# SOME ASPECTS OF BIOLOGICAL CONTROL OF SEED STRORAGE FUNGI

by

**CLAUDIA CALISTRU**

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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 Patricia Berjak, with Dr. Michelle McLean acting as co-superviser.

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.

Claudia Calistru

Claudia Calistru

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## ABSTRACT

Under storage conditions of ambient temperature and relative humidity in South Africa, seed-associated mycoflora proliferates. *Fusarium moniliforme* is ubiquitous in newly-harvested maize, persisting for variable periods in storage, while *Aspergillus flavus* may represent the final group of species in the succession of aspergilli after grain storage under high temperature and/or high humidity. Many strains of these fungi produce toxigenic secondary metabolites (mycotoxins) under local storage conditions. Since pathogenic fungi may be present within the tissues of stored seeds, these contaminants will not be eradicated by external fungicide treatment, therefore a possible alternative is biological control. The aim of the present investigation was to ascertain whether certain strains and/or species of *Trichoderma* have potential as biocontrol agents against the seed-associated pathogenic fungi, *Aspergillus flavus* and *Fusarium moniliforme*.

A study of the fungal growth in dual cultures revealed that from nine isolates of *Trichoderma* spp. (*T. harzianum* and *T. viride*), four had a noticeable inhibitory effect on the growth of the pathogenic fungi. Scanning electron microscopical investigation of fungal interaction demonstrated no obvious hyphal penetration by *Trichoderma* spp. In addition, significant alteration of *Fusarium* hyphae, with pronounced collapse and loss of turgor, and production of aberrant conidial heads and microheads by *A. flavus* were observed. Evidence derived from some biochemical studies revealed that antibiosis (by production of extracellular enzymes, volatile compounds and possible antibiotics) is probably the mechanism involved in the antagonistic effect of the four aggressive *Trichoderma* spp. The *in vitro* studies demonstrated that the use of *Trichoderma* spp. as biocontrol agents against *A. flavus* and *F. moniliforme* appears promising.

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## LIST OF ABREVIATIONS

| | | |
|---|---|---|
| CPD | = | critical point drying |
| EC | = | oesophageal cancer |
| FAO | = | Food and Agriculture Organisation |
| $FB_1$, $FB_2$, $FB_3$ | = | fumonisin $B_1$, $B_2$, $B_3$ |
| HBV | = | hepatitis B virus |
| LEM | = | equine leukoencephalomalacia |
| MSD | = | mystery swine disease |
| PDA | = | potato dextrose agar |
| PGA | = | polygalacturonic acid |
| PLC | = | primary liver cancer |
| ppb | = | parts per billion |
| ppm | = | parts per million |
| SEM | = | scanning electron microscopy |
| TEM | = | transmission electron microscopy |
| UND | = | University of Natal |

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# CHAPTER 1

# INTRODUCTION

# Chapter 1. INTRODUCTION

# INTRODUCTION

## 1.1. GENERAL CONSIDERATIONS

Man depends on plants and their products for the basic necessities of food, shelter and clothing. The development of agriculture by humans about 10 000 years before the present time (Brouk, 1975) was the beginning of man's greatest impact on the ecosystems of the earth. Humans first existed as hunter-gathers, and their remarkable botanical knowledge has been confirmed by many studies. It has been established that the hunter-gathers were applied botanists. They knew the flora of their territories, including the useful or dangerous plants, had knowledge of the detoxification of poisonous food, and about drugs for healing and poisons for killing (Harlan, 1989). Presently, many species have been listed as used or potentially useful crops. In 1967, Jardin compiled the *List of Foods Used in Africa* (Harlan, 1989), and showed that the classes of domesticated plants could be categorised approximately as follows:

| | |
|---|---|
| Grass seeds (actual and potential cereals) | 60 spp. |
| Legume seeds (actual and potential pulses) | 50 spp. |
| Root and tubers | 90 spp. |
| Fruits and nuts | 60 spp. |
| Oil seeds | 550 spp. |
| Vegetables and spices | 600 spp. |
| *Total* | *1 410 spp.* |

## 1.2. MAIZE AND MYCOFLORA

Cereals constitute the most important group of plants consumed by man, with the main food crops of ancient and modern civilisations being rice, wheat and maize and, to a lesser extent, sorghum and barley. In total, some 1 000 plant species are directly important to man, but agriculture has centred on only 15 of them (Lakhanpal, 1989). Maize is currently the world's most important grain crop, with over 350 million metric tonnes produced annually (Berrie, 1977).

The origin of maize (*Zea mays* L.) is still under debate but it is believed that southern Mexico is the most probable locality. 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). The cultivation was simple and planting was achieved apparently by dropping seeds into holes in the soil. The closest relative to maize, *Zea mexicana*, is a plant which grows wild in Central America. Small cobs dating from 7 000 years ago have been found in South Mexico, while cobs dating 3 500 before the present were much larger, indicating that there was deliberate human selection of maize to produce higher-yielding types. Man's greatest impact on maize has occurred in this century, with the introduction of the first hybrid line in 1920 (Langenheim and Thiman, 1982; Jones and Clifford, 1983).

Modern maize is a tall, annual, with flowers occurring on separate male and female inflorescences. The male flowers are borne on a terminal inflorescence 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 inflorescence, which is located half way down the plant, one flower in each spikelet is fertile. The paired spikelets are sessile and are located on a spadix (which is known as the *cob*). Maize has 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. 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 one point, the *micropyle* which is the point of attachment of the caryopsis to the cob (Johann, 1938; Vines and Rees, 1972; Berrie, 1977; Agarwal and Sinclair, 1987). Maize shows a range of varieties, demonstrated by the number of different kernel types. These include dent, flint, sweet, flour and popcorn. Most kernels are yellow but white varieties exist (Duffus and Slaughter, 1980). This cereal is used for food by man for the manufacture of industrial and drinking alcohol, and is processed for starch, oil, and other materials, as well as being used for animal feed. In some of the Latin American countries, and in Africa, maize is the staple diet for humans.

## 1.3 SEED-ASSOCIATED MYCOFLORA

Plant diseases are as old as the plants themselves. There is fossil evidence to support the theory that about 430 million years ago, when colonisation of the land started, parasites adapted with the colonisers to the new environment and ultimately both hosts and pathogens achieved an equilibrium. Over 30 000 species of pathogens have been recorded and of these, about 25 000 are fungi. It is known that about 100 fungi are pathogens of rice, 100 of wheat, another 60 of maize, over 50 of sorghum and an equal number of barley (Lakhanpal, 1989). Neergaard (1977) reported that about 90% of all food crops grown are propagated by seed, and a significant proportion of these seeds is claimed by disease. According to the Food and Agriculture Organisation (FAO) of the United Nations, 5% of all food grains harvested are lost before consumption and in parts of Africa, it is estimated that 30% of the annual harvest is lost (Christensen and Kaufmann, 1969).

The major causes of loss in quality and quantity of stored seeds are rodents, insects, and seed-associated pathogens which include viruses, bacteria, fungi, and nematodes. Fungi, which are adapted to survive in air, soil, water and seeds, and in or on living or dead organic matter, cause most plant diseases, over 8 000 fungal plant pathogens being described (Agarwal and Sinclair, 1987).

Work over the years has established beyond all doubt that the more than 150 species of fungi that invade stored seeds have a major influence on seed condition, and storability of grain. According to Christensen and Kaufmann (1969),
the major changes caused by fungi in stored seeds are:

a. decrease in germinability
b. discoloration of parts or all of the grains
c. heating
d. various biochemical changes
e. loss in weight
f. accumulation of mycotoxins that may be harmful to man and to domestic animals.

### 1.3.1. Classification

Seeds are seldom entirely free of microbial contamination, and the seed-associated fungi may be internal to the tissues or on the surface. Individual species have traditionally been assigned to one of two major categories: field fungi or storage fungi. The field fungi are pathogens which infect the seed prior to harvest. This group comprises species of the genera *Alternaria, Cladosporium, Curvularia, Epicoccum, Fusarium,* and *Verticillium.* Their activity requires high relative humidity (in excess of 95%) and a moisture content of the grain above 25%. The storage fungus group is described as being comprised of xerotolerant species of genera *Aspergillus* and *Penicillium.* These fungi can be metabolically active in seeds at relative humidities of 70% and above, with which grains will attain moisture content of 13% or more (Christensen and Kaufmann, 1969; 1974, Roberts, 1972). However, McLean and Berjak (1987) and Mycock *et al.* (1992) have indicated that propagules of storage fungi may also gain access to seeds in the field, and that at least *Fusarium* species may be facultative storage fungi.

### 1.3.2. The mechanisms of fungal infection

Seeds can be colonised by micro-organisms from the stage of ear emergence to maturation and ripening. The infection process can occur at any time during the preharvest period (ear emergence and milk stage), at harvest, during grain drying, and in storage. Furthermore, any seed placed to germinate into soil is susceptible to attack by both external and internal pathogens (Agarwal and Sinclair, 1987). In the field, there are various mechanisms that facilitate fungal infection. In this regard, insects and nematodes may infect the seeds during feeding and egg laying which cause damage to the seed coat and underlying tissues. The leakage of biomolecules from the seed tissues, which occurs during germination, can, in turn, stimulate the germination of dormant microbial structures within the soil. Under favourable conditions of temperature, pH, water and nutrient availability, any fungal structure will germinate and infect the seeds in its close proximity (Lillehoj *et al.*, 1987; Cotty, 1989).

During invasion of the developing grains, micro-organisms have to tolerate greater extremes of environmental conditions since the aerial plant parts are exposed to low temperatures and significant wind speeds. In the first place, there must be sufficient free water for fungal spores to germinate; then during grain development, the availability of water to fungi is determined by the water present in the underlying tissues, controlled at least partly by rainfall and atmospheric humidity. Additionally, with grain maturation, the nutrient availability may change.

Some fungi can enter plants and seeds through natural openings (hilum, lenticels, micropyle, and stomata), and through wounds made by various abiotic and biotic agents. In this regard, *Aspergillus flavus* has been shown to invade wounds and infect the seedling tissues (Mycock *et al.*, 1990). Other fungi use mechanical pressure or enzymatic action or both to penetrate directly into plant and seed tissues. The invasive process is facilitated by the production of extracellular secretions, which include lytic enzymes. Some of these enzymes have the ability to degrade the cellulose and polygalacturonic acid of the plant cell wall (Raper and Fennell, 1965; McLean *et al.*, 1985).While the plant is still green, chlorophyll and proteins are lost as the concentration of degradative enzymes increases, and nutrients are also lost from the cells, as membranes lose their integrity. All these changes can be reflected in the rate and extent of development and persistance of the seed-associated mycoflora (Lacey *et al.*, 1991).

Infection may occur by external invasion of the caryopses or by systemic infection, and the systemic transmission of pathogens from one generation to the other has been reported for some species of fungi (Agarwal and Sinclair, 1987; Neergaard, 1977; Mycock *et al.*, 1992). In 1987, McLean and Berjak showed that a succession of fungi is manifested during storage. As the propagules of the species appearing in the succession could not have invaded the seeds after harvest under the conditions of that experiment, those authors suggested that pre-harvest, systemic proliferation had occurred during storage. Mycock *et al.* (1988) reported that the invasion of maize caryopses by *A. flavus* under storage conditions also occurs. Infection of the internal

tissues of the emerging shoots (through lesions and stomata) by hyphae of this fungus has been reported (Mycock *et al.*, 1990). The same group of researchers later confirmed the occurrence of systemic infection of seeds, during development on the parent plant (Mycock *et al.*, 1992). Thus, despite previous categorisation of the xerotolerant aspergilli as storage fungi (Christensen and Kaufmann, 1969, 1974) it would appear that propagules of these species are able to gain access to the seed tissues in the field.

### 1.3.3. Contamination by *Aspergillus flavus* Link

As discussed above, *A. flavus,* although a characteristic storage fungus, can colonise maize grains before harvest. The incidence of this fungus in ears is correlated with weather conditions, being favoured by high temperatures and drought at the late milk to dough stage, when maize is most susceptible, and by insect damage. Lillehoj *et al.* (1980) reported that *A. flavus* infection was associated with insects isolated from developing ears and with soil from maize fields in south-eastern regions of the United States. Hill *et al.* reported that 92% of the colonies of *Aspergillus* species isolated from naturally infected maize in Georgia were *A. flavus* (Hill *et al.*, 1985). The production of sclerotia as a survival mechanism in *Aspergillus* species may play an important rôle in their dissemination (Wicklow and Horn, 1982). The same authors described the formation of the sclerotia during harvest. In 1989, in the Mississippi region of the USA, the incidence of *A. flavus* infection of grains was predominant (Zummo and Scott, 1990). In flooded areas of India, in a survey showing a number of fungi associated with either the individual maize kernels or with cobs, *A. flavus* had an incidence of 89% in cobs samples, and 100% occurrence in kernel samples (Sinha, 1987).

In the low desert areas of south-western USA, *A. flavus* has been identified as one of the fungi infecting cotton seeds (*Gossypium hirsutum* L.) as they develop in the field (Klich, 1987). Field studies have been conducted in Arizona to elucidate the routes by which *A. flavus* enters cotton seeds and the factors influencing this process. The fungus appears to enter uninjured bolls through vascular tissue, damaging the site of the injury (Huizar *et al.*, 1990). The development of this injury appears to be a

pathogenic capability of *A. flavus*, which is not generally characteristic of this fungus. Infection and entry may also occur in cotton seed as a result of insect and mechanical injury to the developing fruit and/or its seeds. However, when insect damage was minimised by insecticidal applications, the percentage of fungal contamination was still high (Diener, 1989).

Mycological studies involving peanut seeds have shown that growth of *A. flavus* is greatly enhanced by drought and elevated soil temperatures during the latter third of the growing season. Viable conidia of this fungus have been isolated from peanut field soil and have been shown to germinate in the geocarposphere in response to seed injury and it was found that *A. flavus* may also persist in soil as mycelium (Horn *et al.*, 1994). As peanut seeds develop below the soil surface, soil-borne propagules of *A. flavus* are ideally poised for invasion.

### 1.3.4. Contamination by *Fusarium moniliforme* Sheldon

*Fusarium* species constitute a cosmopolitan group of fungi which colonise aerial and subterranean plant parts, either as primary or secondary invaders (Sayer, 1991; Sayer and Lauren, 1991; Muller and Schwadorf, 1993). Most *Fusarium* species invade vegetative plant tissues and developing seeds in the field and produce damage before harvest. However, some species invading seeds in the field continue to develop if the harvested seeds are stored at high moisture content. The extent of invasion by spores or mycelial fragments is influenced by the amount of inoculum, invertebrate infestation, damage by other fungi, plant resistance, ambient temperature and mechanical damage. In storage, fungal growth is influenced by many of these factors as well as: moisture levels determined by relative humidity, temperature of the air, aeration, microbiological interaction, internal infection of seeds, insects, rapidity of drying, and accidental wetting (Mills, 1989).

*Fusarium moniliforme* is a soil-borne fungus and is a major plant pathogen of maize, and sorghum and also rice on which it causes bakanae disease, one of the most serious diseases of this grain in Asia (Booth, 1971, Rice and Ross, 1994). In New Zealand, fungal isolates have been identificated in local wheat (Agnew *et al.*, 1986),

maize (Hussein *et al.*, 1989), and pasture herbage (di Menna *et al.*, 1987), while in the south-western area of Germany, in 1993, wheat samples showed a high degree of occurrence of fusaria. Kernels with fusaria were found in all wheat samples, and the percentage of kernels with fusaria was 60% to 100% (Muller and Schwadorf, 1993). Several workers have isolated *Fusarium* species from medic seeds in Australia (Wan Zainum and Parbery, 1977). *Fusarium* species were reported to contaminate maize in climates as different as those of north east Mexico (Desjardins *et al.*, 1994), and Finland (Tahvonen and Sorri, 1989). In 1994, Toyota *et al.* reported that the one of the major soil-borne plant diseases in Japan, the now-called fusarium-wilt of radish, is caused by a variety of *Fusarium* species. The pathogens survive in soil as chlamydospores, which germinate in the vicinity of plant roots and infect the host plant. Another disease of cereals seeds, caused by *Fusarium* species, is fusarium head blight (Atanassov *et al.*, 1994).

The fusaria produce symptomless infection of maize plants, as well as infection of seeds, where the fungi invade the tissues beneath the pericarp giving infection as high as 100% (Bullerman and Tsai, 1994). High levels of asymptomatic kernel infection by *F. moniliforme* in commercially grown maize were reported in Mississippi (Zummo and Scott, 1992). Infection occurs about 2 weeks after mid-silk, and by 10 weeks after, up to 66% of kernels may carry the fungus (King, 1981). In 1994, a team from the USA reported the incidence of *F. moniliforme* in maize and maize-based foods and feeds. They found that with the yellow dent corn grown in 1991, *F. moniliforme* was the most commonly found species. Higher levels of this species were found in yellow and milling maize grown in 1991, than in yellow maize grown in 1992. With popcorn, *F. moniliforme* was the predominant *Fusarium* species isolated from all samples. While the number of infected kernels was low, this fungus constituted from 70.9% to 91.7% of the isolates. In sweet maize, which had a very low kernel infection rates, the levels of *F. moniliforme* were low to zero (Bullerman and Tsai, 1994).

In South Africa, *F. moniliforme* is one of the most prevalent seed-borne fungi of maize. An increase in contamination by *F. moniliforme* has been reported during

commercial storage (up to 8 months), indicating its status as a facultative storage fungus (Mycock *et al.*, 1992). The fungus is frequently found in the temperate, subtropical as well as intermediate areas of this country (Mills, 1989). In 1988, a study found that in South African maize, the infection by *F. moniliforme* was 49.6% (Rheeder *et al.*, 1995). The fungus was found to be most prevalent in the northern Transvaal which has a subtropical climate and is the warmest and driest of maize-growing areas (Marasas *et al.*, 1979).

## 1.4. MYCOTOXINS AND RELATED MYCOTOXICOSES

### 1.4.1. History of mycotoxins

The toxins produced by fungi are termed *mycotoxins*, and the conditions they produce are known as *mycotoxicoses*. The high incidence of mycotoxins in food materials is facilitated by the ability of the omnipresent fungal spores to germinate at temperatures ranging from 15 °C to 40 °C. Mycotoxin production by seed-associated mycoflora is influenced by several factors: seed moisture content (relative humidity in the field, drying or re-wetting after harvest), temperature, mechanical damage (bruising, or by insects, or birds), length of the storage period, gaseous environment (carbon dioxide and oxygen), chemical composition of the substrate, nutrients, chemical treatment of the crop, plant stress, fungal strain differences and the nature of the microbiological ecosystem (Patterson, 1979).

Man may become exposed to mycotoxins (Fig. 1.1) directly, by consumption of cereals, nuts, and other vegetable products that are contaminated by toxigenic fungi, or indirectly, by consuming meat, milk, and eggs that may contain mycotoxins. Additionally, workers in the food and feed industries may inhale dust containing spores or be exposed to other contaminated ingredients, and laboratory workers may face similar hazards when preparing crude and pure toxins from fungal cultures.

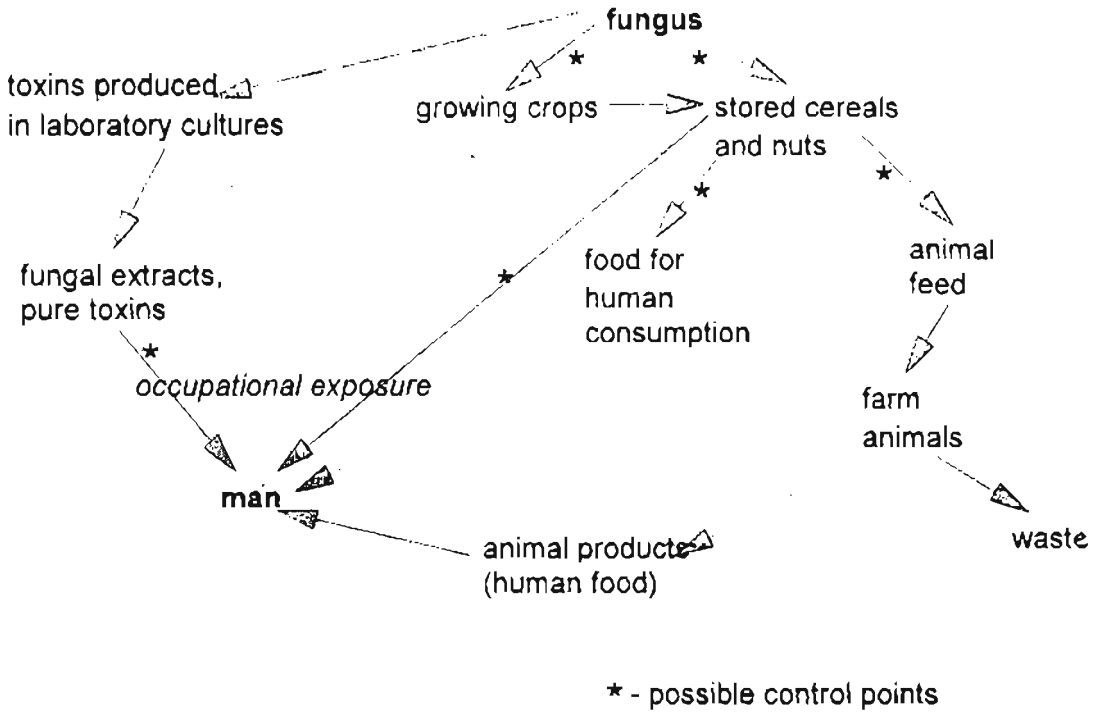



Fig. 1.1. Mycotoxins in the environment. Growing or harvested crops may be infected with a fungus and the toxin it produces passed directly or indirectly to man's food. Pathways for occupational exposure to mycotoxins are also indicated (Patterson, 1979).

In the past, in order to satisfy the demands of the rapidly expanding world population, many measures have been taken (intensive agriculture; improvements in the processing methods; and development of unconventional food) to provide vast stocks of food grains. Associated with storage there have been public health problems of which mycotoxin contamination is just one. From the stage of preharvest until they reach the consumer, food grains may be contaminated by a succession of microflora, each producing an array of metabolites that may result in spoilage of the seeds or accumulation of mycotoxins.

Records of the toxigenicity of mouldy food is more than two centuries old. The first report of mycotoxicoses dates from the 16th century: 'St. Anthony's fire', or ergotism. The disease is caused by ingestion of grain infested with the ergot-producing *Claviceps purpurea* (Fr.) Tul.. It was, however, only in the early 1930s, that the substances responsible for the syndrome were identified as a group of alkaloids (Murthy, 1989). Toxicity associated with 'yellowed' rice has been a subject

of study in Japan for some 80 years, the fungus responsible for this disease being *Penicillium islandicum*, producing islandiotoxin and luteoskyrin (Spensley, 1970).

During World War 2, in Western Siberia, alimentary toxic aleukia (ATA) developed. The disease was favoured by the poor conditions, when the Russian people were forced to use for bread-making the unharvested wheat grains which were in the fields under snow, but contaminated by *Fusarium* spp. It is now known that *F. sporotrichioides* was responsible for this disease (Spensley, 1970). At the same time in the 1940s and 1950s, when fungal metabolites were found to possess useful properties, and particularly with the discovery of penicillin, the illnesses associated with mouldy food or feed were described as 'neglected diseases' (Spensley, 1970).

The relationship between food-borne mycotoxins and the problems in human and domestic animal health was established in 1958, when facial eczema, a disease of sheep in New Zealand, was found to be due to a toxin. This toxin, sporidesmin, was produced in the spores of *Pithomyces chartaram*, on the grass litter of pastures (Spensley, 1970). An oestrogenic syndrome in pigs has been traced to a toxin, zearalenone, produced by *Fusarium graminearum*. This fungus grows on maize stored in cribs, and can also occur on wheat and barley (Spensley, 1970). The mycotoxin field changed completely when in 1960, in the UK, 'Turkey X disease' occurred. It was established that the cause of this outbreak was the consumption of Brazilian groundnuts which were contaminated with highly toxigenic strains of *Aspergillus flavus*. Microscopical examination revealed that the fungus was present in the kernel tissue at, or before harvest (Austwick, 1975).

Many investigations have been aimed at identifying the toxigenicity of fungal species which contaminate food grains and other foodstuff. These species belong to the genera *Alternaria, Curvularia, Fusarium, Cladosporium, Penicillium, Oidium, Nigrospora, Phoma* and *Aspergillus*. The following table (Table 1.1) gives an account of the some of the more commonly encountered mycotoxicoses associated with fungal pathogens (Murthy, 1989).

Table 1.1. Common mycotoxicoses, their expression and causative fungi (adapted from Murthy, 1989).

| Food or feedstuffs | Toxin identified | Implicated organism | Toxic expression In man | In domestic/other animals |
|---|---|---|---|---|
| Groundnut | Aflatoxin | *Aspergillus flavus* Link. *A. parasiticus* Spear | Liver carcinoma | Toxic hepatitis, carcinoma |
| Maize | Ochratoxin | *Aspergillus ochraceus* Wilh. *Penicillium viridicatum* Westl. | Not clear | Nephropathy |
| | Fumonisins | *Fusarium moniliforme* Sheldon | Oesophageal carcinoma | leukoencephalomalacia, (LEM), pulmonary oedema, hydrotorax |
| Maize and barley | $F_2$ toxin, or zearalone | *Fusarium roseum* (Link) Sn. & H. *F. tricinctum* (Cda.) Sn. & H. *F. graminearum* Schw. | Not reported | Hyperoestrogenism, atrophy of ovary, infertility |
| Corn and barley | Trichothecenes ($T_2$ toxin) | *F. tricinctum* (Cda.) Sn. & H. *F. sporotrichioides* Sherb. *F. poae* (Peck.) Wr. | No reports | Growth repression, teratogenic activity, neural disturbances, ATA (alimentary toxic aleukia) death |
| Ergotized seeds of cereals and grasses | Ergot alkaloids | *Claviceps purpurea* (fr.) Tul. | Very rare | Abortion, gangrene of extremities |
| Rice | Luteoskyrin, islanditoxin and cyclochlorotine | *Penicillium islandicum* Sopp. | Very rare | Cirrhosis of liver |
| Maize | Rubratoxins | *P. rubrum* Stoll | Very rare | Hepatic lesions |
| Hay, grains, processed feeds | Cyclopiazonic acids | *P. cyclopium* Westl. | Very rare | Diarrhoea and convulsions |
| Germinating wheat or barley | Patulin | *P. urticae* Bain. | Very rare | Carcinogenicity in rats |
| Pasture grass | Sporidesmins | *Pithomyces chartarum* Berkeley & Curtis | Very rare | Facial eczema or dermatitis |
| Red clover hay | Salframine | *Rhizoctonia legumicola* Gough & Elliot | Very rare | Excessive salivation and a reduction in milk yield |
| Fodder | Satratoxins and verrucarin | *Stachybotrys atra* Corda | Very rare | Haemorrhage, necrosis, excessive salivation |

### 1.4.2. Aflatoxins

Aflatoxins are the most widely distributed and intensively studied group of mycotoxins. In 1961, the toxin extracted from the highly toxigenic strains of *Aspergillus flavus* (isolated from groundnuts) was named **aflatoxin**, from a hybridisation of the generic and specific names of the fungus (Austwick, 1975). The discovery of aflatoxin lead to the examination of other *Aspergillus* species and other

fungi to determine their toxin-producing capabilities. In the years that followed, research focused on the ecology of fungi, their metabolism, the chemical and physical properties of their secondary metabolites and the biological effects of these metabolites.

The two species of *Aspergillus* that produce aflatoxins, *A. parasiticus* and *A. flavus,* can grow and synthesise aflatoxin on a number of substrates, but the contamination of maize, peanuts, cottonseeds, and nuts is of major concern. The aflatoxins are a group of four primary compounds, differentiated by their chromatographic movement and the nature of their fluorescence. They are named aflatoxin $B_1$, $B_2$, $G_1$ and $G_2$. The distinguishing letters for the aflatoxins are related to the colour of the fluorescence exhibited on chromatograms, B being for blue and G for green. The structures of the aflatoxins were established at the Massachusetts' Institute of Technology (Spensley, 1970). *Aspergillus flavus* produces the $B_1$ and $B_2$ toxins predominantly, and *A. parasiticus* the $G_1$ and $G_2$ toxins. Not all strains of *A. flavus* produce aflatoxin, and even toxigenic strains will not yield aflatoxin under all conditions of growth. The presence of the fungi does not, therefore, necessarily parallel high aflatoxin concentrations. Different strains and distinct growth conditions may also affect the ratio and quantity of the four aflatoxins produced. However, as no connection has been made between aflatoxin production and any morphological characteristics of the fungi, the estimation of the toxigenicity of strains is being carried out by chemical tests (Spensley, 1970).

**1.4.2.a. Aflatoxin contamination**

Aflatoxin contamination of grain may occur in the field before harvest and during storage. There is ample evidence that serious aflatoxin problems have occurred in years with above-average temperatures and below-average rainfall (Payne, 1992). Drought stress may also contribute to increased aflatoxin levels (Payne, 1992), as happened in the USA in 1979, where in excess of 90% of the maize crop was contaminated with aflatoxins (Zuber and Lillehoj, 1979). Field studies have also shown a positive correlation between insect damage and aflatoxin contamination (Lillehoj *et al.,* 1976). Insects may facilitate infection of ears with *A. flavus* before

harvest by transporting primary inoculum to the ears, facilitating movement of inoculum already on the silk into the kernel region, disseminating inoculum within the ear, and by predisposing kernels to infection though feeding injury to the protective pericarp (Payne, 1992). It has, however, been difficult to prove conclusively an association of insects with aflatoxin contamination of kernels (Marsh and Payne, 1984).

Aflatoxin contamination occurs world-wide, although in many countries, the extent of contamination is unknown because of the reluctance to report the occurrence. Aflatoxin contamination of peanuts, maize and cottonseeds by *A. flavus* and *A. parasiticus* may occur before or after harvest, during drying, handling and storage. It appears that *A. flavus* is adapted to the aerial environment, and is frequently found contaminating maize, cottonseed and nuts, while *A. parasiticus* is the primary invader of peanut pods and kernels in the soil (Diener, 1989). *Aspergillus* species may cause deterioration of the kernels of different types of nuts: almonds, chestnuts, pistachios, pecans and walnuts (Doster and Michailides, 1994). There are also reports of aflatoxin contamination of groundnut in West African countries, where an average seed infection during 1989-1990 varied with site and year from 5 to 37% (Waliyar *et al.*, 1994). In Japan, the natural occurrence of aflatoxins has been reported in imported agricultural products (nuts, maize and some spices). More than 10% of the strains of *A. flavus* which were isolated from spices (black, white and red pepper) demonstrated the capacity to produce aflatoxin $B_1$ (Ito *et al.*, 1994). In the USA, aflatoxin contamination of maize is chronic in the south eastern states and occurs almost each year (Payne, 1992). In South Africa, strains of *A. flavus* and mycotoxins were isolated from commercial maize harvested in 1989 and 1990 from the five major production areas and this is likely to be an annual occurrence. Fungal isolations and mycotoxin levels were higher in 1990 than 1989, (Rheeder *et al.*, 1995).

### 1.4.2.b. Human disease and aflatoxin exposure on a global basis

Since the discovery of the aflatoxins, many studies have been carried out to investigate the relationship between aflatoxin exposure and the effect on humans. The recognisable health problems associated with aflatoxins range from acute hepatic

toxicities to liver cancer. Human populations are exposed to aflatoxins by the consumption of grains and foodstuff that have been directly contaminated by the strains of *A. flavus* or *A. parasiticus* during growth, harvest and/or storage.

Contamination by fungi is universal, but within a given geographical area, the levels or the final concentration of aflatoxins in the grain product can exceed 12 000 $\mu g\ kg^{-1}$ (12 ppm). The present permissible levels of aflatoxins in agricultural commodities in the USA is 20 $\mu g\ kg^{-1}$ (20 ppb), in South Africa is 10 $\mu g\ kg^{-1}$ (van Egmond, 1989), while in Europe, the minimum acceptable level is lower, at 5 ppb (Groopman and Kensler, 1988). Aflatoxin $B_1$, which is a common component in natural contamination of foodstuffs, is the most potent naturally occuring hepatocarcinogen known for experimental animals. The concern is to determine to what extent individual aflatoxins are related to human disease.

The evidence suggests that the aflatoxins may play a rôle in the aetiology of primary liver cancer in human populations of Africa and south east Asia where fungal contamination of grains and foodstuffs reaches high levels. Shank (1979) suggested the connection between heavy contamination with aflatoxins and liver cancer, particularly in the Karamoja district of Uganda. This area was of interest because, despite the fact that the climate was dry, in fact semi-desert, the levels of aflatoxins were very high. High aflatoxin levels may be explained by the fact that the rainfall of the area is concentrated into a short season, providing temperature and humidity for the proliferation of fungi on stored or freshly harvested food (Alpert *et al.*, 1971). The incidence of primary liver cancer in Swaziland is higher among the male population, and the incidence of the disease decreases with increasing altitude (Keen and Martin, 1971). The probable cause of the hepatoma in Swaziland is the consumption of contaminated groundnuts. Culinary habits were found to have, to a large extent, an impact on ingestion of aflatoxins in large quantities: the groundnuts were pounded in a wooden mortar, and the resulting powder sprinkled on the food as a condiment in the same way as salt and pepper are used. Enough powder was made to last for a week and kept in the kitchen. Both groundnuts and the wooden mortars were tested for aflatoxins and the results showed fungal infestation (Keen and Martin, 1971). A few

years later, another group of researchers extended the study in Swaziland, which supported the hypothesis that aflatoxin ingestion is a factor in the development of primary liver cancer in Africa (Peers *et al.*, 1979).

In Ivory Coast, liver cancer is more frequent among inhabitants in the savanna area than among inhabitants of coastal tropical forests. The aflatoxin contamination in food is more prevalent in the savanna due to the consumption of peanuts and other stored crops and 30% of males in such regions had primary hepatomas (Tuyns *et al.*, 1971). Peers designed another study, but this time in Kenya, to ascertain whether an association existed between the aflatoxins and cancer incidence. The results reported showed a statistically significant relationship between aflatoxin levels and the liver cancer cases (Peers and Linsell, 1973).

As the initial phase of a survey to evaluate the relationship between consumption of fungally contaminated food and the incidence of liver cancer in south east Asia, a study was undertaken to demonstrate the distribution of moulds in food and distinguish those capable of mycotoxin production (Shank *et al.*, 1972). In general, grains, beans and legumes were most extensively sampled because they represent the major components of the diet. Contamination of food by fungi was variable, from 13% to 81%. *Aspergillus flavus* was found with high frequency in almost all food, except fresh vegetables, garlic, onions and spices. In the market food of Hong Kong, maize samples had 88% fungal contamination, of which 43% were infected with *A. flavus*. In Thailand, maize samples had 58% contamination, 53% being by *A. flavus* (Shank *et al.*, 1972). In parts of China, primary liver cancer (PLC) is the major cause of cancer deaths, with almost one in ten people affected. A positive correlation was observed between liver cancer incidence and the consumption of mouldy maize, the results of the survey showing that mouldy maize was consumed in only a few areas, which had a very high rates of primary liver cancer. For areas reporting consumption of mouldy maize, the average cumulative mortality rates were 33.84 and 8.33 per 1 000, for men and women, respectively. A strong relationship between PLC and dietary aflatoxin $B_1$, which presumably came from the ingestion of mouldy food, has been reported in southeast China (Hsing *et al.*, 1991).

The possible rôle of aflatoxins as an occupational risk factor has been considered in Denmark, the exposure to aflatoxins in imported animal feed being the most probable explanation for incidence of liver cancer and cancers of the biliary tract (Oslen *et al.*, 1988). Evidence of aflatoxins in liver tissues was also presented in reports from United States (Phillips *et al.*, 1979). In Mozambique and South Africa (Eastern Cape Province) there is convincing evidence of a distinct association between the intake of aflatoxin by humans and the development of primary liver cancer (van Rensburg *et al.*, 1974). The same senior author, in 1985, identified three major factors which may have a causal rôle in the development of liver carcinoma: 1) the ingestion of foodstuffs contaminated by aflatoxins; 2) chronic active infection with hepatitus B virus (HVB); and 3) an excessive intake of alcohol. However, there is very little evidence to indicate that alcohol plays a rôle in the high incidence areas of hepatocarcinoma in Africa (van Rensburg *et al.*, 1985).

In the past years, many studies have been conducted to understand the aflatoxins and their possible rôle in carcinogenesis. Their influence in human cancer development is still under debate and requires further investigations. The results presented in 1993 by Harrison *et al.*, indicated that the aflatoxins may, in fact, be a risk factor for cancer induction in human organs, in the same way as that of cigarette smoking (Harrison *et al.*, 1993).

### 1.4.3. Fumonisins

Many *Fusarium* species produce a number of secondary metabolites that cause different physiological and pharmacological responses in plants and animals. *Fusarium* species are well known for the production of the trichothecene mycotoxins, but they may also produce a variety of other compounds such as other mycotoxins, pigments, antibiotics and phytotoxins.

*Fusarium moniliforme* produces a group of toxins known as fumonisins, of which fumonisin $B_1$ ($FB_1$), $B_2$ ($FB_2$) and $B_3$ ($FB_3$) are the most common contaminants of maize samples. Fumonisins were discovered in 1988, as a result of a decade of

study by a group of researchers from South Africa, lead by Marasas (Gelderblom *et al.*, 1988, 1990).

### 1.4.3.a. Fumonisin contamination

Fumonisins have been reported in commercial maize meal from many countries including Canada, Egypt, Peru, South Africa and United States (Nelson *et al.*, 1993; Bullerman and Tsai, 1994). In 1994, in the USA, the fungal contamination of maize was higher than normal, but fumonisin levels were abnormally low, probably explained by the mainly cool season which probably lead to the growth of *Fusarium* species but not to the production of fumonisins (Rice and Ross, 1994). In South Africa, the fumonisins were the most frequently recorded mycotoxins, 67.8% and 82.8% detectable contamination of the 1989 and 1990 maize samples, respectively (Rheeder *et al.*, 1995). The same study found that the yellow maize samples had a higher *F. moniliforme* contamination than white maize, but the levels of fumonisins were greater in white maize. The possible explanation for these findings is that yellow maize is more susceptible to infection by *F. moniliforme*, but white samples are a better substrate for the production of the fumonisins (Rheeder *et al.*, 1995).

In Switzerland, in 1991, samples of maize-based products were shown to have very low percentages of fumonisins (lower than 1μg $g^{-1}$) (Pittet *et al.*, 1992). The results from a preliminary survey of 124 samples of various maize-based food from the USA and South Africa, in 1990 and 1991, gave 74% of samples with detectable fumonisins. Only the American samples had concentration of fumonisins greater than 1μg $g^{-1}$ (Nelson *et al.* 1993).

### 1.4.3.b. Diseases associated with *Fusarium moniliforme* and fumonisins

Since the discovery of fumonisins by the South African team, these mycotoxins have been implicated in a number of animal and human diseases.

Equine leukoencephalomalacia (LEM), a neurotoxic disease of horses, donkeys and mules, is characterised by necrosis of the white matter of one or both cerebral hemispheres (Kellerman *et al.*, 1990). The disease was associated in the early

part of this century with the ingestion of mouldy maize, and was referred to as 'mouldy maize poisoning' (Norred, 1993). Proof that LEM is caused by ingestion of maize contaminated by *F. moniliforme* came later, when *F. moniliforme*-contaminated maize was fed to horses, and the animals developed the characteristic symptoms (Marasas *et al.*, 1976).

Additionally, fumonisin $B_1$ causes pulmonary oedema and hydrothorax in pigs, frequently resulting in death (Harrison *et al.*, 1990). More recently, $FB_1$-induced hepatic nodular hyperplasia in pigs suggested that $FB_1$ may also be carcinogenic in non-rodent species (Casteel *et al.*, 1993). Fumonisins have also been implicated as causative agents in mystery swine disease (MSD) (Bane *et al.*, 1992). Fumonisin $B_1$ has also been linked to *Fusarium*-related syndromes in young broiler chickens, the level necessary to promote toxicosis being less then 100 mg $kg^{-1}$ (Ledoux *et al.*, 1992).

In humans, fumonisins have been implicated as a possible aetiological agent in oesophageal cancer. In South Africa, the highest incidence of human oesophageal cancer (EC) has been reported in the south western districts of the Eastern Cape Province, while only 200 km away, in the north eastern parts, the incidence of EC was relatively low. An association between the consumption of *Fusarium moniliforme*-contaminated maize and the high level of oesophageal cancer has been established (Gelderblom *et al.*, 1988): an analysis of samples of mouldy and healthy maize from high and low EC rate areas of Eastern Cape Province demonstrated 35% *F. moniliforme* occurrence in the low EC rate areas, while in high-EC region this fungus had an 86% incidence (Sydenham *et al.*, 1990).

Whether fumonisins are complete carcinogens has not been established, but they appear to be potent tumour promoters (Gelderblom *et al.*, 1988). The International Agency for Research in Cancer has published a monograph in which fumonisin $B_1$ is classified as a '2B' carcinogen, indicating that there is sufficient evidence of carcinogenicity in test animals, but not enough data to draw definite conclusions for humans (Norred, 1993).

## 1.5. ASPECTS OF BIOLOGICAL CONTROL OF FUNGAL PLANT PATHOGENS

The above overview demonstrates the major problems imposed by *A. flavus* and *F. moniliforme*, two fungal species important in effecting plant/seed deterioration. In recent years there have been considerable changes in attitude towards the use of chemicals in agriculture for the eradication of plant pathogens. Public awareness concerning the types and quantities of chemicals used and their impact on the environment has led to regulation of their use and, in some cases, removal from the market (Whipps and McQuilken, 1993). Therefore, interest has focused on alternatives to chemicals, especially for pest and disease control. One of the strategies available is the biological control of pathogens. The greatest research effort in biological disease control has been concerned with the direct use of specific antagonists. Viruses, bacteria, fungi and microfauna have all been observed to give some level of disease control, but the greatest interest is focused on the use of bacteria and fungi for the control of fungal pathogens (Whipps and Lumsden, 1991).

The word *antagonism* was introduced into microbiology in 1874 by Roberts (Baker, 1987), who demonstrated antagonistic action between *Penicillium glaucum* and bacteria in liquid cultures. Inhibition of plant pathogens by their own metabolites was reported later, *Erwinia carotovora* growing on tulip tissue and *Penicillium italicum* in orange rind being killed by liquid media in which these pathogens had grown (Baker, 1987).

### 1.5.1. *Trichoderma* species as biological control agents

There are a number of examples of fungi that parasitise plant pathogens (Adams, 1990) but only a few have been studied to any extent with the aim of biological control. *Trichoderma* species are probably the most studied because they appear to have some potential for use in biological control. The first report of *Trichoderma* spp. used as biocontrol agents was dated as early as 1932, by Weindling who related mycoparasitism to the control of plant disease in *Trichoderma viride* and *Rhizoctonia solani* interactions (Baker, 1987). These anamorphic, filamentous fungi,

*Trichoderma* spp., are widely distributed all over the world and occur in nearly all soils and other natural habitats, and comprise a group of rapid growing Hyphomycetes that can utilise a variety of complex substrates (cellulose, chitin, pectin and starch) as well as simple carbon compounds. Many strains grow rapidly over a wide range of temperatures on solid or liquid media. This genus is resistant to many chemical fungicides and tends to be antagonistic to other fungi (Papavizas, 1985). Many of the *Trichoderma* spp. tested as potential biocontrol agents against plant pathogens have been applied directly to seeds (Table 1.2), or have been incorporated into soil or the growing media (Table 1.3).

Very few researchers have studied the effect of *Trichoderma* spp. on *A. flavus* and *F. moniliforme*. Under storage conditions of ambient temperature and relative humidity in South Africa, the seed-associated mycoflora proliferates with *F. moniliforme* being virtually ubiquitous in newly-harvested maize, and *A. flavus* present after storage (McLean and Berjak, 1987). Both fungal species are generally toxigenic under local conditions. Most of the rural communities use the visibly mouldy maize for beer-making, therefore an association between the prevalence of oesophageal cancer and mycotoxin-contamination has been established, particularly in regular beer drinkers (Rheeder *et al.*, 1992). Because it is almost impossible to convince the rural community about the harmful effects of consumption of mouldy maize, the aim of the present study was to ascertain the possibility of ultimately producing similarly visibly infected grains, but replacing toxigenic fusaria and aspergilli by non-toxigenic *Trichoderma* species.

Table 1.2. *Trichoderma* spp. (applied to seeds) used as antagonists of soil-borne pathogens.

| ANTAGONIST | PATHOGEN | HOST | REFERENCE |
|---|---|---|---|
| *Trichoderma* spp. | *Rhizoctonia solani* | | Elad *et al.*, 1983 |
| | *Sclerotium rolfsii* | | |
| | *Sclerotinia sclerotium* | Celery, lettuce | Budge and Whipps, 1991 |
| *T. harzianum* | *Pythium ultimum* | Cotton | Whipps and McQuilken, 1993 |
| | | Cucumber | Paulitz *et al.*, 1990 |
| | | Wheat | Whipps and McQuilken, 1993 |
| | | Maize | Whipps and McQuilken, 1993 |
| | *Pythium* spp. | Cucumber | Taylor *et al.*, 1991 |
| | | Pea | Lifschit *et al.*, 1986 |
| | *Fusarium oxysporum* f.sp. *cucumerinum* | Pea | |
| | *F. oxysporum* ff. spp. *melosis* and *vasinfectum* | | Sivan and Chet, 1989 |
| | *Cochliobolus sativus* | | |
| | *Aphanomyces euteiches* f. sp. *pisi* | Wheat | Biles and Hill, 1988 |
| | | Pea | Dandurand and Knudsen, 1993. |
| *T. hamatum* | *Pythium* spp. | | Chet *et al.*, 1981 |
| | | Pea | Harman *et al.*, 1980 |
| | | Radish | Harman *et al.*, 1980 |
| *T. viride* | *Pythium* spp. | Sugar beet | Liu and Vaughan, 1965 |
| | *P. ultimum* | White mustard | Wright, 1956 |
| | | Pea | Papavizas and Lewis, 1983 |
| | | | Lifschit *et al.*, 1986 |
| *T. koningii* | *Pythium* spp. | Pea | Hadar *et al.*, 1984 |

Table 1.3. *Trichoderma* spp.(incorporated into soil or growing media) used as antagonists of soil-borne pathogens

| ANTAGONIST | PATHOGEN | HOST | REFERENCE |
|---|---|---|---|
| *Trichoderma spp.* | *Phytophtora cactorum* | Apple | Roiger and Jeffers, 1991<br>Smith *et al.*, 1990 |
| | *Rhizoctonia solani* | Radish | Mihuta-Grimm and Rowe, 1986 |
| *T. hamatum* | *P. ultimum* | Pea | Reyes and Dirks, 1985 |
| | *S. rolfsii* | Bean | Papavizas and Lewis, 1989 |
| | *R. solani* | Cotton | Lewis and Papavizas, 1991 |
| | | Bark | Chung and Hoitink, 1990 |
| | | Beet | Lewis and Papavizas, 1987 |
| *T. harzianum* | *P. aphanidermatum* | Cucumber,gypsophila,pepper, tomato | Sivan *et al.*, 1984 |
| | *P. ultimum* | Lettuce | Lumsden *et al.*, 1990 |
| | *Sclerotium cepivorum* | Onion | Abd-El Moity *et al.*, 1982 |
| | *S. rolfsii* | Peanut | Beckerman and Rodriguez-Kabana, 1975 |
| | | Beans, cotton, tomato | Elad *et al.*, 1980 |
| | *Sclerotinia sclerotioum* | Beans | Bin *et al.*, 1991<br>Trutmann and Keane, 1990 |
| | *R. solani* | Lettuce | Maplestone *et al.*, 1991 |
| | | Tomato, bean, eggplant | Hadar *et al.*, 1979 |
| | *Phytophtora* spp.<br>*Pythium* spp. | Pea | Papavizas, 1981 |
| *T. viride* | *R. solani* | Lettuce | Coley-Smith *et al.*, 1991 |
| *T. koningii* | *Sclerotium rolfsii* | Tomato | Latunde-Dada, 1993 |

# CHAPTER 2

## MACROSCOPICAL AND MICROSCOPICAL OBSERVATIONS OF THE INTERACTION BETWEEN *Aspergillus flavus*, *Fusarium moniliforme* AND *Trichoderma* spp.

# CHAPTER 2

## MACROSCOPICAL AND MICROSCOPICAL OBSERVATIONS OF THE INTERACTION BETWEEN *Aspergillus flavus*, *Fusarium moniliforme* AND *Trichoderma* spp.

# 1. INTRODUCTION

*Aspergillus flavus* and *Fusarium moniliforme* are widespread soil-borne pathogens responsible for serious damage to developing and stored seeds. Since these pathogenic fungi are usually within the tissues of the stored seed, they cannot be eradicated using external fungicide treatments. As a consequence, efforts have been directed towards developing alternatives for more effective fungal eradication (Chet and Baker, 1981). Considerable attention has been paid to fungal antagonists mainly because of their potential for reducing the inoculum density of plant fungal pathogens (Elad *et al.*, 1980; Lewis and Papavizas, 1987).

Various *Trichoderma* species have been reported to inhibit fungal pathogen growth and development successfully (Elad *et al.*, 1983). The ability of these antagonists to attack fungal pathogens at different stages of development has led to the use of *Trichoderma* spp. as potential biocontrol agents.

Although there have been numerous recent attempts to use *Trichoderma* spp. for experimental biological control of certain fungal-induced plant diseases (Chet and Baker, 1981; Cole and Zvenyika, 1988; Coley-Smith *et al.*, 1991), very few researchers have studied the effect of *Trichoderma* spp. on *Aspergillus* and *Fusarium* spp.. Successful results have, however, been reported for the biological control of *Fusarium* crown rot of tomatoes induced by *F. oxysporum* f. sp. *radicis lycopersici* (Sivan *et al.*, 1987). Chlamydospore germination of *F. melonis* and *F. vasinfectum* in melon and cotton rhizosphere soil was significantly inhibited after *T. harzianum* application to the seed (Sivan and Chet, 1989 b). Additionally, several isolates of *T. harzianum* reduced the growth of populations of *Rhizoctonia solani* and, to a lesser extent, of *F. solani* in soil (Cole and Zvenyika, 1988). Under field conditions, *R. solani* and *F. solani* infection of tobacco transplants was reduced following addition of *T. harzianum* inoculum to methyl-bromide-fumigated seedbeds (Cole and Zvenyika, 1988).

The possible mechanisms of *Trichoderma* antagonism have been studied. Such mechanisms have been suggested to involve antibiotic and enzyme production, as well as parasitism (Elad *et al.*, 1983; Benhamou and Chet, 1993). The present study was undertaken in an attempt to elucidate the nature of the interaction between *A. flavus* and *F. moniliforme* with *Trichoderma* spp. Use of dual cultures should demonstrate whether or not particular cultures of *Trichoderma* spp. do, in fact, inhibit growth of *F. moniliforme* and *A. flavus*. Such observations should also yield information on the extent of the interaction. Scanning electron microscopical observations have potential to demonstrate whether or not actual parasitism occurs (i.e. penetration of host hyphae by *Trichoderma* spp.), or whether growth inhibition of *F. moniliforme* and/or *A. flavus* is achieved at a distance from the hyphae of *Trichoderma* spp. In the latter case, inhibition, whether by defined antibiotics or enzymes, may be suggested.

# 2. MATERIALS AND METHODS

## 2.1. Fungal species

The fungal species used in this investigation were:

- *Aspergillus flavus* Link
- *Fusarium moniliforme* Sheldon
- *Trichoderma* species:

| Fungus | Strain | Designated code |
|---|---|---|
| *T. harzianum** | PPRI 5145 | T1 |
| *T. harzianum** | PPRI 3295 | T2 |
| *T. harzianum** | PPRI 4268 | T3 |
| *T. viride** | PPRI 3774 | T4 |
| *T. viride** | PPRI 3775 | T5 |
| *T. viride** | PPRI 4705 | T6 |
| *Trichoderma* sp.* | UND | T7 |
| *Trichoderma* sp.* | UND | T8 |
| *Trichoderma* sp.* | UND | T9 |

* Authorities do not seem to be cited for *Trichoderma* spp., probably because the taxonomy has been in disarray for decades (Papavizas, 1985).

*A. flavus* and *F. moniliforme* were isolated from local maize seeds, while *Trichoderma* spp. T1-T6 were provided by the Plant Protection Research Institute (PPRI), Pretoria. *Trichoderma* spp. T7-T9 were obtained from the Department of Physiology, University of Natal, Durban.

## 2.2. Media

The fungal cultures were maintained on potato dextrose agar (PDA).

Formula:

| | |
|---|---|
| Potato dextrose agar | 25.0 g |
| Bacteroagar | 15.0 g |
| Sodium chloride | 40.0 g |
| Distilled water | 1 000 ml |

(The Oxoid Manual, 1990)

- The ingredients were mixed, then sterilised by autoclaving at 121 °C for 20 min.
- The sterilised solution was allowed to cool to approximately 60 °C, poured aseptically into sterile plastic Petri dishes and allowed to set.
- Plates were stored at 2 - 8 °C in sealed plastic bags.
- Before use, the plates were allowed to equilibrate to ambient temperature.

**2.3. The subculture technique**

Subculturing was carried out under sterile conditions, in a positive pressure chamber, to avoid contamination of plates. Using sterile inoculation needles and a stereomicroscope, fungal spores were transferred from the stock culture to the agar plates. Petri dishes were sealed with Parafilm (American Can Company), and incubated in the dark, at 25 °C for 12 days.

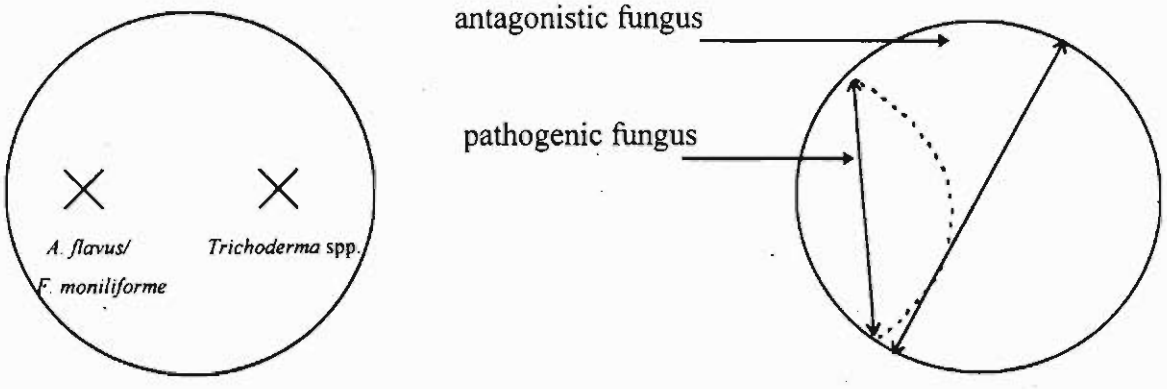
**2.4. Colony description**

- *Colony colour*: the colour of the colonies was recorded in daylight.
- *Colony texture*: the morphology of the fungal mycelium was noted.
- *Pigment production*: the production and colour of pigments were recorded.
- *Exudate droplets*: if present, the relative quantity and colour were noted.

**2.5. Interaction studies**

Nine strains of *Trichoderma* spp. (*T. harzianum* T1-T3, *T. viride* T4-T6 and *Trichoderma* spp. T7-T9) were challenged by one strain of *F. moniliforme* and one strain of *A. flavus*. The fungi were inoculated at opposite edges of the agar plate and

incubated for ten days at 25 °C. Twenty replicates were used per combination. Fungal growth was measured as described in Fig. 2.a following three, five and seven days after inoculation.




Inoculation points

Fig. 2.a. Measurement of fungal growth.

## 2.6. Scanning electron microscopy

Fungal samples of both combinations, pathogen-aggressive antagonist (aggressiveness based on the observations on dual cultures) and pathogen-non-aggressive *Trichoderma* spp. were investigated by scanning electron microscopy. Discs (10 mm diameter) were excised from the inter-species contact zone of five-day old dual colonies. Plugs were taken from plates showing the most inhibitory effect on pathogenic growth (*A. flavus/F. moniliforme*-T1, T2 T5 and T6) and the most non-inhibitory effect (*A. flavus/F. moniliforme*-T4 and T7). All samples were processed according to the protocol of Mycock and Berjak (1991) in the following manner:

1. Plugs were fixed for 2 - 24 hours in 2.5% glutaraldehyde.
2. After fixation, the samples were gently washed three times for 5 minutes in 0.5 M phosphate buffer.
3. Material was postfixed for one hour in 0.5% aqueous osmium tetroxide.

4. Specimens were washed three times for 5 minutes in 0.5 M phosphate buffer.

5. The plugs were dehydrated using an alcohol series ( 25%, 50%, 75% and 100%), with 10 - 30 minutes immersions, at each concentration.

6. Material was transferred from absolute alcohol to a critical point drier (Hitachi C.P.D.).

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

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

# 3. RESULTS

## 3.1. Macroscopic observations of dual cultures

In studying *Trichoderma* spp. and *A. flavus/F. moniliforme* in dual cultures (Figs 2.1-2.3), four strains of *Trichoderma* species (*T. harzianum* T1, T2 and *T. viride* T5, T6) had an inhibitory effect on the growth of *F. moniliforme* and *A. flavus* (Tables 2.1 and 2.2) and will be referred to as aggressive *Trichoderma* spp. The first contact between both *Trichoderma* species and either of the two pathogenic fungi was observed within five days of inoculation. Following 10 days, vigorous growth on agar by aggressive species/strains of *Trichoderma* had occurred (Fig. 2.1), with the mycelium of *A. flavus/F. moniliforme* appearing almost encircled by the aggressive *Trichoderma* colony.

In dual cultures of *F. moniliforme* and *T. harzianum* T2, or *T. viride* T6, an inhibition zone around the pathogen was observed, without there being macroscopically-visible contact of the hyphae of the two colonies. When *F. moniliforme* was co-cultured with *T. harzianum* T1, the pink colonies of the pathogen produced white exudate and a yellow pigment (i.e. reverse of plate) which had not been observed in single culture (Table 2.3). The same pattern was observed in the dual culture of *F. moniliforme* and *T. viride* (T5). This pigmentation and exudate were absent from the *Fusarium*-non-aggressive *Trichoderma* spp. (T3, T4, T7, T8 and T9) combinations. In addition, the clear inhibition zone was also absent, *F. moniliforme* colony morphology appearing macroscopically similar to that of the fungus in single culture.

The response of *A. flavus* to the aggressive *Trichoderma* mycelium (Fig. 2.1) was the production of yellow pigment around the edges of the colony. This was observed when *A. flavus* was co-cultured with *T. harzianum* T1, T2 and *T. viride* T6 (Table 2.3). In addition, an inhibition zone was observed when *A. flavus* was co-cultured with aggressive *T. harzianum* T2 (Fig. 2.1) and with non-aggressive *T. viride* T4 (Fig. 2.2) and *T. viride* T9 (Fig. 2.3). The dual cultures of *A. flavus* and non-

aggressive *Trichoderma* spp. (T3, T4, T7, T8 and T9) revealed no morphological colony alteration in *A. flavus*, the appearance being similar to that in single culture.

Table 2.1. Colony diameter of *Trichoderma* spp. and *F. moniliforme* in dual cultures.

| Fungal species | Diameter of fungal colony (mm)[a] | | |
|---|---|---|---|
| | Day 3 | Day 5 | Day 7 |
| *F. moniliforme* | 27.1 ± 1.37 | 35.5 ± 1.28 | 35.8 ± 0.98 |
| *T. harzianum* (T1) | 32.4 ± 3.52 | 72 ± 2.52 | 78.8 ± 1.16 |
| *F. moniliforme* | 20.3 ± 1.21 | 32.1 ± 2.31 | 34 ± 1.26 |
| *T. harzianum* (T2) | 39.8 ± 1.29 | 42.6 ± 2.8 | 50.5 ± 1.81 |
| *F. moniliforme* | 14.3 ± 1.36 | 24 ± 2.28 | 36 ± 4.81 |
| *T. harzianum* (T3) | 6.7 ± 0.41 | 11.8 ± 3.97 | 18.2 ± 4.91 |
| *F. moniliforme* | 18.1 ± 0.98 | 31.1 ± 0.98 | 46 ± 2.82 |
| *T. viride* (T4) | 2.5 ± 2.24 | 10 ± 2.28 | 25.8 ± 2.31 |
| *F. moniliforme* | 25.6 ± 2.3 | 40.6 ± 3.61 | 44.1 ± 2.31 |
| *T. viride* (T5) | 25.3 ± 2.50 | 21.5 ± 1.37 | 57.5 ± 1.51 |
| *F. moniliforme* | 18.5 ± 1.37 | 29.5 ± 0.49 | 38 ± 1.0 |
| *T. viride* (T6) | 32.8 ± 2.56 | 55.9 ± 6 | 67.8 ± 2.4 |
| *F. moniliforme* | 18.5 ± 1 | 32.1 ± 0.75 | 44 ± 1 |
| *Trichoderma* (T7) | 6.8 ± 0.4 | 15.6 ± 2.58 | 21.1 ± 1.32 |
| *F. moniliforme* | 23.8 ± 1.1 | 40.3 ± 2.5 | 49.6 ± 4.45 |
| *Trichoderma* (T8) | 18.6 ± 2.33 | 27.3 ± 3.2 | 38.8 ± 6.4 |
| *F. moniliforme* | 21.5 ± 0.49 | 37 ± 2.44 | 44 ± 1.26 |
| *Trichoderma* (T9) | 8.41 ± 1.42 | 12.5 ± 0.49 | 23.6 ± 4.17 |

[a] Values represent means ± SE of twenty replicates

Table 2.2. Colony diameter of *Trichoderma* spp. and *A. flavus* in dual cultures.

| Fungal species | Diameter of fungal colony (mm)[a] | | |
|---|---|---|---|
| | Day 3 | Day 5 | Day 7 |
| *A. flavus* | 23 ± 0.89 | 32.6 ± 0.40 | 47.3 ± 3 |
| *T. harzianum* (T1) | 28 ± 0.89 | 45 ± 0 | 58.8 ± 1.16 |
| *A. flavus* | 22.3 ± 0.87 | 31.1 ± 0.49 | 45.8 ± 0.98 |
| *T. harzianum* (T2) | 35.9 ± 3 | 55.3 ± 0.51 | 70.5 ± 1 |
| *A. flavus* | 20.3 ± 0.81 | 26.1 ± 0.68 | 42 ± 1 |
| *T. harzianum* (T3) | 13.5 ± 1.42 | 16.9 ± 2.1 | 38.1 ± 2.2 |
| *A. flavus* | 21.4 ± 0.9 | 29 ± 1 | 50.1 ± 2.63 |
| *T. viride* (T4) | 20.8 ± 0.51 | 34 ± 0 | 41.1 ± 1.49 |
| *A. flavus* | 22.5 ± 0.54 | 30.5 ± 0.49 | 47.8 ± 3.1 |
| *T. viride* (T5) | 15.5 ± 2.72 | 34.58 ± 2.8 | 68 ± 3.2 |
| *A. flavus* | 19.5 ± 2.97 | 31.2 ± 1.4 | 49.5 ± 1.87 |
| *T. viride* (T6) | 21.9 ± 0.2 | 36 ± 0 | 68 ± 0 |
| *A. flavus* | 21.9 ± 1.56 | 33 ± 0 | 49 ± 3.8 |
| *Trichoderma* (T7) | 17 ± 1.1 | 32.8 ± 1.5 | 32.8 ± 1.9 |
| *A. flavus* | 21.5 ± 0.4 | 30.5 ± 0.4 | 45.6 ± 2.25 |
| *Trichoderma* (T8) | 19.2 ± 4 | 30.4 ± 0.8 | 47.1 ± 1.32 |
| *A. flavus* | 32.1 ± 3.37 | 31.5 ± 1.9 | 45.6 ± 2.22 |
| *Trichoderma* (T9) | 16 ± 7.2 | 23 ± 0.8 | 34.4 ± 1.35 |

[a]Values represent the means ± SE of twenty replicates

Table 2.3. Colony description of fungi in single culture and in dual cultures.

| Fungal species | Colony description | | | | Fungal species |
|---|---|---|---|---|---|
| | Dual culture | Individual culture | | Dual culture | |
| *A. flavus* | floccose, green, **yellow edges** | floccose, green | fluffy, white | fluffy, white | *T. harzianum* (T1) |
| *A. flavus* | floccose, green, **yellow edges, yellow pigment inhibition zone** | floccose, green | fluffy, light-yellow | fluffy, light-yellow | *T. harzianum* (T2) |
| *A. flavus* | floccose, green | floccose, green | velvety, light-green | velvety, light-green | *T. harzianum* (T3) |
| *A. flavus* | floccose, green inhibition area | floccose, green | fluffy, white | fluffy, white | *T. viride* (T4) |
| *A. flavus* | floccose, green | floccose, green | aerial, white | aerial, white | *T. viride* (T5) |
| *A. flavus* | floccose, green, yellow edges, **yellow pigment** | floccose, green | fluffy, white | fluffy, white | *T. viride* (T6) |
| *A. flavus* | floccose, green | floccose, green | fluffy, white | fluffy, white | *Trichoderma* sp. (T7) |
| *A. flavus* | floccose, green | floccose, green | aerial, light-green | aerial, light-green | *Trichoderma* sp. (T8) |
| *A. flavus* | floccose, green inhibition zone | floccose, green | light-green | light-green | *Trichoderma* sp. (T9) |
| *F. moniliforme* | aerial, light-pink, **yellow edges, yellow pigment, white exudate** | aerial, light-pink | fluffy, white | fluffy, white | *T. harzianum* (T1) |
| *F. moniliforme* | aerial, light-pink, **clear surrounding zone** | aerial, light-pink | fluffy, light-yellow | fluffy, light-yellow | *T. harzianum* (T2) |
| *F. moniliforme* | aerial, light-pink | aerial, light-pink | velvety, light-green | velvety, light-green | *T. harzianum* (T3) |
| *F. moniliforme* | fluffy, light-pink | fluffy, light-pink | fluffy, white | fluffy, white | *T. viride* (T4) |
| *F. moniliforme* | aerial, light-pink, **yellow edges, exudate present** | fluffy, light-pink | fluffy, white | fulffy, white | *T. viride* (T5) |
| *F. moniliforme* | light-pink, **clear zone around** | aerial, light-pink | fluffy, white | fluffy, white | *T. viride* (T6) |
| *F. moniliforme* | fluffy, lifht-pink | aerial, light-pink | fluffy, white, light-yellow pigment | fluffy, white | *Trichoderma* sp. (T7) |
| *F. moniliforme* | fluffy, light-pink | aerial, light-pink | aerial, light-green | aerial, light-green | *Trichoderma* sp. (T8) |
| *F. moniliforme* | light-pink | light-pink | aerial, light-green | aerial, light-green | *Trichoderma* sp.(T9) |

In all combinations, *Trichoderma-A. flavus/F. moniliforme*, no morphological changes of *Trichoderma* spp. colonies were recorded.

### 3.2. Scanning electron microscopy

#### 3.2.1. Individual cultures

The aspergilli are filamentous fungi (Cole and Samson, 1979) with the conidial apparatus developing from specialised, thick-walled cells, the foot cells (Fig. 2.4). The uniformly decorated conidiophore, which is usually not septate, enlarges upwards to form a globose or subglobose vesicle (Fig. 2.5). The fertile area of the mature vesicle gives rise to a layer of conidium-producing cells, the phialides (Fig. 2.6), which usually develop simultaneously and approximately perpendicular to their point of origin (Fig. 2.7). The production of primary conidia occurs more or less synchronously (Fig. 2.8), followed by production of chains of secondary conidia (Fig. 2.9). Emergence of conidia occurs on the phialide apex, where the remains of the sporogenous cell wall forms a collarette (Fig. 2.10). The conidia of *A. flavus* are subglobose or globose with a characteristic roughed decoration when mature (Fig. 2.11).

Scanning electron micrographs of control colonies of *F. moniliforme* cultured on PDA illustrate the aerial, fluffy hyphae (Fig. 2.12). The conidiophore may be branched or unbranched (Fig. 2.13), forming lateral monophialides (Fig. 2. 14). The phialidic system may give rise to only a single conidium (Fig. 2.14), or may produce a succession of conidia. When the conidium reaches full size, the enveloping wall of the apex of the phialide becomes ruptured, leaving a collar, the collarette (Fig. 2.14). Apart from the disposition in long chains, the oval-shaped microconidia formed on monophialides often become aggregated into false heads (Fig. 2.15). Although macroconidia are sometimes produced by this fungus (Nelson *et al.*, 1983), in the present study, only microconidia were observed.

The Hypomycete genus *Trichoderma* is characterised by a fast-growing mycelium (Fig. 2.16 and 2.22) bearing branched conidiophores (Figs. 2.17 and 2.21).

Cultures of *Trichoderma* spp. quickly lose the capacity to sporulate when grown in the dark (Figs 2.16 and 2.22). Conidiophores of *T. harzianum* are long, with short side branches (Fig. 2.18), while *T. viride* has conidiophores that are typically divergently branched (Fig. 2.23), with slender side branches occurring near the conidiophore tip (Fig. 2.23). Conidiophores of *T. harzianum* may have sterile hyphal elongations, in contrast to *T. viride*, where these sterile ends are rare. The short and plump phialides of *T. harzianum* bear relatively smooth, subglobose conidia (Fig. 2. 19), while the long, slender phialides of *T. viride* are arranged in divergent groups of 2 to 4, carrying mostly distinctly roughened conidia ( Fig. 2. 24) (PPRI, 1994) .

### 3.2.2. Dual cultures

Scanning electron micrographs of the mycelial samples collected from the interaction regions between aggressive or non-aggressive *Trichoderma* spp. and the pathogenic fungi revealed different interaction patterns. *Trichoderma viride* T4, and *T. harzianum* T2 or *T. viride* T5 have been chosen as non-aggressive and aggressive strains, respectively, to illustrate the microscopical interaction with *A. flavus* and *F. moniliforme*.

#### a. *Trichoderma* spp. - *A. flavus*

Despite the consistent occurrence of the inhibition zone (Figs 2.1-2.3), aerial hyphae of the two species intermingled. However, microscopical differences were recorded in the interaction between aggressive or non-aggressive *Trichoderma* spp. and *A. flavus*.

Non-aggressive *T. viride* T4-*A. flavus*

In dual cultures between non-aggressive *T. viride* T4 and *A. flavus*, the hyphae of the former intermingled with those of the *A. flavus* colony (Fig. 2.25). In comparison with the appearance of *A flavus* in single culture, there were no apparent alterations in hyphal or sporulating conidial head morphology (Fig. 2.26).

Aggressive *T. harzianum* T2-*A. flavus*

In the aggressive T2-*A. flavus* combination, the antagonist fungus grew towards *A. flavus* mycelium, and by day 5, had over-run the *A. flavus* hyphae (Fig. 2.27). During the interaction process, the aggressive *T. harzianum* T2 hyphae established close contact with the host by coiling around the hyphae and conidiophores (Fig. 2.28). Despite the coiling of the hyphae, there was no visible evidence of hyphal penetration by *T. harzianum.* In the contact region of the dual culture, some aberrant conidial heads (e.g. absence of vesicle or fewer phialides) of the *A. flavus* colony were observed (Figs 2.31-2.32). Whether aberrant or not, conidial heads produced seemingly normal spores (Figs 2.29-2.32), but it is not known whether such spores would germinate or produce viable mycelium. Normal conidial heads (Figs 2.27-2.28) and microheads (Fig. 2.33) were also produced.

**b. *Trichoderma* spp. - *F. moniliforme***

Non-aggressive *T. viride* T4-*F. moniliforme*

When *F. moniliforme* was co-cultured with non-aggressive strains of *Trichoderma* spp., no morphological changes of *F. moniliforme* were observed (Fig. 2.34). Aerial hyphae predominated (Fig. 2.35), reflecting the fluffy, well-developed appearance of the colony (Fig. 2.36). The morphological features of the hyphae of *F. moniliforme* (Fig. 2.37) appeared similar to those of the fungus in single culture (Fig. 2.12).

Aggressive *T. viride* T5-*F. moniliforme*

The reaction between *T. viride* T5 and *F. moniliforme* has been chosen as illustrative. In the growth-restricted colonies of *F. moniliforme* co-cultured with aggressive strains of *T. viride* T5, the hyphae of *F. moniliforme* were generally wrinkled, prostrate and adpressed (Fig. 2.39). As early as five days after inoculation, these alterations of hyphal morphology were evident (Figs 2.40-2.41). Pronounced collapse of the *F. moniliforme* hyphae was observed frequently ( Fig. 2.44) together with the adpressed and aggregated aspect of the mycelium (Figs 2.42-2.43). Alteration in hyphal morphology of *F. moniliforme* was also observed in areas where the

antagonist was not in close proximity (Fig. 2.38). In none of the examined samples, were the hyphae of *Trichoderma* spp. observed to penetrate those of *F. moniliforme*.

# 4. DISCUSSION

Hyphal interaction between *Trichoderma* spp. and some plant pathogenic fungi has been previously studied at the light and electron microscope levels (Elad *et al.*, 1983), and the parasitic ability of a number of *Trichoderma* isolates has been convincingly demonstrated (Benhau and Chet, 1993; Elad *et al.*, 1983). In an attempt to ascertain the nature of the interaction between aggressive and non-aggressive *Trichoderma* spp., and *A. flavus* and *F. moniliforme*, nine strains of two *Trichoderma* spp. were investigated macroscopically. The contact zone between the most and least aggressive of the antagonist isolates and each pathogen were then selected for investigation by scanning electron microscopy. The contact zone between *A. flavus* and *T. harzianum* T2, and *F. moniliforme* and *T. viride* T5, respectively, are presented to illustrate the SEM aspects of aggressive interactions, while the non-aggressive *T. viride* T4 illustrates the interactions for both pathogens.

The investigation of fungal development in dual cultures clearly showed that despite the fact that *Trichoderma* spp. may be differentially selective against different fungi (Hadar *et al.*, 1979), the same four isolates of the *Trichoderma* spp. under investigation curtailed the growth of both *A. flavus* and *F. moniliforme* in culture. *Trichoderma harzianum* T2 demonstrated antagonistic patterns, a clear inhibition zone and induction of pigment production by the *A. flavus* colony, and also growth inhibition of *F. moniliforme* (Table 2.3; Fig. 2.1). The presence of the inhibition zone in dual culture without there being hyphal contact, may possibly indicate the liberation of diffusable inhibitory substances by *Trichoderma* strains (Chapter 3). Similar morphological changes as pigment production by *A. flavus* when co-cultured with *T. harzianum* T1 or T2 and *T. viride* T6 (not illustrated) have been exhibited by *Aspergillus* spp. growing under certain stressful nutritional and environmental conditions (Raper and Fennell, 1965), suggesting that the aggressive strains of *Trichoderma* spp. may have imposed some stress (nutritional or other) on *A. flavus*.

Although *Trichoderma* spp. have been reported to be parasitic on various plant pathogens (Elad *et al.*, 1983; Doi *et al.*, 1994), the present scanning electron

micrographs of the interaction zones of *A. flavus/F. moniliforme-Trichoderma* spp. did not suggest parasitic activity (i.e. penetration of hyphae). Scanning electron microscopical investigations of dual cultures confirmed the apparently differing pattern of fungal interaction suggested by macroscopical observations. In *A. flavus-T. harzianum* T2 combination, aerial fungal mycelia were interdigitated, while in *F. moniliforme-T. viride* T5 interaction, hyphae of the former became aggregated and/or collapsed, even at a distance from the mycelium of *T. viride* T5.

When aggressive *Trichoderma* spp. were grown with *F. moniliforme*, no physical contact between the colonies was observed, whereas co-cultured with *A. flavus*, aggressive *Trichoderma* spp. coiled around the host but no obvious penetration was observed. This is in contrast to the observations for the *Rhizoctonia solani-Trichoderma* spp. interaction where penetration was reported (Elad *et al.*, 1983; Ordentlich and Chet, 1989).

Collapse and loss of turgor of *F. moniliforme* hyphae, despite the fact that the hyphae of *Trichoderma* spp. were not in direct contact with the former, suggested that extracellular metabolites and/or volatile compounds could be responsible for the observed degradation (Chapter 3). The presence of abnormal conidial heads and microheads in *A. flavus* colonies co-cultured with aggressive *Trichoderma* spp. suggested that stress conditions had been imposed on the pathogen. These observations may be interpreted in terms of the action of metabolites elaborated by *Trichoderma* spp. causing physiological and morphological abnormality in *A. flavus*, and the severity of the response in *F. moniliforme* causing actual hyphal collapse.

In summary, of nine strains of *Trichoderma* spp. used in this study, two strains of *T. harzianum* (T1 and T2) and two of *T. viride* (T5 and T6) were capable of curtailing the growth of both *A. flavus* and *F. moniliforme*. In both the *T. harzianum* T2-*A. flavus* and *T. viride* T5-*F. moniliforme* interactions which are reported in detail, scanning electron microscopy revealed morphological changes to the pathogen colonies (i.e. production of aberrant conidial heads by *A. flavus* and adpression and aggregation of the *F. moniliforme* hyphae) in co-culture. In no samples investigated,

was hyphal penetration observed, indicating that parasitism by *Trichoderma* spp. does not appear to be the mechanism causing growth inhibition of either pathogen in the present case. This aspect, however, should be scrutinised in futher studies using TEM and light microscopy, for the *Trichoderma* spp.-*A. flavus* situation.

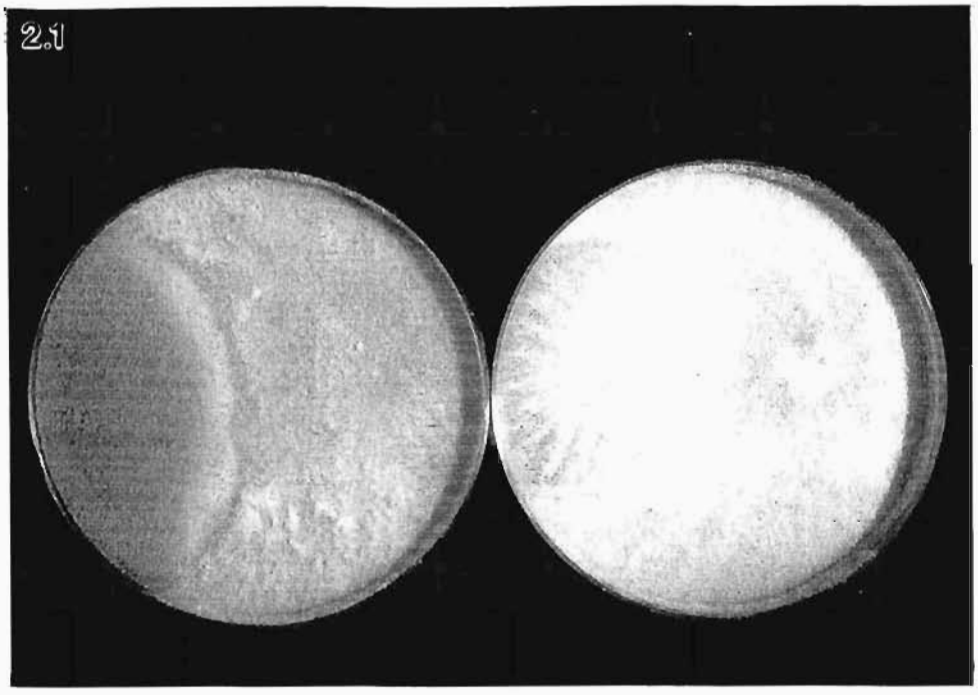
In addition, the collapse and loss of turgor of *F. moniliforme* in areas where *T. viride* T5 was not in direct contact, and the production of abnormal conidial heads by *A. flavus* co-cultured with aggressive *T. harzianum* T2, suggests that the production of diffusable substances by *Trichoderma* spp. (extracellular enzymes and/or antibiotics and/or volatile compounds) may be involved in the observed inhibitory effects.

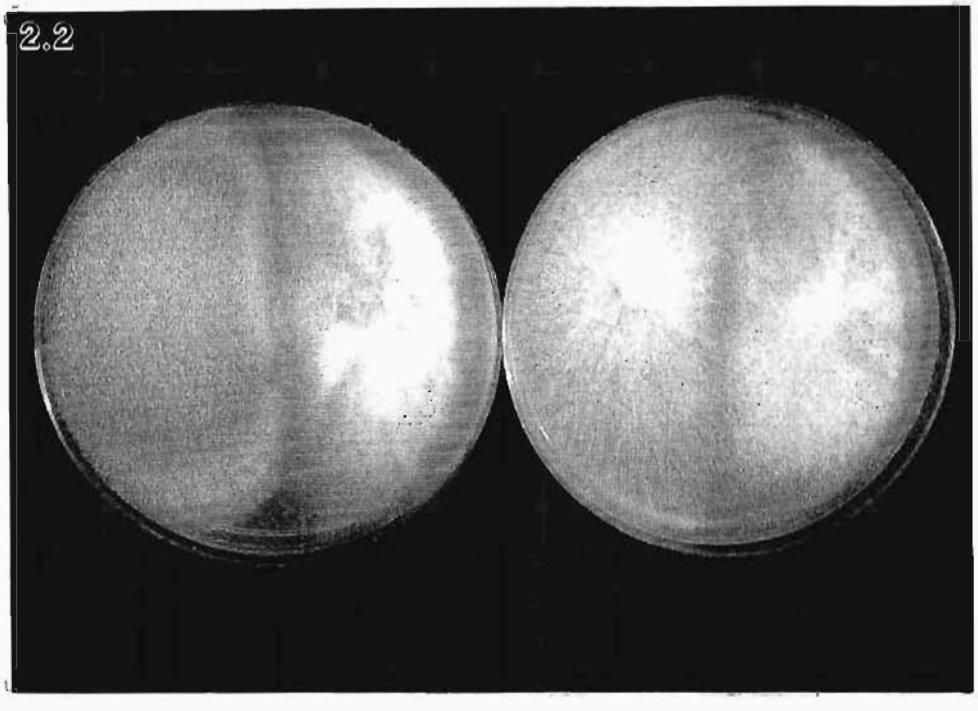
Figs 2.1-2.3. Dual culture of *A. flavus*, *F. moniliforme* and *Trichoderma* spp. In each case the *Trichoderma* spp. was inoculated on the right-hand side of the plate.

Fig. 2.1. Dual culture of *A. flavus* (left), *F. moniliforme* (right) and *T. harzianum* T2.

Fig. 2.2. Dual culture of *A. flavus* (left), *F. moniliforme* (right) and *T. viride* T4.

Fig. 2.3. Dual culture of *A. flavus* (left), *F. moniliforme* (right) and non-aggressive *T. viride* T9. It is suggested that this strain of *T. viride* may also be characterised by slow growth.


2.1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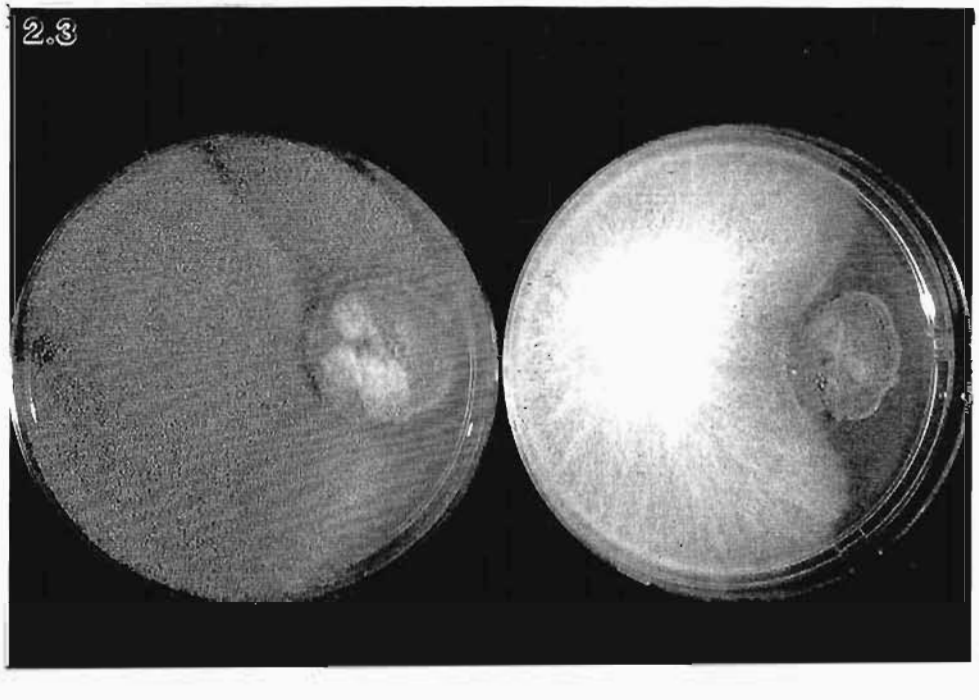
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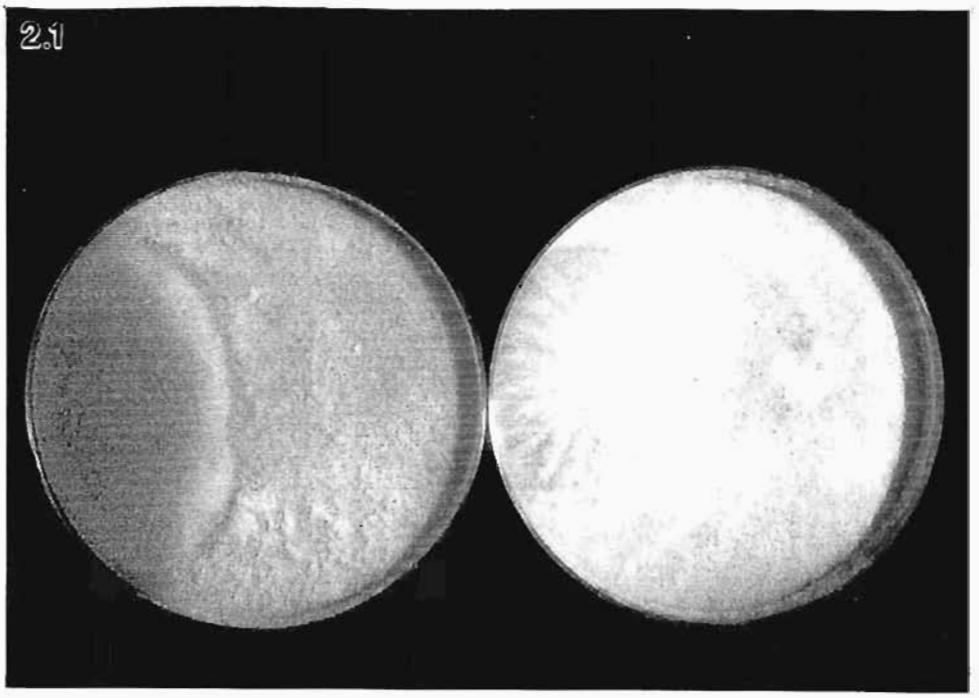
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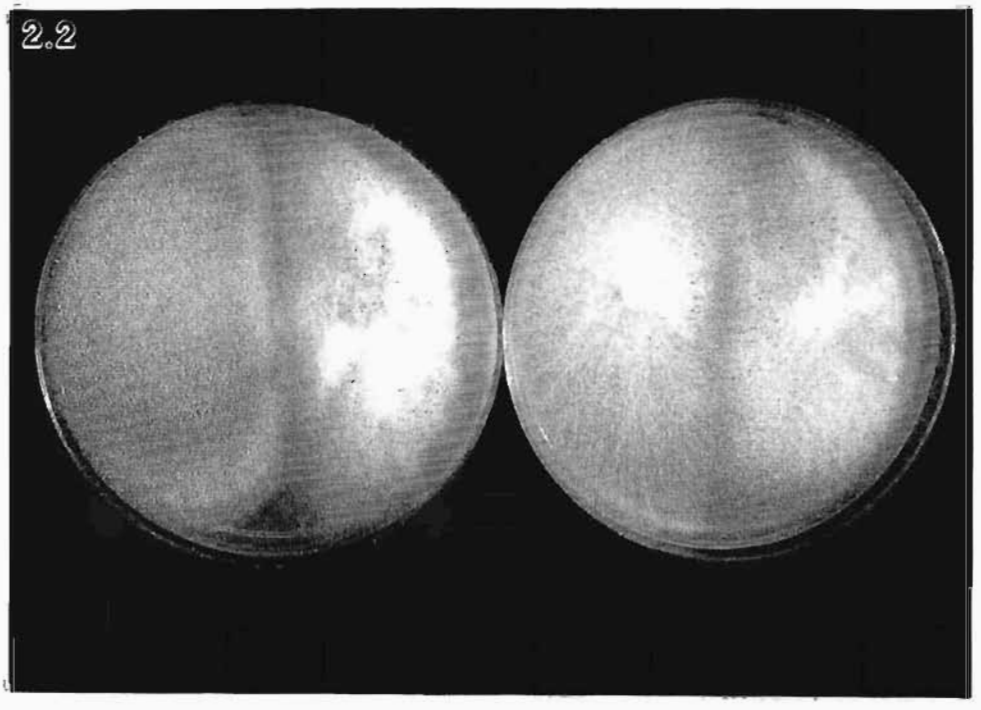
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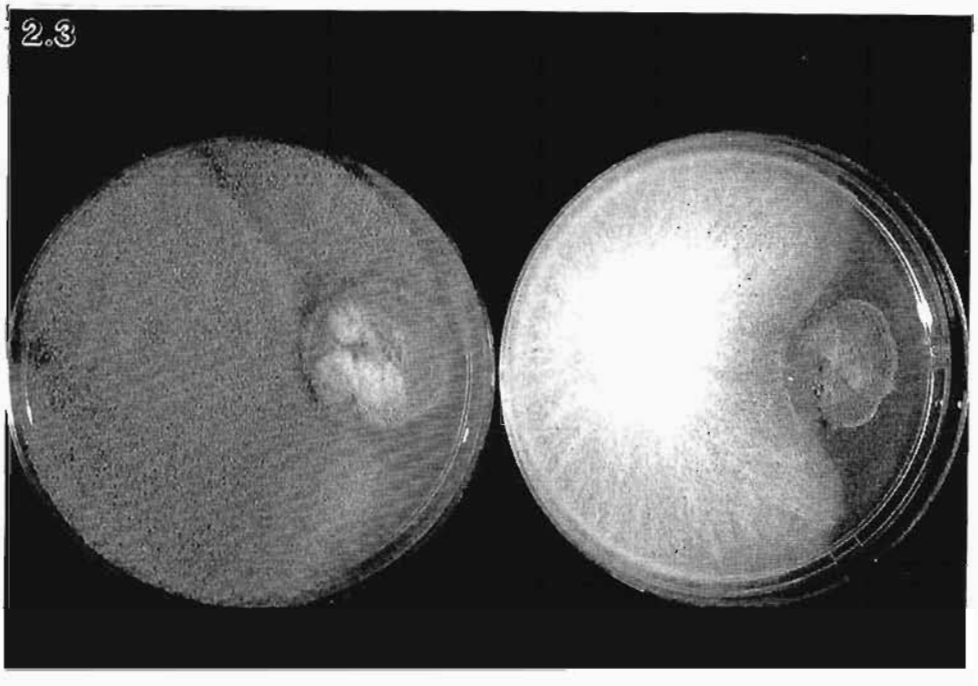

2.3

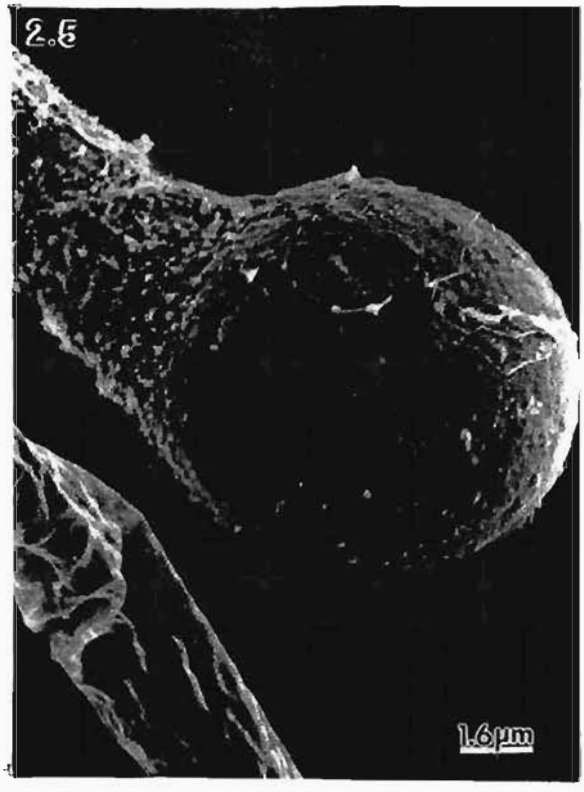
Figs 2.4 - 2.7. Scanning electron micrographs of *A. flavus* in single culture.

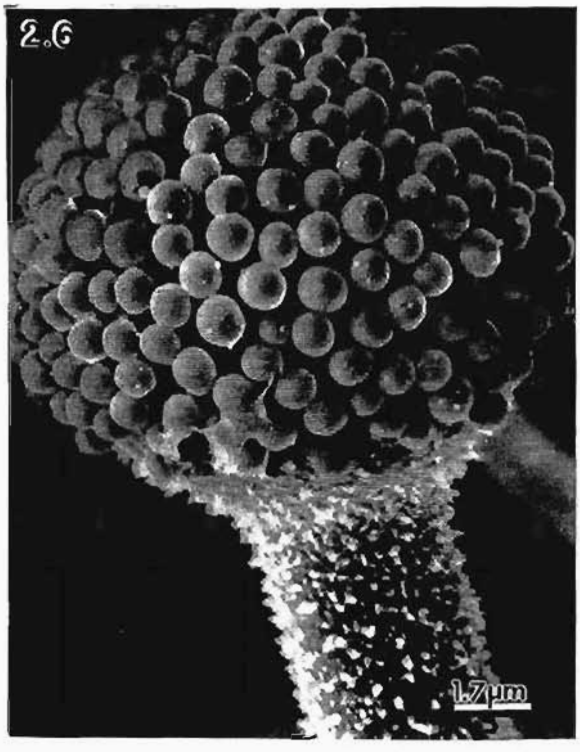
2.4. Formation of a conidiophore from a foot cell. Note the verrucose decoration of the conidiophore (arrow).

2.5. After the conidiophore has fully developed, apical blastogenesis occurs, resulting in the formation of a subglobose vesicle.

2.6 - 2.7. The mature vesicle gives rise to a layer of phialides, the conidiogenous cells.


2.4
1.8µm


2.5
1.6µm


2.6
1.7µm


2.7
1.6µm

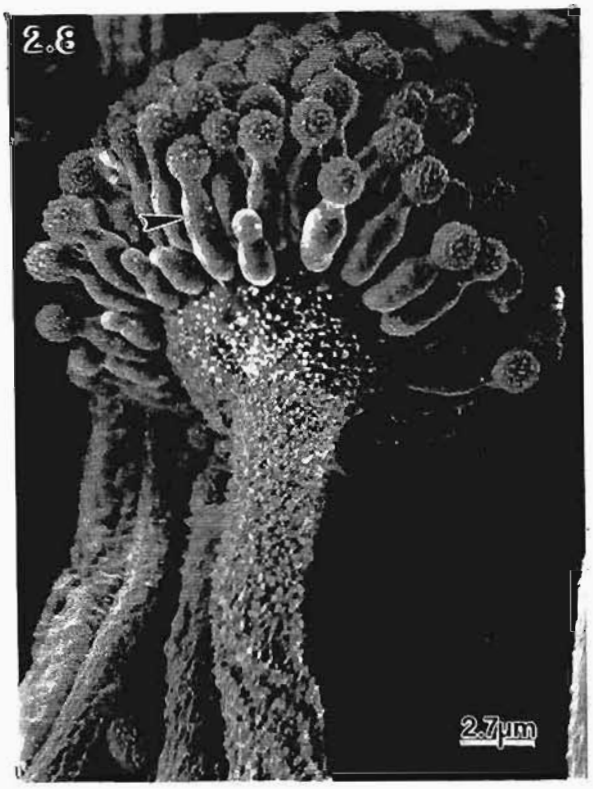
Figs 2.8 - 2.11. Scanning electron micrographs of *A. flavus* in single culture.

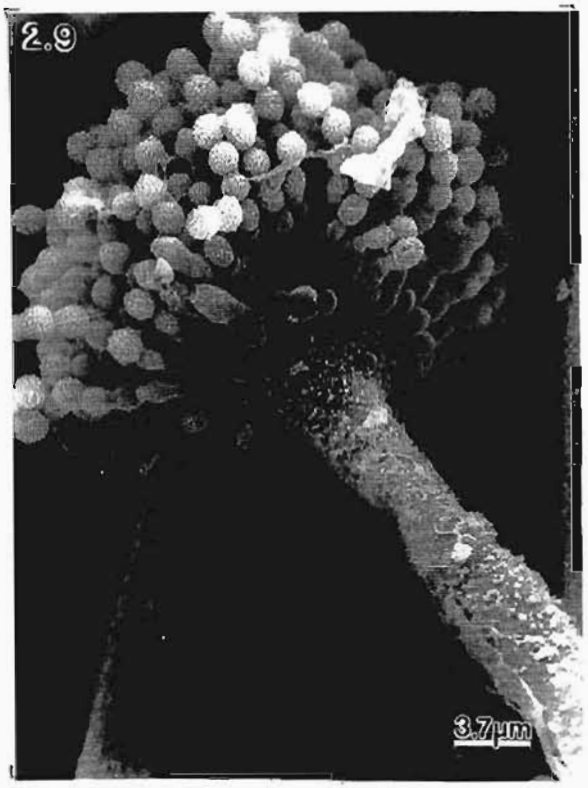
2.8. The production of primary conidia occurs more or less synchronously. Note the ampulliform phialides (arrows).

2.9. Formation of secondary conidia.

2.10. Presence of a collarette (arrow).

2.11. Characteristically roughened mature conidia.


2.8
2.7µm


2.9
3.7µm


2.10
0.4µm


2.11
1.3µm

Figs 2.12 - 2.15. Scanning electron micrographs of *F. moniliforme* in single culture.

2.12. General view of a colony of *F. moniliforme.*

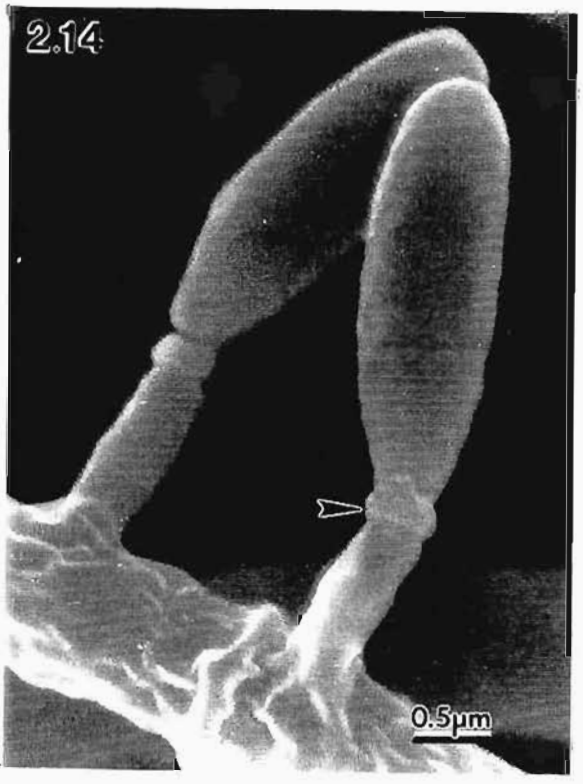
2.13. Septate conidiophores forming lateral monophialides.

2.14. The phialidic system giving rise to conidia. Note the presence of the collarette (arrow).

2.15. Aggregation of microconidia into a false head.


2.12
8.3µm


2.13
1.5µm


2.14
0.5µm


2.15
1.2µm

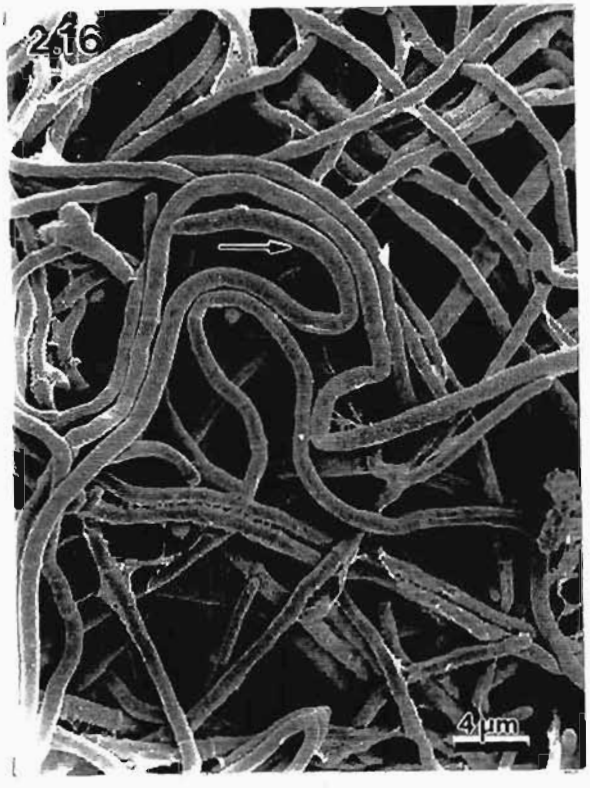
Figs 2.16 - 2.19. Scanning electron micrographs of *T. harzianum* in single culture.

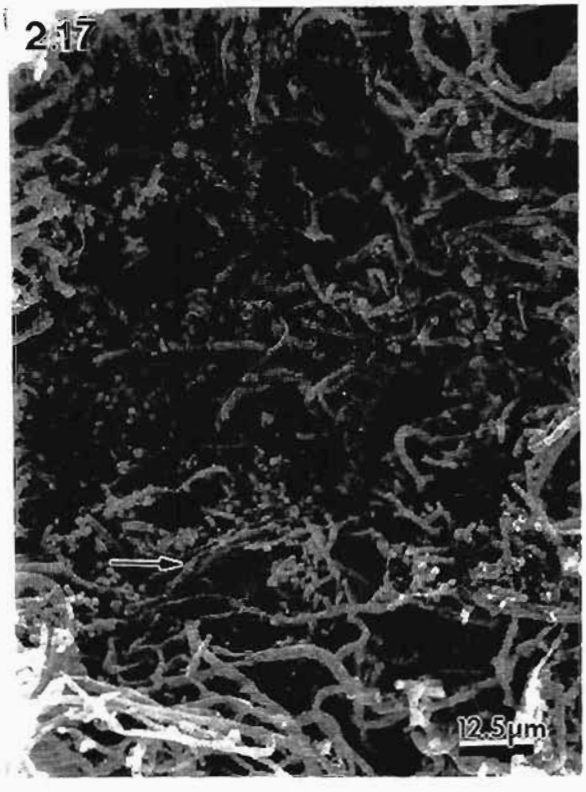
2.16. Culture grown in dark for seven days. Note the absence of sporulation and the occasional cohesion between adjacent hyphae (arrow).

2.17. Sporulating colony grown in the light. Note occasional apparently cohesive hyphae (arrow).

2.18. Conidiophore with short side branches.

2.19. Short phialides giving rise to smooth, subglobose conidia.


2.16
4 µm


2.17
12.5 µm


2.18
1.2 µm


2.19
0.8 µm

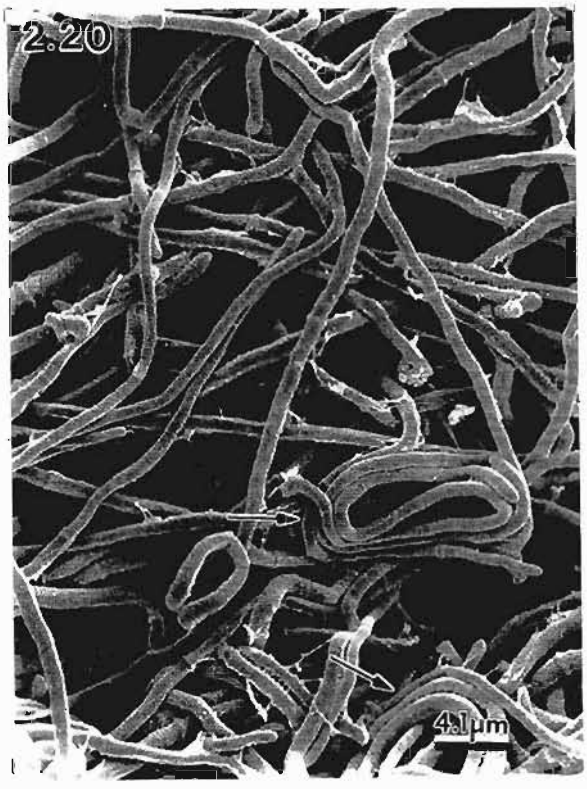
Figs 2.20 - 2.23. Scanning electron micrographs of *T. viride* in single culture.

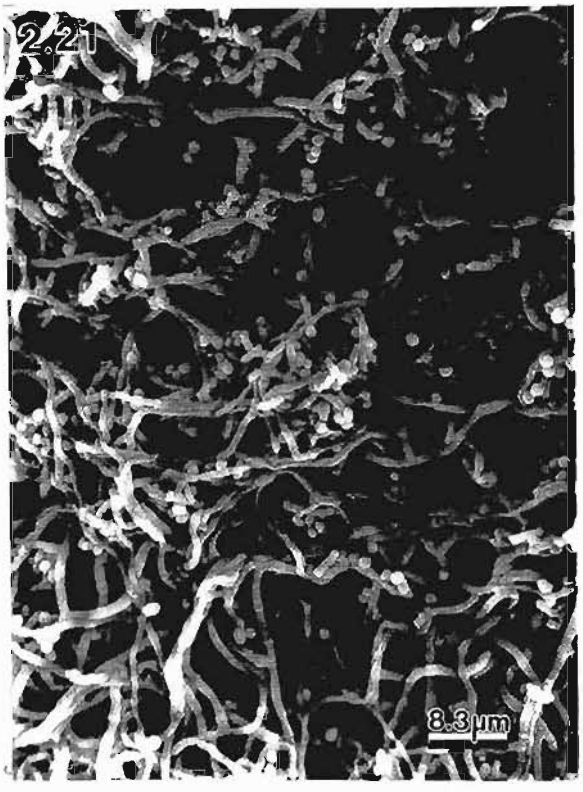
2.20. Colony grown in dark for seven days. Note the absence of sporulation and apparently cohesive hyphae (arrow).

2.21. Sporulating mycelium of *T. viride*.

2.22. Hypha giving rise to a conidiophore. Note the fine filamentous material that appears loosely binding adjacent hyphae.

2.23. Conidiophore with typically divergent and long phialides.


2.20
4.1µm

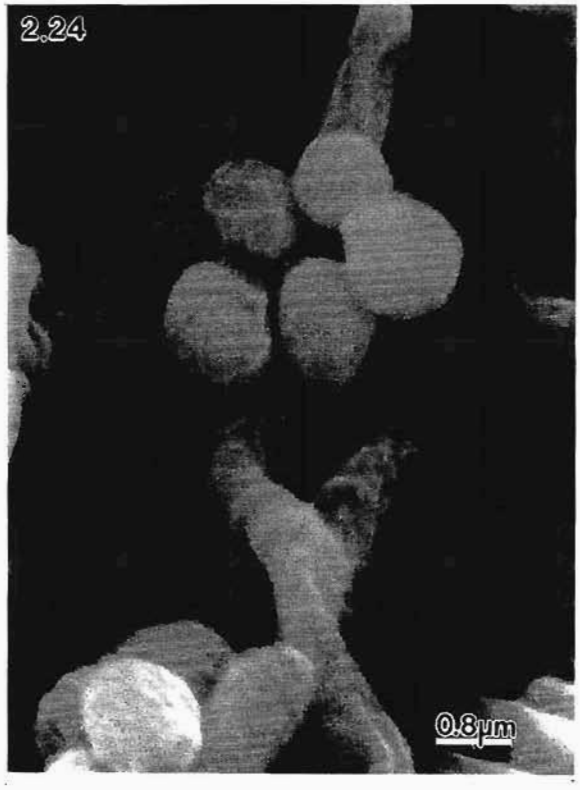

2.21
8.3µm


2.22
0.9µm


2.23
1.3µm

Fig. 2.24. Scanning electron micrographs of *T. viride* in single culture.

2.24. Characteristic mature, roughened conidia.


2.24
0.8µm

Figs 2.25 - 2.26. Scanning electron micrographs of dual culture of *A. flavus* and non-aggressive *T. viride* T4.

2.25. In dual culture, the non-aggressive *T. viride* T4 hyphae intermingled with those of *A. flavus* at the contact zone.

2.26. Morphology of *A. flavus* appeared normal. No apparently abnormal heads were observed.


2.25
T
A
33 µm


2.26
20 µm

Figs 2.27 - 2.29. Scanning electron micrographs of the interaction zone between *A. flavus* and aggressive *T. harzianum* T2.

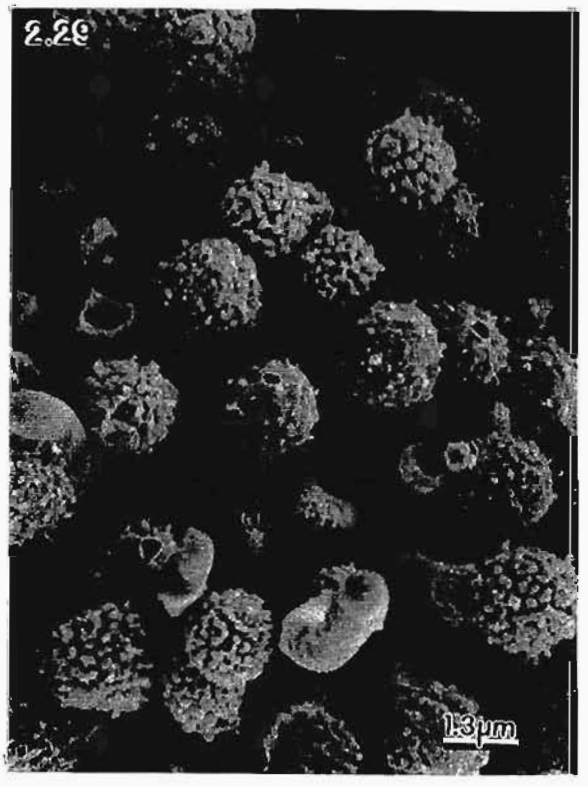
2.27. By day five, the *T. harzianum* T2 hyphae had intermingled with those of *A. flavus*.

2.28. *T. harzianum* T2 hyphae coiling around *A. flavus* hyphae and conidiophores (arrow). Note the occurrence of an apparently normal sporulating head.

2.29. Apparently normal production of mature conidia by *A. flavus* in dual culture with aggressive *T. harzianum* T2.


2.27
A
T
100µm


2.28
f
a
f
a
8.3µm

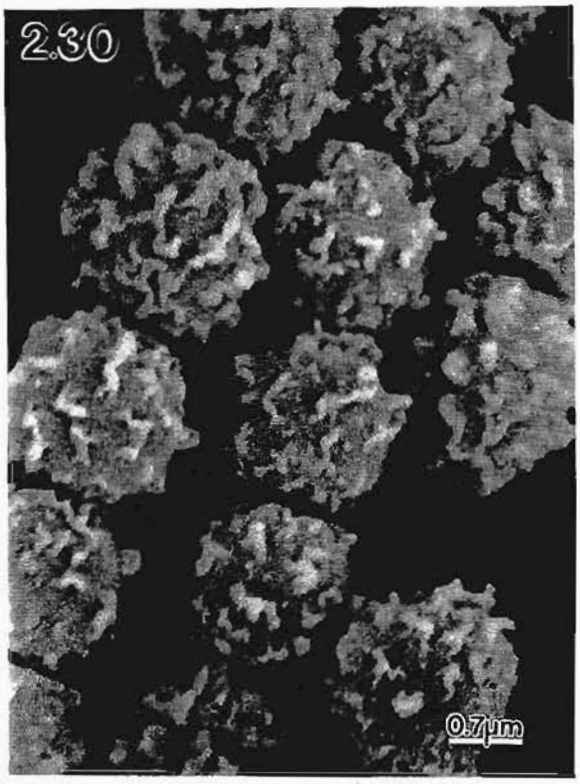

2.29
1.3µm

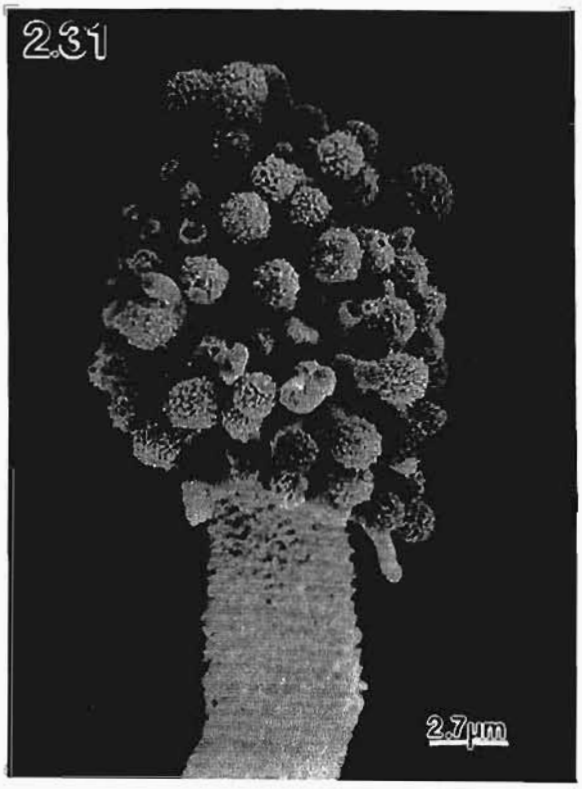
Figs 2.30 - 2.33. Scanning electron micrographs of *A. flavus* colony co-cultured with aggressive *T. harzianum* T2.

2.30. Mature conidia of *A. flavus*.

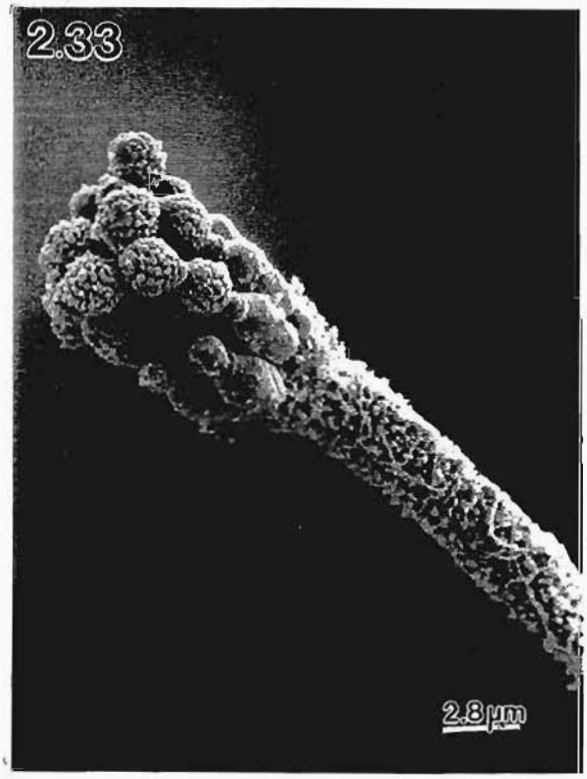
2.31 - 2.32. Abnormal mature conidial heads of *A. flavus*.

2.33. Microhead of *A. flavus*.


2.30
0.7µm


2.31
2.7µm

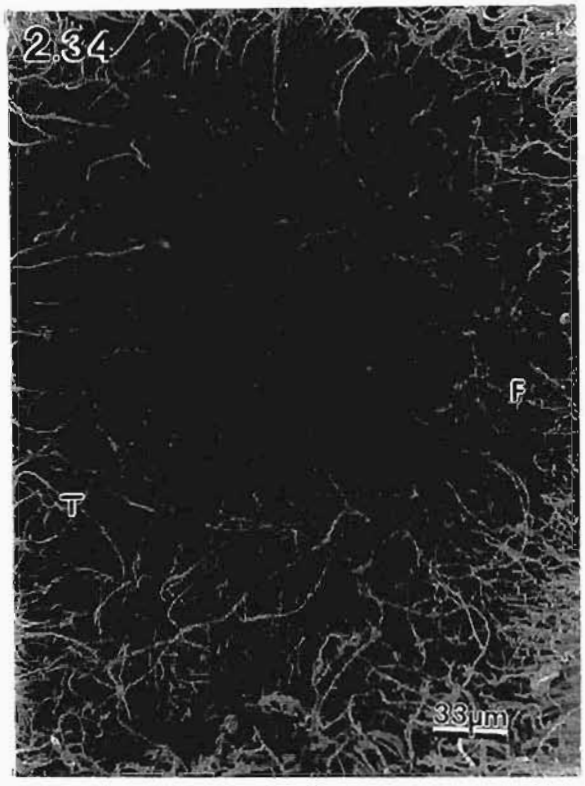

2.32
2.7µm

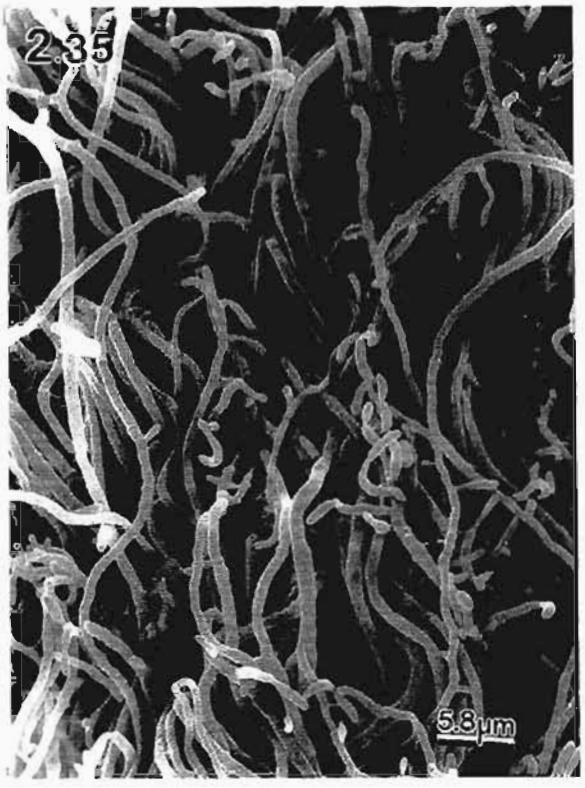

2.33
2.8µm

Figs 2.34 - 2.37. Scanning electron micrographs of dual culture of *F. moniliforme* (F) with the non-aggressive *T. viride* T4 (T).

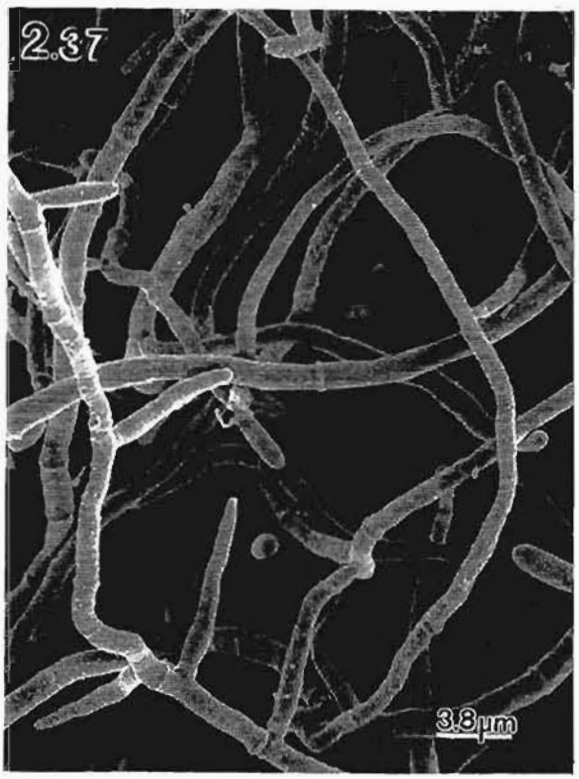
2.34. No morphological changes in the mycelium of *F. moniliforme* (F) were observed when co-cultured with *T. viride* T4 (T).

2.35 - 2.37. Normal, well-developed aerial hyphae of *F. moniliforme*.


2.34
F
T
33μm


2.35
5.8μm


2.36
8.3μm

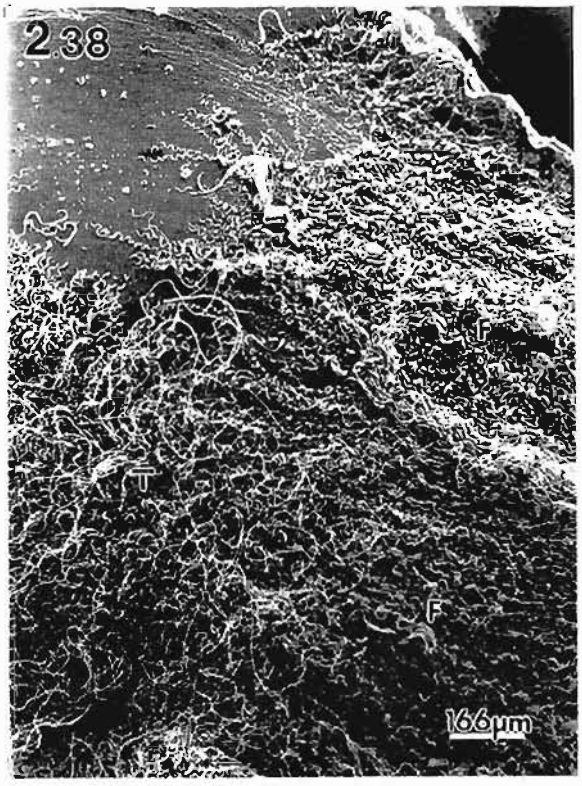

2.37
3.8μm

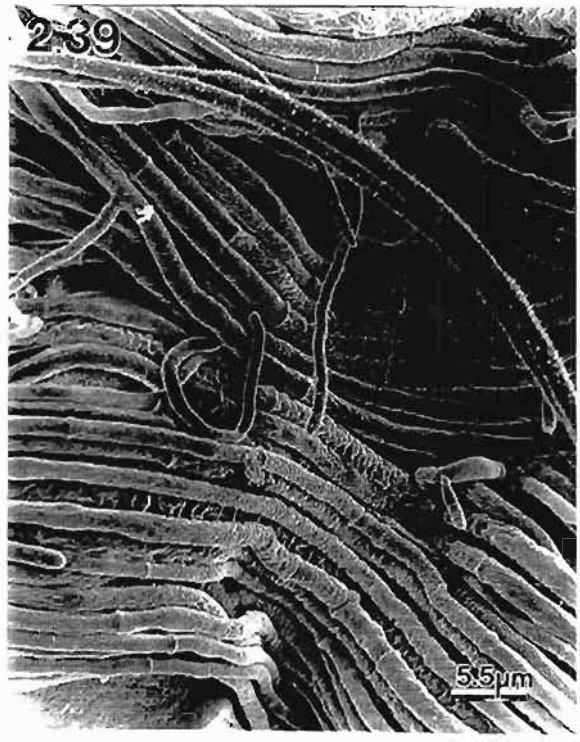
Figs 2.38 - 2.41. Scanning electron micrographs of dual cultures of *F. moniliforme* and aggressive *T. viride* T5.

2.38. General view of the dual culture. Note the *F. moniliforme* hyphal aggregation in areas where there is no contact with *T. viride* T5 hyphae (arrows).

2.39-2.40. Aggregation of *F. moniliforme* hyphae, underlying abnormal mycelium morphology.

2.41. Illustrates collapse of individual *F. moniliforme* hyphae.


2.38
F
T
F
166µm


2.39
5.5µm


2.40
4µm


2.41
5.5µm

Figs 2.42-2.44. Scanning electron micrographs of *F. moniliforme* when co-cultured with the aggressive *T. viride* T5.

2.42 - 2.43. Adpressed and aggregated colony of *F. moniliforme*.

2.44. Collapsed hyphae of *F. moniliforme*.


2.42
5.5µm


2.43
5.5µm


2.44
1.3µm

# CHAPTER 3

# A STUDY OF THE PRODUCTION OF EXTRACELLULAR METABOLITES BY *Trichoderma* spp.

# CHAPTER 3

# A STUDY OF THE PRODUCTION OF EXTRACELLULAR METABOLITES BY *Trichoderma* spp.

# 1. INTRODUCTION

Fungi used as antagonists in biological control of other micro-organisms have been suggested to inhibit growth by three mechanisms of action. These mechanisms include: *parasitism* (antagonist derives some or all of its nutrients from the host); *competition* (for space and nutrients), and *antibiosis* (the production of an inhibitory metabolite or antibiotic) (Whipps and Lumsden, 1991). While one mechanism predominates, this does not exclude the possibility that one or both of the other two mechanisms also operate.

*Mycoparasitism* of plant pathogenic fungi by other fungi has been frequently reported from *in vitro* studies. *Trichoderma hamatum* was found to grow towards host hyphae and, after contact, formed coils and appressoria-like structures from which penetration took place (Chet and Baker, 1981). Similarly, coiling of *T. harzianum* around *Rhizoctonia solani* hyphae was an early event preceding hyphal damage of the host (Benhamou and Chet, 1993). Although the parasitic activity of *Trichoderma* spp. has been demonstrated for other fungal interactions, it would appear that in *A. flavus-Trichoderma* spp. and *F. moniliforme-Trichoderma* spp. interactions, *Trichoderma* spp. did not exhibit parasitic activity (Chapter 2). However, this aspect must be checked using other microscopical modes.

*Competition* may play an important rôle in fungal interactions; it may be an independent phenomena or it may be combined with other mechanisms (Whipps and McQuilken, 1993). Competition, as a co-action, has been studied relatively little, possibly because it cannot be easily recognised or differentiated from antibiosis in agar tests (Lockwood, 1988). In fact, inhibition zones indistinguishable from those caused by antibiosis can be produced by depletion of nutrients (Lockwood, 1988).

The finding that some antagonist *Trichoderma* spp. used as biocontrol agents, are capable of producing either antibiotics or extracellular enzymes, or both, has

provided key information in understanding of the degradation events associated with parasitism (Cherif and Benhamou, 1990). In this regard it is important that parasitism be appreciated not merely in terms of physical penetration, but also (and perhaps exclusively) as a result of the effects of externally secreted substances from the parasite, on the host. Certain species of fungi used in biocontrol produce antibiotics active against plant pathogens. For example, a strain of *Gliocladium virens*, a fungus similar to *Trichoderma* spp., which suppressed growth of *Pythium ultimum*, produced the antibiotic, glioviridin, in culture (Howell, 1982). A large number of antibiotics are produced by *Trichoderma* species, some of which have been characterised, e.g. several peptide antibiotics (Graeme-Cook and Faull, 1991). Trichodermin has been isolated from culture fluid of a strain of *T. viride* (Godtfredsen and Vangedal, 1965), while gliotoxin has been associated with a *T. viride* strain effective against a *Pythium* sp. (Whipps and Lumsden, 1991). In addition, Lifshitz *et al.* (1986) demonstrated that localised antibiotic production by *Trichoderma* spp. was probably the only mechanism of action sufficiently rapid to prevent infection of pea seeds by *Pythium* spp. Of these, *T. viride* showed the strongest antibiotic action. Two other cyclic polypeptides, trichotoxin A (Hou *et al.*, 1972) and suzukacilin (Domsch *et al.*, 1980) have been isolated from *T. viride*. In contrast, *T. harzianum*, which is considered to be a "good antagonist", produces relatively few antibiotics (Chet and Baker, 1980).

Apart from antibiotic production, *Trichoderma* spp. are known to be prolific producers of polysaccharide lyases, proteases and lipases, all of which may be involved in host cell degradation (Benhamou and Chet, 1993). Of the enzymes most commonly suggested to be active against plant pathogens, chitinases, cellulases and β-glucanases are considered important (Chet and Baker, 1981). Chitinolytic enzymes have been purified and characterised from *Trichoderma* spp. (Harman *et al.*, 1993; Lorito *et al.*, 1993). Additionally, *T. viride* strains have been successfully tested *in vitro* for chitinase activity (Domsch *et al.*, 1980) while *T. harzianum* has been reported to produce cellulolytic enzymes (Ahmad and Baker, 1987; Peterbauer and Heidenreich, 1992; Harman *et al.*, 1993).

In the electron microscopical investigation of *A. flavus*/*F. moniliforme*-*Trichoderma* spp. interactions (Chapter 2) parasitism appeared not to contribute to the antagonist nature of the four *Trichoderma* isolates deemed to be 'aggressive'. In the light of these observations, the present study investigates the ability of the four aggressive *Trichoderma* isolates to produce inhibitory substances (extracellular enzymes, possible volatile compounds and antibiotics).

# 2. MATERIALS AND METHODS

## 2.1. Agar studies of antifungal activities of filtrates from cultures of *Trichoderma* spp.

### 2.1.1. Macroscopic observations

The effects of culture filtrates of aggressive *T. harzianum* (T1 and T2) and *T. viride* (T5 and T6) on the growth of *A. flavus* and *F. moniliforme* were studied using agar plates. *Trichoderma* strains were grown in 75 ml of Malt Extract Broth (17 g malt extract and 3 g mycological peptone added to 1 litre distilled water) in constant light, for 12 days at 26 °C. The mycelial mats were removed by filtration. The culture filtrates were sterilised by passing them through a 0.45 μm-pore membrane filter.

Culture filtrates (0.2 ml) were pipetted into a 10 mm diameter well, centrally placed in a PDA plate. *Aspergillus flavus* or *F. moniliforme* were then inoculated, around the wells containing the culture filtrates of *Trichoderma* spp., at a distance of 30 mm. The possible inhibitory effects on *A. flavus* and *F. moniliforme* growth were assessed 10 days after inoculation. (It is appreciated retrospectively that control cultures with wells containing an equivalent volume of culture solution in which no fungi had been grown, should have been set up).

### 2.1.2. Scanning electron microscopy

After 6 days, mycelial plugs of pathogenic colonies were taken from the contact zone between the well containing filtrate of the *Trichoderma* spp. and the hyphae of *A. flavus* and *F. moniliforme*. All samples were prepared for scanning electron microscopy as described in Chapter 2.

### 2.1.3. Assay for presence and relative activity of antifungal metabolites in culture filtrates

The separate effects of different carbon or nitrogen sources on antifungal metabolite production by the four *Trichoderma* isolates were studied using liquid culture (adapted from the method of Jackson *et al.*, 1991). The basal medium used in

this study was Czapek Dox medium containing (g $l^{-1}$): 1 g $KH_2PO_4$; 0.5 g $MgSO_4.7H_2O$; 0.5 g KCl; 0.01 g $FeSO_4.7H_2O$; 0.01 g $ZnSO_4.7H_2O$; 0.005 g $CuSO_4.5H_2O$). Forty g $l^{-1}$ glucose or maltose or sucrose as carbon source or one of two nitrogen sources, L-alanine and $KNO_3$ (2 g $l^{-1}$) were added to the basal medium.

Fifty ml of medium (basal medium with the carbon or nitrogen source) were inoculated with each of the four aggressive *Trichoderma* spp., and incubated for 7 days, in the light, at 25 °C. Culture filtrates were used in assays to determine the presence of antifungal metabolites and their activity on *A. flavus* and *F. moniliforme*.

Culture filtrates were sterilised by passing them through Whatman cellulose-nitrate filters (0.2 µm pore size) and then added at 10% (v/v) to PDA. The filtrate supplemented with PDA was dispensed in 10 ml volumes to 90 mm diameter Petri dishes. Control plates contained no culture filtrates incorporated into the medium. *Aspergillus flavus* or *F. moniliforme* was then inoculated at the centre of the agar plates. The radial diameter of the colonies was measured for six replicate plates per treatment, after 6 days of incubation in the light, at 25 °C.

**2.2. Possible volatiles produced by *Trichoderma* isolates**

The production of volatiles by aggressive *Trichoderma* isolates (T1, T2, T5 and T6) was tested using the technique of Dick and Hutchinson (1966). Plugs (4 mm diameter) were aseptically removed from the actively growing margin of each *Trichoderma* spp. colony and each placed in the centre of a Petri dish containing PDA. Plates which had been inoculated 4 days previously separately with either *A. flavus* or *F. moniliforme* were then inverted over the plates containing the plugs of the *Trichoderma* spp. A membrane (0.45 µm - pore size) was placed between the two Petri dishes. Plates were sealed together with adhesive tape. Control plates consisted of *A. flavus* or *F. moniliforme* inverted over plates containing just the agar medium. The colony diameter of *A. flavus* and *F. moniliforme* was assessed after 6 days, for six replicates incubated in the light, at 25 °C.

**2.3. Extracellular enzyme production by *Trichoderma* spp.**

The production of extracellular enzymes by four aggressive *Trichoderma* strains was investigated using solid media (methods adapted from Hankin and Anagnostakis, 1975; El Azzabi *et al.*, 1981). The ability to produce extracellular lipase, protease, amylase, pectinase, cellulase and chitinase was investigated using the solid media:

Lipid agar: To 1 l distilled water, the following were added: 20 g bacteriological agar, 5 g tryptone, 80 ml maize seed oil, 40 ml 0.1% Nile Blue sulphate and 30 g NaCl. To ensure that the oil droplets were distributed evenly on the surface of agar, the agar dishes were rolled continuously during agar solidification.

Protein agar: 15 g bacteriological agar, 4 g gelatine and 30 g NaCl were added to 1 l distilled water.

Starch agar: This medium consisted 15 g bacteriological agar, 2 g soluble starch and 30 g NaCl, made up to 1 l with distilled water.

Pectin agar (PGA): 500 ml of mineral solution (per litre: 2 g $(NH_4)_2SO_4$; 4 g $KH_2PO_4$; 6 g $Na_2HPO_4$; 0.2 g $FeSO_4.7H_2O$; 1 mg $CaCl_2$; 10 µg $H_3BO_3$; 10 µg $MnSO_4$; 70 µg $ZnSO_4$; 50 µg $CuSO_4$; 10 µg $MoO_3$), 1 g yeast extract, 15 g bacteriological agar, 5 g polygalacturonic acid and 30 g NaCl were added to 1 l distilled water.

Cellulose agar: This medium was prepared by adding 2 g carboxymethyl cellulose and 15 g bacteriological agar to 1 l distilled water.

Chitin agar: This medium containing 2 g chitin and 15 g bacteriological agar, was made up to 1 l with distilled water.

In a second experiment, osmotic pressures were elevated by adding sodium chloride to each of the media described above, such that concentrations of 5, 10 and 15% were achieved.

Each of the four aggressive strains of *Trichoderma* spp. was inoculated in the centre of plates of each of the above media. Fungal colony diameter of 15 replicates per treatment was measured after 10 and 15 days of incubation (25 °C, 24 h photoperiod). Depletion of the nutrient source (hence presence of appropriate extracellular enzyme) by *Trichoderma* spp. was assessed as follows (Gahan, 1984; McLean *et al.*, 1985):

Lipolytic activity: The increase of fat acidity following lipid breakdown is indicated by a change in colour of the Nile Blue indicator in the medium from an orange-pink to blue. Any change in colour towards blue thus represents lipolytic activity. The reverse colony colouration was considered as a diffusable blue which could be measured beyond the colony edges.

Proteolytic activity. By flooding plates with a saturated solution of ammonium sulphate, the gelatine was precipitated, producing an opaque medium. The presence of clear zones would therefore indicate utilisation of the gelatine.

Pectinolytic activity. The presence of pectins was demonstrated by flooding plates with toluidine blue (1% aqueous solution). Depletion of pectin was indicated by a pink coloration.

Amylolytic activity. By flooding plates with an iodine solution (0.2 g $I_2$; 2 g KI in 100 ml distilled water), a clear yellow zone in an otherwise dark blue surrounding would indicate starch depletion.

Cellulolytic activity. Cellulose was identified by means of a zinc chlor-iodide and potassium iodide test (30 g zinc chloride; 5 g potassium iodide; 1 g iodide and 14

ml distilled water). A clear zone in an otherwise dark brown surrounding would indicate cellulose depletion.

Chitinolytic activity. The presence of chitin can be demonstrate by staining the fungal plates with potassium iodide in 1% sulphuric acid to yield a violet colour. A brown reaction would indicate the absence of chitin.

**2.4. Possible antibiotic production by *Trichoderma* spp.**

The possible antibacterial effects of the four *Trichoderma* isolates (T1, T2, T5 and T6) were investigated using solid medium. Two bacterial strains, *Escherichia coli* (Gram -) and *Staphylococcus aureus* (Gram +) were incubated in 20 ml nutrient broth (2 g yeast extract; 5 g peptone and 5 g NaCl in 1 l distilled water) for 48 h at 37 °C.

The two strains of bacteria were transferred and evenly spread onto PDA plates containing wells with culture filtrates of each *Trichoderma* sp. After 24 h incubation at 37 °C, the possible antibiotic production by filtrates from *Trichoderma* spp. ascertained to be antagonistic to the fungal pathogens (Chapter 2) was assessed in terms of the presence of inhibition zones around the wells containing the extracts. The measurement of the inhibition zones was carried out as described in Fig. 3.a.

well containing filtrate

bacterial growth

inhibition zone

Fig. 3.a. Measurement of the inhibition zones.

# 3. RESULTS

## 2.1. Agar studies of antifungal activities of filtrates from *Trichoderma* spp. cultures

In the agar plate studies (Figs 3.1-3.8) filtrates of *Trichoderma* spp. generally inhibited *A. flavus* and *F. moniliforme* growth, with *F. moniliforme* being the most susceptible to the filtrates. In one instance, however (with *T. harzianum* T2 filtrate), *F. moniliforme* hyphae proliferated unusual aerial hyphae over the well containing the filtrate (Fig. 3.4). The filtrate of *T. harzianum* T2 had a marked effect on the *A. flavus* colony. A distinct inhibition zone was apparent, as well as a yellow pigmentation of the *A. flavus* colonies around the filtrate-containing well (Fig. 3.3). The filtrates of *T. viride* T5 exhibited inhibitory effects on both *A. flavus* (Fig. 3.5) and *F. moniliforme* (Fig. 3.6), while filtrates of *T. harzianum* T1 and *T. viride* T6 demonstrated a less marked inhibitory effect (Figs 3.1-3.2 and Figs 3.7-3.8).

Scanning electron micrographs of mycelial samples of *A. flavus* and *F. moniliforme* grown on plates with T2 and T5 filtrates (chosen because of their marked inhibitory effects, Chapter 2), revealed that extracts of *Trichoderma* spp. were able to induce alterations in *A. flavus* and *F. moniliforme* morphology. In the case of *A. flavus*, abnormal vesicles were observed for both the T2 and T5 filtrates (Figs 3.9-3.10), and change of conidiophore shape was demonstrated by hyphae grown on plates with T5 extract (Fig. 3.10). In addition, the *A. flavus* colony contained numerous aberrant conidial heads (Figs 3.11-3.12).

When *F. moniliforme* was exposed to culture filtrates of *T. harzianum* T2 or *T. viride* T5, pronounced morphological hyphal alteration was observed (e.g. cell wall abnormality [Fig. 3.14]). Microconidia were produced in chains when *Fusarium* was exposed to T2 filtrate (Fig. 3.13) while, on exposure to T5 filtrate, false heads of microconidia were observed (Figs 3.15-3.16). When co-cultured against the antagonistic *T. viride* T5 filtrate, abnormal structures were also observed associated with *F. moniliforme* hyphae (Fig. 3.17). The structure illustrated in this figure appears

to be an actual morphological abnormality of a hypha, with a remarkable resemblance to Hülle cells produced by aspergilli in responce to stress..

### 2.1.3. Antifungal metabolite occurrence in culture filtrates

Depending on the carbon or nitrogen sources, some culture filtrates of *Trichoderma* spp. had noticeable effects on growth of *F. moniliforme* and *A. flavus*, as measured by colony diameter (Table 3.1). In general, *F. moniliforme* was more susceptible to *Trichoderma* filtrates than was *A. flavus*. Of the three carbon sources, *F. moniliforme* or *A. flavus* growth was most restricted on the medium incorporating glucose (with exception of *T. viride* T6), whereas no inhibition was observed on plates with sucrose as carbon source. Significant inhibitory effects were demonstrated by some *Trichoderma* spp. filtrates when introduced into the L-alanine medium. When *Trichoderma* isolates were grown (to obtain culture filtrates) on $KNO_3$ as the nitrogen source, no growth was recorded.

Metabolites of *T. viride* T5 and *T. harzianum* T2 were generally more effective in reducing *A. flavus* and *F. moniliforme* growth. The filtrates of the other two aggressive strains, *T. harzianum* T1 and *T. viride* T6 exhibited no inhibitory effects on the *A. flavus* colonies (Table 3.1).

## 2.2. Antifungal properties of volatiles released from Trichoderma isolates

Colony growth of *A. flavus* and *F. moniliforme* was inhibited when exposed to the trapped atmosphere from cultures of *Trichoderma* spp. (Table 3.2). *Trichoderma viride* T5 and *T. viride* T6 had significant inhibitory effects in reducing colony diameter of both fungal pathogens. On the other hand, any volatile compounds emanating from *T. harzianum* T1 had no effect in curtailing the growth of *F. moniliforme* or *A. flavus* colonies, while *T. harzianum* T2 volatiles exhibited a small, but significant inhibition of *A. flavus* colony expansion.

Table 3.1. Colony diameters of *F. moniliforme* and *A. flavus* grown on different media incorporating a variety of carbon and nitrogen sources, and liquid culture filtrates of *T. harzianum* and *T. viride* strains.

| Medium | Pathogen[a] | | | Antagonist incorporated |
|---|---|---|---|---|
| | Control | | Day 6 | |
| | | *F. moniliforme* | | |
| Maltose | 62.2 ± 0.33 | ≠ | 60.2 ± 1.47 | *T. harzianum* T1 |
| | | | 60.9 ± 2 | *T. harzianum* T2 |
| | | | 64.9 ± 1.27 | *T. viride* T5 |
| | | ≠ | 59.9 ± 1.59 | *T. viride* T6 |
| Sucrose | 58.2 ± 0.46 | | 59.9 ± 0.94 | *T. harzianum* T1 |
| | | | 57.9 ± 1.46 | *T. harzianum* T2 |
| | | | 60.2 ± 0.56 | *T. viride* T5 |
| | | | 58.0 ± 0.72 | *T. viride* T6 |
| Glucose | 61.1 ± 0.78 | ≠ | 57.3 ± 0.62 | *T. harzianum* T1 |
| | | ≠ | 58.2 ± 0.36 | *T. harzianum* T2 |
| | | ≠ | 57.6 ± 0.79 | *T. viride* T5 |
| | | | 60.3 ± 0.75 | *T. viride* T6 |
| L-alanine | 64.5 ± 0.48 | ≠ | 61.0 ± 0 | *T. harzianum* T1 |
| | | | 64.8 ± 0.83 | *T. harzianum* T2 |
| | | ≠ | 63 ± 1.55 | *T. viride* T5 |
| | | ≠ | 62.8 ± 0.42 | *T. viride* T6 |
| $KNO_3$ | no growth | | no growth | no growth |
| | | *A. flavus* | | |
| Maltose | 54.7 ± 0.52 | | 54.7 ± 0.88 | *T. harzianum* T1 |
| | | | 57.4 ± 0.96 | *T. harzianum* T2 |
| | | ≠ | 49.8 ± 0.73 | *T. viride* T5 |
| | | | 59.9 ± 0.69 | *T. viride* T6 |
| Sucrose | 57.9 ± 0.67 | | 64.5 ± 0.95 | *T. harzianum* T1 |
| | | | 60.0 ± 0.63 | *T. harzianum* T2 |
| | | | 59.9 ± 0.69 | *T. viride* T5 |
| | | | 61.7 ± 2.18 | *T. viride* T6 |
| Glucose | 58.9 ± 0.19 | | 60.8 ± 0.54 | *T. harzianum* T1 |
| | | | 63.9 ± 0.67 | *T. harzianum* T2 |
| | | | 61.0 ± 0.66 | *T. viride* T5 |
| | | | 66.0 ± 0.78 | *T. viride* T6 |
| L-alanine | 54.1 ± 0.51 | | 59.4 ± 1.21 | *T. harzianum* T1 |
| | | ≠ | 51.8 ± 1.65 | *T. harzianum* T2 |
| | | | 59.4 ± 0.65 | *T. viride* T5 |
| | | | 59.4 ± 0.53 | *T. viride* T6 |
| $KNO_3$ | no growth | | no growth | no growth |

[a] Values represent means standard deviation of six replicates

≠ Statistically significant difference (Student t Test)

Table 3.2. Colony diameter of pathogenic fungi grown on agar plates inverted over plates of *Trichoderma* spp.

| Antagonist | Hyphal growth of pathogens[a] | | | | | |
|---|---|---|---|---|---|---|
| | *A. flavus* | | | *F. moniliforme* | | |
| | Control | | Day 6 | Control | | Day 6 |
| *T. harzianum* (T1) | 74.6 ±0.51 | | 74 ±0.7 | 74 ±0.8 | | 74.1 ±0.51 |
| *T. harzianum* (T2) | 74.6 ±0.51 | ≠ | 71.9 ±0.66 | 74 ±0.8 | | 75.3 ±0.4 |
| *T. viride* (T5) | 74.6 ±0.51 | ≠ | 67.3 ±0.87 | 74 ±0.8 | ≠ | 66.4 ±0.97 |
| *T. viride* (T6) | 74.6 ±0.51 | ≠ | 71.5 ±0.8 | 74 ±0.8 | ≠ | 69.9 ±0.8 |

[a] Values represent means ± standard deviations of six replicates

≠ Statistically significant difference (Student t Test)

## 2.3. Extracellular enzyme production by *Trichoderma* spp.

The ability of four strains of *Trichoderma* spp. to produce extracellular enzymes is summarised from the solid media studies in Table 3.3. Although the fungi grew well on all media, when stained appropriately to demonstrate depletion of nutrient source (hence demonstrating extracellular enzyme production and activity), only amylase (Figs 3.18-3.19) and cellulase activity (Fig. 3.21) were exhibited by all four *Trichoderma* spp. isolates. Lipase activity (Figs 3.22-3.23) was demonstrated by *T. harzianum* T1, T2 and *T. viride* T5, while *T. harzianum* T2 and *T. viride* T5 exhibited proteolytic activity (Fig. 3.20). All fungi grew prolifically on pectin agar, but only *T. harzianum* T1 and *T. viride* T5 demonstrated pectinolytic activity. Based on these results, it would appear that *T. viride* T5 produced the widest spectrum of extracellular enzymes of the four *Trichoderma* spp. isolates tested. Fungal growth appeared uniformly vigorous on agar incorporating chitin as the substrate, but no indications of extracellular chitinase activity could be obtained by the method presently used.

Table 3.3. Extracellular enzyme production by *Trichoderma* spp.

| | *T. harzianum* | | *T. viride* | |
|---|---|---|---|---|
| Extracellular enzyme | T1 | T2 | T5 | T6 |
| Amylase | + | + | + | - |
| Cellulase | + | + | + | + |
| Pectinase | + | - | + | - |
| Lipase | + | + | + | - |
| Protease | - | + | + | - |
| Chitinase | - | - | - | - |

+ enzymatic activity detected
- no detectable enzymatic activity

With increasing osmotic potential, the general trend was for a decrease in enzyme production (Table 3.4). Pectinolytic activity appeared to be the most affected by the imposed water stress. *Trichoderma viride* T5 demonstrated lipolytic, proteolytic, pectinolytic and cellulolytic activity (Fig. 3.21) at all salt concentrations, reflecting a marked osmotolerance. In assessing lipolytic activity, for *T. viride* T5 (Fig. 3.22), the blue coloration presented beyond the colony bounds, demonstrated the production of diffusable lipase in contrast to that of *T. harzianum* T2, where no diffusable lipase was observed (Fig. 3.23).

Table 3.4. The growth of four *Trichoderma* spp. strains at different osmotic potentials, measured as a diameter (mm) in agar plate culture.

| Medium | Fungal species[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *T. harzianum* (T1) | | *T. harzianum* (T2) | | *T. viride* (T5) | | *T. viride* (T6) | |
| | Days of incubation | | | | | | | |
| | 10 | 15 | 10 | 15 | 10 | 15 | 10 | 15 |
| Lipid 0 % NaCl | 38.3 ± 0.65 | 55 ± 0.38 | 39.7 ± 0.80 | 44 ± 0.24 | 50.7 ± 0.46 | 64.2 ± 0.28 | 4.9 ± 0.62 | 5.8 ± 0.34 |
| Lipid 5% NaCl | 22.6 ± 0.52 | 28.2 ± 0.64 | 22.4 ± 0.64 | 29.6 ± 0.70 | 50.3 ± 0.65 | 58.7 ± 0.79 | 3.7 ± 0.43 | 4.5 ± 0.36 |
| Lipid 10% NaCl | - | - | 5.8 ± 0.28 | 5.8 ± 0.28 | 24.6 ± 0.81 | 30 ± 0.2 | - | - |
| Protein 0 % NaCl | 30.4 ± 0.70 | 42.2 ± 0.54 | 28 ± 0.65 | 45.6 ± 0.87 | 42.5 ± 0.66 | 58.4 ± 0.23 | 24.6 ± 0.46 | 37 ± 0.19 |
| Protein 5% NaCl | 17.8 ± 2.23 | 27.9 ± 0.3 | 25.7 ± 0.4 | 30.9 ± 1 | 40 ± 0.15 | 55.7 ± 1.15 | 24.4 ± 0.87 | 35.5 ± 0.51 |
| Protein 10% NaCl | - | - | 1.6 ± 0.57 | 1.6 ± 0.57 | 7.6 ± 0.69 | 19.3 ± 0.57 | 1 ± 0 | 1 ± 0 |
| Starch 0% NaCl | 44 ± 0.19 | 55.2 ± 0.54 | 17.8 ± 0.92 | 26.3 ± 0.23 | 7.6 ± 0.81 | 10.7 ± 0.34 | 3 ± 0.21 | 4.6 ± 0.81 |
| Starch 5% NaCl | 43.9 ± 1 | 50.9 ± 0.83 | 15.6 ± 0.57 | 20.8 ± 0.23 | 6.7 ± 0.46 | 8 ± 0 | 3 ± 0.1 | 3 ± 0.1 |
| Starch 10% NaCl | - | - | - | - | 3.8 ± 0.23 | 6.8 ± 0.2 | 2 ± 0 | 2 ± 0 |
| PGA 0% NaCl | 1 ± 0 | 3.4 ± 0.62 | 5.6 ± 0.26 | 7.4 ± 0.51 | 9.5 ± 0.87 | 17.2 ± 0.64 | 2.4 ± 0.21 | 4 ± 0 |
| PGA 5% NaCl | 1 ± 0 | 3 ± 0.2 | 5.9 ± 0.11 | 7.1 ± 0.32 | 8.1 ± 0.96 | 15.4 ± 0.51 | 2 ± 0 | 3.4 ± 0.52 |
| PGA 10% NaCl | - | - | - | - | 14.3 ± 0.61 | 21.6 ± 0.57 | - | - |
| Cellulose 0% NaCl | 20 ± 0.1 | 28.4 ± 0.66 | 27.7 ± 1.15 | 34.8 ± 0.46 | 25 ± 1.0 | 37.5 ± 0.77 | 22.4 ± 0.81 | 28.2 ± 0.57 |
| Cellulose 5% NaCl | 19.6 ± 0.52 | 25.5 ± 0.77 | 26.6 ± 1.16 | 32.6 ± 1.18 | 25.6 ± 0.69 | 35.9 ± 0.36 | 20.9 ± 0.17 | 25.9 ± 1 |
| Cellulose 10%NaCl | - | - | 2.6 ± 0.57 | 2.6 ± 0.57 | 14.2 ± 0.66 | 22.2 ± 0.64 | - | - |

[a] Values represent the mean ± standard deviations of three replicates
- no growth

### 3.4. Possible antibiotic production by *Trichoderma* spp.

A possible antibiotic production by four aggressive *Trichoderma* spp. isolates (T1, T2, T5 and T6) was tested using one Gram positive bacterium, *Staphylococcus aureus* and one Gram negative bacterium, *Escherichia coli* (Table 3.5).

When *E. coli* was grown on plates with wells containing filtrates from colonies of *Trichoderma* spp., it was observed that *T. viride* T5 filtrate gave the greatest inhibition zone, while *T. harzianum* T2 had a far lesser inhibitory effect (Table 3.5; Fig. 3.24). In the case of the Gram positive bacterium, *S. aureus*, *T. viride* T5 gave once again the largest inhibition zone, and *T. harzianum* T2 demonstrated a relatively small inhibition zone. Bacterial growth was inhibited by *T. harzianum* T1 and *T. viride* T6 culture filtrates (Table 3.5; Fig. 3.25).

Table 3.5. Effects of culture filtrates of *Trichoderma* spp. on bacterial growth, as measured by inhibition zones (mm).

| Bacteria[a] | Culure filtrates of *Trichoderma* spp. | | | |
|---|---|---|---|---|
| | *T. harzianum* (T1) | *T. harzianum* (T2) | *T. viride* (T5) | *T. viride* (T6) |
| *E. coli* | 27.1 ± 0.60 | 17.5 ± 0.87 | 29.7 ± 0.63 | 24.9 ± 0.60 |
| *S. aureus* | 28.2 ± 0.58 | 22.1 ± 0.60 | 29.8 ± 0.51 | 28.1 ± 0.72 |

[a] Values represent mean of 15 replicates ± standard deviation

The results of this experiment demonstrated antibiotic production by all *Trichoderma* isolates, but more notably by *T. viride* T5 which exhibited the greatest inhibition on bacterial growth.

# 4. DISCUSSION

Understanding the mechanisms involved in the antagonistic effect of e.g. *Trichoderma* spp. on plant pathogens is important for the eventual improvement of biocontrol methods. While the evidence for the rôle of mycoparasitism in biological control is convincing (Papavizas, 1985; Chet and Inbar, 1994), definite evidence establishing the importance of antibiosis has been more elusive. According to the generally accepted definition, *antibiosis* is the mechanism mediated by specific metabolites such as enzymes, volatile compounds and antibiotics (Fravel, 1988).

Culture filtrates have been used to demonstrate the possible rôle of fungal metabolites in the process of biocontrol, and filtrates from *Trichoderma* spp. have been reported to have antifungal activities. Doi and Mori (1994) reported successful antifungal potential of culture filtrates of two *Trichoderma* spp. on wood decay fungi, and filtrates of various *T. harzianum* strains were found to be suppressive of the white-rot pathogen, *Sclerotium cepivorum* (Papavizas *et al.*, 1982).

In the present study, culture filtrates of four *Trichoderma* spp. inhibited the growth of both *A. flavus* and *F. moniliforme* to some extent, with the latter being more susceptible. Although there is a statistical significant difference between the growth measurements for control colonies and the inhibited colonies of the pathogens, it was observed that in general, *Trichoderma* filtrates had only marginally curtailed pathogen growth, in contrast with, e.g. metabolites of *Gliocladium virens* which completely inhibited the growth of *Sclerotium cepivorum* (Jackson *et al.*, 1991). The explanation for these results may be that the 1:10 dilution presently used may have resulted in a too-low concentration of inhibitory metabolites to effect marked inhibitory response. In addition, Jackson *et al.* (1991) using the same dilution (1:10) of culture filtrates of several isolates of *T. viride*, found that no inhibition of growth occurred of three pathogens.

Based on macroscopic and microscopic observations (Chapter 2), it was apparent that *T. harzianum* (T1 and T2) and *T. viride* (T5 and T6) were aggressive towards *A. flavus* and *F. moniliforme*. The macroscopic observations of plates with the pathogens cultured with filtrates of *Trichoderma* spp. and scanning electron microscopy, confirmed the antifungal activities of *Trichoderma* filtrates. Similar morphological hyphal alterations, which were more marked in the case of *F. moniliforme*, were observed in dual cultures of *A. flavus-Trichoderma* spp. and *F. moniliforme-Trichoderma* spp. The observations on co-cultures indicated that hyphal penetration certainly does not appear to be the main mechanism of action of *Trichoderma* spp, but the presence of inhibitory metabolites may possibly contribute to the observed antifungal effects.

Antibiosis mediated by volatile substances has received less attention than antibiosis through the production of enzymes. Doi and Mori (1993) showed that volatiles from *Trichoderma* spp. were able to arrest the hyphal growth of different fungal pathogens on agar plates. Those authors reported that *T. viride* produced volatiles which had potential to inhibit the hyphal growth of *Lentinus lepidus* and *Coriolus versicolor*. To date, alkyl pyrones have been identified as volatile metabolites produced by *T. harzianum* (Fravel, 1988).

The results of the present study suggest, firstly, that *Trichoderma* spp. produce volatile compounds and secondly, that such compounds may play rôle in the inhibitory effects observed on colony growth and morphology of *A. flavus* and *F. moniliforme*. For *F. moniliforme-T. viride* T5 co-cultures, the observed inhibition zone, where SEM demonstrated no hyphal contact (Chapter 2), may have resulted partly from the production of some volatile/s by the *Trichoderma* spp.

The involvement of enzymes in biological control complicates the distinction between mycoparasitism and antibiosis. In some instances, production of a cell wall degrading enzyme by an antagonist may be involved simultaneously in both parasitism and antibiosis. Elad *et al.* (1983) demonstrated hyphal penetration by *Trichoderma* spp. which was mediated by enzymatic activity. In addition, the

investigation of Benhamou and Chet (1993) revealed that *T. harzianum* was able to parasitise *Rhizoctonia solani* hyphae by producing chitinase. In other instances however, *Trichoderma* spp. produce enzymes but without there being evidence of parasitism (Benhamou and Chet, 1993).

Since fungal cell walls are composed mainly of chitin and β-1,3-glucans embedded in a matrix of amorphous material (Cherif and Benhamou, 1990), successful wall degradation requires the activity of more than one enzyme. Sivan and Chet (1989 a) have speculated that a co-ordinated action of polysaccharidases, lipases and proteases is an important determinant in the antibiosis process.

The present investigation demonstrated that *T. harzianum* (T1 and T2) and *T. viride* T5 produce a spectrum of extracellular enzymes. These results confirm the macro- and microscopical demonstration of the aggressive behaviour of *T. harzianum* T2 and *T. viride* T5 against *A. flavus* and *F. moniliforme*. As has been documented previously by Domsch *et al.* (1980), *T. viride* T5 in the present study produced pectinase and proteinase, and *T. harzianum* T2 exhibited a marked proteinase activity. Although chitinase activity has been reported for *T. viride* (Domsch *et al.*, 1980), chitinolytic activity could not be demonstrated for any of the fungi used in the present investigation. These negative results may, however, have resulted from some methodological error, especially in view of the cell wall abnormality seen when *F. moniliforme* was cultured with filtrate derived from *Trichoderma* spp., and the vigorous growth of the fungi on a medium incorporating chitin as the substrate. The general spectrum of enzymatic activity demonstrated by *Trichoderma* spp., notably *T. harzianum* T2 and *T. viride* T5 provides evidence that extracellular enzymes may play a important rôle in antibiosis against *A. flavus* and *F. moniliforme*.

Antibiosis may also involve the production of antibiotics (Fravel, 1988), *Trichoderma* spp. being reported to produce such compounds (Godtfredsen and Vangedal, 1965; Howell, 1982; Graeme-Cook and Faull, 1991). All four *Trichoderma* spp. used in the present investigation inhibited bacterial growth, but antibiotic screening *per se* was not conducted. This observed antibacterial activity may have

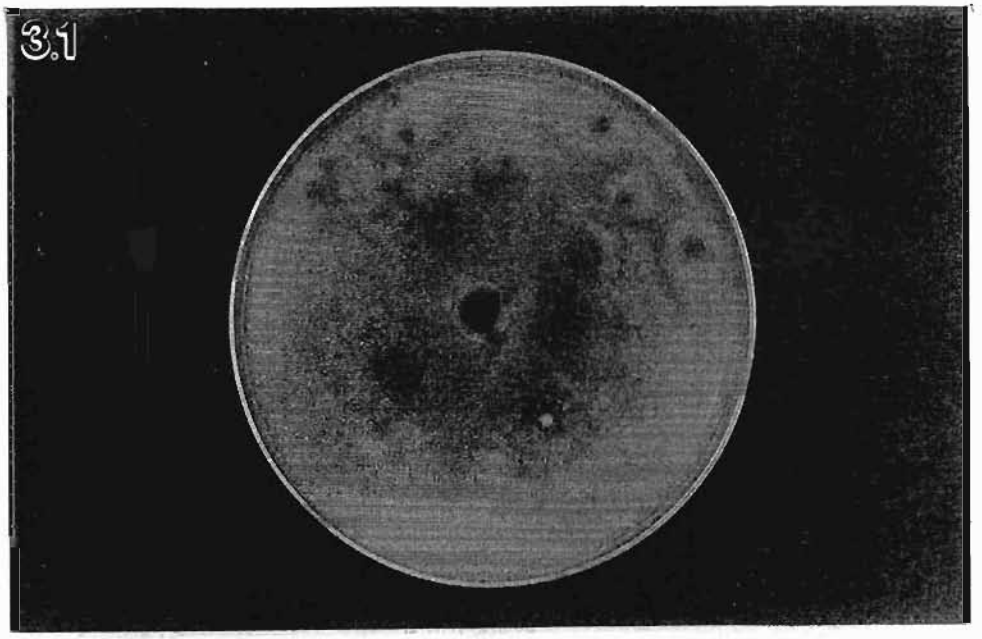
been induced by other metabolites (excluding antibiotics). For example, one strain of *T. hamatum* was described to produce isocyanide and polypeptide metabolites (Brewer *et al.*, 1987). Those metabolites were inhibitory to bacteria that digest cellulose in ruminants, and *T. hamatum* has been implicated in the suppression of these bacterial populations.

In summary, the observations of this study provide evidence that antibiosis by elaboration of volatile compounds and/or extracellular enzymes and perhaps antibiotic production are possible contributory factors involved in the observed inhibitory effects of *Trichoderma* spp., notably *T. viride* T5 and *T. harzianum* T2, against *A flavus* and *F. moniliforme*. It is, however, important to appreciate that all the observations made, and conclusions drawn, from this study, pertain to the *in vitro* situation. Not only is it now necessary to determine whether *Trichoderma* spp. are capable of out-competing *A. flavus* and *F. moniliforme* within seeds, but to ascertain whether *Trichoderma* spp. are capable of surviving at all, within the air-dry seed. The observation presently made, that the *Trichoderma* spp. are adversely affected by the increasing osmotic potential of the media, is not promising regarding the survival and/or aggressiveness of these species within air-dry seeds, with the exception perhaps of *T. viride* T5.

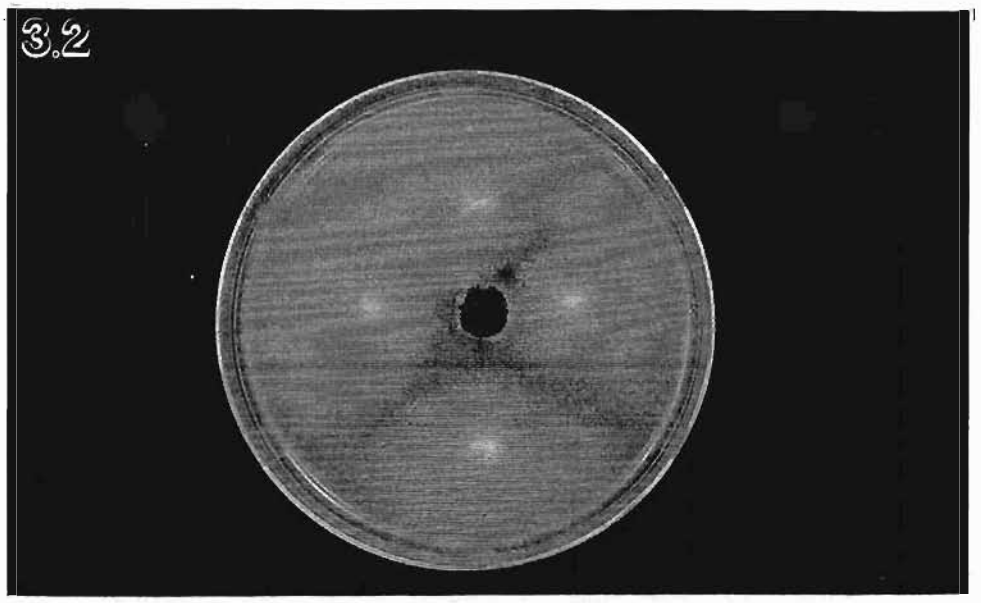
Figs 3.1-3.2. Response of *A. flavus* and *F. moniliforme* to exposure to filtrates of *T. harzianum* T1.

3.1. Inhibition of *A. flavus* growth surrounding the well containing *T. harzianum* T1 culture filtrate.

3.2. Inhibition of growth of *F. moniliforme* surrounding the well containing *T. harzianum* T1 culture filtrates.




*Aspergillus flavus - extract of T. harzianum (T1)*

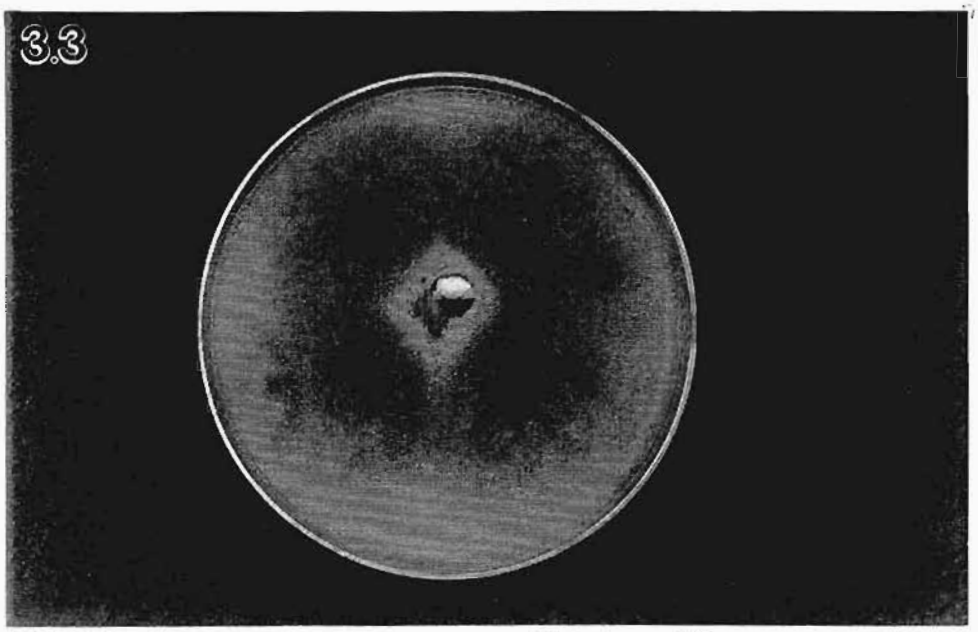



*Fusarium moniliforme - extract of T. harzianum (T1)*

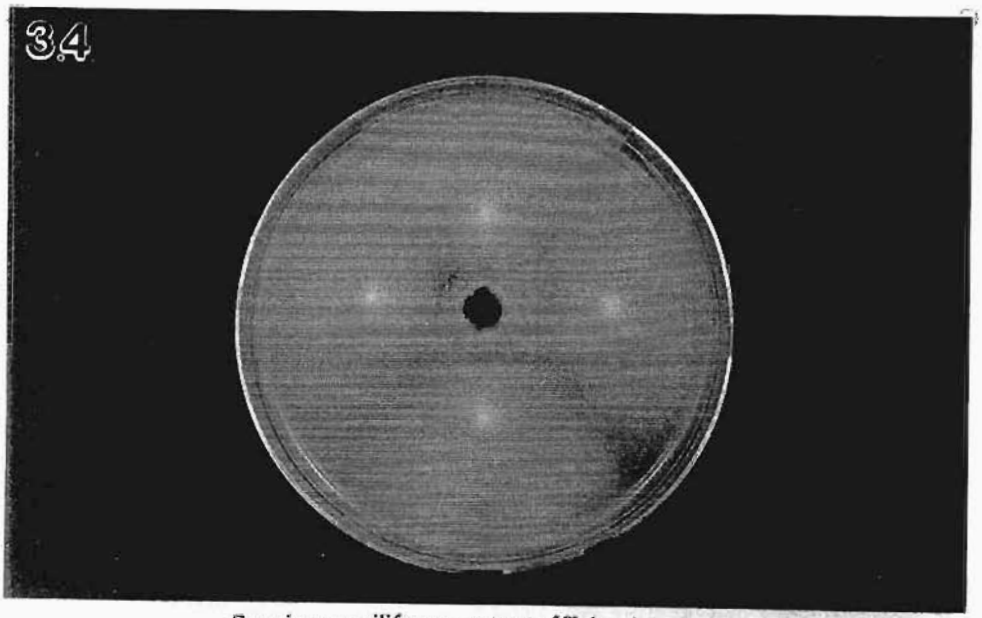
Figs 3.3-3.4. Response of *A. flavus* and *F. moniliforme* to exposure to filtrates of *T. harzianum* T2.

Fig. 3.3. Inhibition of *A. flavus* colony growth by filtrate of *T. harzianum* T2. Note the yellow pigmentation exhibited by *A. flavus* in contact with the extract of *T. harzianum* T2.

Fig. 3.4. *Fusarium* colony grown on plate containing well with *T. harzianum* T2. Note that *F. moniliforme* hyphae had grown over well containing *Trichoderma* filtrate. Note the change (more aerial hyphae) in morphology of the mycelium in close contact with the well.




*Aspergillus flavus - extract of T. harzianum (T2)*

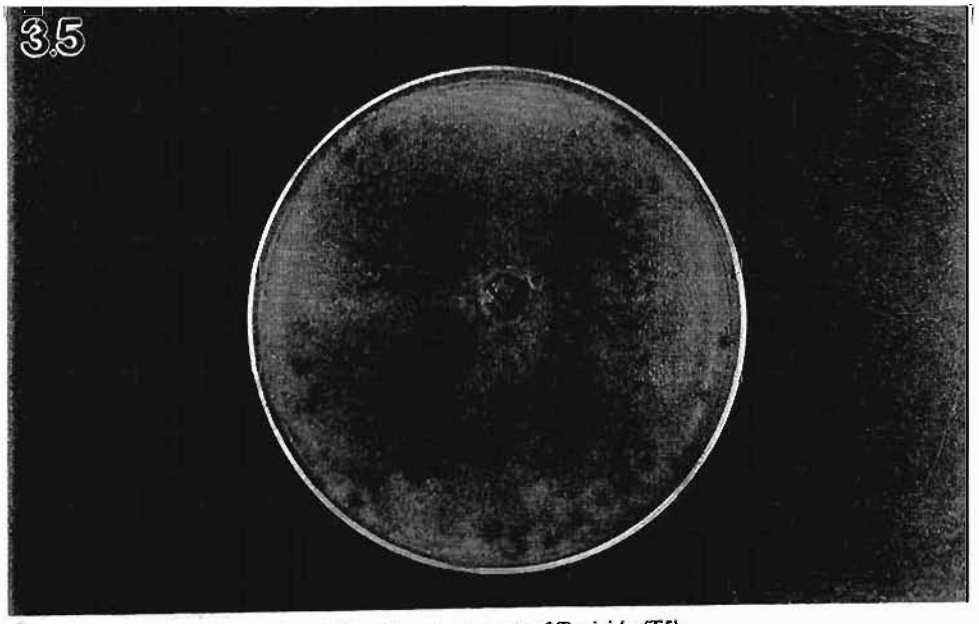



***Fusarium moniliforme - extract of T. harzianum (T2)***

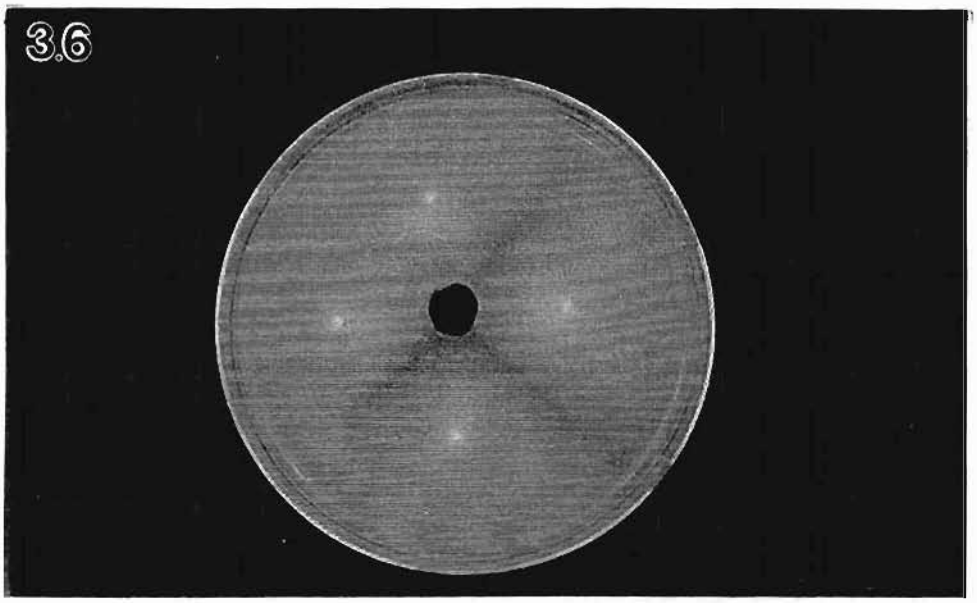
Figs 3.5-3.6. Response of *A. flavus* and *F. moniliforme* to exposure to filtrates of *T. viride* T5.

Fig. 3.5. Inhibition of *A. flavus* colony growth exhibited by culture filtrate of *T. viride* T5.

Fig. 3.6. Inhibition of *F. moniliforme* growth demonstrated around the well containing *T. viride* T5 filtrate.




*Aspergillus flavus - extract of T. viride (T5)*

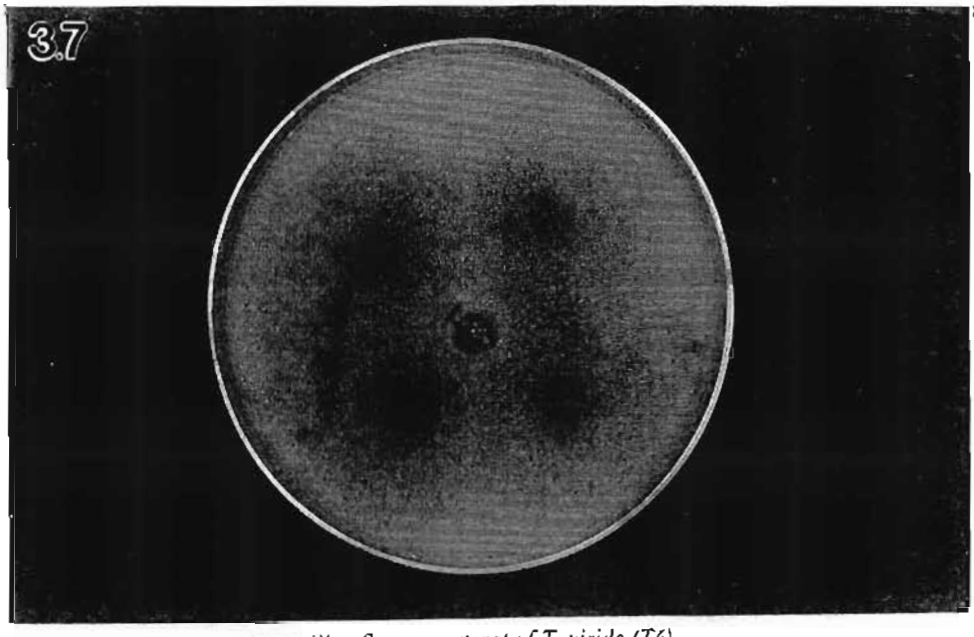



*Fusarium moniliforme - extract of T. viride (T5)*

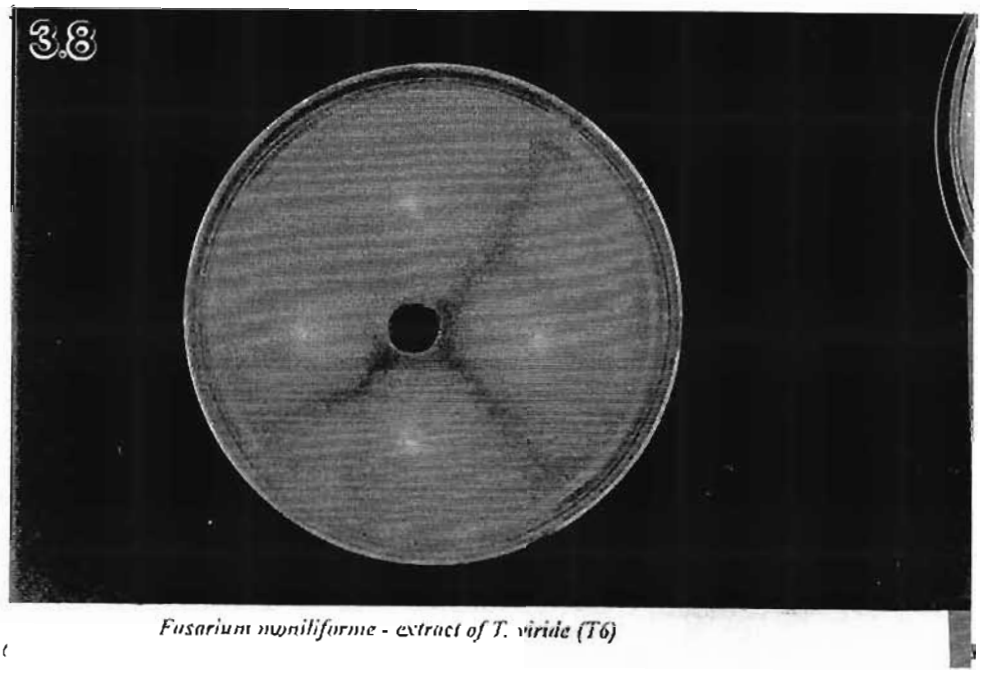
Figs 3.7-3.8. Response of *A. flavus* and *F. moniliforme* to exposure to filtrates of *T. viride* T6.

Fig. 3.7. Marginal growth inhibition of *A. flavus* around the well containing the filtrate of *T. viride* T6.

Fig. 3.8. Inhibition of *F. moniliforme* growth by *T. viride* T6 culture filtrate.




*Aspergillus flavus - extract of T. viride (T6)*




*Fusarium moniliforme - extract of T. viride (T6)*

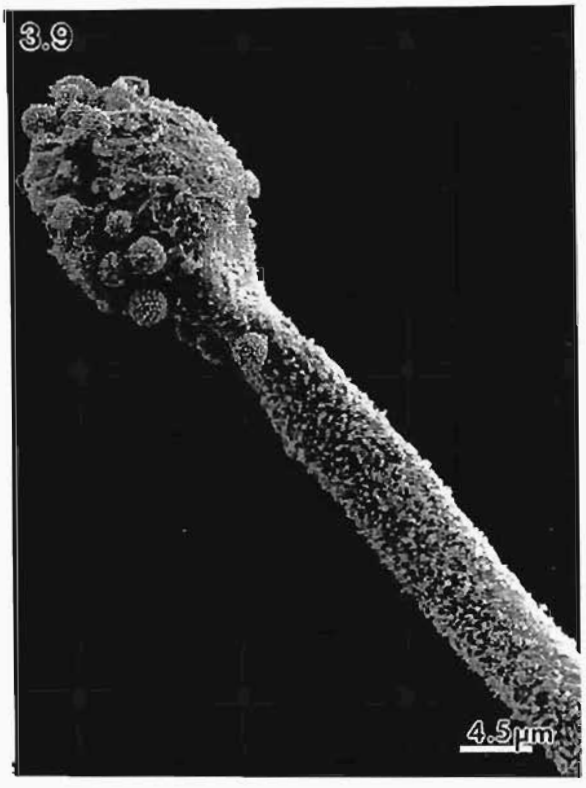
Figs 3.9-3.12. Scanning electron micrographs of *A. flavus* colony grown on plates containing wells with *T. harzianum* T2 and *T. viride* T5 culture filtrates (c.f. Figs 2.4-2.7).

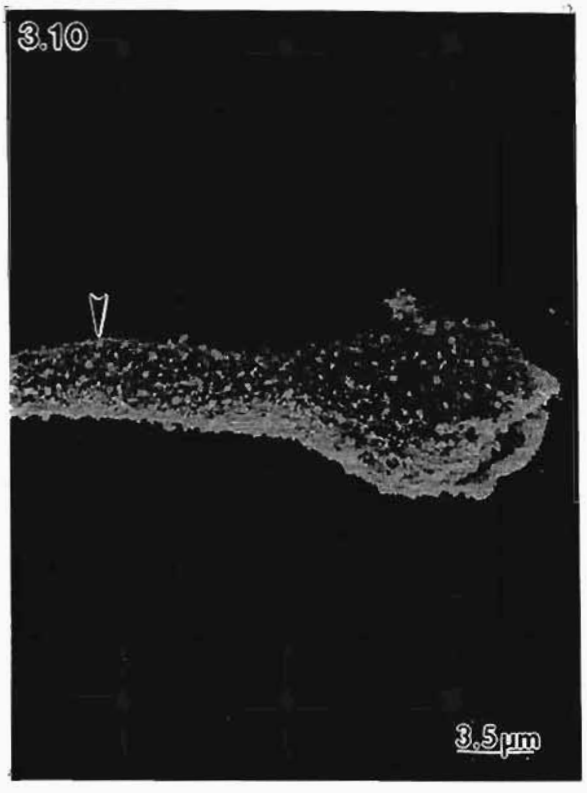
Fig. 3.9. Abnormal vesicle of *A. flavus*; filtrate of *T. harzianum* T2.

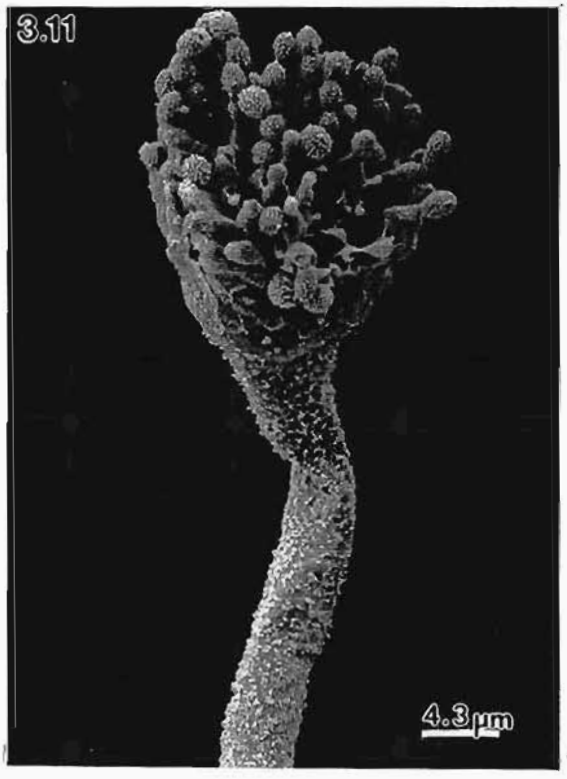
Fig. 3.10. Aberrant vesicle of *A. flavus*. Note the change of shape of conidiophore (arrow); filtrate of *T. viride* T5.

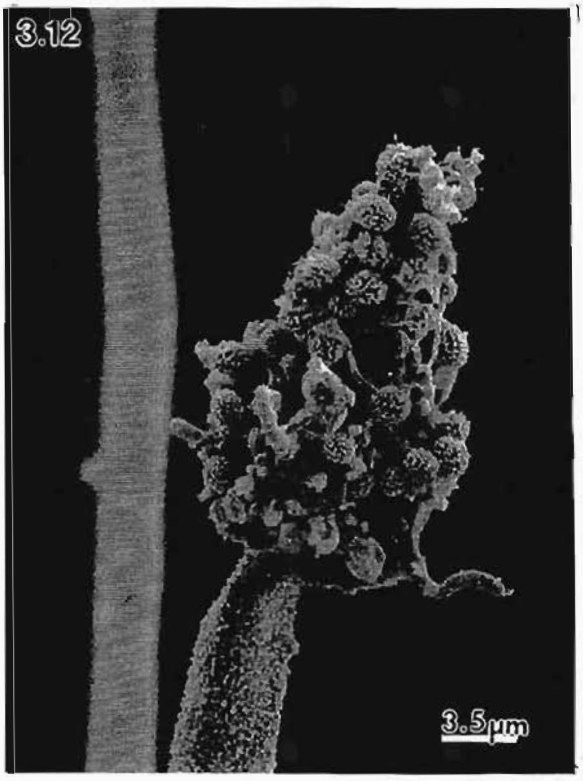
Fig. 3.11. Abnormal conidial head of *A. flavus*; filtrate of *T. viride* T5.

Fig. 3.12. Aberrant conidial head of *A. flavus*; filtrate of *T. harzianum* T2.


3.9
4.5µm


3.10
3.5µm


3.11
4.3µm


3.12
3.5µm

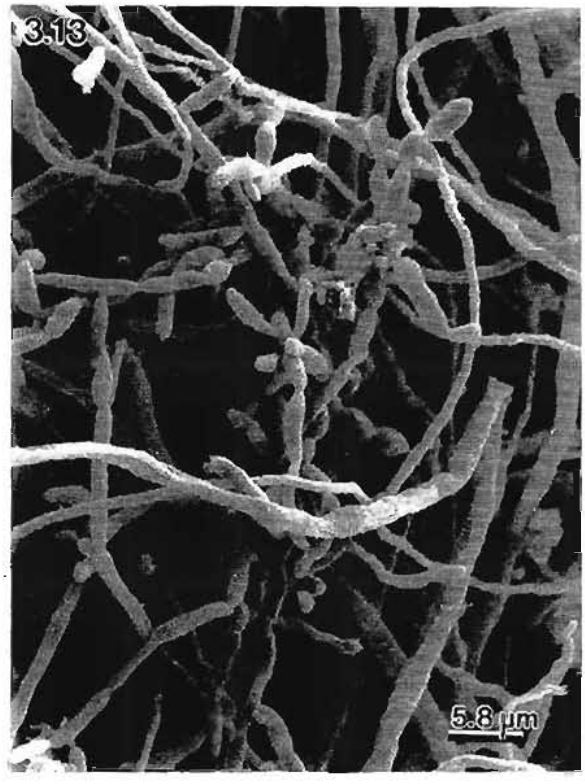
Figs 3.13-3.16. Scanning electron micrographs of *F. moniliforme* colony grown on plates containing wells with culture filtrates of *T. harzianum* T2 and *T. viride* T5 (c.f. Figs 2.12-2.15).

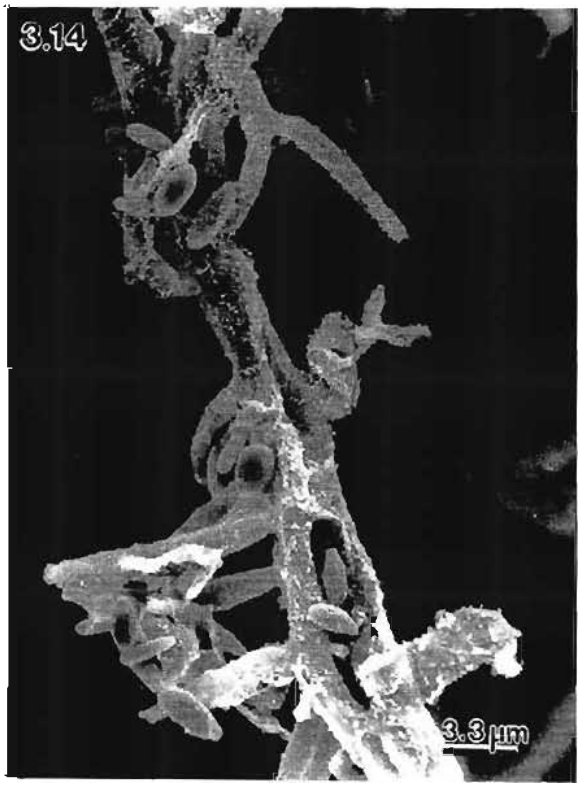
Fig. 3.13. Hyphae of *F. moniliforme* with microconidia produced in chains; filtrate of *T. harzianum* T2.

Fig. 3.14. Mycelium of *F. moniliforme* exhibited a pronounced wall abnormality; filtrate of *T. harzianum* T2.

Fig. 3.15. Microconidia of *F. moniliforme* arranged in false heads; filtrate *of T. viride* T5.

Fig. 3.16. False head of microconidia together with abnormal hyphae; filtrate of *T. viride* T5.


3.13
5.8 µm


3.14
3.3 µm


3.15
1.5 µm

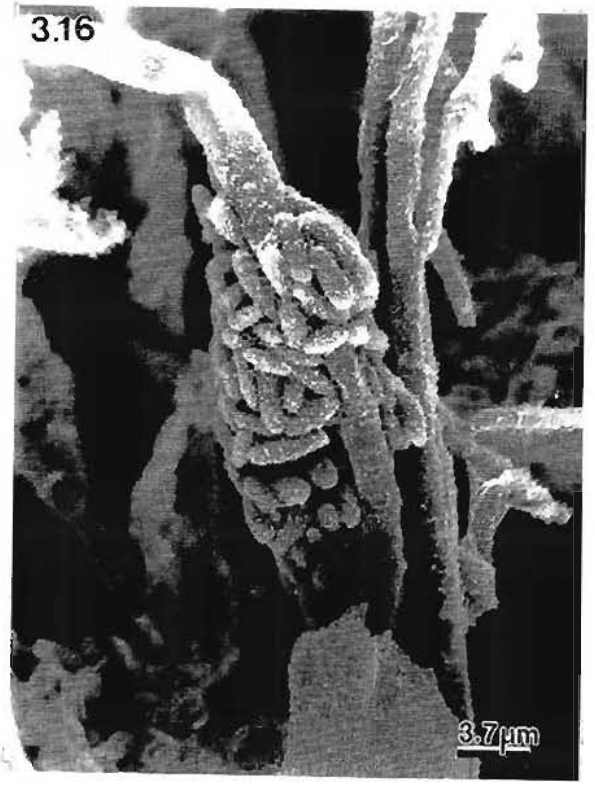

3.16
3.7µm

Fig. 3.17. Scanning electron micrographs of *F. moniliforme* colony grown on plates containing well with *Trichoderma* spp.

Fig. 3.17. Abnormal structure with associated hyphae exhibited by *F. moniliforme* when exposed to filtrate of *T. viride* T5.

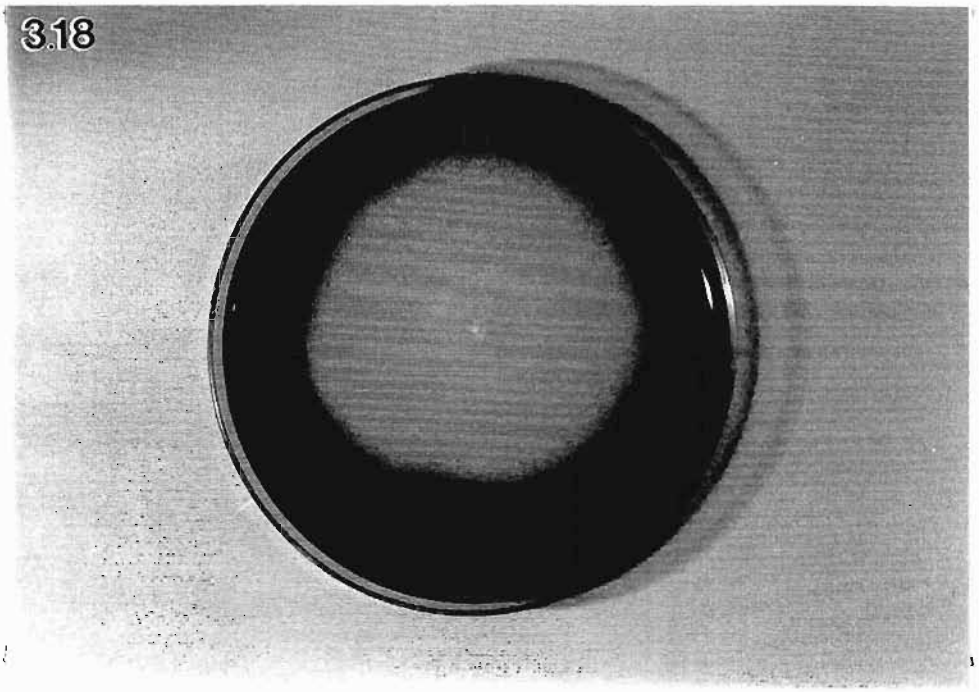

3.17
1.2 μm

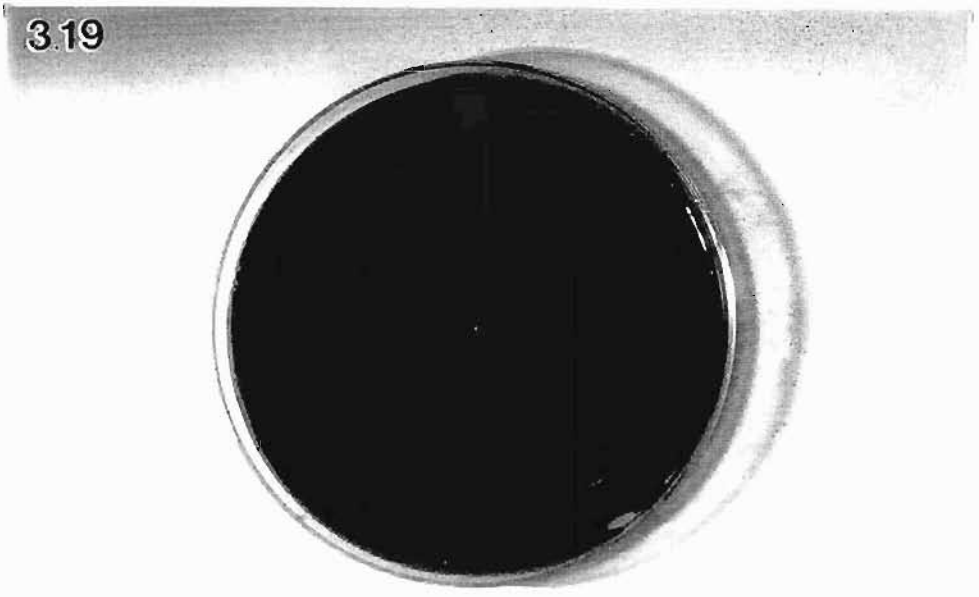
Figures 3.18-3.23 represent a selection of illustrations demonstrating extracellular enzyme activity for the *Trichoderma* spp. strains investigated. Note that each reaction for every strain has not been illustrated.

Figs 3.18-3.19. Extracellular enzyme production by *Trichoderma* spp.

Fig. 3.18. Amylolytic activity of *T. harzianum* T1.

Fig. 3.19. Absence of amylolytic activity by *T. harzianum* T6.

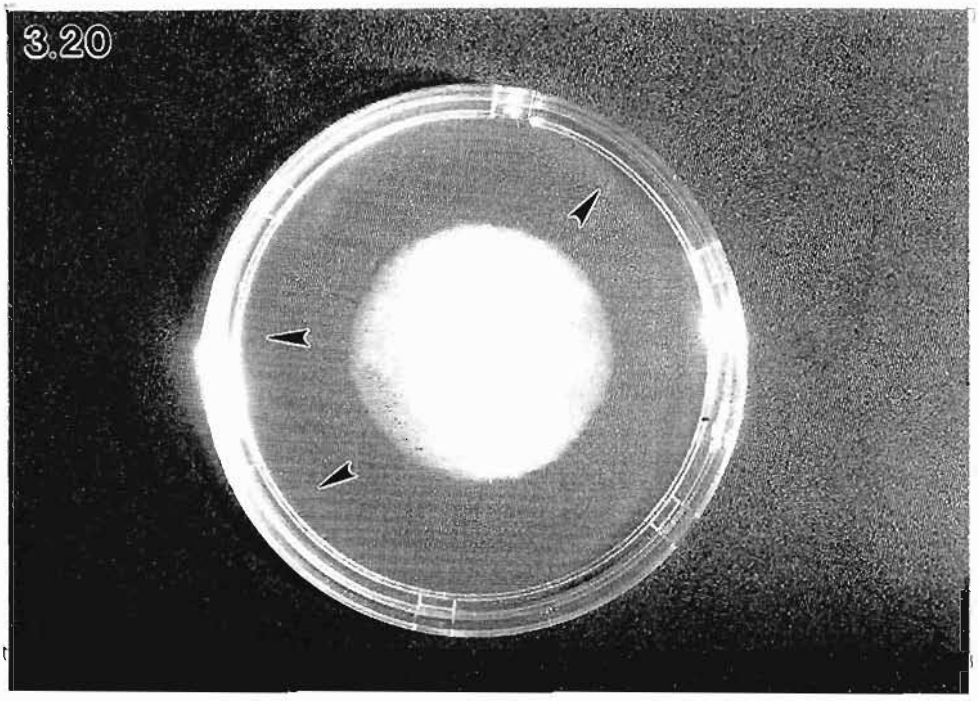

3.18

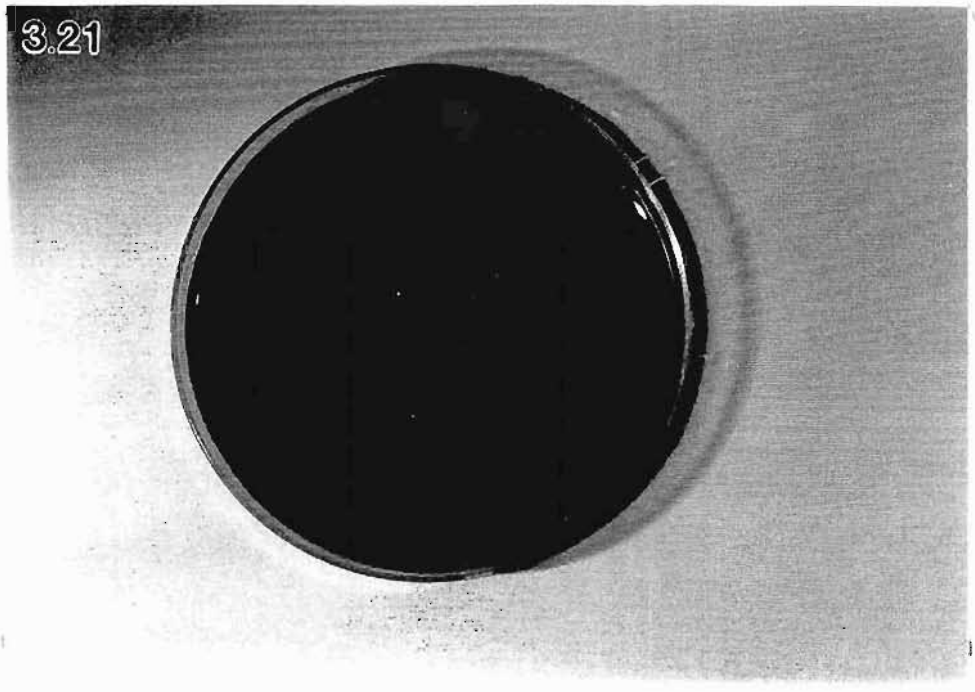

3.19

Figs 3.20-3.21. Extracellular enzyme production by *Trichoderma* spp.

Fig. 3.20. The clear zone would indicate gelatine depletion from the medium, suggesting proteolytic activity by *T. harzianum* T2.

Fig. 3.21. Plate demonstrating production of cellulase by *T. viride* T5. The clear zone indicates the depletion of cellulose.

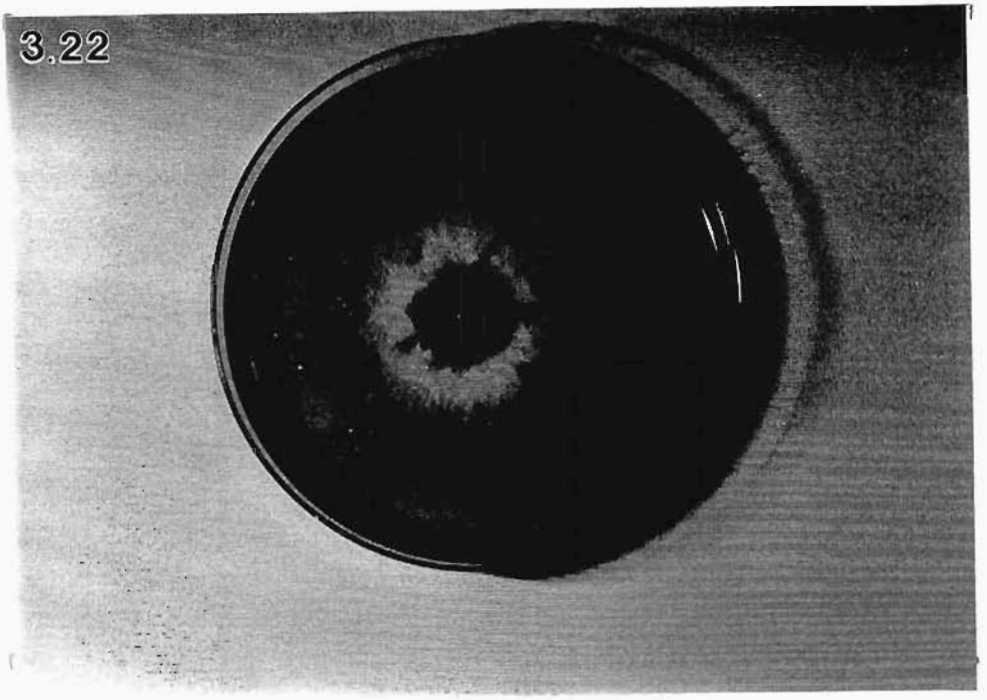

3.20

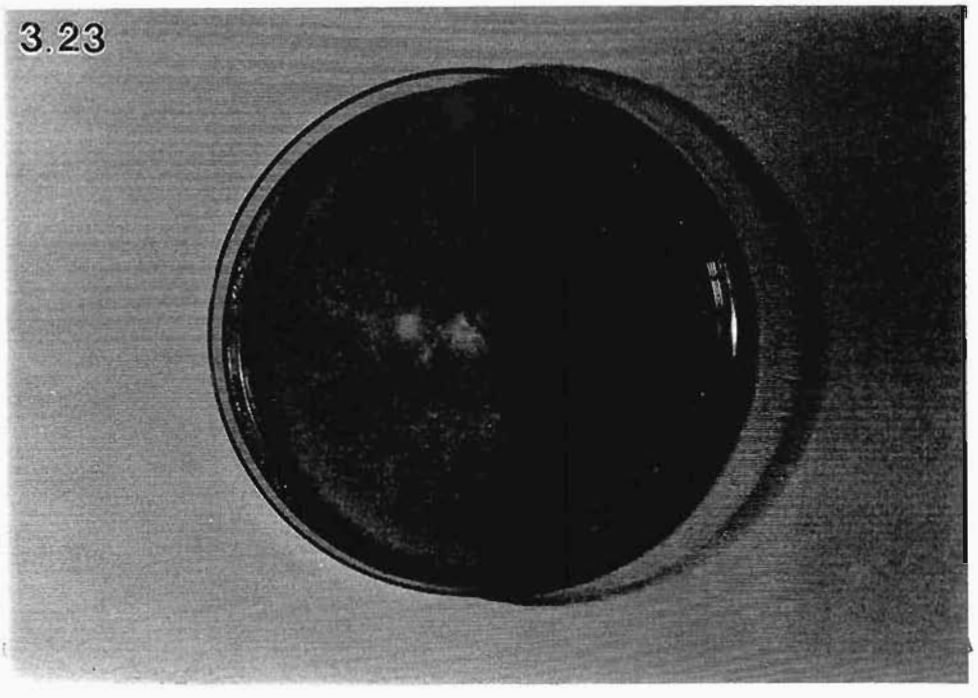

3.21

Figs 3.22-3.23. Extracellular enzyme production by *Trichoderma* spp.

Fig. 3.22. Lipase activity by *T. viride* T5. The blue colour indicates the activity of diffusable lipase.

Fig. 3.23. Lipase activity of *T. harzianum* T2 restricted to colony limits.

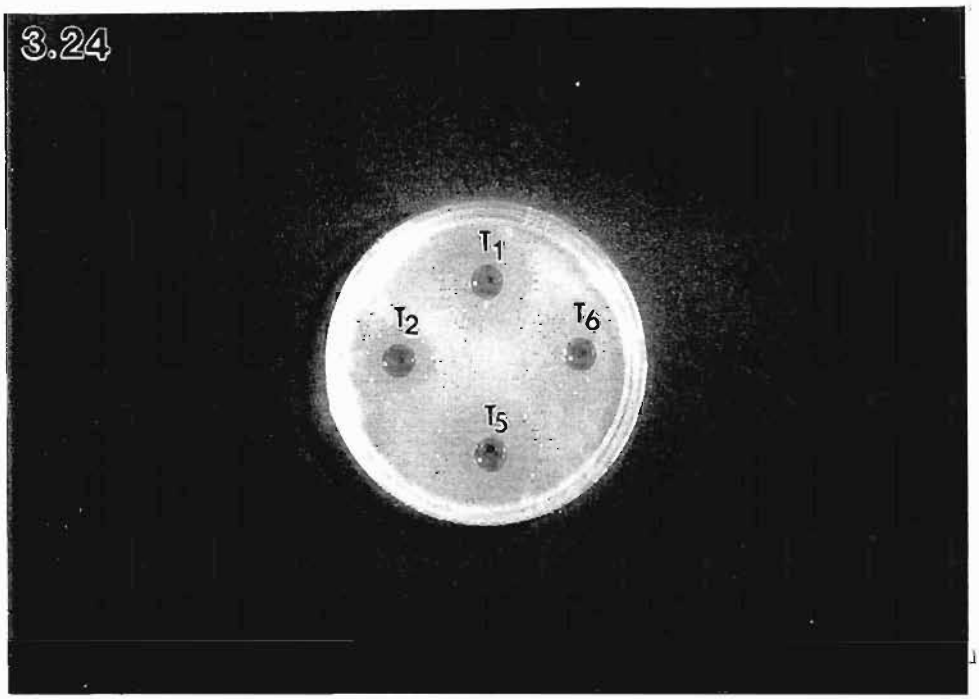

3.22

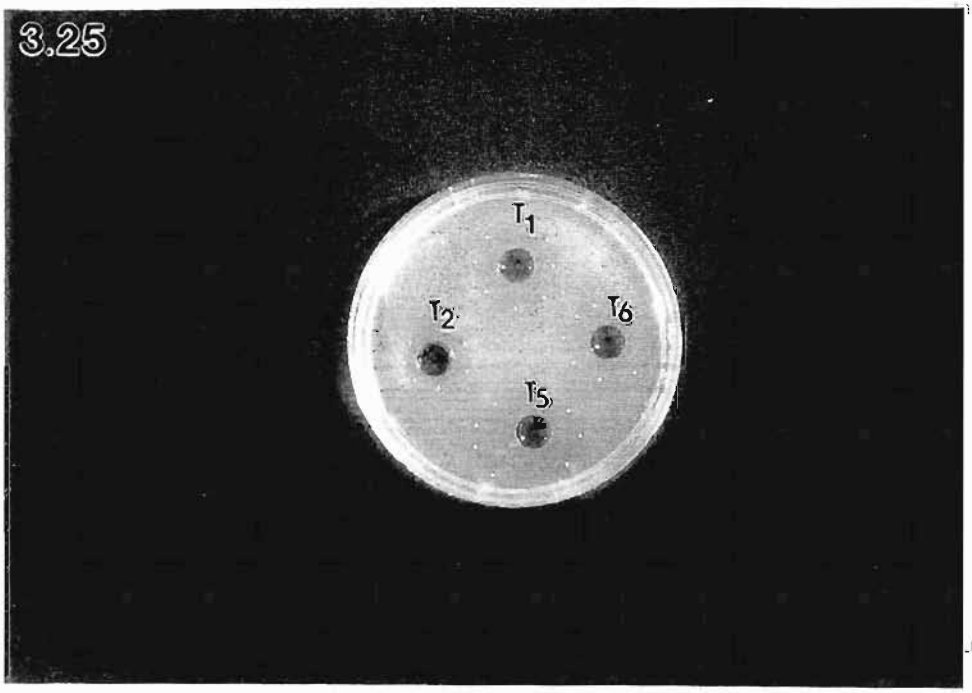

3.23

Figs 3.24-3.25. Possible antibiotic production by *Trichoderma* isolates.

Fig. 3.24. Note the inhibition zones of *E. coli* around the wells containing filtrates of *Trichoderma* spp.

Fig. 3.25. Note the inhibition zones of *S. aureus* around the wells containing filtrates of *Trichoderma* spp.


3.24
T1
T2
T6
T5


3.25
T1
T2
T6
T5

# CHAPTER 4

# SUMMARY AND FURTHER STUDIES

# CHAPTER 4

# SUMMARY AND FURTHER STUDIES

## 4.1. CONCLUDING REMARKS

The present investigation contributes towards the elucidation of the mode of action of *Trichoderma* spp. against *A. flavus* and *F. moniliforme*, and has advanced the understanding of the mechanisms that could be involved in biological control of seed-associated fungal pathogens. Research has involved the use of scanning electron microscopy and several biochemical techniques to reveal some morphological and physiological parameters assessed for *A. flavus/F. moniliforme* and *Trichoderma* spp. interactions.

Based on the findings of the macroscopic observations of the dual cultures, four out of nine *Trichoderma* strains exhibited aggressiveness, being able to curtail the growth of *A. flavus* and *F. moniliforme*. Scanning electron microscopical evaluation of the fungal interaction has demonstrated morphological hyphal alterations for both *A flavus* and *F. moniliforme* when co-cultured with aggressive *Trichoderma* spp. As assessed by SEM, mycoparasitism would appear not to be the mechanism involved in *A. flavus/F. moniliforme-Trichoderma* spp. interaction, although this requires verification using light and transmission electron microscopy. Finally, the results of biochemical studies have substantiated the concept of antibiosis being a major mechanism by which aggressive *Trichoderma* spp. inhibit the growth of *A. flavus* and *F. moniliforme*. It would appear from the present study, that antibiosis by *Trichoderma* spp. occurs by production of volatile compounds, extracellular enzymes and possible antibiotic elaboration.

## 4.2. FUTHER STUDIES

Before several *Trichoderma* strains become available commercially for biocontrol, major research priorities must include the following broad areas:

a. studies to test the efficacy of *Trichoderma* spp. as a biocontrol agent in maize seeds. This is particularly important in view of the deleterious effects of increasing salt concentration on growth of *Trichoderma* spp., as the interior of a dry seed is an osmotically stressful environment;

b. elucidation of the mechanism of biocontrol at the molecular level with the object of using the information to predict biocontrol capabilities;

c. genetic manipulation to enhance the ability of *Trichoderma* spp. to control diseases, to adapt to various environmental conditions and to tolerate pesticides;

d. development of models to understand the effect of the micro-environment on the survival and proliferation of *Trichoderma* spp. in the soil and plant rhizosphere;

e. interaction of *Trichoderma* spp. with other control measures, to develop integrated management systems for disease control;

f. an appreciation of attitudinal and economic factors such as acceptability by growers and consumers;

g. finally, but very importantly, testing for toxicological aspects, implying in-depth studies of mammalian toxigenicity.

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