occurrence of these enzymes suggests an essential rôle for them in the infective process (English and Albersheim, 1969; Jones and Clifford, 1983). Additionally, it is now becoming clear that pathogens sequentially release enzymes capable of degrading the various components of the seed (Agarwal and Sinclair, 1987). This however, does not mean that each pathogen secretes an entire spectrum of enzymes; most appear to liberate enzymes specific to certain components.

A number of the saprophytic fungal species involved in the degradation of stored seeds are also renowned for their production of extracellular enzymes. Aspergillus oryzae for instance, is used in the orient in the fermentation of sakè, whilst the amylolytic capabilities of A. niger are used by the Germans in the conversion of inulin to ethanol (Raper and Fennell, 1965). This chapter investigated the ability of A. flavus Link var. columnaris Raper and Fennell, A. versicolor (Vuill.) Tiraboschi, A. candidus Link and an A. glaucus group member to produce extracellular enzymes which would be capable of degrading seed tissues.

5:2 Materials and Methods

5:2:1 Agar Studies

Two sets of experiments were conducted. In the first, malt extract agar (MEA), potato dextrose agar + 6% NaCl (PDA), Czapek-Dox agar (CDA) and Czapek-Dox agar + 20% sucrose (CDA + 20% sucrose) provided mono- or disaccharides in the form of dextrose, dextrose, sucrose and sucrose, respectively. The more complex carbon sources included pectin, protein (gelatin), starch and lipid (corn seed oil). The first three complex media were prepared according to Hankin and Anagnostakis (1975) and the lipid agar was adapted from the method of El Azzabi, Clarke and Hill (1981).

5:2:1:1 Pectin agar

This medium comprised:

Mineral solution	500 ml
Yeast extract	1 g
Bacteriological agar	15 g
Citrus pectin	5 g
NaCl	30 g
Distilled water	500 ml
pH adjusted to	5.75

Prepared and poured as previously described in 2:2:1.

The mineral solution comprised

$(NH_4)_2SO_4$	2 g/l
KH_2PO_4	4 g/l
Na_2HPO_4	6 g/l
$FeSO_4.7H_2O$	0.2 g/l
$CaCl_2$	1 mg/l
H_3BO_3	10 µg/l
$MnSO_4$	10 µg/l
$ZnSO_4$	70 µg/l
$CuSO_4$	50 µg/l
MoO_3	10 µg/l

5:2:1:2 Protein agar

Nutrient agar	15 g
Gelatin	4 g
NaCl	30 g
Distilled water	1 000 ml

Autoclaved, and poured as previously described in 2:2:1

5:2:1:3 Starch agar

Nutrient agar	15 g
Soluble starch	2 g
NaCl	10 g
Distilled water	1 000 ml

Autoclaved and poured as previously described 2:2:1.

5:2:1:4 Maize Meal agar

One hundred grams of maize caryopses were autoclaved with 200 ml distilled water. The mixture was then homogenised, 15 g of bacteriological agar were added and made up to the final volume of 1 000 ml with distilled water.

Autoclaved and poured as previously described in 2:2:1.

5:2:1:5 Lipid agar

Bacteriological agar	20 g
Tryptone	5 g
Corn seed oil	80 ml
0.1% Nile Blue sulphate	40 ml
NaCl	30 g
Distilled water	880 ml
pH was adjusted to	7.2

During the pouring of this solution into precooled, sterile Petri dishes, the medium was rolled continously to ensure that the oil droplets were distributed evenly in the molten agar.

In the second set of experiments, similar media were used but citrus pectin was substituted by the more purified polygalacturonic acid, and the nutrient agar in the protein and starch agars was replaced by bacteriological agar. Additionally, a cellulose agar consisting of 15 g bacteriological agar and 2 g carboxymethyl cellulose made up to 1 000 ml with distilled water was prepared. These experiments were conducted to assess the ability of the fungi to produce the various enzyme types on media containing only a single carbon source.

In this second set of experiments, osmotic potentials were elevated by adding NaCl such that concentrations of 5, 10 and 15% were achieved.

5:2:2 Detection of Extracellular Enzyme Production

The ability of the four Aspergillus species to produce extracellular lipase, protease and amylase was investigated. The media described for the second set of experiments (5:2:1) were treated in the following manner.

5:2:2:1 Amylolytic activity

After the desired number of days of incubation, the plates were flooded with an iodine solution (0.2 g I, and 2 g KI 100 ml^{-1} distilled water). A clear yellow zone in an otherwise blue-black surrounding showed a depletion of starch.

5:2:2:2 Proteolytic activity

The plates were flooded with a saturated solution of ammonium sulphate, thus any remaining gelatin was precipitated to produce an opaque medium. Clear zones in the opaque area indicated utilization of the gelatin.

5:2:2:3 Lipolytic activity

Lipid breakdown results in an increase of fatty acidity, as indicated by a change in colour of the Nile Blue indicator in the medium from an orange-pink to blue. Any changes in colour towards blue thus represent lipolytic activity. The reverse colouration was considered and in some instances, depending on the amount of lipase produced, a diffusible blue line could be measured beyond the colony bounds.

The plates were poured such that each contained approximately 15 ml of medium, and were incubated at 25 - 30 °C. For each colony (30 replicates) two diameters (mm), at right angles, were measured at 5, 7, 10, 12 and 15 days of incubation. For the detection of extracellular enzymes, the same measurements were made but at 10 and 15 days.

5:2:3 Detection of Extracellular Enzyme Production Using Liquid Culture

A. flavus var. columnaris and A. versicolor were inoculated onto 50 ml of maize broth (100 g autoclaved, homogenised maize caryopses per 1 000 ml distilled water) and incubated at 25 - 30 °C. Uninoculated broth served as controls. At intervals of 5, 7, 10, 12 and 15 days the mycelial mat was removed and the remaining contents of the Erlenmeyer flask centrifuged for ten minutes at 3 000 g. The supernatant, containing the crude enzyme extract, was collected.

The following assays were carried out on the crude extract:

i. Protease activity (Bergmeyer, 1974), expressed as Tu^{cas} ml^{-1} (amount of casein hydrolysed per ml of extract per minute).

ii. Cellulase activity (Mandels, Andreotti and Roche, 1976), expressed as mg glucose ml^{-1} h^{-1}.

iii. Reducing sugar levels, expressed as mg glucose ml^{-1} (Mandels. Andreotti and Roche, 1976).

5:3 Results and Discussion

Aspergillus flavus var. *columnaris* and *A. candidus* were more prolific enzyme producers than *A. versicolor* and the *A. glaucus* species. At the one extreme, both *A. flavus* var. *columnaris* and *A. candidus* exhibited vigorous pectinolytic, cellulolytic, amylolytic, lipolytic and proteolytic activity. *A. versicolor* was capable of amylolytic and cellulolytic activity, weak lipolytic and pectinolytic activity and a possibly very weak proteolytic activity (Tables 5.1, 5.2, 5.3 and Figs 5.1 and 5.2). At the other extreme, the *A. glaucus* species, under nutrient-deficient conditions, could not produce amylase, cellulase or protease. However, this fungus did exhibit pectinolytic and a very weak lipolytic activity (Table 5.2).

With increasing osmotic potential, the general trend was for a decrease in enzyme production (Table 5.3). Pectinolytic enzyme production appeared to be the least affected by the imposed water stress (Table 5.2). Both *A. flavus* var. *columnaris* and *A. candidus* produced lipolytic activity at all three salt concentrations, the activity of the former exceeding that of the latter at 5 and 10% NaCl, but at 15% NaCl the difference was not as marked. *A. candidus*, although not producing as much extracellular amylase or protease as *A. flavus* var. *columnaris*, was capable of producing these enzymes at the higher salt concentrations. This reflected the greater osmotolerance of *A. candidus* compared with that of *A. flavus* var. *columnaris* (See Chapter 3). *A. versicolor* was by far the most active amylase producer at the highest water activity. If colony diameter (Table 5.2) and the diameter of the clear zone on the medium (Table 5.3) were considered, this species grew slowly, but the effects of the diffusible extracellular enzyme were more marked than those of the other fungi.

In the context of fungal activity in air-dry seeds, it may be possible that lipase and protease production were the most important for *A. flavus* var. *columnaris*, lipase, protease and

Table 5.1 Colony diameter (mm) of three species of *Aspergillus* on a variety of carbon sources.

	1. *A. flavus*			2. *A. versicolor*				3. *A. glaucus* sp.			
Days	5	12	15	5	12	15	1:2	5	12	15	1:3
Medium											
Malt extract agar	38,3 ±12,6	75,3 ±5,0	*Full	13,6 ±1,0	23,7 ±2,6	25,0 ±3,8	3,5	13,3 ±1,0	**	34,5 ±0,7	2,6
Czapek-Dox agar	21,0 ±6,8	56,5 ±8,5	74,5 ±17,6	8,7 ±1,3	24,0 ±1,5	31,5 ±1,9	2,4	7,4 ±0,9	**	38,8 ±1,5	1,9
Czapek-Dox + 20% sucrose	27,0 ±13,5	80,7 ±12,7	Full	9,2 ±1,2	25,6 ±1,6	31,5 ±4,4	2,8	26,2 ±1,2	**	Full	0,0
Potato dextrose	31,3 ±7,0	67,3 ±7,4	82,3 ±9,8	11,3 ±1,1	27,1 ±1,1	33,0 ±1,8	2,5	42,8 ±3,1	**	Full	-1,3
Lipid agar	31,6 ±10,4	83,6 ±4,6	Full	4,5 ±1,0	13,0 ±2,0	16,2 ±3,2	5,4	9,0 ±1,0	11,8 ±1,0	**	7,1
Protein nutrient	20,2 ±6,2	57,8 ±6,3	72,6 ±9,0	5,7 ±1,2	10,8 ±1,1	13,2 ±1,1	5,5	8,0 ±0,8	21,0 ±3,4	**	2,8
Starch nutrient	33,6 ±9,7	85,6 ±5,4	Full	2,7 ±0,7	9,2 ±1,2	13,1 ±1,1	6,7	10,0 ±1,0	16,7 ±0,6	**	5,1
Pectin agar (pH 5,75)	30,8 ±11,6	Full	Full	12,0 ±2,2	29,4 ±5,0	35,5 ±6,7	3,0	27,0 ±1,6	55,8 ±6,6	**	1,6
Maize meal	31,0 ±7,8	Full	Full	11,3 ±1,2	34,2 ±8,4	50,3 ±3,7	2,6	6,3 ±0,3	20,8 ±0,5	26,5 ±1,4	4,2

Table 5.2 Colony diameter (mm) of four species of Aspergillus grown on nutrient-deficient media. NT, not tested.

	1. A. flavus			2. A. versicolor				3. A. candidus				4. A. glaucus sp.			
Days	5	12	15	5	12	15	1:2	5	12	15	1:3	5	12	15	1:4
Medium															
LIPID 5 % NaCl	42,3 ±2,1	70,0 ±2,6	Full	2,4 ±0,6	8,4 ±2,5	13,0 ±4,6	6,8	6,8 ±0,3	32,3 ±1,0	41,4 ±0,8	2,1	-	1,9 ±0,5	4,9 ±3,9	18,0
10% NaCl	19,8 ±1,6	33,7 ±3,6	38,4 ±2,4	-	-	-		2,2 ±0,6	22,7 ±1,6	31,5 ±0,9	1,2	-	-	-	
15% NaCl		2,6 ±0,5	3,0 ±0,7	-	-	-			2,6 ±0,5	3,8 ±0,5	-1,3	-	-	-	
PROTEIN															
5% NaCl	8,2 ±0,8	23,4 ±2,6	26,6 ±2,2	-	-	-		2,2 ±0,2	8,2 ±0,1	9,9 ±0,5	2,7	-	-	-	
10% NaCl	-	-	-	-	-	-		*NT	NT	NT					
15% NaCl	-	-	-	-	-	-			6,2 ±0,4	7,1 ±0,9		-	-	-	
STARCH															
5% NaCl		20,2 ±0,6	25,1 ±1,1	2,3 ±0,4	5,2 ±0,4	6,4 ±0,5	3,9	1,7 ±0,1	21,8 ±0,3	27,9 ±0,7	-1,1	-	-	-	
10% NaCl	-	-	-	-	-	-			19,3 0,3	24,7 0,3		-	-	-	
15% NaCl	-	-	-	-	-	-		-	-	-		-	-	-	
PGA (pH 5,75)	57,3	Full	Full	10,6	23,6	28,5	3,7	16,2	38,2	44,4	2,3	29,9	64,2	Full	1,4
5% NaCl	±1,8			±1,2	±0,9	±1,1		±0,9	±0,6	±2,3		±2,4	±1,3		
10% NaCl	28,9 ±4,1	Full	Full	7,1 ±0,4	15,7 ±0,6	18,5 ±0,5	5,6	9,5 ±1,6	32,4 ±3,2	40,8 ±3,4	2,7	25,9 ±1,0	63,6 ±1,3	Full	1,4
15% NaCl	12,1 ±2,0	25,5 ±1,1	25,8 ±1,0			1,5 ±0,4	17,2	5,7 ±0,3	20,1 ±0,5	25,6 ±0,8	1,0	11,9 ±0,2	32,3 ±1,8	34,9 ±1,4	-0,96
PGA (pH 8,0)	6,5	19,4	32,1	-	-	-		NT	NT	NT		NT	NT	NT	
5% NaCl	±1,9	±0,9	±0,5												
10% NaCl	2,9 ±0,4	9,5 ±0,4	10,3 ±1,1	-	-	-		NT	NT	NT		NT	NT	NT	
15% NaCl	-	-	-	-	-	-		NT	NT	NT		NT	NT	NT	
CELLULOSE															
5% NaCl	11,7 ±2,3	44,7 ±1,8	57,5 ±1,0	4,7 ±1,3	16,1 ±1,6	16,8 ±4,8	3,4	9,1 ±0,1	17,2 ±0,2	21,8 ±1,7	2,6	-	-	-	
10% NaCl	-	-	-	-	-	-		-	-	-		-	-	-	
15% NaCl	-	-	-	-	-	-		-	-	-		-	-	-	

Table 5.3 Extracellular enzyme production of four species of *Aspergillus* measured as a diameter (mm) in agar-plate cultures.

	Aspergillus flavus		*Aspergillus versicolor*		*Aspergillus candidus*		*Aspergillus glaucus* sp.	
Days	10	15	10	15	10	15	10	15
Medium LIPID								
5% NaCl	>88	>88	-	-	26,9 ±0,8	56,6 ±0,9	-	-
10% NaCl	45,3 ±1,3	88	-	-	18,7 ±1,7	40,9 ±2,1	-	-
15% NaCl	2,9 ±0,6	4,5 0,6	-	-	1,9 ±0,1	3,8 ±0,5	-	-
"Reverse: Blue								
5% NaCl	✓	✓	x	✓	✓	✓	x	✓
10% NaCl	✓	✓	x	x	✓	✓	x	x
15% NaCl	✓	✓	x	x	x	x	x	x
PROTEIN								
5% NaCl	55,3 ±0,7	76,3 ±1,5	-	-	9,8 ±0,3	55,4 ±1,5	-	-
10% NaCl	-	-	-	-	NT	NT	NT	NT
15% NaCl	-	-	-	-	7,9 ±0,1	27,1 ±1,6	-	-
STARCH								
5% NaCl	19,9 ±0,1	40,1 ±1,1	28,2 ±0,1	42,3 ±0,2	20,1 ±0,4	32,3 ±1,1	-	-
10% NaCl	-	-	-	-	16,5 ±0,5	27,5 ±0,8	-	-
15% NaCl	-	-	-	-	-	-	-	-
CELLULOSE								
5% NaCl	>88	>88	29,5 ±0,5	50,5 ±0,9	NT	NT	NT	NT
10% NaCl	28,7 ±4,4	35,0 ±7,4	9,7 ±0,3	12,6 ±4,9	NT	NT	NT	NT
15% NaCl	-	-	-	-	NT	NT	NT	NT

amylase for A. candidus, amylase and cellulase for A. versicolor, with the A. glaucus species exhibiting no preferential secretory abilities. All four species exhibited pectinolytic activity.

Considered in the context of enzyme production, it may be possible to explain the sequential processes involved in the deterioration of stored seeds as a consequence of fungal contamination. Depending on the seed moisture content a succession of Aspergillus species occurs (See Chapter 3).

A. restrictus (seed moisture content 13.5 - 14.5%)
A. glaucus (seed moisture content 14.0 - 14.5%)
A. versicolor (seed moisture content 14.2 - 15%)
A. versicolor (seed moisture content 15.0 - 15.5%)
A. ochraceus (seed moisture content 15.0 - 15.5%)
A. candidus (seed moisture content 15.0 - 15.5%)
A. flavus (seed moisture content 18.0 - 18.5%)
(Christensen and Kaufmann 1969, 1974)

When comparing the effects of various Aspergillus species on pea seed germination, Christensen and Kaufmann (1974) concluded that the more xerotolerant species, such as A. restrictus have less effect on germination than the less xerotolerant species such as A. flavus. These differences in deteriorative ability may be explicable in terms of the enzymatic potentials of the fungi involved.

Incipient deterioration in seeds has been attributed to two group species - A. restrictus and A. glaucus. A. restrictus may remain active in grain for several months, provided the moisture content does not increase above a level at which it cannot compete with the other fungi. A. restrictus does not cause heating of grain, unlike A. glaucus, which may cause slight increases in temperature (Christensen and Kaufmann, 1969, 1974). It has been reported that A. amstelodami, an A. glaucus group member, was responsible for an initial increase in the fat acidity of maize meal, perhaps causing the slight increase in grain temperature recorded, but the free fatty acid levels decreased rapidly (Goodman and Christensen, 1952). Bottomley, Christensen and

Geddes (1952) and McGee and Christensen (1970) found very little increase in fat acidity with A. glaucus group members, fat acidity increasing only when A. candidus and A. flavus appeared. Flannigan and Bana (1980) and El Azzabi, Clarke and Hill (1981) found A. glaucus group members to be poor lipase producers on corn seed and rape seed oil, respectively. The results of the present work (Tables 5.2 and 5.3) support those findings.

Bottomley, Christensen and Geddes (1952) did notice, however, that with infection by an A. glaucus group member there was a rapid loss in the level of non-reducing sugars in the seed, while Ramsted and Geddes (1942) using similar infections found a decrease in both the reducing and non-reducing sugar levels. The observations presently reported are in accordance with those findings, the A. glaucus species being more efficient at utilizing mono- and disaccharides than at producing enzymes to break down the starch, lipid and protein. Although this species did not establish on the nutrient-deficient starch agar (Table 5.2) thus suggesting an inability to produce amylases [also found by Flannigan and Bana (1980)] it may produce glucosidases which reduce the dextrins present in the seed to glucose. The reported heating may be explained in terms of such activity.

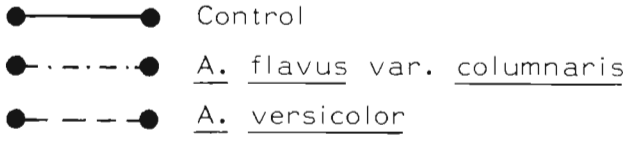
A. glaucus species may gradually increase the moisture content of the grain by their metabolic activities, and if the moisture content increases sufficiently, less xerotolerant species may establish. Thus an increase of A. glaucus activity may not be highly damaging in itself, suggesting weak pathogenicity, but such an increase in moisture content bodes future trouble.

Invariably A. candidus succeeds A. glaucus but a number of less xerotolerant species such as A. versicolor and A. ochraceus may further modify the micro-environment of the seed. This investigation has shown that A. versicolor is not a prolific extracellular enzyme producer (Table 5.3). There was little lipolytic activity, as also found by El Azzabi et al., (1981), although Dirks, Boyer and Geddes (1955) found that A. versicolor produces an active lipase on wheat germ oil. A. versicolor was

also only weakly proteolytic (Fig. 5.1) and on nutrient-deficient protein agar no growth was observed (Table 5.2). The enzymatic capabilities of this species lay in its ability to produce amylase and cellulase (Table 5.3 and Fig. 5.2). A. versicolor produced more amylolytic ativity than did A. flavus var. columnaris or A. candidus under conditions of mild osmotic stress. However, only A. candidus continued to show amylolytic activity when grown on a medium containing 10% NaCl. The cellulase activity of A. versicolor surpassed that of A. flavus var. columnaris (Fig. 5.2). The total reducing sugar levels of the extract of A. versicolor inoculum exceeded that of A. flavus var. columnaris indicating that the enzymes produced by A. versicolor were prolific on media where simple sugars had been incorporated (Table 5.1). This suggested that A. versicolor utilised these carbon sources with a greater efficiency than the other sources, e.g. protein and lipid. However, the relatively rapid growth on maize meal agar which contained a large proportion of carbohydrate also pointed to the ability of A. versicolor to produce the polysaccharide-hydrolysing enzymes (Table 5.1).

According to Christensen and Kaufmann (1969, 1974), A. candidus infection may bring about seed spoilage in four days. Furthermore, the fungus is capable of increasing the temperatures within the seed pile to 55 °C. Since A. flavus (seed moisture content 18 - 18.5%) invariably follows A. candidus (seed moisture content 15 - 15.5%), it appears that A. candidus is capable of increasing the seed moisture content by up to 3% in a short time. Heating and water liberation are an indication of high metabolic rate. A. candidus, like A. flavus, is responsible for increasing the free fatty acid content of grain (Christensen and Kaufmann, 1969, 1974; McGee and Christensen, 1970) and also produces an active lipase on wheat germ oil (Dirks et al., 1955) and on rape seed oil (El Azzabi et al., 1981). From the results (Table 5.3) obtained in the present investigation, it would appear that A. candidus in culture produces active lipase, protease and amylase (even at the higher osmotic potentials), the first of which, particularly, would result in large quantities of water and heat liberation (Kent-Jones and Amos, 1957; Lehninger, 1975). There is

Fig. 5.1 Protease activity, measured as the ability to hydrolyse casein, of the crude extract of the control and Aspergillus versicolor and Aspergillus flavus var. columnaris maize broth.



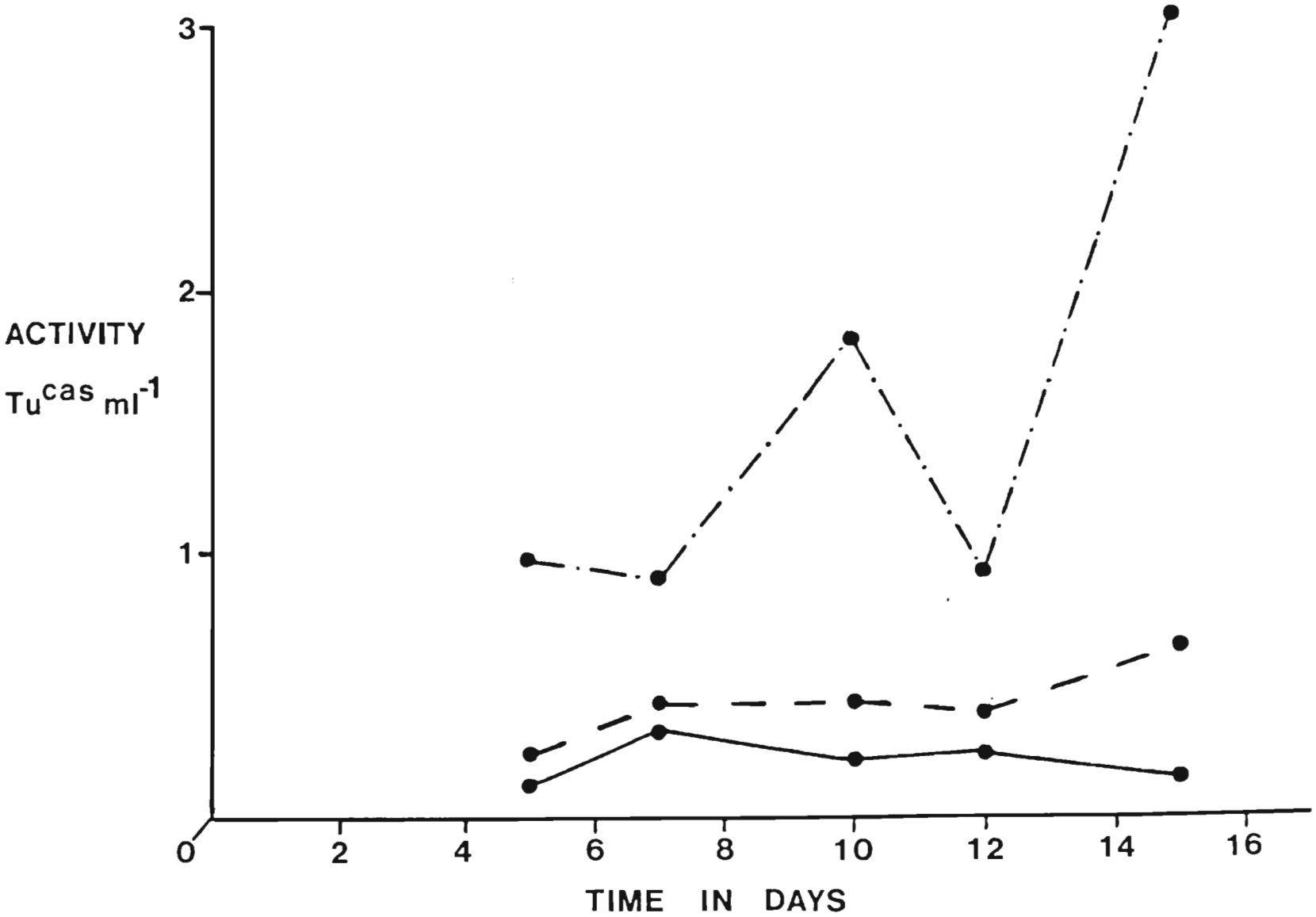
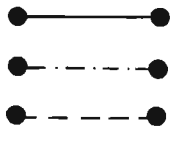

ACTIVITY
Tu^cas ml^-1
3
2
1
0
2
4
6
8
10
12
14
16
TIME IN DAYS

Fig. 5.2 Cellulase activity, measured as the ability to liberate glucose from cellulose filter paper, of A. flavus var. columnaris, A. versicolor and the control extracts.



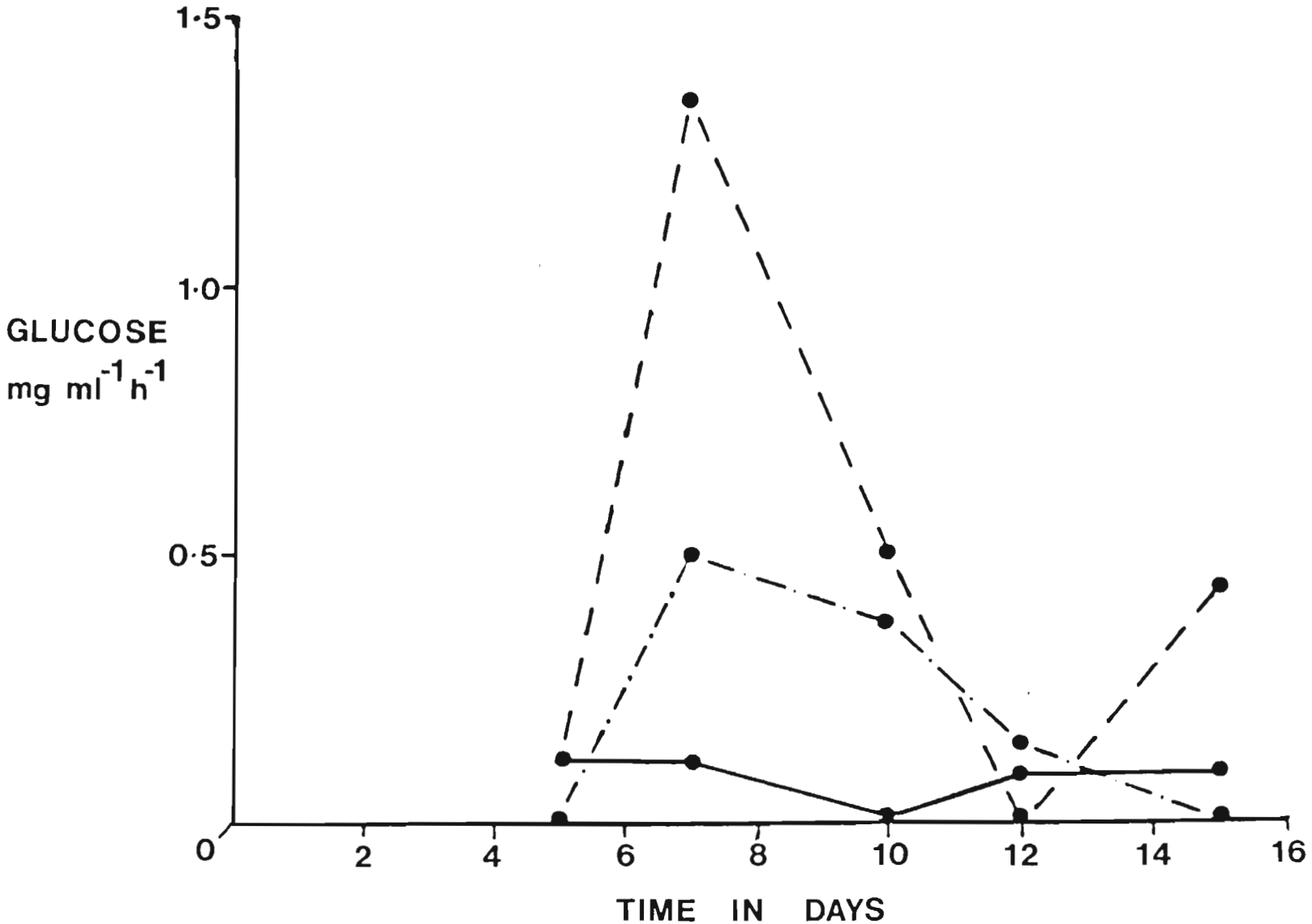
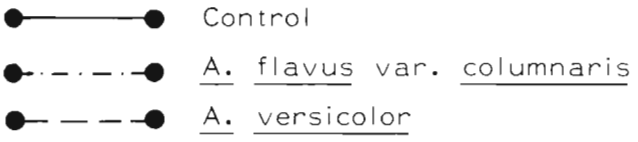
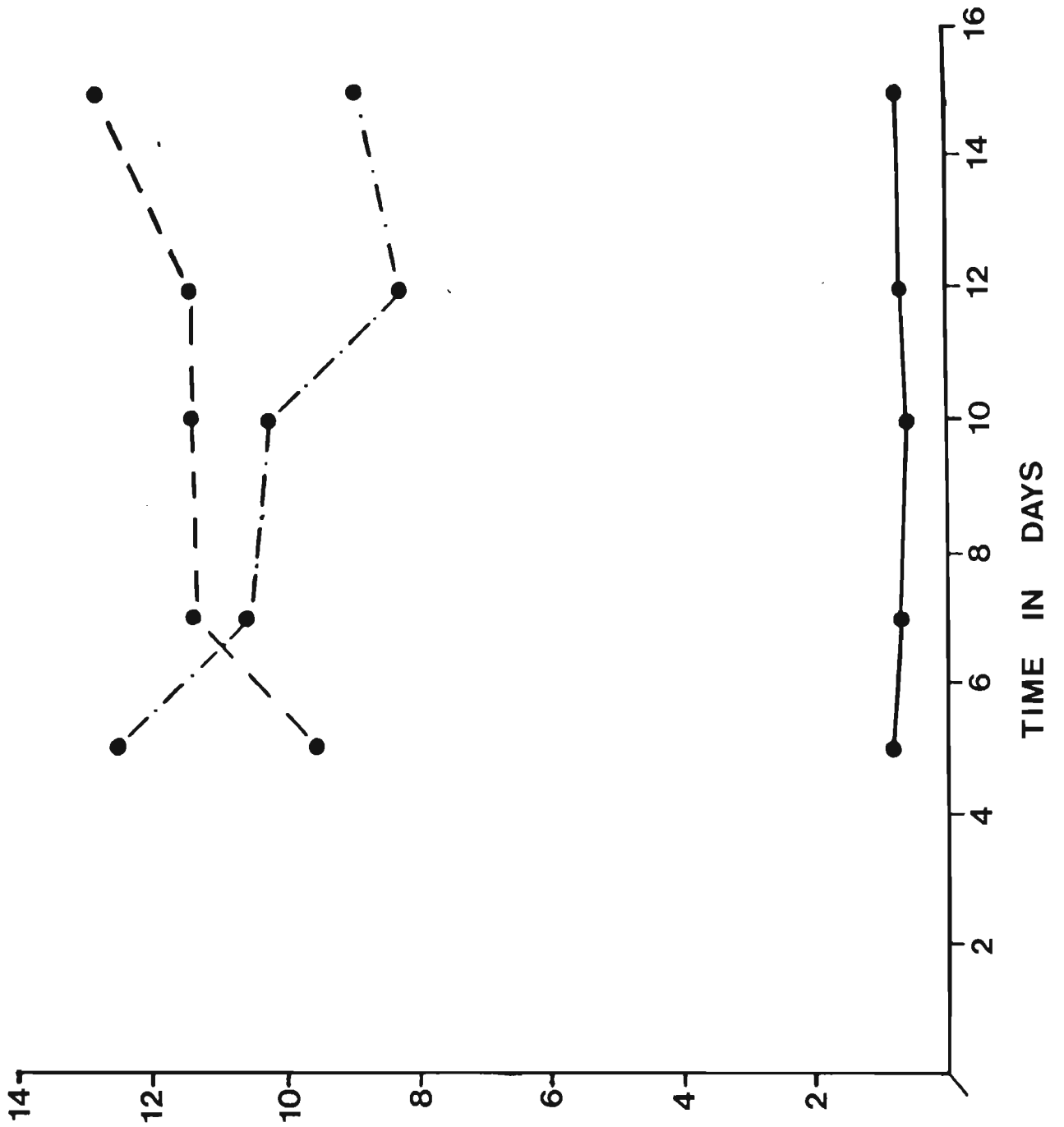

1·5
1·0
0·5
0
GLUCOSE
mg ml-1 h-1
2
4
6
8
10
12
14
16
TIME IN DAYS

Fig. 5.3 Total reducing sugar levels (mg/ml) of crude extracts of the control and A. flavus var. columnaris - and A. versicolor - inoculated maize broth.




GLUCOSE
mg ml-1
14
12
10
8
6
4
2
2
4
6
8
10
12
14
16
TIME IN DAYS

little information on the levels of free amino acids in deteriorating seeds, but Zeleny and Coleman (1939) suggested that protease activity is of little consequence in the early stages of seed deterioration. According to those authors, amino acids are released slowly and are not detectable until grain has reached an advanced stage of deterioration.

A. flavus, which is well known for its lipase and protease potentials, is also responsible for the heating of stored grain and increases in free fatty acid levels (Christensen and Kaufmann, 1969, 1974; McGee and Christensen, 1970). In sealed storage, the large amount of water liberated from grain infected with this fungus points to a high metabolic turnover, and to lipid and protein oxidation. The present investigation attributes vigorous lipolytic and proteolytic activities to A. flavus var. columnaris, with the former occurring at low water activities. Moderate amylolytic and cellulase activities (Table 5.3 and Figs. 5.2 and 5.3) also appear to characterise this species. Although this fungus is obviously capable of degrading carbohydrates, its greatest deteriorative potential lies in the proteolytic and particularly, in its lipolytic activities.

Several features regarding extracellular enzyme production became apparent from these studies:

i. The fungi differ in their ability to produce extracellular enzymes.

ii. The level of enzyme activity varies with the fungus involved.

iii. Osmotic potential affects enzyme production as well as the ability of the fungus to grow.

iv. The spectrum of enzymes produced increases from the more xerotolerant to the less xerotolerant of the fungal species.

These features may be related to the succession of Aspergillus species (Christensen and Kaufmann, 1969, 1974) which have been described as being associated with the spoilage of stored seed.

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Chapter 6

Relationship between extracellular enzyme production and location of storage fungi in maize seed.

Conference Presentation

Mycock, D.J., Lloyd, H.L. and Berjak, P. (1987). The relationship between extracellular enzyme production of four seed storage fungi and their location in maize seed. Proceedings of the South African Association of Botanists.

Publication

Mycock, D.J. and Berjak, P. Location of some storage fungi in maize seeds in relation to their extracellular enzyme capabilities (In prep).

6 Relationship between extracellular enzyme production and location of storage fungi in maize seed.

6:1 Introduction

It has long been contested that moisture content is the major factor determining the succession of fungal species associated with stored seed (Christensen and Kaufmann, 1974). However, other factors may play an equal, or even more important rôle. In this regard interspecific fungal interactions and competition are major factors, and these in turn may depend on the spectrum of extracellular enzymes elaborated by each species (McLean and Berjak, 1987). Furthermore, the precise location of the mycelium of a particular storage fungus within a seed may also be dependent on its enzymic capabilities. It was this aspect which was examined and reported in this chapter. Four fungal species viz. Aspergillus chevalieri (Mangin), an A. glaucus group member; Aspergillus sydowi (Bain and Sart.), an A. versicolor group member; Aspergillus oryzae (Ahlb.) Cohn a member of the A. flavus group and Penicillium pinophilum were used. These species were chosen because they each came from a different group within the succession of storage fungi. Additionally, the results obtained in the previous chapter indicated that the less xerotolerant fungal species were more prolific extracellular enzyme producers than the more xerotolerant species, thus the choice of the present species also served to verify the observed trends.

6:2 Materials and Methods

6:2:1 Seed

Caryopses (seeds) of Zea mays (var. Hickory King) were obtained from the Pioneer Seed Company, Greytown, Natal, South Africa and stored at 4 °C until use. Moisture content on removal from this storage was 10.2%.

6:2:2 Hot Water Treatment

The seeds were hot-water-treated and surface-sterilised to reduce as far as possible the inherent infection. The procedure described in 4:2:2 was utilised.

6:2:3 Internal Infection

Seeds were tested both prior to and after the hot water treatment, using the procedures outlined in 4:2:3.

6:2:4 Storage

After hot-water treatment and surface sterilisation (see 4:2:2), excess water was blotted with sterile filter paper and the seed dried to a moisture content of 11% in a stream of dry air. Experimental seeds were infected by dusting with the spores of Aspergillus chevalieri, Aspergillus oryzae, Aspergillus sydowi and Penicillium pinophilum prior to storage. Uninoculated seed acted as a control. All the fungal species had been isolated from maize seed locally and had been identified by the Mycological Research Unit, of the Department of Plant and Seed Protection, Pretoria. A sufficient quantity of seed was stored at 95% RH (Thewlis, Glass, Hughes and Meetham, 1961) and 25 °C to allow fortnightly sampling for six weeks.

6:2:5 Microscopy

Twenty seeds were removed from the storage bins every fortnight, each seed was halved longitudinally through the embryo and prepared for scanning electron microscopy (procedures detailed in 2:2:5).

6:2:6 Internal Infection

At each sampling, 50 seeds from each storage bin were tested for internal infection.

6:2:7 Germination Studies

6:2:7:1 Moisture content

The moisture content of 30 seeds was determined gravimetrically and expressed on a wet mass basis at each sampling (see 3:2:2 for methodology).

6:2:7:2 Germination and Establishment

Fifty seeds were set to germinate under sterile conditions. Percentage germination was scored after 96 hrs. These seeds whether germinated or not were then planted in sterile vermiculite. Seedling dry mass was determined after 14 days.

6:2:8 Studies using Agar Plates

The ability of the four seed-storage fungi to utilise protein, starch, lipid, polygalacturonic acid (PGA), cellulose, and glucose as sole carbon sources was tested using solid medium techniques (5:2:1). The plates were poured such that each contained 15 ml of medium, and were incubated at 25 °C. For each species (25 replicates) two diameters (mm), at right angles, were measured after 10 days of incubation.

6:3 Results and Discussion

Of the four species investigated, A. oryzae grew most rapidly on all media tested, thereby demonstrating its ability to utilise efficiently carbon sources as diverse as protein, starch, lipid, PGA, cellulose and glucose (Table 6.1). A. oryzae, although capable of sustained growth on PGA and cellulose did not utilise those carbon sources as efficiently as A. sydowi, in that only the latter had reached the stage of sporulation after 10 d. A. sydowi also grew well on protein, starch and glucose enriched media (Table 6.1). However, A. sydowi grew slowly on the lipid medium only. In contrast to these two species, A. chevalieri and P. pinophilum grew slowly on all media tested.

As was found previously (Chapter 5), the less xerotolerant species of storage fungus have greater extracellular enzyme capabilities. A. oryzae (an A. flavus group member) exhibited a wide range of extracellular enzyme capabilities, whereas A. sydowi (a member of the A. versicolor group) which is more xerotolerant, efficiently utilised a narrower range of substrates. A. chevalieri (a member of the A. glaucus group) had the weakest potential of the aspergilli tested and is also known to be the most xerotolerant (Raper and Fennell, 1965; Christensen and Kaufmann, 1974). The penicillia are said to invade seed with moisture contents between 15 and 18%, and as such are moderately xerotolerant (Christensen and Kaufmann, 1969, 1974). It could therefore be expected that Penicillium species are capable of utilising a fairly wide range of substrates. However, the present results do not support this, and it is possible that P. pinophilum is more xerotolerant than expected. Cazalet and Berjak (1983) have found that Penicillium species can grow in sugar cane seed at moisture contents below 15%.

The untreated seeds showed an internal infection level of 75% comprising Aspergillus 61%, Fusarium 11% and Penicillium 3%. Hot-water treatment reduced this internal infection level to 25% comprising Aspergillus 5%, Fusarium 9% and bacteria 11%. Heat treatment had no effect on germination which was maintained at 98%.

Table 6.1 Average of 50 diameters (mm) of the four seed-storage fungi grown on media containing single carbon. Twenty five colonies of each species were measured after 10 days incubation at 25°C.

	A.oryzae	*A.sydowi*	*A.chevalieri*	*P.pinophilum*
PROTEIN	57.0 ±1.3	42.0 ±0.6	20.0 ±0.7	19.0 ±0.1
STARCH	88.0 ±4.4	55.0 ±1.6	24.0 ±1.1	20.0 ±0.5
LIPID	57.0 ±1.9	23.0 ±0.8	31.0 ±0.5	21.0 ±1.0
PGA	51.0 ±1.0	60.0 ±2.1	22.0 ±1.1	22.0 ±0.9
CELLULOSE	58.0 ±2.3	50.0 ±1.7	22.0 ±0.3	22.0 ±1.0
GLUCOSE	84.0 ±4.9	78.0 ±3.2	30.0 ±1.5	19.0 ±0.3

Despite the remaining inherent infection, there was no detectable fungal growth on the hot watered treated control stored seeds at any stage, nor were any of the four fungi used experimentally isolated from this material. However, the four storage fungi under investigation were each isolated at every sampling from the appropriate experimental seed batch.

In all cases, after 14 days of storage, some of the spores with which the seeds had been dusted had germinated and hyphae were seen ramifying over the seed surfaces (Fig. 6.1) and in most cases asexual reproductive structures had developed (Fig. 6.2). The peduncular tissue was also infected (Fig. 6.3). At this stage, hyphae of P. pinophilum, A. chevalieri and A. sydowi had not penetrated the internal seed tissues, but there was evidence of invasion of the cotyledon and peri-embryonic tissues by A. oryzae (Fig. 6.4). The observed damage to the embryonic tissues was corroborated by the increasing decline in germination totality (Fig. 6.11) and the decline in dry mass of plants developed from those seeds (Fig. 6.12). Collectively these results could be correlated with the greater enzymatic versatility of A. oryzae.

After one month in storage, the hyphae of the other three species had advanced into the cotyledonary tissue (Fig. 6.5). But at that point the more vigorous A. oryzae had invaded all the internal seed tissues and these were already showing considerable deterioration (Fig. 6.6). After a further two weeks, the mycelium of that species was firmly established and the seeds were extensively degraded (Fig. 6.7). By this final sampling stage, the hyphae of A. sydowi (the second most vigorous of the fungi tested on the various carbon sources) were present in the peri-embryonic tissues but not in the endosperm. A. chevalieri, which generally exhibited a far slower growth on the various carbon sources, had advanced into the seed but not invaded the embryonic axis proper, or the endosperm (Fig. 6.8). No hyphae of Penicillium mycelium were apparent in either the embryonic axis or the endosperm, with the species appearing to be limited to the scutellum (Fig. 6.9).

Two Weeks In Storage

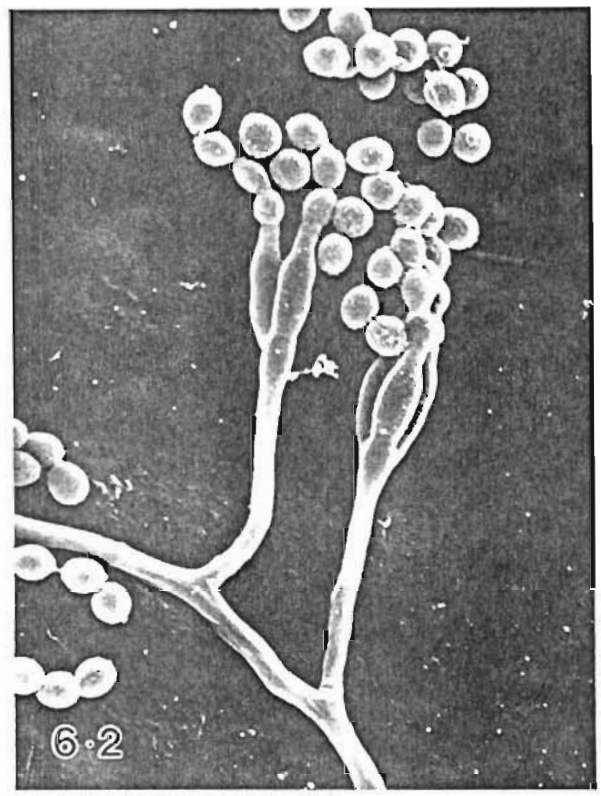
Fig. 6.1 After 14 days in storage some spores of all species tested had germinated. In this micrograph hyphae of A. sydowi are seen ramifying over the surface of a seed (× 300).

Fig. 6.2 Asexual reproductive structures of P. pinophilum (× 4 400).

Fig. 6.3 Hyphae of A. oryzae on the surface of the peduncle (× 3 000).

Fig. 6.4 Hyphae (h) of A. oryzae infecting the peri-embryonic (e) tissues (× 60).

6·1



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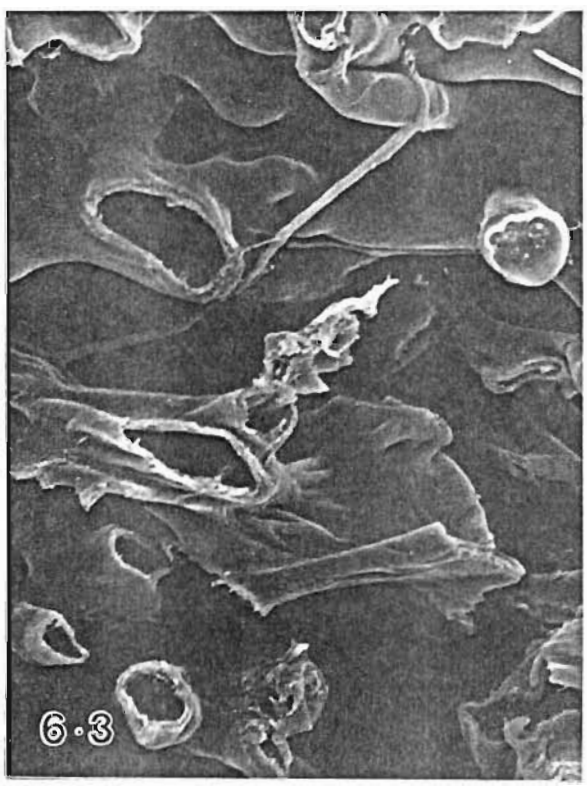

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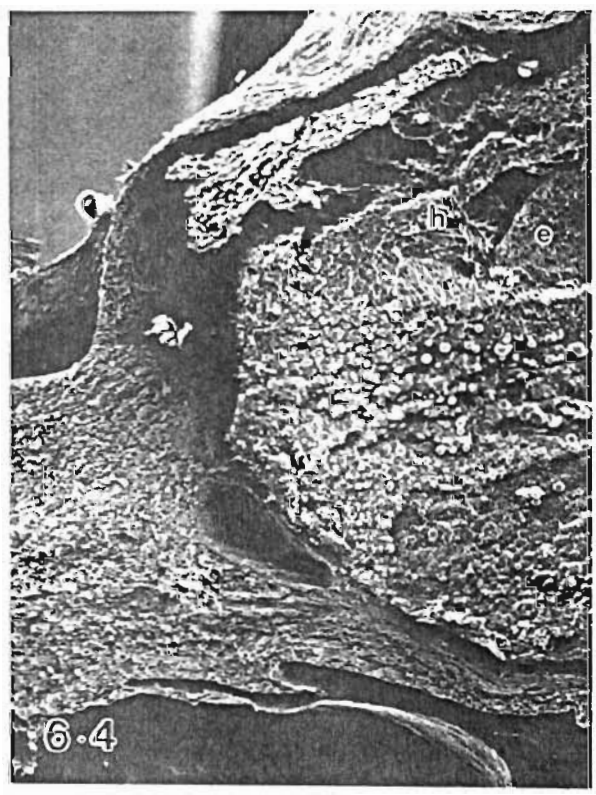
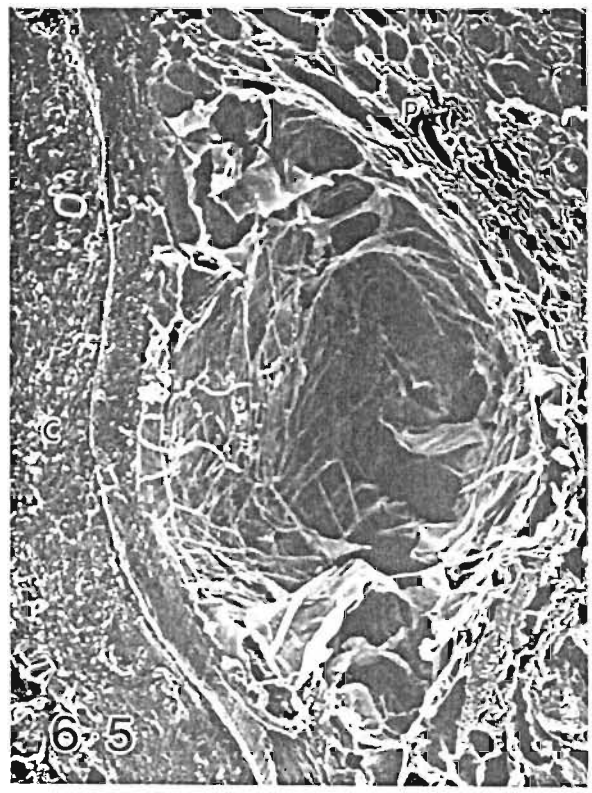


6·4

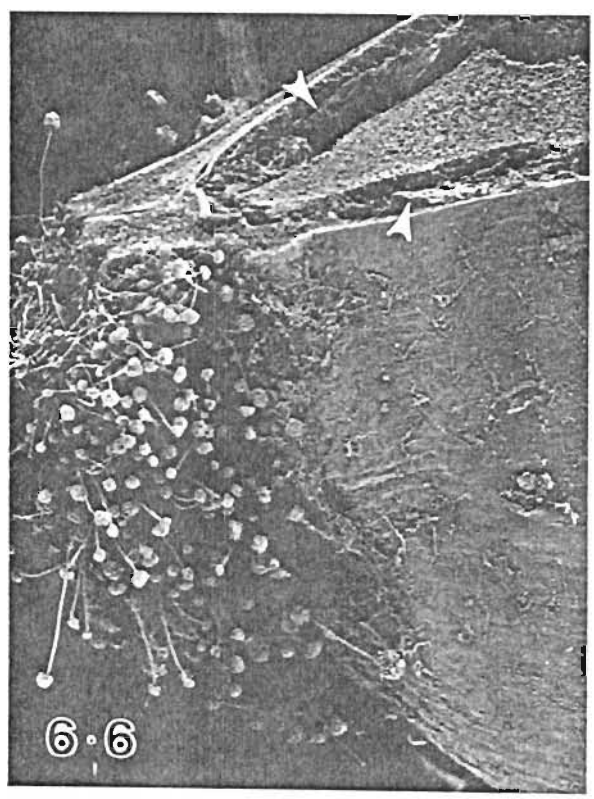
Four Weeks In Storage

Fig. 6.5 Hyphae of A. sydowi penetrating from the peduncle (p) into the cotyledon (c) (x 600).

Fig. 6.6 Degradation (arrows) of the internal tissues by A. oryzae. Note also the abundance of reproductive structures (x 40).



6·5

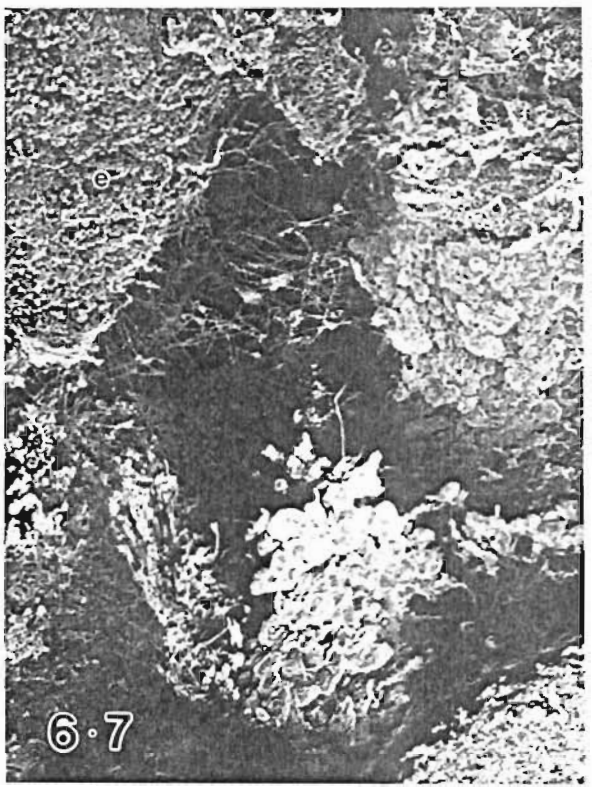


6·6

Fig. 6.7 Remains of the embryonic axis [e]. A. oryzae infection (x 100).

Fig. 6.8 Infection of the cotyledons [c] and peri-embryonic [pe] axis by A. chevalieri (x 80).

Fig. 6.9 Hyphae of the P. pinophilum penetrating into the scutellum [s] (x 300).



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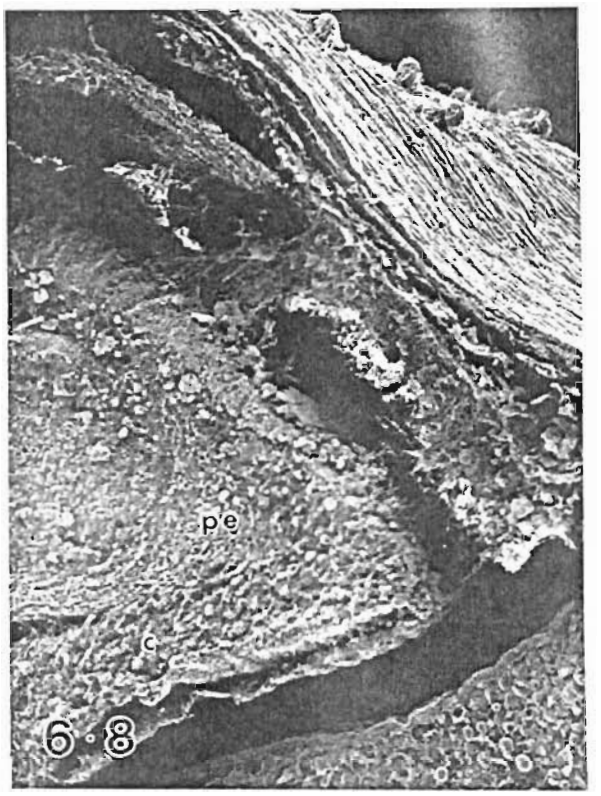

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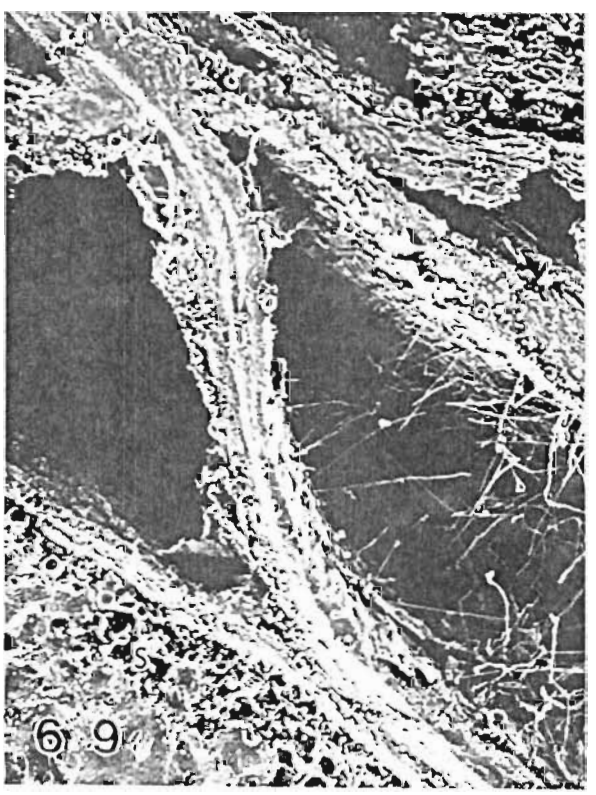


6·9

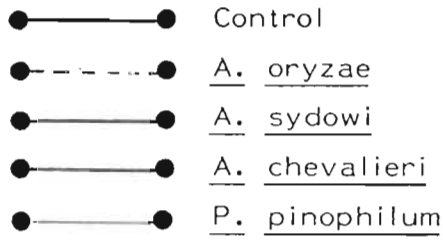
With increasing storage time there was an increase in seed moisture content and in all cases the infected seeds were wetter than those of the controls (Fig. 6.10). Further, the increase in moisture content of the various batches relative to the controls, declined in parallel with the degree of invasion of the seed by each specific fungus (Fig. 6.10). These data emphasize that the storage fungi not only have a significant effect on moisture content but also that the extent to which moisture content is elevated may be correlated with the metabolic activities of the fungal species involved. This in turn appears to determine the rate of invasion and the location of the mycelium within the seed.

The seed viability (assessed by germination potential) also declined during the experiment (Fig. 6.11). The drop in viability of the control material was more than likely due to the storage conditions, which were akin to those used in accelerated ageing (Justice and Bass, 1978). Over and above this decline, the loss paralleled the extent to which each seed batch was infected by each fungal species (Fig. 6.11). The viability of the seed sample infected by A. oryzae (the most vigorous fungus) dropped by about 90% to 10%, whereas that of the P. pinophilum (the least vigorous fungus) dropped by only some 30%.

The effect of each storage fungus on the vigour of the surviving seeds was shown by the reduction in dry mass of the seedlings (Fig. 6.12). Once again the seedlings developed from seeds infected with A. oryzae showed the greatest reduction compared with the control. Similarly with the other fungal species the less vigorous the seed infection the less the effect on the plants.

All the species tested in this study gained access to the interior of the intact seed via the peduncle and then through the micropyle. This supported and emphasized the results obtained in Chapter 4 and further indicated that externally located seed storage fungi utilise the path of least resistance in their invasion of the intact seed. Infection in this manner allows access to the scutellum, the embryonic axis and the endosperm in that order.

Fig. 6.10 Moisture content of both the control and infected seeds over the test period.



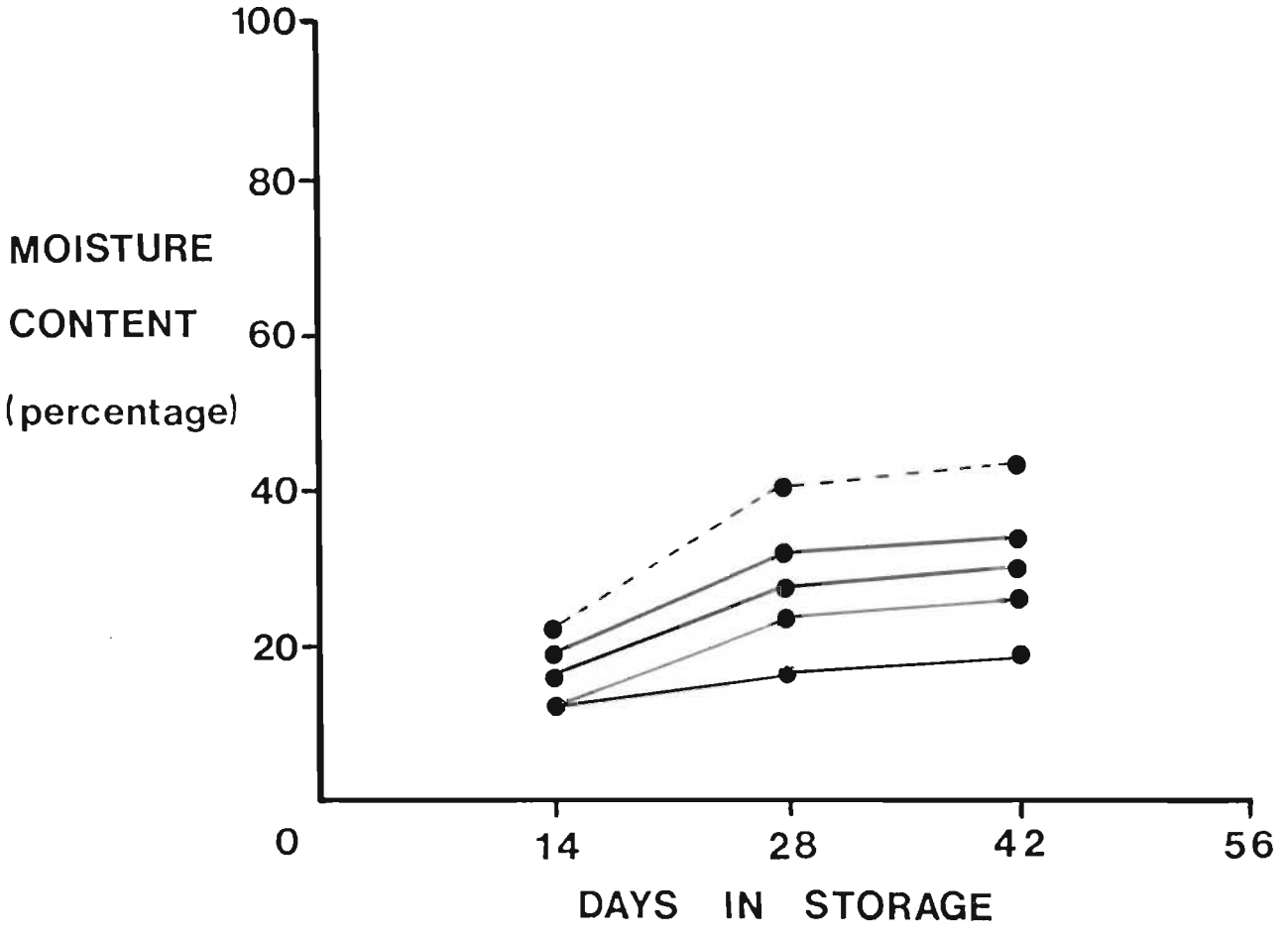
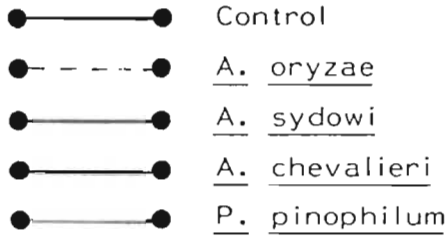

100
80
60
40
20
0
MOISTURE
CONTENT
(percentage)
14
28
42
56
DAYS IN STORAGE

Fig. 6.11 Germination potential of the stored seeds over the experimental period.



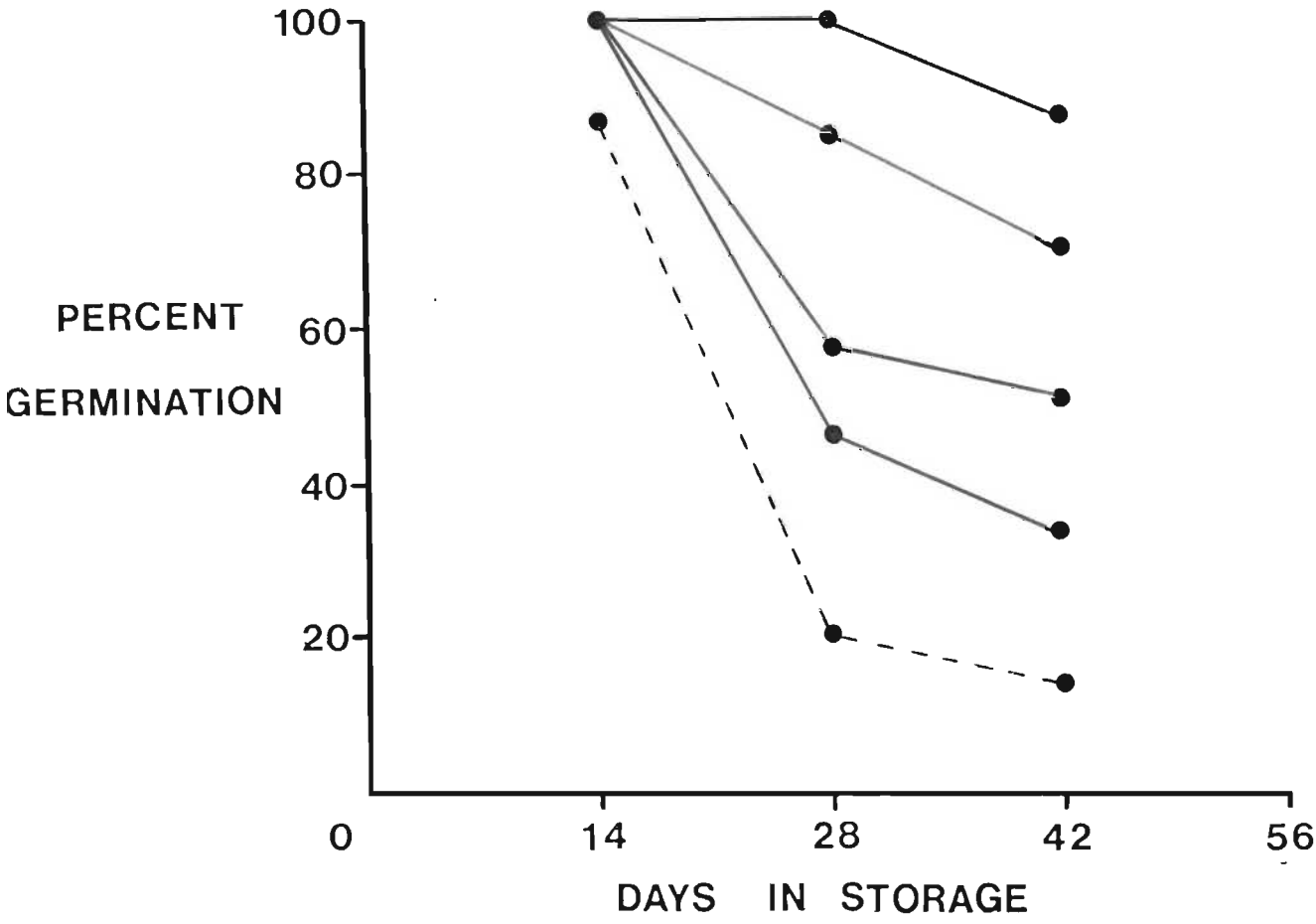
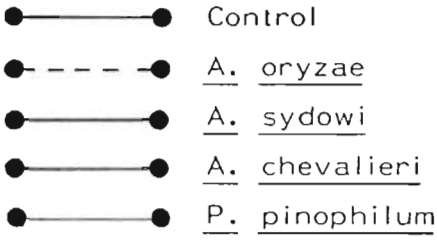
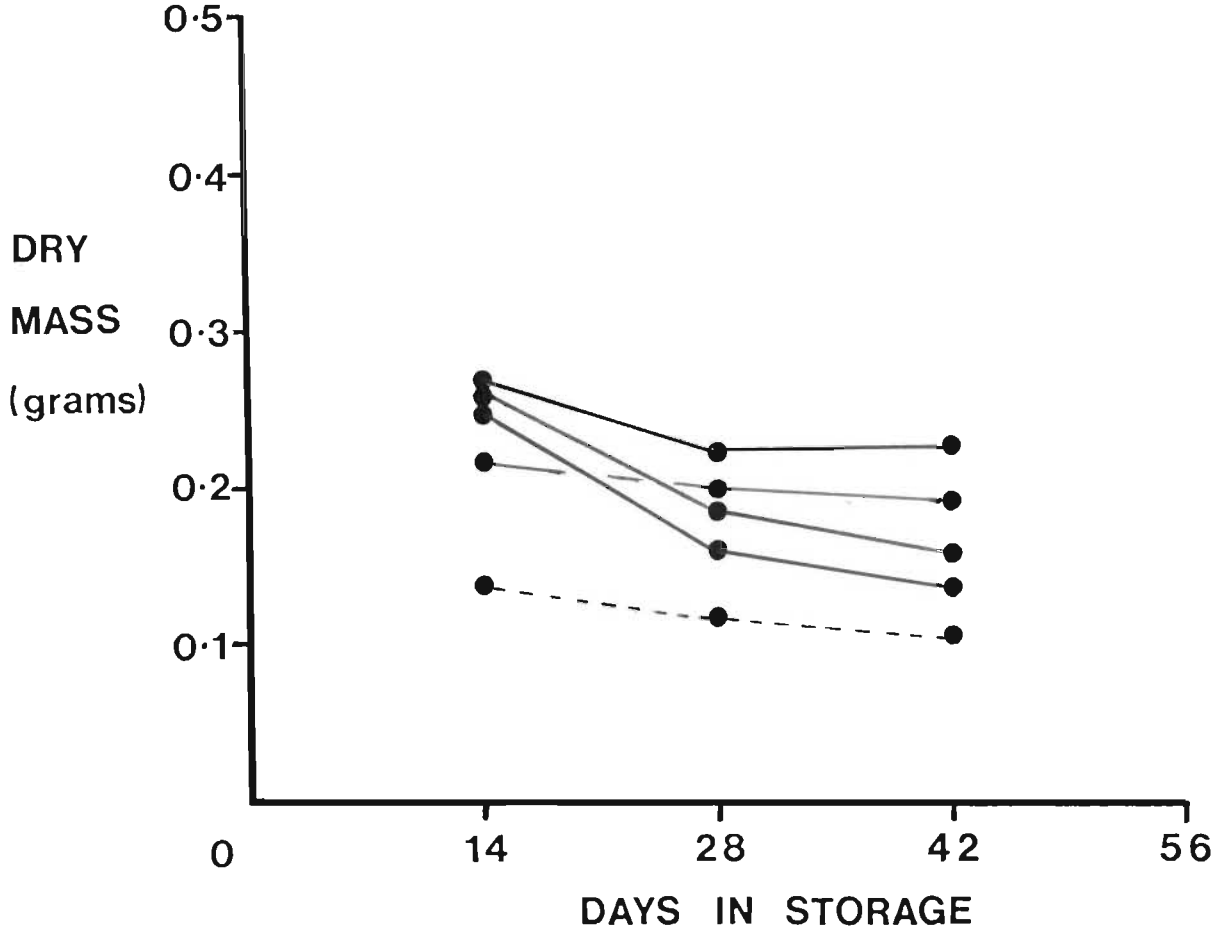

100
80
60
40
20
PERCENT
GERMINATION
0
14
28
42
56
DAYS IN STORAGE

Fig. 6.12 Dry mass of 14-day-old seedlings developed from both the stored control and infected seeds.




0·5
0·4
0·3
0·2
0·1
DRY
MASS
(grams)
0
14
28
42
56
DAYS IN STORAGE

However, the rate of invasion and the location of the mycelium vary on a fungal species basis, and this in turn appears to be related to the enzymatic capabilities of the pathogens involved.

Under the experimental conditions used in these experiments only one fungal species was associated with each seed batch, consequently the degree of deterioration was dependent on the capabilities of that particular fungus. However, in the natural situation seeds appear to harbour a wide spectrum of fungal propagules when they are harvested (McLean and Berjak, 1987) and an equally wide spectrum of spores is found in the storage environment (Christensen and Kaufmann, 1969, 1974). It is, therefore, highly unlikely that a single invasion will occur under natural conditions. Furthermore, the succession of fungal species associated with stored grain may well be achieved not only by the increasing seed moisture content and temperature due to fungal metabolism, but also by each participant in the pathway making available substrates for its successor.

In summary therefore;

i. The rate and location of infection within the seed is related to (and probably dependent on) the extracellular enzyme capabilities of individual species of seed-storage fungus.

ii. Apart from its elevating seed-moisture content through the release of metabolic water, the metabolism of a particular fungus may also make substrates available for its successor.

iii. In terms of extracellular enzyme capabilities, the less xerotolerant species are more aggressive than the xerotolerant fungal species, thus verifying the observations of Chapter 5.

iv. Infection results in the decline in both seed vigour and plant mass.

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Chapter 7

Infection of maize seedlings by *Aspergillus flavus* var. *columnaris*

Published Article

Mycock, D.J., Rijkenberg, F.H.J. and Berjak, P. (1990). Infection of maize seedlings by Aspergillus flavus var. columnaris var. nov. Seed Science and Technology, 18 (In Press).

Conference Proceedings

Mycock, D.J. Lloyd, H.L. and Berjak, P. (1986) Infection of maize by Aspergillus flavus var. columnaris. Proceedings of the Electron Microscopy Society of Southern Africa, 16, 121 - 122.

Mycock, D.J., Lloyd, H.L. and Berjak P. (1987) Infection of Zea mays seedlings by Aspergillus flavus var. columnaris. Proceedings of the South African Society for Plant Pathology, Phytophylactica, 19, 121.

Mycock, D.J., Rijkenberg, F.H.J. and Berjak, P. (1989) Infection of maize seedlings by Aspergillus flavus var. columnaris var. nov. Proceedings of the 22nd Congress of the International Seed Testing Association, Edinburgh, Scotland.

7 Infection of maize seedlings by *Aspergillus flavus* var. *columnaris*

7:1 Introduction

Apart from reducing the nutritional value and germinability of stored grains, the invasion of seed tissues by originally externally located seed storage fungi causes the loss of vigour of plants developed from those seeds (Chapters 4 and 6). Even if storage conditions are such that this mycoflora is not metabolically active, it appears that at least some of the storage fungi are present during seed development and those that are internally located can survive protracted storage (Hesseltine and Bothast, 1977; Marsh and Payne, 1984; McLean and Berjak, 1987; Chapter 3). It is therefore possible that, on seed imbibition during early germination, the concomitant germination and growth of the fungal inoculum (be it internal or external) may also affect the seed embryo growth and seedling establishment. This chapter reports an investigation on the possibility of transmission of an externally located inoculum of Aspergillus flavus var. columnaris into germinating maize seeds and thence into the establishing plants.

7:2 Materials and Methods

7:2:1 Seeds

Caryopses (seeds) of Zea mays L. (moisture content 9.6%) (q.v. Chapter 4) were obtained one month after harvest from the Pioneer Seed Company, Greytown, Natal, South Africa. Prior to the experiments, the seeds were treated with hot water and surface-sterilised to reduce possible inherent infection.

7:2:2 Hot Water Treatment

As described 4:2:2.

7:2:3 Internal Infection

As described 4:2:3.

7:2:4 Seed Inoculation

Experimental seeds were infected by dusting (using a fine brush) with the spores of Aspergillus flavus Link var. columnaris, prior to being set to germinate, the controls not being inoculated. The culture used was originally isolated from maize seed, maintained on potato dextrose agar (PDA) plus 6% NaCl and identified by the Mycological Research Unit of the Plant Protection Institute, Pretoria.

7:2:5 Infection Studies

Immediately after inoculation, seeds were set to germinate in sand or vermiculite, or on filter paper held in glass Petri dishes containing 8 ml distilled water, all substrates and containers having been sterilised. After 24, 48 and 72 h the emerged shoot was excised and aseptically halved down the long axis. One half of the shoot was surface-sterilised, (see 3:2:4:1) sliced into sections 3 mm thick, plated aseptically onto PDA and incubated at 25 °C. The remaining half was prepared for microscopy (Berjak, 1984) [See also 2:2:5].

7:2:6 Establishment Studies

Seeds were treated and infected as described above and then set to germinate on sterile filter paper. The germinated seeds were then planted in sterile soil and seedlings were allowed to establish. Five portions each of root (mid section), stem (10 mm on either side of the first node) and tissue from the apex of the youngest leaf were excised from each of ten plants at weekly intervals over a six-week period. Each tissue portion was surface-sterilised (see 3:2:4:1) under gentle vacuum, sliced into sections 3 mm thick, plated aseptically onto PDA and then incubated at 25 °C.

7:2:7 Analyses for Soil- and Air-borne Contaminants

These were conducted at weekly intervals, the former by plating out ten fresh 0.1 g samples of soil from the pots used to grow the plants in, onto PDA. Air-borne contamination was assessed by exposing ten PDA plates for 30 minutes in the immediate vicinity of the plants in the greenhouse.

7:4 Results and Discussion

As it has proved seemingly impossible to obtain totally uninfected maize seed from commercial sources, the hot water treatment was used to reduce the inherent infection as far as possible. Untreated seeds had a pathogen level of 83%, comprising species of Fusarium (75%), Aspergillus (4%) and Penicillium (4%). Hot water treatment reduced this to 10% comprising 7% Aspergillus spp. and 3% Fusarium spp. In no case, however, was A. flavus Link var. columnaris isolated from the control seeds during the investigation. Heat treatment appears to have more effect on Fusarium and Penicillium species than on the aspergilli. Since some of the aspergilli are thermotolerant (Raper and Fennell, 1977), it is to be expected that they could better tolerate elevated temperatures, at least in the short-term. However, overall survival, even of Aspergillus species, was reduced by this treatment that had no effect on seed germinability, which was maintained at 98 %.

Aspergillus flavus var. columnaris was not isolated from newly emerged shoots 24 hours after the seeds were set to germinate (Table 7.1). However, scanning electron microscopy of the experimental material at this stage revealed that there were ungerminated spores of the fungus on the emerging shoot surface. It can be assumed that these had been carried onto the shoot during germination, but were subsequently removed during the surface-sterilisation which preceded isolation trials. Furthermore, within the first 24 hours, no internal infection by A. flavus var. columnaris had yet become established within the shoot tissues. However, at the 48 and 72 hour samplings, this fungal species was isolated from the internal tissues of the shoots formed from only the inoculated seeds. The seedlings grown from the control, uninoculated seeds were not internally infected by any fungus at all (Table 7.1); however, a bacterial infection occurred in a single instance at the 72 hour sampling.

Table 7.1

Internal contaminants of shoot tissues 24, 48 and 72 hours after maize seeds had been set to germinate on 3 different substrata.

	FILTER PAPER		VERMICULITE		SAND	
	Control	Infected	Control	Infected	Control	Infected
24 hrs	—	—	—	—	—	—
48 hrs	—	*A. flavus columnaris*	—	*A. flavus columnaris*	—	*A. flavus columnaris*
72 hrs	—	*A. flavus columnaris*	—	*A. flavus columnaris*	Bacteria	*A. flavus columnaris* Bacteria

After 48 hours the surface spores had germinated and hyphae were observed ramifying over the surfaces of the shoots (Fig. 7.1). Some hyphal elements were noted apparently penetrating the stomata of the shoot surfaces (Fig. 7.2). The shoot surfaces of the seedlings germinated from seed in vermiculite or in sand, had incurred some superficial physical injury and invasion had occurred, particularly in the latter case. Hyphae were seen penetrating the lesions in these surfaces (Fig. 7.3) Presumably similar injury and invasion would occur in field germination.

After 72 hours, hyphae of A. flavus var. columnaris were observed penetrating not only the stomata and the lesions, but also apparently the intact cuticle (Fig. 7.4). However, the presence of submicroscopic lesions, not resolved by S.E.M., cannot presently be precluded. No matter what the pathway of entry, hyphae of this variety of A. flavus had penetrated into and through the coleoptile and the space between the coleoptile and primary leaves had been colonised. A particularly significant observation was that the primary leaves had been infected (Fig. 7.5), the fungus therefore having gained access to the embryonic axis. In extreme cases conidiophores and sporulating heads characteristic of this variety of A. flavus (Mycock, McLean and Berjak, 1984) were produced on the shoot surfaces (Fig. 7.6) [See also Chapter 2].

As a group the storage fungi are believed not to colonise actively metabolising plant tissue, their activity being reported as being confined to dying, dead or naturally dried organic matter (Raper and Fennell, 1977). The results of the present investigation, however, clearly implicate the storage fungi as also being opportunistic invaders of the coleoptile, and of the seedlings tissue via the stomata and surface lesions.

Coleoptile tissues begin to senesce during shoot emergence from the seed, consequently this tissue is vulnerable to invasion by a seed storage fungus. An anomaly, however, is the fact that this fungal species appears capable of colonising the actively-growing and apparently intact primary leaves. Klich, Lee and

Figure 7.1

Hyphae ramifying over the surface of shoot material developed from an artificially infected seed (× 1 000).

Figure 7.2

A hyphal element in an apparent attempt to gain access to the underlying tissues via a stoma (× 3 000).

Figure 7.3

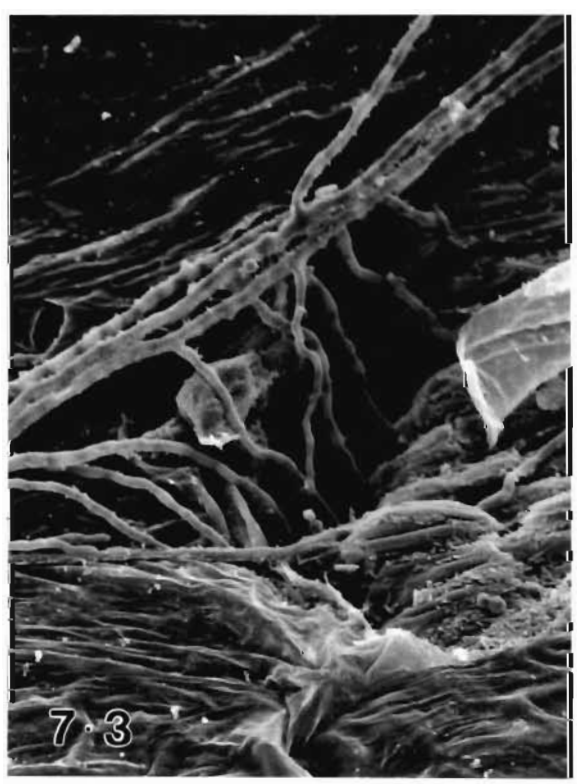
Hyphae penetrating surface lesions of the shoot (× 1 000).

Figure 7.4

Hyphal elements penetrating apparently intact cuticle (× 3 000).


7.1


7.2


7.3

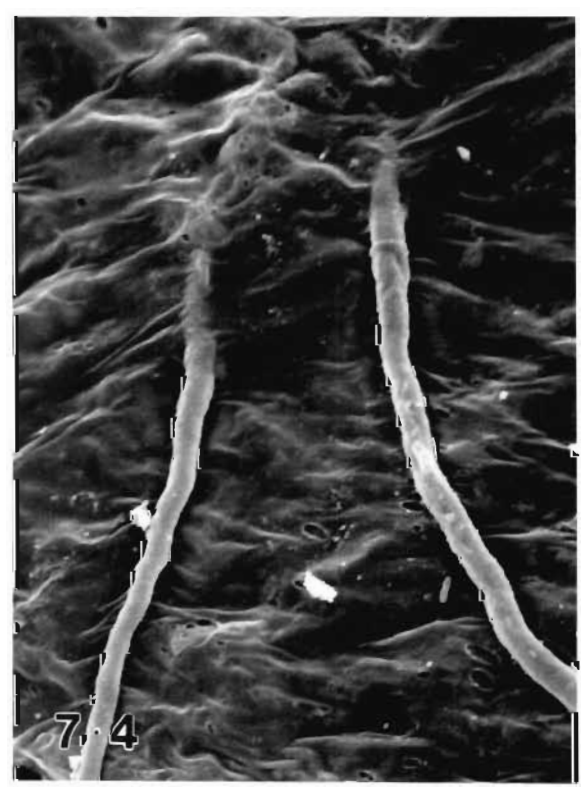
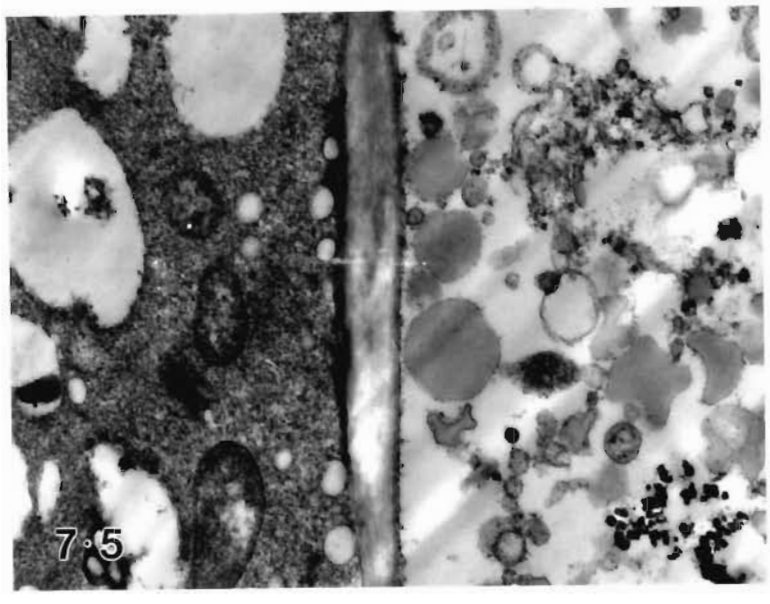

7.4

Figure 7.5

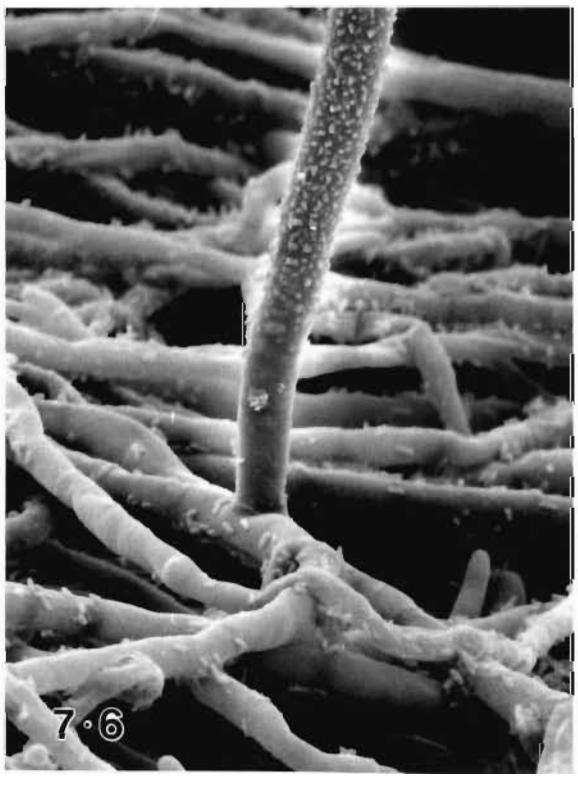
Infected leaf tissue. Note presence of cell degradation (× 11 000).

Figure 7.6

Shoot surface, showing developing conidiophore, typical of *A. flavus* var. *columnaris* (× 2 400).



7·5



7·6

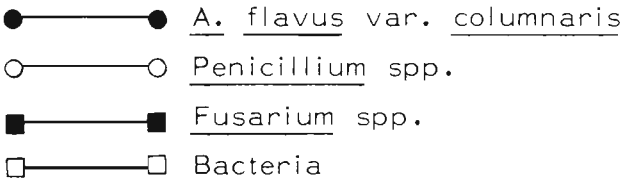
Huizar (1986) have reported the ability of A. flavus to grow in the vegetative tissues of mature cotton plants, the infection moving away from the point of inoculation. Similarly Cole, Hill, Blankenship and Sanders (1986) have found that colour mutants of A. flavus and A. parasiticus are capable of invading peanut plants.

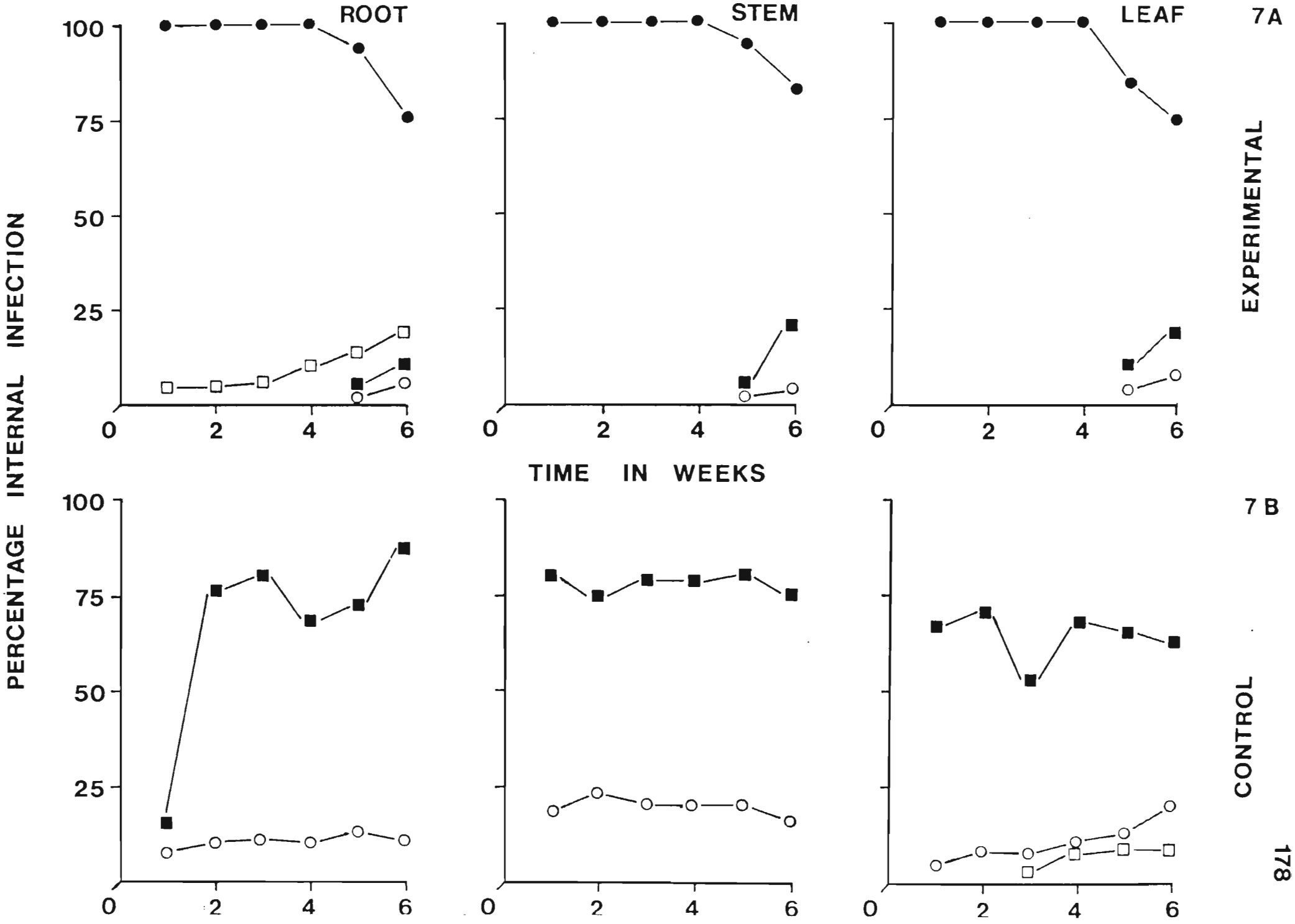
The present studies have shown that A. flavus var. columnaris can not only colonise the actively developing maize seedling shoot tissue, but that infection can also be carried through to the growing plants. In the establisment studies the seeds were allowed to germinate on sterile filter paper in order to minimise physical damage to the emerging shoots and roots, thereby reducing the possibility of opportunistic invasion of the plant tissues. Care was taken also during planting, to prevent physical damage to the seedling tissues. Throughout the test period no A. flavus var. columnaris was isolated from the soil, nor the air in the vicinity of the plants. However, other Aspergillus species, mainly A. niger, and also species of Fusarium, Penicillium and Cladosporium were isolated. Nevertheless, during the first month after planting, A. flavus var. columnaris was the only fungal species isolated from the internal tissues of root, stem and leaves of plants grown from the inoculated seed (Fig. 7.7a). After this period there was a small drop in the incidence of this isolate correlated with an increase in the isolation of Fusarium and Penicillium species. This variety of A. flavus, however, remained the dominant species in all experimental plant tissues over the six week growing period. Plants grown from the uninoculated control seeds were contaminated with both Fusarium and Penicillium species (Fig. 7.7b) but neither with A. flavus var. columnaris nor any other Aspergillus species.

Seed to seedling infection by A. flavus var. columnaris has previously been shown to take place (Mycock et al., 1988). However, in the present case it is unlikely that fungal establishment had occurred within the seeds. Instead, inoculum originally present on the seed surface is suggested to have been carried onto the surface of the emerging shoot. Precautions were

Figure 7.7

Internal contaminants of maize tissues (root, stem and leaf) over a six week period. Experimental material (7A) was grown from artificially infected seeds. Control material (7B) was not infected.




ROOT
STEM
LEAF
7A
EXPERIMENTAL
100
75
50
25
0
2
4
6
PERCENTAGE INTERNAL INFECTION
TIME IN WEEKS
7 B
CONTROL

taken to minimise opportunistic invasion via surface lesions of the plant by air- or soil-borne inoculum, thus it can be assumed that the A. flavus infection had originated from spores on the seed surface and that this fungus had invaded the tissues mainly through the stomata and via surface lesions (Fig. 7.2). Additionally perhaps, invasion may have occurred by penetration of the intact cuticle (Fig. 7.4). Invasion by this latter mode would require far more complex processes: A. flavus var. columnaris is known to produce a spectrum of extracellular enzyme types (Raper and Fennell, 1977; McLean, Mycock and Berjak, 1985; Chapter 5) certain of which would presumably be involved should such an invasion process occur.

The penetration of the cuticle and the cell wall is a common occurrence where obligate biotrophs and facultative parasites are concerned, both mechanical and enzymatic penetration into the plant cells taking place (Hudson, 1986). However, this phenomenon has not been previously reported for the aspergilli.

It therefore appears that the seed storage fungus Aspergillus flavus Link var. columnaris can colonise the tissues of a maize seedling grown from surface-contaminated (inoculated) seed and that this infection can be carried into the maturing plant. Further, penetration into the plant tissues may include more complex mechanisms than those previously attributed to a saprophyte.

The pathway of movement of this fungus in the plant tissues is yet to be determined. Scanning electron microscopical studies on this fungus have shown hyphae in the xylem elements of the peduncle of maize seed (Mycock et al., 1988), and Klich et al. (1986) have reported the presence of hyphae of this species in the xylem of the outer pigment layer of cotton seed. It is therefore possible that these passages are used by this fungus in its movements through tissues of the growing (maize) plant as well.

The results of the present investigation indicate that inoculated seed can give rise to infected plants and that infection can remain internal to those plants for at least six weeks. It can thus be postulated that aspergilli as exemplified by A. flavus var. columnaris are capable of remaining as internal contaminants of maize plants throughout the life cycle and consequently the developing seed could also be infected. This aspect is investigated in the following Chapter.

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Chapter 8

Systemic transmission of *Aspergillus flavus* var. *columnaris* from one seed generation to the next

Conference Proceedings

Mycock, D.J. and Berjak, P. (1990). Seed-to-seed transmission of Aspergillus flavus var. columnaris. Proceedings of the Annual Congress of the South African Association of Botanists.

Paper in Preparation

Mycock, D.J., Rijkenberg, F.H.J. and Berjak, P. Systemic transmission of Aspergillus flavus var. columnaris from one maize seed generation to the next.

8. Systemic transmission of *Aspergillus flavus* var. *columnaris* from one seed generation to the next

8:1 Introduction

The seed storage fungi, which comprise several species of Aspergillus and Penicillium, have traditionally been viewed as invading post-harvest seeds once moisture contents acceptable for storage have been achieved. On the other hand, species of genera such as Fusarium, Cladosporium and Alternaria (i.a.) have been classified as field fungi on the basis that they gain access to a seed during its development or, if harvested, when the moisture content (mc) is still relatively high (Christensen and Kaufmann, 1969; 1974; Christensen and Sauer, 1982). It has been accepted that the storage fungi come to replace field fungal species, the latter dying out as seed mc becomes limiting (Christensen and Kaufmann, 1974). The storage fungi appear in a predictable succession, from the most to the least xerotolerant species, and, according to Magan and Lacey (1984a; 1984b) a species that functions optimally under a certain set of conditions, is the one that is likely to predominate under those conditions.

M^{C}Lean and Berjak (1987) have suggested that, while invasion of post-harvest seed by storage fungi is certainly one means by which the species concerned gain access to the inner tissues of the seeds, there is a possibility of systemic infection as well. Those authors based this assertion on the finding that storage fungal species were present in premature maize caryopses (seeds) that were harvested and stored under conditions precluding their invasion after removal from the parent plant. Work on peanuts by Hanlin (1966) also suggested that a measure of systemic transmission of Aspergillus and Penicillium might occur, while it has also been proposed that particular A. flavus populations may invade pre-harvest corn (Guthrie, Lillehoj, Barry, M^{C}Millian, Kwolek, Franz, Catalano, Russell and Widstrom, 1982). In this regard, M^{C}Lean and Berjak (1987) have shown that a succession of storage fungal species, correlated with the spectrum of fungal propagules contaminating the seed at harvest, is manifested during storage.

Mycock, Lloyd and Berjak (1988) have demonstrated microscopically that A. flavus var. columnaris var. nov. (one of the least xerotolerant of the storage Aspergilli) will invade maize caryopses through both the peduncle and pericarp lesions, under suitable storage conditions (Chapter 4). Additionally, hyphae of this fungus originating from spores present on the surface of the germinating seed, have been shown to invade the internal tissues of the emerging shoot through lesions and stomata (Mycock, Rijkenberg and Berjak, 1990). Those authors also indicated that A. flavus var. columnaris persisted in the young plants for at least the six weeks during which they were monitored. Although the species of Aspergillus that are characteristically associated with stored seeds are generally considered to be saprophytes, various investigations have suggested that certain species are able to invade and infect growing plants, e.g. A. flavus in cotton (Klich, Lee and Huizar (1986) and colour mutants of both A. flavus and A. parasiticus in peanut plants (Cole, Hill, Blankenship and Sanders, 1986).

Considered collectively, the various reports cited above suggest that systemic transmission of storage fungi might well be one mode of seed infection. If this is the case, then the anomaly of the sudden appearance of such fungi in stored seeds is explicable in terms other than (or additional to) the persistence of their propagules in the store. While transmission of pathogens from one seed generation to the next has been reported for a number of fungal species (Hudson, 1986; Agarwal and Sinclair, 1987), there is no unequivocal evidence for systemic transmission of storage fungi. The present contribution reports on an investigation designed to ascertain whether or not seeds infected by a specific seed storage fungus will give rise to plants that transmit the infection to the following seed generation.

8:2 Materials and Methods

8:2:1 Seeds

Caryopses of a yellow maize variety (Spc 7001) were obtained one month after harvest from the Department of Agricultural Economics and Marketing of the Directorate of Plant and Liquor Control, Pretoria. The seed was stored at 4 °C, at the original moisture content of 8% until use.

8:2:2 Hot Water Treatment and Surface Sterilisation

The seed was hot-water treated at 55°C for 45 min, following a 4h pre-soak in sterile water at ambient temperature. After redrying to a moisture content of 12.5 % the seed was surface-sterilised as described previously (Mycock et al., 1990 [see also 4:2:3]).

8:2:3 Infection and storage

Two thirds of the seeds were dusted with the spores of Aspergillus flavus Link var. columnaris (WB 4818) obtained from the C.A.B. International Mycological Institute, Kew, U.K. The remaining third were not inoculated and acted as controls. In order for infection of the internal seed tissues to occur, all the seeds were stored at 85% RH and 25 °C for one month.

8:2:4 Germination and establishment:

After storage all the seeds were set to germinate under sterile conditions. Germination rate and totality were assessed every 24 h until 96 h when all the seeds were planted in sterilised soil and allowed to establish into mature plants. Half the infected material and the control were treated with a commercial systemic fungicide mixture (Ridomil MZ [Ciba Geigy] and Benlate [du Pont]) at regular intervals throughout the plant life cycle. Each of the treatments was grown in a separate greenhouse at the Durban Botanic Gardens. All the plants were

treated with insecticide (Malasol [Efekto]), acaricide (Tedion, [Efekto]) and macro- and micro-nutrients (Nitrosol [Fleuron]) at regular intervals.

8:2:5 Internal contaminants:

At weekly intervals 10 portions of root (midsection), stem (10 mm on either side of the second node) and youngest leaf apex (plus reproductive structures when formed) were taken from each of 10 separate plants from each treatment. The tissues were surface sterilised in 2% sodium hypochlorite for 20 min, rinsed with sterile distilled water, aseptically cut into 3 mm portions and plated onto Czapek - Dox agar. Contaminants were identified after incubation for one week at 25 °C.

8:2:6 Soil and air contaminants:

Soil and air contaminants were monitored every 14 d, as previously described (Mycock et al., 1990 [see also 7:2:7]).

8.3 Results and Discussion

Prior to hot water treatment the maize seeds were soaked in sterile distilled water for four h during which their moisture content was elevated from 8 to 22%, further increasing in the 45 min exposure to water at 55 °C (Fig. 8.1). Thereafter the caryopses were redried in a stream of air to a moisture content of 12.5%. However, and presumably partly as a result of the seeds coming to equilibrium with the environment, during the following month in storage at 85% RH both the control and infected seed increased in moisture content to 18 and 18.8%, respectively (Fig. 8.1). The differential increase was unexpected as, at 85% RH, the moisture content of the caryopses all originally at 12.5% should have increased by the same amount as a result of equilibration. It is noteworthy that the moisture content of the seeds experimentally infected with A. flavus var. columnaris was consistently slightly higher than that of the uninoculated, control seeds. As the storage environment was conducive to the germination of the spores and subsequent growth of A. flavus var. columnaris, the consistently higher moisture content of the experimental seeds could be attributed to metabolism of this fungus.

The hot water treatment reduced the original total infection status from 80 to 12%, predominantly by a reduction in the level of Fusarium spp. although, after one month in storage at 25 °C and 85% RH, the percentage of seeds from which these species could be isolated had risen to 20 (Table 8.1). However, this was in marked contrast to those seeds experimentally inoculated immediately prior to storage, from all of which only A. flavus var. columnaris was isolated after storage under identical conditions (Table 8.1).

Hot water treatment had no effect on germination totality which was maintained at 98%, although the rate of germination was slightly retarded until 96 h after the start of imbibition (cf. Figs. 8.2a & b). These findings are in agreement with those reported by Berjak, Whittaker and Mycock (1990) for seeds of two other maize

Fig. 8.1

Moisture content of the maize caryopses prior to, and at various stages during, treatment and storage.

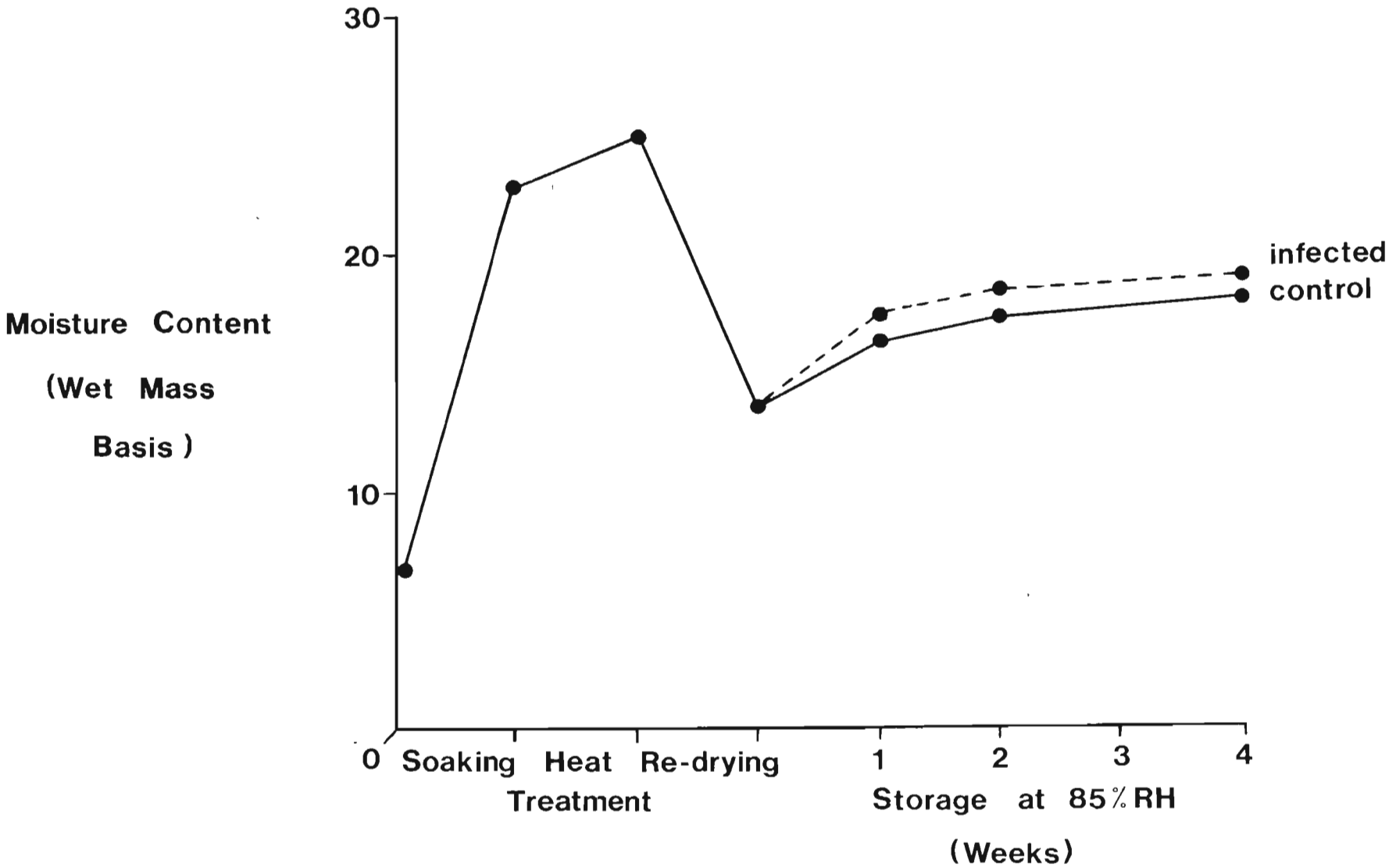
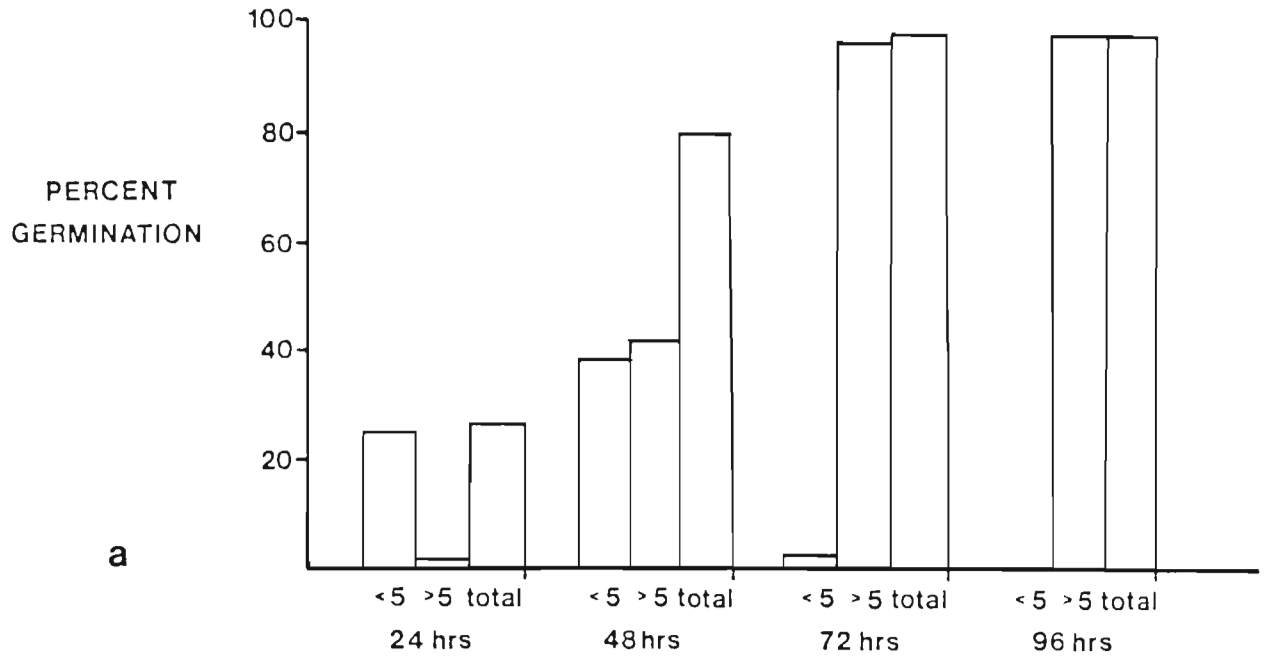

Moisture Content
(Wet Mass
Basis)
30
20
10
0
Soaking
Heat
Re-drying
Treatment
1
2
3
4
Storage at 85% RH
(Weeks)
infected
control

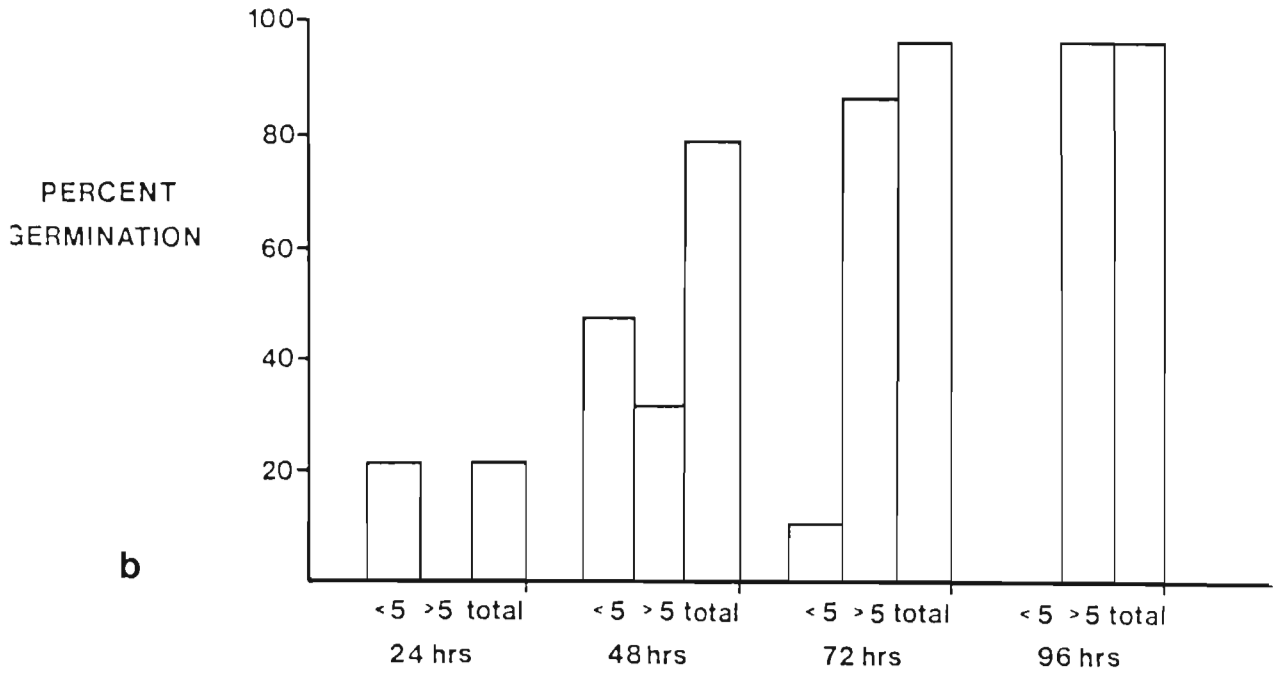
Table 8.1 Seed contaminants (%).

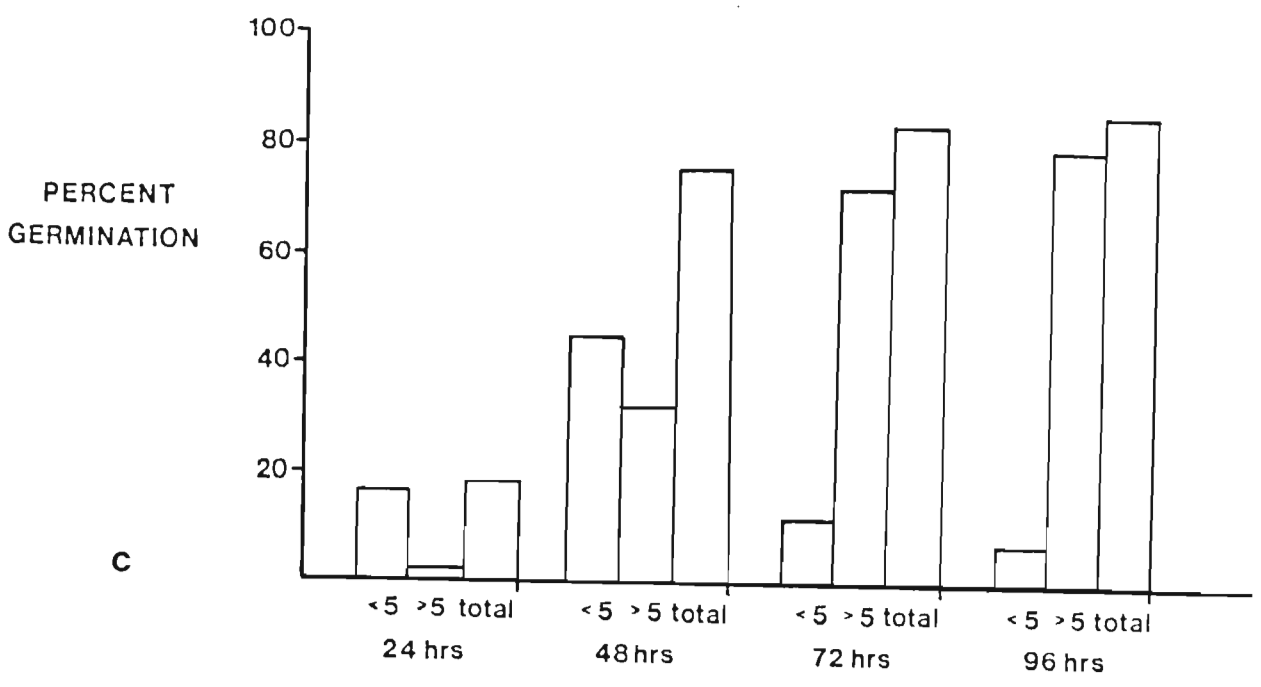
			Total	*Fusarium* spp.	*Penicillium* spp.	*A. flavus* var. *columnaris*	Bacteria
1st GENERATION SEEDS	original condition. mc: 8%		80	60	10	-	10
	immediately following hot water treatment. mc: 12.5%		12	6	4	-	2
	immediately after storage.	control mc: 18.0%	25	20	2	-	3
		experimentally infected mc: 18.8%	100	-	-	100	-
2nd GENERATION SEEDS, MILK STAGE	control seeds: plants treated with systemic fungicide. seed mc: 40%		96	60	21	-	15
	experimental seeds: fungicide-treated plants grown from seeds infected with *A. flavus* var. *columnaris*. seed mc: 38%		97	50	25	12	10
	experimental seeds: plants grown from seeds infected with *A. flavus* var. *columnaris*. seed mc: 41%		97	64	-	29	4
2nd GENERATION MATURE SEEDS	control seeds: plants treated with systemic fungicide. seed mc: 12%		94	72	10	-	12
	experimental seeds: fungicide-treated plants grown from seeds infected with *A. flavus* var. *columnaris*. seed mc: 12%		95	63	4	20	8
	experimental seeds: plants grown from seeds infected with *A. flavus* var. *columnaris*. seed mc: 13%		97	60	-	35	2

Fig. 8.2

Germination rate and totality of maize seeds: a. Prior to hot water treatment; b. Immediately after drying-back following hot water treatment; c. After one month of storage, following hot water treatment and re-dehydration.


PERCENT GERMINATION
100
80
60
40
20
a
<5 >5 total
24 hrs
<5 >5 total
48 hrs
<5 >5 total
72 hrs
<5 >5 total
96 hrs


PERCENT GERMINATION
100
80
60
40
20
b
<5 >5 total
24 hrs
<5 >5 total
48 hrs
<5 >5 total
72 hrs
<5 >5 total
96 hrs


PERCENT GERMINATION
100
80
60
40
20
c
<5 >5 total
24 hrs
<5 >5 total
48 hrs
<5 >5 total
72 hrs
<5 >5 total
96 hrs

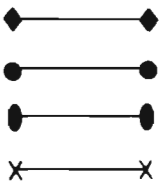
varieties. The control stored seeds exhibited germination rates and totals much the same as those immediately after hot water treatment. However, both these parameters were reduced in the stored infected seeds, which was presumably due to the fungal infection (Fig. 8.2c).

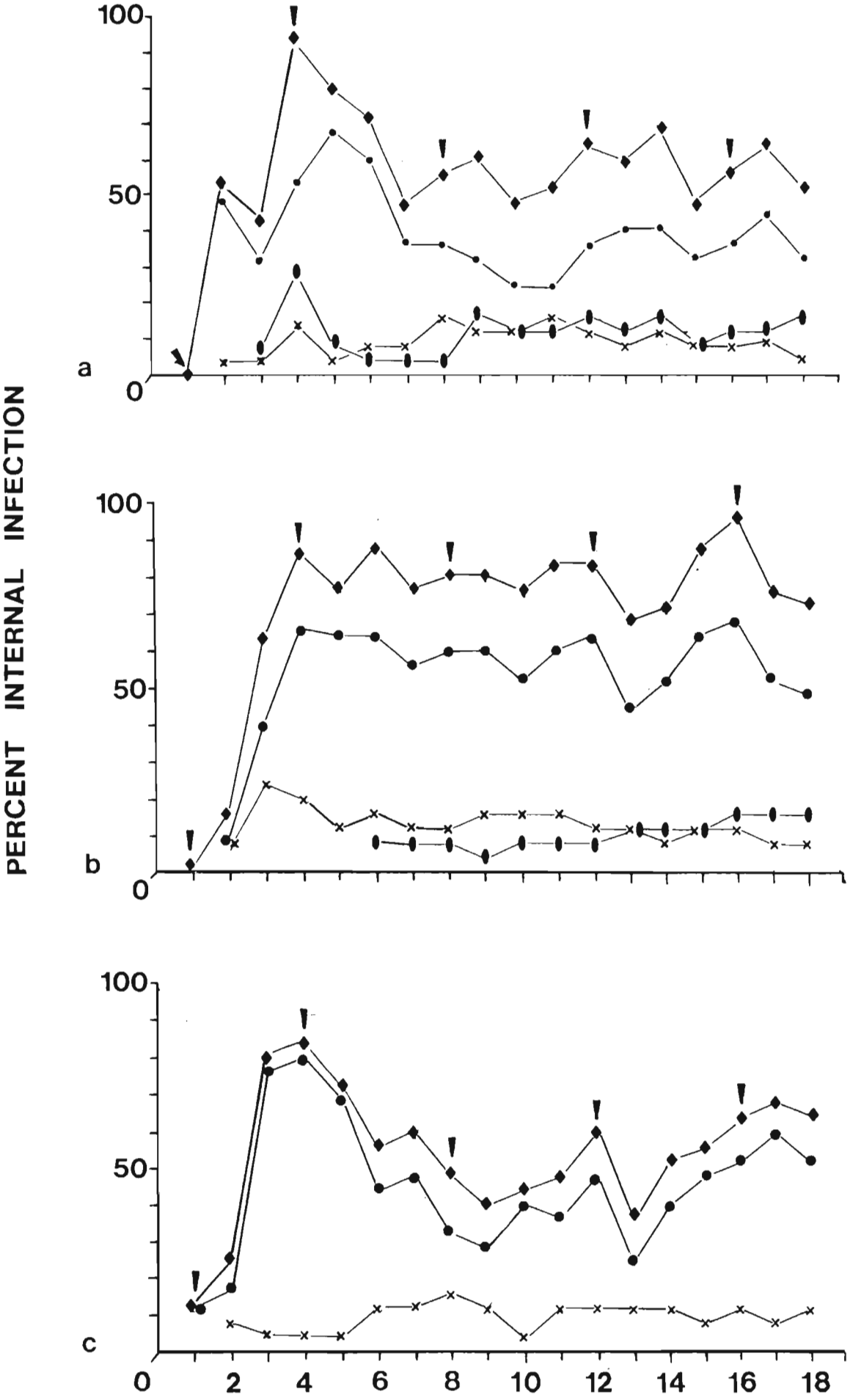
At 14-d intervals the soil and air in the greenhouses were tested for microbial contamination. Although Aspergillus niger and A. glaucus were found to be present, at no point during the experiment was A. flavus var. columnaris isolated from either of these two media. Fusarium spp. were isolated from the soil but only after the first two weeks of the experiment. Since occurrence of this genus is ubiquitous in Southern Africa (Marasas, Burgess, Anelich, Lamprecht and van Schalkwyk, 1988) their introduction into the soil could be assumed to be from air-borne spores.

Plants developed from control seeds were predominantly contaminated by Fusarium spp. with Penicillium and bacterial spp. occurring at lower levels (Figs. 8.3a - c). The Fusarium spp. appeared in the tissues at the same time as they were first isolated from the soil. It is known that F. moniliforme can infect maize seed, germinating seedlings and plants through damaged tissues (particularly via injury caused by insects) and further, the species can also be systemically transmitted through maize plants developed from infected seeds [Foley, 1962; Lawrence, Nelson and Ayers, 1981]. Both modes of contamination were possible in the present case. Since the plants were isolated in screened greenhouses and treated with insecticide at regular intervals the possibility of insect-induced damage and infection was kept minimal. However, it is likely that some physical injury occurred during establishment in the soil (Mycock et al., 1990) and this may have provided lesions through which invasion took place. Alternatively, as at sowing 20% of the seeds harboured Fusarium spp., systemic transmission might have been expected in the resultant plants. However, as Fusarium spp. were not isolated at significant levels until two and three weeks (root; stem and leaf, resp.) after planting, if systemic transmission had occurred in 20% of the sample, the delay in establishment of the infection is

Fig. 8.3

Internal contaminants of roots (a), stems (b) and leaves (c) of plants grown for 18 weeks from control (heat treated and re-dehydrated) seeds. ▼ indicate timing of application of systemic fungicide mixture.




PERCENT INTERNAL INFECTION
100
50
0
a
b
c
0
2
4
6
8
10
12
14
16
18
TIME IN WEEKS

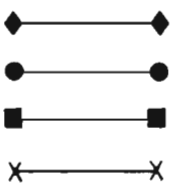
difficult to explain. Further, the incidence of the fusaria rose later in the experiment, isolates being obtained from well over half the plants after four weeks, suggesting that there must have been an input of the species concerned. It is probable that this was at least partly through injury at the root level, where the infection was first manifested (Fig.8.3a) later moving through the growing plant. Additionally, invasion of the aerial portions cannot be precluded, considering the high infection levels occurring later in stem and leaves (Figs 8.3b & c).

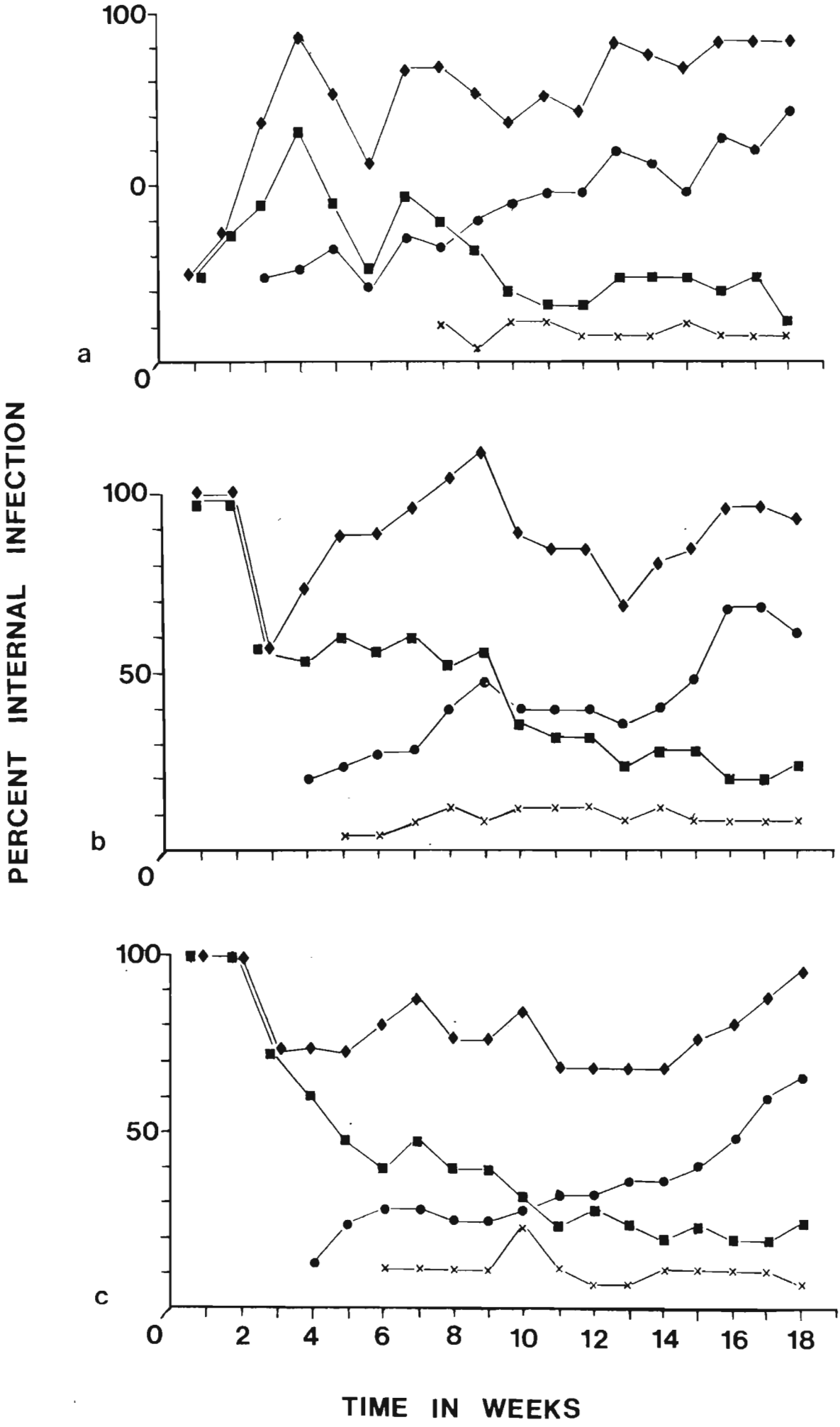
No matter what the source of the infection, by four weeks the majority of the control plants were infected by Fusarium species. The stem nodal sections were almost always more contaminated by this fungal genus than the roots or leaves (Figs 8.3a - c). This was in accordance with the observations of Foley (1962) who reported that F. moniliforme was at a higher frequency in maize nodal tissue than internodal tissue. The mixture of systemic fungicides (active ingredients mancozeb and benomyl) had, not surprisingly, only a transitory effect on the status of the fusaria (and none on the penicillia) in the control plants. However, this particular combination was used to ascertain whether or not it would have an effect when applied systemically, on A. flavus var. columnaris. In this regard, there appears to be no information as to what fungicide(s) might be effective, presumably because A. flavus var. columnaris, like the other storage fungi, has not previously been considered to be systemically transmitted.

The plants grown from the infected seeds had consistently higher total infection levels (Figs 8.4a - c) than plants of any of other treatments. A. flavus var. columnaris was the dominant isolate of the various plant tissues for the first nine weeks of the experiment. In the roots this species initially increased in frequency to 66% before it declined to levels lower than the Fusarium species (Fig. 8.4a), whereas in the stems and leaves A. flavus var. columnaris was initially isolated from all the plants, then declining in frequency (Figs 8.4b & c). This suggests that A. flavus var. columnaris had infected the plumules of the caryopses more effectively than the radicles during storage. Hence on

Fig. 8.4

Internal contaminants of plants grown for 18 weeks from seeds that were heat treated, re-dehydrated and then experimentally infected with spores of A. flavus var. columnaris. a, roots; b, stems; c, leaves.




100
0
a
100
50
0
b
100
50
c
0
2
4
6
8
10
12
14
16
18
PERCENT INTERNAL INFECTION
TIME IN WEEKS

germination, the infection had readily moved into the aerial parts of the seedlings and only later invaded the root system. This was unexpected in that Mycock et al. (1988) noted that A. flavus var. columnaris invades the radicle tissues of stored maize caryopses prior to the plumule. Additionally, it has been noted that this fungal species may remain in all the tissues (including the roots) of all plants developed from infected seeds for up to four weeks (Mycock et al., 1990) whilst in the present investigation the A. flavus var. columnaris infection level remained at 100% in shoots and leaves for only two weeks.

The abrupt decline in the incidence of A. flavus var. columnaris in the stem and leaf tissues (over three and six weeks, respectively) may have been due to a number of inter-related factors. Firstly, the fungal species is not an aggressive parasite. In fact, it has hitherto not been recognised as a plant parasite, sensu stricto. Being more adapted to the reduced moisture conditions of storage, under the conditions within a growing, actively metabolising plant this species is likely to be out-competed. That is, its establishment in the plant tissues would be less vigorous than that of the field fungi which are better suited to such conditions [inter- specific fungal competition for a niche]. However, it must be noted that the decline in the frequency of A. flavus var. columnaris commenced prior to the establishment of the fusaria within the tissues (compare Figs 8.3a - c with 8.4a - c).

Secondly, and possibly more importantly, seeds of different cultivars or varieties of maize are known to have differing resistance levels to infection by Aspergillus species, with inbreds being more susceptible than hybrids (Zuber, Calvert, Kwolek, Lillehoj and Kang, 1978; Cantone, Tuite, Bauman and Stroshine, 1983; Tucker, Trevathan, King and Scott, 1986). Those authors maintain that A. flavus infects developing maize caryopses via lesions caused by physical damage to the seed, and it is at this level that resistance or susceptibility is expressed. This may explain the differences between the presumed location of the infection of the stored seeds (as indicated by the seedling studies) in the present investigation and those previously described for

another maize variety (Mycock et al., 1990). However, it is also probable that the resistance exhibited by seeds of a particular variety (of maize) will be expressed in all the tissues of the plant developing from that seed. Thus the more rapid decline in the incidence of A. flavus var. columnaris in the stem and leaf tissues in the present case, compared with the situation in seedlings of the maize variety previously decribed (Mycock et al., 1990), could perhaps be a manifestation of different resistance levels in these plants.

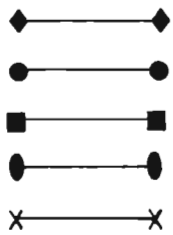
Fusarium species were first isolated from the root tissues of plants from experimentally-infected seeds after three weeks growth and, after a further week, these fusaria were in the stem and leaf tissues. Although the presence, but total supression, of the fusaria in these seeds at sowing is a very real possibility, this sequence of their appearance suggests that these fungal species were infecting the plants from the soil. The fusaria are field fungi and therefore must be assumed to be better adapted to microbial competition (than the xerotolerant storage fungi) in developing plant tissues. These more aggressive fungi came to dominate the plant tissues by 11 weeks and by the end of the experiment over 60% of the plants harboured this fungal type.

However, despite the switch from the domination of the tissues during plant establishment by a so-called storage fungus to that of a field fungus in the mature plants (Figs 8.4a - c), A. flavus var. columnaris remained in all the plant tissues throughout the entire life cycle of the latter. Additionally and more importantly, A. flavus var. columnaris was isolated from the floral structures (Table 8.2), the developing seed [mc 41%] and the mature seed [mc 13%] (Table 8.1), indicating that this species of Aspergillus can be transmitted from one generation to the next.

The plants grown from infected seed but treated with the systemic fungicides had total infection levels similar to the controls. The decline in the frequency of A. flavus var. columnaris was more rapid than in the untreated, infected experimental plants (cf. Figs 8.4a - c with 8.5a - c). When

Fig. 8.5

Internal contaminants of roots (a), stems (b) and leaves (c) of plants grown from experimentally-infected seeds for 18 weeks, with periodic irrigation with a Ridomyl-Benlate solution (▌).



◆——◆ total

●——● *Fusarium* spp.

■——■ *A. flavus* var. *columnaris*

●——● *Penicillium* spp.

×——× bacteria

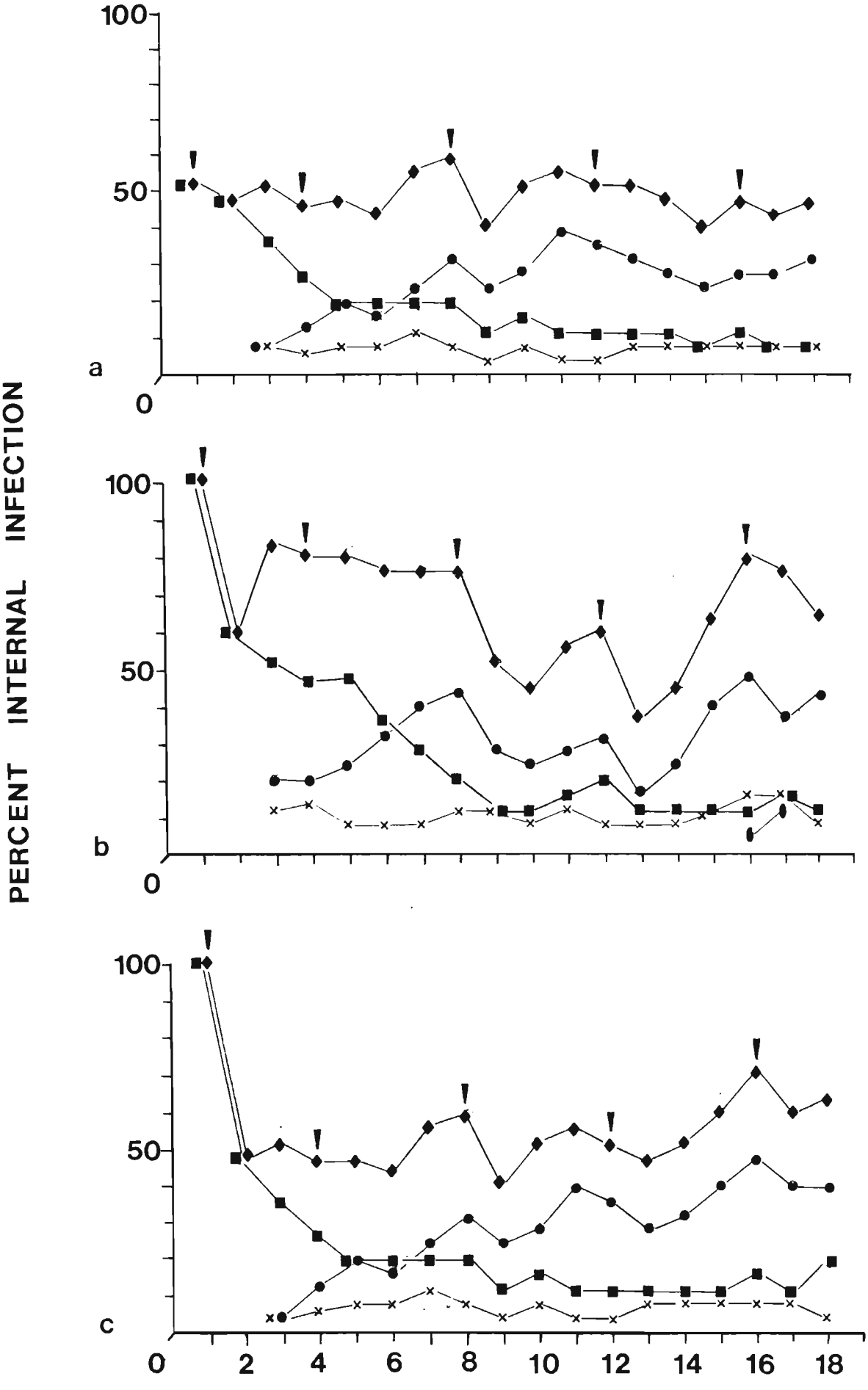

PERCENT INTERNAL INFECTION
100
50
0
a
b
c
0
2
4
6
8
10
12
14
16
18
TIME IN WEEKS

Table 8.2 Contaminants from male and female inflorescences. Note that Fusarium spp. were isolated from all inflorescences tested, and A. flavus var. columnaris was present in 100% of the inflorescences from the experimental plants, irrespective of whether or not systemic fungicide had been administered.

		Total %	Fusarium spp.	Penicillium spp.	Aspergillus niger	A. flavus var. columnaris	Bacteria
control	male	100	+	-	+	-	-
	female	100	+	+	-	-	+
experimental plants (fungicide treated)	male	100	+	-	-	+	+
	female	100	+	-	-	+	+
experimental plants	male	100	+	-	-	+	+
	female	100	+	-	-	+	+

fungicide was administered the fusaria came to dominate the plant tissues after only six weeks, as opposed to nine weeks in the experimentally-infected plants. After this transition and despite the continued treatment with systemic fungicides, Fusarium spp. continued to increase in frequency and, as observed earlier, A. flavus var. columnaris remained at a low but constant level within all the tissues. As in the control material, the systemic fungicides did have a transitory effect, there being a slight decline in the incidence of the Fusaria after each application. The fungicide mixture was effective against A. flavus var. columnaris to a certain degree in that the transition between the fusaria and the storage fungus occurred earlier in the experiment and the drop in frequency of the isolate was faster. However, the fungicide mixture used was not effective in eliminating the species (Figs 8.5a - c). As a consequence, A. flavus var. columnaris was isolated from the male and female inflorescences, (Table 8.2), the immature seed [mc 38%] and the mature seed [mc 12%] (Table 8.1).

Maize seeds internally infected with A. flavus var. columnaris, if set to germinate are capable of giving rise to plants that are internally infected with the same fungus. Although its incidence declines with respect to the better suited and more aggressive field fungi, A. flavus var. columnaris remains a contaminant of the plant. Consequently the infection not only can be, but is, carried through to the developing seed, remaining there as the plant propagules mature, and therefore being present at harvest.

Collectively these results help to explain the enigma of the presence of storage fungi within the tissues of hand-harvested and surface sterilised maize caryopses (M^{C}Lean and Berjak, 1987). In that investigation, it was suggested that the seed was systemically infected whilst developing on the parent plant. The present results confirm this suggestion. It is, however, essential to not ignore the possibility of infection via the silks (as reported by Marsh and Payne, 1984) or physical damage to the nascent seed surface suggested by several groups of authors (Zuber et al., 1978; Cantone et al., 1983; Tucker et al., 1986).

In the present investigation the form which A. flavus var. columnaris assumed within the plant tissues was not known, nor was it established how the fungus travelled through the plant. Earlier observations (Mycock et al., 1988) indicated that the mycelium can move through the vascular tissue of the peduncle. Similarly, Cole, Hill, Blankenship and Sanders (1986) have reported movement through the vascular tissue of peanut kernels. Movement of A. flavus through the vegetative tissues of cotton has also been reported (Klich, Lee and Huizar, 1986). Together these observations indicate that the fungus may be in the mycelial (as opposed to a dormant) form.

The ability of A. flavus var. columnaris to grow in actively metabolising maize plant tissues indicates that apart from being a highly specialised, xerotolerant saprophyte, the fungus is also a parasite. This further emphasises the necessity for reconsideration of the concept of field and storage fungi. Additionally, the implication of this work is that despite the application of the particular systemic fungicides used, A. flavus var. columnaris is capable of infecting nascent seeds. As a consequence, the post-harvest application of topical fungicides may help prevent the spread of the fungus within the store, but, assuming conditions are suitable for its growth, fungal destruction of the seed will not be halted, as the fungus is already within the seminal tissues. In this regard, the term, 'pathogen', is appropriate.

8.4 References

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CHAPTER 9

OVERVIEW

9 OVERVIEW

9:1 The Field

Seeds placed into the soil are open to microbial attack from both internally and externally located pathogens (Agarwal and Sinclair, 1987). During feeding and egg laying, insects and nematodes may injure the seed coat and underlying tissues, and such damaged areas may be infected by micro-organisms situated in the rhizosphere (Neergaard, 1977; Agarwal and Sinclair, 1987). Additionally, during seed germination the leakage of carbohydrates, amino acids and other biomolecules from the seed tissues can cause germination of dormant microbial structures within the soil. The developing seedling structures may then be infected by specialised biotrophs or necrotrophs (Jones and Clifford, 1983). Alternatively, seeds may harbour internal inocula of fungal pathogens; these fungal structures may be associated with one or more of the seed tissues and, on germination, infect the developing seedling (Neergaard, 1977).

Propagules of the aspergilli are ubiquitous and the conidia and sclerotia of A. flavus are frequently isolated from soil (Raper and Fennell, 1965; Wicklow, 1983; Kozakiewicz, 1989). Additionally, these propagules are associated with the surfaces of seed, particularly seed that has been in storage (Christensen and Sauer, 1982). Under the correct conditions of temperature, water availability, pH, and nutrient supply these fungal structures will germinate and any seed in proximity could become infected (Holmquist, Walter and Stahr, 1983; Wicklow, 1983; Sanders, Blakenship, Cole and Hill, 1984; Wicklow and Donahue, 1984; Niles, Norman and Pimbley, 1985; Lillehoj, Wall and Bowers, 1987; Cotty, 1989). However, a number of factors, such as host genotype and resistance, the presence of insects and the level of the inoculum may influence the infection process (Neergaard, 1977; Agarwal and Sinclair, 1987).

Crop cultivars may react differently to fungal contamination for a number of reasons: for example, the level of phenolics in the testa may vary between cultivars and this will affect the infection process (Jones and Clifford, 1983; Agarwal and Sinclair,

1987). Similarly the thickness of the waxy coating on the seed coat influence its infectibility (Jones and Clifford, 1983; Agarwal and Sinclair, 1987). Since these barriers are under genetic control, it becomes obvious that the selection of resistant, yet vigorous and high yielding, cultivars is highly desirable (Agarwal and Sinclair, 1987). At the seed development stage, maize cultivars are known to vary in their resistance to invasion by A. flavus (Zuber, Calvert, Kwolek, Lillehoj and Kang, 1978; Cantone, Tuite, Baumann and Stroshine, 1983; Tucker, Trevathan, King and Scott, 1986). Whether this is also a characteristic of the mature seed is not known. However, aspects of the present investigation have suggested that such variations in resistance may be a characteristic of the entire plant (Chapters 7 and 8).

The presence of insects in the soil may also affect the seed contamination process. Numerous examples of increased levels of infection due to the action of insects are cited (see Neergaard, 1977; Jones and Clifford, 1983; Agarwal and Sinclair, 1987). For example, the infection levels of asparagus seed by Fusarium oxysporum f. sp. asparagi and F. moniliforme are greatly increased by the tunnels produced by the asparagus beetle (Inglis, 1980).

Seeds in the soil may also be the sites of microbial competition, and this is greatly affected by the levels of inocula associated with the seed. Aspergillus flavus has been shown to be an aggressive fungus in that its presence reduces the extent of peanut kernel infection by Sclerotium bataticola (Jackson, 1965). Similarly, its growth reduces the level of Drechslera maydis associated with maize seed (Doupnik, 1972).

Since soil particles are abrasive during the germination process, the protruding radicle and extending plumule are prone to physical injury. In keeping with its opportunistic invasive nature, the storage fungus A. flavus var. columnaris has been shown to invade such wounds (Chapter 7), and hyphal penetration through the stomata also exemplified this characteristic (Chapter 7). Seenappa, Stobbs and Kempton (1980) have similarly reported A.

flavus infection of red pepper pods via the stomata. However, the direct penetration of the apparently intact seedling cuticle by A. flavus var. columnaris was indicative of a more specialised mode of infection (Chapter 7). As this fungus is a prolific producer of extracellular secretions (Chapter 5), it can be postulated that these include lytic enzymes for the invasive process. The ability of the species to degrade the components of the cuticle is unknown. This fungus, however, can readily degrade cellulose and polygalacturonic acid the major components of plant cell wall (Chapter 5) a capability which would greatly facilitate invasion. Similarly, A. niger, a recognised plant pathogen, is renowned for its pectinolytic and cellulolytic capabilities (Raper and Fennell, 1965; Czajkowska, Hornecka and Ilnicka-Olejniczak, 1988; Lyutskanov, Pishtiyski, Glinka, Gladkikh, Shaposhnikov and Vassileva, 1988). Dean and Timberlake (1989), using A. nidulans as a model, have linked the production of cell-wall-degrading enzymes to fungal pathogenesis.

Alternatively, when a seed is set to germinate, it may already be internally infected. Maize seed stored at temperatures above 15 °C and in equilibrium with relative humidities above 80% are prone to infection by storage fungi (Christensen and Kaufmann, 1969, 1974; Christensen and Sauer, 1982; Chapters 4, 6 and 8). The planting of maize seed internally infected with A. flavus var columnaris allows for the systemic infection of the resultant plant (Chapter 8).

Once infection has occurred, be it from an external or an internal source, the fungus may stunt seedling growth, induce wilting or even cause plant death (Neergaard, 1977; Agarwal and Sinclair, 1987). A. flavus var. columnaris was capable of infecting maize seedlings both via injured areas and by internally infecting the tissues prior to seed germination. Although the species did not cause wilting or kill the maize plants it did reduce vigour, with plants grown from infected seeds having a consistently lower dry mass than the uninfected controls (Chapters 4 and 7). The other storage fungi also appear to affect seedling vigour; plants grown from seeds infected by A. sydowi, A. oryzae and an A. glaucus

group species consistently produced smaller plants than uninfected material (McLean and Berjak, 1987; Chapter 6). The vigour of soybean seedlings is similarly affected when the seeds are infected by A. melleus (Ellis, Ilyas and Sinclair, 1974).

Fusarium species, which are known to cause a number of plant diseases, were also associated with the plants grown in the present investigation. Usually the maize plant becomes contaminated by externally located fusaria during, or immediately after, seed germination (Lawrence, Nelson and Ayers, 1981). Fusarium moniliforme is believed to infect the seed tissues when the expanding embryonic axis breaks through the pericarp during germination and only later moves into the growing plant (Agarwal and Sinclair, 1987). Alternatively, this fungal pathogen may penetrate the axis during storage and subsequently move through the developing plant (Lawrence et al., 1981). In the present investigation it appeared that infection occurred via the roots two to three weeks after germination (Chapter 8). Fusarium species have also been found associated with maize seed tissues (Marasas, Kriek, Wiggins, Steyn, Towers and Hastic, 1979; McLean and Berjak, 1987; this thesis) and these can survive storage (Russell, Murray and Berjak, 1982; Chapter 3). It is known that F. moniliforme is associated with the pericarp tissues (Russell and Berjak, 1983) and it is possible that, at germination, the fungus can become internal to the developing plant. No matter the mode of infection, Fusarium species can be systemically transmitted through maize plants (Foley, 1962; Neergaard, 1977; Lawrence et al., 1981; Agarwal and Sinclair, 1987; Chapters 7 and 8).

Apart from affecting seed and plant vigour and viability, Fusarium species, like A. flavus may also produce mycotoxins which can be harmful to man and his livestock (Marasas, 1989). Although the toxicity varies between the Fusarium species, their presence must be considered important. In the present studies, however, these fungi were not identified to species level and hence no direct correlations between their presence and toxicity could be drawn.

The mechanism by which fungal pathogens move through plant tissues varies with the particular pathogen involved and many can assume more than one form (Neergaard, 1977; Jones and Clifford, 1983; Agarwal and Sinclair, 1987). Most fungal species move through the plant tissues in the mycelial form, for example Ustilago nuda in barley (Neergaard, 1977) and Fusarium moniliforme in maize (Foley, 1962; Lawrence et al., 1981). Others, also in the mycelial form, move specifically through the plant vascular system. In these cases if infection levels are high, the pathogens can cause wilting and death by blocking the transpirational stream: an example is Fusarium oxysporum in pea (Neergaard, 1977). Some fungi can assume a mycoplasmal form and move through the plant in this state; an example being F. moniliforme in maize (Lawrence et al., 1981). Ascochyta pisi, which causes leaf spot or blight in pea, is believed to become associated with the meristematic tissue of the embryonic axis of the seed, therefore during germination and plant growth the pathogen is able to move with this particular tissue (Neergaard, 1977).

Observations from the present investigation indicate that A. flavus var. columnaris is in the mycelial form. Hyphae of the species were observed in the vascular tissue of the peduncle (Chapter 4) and in the coleoptile of germinating seeds (Chapter 7). Similarly, Cole, Hill, Blakenship and Sanders (1986) have reported hyphal elements of A. flavus and A. parasiticus in the vascular tissue of peanut kernels. These observations are further supported by the findings of Marsh and Payne (1984) and Klich, Lee and Huizar (1986) of hyphae of A. flavus in the silks of maize inflorescences and the vegetative tissues of cotton, respectively.

During plant growth, injury of any sort can facilitate additional infection of the tissues from external sources. Apart from insect-induced damage, seedlings are also prone to damage by birds and other animals, winds, heavy rains and hail (Neergaard, 1977; Jones and Clifford, 1983; Agarwal and Sinclair, 1987). In the present investigations the plants were grown in greenhouses,

thus minimising the effects of the weather and the regular application of insecticides minimised the factor of damage caused by insects.

As the plants matured, there was a decline in the level of A. flavus var. columnaris in the tissues and an increase in the level of Fusarium species (Chapters 7 and 8). The rate at which A. flavus var. columnaris declined, appeared to be dependent on the maize cultivar concerned and on the application of fungicide. The plants grown from experimentally infected seeds of one cultivar (Chapter 7) appeared to harbour the A. flavus var. columnaris infection for longer than those grown from another (Chapter 8), indicating the possibility of varying degrees of resistance. In this regard, Cantone et al., (1983) have indicated that inbred maize cultivars are generally more susceptible than hybrids, and it is not unreasonable to assume variation among different hybrids. Zea mays (Spc 7001) used in Chapter 8 is a hybrid and appeared to be more resistant to infection by A. flavus var. columnaris thus supporting the observations of those authors. Although the hybrid cultivar used in the investigations described in Chapter 7 was not known, the fact that A. flavus var. columnaris appeared to remain in the tissues for a longer period indicated a lower resistance.

The fungicides used in these experiments were not successful in eliminating A. flavus var. columnaris from the maize tissues. However, their application did increase the rate of decline of that fungal species. The switch from dominance in the maize tissues of A. flavus var. columnaris to that of fusaria, occurred more rapidly in plants treated with fungicides than those not treated (Chapter 8).

Despite the variations in resistance to infection by A. flavus var. columnaris suggested to exist between the different hybrids presently used, and the use of fungicides, this fungus remained as a contaminant of the maize tissues, even though at lowered frequencies. The vigorous field fungi became the dominant isolates from the tissues, and remained such for the entire experimental

period. However, both fungal types were capable of infecting the flowers and developing seeds and, as a consequence, at harvest were present as internal contaminants of the mature seed. It is interesting to note that as the seed matured and lost water the frequency of isolation of A. flavus var. columnaris increased. Presumably this was due to its ability to metabolise at lower water availabilities than the Fusarium species.

As orthodox seeds dry on the parent plant, there are changes in seed metabolism from growth and development to reserve deposition, and finally there is a decline in metabolism to a state of quiescence (Bewley and Black, 1985). It can be postulated that during reserve deposition (maize caryopses store starch, a hydrophobic substance) and the slowing down of metabolism, the gradual loss of water imposes a stress on the field fungi (Fusarium species). A. flavus var. columnaris, a storage fungus, is xerotolerant and is therefore better adapted to growth under such conditions. This may explain the increase in the isolation of this species as the seeds dried. Lillehoj et al. (1987) have noted that maize seed (28 days after fertilisation) is readily invaded by A. parasiticus, and aflatoxin can be produced. Despite the seed moisture content being above 50% these authors maintained that the water availability due to the substrate composition allows storage fungal development. This indicates that factors other than moisture content per se, are also important in the initiation of the infection process.

The changes in the physiological status of the seed tissues may well provide suitable growth conditions for all seed-storage fungi. It can be postulated that the succession of seed storage fungi associated with stored seed (Christensen and Kaufmann, 1969, 1974; Christensen and Sauer, 1982; Agarwal and Sinclair, 1987; Chapters 3, 5 and 6) may well occur in reverse order in naturally drying seed. The A. flavus group species may establish at the higher moisture contents, but as the seed tissues lose water, the more xerotolerant A. candidus may also establish. Further drying would allow proliferation of A. versicolor and finally A. glaucus

group species at lower seed moisture contents. This succession may also be dependent on substrate availability, fungal extracellular enzyme capability and inter-fungal competition (Chapter 6).

The systemic transmission of A. candidus, A. versicolor and A. glaucus group species has not as yet been proven. However, in view of the present findings, and the apparently spontaneous appearance of the succession of storage fungi in hand-harvested, surface-sterilised and aseptically stored maize caryopses (McLean and Berjak, 1987), such a possibility cannot be ruled out.

The seed-storage fungi are not metabolically active at moisture contents lower than 12%, therefore the continued loss of water from the maturing seed would also ultimately impose a limiting water stress on them. This restricted water availability would be unlikely to kill them, but would probably cause the fungi to assume more dormant forms, for example encysted hyphae (Jones and Clifford, 1983). It is probable that it is in this dormant form that these fungi enter storage.

Coupled with the presently observed ability of A. flavus var. columnaris to infect maize caryopses systemically, is its ability to contaminate the developing seed via the silks (Marsh and Payne, 1984). Those authors report that A. flavus can penetrate the developing seed by fungal growth on the surface, and via the internal tube of the silks.

Additionally, during seed development, the nascent seed structures are vulnerable to injury by birds, insects and the environment, and any wounds are readily infected (Neergaard, 1977; Jones and Clifford, 1983; Agarwal and Sinclair, 1987). Payne (1983) reported that in the south eastern states of the U.S.A., where aflatoxin contamination of Zea mays is a severe problem, the routes of infection utilised by A. flavus are wounds and the silks. Wicklow (1983) maintained that in that same region the primary source of inoculum of A. flavus is in the soil. Insects are believed to be the major form of transport from that medium to the developing seed (Neergaard, 1977; McMillian, 1983;

Rodriguez, Patterson, Potts, Poneleit and Beine, 1983; Barry, Zuber, Lillehoj, McMillan, Adams, Kwolek and Widstrom, 1985) and wind and rain may also move the fungal propagules (Agarwal and Sinclair, 1987). No matter what the source of injury or the mode of transport, infection can, and does, occur readily and aflatoxin is produced (Zuber et al., 1978; Cantone et al., 1983; Lillehoj, 1983; Lillehoj, McMillian, Widstrom, Guthrie, Jarvis, Barry, Kwolek, 1984; Tucker et al., 1986; Lillehoj et al., 1987). The level of infection and the amount of toxin produced is dependent on a number of parameters, but primarily on the level of inoculum (Wicklow, 1983) and the prevailing environmental conditions. Sclerotia and conidia can germinate only under the correct conditions of temperature, moisture and nutrient availability (Rudolph, 1962; Holmquist et al., 1983; Wicklow, 1983; Wicklow and Donahue, 1984; Cotty, 1989). Further, each strain/isolate of A. flavus varies in its requirements for germination, growth and aflatoxin production (Zuber et al., 1978; Wicklow and Shotwell, 1982) Cotty (1989) found that there was tremendous variation among 70 isolates of A. flavus in terms of culture characteristics and aflatoxin production. Therefore, only if the correct conditions for growth on/in the seed tissues prervail, will infection occur (Holmquist et al., 1983; Lillehoj et al., 1986).

Under the present conditions, infection via the silks and superficial wounds was obviated as far as possible in that the plants were grown in isolated greenhouses and regularly treated with insecticides. At no point was A. flavus var. columnaris isolated from the soil or the air indicating that infection from external inocula did not occur.

Until the late 1970s it was assumed that the storage fungi became associated with seed only during storage. The subsequent discovery that pre-harvest contamination can occur (McLean and Berjak, 1987), indicated that at least A. flavus is able to establish under defined conditions within the developing seed tissues. The present findings, however, clearly indicate that apart

from being a highly specialised xerotolerant seed storage fungus, A. flavus var. columnaris is also a biotroph which can be transmitted from seed generation to seed generation.

Storage

Ideal storage conditions- that is, storage bins which are free of pests and that are at low temperatures and relative humidities are economically precluded for the average farmer, and particularly for subsistance farmers. Even in commercial seed stores, conditions are far from optimal, and it is only really in sophisticated seed storage facilities that these requirements are met (Neergaard, 1977; Bailey, 1982; Berjak, 1987).

However, even under ideal conditions, stored seeds will deteriorate and this is primarily due to natural ageing. The rate at which any batch of seed will age is dependent on the species, the seed moisture content and the temperature of storage (Berjak and Villiers, 1970; Roberts, 1972, 1983; Berjak, Dini and Gevers, 1986). Ageing is usually manifested as a narrowing of the environmental range over which the seed will germinate, reductions in seedling vigour and ultimately, as total loss of viability (Berjak and Villiers, 1970, 1972a, b, c; Roberts, 1972, 1983). At the cellular level this process is characterised by changes in membrane composition and integrity, genetic composition and reductions in enzyme capacity (Pomeranz, 1974; Osborne, 1980; Roberts, 1983; Berjak *et al.*, 1986). If storage is over a short period these sub-cellular alterations can be repaired, and it is the time required for these repair processes that causes the observed delays in germination (Berjak and Villiers, 1970; 1972a, b, c). If, however, storage is protracted the, accumulated damage becomes too great and germination capacity is lost (Berjak and Villiers, 1970; 1972a, b, c; Roberts, 1972; 1983).

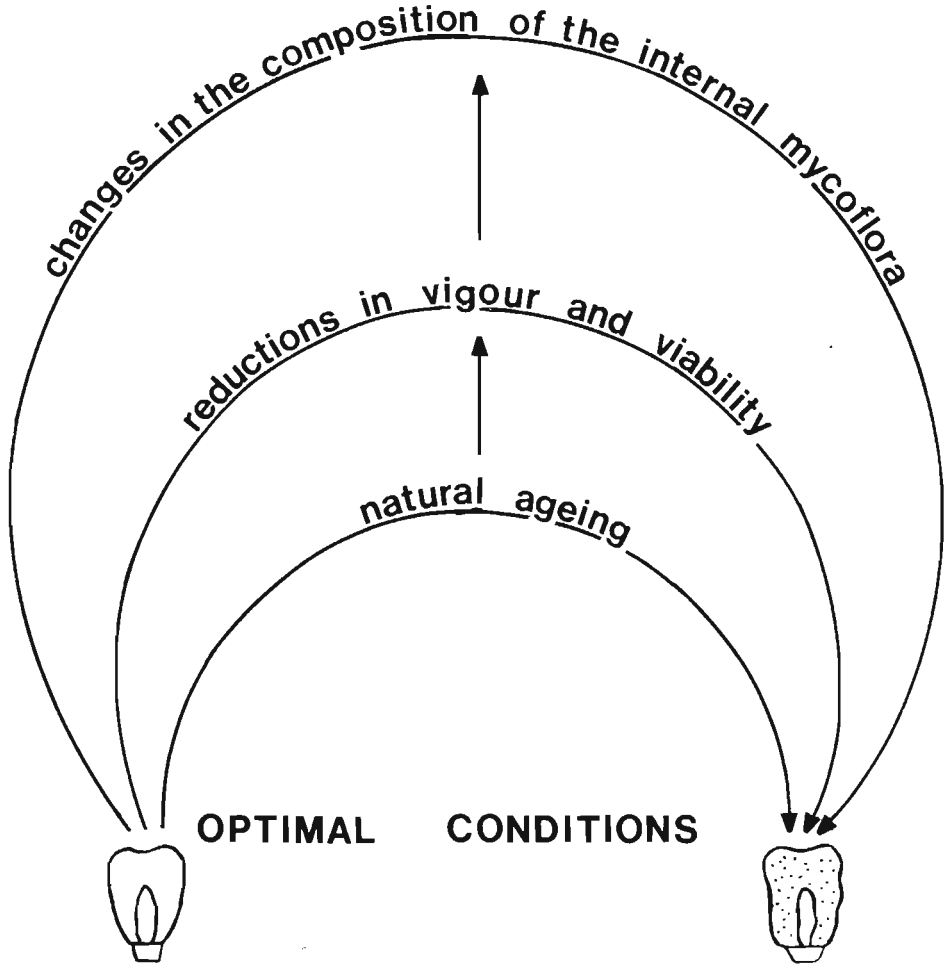
Although the environmental ranges required for germination were not tested in the present investigation, reductions in seed vigour and viability were detected in two batches of maize seed stored for a number of years (Chapter 3). The observed drop in the rate of germination, reduction in the dry mass of seedlings produced from the seed, and the loss of the capacity to germinate by some of the seeds, clearly illustrated that ageing is a very real problem.

The ageing processes were also accompanied by alterations in the composition of the fungal flora associated with the seed tissues (Chapter 3). As storage time increased, the frequency of isolation of Fusarium species declined and there was a concomitant increase in the isolation of Aspergillus and Penicillium species. Since the seeds were stored under conditions which precluded fungal growth and the additional imput of fungal species (sterile storage below 6 °C and 65% RH), it was assumed that the propagules of the storage fungi within the seed tissues were better suited to survive storage than those of the field fungi. It could be postulated that this difference was related to the long-term survival under xeric conditions of the species concerned (Fig. 9.1).

These alterations in the inherent mycoflora could have had a profound effect if the storage conditions had changed. The slightest increase in temperature and seed moisture content could have allowed the most xerotolerant of the seed storage fungi to germinate and in so doing set into motion a chain of deleterious events. As the seeds were also ageing and therefore becoming more debilitated, degradation by the fungi would have been further facilitated. The leakage of biomolecules from cells of aged seeds (as a result of the loss of membrane integrity) would have provided a readily available nutrient supply for the fungi when conditions became suitable for their growth.

Generally, when seed is introduced into commercial storage it is not uniformily sound, and is placed under conditions which are far from optimal. At harvest the seed could have been damaged, particularly if it was mechanically harvested (Qasem and Christensen, 1960; Christensen and Kaufmann, 1974; Neergaard, 1977), and reaping and threshing often inflicts further injury (Christensen and Kaufmann, 1974). Drying, if required, can cause cracking, and if incomplete, renders the seed open to fungal attack (Neergaard, 1977; Foster, 1982). The processing and transport of seed into storage, apart from subjecting the seed to damage, is often associated with high levels of propagules of storage fungi (Christensen and Kaufmann, 1969; Neergaard, 1977; Silas, Harrison, Carpenter and Roth, 1987). A large amount of

Fig. 9.1 A schematic account of the events occurring in seeds stored under conditions of low temperature and relative humidity.


changes in the composition of the internal mycoflora
reductions in vigour and viability
natural ageing
OPTIMAL CONDITIONS

debris and broken grain is associated with the seed in storage, and these too may harbour propagules of storage fungi (Berjak, 1987). Many insect species and mites, which can also carry fungal spores, may accompany the seed into storage and, if not controlled by lowered temperatures or fumigants, can inflict further physical injury (Christensen and Kaufmann, 1969; Neergaard, 1977; Cotton and Wilbur, 1982; Agarwal and Sinclair, 1987). Additionally, the metabolic activity of these animals can elevate seed moisture content to levels where the storage fungi can establish (Fig. 9.2).

Any seed placed into storage is highly unlikely to be free of storage fungal propagules: these may be internal to the seed tissues as a result of a systemic infection in the field; internal to the seed tissues due to contamination of the silks or physical injury during development; on the seed surfaces; or derived from the storage bin. If the storage conditions are, or become, conducive for the germination and establishment of, or resumption of activity by, the storage fungi a succession of species will utilise the seed as a nutrient source.

Externally located seed-storage fungi, being true to their opportunistic saprophytic nature, will invade the seed tissues via any physically injured area (Fig. 9.3). Alternatively, if the seed is intact, the fungi move through the loose peduncular tissue and penetrate through the micropyle into the underlying seed tissues (Chapter 4).

In southern Africa the first storage fungi to utilise the seed tissues are the xerotolerant A. glaucus group species (seed mc 14 to 15%). Members of this group are not fast-growing but their continued activity can be deleterious, with significant reductions in germinability and seedling viability being reported (Christensen and Kaufmann, 1974; Christensen and Sauer, 1982; Agarwal and Sinclair, 1987; Chapter 6). Aspergillus ruber a member of the A. glaucus group for example, can reduce the germination capacity of pea seeds stored at 85% RH, to zero within eight months (Field and King, 1962). Although species within this group have been

Fig. 9.2 Scanning electron micrograph of the head of a weevil, one of a substantial population, found in association with maize seed in a local storage bin (x 200). Note, the fungal spores (s) on the head, mouthparts and legs.

Fig. 9.3 Surface cracks in maize caryopses are points of fungal infection (x 400).

9·2

9·3

reported to utilise a wide range of substrates (Flannigan and Bana, 1980), the growth of those tested on the specialised solid media in the present investigation was slow (Chapters 5 and 6). However, the species under investigation did exhibit the ability to degrade simple sugars readily. The activity of this group increases the seed moisture content and temperature such that less xerotolerant, but more aggressive, storage fungi can establish (Christensen and Kaufmann, 1969, 1974; Christensen, 1972; Christensen and Sauer, 1982; Agarwal and Sinclair, 1987). Members of the A. glaucus group may also liberate mycotoxins which can be dangerous to man (Moreau, 1979; Kozakiewicz, 1989). Aspergillus ruber also releases a toxin which kills the embryonic axis of the seed in advance of infection (Harman and Nash, 1972).

Aspergillus versicolor group species are the next to invade/establish in the seed tissues [seed moisture content 14.2 - 15%] (Christensen and Sauer, 1982). The ability of these fungi to degrade more complex sugars such as cellulose and starch, as well as the simple sugars ,allows them to out-compete the less vigorous A. glaucus group species (McLean, Mycock and Berjak, 1986; Chapter 5). These capabilities also allow more rapid and profound effects on the seed tissues (Chapters 5 and 6). Aspergillus versicolor is also renowned for its production of sterigmatocystin, which is a precursor of the carcinogenic aflatoxins (Moreau, 1979).

A. candidus and A. ochraceus group species (seed moisture content 15 - 15.5%) follow A. versicolor in the succession. A. candidus, apart from being able to degrade simple and complex sugars, was also capable of degrading lipids (Chapter 5). Consequently this species may discolour and kill seeds very rapidly, and the rapid growth can produce heating up to 55 °C (Christensen and Kaufmann, 1974; Christensen and Sauer, 1982). Although A. ochraceus group members were not tested in the present investigation, they are known to produce cellulase, pectinase and proteases (Raper and Fennell, 1965; Flannigan and Bana, 1980), and on several occasions, have been isolated from

maize seeds of local origin in this laboratory. These two groups also produce toxins, with A. candidus being reported to release citrinin, candidulin and kojic acid (Kozakiewicz, 1989) and A. ochraceus, ochratoxin (Moreau, 1979).

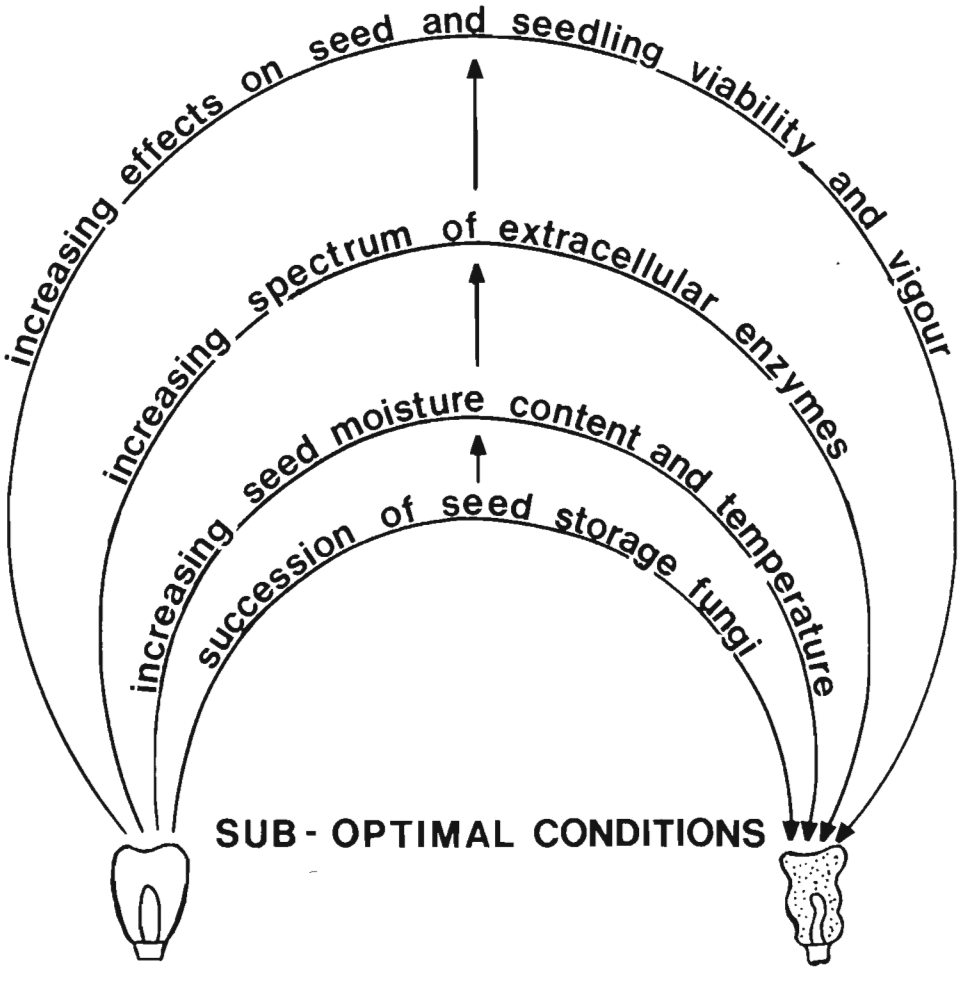
At seed moisture contents above 18% A. flavus group members may infect the grain. Apart from the elaboration of aflatoxin (Goldblatt, 1969; Moreau, 1979; Steyn, 1980), members of the group are also renowned for their prolific production of extracellular enzymes (Raper and Fennell, 1965; Christensen and Kaufmann, 1969, 1974; McGee and Christensen, 1970; Chapters 5 and 6). As a consequence, these species are able to degrade all the component tissues of stored caryopses with the seed rapidly progressing through the symptoms of discolouration, mustiness, loss of germinability to total decay (Christensen and Kaufmann, 1969, 1974; Christensen and Sauer, 1982; Agarwal and Sinclair, 1987; Kozakiewicz, 1989; Chapters 5 and 6).

The last members of the succession are species of the genus Penicillium. At moisture contents above 18.5% these species cause discolouration, grain mustiness and ultimately total decay. They are also capable of growth at lower temperatures than the aspergilli and therefore can cause problems in cool storage. In addition, members of the group can produce mycotoxins; for example P. viridicatum can produce ochratoxin (Christensen and Sauer, 1982). Additionally, the findings of these investigations and those of Cazalet and Berjak (1983) indicate that members of this genus may be active in stored seed with moisture contents lower than 18.5% (Chapter 6).

It therefore appears that the particular tissue utilised, and the rate at which it is degraded, by the seed-storage fungi are not only controlled by seed moisture content and temperature (Christensen and Kaufmann, 1969, 1974; Christensen and Sauer, 1982; Agarwal and Sinclair, 1987) but also by the extracellular enzyme capabilities of each species (Chapters 5 and 6). The characteristics of the fungal structures are also dependent on carbon source and water availability (Chapter 2). Additionally,

apart from elevating seed moisture content through the release of metabolic water , the metabolism of each member of the succession may also liberate substrates suitable for its successor (Chapter 5). Most importantly, no matter which of the species are involved, infection results in the decline in both seed vigour and viability and the plants produced from infected seed also have reduced vigour. Seed destined for consumption will more than likely be contaminated with mycotoxins if it has been in storage under conditions, and for periods, allowing for storage fungal proliferation (Fig. 9.4).

Fig. 9.4 A schematic representation of the events occurring in seeds stored under sub-optimal conditions.


increasing effects on seed and seedling viability and vigour
increasing spectrum of extracellular enzymes
increasing seed moisture content and temperature
succession of seed storage fungi
SUB - OPTIMAL CONDITIONS

9:3 Final Comments

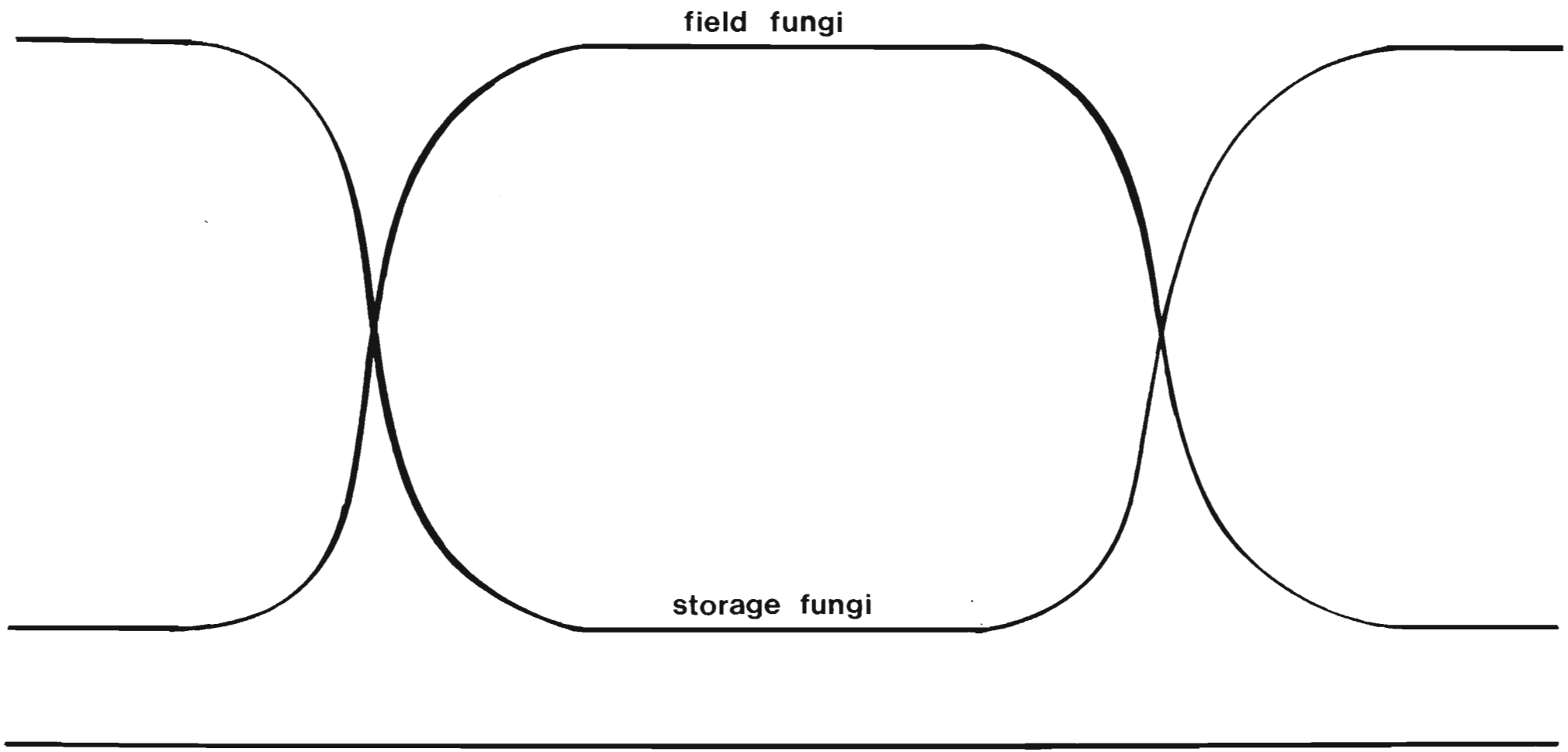
In the light of the findings of the present investigations it can be postulated that during the life cycle of a maize plant there are switches of the dominating fungal species within the tissues.

A seed set to germinate may be internally infected by fungi. If the seed has come from protracted storage, the dominant pathogens will probably be storage fungi (Chapter 3 and Fig. 9.5). During germination and early establishment this mycoflora will remain dominant, but field fungi will occur at lower levels (Chapter 8). Alternatively, the seed or the seedling tissues may become infected by a storage fungus located in the soil or on the seed surface (Chapter 7). In either case, as the plant matures, the vigorous field fungi (Fusarium species in this case), ultimately come to dominate because they are better suited to growth in hydrated plant tissues (Chapter 8 and Fig. 9.5).

The developing seeds on the parent plant are therefore open to systemic infection by the internally located fungi. However, as the seed losses moisture during the natural drying process, the conditions within the seed tissues become more favourable for the storage fungal component (A. flavus var. columnaris in this case). Additionally, externally located inocula of A. flavus may infect the seed via the silks and wounds. Consequently, the frequency of isolation of A. flavus var. columnaris increases and concomitantly that of fusaria declines (Fig. 9.5). Hence at harvest, there is a mixture of both field and storage fungi associated with the seed tissues (Fig. 9.5).

If the seed is placed into relatively good storage conditions, the field fungi will continue to decline in frequency. The propagules of the storage fungi associated with the seed tissues, presumably because of their xerotolerance, are better suited to survive the storage environment (Chapter 3 and Fig. 9.5). If, however, the storage conditions are sub-optimal the storage fungi associated with the seed tissues will rapidly degrade the seed

Fig. 9.5 Relative levels of field and storage fungi associated with Zea mays. The levels of each fungal type is dependent on resistance levels of the host and virulence of the pathogens involved.


field fungi
storage fungi
STORAGE
SEED GERMINATION
PLANT ESTABLISHMENT AND GROWTH
SEED DEVELOPMENT
HARVEST
STORAGE

(Chapter 5 and 6). Additionally, propagules of the storage fungi associated with the seed surfaces and in the storage bin, may germinate and invade the seed tissues (Chapters 4, 5 and 6).

The implications of this may be far-reaching and manifold:

i Even under storage conditions were there is no extraneous input of fungal propagules, loss of stored grain is possible. Under suitable environmental conditions, the dormant internal fungal structures will germinate/resume development, establish and destroy the seed. Such environmental conditions are readily available in the storage bins of subsistence farmers in tropical and sub-tropical regions.

ii The application of contact-fungicide dressings to the seed will only reduce the spreading of the fungal infection, but not the destruction of the mycoflora within the already-infected seed.

iii Systemic fungicides other than those presently used may be found to be effective in eliminating A. flavus from the plant tissues. However, apart from being costly these chemicals may only be used when grain is destined for seed. No contact or systemic fungicides may be used if grain is destined for consumption.

iv The use of other treatments, such as hot water (Berjak, Whittaker and Mycock, 1990), to reduce the inherent infection before seed is placed into storage or is planted may reduce infection levels in the field, such treatments if carried out immediately prior to storage should render the stored grain far less mycotoxin-contaminated when eventually consumed. However, drying subsequent to treatment is very important.

v The apparently most effective approach for the control of A. flavus infections would be the selection and development of resistant maize genotypes. Such procedures, although both costly and time consuming, merit ongoing investigation.

vi It is clear that the terminology of 'Field and 'Storage' fungi is artificial and, to some extent misleading, and should be replaced. A reasonable alternative, embracing the entire spectrum of species involved would be the seed-associated fungi.

vii Finally, the aspergilli (and penicillia) involved should be accorded pathogen status. In the light of the seed degradation and plant debilitation resulting from the activity and persistence of the species concerned, their consideration as mere saprophytes (Hudson, 1986) is outdated and incorrect.

9.4 References

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Appendix 1

Identification numbers of the various fungal isolates used throughout the study (Chapters 2,4,5,6,7). Two numbers are listed *viz.* those of the University of Natal (NUD) and the PREM number which was allocated when a sample of each species was submitted for the National Fungal Collection housed in Pretoria.

Species	NUD No.	PREM No.
Aspergillus candidus	8	Not given *
Aspergillus chevalieri	345	47924
Aspergillus flavus	21	Not given *
Aspergillus flavus var.*columnaris*	24	Not given *
Aspergillus glaucus sp	16	47516
Aspergillus oryzae	170	47625
Aspergillus parasiticus	401	48335
Aspergillus sydowi	92	47555
Aspergillus versicolor	33	Not given **
Penicillium pinophilum	155	47638

* The identity of these species was confirmed by Dr. G.C.A. van der Westhuizen of the Plant Protection Research Institute, Pretoria in 1983. No PREM numbers were issued.

** Idenification of this isolate was confirmed by Dr. G.C.A. van der Westhuizen of the Plant Protection Research Institute, Pretoria in 1982. A PREM numbers was not issued.