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THE ASSOCIATION BETWEEN SOME *FUSARIUM* SPP. AND SEED QUALITY IN MAIZE (*ZEA MAYS* L.)

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ABSTRACT

The effect of delayed harvest on the occurrence and incidence of seed-borne *Fusarium* spp. and their effects on seed quality was investigated using four maize cultivars (Pioneer 3551, 3591, 3709 and 3475) over two seasons (1989/90, 1990/91) at Massey University, Palmerston North. As harvest was delayed from April to July, the percentage of cobs showing *Fusarium* mould increased. Cultivar 3551 tended to develop *Fusarium* cob mould later in the season (June) than the other three cultivars.

In both seasons the percentage of seeds of all four cultivars infected with *Fusarium* spp. increased as harvest was delayed. However, there was a difference between the two seasons; in 1989/90 the mean percentage of seeds carrying *Fusarium* spp. was 26%, 39%, 70% and 82% for April, May, June and July harvests respectively, while the corresponding levels for 1990/91 were 1%, 9%, 31% and 40% respectively. Between season differences were ascribed to climatic differences, the former season being wetter and warmer than the latter. There were only minor differences among cultivars for the percentage of seeds carrying *Fusarium* spp. *F. graminearum* was the species most consistently detected in all cultivars in both seasons, being recorded from 16%, 31%, 53% and 72% of seeds from the 1989/90 April to July harvests respectively, and from 0%, 6%, 25% and 30% of seeds from the same harvest times in 1990/91. *F. subglutinans*, *F. poae* and other *Fusarium* spp. were also detected, but their incidence was generally low.

Seed-borne *Fusarium* did not significantly reduce seed germination or vigour. In both seasons germination was between 86-99% for all cultivars. However, any dead seeds bore evidence of *F. graminearum* mycelial growth. Mycotoxins were recorded in seeds from all harvests in both seasons and mycotoxin levels increased as harvest was delayed. However, there were differences between seasons, as mean levels of Zearalenone, αZearalenol, Nivalenol and Deoxynivalenol ranged from 0.06 - 1.42 mg/kg seed in 1989/90, but from 0.0 - 0.54 mg/kg seed in 1990/91. In all cultivars and at most harvests in both years, levels of αZearalenol and of Nivalenol increased earlier than those of Zearalenone and Deoxynivalenol. Mycotoxin

differences among cultivars and the precise nature of the relationship between specific *Fusarium* species and mycotoxin development urgently requires further study, because of the potential for human and animal health problems.

Fusarium spp. from seed-culture colony were initially identified macroscopically on Malt Agar (MA), with pure cultures later being verified by the International Mycological Institute (UK). Subsequently, cultures were studied on Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and on Carnation Leaf Agar (CLA), with the final identity of seed-culture colonies being verified on CLA.

Colony texture and colour (including agar pigmentation) were initially used to separate *Fusarium* species detected on MA from infected seeds after harvest into a series of groups, ie 'red and fluffy', 'red centre', 'red and lobed', 'cream and fluffy', and 'cream and lobed' for *F. graminearum*. *F. crookwellense* was also separated as a 'red centre' type of colony while *F. culmorum* was separated as 'cream and flat', *F. subglutinans* 'purple and strands' type, and *F. poae* as 'purple/white/cream and powdery' type. While it was possible to differentiate the five types of *F. graminearum* on MA, it was not possible to distinguish *F. graminearum* 'red centre' type from *F. crookwellense*, although *F. culmorum* was relatively easy to differentiate from *F. graminearum* and *F. crookwellense*. Use of PDA or MEA pure cultures to differentiate *F. graminearum* from *F. crookwellense* or *F. culmorum* was not successful because the colony morphology of these three species was similar. However, *F. subglutinans* and *F. poae* were readily identified macroscopically on MA and MEA.

F. graminearum seed-culture colonies which did not sporulate on MA or MEA in most cases readily formed perithecia of *Gibberella zeae* on CLA (in the presence of 40W NUV light) regardless of whether the cultures were initiated by single germinated spores or by mass transferred inoculum. Those colonies which did sporulate on MA or MEA formed abundant sporodochia on CLA but not perithecia. CLA was also used to identify *F. graminearum* (*G. zeae*) from maize seeds or

seedlings by direct plating of these structures after surface disinfection. Full descriptions of the *Fusarium* colonies on the various media used are presented.

Fusarium survival in seed during storage depended upon seed moisture content (SMC) and storage temperature. *F. graminearum* was eliminated from seed at 14% SMC stored at 30°C and 25°C after 3 or 6 months storage, respectively, but survived at low levels (1-5%), together with *F. subglutinans* (1-7%), *F. poae* (1-2%) at these temperatures and 10% SMC. *F. subglutinans* and *F. poae* in seeds at 14% SMC did not survive after 9 months storage at 30°C. In seed stored at 5°C, *Fusarium* spp. infection levels did not decline after 12 months of storage at both 10 and 14% SMC. These results suggest a possible control strategy for producing *Fusarium* free seed, providing seed moisture content is not greater than 10%. At a storage temperature of 30°C, the post-storage germination of seed at 14% SMC had dropped to under 10% within 3 months, but seed at 10% SMC maintained its germination (88-97%) throughout the storage trial. After 12 months seed storage at 5°C (sealed storage) or 25°C (open storage), mycotoxin levels were similar to pre-storage levels.

The requirements of Koch's postulates were fulfilled in demonstrating that seed-borne *F. graminearum* was transmitted from maize seeds to seedlings under aseptic conditions in a glasshouse maintained at a temperature of 14°C to 17°C. The mean transmission rate (48%) was similar to the original seed-borne inoculum which suggests that under favourable environmental conditions, the pathogen will be effectively transferred from the seed to seedlings. *F. graminearum* had little effect on seedling emergence or survival, but was associated with a high percentage of seedlings with scutellum-mesocotyl/scutellum-main root lesioning. In the field, *F. graminearum* was consistently isolated from seedlings, but seed transmission could not be confirmed because of the presence of soil-borne inoculum, ie the pathogen was isolated from up to 37% of seedlings from a seed lot which carried only 1% seed-borne inoculum.

F. subglutinans was also proved to be seed transmitted under the same glasshouse conditions as described for *F. graminearum*. The significance of surface-

borne inoculum of this pathogen was demonstrated in that the mean transmission rate for non-surface disinfected seed lots was 81%, whereas it was only 7% for surface disinfected seed lots. *F. subglutinans* was associated mainly with 'above sand level' seedling infection (coleoptile-node infection, leaf/shoot blight, shoot wilt and seedling stunting). However, *F. subglutinans* was rarely detected in seedlings from the field, possibly because of the antagonistic effects of mycopathogenic fungi such as *Gleocladium roseum.*

These results are discussed, particularly in relation to the significance of *F. graminearum* and *F. subglutinans* as seed-borne pathogens of maize, and the difficulties inherent in the identification of *Fusarium* spp. following seed health testing. It is likely that these seed-borne *Fusarium* spp. are more important because of their association with mycotoxins, than with any effects they have as an inoculum source for diseases of maize.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 MAIZE AND ITS IMPORTANCE

Maize (*Zea mays* L.), also known as corn or indian corn (Benson and Pearce, 1987) apparently originated in Mexico, where it is thought to have been cultivated by the year 5,000 BC (Jugenheimer, 1976; Jones and Clifford, 1983; Benson and Pearce, 1987; FAO, 1992). Following European discovery of the Americas, maize was moved quickly to Northern America, Europe, Africa and Asia, including Australasia (Benson and Pearce, 1987; FAO, 1992). Maize is believed to have developed from its botanical relative, toesinte (*Euchlaena mexicam* Schrod.) (Jugenheimer, 1976; Jones and Clifford, 1983; Benson and Pearce, 1987; FAO, 1992).

There are many types of maize (Jugenheimer, 1976; Jones and Clifford, 1983; Kommedahl and Windels, 1986; Zuber and Darrah, 1987), the differences depending on the type of the endosperm (Zuber and Darrah, 1987). The recognised types which are also regarded as sub-species of *Zea mays* L. (Jones and Clifford, 1983) are:

The dent type - owes it names to the depression which develops in the seed when the soft starch (in the seed) dries.

The flint type - does not become indented and has a lower soft starch content. Popcorn is a type that will pop upon heating (Kommedahl and Windels, 1986).

The flour type - contains a very high proportion of soft starch.

The sweetcorn type - is sweet because the sugars within the seeds are not converted to starch during ripening. Several mutant genes condition the endosperm

making it sweet when consumed about 18-20 days after pollination (Zuber and Darrah, 1987).

Maize, wheat and rice are the three most important cereal grains of the world but maize is second to wheat (Benson and Pearce, 1987; FAO, 1992). Most of the world's maize production is concentrated in North America with the Corn Belt region of the United States (USA) producing about half of the world's total production (Jones and Clifford, 1983; Benson and Pearce, 1987).

The world maize production increased during 1979-1986 with land planted with maize increasing from 105 million ha to 127 million ha and harvested maize from 421,751 to 486,198 million metric tonnes (FAO, 1992). This increase is thought to have resulted from additional land increase but more significantly from genetic improvement, including introduction of high yielding varieties, especially hybrids and more efficient technological field practices, including fertiliser application and pest and disease control and harvesting techniques (FAO, 1992).

While in developing countries maize is grown mainly for human consumption, in developed countries it is mainly grown for animal feed and industrial uses such as starch production (FAO, 1992). Benson and Pearce (1987) and Watson (1987) state that over 90% of maize produced or imported in developed countries is used for animal feed. Maize forms the staple diet in many parts of Africa and Latin America (Jones and Clifford, 1983). As food, the whole grain either mature or immature may be used (cooked or roasted on cob) or the dry shelled grain may be processed by dry and wet milling technologies to give large intermediary products such as grits, maize meal, maize flour and flaking grits (FAO, 1992), jam, ice cream, etc (Chappell, 1985).

Maize is currently the third largest arable crop in New Zealand after wheat and barley and is primarily used as poultry and pig feed and as a supplement feed to other animals such as deer, horses and other livestock (Chappell, 1985; Sayer, 1991; Sayer and Lauren, 1991; Hardacre *et al.*, 1991). While about 70% of the production

in New Zealand is used for animal feed, the remaining 30% is milled and used to produce food products including breakfast cereals, cakes, breads, biscuits, cooking oils, etc and industrial products such as starch and glue (Chappell, 1985; Hardacre *et al.*, 1991).

1.2 GROWING A MAIZE CROP

Maize is grown over a wide range of climatic conditions but most production is in the latitudes 30° and 47° north and south of the equator (Benson and Pearce, 1987). Maize grown far north or south in temperate climates is used mainly for forage and not grain (Benson and Pearce, 1987; Hardacre *et al.*, 1991). Temperature and moisture are the key factors that determine the growing of maize (Benson and Pearce, 1987) and maize is known to be a warm weather crop requiring frost free conditions during the growing period for reliable yields (Berger, 1962; Shaw, 1977). Maize growth and development occurs with temperatures between 10°C and 30°C (Duncan, 1975), optimal temperatures being between 21°C and 27°C (Shaw, 1977), with the highest corn yields being obtained at daytime maximum of 24-30°C (Benson and Pearce, 1987). Maize requires considerable moisture during the growing period (Berger, 1962). However, high rainfall of 70-80 mm falling within 6-9 days just after silking favours maize infection by *Fusarium graminearum* Schwabe (Sutton *et al.*, 1980a), particularly when cool to warm temperatures of about 21-24° prevail (Tuite *et al.*, 1974; Sutton, 1982; Lacey and Magan, 1991). Since maize requires warm, frost free conditions during the growing season for reliable yields, in New Zealand this restricts the majority of grain production to the Auckland, Waikato, Bay of Plenty, Poverty Bay, Hawkes Bay and Manawatu regions, and small areas in the South Island (Hardacre *et al.*, 1991).

Choosing the right hybrid is important for maize production in New Zealand and Hardacre *et al.* (1991) recommend that hybrid maturity should be among the first factors to be considered when selecting hybrids. To avoid early autumn frost crop damage, the maize crop should mature/be harvested before May/in May and the grain should have a moisture content below 25% (Hardacre *et al.*, 1989; Hardacre *et al.*,

1991). The crop should be sown as early as possible in the season (but avoiding late frost and soil temperatures below about 8°C), for example in late October/early November in the Manawatu (Hardacre *et al.*, 1989; 1991).

In most seasons, the maximum yields will be obtained by growing a hybrid which occupies the full growing season (Bansal and Eagles, 1985). Late maturing hybrids (characterised by high grain moisture content (27%) at harvest) tend to be higher yielding than early or mid-season hybrids (Hardacre *et al.*, 1991) but when grown in the Manawatu region will not reach grain moisture content below 25% until much later than May (if at all) (Hardacre *et al.*, 1991).

In cooler areas such as the Manawatu or those with shorter growing seasons, early maturing hybrids provide a full season of growth (Bansal and Eagles, 1985). However, mid-season hybrids (suited for intermediate growing temperature areas such as Waikato) are sometimes grown in cooler regions, and when this happens maize crops harvesting is delayed because of slow grain drying until July or even August (Bansal and Eagles, 1985). Hardacre *et al.* (1991) recommended that late maturing hybrids should not be grown in the Manawatu region because when grown there they showed a high incidence of cob rotting.

1.3 MAIZE DISEASE PROBLEMS CAUSED BY FUSARIUM

Kruger (1989) states that maize diseases caused by *Fusarium* spp. occur worldwide and cause severe damage by attacking roots, stalks, cobs and seedlings, the most important diseases being stalk rot and cob rot. Teich (1989) reported that cob rot disease results in reduced yields, shrunken grain and accumulation of several mycotoxins which make the grain unfit for man and livestock. The occurrence and significance of maize diseases caused by *Fusarium* spp. in different parts of the world has been discussed in detail by Shurtleff (1980), Burgess *et al.* (1981), Cassini (1981), Maric (1981) and Kommedahl and Windels (1981). *Fusarium* species that commonly cause ear rot in maize are *F. graminearum* Schwabe (teleomorph *Gibberella zeae* (Schw.) Petch); *F. moniliforme* Sheldon (teleomorph *G. fujikuroi*

(Saw.) Wr.); *F. culmorum* (W.G. Smith) Sacc.; *F. moniliforme* Sheldon var *subglutinans* Wollenw. & Reinking (teleomorph *G. fujikuroi* (Saw.) Wr. var *subglutinans* Edwards) [Syn. *F. subglutinans* (Wollenw. & Reinking), Nelson, Toussoun & Marasas (teleomorph *G. subglutinans* (Edwards) Nelson *et al.*]. The epidemiology of cob rot disease caused by *F. graminearum* has been discussed in detail by Sutton (1982). *F. sporotrichoides* Sherb, *F. poae* (Peck) Wollenw. and *F. tricinctum* (Corda) Sacc. may also occur in overwintered cereals (Neish *et al.*, 1983; Lacey and Magan, 1991).

The time of harvest of maize seed is important because the seed is infected while still in the field on the mother plant, starting from the silking stage of the crop (Edwards, 1936; Enerson and Hunter 1980a; Sutton, 1982), and increasing through the seed maturation period (Koehler, 1942; Hesseltine and Bothast, 1977; Sutton and Baliko, 1981). Delayed harvest is thought to increase disease infection (Sutton, 1982).

The climate throughout New Zealand is generally favourable for the growth of *Fusarium* spp. (Lauren *et al.*, 1991), favouring cob rot disease of maize (Fowler, 1985; Hardacre *et al.*, 1991). *F. graminearum* and *F. moniliforme* var *subglutinans* were reported (Fowler, 1985) in maize crops in New Zealand, causing stalk rot and cob rot together with *Diplodia zeae* (Schw.) Lev. and *Nigrospora* spp. Hussein and Baxter (1985) reported *F. graminearum* to be the most common *Fusarium* sp. isolated from maize seed samples from the Manawatu region two weeks before harvest. Later in 1991, Lauren *et al.* (1991) also reported the occurrence in New Zealand-grown maize grain of several *Fusarium* spp, with *F. graminearum* and *F. crookwellense* Burgess, Nelson and Toussoun, occurring in high levels (56-65% and 18-20%, respectively) in the Manawatu region while *F. graminearum*, *F. crookwellense* and *F. semitectum* Berk. & Rav. were the most common species isolated from field and stored maize seeds in the Waikato region (Sayer, 1991).

Colonisation of maize grain by *Fusarium* spp., especially *F. graminearum* has been reported to continue in storage under conditions favourable for its growth and

extensive growth has been observed on dehusked ears stored in cribs (Sutton, 1982; Lacey and Magan, 1991). *F. graminearum* grows on kernels with moisture contents of 20-22% (Eugenio *et al.*, 1970; Sutton, 1982), moisture contents at which harvest may be carried out (Aldrich *et al.*, 1976; Jugenheimer, 1976). However, Christensen and Kaufmann (1969) stated that "*Fusarium* dies relatively rapidly in grain stored at moisture contents of about 12-13% and temperatures above 70°F (approximately 21.1°C)", while Watson (1987) states that *Fusarium* dies rapidly in maize at 16-20% moisutre, but Abbas and Mirocha (1986) found that *F. graminearum* survived in maize seeds stored at 0°C for 13 years.

Fusarium spp. produce a variety of mycotoxins in the maize grain they infect (Kruger, 1989; Teich, 1989; Chelkowski, 1989, 1991; Logrieco *et al.*, 1993) and these cause mycotoxincoses of animals and man upon ingesting the grain (Joffe, 1986; Marasas and Nelson, 1987). Mycotoxins, in particular zearalenone and trichothecenes, have been reported to occur naturally in maize grain produced in New Zealand (Hussein *et al.*, 1989; Lauren *et al.*, 1991), and contamination of pasture by zearalenone has been shown to be a limiting factor in sheep reproduction in some areas of New Zealand (Towers *et al.*, 1987). Delayed maize harvest or no harvest (overwintering) under cool or cold, and wet conditions favour an increase in the production of zearalenone and trochothecenes (Caldwell and Tuite, 1974; Wicklow *et al.*, 1990). T-2 toxin which caused the haemorrhagic syndrome (alimentary toxic aleukia-ATA) in Russia during World War II was produced by *F. poae* and *F. sporotrichoides* in cereal grain which overwintered (Joffe, 1986). In New Zealand, Bansal and Eagles (1985) reported that harvest may be delayed until July or August, in which case cob rotting may have increased, resulting in an increase of mycotoxins and rotten grains contain higher levels of mycotoxins than sound ones (Chelkowski, 1989, 1991; Perkowski *et al.*, 1991).

The production of *Fusarium* mycotoxins especially zearalenone, is known to continue during maize grain storage, especially under adverse storage of wet or humid conditions, for example when maize cobs (ears) are stored in cribs without proper or no cover from the rain under cool conditions (Sutton, 1982; Wicklow *et al.*, 1990;

Lacey and Magan, 1991). *Fusarium* mycotoxins have been found in stored maize in New Zealand (Hussein *et al.*, 1989; Sayer and Lauren, 1991).

1.4 OBJECTIVES OF THE STUDY

Neergaard (1979) states that 25% of maize production losses in the world is due to seed-borne *F. graminearum* and *Diplodia zeae*, yet Rheeder *et al.* (1990) found seed-borne *F. graminearum* to have little influence on germination in the laboratory. The significance of *F. moniliforme*, the most commonly detected seed-borne *Fusarium* in maize and of *F. subglutinans* in regard to their role in seeds for planting is very much controversial (King and Scot, 1981; Naik *et al.*, 1982; Ochor *et al.*, 1987; Van Wyk *et al.*, 1988). Despite accumulated knowledge about the epidemiology and mycotoxicology of *F. graminearum*, its seed to seedling transmission, as well as the value of the infected seeds after storage, have received very little attention. Additionally, the effects of delayed maize harvesting on *Fusarium* infection levels on seed quality have not been studied in New Zealand.

Therefore, the objectives of the study were:

1. To evaluate the effect of delayed harvest on the incidence of *Fusarium* spp. in the seed and their effects on seed quality.

2. To identify those species of *Fusarium* most commonly associated with maize seed in the field.

3. To study the persistence of *Fusarium* spp. in seeds at different temperatures and moisture contents during storage, and the quality of the seeds after storage.

4. To demonstrate the seed transmission of *Fusarium* spp. under glasshouse and field conditions.

CHAPTER TWO

LITERATURE REVIEW

The volume of literature on *Fusarium* spp. and on maize seed production and storage is extensive. For this reason, only those aspects directly related to the present study will be covered in this review.

2.1 THE GENUS *FUSARIUM* LINK AND ITS TAXONOMY

2.1.1 The genus *Fusarium* Link

The definitive character of the genus *Fusarium* is the production of septate, fusiform to sickle-shape conidia, termed macroconidia, with a foot-shaped basal cell and a more or less beaked apical cell (Fig. 2.1). Macroconidia may be produced in discrete pustules, called sporodochia, or in confluent, slimy masses, known as pinnotes. Some species of *Fusarium* also produce smaller 1-2 celled conidia, the microconidia, of various shapes. Chlamydospores, either terminal or intercalary, are characteristic of some species also. *Fusarium* colonies are usually fast growing and consist of felty aerial mycelium which may be pale, or brightly coloured in shades of pink, red, violet or brown (Booth, 1971; Nelson *et al.*, 1983; Pitt and Hocking, 1985; Burgess *et al.*, 1988).

The genus *Fusarium* is one of the most economically important genera of fungi because it includes many pathogenic species which cause a wide range of plant diseases (Table 2.1), (Nelson *et al.*, 1981a); some species which are highly mycotoxigenic, causing both animal and human diseases (Marasas *et al.*, 1984; Burgess, 1985; Joffe, 1986; Marasas and Nelson, 1987); and a number of species

which cause opportunistic infections also of humans and animals (Rebell, 1981). In addition, many species are common soil saprophytes (Burgess, 1981) and active in the decomposition of cellulosic plant materials (Pitt and Hocking, 1985).

Table 2.1 Some examples of the plant diseases caused by *Fusarium* species.

Species	Disease	Reference
oxysporum	Vascular wilts Crown rot of tomato	Beckman, 1987 Booth, 1971 Nelson, 1981 Nelson *et al.*, 1981b Jarvis & Shoemaker, 1978
solani	Root rot of legumes and other crops	Burkholder, 1919 Nelson *et al.*, 1981b
moniliforme	Stalk, root and cob rot of corn Stalk and root rot of sorghum	Marasas *et al.*, 1979 Nelson *et al.*, 1981b Trimboli & Burgess, 1983, 1985
subglutinans	Stalk and cob rot of corn Pitchcanker of pine Fruit rot of pineapple	Nelson *et al.*, 1981b Dwinell *et al.*, 1981 Bolkan *et al.*, 1979
culmorum	Foot and root rot of wheat	Cook, 1980 Nelson *et al.*, 1981b
graminearum Group 1	Crown rot of wheat, barley, triticale, oats and grasses	Burgess *et al.*, 1981 Burgess *et al.*, 1987b
graminearum Group 2 (*Gibberella* *zeae*)	Stalk and cob rot of maize Head scab of wheat Stub dieback of carnations	Nelson *et al.*, 1981b Atanasoff, 1920 Sutton, 1982 Nelson *et al.*, 1975
avenaceum	Root rot of medics, and other legumes Stem rot of carnations	Lamprecht *et al.*, 1984 Burgess *et al.*, 1973 Nelson *et al.*, 1981a
lateritium	Storey's bark disease, scaly bark and collar rot of coffee	Siddiqui & Corbett, 1963

Source: Burgess *et al.* (1988)

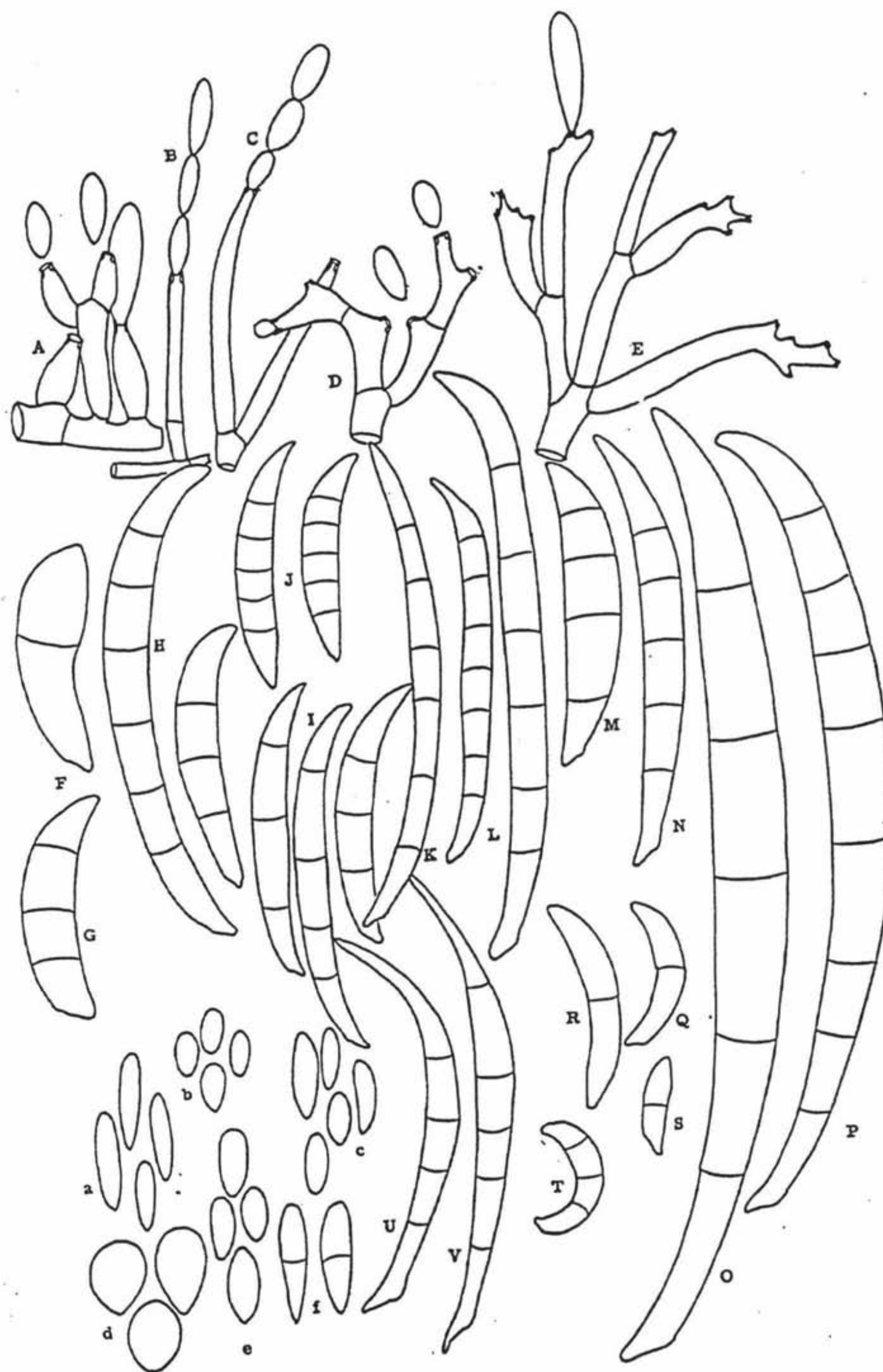


Figure 2.1 Spore form and conidiogenous cells. A-E, microconidia, conidiogenous cells; A, *F. oxysporum*; B, *F. moniliforme*; C, *F. decemcellulare*; D, *F. moniliforme* var. *subglutinans*; E, *F. semitectum*; F-V, macroconidia; F, *F. tumidum*; G, *F. trichothecioides*; H, *F. solani*; I, *F. oxysporum*; J, *F. semitectum*; K, *F. avenaceum*; L, *F. lateritium*; M, *F. culmorum*; N, *F. graminearum*; O, *F. coccophilum*; P, *F. decemcellulare*; Q, *F. dimerum*; R, *F. nivale*; S, *F. tabacinum*; T, *F. larvarum*; U, *F. acuminatum*; V, *F. equiseti*.

a-f, microconidia; a, *F. moniliforme*; b, *F. moniliforme* var *subglutinans*; c, *F. oxysporum*; d, *F. poae*; e, *F. solani*; f, *F. fusarioides*.

Source: Booth, 1971.

The genus has a wide distribution and representatives occur in all major agronomic regions of the world, some species having a cosmopolitan geographic distribution, whereas others tend to occur predominantly in tropical and subtropical regions, or cool to warm temperate regions (Burgess, 1981).

Burgess (1981) and Mills (1989) reviewed the ecological aspects of *Fusarium* spp., and Burgess (1981) differentiated three modes of existence; the soil-borne fusaria, such as *F. oxysporum*, *F. solani*, *F. culmorum*, *F. equiseti* (Cda) Sacc. and *F. graminearum* Group 1; air-borne fusaria, eg *F. ridigidiusculum*, *F. lateritium* and *F. graminearum* Group 2; and fusaria which live in both the subterranean and aerial plant parts, typified by *F. moniliforme*, *F. subglutinans* and *F. semitectum*. Because of progression succession of mycoflora (including *Fusarium* spp.) in seeds during storage, McLean and Berjak (1987) have considered maize grains as a micro-ecological niche. Seed-borne Fusaria have been discussed by Neergaard (1979) and Richardson (1990) has listed those found in maize.

The genus *Fusarium* is notorious for the high degree of variability in cultural morphology and physiological characteristics of some species, and this characteristic has caused great difficulties in the taxonomy of the genus (Booth, 1971; Marasas *et al.*, 1984; Booth, 1984; Pitt and Hocking, 1985; Burgess *et al.*, 1988; Nirenberg, 1989; Nelson, 1991; Windels, 1991). The morphology of the macroconidia is the basis for identification of *Fusarium* species (Booth, 1971; Nelson *et al.*, 1983; Pitt and Hocking, 1985; Burgess *et al.*, 1988; Nelson, 1991).

2.1.2 Taxonomy

Marasas *et al.* (1984) have stated that the taxonomy of the genus *Fusarium* is complex and difficult to apply because of the use of different taxonomic systems in different countries. This situation has led to great confusion in the extensive literature on plant pathogenic species (Booth, 1971; Marasas *et al.*, 1979; Nelson, 1992) and on *Fusarium* mycotoxicology (Marasas *et al.*, 1984). The confusion has occurred because the same fungus is known under a variety of different names or different

species are lumped together under one name (Marasas *et al.*, 1984; Nelson, 1991). These problems have arisen because of misidentification of species as a result of complications created by the extreme variability of *Fusarium* species in culture, and the fact that they mutate and degenerate rapidly, particularly under conditions of repeated subculturing on common laboratory media (Nelson *et al.*, 1983; Nelson *et al.*, 1984; Pitt and Hocking, 1985; Burgess *et al.*, 1988; Nelson, 1991; Windels, 1991).

The different taxonomic systems which have been used in the identification of *Fusarium* species have been reviewed by various authors, eg Booth (1971, 1981, 1984); Messiaen and Cassini (1981); Joffe (1986); Burgess *et al.* (1988); Nirenberg (1989) and Nelson (1991). Windels (1991) has discussed the gaps and problematic areas in the current systems of *Fusarium* taxonomy and identification.

According to Burgess *et al.* (1988) and Nelson (1991), approximately 1000 *Fusarium* species had been described by 1900, based largely on examination of sporodochia on plant materials, but Wollenweber and Reinking (1935) as cited by Nelson (1991), were the first scientists to produce a major taxonomic system on *Fusarium*. In their "monumental" (Nelson, 1991) publication "Die Fusarien", Wollenweber and Reinking classified *Fusarium* into 16 sections, 65 species, 55 varieties and 22 forms (Booth, 1971). Since 1935, other taxonomic systems have been published (Nelson, 1991), all based, to various degrees, on that of Wollenweber and Reinking (1935), but names/number of species have varied from 78 species (Gerlach and Nirenberg, 1982) to 9 species (Snyder and Hansen, 1941a, 1945; Messiaen and Cassini, 1968): for example, in section Discolor (see Joffe, 1986). Particular systems have tended to be more accepted in particular regions of the world than others (Burgess *et al.*, 1988; Liddell, 1991); for example the nine species system of Snyder and Hansen (1940s) was adopted in the USA but not widely accepted elsewhere (Burgess *et al.*, 1988).

In the publications of Snyder and Hansen (1941a, 1945) all species previously in the Sections Roseum, Arthrosporiella, Gibbosum and Discolor were all referred

to as *F. roseum*. Strains which were pathogenic to cereals were labelled *F. roseum* f. sp. *cerealis* (Booth, 1971). Later, Snyder *et al.* (1957) introduced the cultivar concept and what they referred to as morphologically different strains of *F. roseum* were given cultivar names - such as *F. roseum* 'Graminearum' or if pathogenic, as *F. roseum* f. sp. *cerealis* cultivar 'Graminearum', 'Culmorum' or 'Avenaceum'. According to Booth (1971), Burgess *et al.* (1988) and Nelson (1991), this system introduced a rather complex, three-or four stage nomenclature for *Fusarium* without any simplification of identification. Based on the taxonomic system of Snyder and Hansen (1941a, 1945), Messiaen and Cassini (1968) introduced a system which involved use of varieties resulting in nomenclature such as *F. roseum* var *graminearum* (Booth, 1971).

The taxonomic confusion has also occurred in the Section Lisiola. Booth (1971) and Nirenberg (1989) have illustratively reviewed synonyms (Table 2.2) of members of *Fusarium* in this section. Notable is the misidentification of *Fusarium subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas comb. nov. as *Fusarium moniliforme* Sheldon (Koehler, 1959; Marasas *et al.*, 1979; Bottalico *et al.*, 1989a; Nelson, 1992). Booth (1971) and Nirenberg (1989) have reviewed the synonyms of *F. moniliforme*/*F. subglutinans* and Nelson *et al.* (1983) elevated *F. subglutinans* to a species status, the main difference between *F. subglutinans* and *F. moniliforme* being the absence of microconidial chains and the presence of polyphialides in *F. subglutinans*.

In Section Sporotrichiella, the reduction (Snyder and Hansen, 1945) of all the species, ie *F. poae*, *F. sporotrichiodes*, *F. sporotrichiodes* var *minus*, *F. chlamydosporum*, *F. tricinctum* of Wollenweber and Reinking (1935) to only one species, *F. tricinctum* (Joffe, 1986) has complicated the literature on members in this Section.

The current taxonomic system was published by Nelson *et al.* (1983) who selected what they considered the best parts of several systems and combined them with results of their own research to develop a compromise system for practical *Fusarium* spp. identification (Nelson *et al.*, 1983; Nelson, 1991). In their

Table 2.2 Summary of synonymy within the Liseola section.

Wollenweber 1931	Wollenw. and Reink. 1935	Bilay 1955	Booth 1971
F. moniliforme F. moniliforme var. crumpens F. moniliforme var. majus F. samoense	F. moniliforme	F. moniliforme	F. moniliforme
F. moniliforme var. minus	F. moniliforme var. minus		
	F. lactis	F. moniliforme var. lactis	
F. moniliforme var. subglutinans F. neoceras	F. moniliforme var. subglutinans F. neoceras	F. moniliforme var. subglutinans	
F. moniliforme var. anthophilum	F. moniliforme var. anthophilum	[F. tricinctum.] var. anthophilum	F. moniliforme var. subglutinans

Gerlach & Nirenberg 1982	Nelson *et al.* 1983	Booth 1971
F. verticillioides (Sacc.) Nirenberg	F. moniliforme Sheld.	F. moniliforme Sheld.
F. proliferatum (Matsushima) Nirenberg var. proliferatum	F. proliferatum (Matsushima) Nirenberg	F. moniliforme Sheld.
F. proliferatum (Matsushima) Nirenberg var. minus (Wollenw.) Nirenberg	F. moniliforme Sheld.	F. moniliforme Sheld.
F. sacchari (Butler) W. Gams var. subglutinans (Wollenweber & Reinking) Nirenberg	F. subglutinans (Wollenw. & Reinking) Nelson *et al.*	F. moniliforme Sheld. var. subglutinans Wollenw. & Reinking
F. sacchari (Butler) W. Gams var. elongatum Nirenberg	F. subglutinans (Wollenw. & Reinking) Nelson *et al.*	-
F. succisae (Schroter) Sacc.	F. succisae (Schroter) Sacc.	F. moniliforme Sheld.
F. anthophilum (A. Braun) Wollenw.	F. anthophilum (A. Braun) Wollenw.	F. moniliforme Sheld. var. subglutinans Wollenw. & Reinking

Sources: Booth, 1971; Nirenberg, 1989.

"Laboratory Manual for *Fusarium* Research", which is based on the taxonomic approach of Nelson *et al.* (1983), Burgess *et al.* (1988) have incorporated a key and descriptions of common species found in Australasia.

2.2 *FUSARIUM* SPP. IN CULTURES AND THEIR IDENTIFICATION

2.2.1 Cultures

The genus *Fusarium* is notoriously characterised by a considerable degree of morphological and physiological variability (Booth, 1971; Burgess and Liddell, 1983; Pitt and Hocking, 1985; Burgess *et al.*, 1988; Windels, 1991; Nelson, 1991). This is a result of the genetic nature of the genus and its ability to vary morphologically in response to changes in the environment (Booth, 1971; Puhalla, 1981; Nelson *et al.*, 1983; Burgess *et al.*, 1988).

Morphological characteristics, especially those of the macroconidia, provide the basis for identification and taxonomy of *Fusarium* (Nelson *et al.*, 1983; Burgess *et al.*, 1988; Nelson, 1991; Windels, 1991). Thus it is essential that standardised culturing procedures be adopted in taxonomic and identification studies of *Fusarium* to minimise variability that occurs due to environmental factors (Nelson *et al.*, 1983; Pitt and Hocking, 1985; Burgess *et al.*, 1988).

Nelson *et al.* (1983) stated that the majority of *Fusarium* species isolated from nature produce macroconidia in sporodochia and that the sporodochial type species mutate in nature and in culture. The basic mechanism of cultural mutation or degeneration is not much understood (Burgess *et al.*, 1988) but frequent subculturing on media rich in carbohydrates such as PDA and similar media (Nelson *et al.*, 1983) and mass-transfer of mycelium, promote culture mutation (Pitt and Hocking, 1985; Burgess *et al.*, 1988) resulting in cultural degenerate variants. Some isolates will degenerate more quickly than others even after only one or two subcultures (Nelson *et al.*, 1983; Pitt and Hocking, 1985). It is known that culturally degenerate variants

may differ significantly from their "wild-type" parent cultures in respect of morphological and physiological attributes. The occurrence of degenerate cultural mutants is more frequent in some species than others (Toussoun and Nelson, 1975; Nelson *et al.*, 1983).

Nelson *et al.* (1983) and Burgess *et al.* (1988) stated that there are two types of degenerate cultural variants, namely, pionnatal and mycelial types. The pionnatal type has a flat, shiny, wet appearance, with little or no aerial mycelium, is usually more brightly coloured than the sporodochial type and has abundant macroconidia which may be distorted. The mycelial type consists of abundant sterile aerial mycelium which is usually white with few macroconidia.

The occurrence of degenerate cultural variants can be minimised by subculturing on natural substrates, such as soil and straw, or low nutrient media such as Carnation Leaf Agar (CLA) or Water Agar (WA), by initiating a culture from a single germinating spore or hyphal tip, and by minimising subculturing (Booth, 1971; Nelson *et al.*, 1983; Pitt and Hocking, 1985; Burgess *et al.*, 1988).

The other advantages of initiating a culture from single germinated spores or single hyphal tips are that the culture is uniform, because it is composed of one genotype, and by this procedure mixed cultures can be separated and the mutants discarded (Nelson *et al.*, 1983; Burgess *et al.*, 1988).

2.2.2 Isolation of *Fusarium* spp. from natural substrates

Methods for isolating *Fusarium* species from different substrates have been described by Booth (1971, 1977), Nelson *et al.* (1983), Burgess and Liddell (1983) and Burgess *et al.* (1988). They include procedures for isolation from soil, plant debris from the soil, infected plants and from the air. The procedure for isolation of *Fusarium* spp. from infected plants and seedlings has been described in detail by Burgess *et al.* (1988) and the main aspects involved include:

(a) Selection of a plant or seedling with typically infected plant tissue, avoiding old necrotic areas which may be invaded by secondary invaders.

(b) Surface disinfection of the plant material to remove surface contaminants and to reduce the number of secondary invaders.

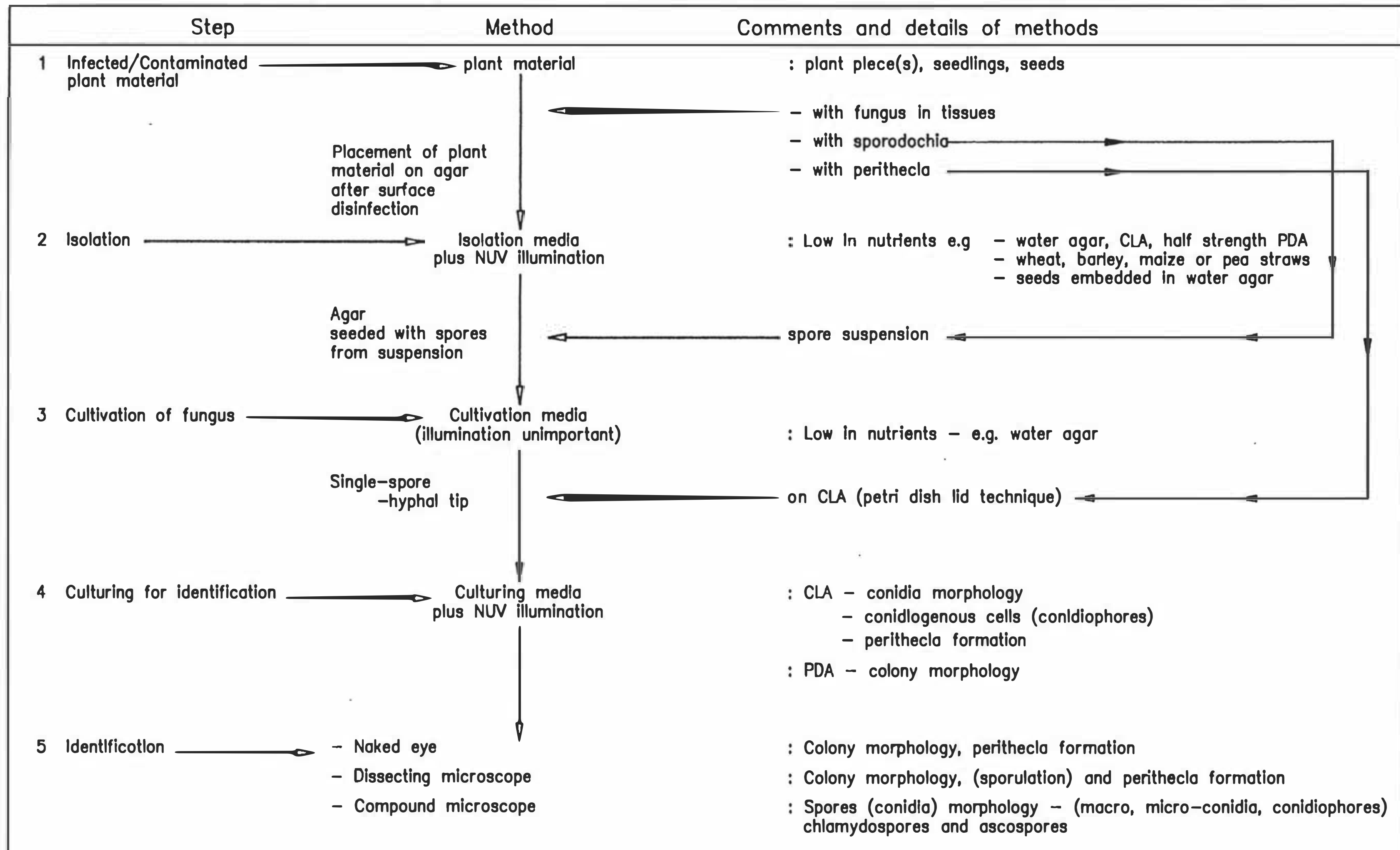
(c) Damp drying of the disinfected plant material before plating on to agar to prevent saprophytic bacteria from growing on the agar and suppressing the growth of the pathogen.

(d) The plating of very small pieces of infected plant tissue onto the appropriate agar in order to promote rapid growth out of the tissue by the pathogen without it being overgrown by saprophytes, and to simplify subculturing.

Procedures for preparing cultures for the identification of *Fusarium* spp. as described and discussed by Nelson *et al.* (1983) and Burgess *et al.* (1988) are summarised in Fig. 2.2.

2.2.3 Recommended culture media for isolation

Isolation of *Fusarium* species from plant tissue should be done on a medium low in nutrients, such as the natural media recommended by Hansen and Snyder (1947), and Snyder and Hansen (1947), which include wheat straw, barley straw, pea straw, and seeds embedded in water agar. A more recent recommendation is for the use of carnation leaf agar (Nelson *et al.*, 1983).



Constructed from: Nelson *et al.* (1983) and Burgess *et al.* (1988)

Figure 2.2 Diagram of procedures for preparing cultures for *Fusarium* species identification

2.2.4 *Fusarium* culturing media

A wide range of media has been developed for isolation of Fusaria from different substrates, including those for growth, stimulating sporulation and preservation of *Fusarium* species (Booth, 1971, 1977; Nelson *et al.*, 1983, Burgess and Liddell, 1983; Pitt and Hocking, 1985; Burgess *et al.*, 1988). Burgess *et al.* (1988) have described these media under four groups:

General purpose media

(a) Carnation leaf agar (CLA)
(b) Chaff-grain medium
(c) Potato dextrose agar (PDA)
(d) Pea seed soil extract agar (PSSA)
(e) Soil agar (SA)
(f) V_8-juice agar
(g) Water agar

Selective media

(a) Dichloran chloramphenicol peptone agar (DCPA)
(b) Komada's medium
(c) Modified potato dextrose agar (MPDA)
(d) Peptone pentanchloronitrobenzene (PCNB) agar (PPA)
(e) Selective *Fusarium* agar (SFA)

Defined Medium

(a) Defined buffered agar

Pitt and Hocking (1985), however, studied and described *Fusarium* spp on other media which were:

(a) Czapek Yeast Extract Agar (CYA)

(b) 25% Glycerol Nitrate Agar (G25N)

(c) Malt Extract Agar (MEA)

2.2.5 General purpose media: CLA and PDA

Conidia formed on weak media such as CLA are more uniform in respect to shape than those produced on carbon rich media (Burgess *et al.*, 1988). Fisher *et al.* (1982) and Nelson *et al.* (1983) observed that the advantage of using CLA is that it promotes sporulation rather than mycelial growth. Conidia and conidiophores are produced in abundance, their morphology closely approximates that seen under natural conditions, and phenotypic variation is reduced. CLA is low in available carbohydrate and contains complex, natural occurring substances such as vitamins, of the type encountered by *Fusarium* in nature on a host plant or natural substrate (Nelson *et al.*, 1983). Direct observation of a culture of *Fusarium* on CLA under a compound microscope facilitates identification of the manner in which conidia are borne on conidiophores, especially the microconidia in false heads, the presence of chains of microconidia and chlamydospores (Fisher *et al.*, 1982; Burgess *et al.*, 1988). The other advantage of using CLA is that the perithecia of the perfect state of homothallic strains of *Fusarium* species, eg *F. graminearum* (*Gibberella zeae*) and *F. solani* (*Nectria haematococca*) will form readily on this medium when incubated under favourable conditions of light and temperature (Tschanz *et al.*, 1975a, 1976; Fisher *et al.*, 1982; Nelson *et al.*, 1983; Burgess *et al.*, 1988).

Conidia formed on PDA are usually variable in respect to shape and size and are not suitable for use in identification of cultures (Burgess *et al.*, 1988). Nelson *et al.* (1983) explained that because of its high available carbohydrate content PDA generally emphasises mycelial growth to the detriment of sporulation. Cultures grown on PDA sporulate poorly, frequently taking more than a month to do so, and the conidia produced are often misshaped and atypical (Nelson *et al.*, 1983). Consequently, with very few exceptions such as microconidia of species in the Section Sporotrichiella, PDA cultures are not used for microscope observation. Mutations are also enhanced when *Fusarium* spp. are grown on PDA (Nelson *et al.*,

1983; Burgess *et al.*, 1988). However, colony morphology, pigmentation and growth rates of *Fusarium* spp. on PDA are reasonably consistent if the medium is prepared carefully and cultures are initiated from standard inocula and are incubated under standard conditions. In these circumstances, colony characteristics are useful secondary criteria for identification (Burgess *et al.*, 1988).

2.2.6 Selective media

According to Agarwal and Sinclair (1987) selective media which inhibit most fungi (except *Fusarium*) and bacteria and allow even slow growing Fusaria to grow out of the seeds have been used in seed health testing. Some of these include Oxgall-PDA (de Tempe, 1973) for detection of *Fusarium* spp. in wheat; modified Czapeck-Dox agar (Malalasekera and Colhoun, 1969; Agarwal and Singh, 1974) for detection of *F. culmorum* in wheat and for detection of *F. moniliforme* in maize, pearl millet, sorghum and soybean. The disadvantage of using selective media for detection of Fusaria is that colonies which develop from seeds have to be subcultured onto identification media. It is also difficult to choose one selective medium for all *Fusarium* spp. (Hussein, H.M., pers. comm.).

Hussein (1987) plated surface disinfected maize seeds on PDA-D medium (PDA-dichloran) and others on PCNB (peptone pentachloronitrobenzene) medium and incubated the seeds in the dark at 25°C. The plates were examined after 5 and 10 days of incubation, and all *Fusarium* spp. were subcultured onto identification media, ie PDA, CLA and potato sucrose agar (PSA). Neish *et al.* (1983) also plated surface disinfected seeds on PCNB agar and after 5 days of incubation at 25°C the Fusaria growing from the seeds were subcultured onto potato sucrose agar. Sayer and Lauren (1991) plated surface disinfected maize seeds and cereal grains on potato sucrose agar containing chloramphenicol. After 3-5 days of incubation the number of fungi (*Fusarium, Alternaria* and *Penicillium*) were recorded, after which Fusaria were subcultured onto potato carrot agar slopes and the species were then identified by the criteria of Burgess *et al.* (1988). Anderson (1986) and ISTA (1993) have described a lengthy procedure involving the use of an agar medium containing PCNB or blotters

sprayed with a nutrient broth containing PCNB for detection and identification of *F. moniliforme* var *subglutinans* (*F. subglutinans*) from crushed pine (*Pinus* spp.) seeds. In the method described by Anderson (1986) a small part of the colony is examined on a microscope slide to identify the polyphialides, microconidia and macroconidia.

2.2.7 Other media - MA

Rheeder *et al.* (1990) identified *F. moniliforme*, *F. subglutinans*, *F. graminearum* and *Diplodia* spp. on 1.5% malt extract agar. Surface disinfected maize seeds were incubated at 25°C for 5-7 days after which "all cultures that developed from the seeds were identified directly on the plates". Wicklow (1988) examined "microscopically" individual maize seeds on malt extract agar after 6 days of incubation and identified *F. moniliforme* and other fungi. Marasas *et al.* (1981) identified *F. verticillioides* var *subglutinans* (*F. subglutinans*), *F. graminearum*, *F. chlamydosporum*, *F. equiseti*, *F. poae* and other fungi. Surface disinfected seeds were plated and incubated on 1.5% malt extract agar in the dark at 25°C for 5 days. The fungal colonies that developed from the seeds were identified directly and counted. Colonies that could not be identified directly were transferred to PDA for later identification.

2.2.8 Incubation Conditions: Temperature and light requirements

Neergaard (1979) noted that selection of incubation temperature depends largely on the pathogen to be detected as well as the testing procedure. In agar tests for fungi, the incubation temperature must be related to the optimum for mycelial growth, as well as for sporulation. Neergaard (1979) stated that, in general, seeds of temperate species are usually incubated at 20°C.

Toussoun and Nelson (1976) reported that most fungi will grow well in the range of 18-24°C (though *F. nivale* grows best at 5-10°C), and alternating diurnal temperatures are the best (Zacharia *et al.*, 1956), such as 25°C day and 20°C at night (Neergaard, 1979; Nelson *et al.*, 1983; Burgess *et al.*, 1988).

Leach (1962a, 1963a, 1963b, 1964, 1967a, 1967b) demonstrated that many fungi show increased sporulation when subjected to irradiation in the blue and ultraviolet wavelengths of the light spectrum. Many authors, cited by Neergaard (1979), have found that ultraviolet wavelengths present in solar radiation at the earth's surface are almost solely confined to the near ultraviolet range of 300-400 nm.

Chuaiprasit *et al.* (1974) explained that fluorescent black light tubes emit radiation within the range of near ultraviolet (NUV) to visible light (320-420 nm) which gives a much higher proportion of NUV at 360 nm that is thought to be most suitable for routine seed health testing. 360 nm is effective in inducing sporulation without adverse effects even at higher dosages (Leach, 1967a) and a high proportion of the radiation is transmitted through many plastics and certain types of glass, particularly Pyrex (Howell, 1964; Leach, 1967a).

Both light quality and light/darkness cycles markedly influence sporulation of many fungi (Leach, 1962a, 1962b, 1963b). Some light sensitive fungi sporulate when exposed continuously to NUV radiation, but others require subsequent periods of darkness to complete spore production (Leach 1961, 1962).

There is an important interaction of light and temperature which significantly affects sporulation. Formation of conidiophores is stimulated by light (NUV) at relatively higher temperatures, whereas formation of conidia requires lower temperatures and darkness (Neergaard, 1979).

Neergaard (1979) suggested that for many diurnal sporulators, the temperatures critical to the effects of alternating light and darkness lie somewhere between 20°C and 25°C. He also suggested that the sporulation response to alternating periods of light and darkness, particularly when accompanied by high and low temperatures respectively, agrees with the reproductive behaviour of many pathogenic fungi which under natural conditions show a peak of spore dissemination in the morning after a night of spore production.

Light has a marked effect on the morphology of fungi. Snyder and Hansen (1941b) showed that conidia of *Fusarium* of different species produced in light were consistently larger and invariably had more septa than those developed in the dark. Cultures kept in darkness showed consistently greater mycelial growth than those kept in light.

Sporulation and pigmentation of fungal cultures is favoured by light including NUV wavelengths and fluctuating temperatures (Snyder and Hansen, 1941b, 1947; Leach, 1967b). However, Zacharia *et al.* (1956) found that *F. decemcellulare* developed more conspicuous pigments in dark than in light.

Dickson (1923) found that *G. saubinetii (F. graminearum)* in pure culture grew over a wide range of temperatures, namely, 3-32°C, with an optimum for spore germination, vegetative development and sporulation of about 24°C on unacidified, and 28°C on acidified, media. Neergaard (1979) quoting Dickson (1923), Jones (1924) and Tu (1929) reported the approximate peak of optimum and approximate range of optimum for mycelial growth of *F. graminearum* to be 24-27°C and 22-28°C, respectively. Tschanz *et al.* (1976) reported that perithecial production by *F. graminearum* on Carnation Leaf Agar (CLA) medium occurred at temperatures ranging from 15-32°C. There was a sharp decrease in the number of perithecia produced above 28.5°C. Incubation at temperatures between 27-31°C resulted in the production of small, immature, collapsed or aborted perithecia in cultures.

To stimulate sporulation, alternating 12 hour periods of NUV light and darkness during incubation are recommended (ISTA, 1993). The recommended source of light is the "Black Light" fluorescent lamp which has a peak emission of NUV energy at 360 nm (Leach, 1967a, 1971b). Cool, white daylight fluorescent tubes are considered satisfactory for the same purpose (ISTA, 1993). Neergaard (1979) described the appropriate set up for NUV irradiated incubation.

Burgess *et al.* (1988) reported that at the Fusarium Research Laboratory in Sydney, Australia, *Fusarium* cultures are incubated 40 cm below a light bank (75 cm

wide) containing four 40 W Black Light tubes (Philips[R], TL 40W/80 RS F40 BLB) and that all cultures for identification are incubated in an alternating temperature regime of 25°C day/20°C night, with a 12 h photoperiod.

2.2.9 *Fusarium* species identification in pure culture

Due to their capacity for rapid change, and their complexity in culture, identification of *Fusarium* to species has long been difficult and as previously explained, this has led to many taxonomic systems and confusion (Nelson, 1991). Booth (1971) believed that because of this difficulty many plant pathologists approach *Fusarium* spp. with caution or avoid working with *Fusarium* altogether. Nath *et al.* (1970) stated that in routine seed health testing, identification of *Fusarium* to species level presents considerable difficulties and Kruger (1989) stated that "for a plant pathologist the taxonomy and identification of the genus *Fusarium* cause some problems." Liddell (1991) stated that a practical and reliable key to the Fusaria that can be used under most laboratory circumstances is not yet available, although a number of laboratory manuals are available (see Booth, 1971, 1977; Toussoun and Nelson, 1976; Nelson *et al.*, 1983; Burgess and Liddell, 1983; Joffe, 1986; Burgess *et al.*, 1988).

Diagnostic features for *Fusarium* spp. have been described by Booth (1971), Nelson *et al.* (1983), Pitt and Hocking (1985) and Burgess *et al.* (1988). In addition, Nelson *et al.* (1983) provided synoptic keys for identification of the majority of *Fusarium* spp. while Burgess *et al.* (1988) gave keys with guides for identification of common species of *Fusarium* in Australasia.

Although *Fusarium* includes some populations which are quite variable, the identification of most species is not difficult provided consistent and appropriate media and consistent culturing procedures and incubation conditions are adopted and cultures could be identified soon after primary isolation (Pitt and Hocking, 1985; Burgess *et al.*, 1988). Individual cultures must be started from single germinated spores or single hyphal tips (Booth, 1971, 1977; Nelson *et al.*, 1983; Burgess and

Liddell, 1983; Burgess *et al.*, 1988) and should be incubated under standardised environmental conditions (Nelson *et al.*, 1983; Burgess *et al.*, 1988). Use of single spore or hyphal tip culture ensures uniformity and consistency of the colony, ensures pure culture, helps to separate mixed culture and aids in distinguishing cultural variants (mutants) or degenerating cultures.

Burgess *et al.* (1988) summarised the important characters to be used in the identification of *Fusarium* species. They include:

(a) shape of the macroconidia
(b) presence or absence of microconidia
(c) shape and mode of formation of microconidia
(d) nature of the conidiogenous cell bearing microconidia
(e) presence or absence of chlamydospores
(f) colony diameter on PDA after dark incubation for 3 days at 25°C and 30°C
(g) colony morphology on PDA after incubation for 10-14 days at alternating day and night temperatures of 25°C/20°C and a 12 hour photoperiod.

Burgess *et al.* (1988) pointed out that the relative importance of each of these characters used in identification varies between species. The morphology of the macroconidia is the principle diagnostic feature, and the shape of the macroconidia is generally the most important character in the definition and identification of *Fusarium* spp. (Pitt and Hocking 1985; Burgess *et al.*, 1988). Burgess *et al.* (1988) explained that the shape of the macroconidia, and the shape and mode of formation of microconidia, are remarkably consistent characters and are used as primary criteria for identification of *Fusarium* species. The presence of absence of chlamydospores is also a primary criterion for identification of *Fusarium* to species level but is less reliable than the shape of macroconidia and the shape and mode of formation of microconidia. Nelson *et al.* (1983) and Burgess *et al.* (1988) emphasised that morphology and growth rates on PDA are only useful as secondary criteria for identification of *Fusarium* to species level. In addition, Burgess *et al.* (1988) provided a key of diagnostic features of different *Fusarium* spp. in the taxonomic

Section Discolor, which are commonly difficult to differentiate (Table 2.3) and key characters for the identification of *Fusarium* spp. in the other sections (Table 2.4).

Table 2.3 Comparison of key diagnostic features of *F. culmorum*, *F. sambucinum*, *F. crookwellense* and *F. graminearum* Groups 1 and 2.

Species	Colony diameter on PDA[1] 25°C	Central spore mass on PDA slope	Macroconidium (on CLA)		
			General shape	Base of basal cell	Apical cell
culmorum	5.5 - 6.8	+++	Short, stout	Notched	Blunt or slightly papillate
sambucinum	2.4 - 3.5	+++	Short, relatively narrow	Notched	Papillate
crookwellense	5.4 - 6.6	+++	Medium, widened at mid-point	Foot-shaped	Tapered
graminearum Group 1	3.9 - 5.1	+	Medium to long, relatively narrow	Foot-shaped	Tapered
graminearum Group 2[2]	4.7 - 6.1	-	Medium to long, relatively narrow	Foot-shaped	Tapered

[1] Colony diameters determined after 72 hr incubation in the dark, the inoculum being a single germinated macroconidium.

[2] *F. graminearum* Group 2 forms perithecia abundantly on CLA.

Source: Burgess *et al.*, 1988

Table 2.4 Key characters for the identification of some *Fusarium* species in some taxonomic sections.

Section Discolor

F. graminearum : Group 1
(a) The shape of the macroconidium
(b) The absence of microconidia
(c) The colony morphology on PDA
(d) Inability to form perithecia on CLA

F. graminearum : Group 2
(a) The shape of the macroconidium
(b) The absence of microconidia
(c) The colony morphology on PDA
(d) The formation of perithecia on CLA.

F. crookwellense
(a) The shape of the macroconidia
(b) The absence of microconidia
(c) The colony morphology on PDA

F. culmorum
(a) The shape of the macroconidia (being short and stout)
(b) The colony growth rate on PDA (fast growth rate)
(c) The colony morphology on PDA.

F. sambucinum
(a) The papillate apical cell of the macroconidium
(b) The slow growth rate
(c) The colony morphology

Section Liseola

F. moniliforme
(a) Presence of long chains of microconidia produced from monophialides
(b) Absence of chlamydospores

F. proliferatum
(a) Presence of chains of microconidia produced from polyphialides
(b) The chains are often formed in pairs in the shape of a "V"
(c) Absence of chlamydospores

F. anthophilum
(a) The globose ("basketball" shaped) microconidia
(b) Presence of polyphialides
(c) Absence of chlamydospores

Table 2.4 continued

F. subglutinans
(a) Formation of microconidia in false-heads from polyphialides
(b) Absence of chlamydospores

Section Sporotrichiella

F. poae
(a) The abundant globose microconidia formed from urn-shaped monophialides in clusters which resemble a bunch of grapes

F. tricinctum
(a) The shape of macroconidia
(b) The two types of microconidia

F. sporotrichoides
(a) The shape of the macroconidia
(b) The two types of microconidia
(c) The presence of polyphialides

F. chlamydosporium
(a) Abundant comma-shaped microconidia produced from polyphialides

Section Gibbosum

F. equiseti
(a) The shape of the macroconidia
(b) Brown pigmentation of slope cultures
(c) Chlamydospores

Source: Burgess *et al.* (1988)

2.3 MAIZE SEED DEVELOPMENT AND MAIZE CROP DISEASES

2.3.1 Seed development

Aldrich *et al.* (1976) divided maize development into six stages, ie germination and seedling establishment, vegetative development, tassel and ear initiation; flowering, that is pollen shedding and silking (or tasselling to silking - Larson and Hanway, 1977), seed development and maturation, and finally, seed maturity and drying in the field (or dry-down period - Larson and Hanway, 1977).

Pollination and fertilisation occur within 4-10 days after tassel emergence, which may be about 3-5 days after silk emergence (Aldrich *et al.*, 1976). Pollen shading and silking usually take place during the hottest days of the maize growing season and during this period all major vegetative growth of the plant has taken place and metabolic activity of the plant tissues is normally at a peak level.

Tassel and ear initiation stage is the most critical period in the development of the corn plant because of heavy demand for water and nutrients, especially nitrogen, and that conditions during this phase determine the number of ears and kernels per ear (Larson and Hanway, 1977). Water, fertility, competition and light stresses may reduce seed set. Hot, dry weather interferes with pollination and bare cobs may occur (Larson and Hanway, 1977). Moisture promotes pollen germination and growth (Berger, 1962).

Cob, husks and shank development is completed by 14 days after silking, after which starch accumulates in the endosperm. Dry matter accumulation, which is entirely in the kernel by nutrients being translocated from other plant parts, ceases about 50-60 days after silking (Larson and Hanway, 1977).

In studies to show the relationship between seed moisture content and time (days) after pollination to harvest date of maize, Schmidt and Hallauer (1966) divided the maize grain development period into approximately five phases, as follows:

88-75% MC - water blister stage to roasting ear stage

75-50% MC - milky roasting stage through complete dent

50-30% MC - complete dent stage to an approximate average physiological maturity stage

30-25% MC - approximate average physiological maturity stage through a final combination of maturity stage and drying

25-20% MC - a drying stage

Ritchie and Hanway (1982) identified maize seed development stages as blister, milk, dough, dent, and physiological maturity.

(i) Blister stage

Aldrich *et al.* (1976) reported that for the first few days after fertilisation no visible change takes place in the fertilised ear shoot, except that the silks wilt and turn brown. The cob continues to grow and by the time the developing seeds appear as watery blisters the cob has reached its full length and diameter. The blister stage is reached about 10-11 days after fertilisation. The maize embryo is much slower to develop than the endosperm (Watson, 1987). The endosperm starts to develop within 3-5 hours after fertilisation by nuclear division only, but growth continues to maturity by cell enlargement to accommodate the starch and storage proteins synthesised.

(ii) Roasting stage

Within the next two weeks the seeds grow very rapidly and the developing embryo takes shape. At this stage most of the plant's physiological activity is directed towards food storage in the seeds; thus starch formation begins about 2 weeks after fertilisation (Kiesselbach, 1949). By the end of the third week (21 days) after pollination, the seeds are filled with a milky almost fluid substance, high in sugars but containing the beginnings of starch and protein-forming bodies - and this is the "roasting" ear stage (Aldrich *et al.*, 1976). From this time until about the end of the fifth week (35 days), the contents of the seed undergo a marked change - sugars rapidly disappear and are replaced first by gummy dextrins (soft starch) and shortly thereafter by drier starch. The top or crown of the seed is the first area

where hardened dry starch is deposited and by 40 days after fertilisation a definite band can be seen across the seed separating the maturing starchy area from the lower milky region where food storage deposit continues. Dry matter accumulation continues corresponding to loss of moisture and by the end of the 7th week (49 days) the embryo has nearly reached its full size, food storage slows down and seed nears maturity (Aldrich *et al.*, 1976).

(iii) Seed maturity

According to Aldrich *et al.* (1976) by the end of the eighth week (56 days) after pollination, the maize seed usually has reached the greatest dry weight it will have and can be considered physiologically mature. As the starch deposit is completed, the seed becomes harder and gradually becomes fully dented. However, the seed does not reach maximum dry weight until the moisture content is below 35%, ie between 28 and 30-35% (Aldrich *et al.*, 1976). Hybrids differ significantly in the moisture content at which maximum kernel dry weight is obtained (Carter and Poneleit, 1973). Kang and Zuber (1989) state that 95% physiological maturity varies from 53-61 days after silking, while Larson and Hanway (1977) give 50-60 days to physiological maturity.

That physiological maturity is reached when seeds on the ear have achieved maximum dry matter accumulation and that the so-called "black layer" is a good visual indicator to maturity was confirmed by Daynard and Duncan (1969). The black layer is a dark area at the tip of the mature seeds formed by the collapse (outwardly) of seed cells at near the tip of the seed, and its appearance signals that the transport of photosynthate into the seed has stopped because these cells close off the conducting tubes in the pedicel (Aldrich *et al.*, 1976). However, Carter and Poneleit (1973) showed that the black layer can form at moisture contents varying from 15-35%. Aldrich *et al.* (1976) state that an individual ear can be considered to be essentially mature when 75% of the seeds in the central part of the ear have black layer, as from this time onwards maturity of the maize ear and seed is entirely a matter of moisture loss.

2.3.2 Maize seed dry down and harvest time

Maize seed harvest may be started when it is physiologically mature, ie when the kernel moisture is 30-40%, depending on the hybrid (Jugenheimer, 1976), but most authors state that the seed must be left to dry to harvestable moisture level of 21-30% (Delouche, 1980; Hardacre *et al.*, 1991). The rate of drying is reported to depend mainly on the weather and also on the hybrid type (Hardacre *et al.*, 1991). Shaw (1977) and Watson and Ramstand (1987) state that in North America the maturation of maize is more greatly affected by temperature in the Autumn than rainfall, except in very dry years, and that prolonged periods of cloud, alone or combined with abnormally low temperatures, can slow down the maturation process. Schmidt and Hallauer (1966) found that before physiological maturity, seed moisture loss was primarily a physiological process and reported that daily rates of seed moisture reduction varied considerably from year to year within the five phases of seed growth; above 30% seed moisture content the rate of moisture reduction was significantly related to the temperature of the air while below 30% seed moisture reduction rate was significantly related to saturation deficit, wet bulk depression and relative humidity of the air. The greatest variability in the average daily rate of seed moisture reduction occurs in the 30-20% phase (Schmidt and Hallauer, 1966). The maize seed itself dries from the crown downwards so that the part nearest the cob is highest in moisture during most of the drying period - thus the cob has a higher moisture than the grain during maturation and drying (Koehler, 1942).

Maize seed is harvested on the ear/cob by hand or machine (Zuber and Darrah, 1987) in order to facilitate sorting of moulding or insect damaged cobs (Naik *et al.*, 1982) or cobs which are not true to type or cultivar (Thomson, 1979). The other reason for harvesting on the cob is to avoid mechanical damage of wet seed, especially when it has higher than 30% moisture content (Thomson, 1979).

Larson and Hanway (1977) state that seeds to be dried in cribs should be harvested at 20-25% moisture. High moisture seed has to be promptly dried down artificially to safe storage moisture content of 13-14% (Chappell, 1985) in order to

avoid spoilage by invasion of storage fungi (Christensen and Sauer, 1982) and continuation of growth by *Fusarium* spp. in the ears/cobs (Sutton, 1982; Kruger, 1989; Lacey and Magan, 1991).

Since artificial drying of wet harvested seed or grain is costly (Hardacre *et al.*, 1989, 1991), and because dryer capacity may limit the amount of maize to harvest at a given time (Jugenheimer, 1976), maize seed is often left in the field to dry naturally (Aldrich *et al.*, 1976). The other cause of delayed harvest of maize is that wet weather may prolong the field drying period for ears and make paddocks too wet and difficult for mechanical harvesting (Hardacre *et al.*, 1989, 1991). However, while early harvest reduces risks of abnormally low temperatures and frost which damage seeds by reducing germination (Rossman, 1949), too early harvest of immature seeds also results in low seed viability and germination (Austin, 1972).

2.3.3 Pathogens and diseases of maize crops

There are about 120 pathogens causing significant diseases of maize worldwide (Shurtleff, 1980). These include fungi, bacteria, viruses, mycoplasmas and nematodes. These diseases have been classified (McGee, 1988) into four categories, namely, those which are seed-borne only, those seed-borne and seed transmitted, those disease whose seed-borne and pathogen transmission aspects have not been recorded and, finally, pathogens which infect maize by inoculation only. Neergaard (1979) and Richardson (1990) have reviewed seed-borne pathogens of maize while the significance of these pathogens, including the production of mycotoxins, has been reviewed by Neergaard (1979), Shurtleff (1980), Nelson and Cook (1981), Sutton (1982), McGee (1988), Kruger (1989) and Teich (1989). Williams and McDonald (1983) have summarised the damage caused by field fungi to maize grain and Shurtleff (1980) has discussed those pathogens that cause stalk rot of maize. In New Zealand, Fowler (1985) states that twenty eight pathogens of maize have been recorded on maize, but only four diseases (some caused by more than one pathogen) are of significant economic importance, ie northern leaf blight, head smut, stalk rot,

and ear and root rots. Diseases of maize in New Zealand are summarised in Table 2.5.

Table 2.5 Diseases of maize in New Zealand.

Pathogen	Disease
Fungi	
Ascochyta sorghi	Leaf spot
Aureobasidium zeae (= Kabatiella zeae)	Eye spot
* *Botrytis cinerea*	Ear rot
* *Chaetomium* sp.	Seedling root rot
Colletotrichum graminicola	Leaf spot
Cochliobolus heterostrophus (= Drechslera maydis)	Southern leaf blight
Diplodia maydis (= Diplodia zeae)	Stalk rot, ear rot
Fusarium avenaceum	Seedling root rot
F. culmorum	Seedling root rot
F. moniliforme (= Gibberella fujikuroi)	Stalk, ear, sheath, and seedling root rot
F. oxysporum	Root rot, adult plants Seedling root rot
F. roseum	Root rot, adult plants
F. solani	Seedling root rot
Gibberella zeae	Stalk rot, root rot
Nigrospora oryzae (= Khuskia oryzae)	Stalk rot, ear rot
* *N. sacchari*	Kernel blemish
* *Periconia macrospinosa*	Seedling root rot
Puccinia sorghi	Rust
Pythium afertile	Seedling root rot
Pythium monospermum	Seedling root rot
Rhizoctonia solani (= Pellicularia filamentosa = Thanatephorus cucumeris)	Root rot
Sclerophthora macrospora	Crazy top
Setosphaeria turcia (= Drechslera turcia, Helminthosporium turcicum, Exserohilum turcicum)	Northern leaf blight
Sphacelotheca reiliana	Head smut
Trichoderma koningii	Seedling root rot
Bacteria	
Erwinia chrysanthemi pv. zeae	Stem rot
Virus	
Maize dwarf mosaic virus	Mosaic, stunting
Maize leaf fleck virus	Leaf speck

* Pathogenicity not confirmed.

Source: Fowler (1985).

2.3.4 Maize diseases caused by *Fusarium* and their epidemiologies

Literature on *Fusarium* maize diseases is discussed under various names. Shurtleff (1980) groups *Fusarium* maize diseases under 4 categories, viz., Gibberella stalk rot, Fusarium stalk rot, Gibberella or red ear rot, and Fusarium kernel or ear rot. Gibberella stalk and Gibberella ear rots are caused by *Gibberella zeae* (Schw.) Petch (perfect state, also known as teleomorph of *Fusarium graminearum* Schwabe.), while Fusarium stalk rot or Fusarium kernel or ear rot are caused by *Fusarium moniliforme* Sheld (asexual state of *Gibberella fujikuroi* (Saw.) Wr. and *Fusarium subglutinans* Wr. and Reink. McGee (1988) reviewed and summarised seed-borne diseases of maize caused by *Fusarium* species and discussed them under three major names, viz., Gibberella ear and stalk rot, Fusarium stalk and ear rot, and Fusarium stalk and root rot (Table 2.6).

2.3.4.1 Root rots

According to Shurtleff (1980) no clear demarcation exists between seedling blight and root rot and many organisms attack both stem and root tissue. Root rots of maize are widely distributed but do not appear to cause loss except in special situations (Shurtleff, 1980). Infection of the roots takes place throughout the whole season and always increases up to harvest (Kruger, 1989). Early season root rots occur in soils where oxygen is deficient due to poor drainage or compaction and are due mainly to *Pythium gramicola* and late season root rots such as those associated with *Fusarium* spp. increase with maturity, senescence of root tissues and field stress, and root rot is reported to commonly be a prelude to stalk rot (Shurtleff, 1980).

Referring to work done in USA, South Africa, France and Germany, Kruger (1989) states that in warmer districts *F. moniliforme*, *F. oxysporum*, *F. graminearum* and occasionally *F. equiseti* are the most common species associated with root rot. In cooler districts, *F. culmorum* predominates, followed by *F. subglutinans* and *F. oxysporum* and sometimes *F. crookwellense*, *F. solani*, *F. merismoides*, *F. sporotrichoides*, *F. tricinctum*, *F. poae* and *F. sambucinum*. *F. graminearum* which occurs mainly on stalks is less frequently isolated from roots in moderate climates (Kruger, 1989).

Table 2.6 Names of diseases of maize caused by *Fusarium* species.

Common disease name	Gibberella ear and stalk rot	Fusarium stalk and ear rot	Fusarium stalk and root rot
Other disease names	Gibberella stalk rot Gibberella ear rot Red ear rot Pink ear rot	Fusarium stalk rot Fusarium ear rot Fusarium kernel rot	Fusarium stalk rot Fusarium root rot Fusariosis
Pathogen(s)	*Gibberella zeae* *G. saubinetti* *Fusarium roseum f.* sp. cerealis *F. roseum* 'Graminearum' *F. graminearum*	*Fusarium moniliforme* *F. moniliforme var. subglutinans* *F. verticilloides* *F. sacchari var. subglutinans* *Gibberella moniliformis* *G. fujikuroi*	*Fusarium culmorum* *F. semitecum* *F. tricinctum* *F. sporotrichioides* *F. poae* *F. equiseti* *F. solani* *F. oxysporum* *Fusarium* spp.

Constructed from McGee (1988)

2.3.4.2 Stalk rots

Stalk rot is a complex problem involving many plant pathogenic and non-pathogenic fungi and bacteria (Dodd, 1980). Stalk rots caused by *Fusarium* spp. are divided into two separate diseases, namely, Fusarium stalk rots caused by *Fusarium moniliforme* and *F. subglutinans*, and Gibberella stalk rot caused by *F. graminearum* (Shurtleff, 1980; Kommedahl and Windels, 1981; Burgess *et al.*, 1981). McGee (1988) also recognises a third category of stalk rot, ie that caused by other Fusaria.

Throughout the world, *F. graminearum* and *F. moniliforme* (including *F. subglutinans*) are the most frequently reported causes of stalk rot of maize caused by *Fusarium* (Burgess *et al.*, 1981; Cassini, 1981). Other Fusaria have been found associated with stalk rots (McGee, 1988). The probable order of importance is *F. graminearum*, *F. moniliforme*, *F. subglutinans*, *F. culmorum*, *F. oxysporum*, *F. avenaceum* and *F. equiseti* (Kruger, 1989). Generally basal or crown rot of corn is attributed to *F. graminearum* and *F. moniliforme* (Christensen and Wilcoxson, 1966) whereas nodal stalk rot is caused by *F. moniliforme* (Roane, 1950).

Other fungi known to cause stalk rot include *Diplodia zeae*, *Rhizoctonia bataticola*, *Colletotrichum graminicola*, *Phaeocystostoma amibiguum* (Shurtleff, 1980). Kruger (1989) regards *D. zeae* as the most important stalk infecting fungus of the non-Fusarium species.

Stalk rot is reported to be a disease of maturity and senescing plants (Cassini, 1981). Dickson (1956), Maric (1981) and Burgess *et al.* (1981) state that stalk rot becomes evident after flowering, and during the milk and wax stages of seed formation (Maric, 1981). Koehler (1960) found *F. moniliforme* and *F. graminearum* at flowering and after flowering, respectively. *F. moniliforme* was frequently isolated from symptomless corn stalks (Windels and Kommedahl, 1984). The stalk rot syndrome typically starts with permanent wilting of the plant and is followed by the rotting of the pith tissue in the lower internode and finally the collapse of the plant (Dodd, 1980).

In *Fusarium* infection, the rotten pith is pinkish or reddish and the red colour distinguishes this infection from that due to *Diplodia* (Jones and Clifford, 1983). Insects (eg European corn borer) may aid in stalk rot development by spreading inoculum, and early maturing hybrids are more susceptible to stalk rot than full-season hybrids (Christensen and Wilcoxson, 1966).

The significance of stalk rots of corn are that grain yields are reduced because wilted and rotted plants lodge. As a result, cobs may be missed during harvesting, and harvesting lodged plant crops also slows down the machine harvesting rate (Dodd, 1980). In addition, seeds on lodged plants are prone to attack by soil organisms and are likely to dry more slowly. This situation encourages cob rot fungi to continue colonisation (Shurtleff, 1980; Fowler, 1985).

2.3.4.3 Cob rot/ear rot caused by Fusaria

Information on maize cob rot has been reviewed by many authors, but most recently Teich (1989), Kruger (1989) and Chelkowski (1989). Cob rot is a worldwide problem (Kruger, 1989). Most authors state that *Fusarium* species which commonly cause ear rot are *F. graminearum* Schwabe, *F. moniliforme* Sheldon and *F. moniliforme* var *subglutinans*/*F. subglutinans* (see Teich, 1989). *F. graminearum* is considered to be the most important *Fusarium* species pathogenic to maize (Visconti *et al.*, 1990).

F. graminearum is reported to be the main *Fusarium* species on cereals and maize in warmer regions such as the USA corn belt, California, eastern Australia (Cook, 1981), Southern Europe (Italy, Spain, Yugoslavia, Austria, France) (Manninger, 1979) and the Republic of China (Wu *et al.*, 1984). *F. culmorum* is the main species in temperate regions, such as the Prairie and Maritime provinces of Canada, the northern states of the USA, Northern Europe (the UK, Finland, Poland, the Netherlands, Hungary, Germany) (Cook, 1981). *F. moniliforme* is found in humid, semi-humid and sub-humid temperate climates, tropical zones (Teich, 1989) and sub-tropical regions, whereas *F. subglutinans* is isolated more frequently from

temperate and cool areas (Booth, 1971; Marasas *et al.*, 1979; Burgess 1981; Kruger, 1989).

Other Fusaria which have been isolated from diseased cobs include *F. poae*, *F. proliferatum*, *F. crookwellense*, *F. avenaceum*, *F. tricinctum*, *F. sporotrichoides*, *F. sporotrichiella*, *F. equiseti*, *F. semitectum* and *F. solani* (Cassini, 1981; Maric, 1981; Chelkowski, 1989).

2.3.4.4 Symptoms of cob rot caused by *F. graminearum*

Symptoms of cob rot caused by *F. graminearum* have been described by Shurtleff (1980), McGee (1988) and Reid *et al.* (1992a) as being associated with a pinkish to red mould on the cob or seeds. In severe infection or in early infected cobs, the husks commonly adhere to the seeds. The disease normally starts from the tip of the cob and spreads downwards towards the butt of the cob; the extent of spread depending on weather conditions and the time of infection (Dickson, 1956), but usually does not progress further than halfway down the ear (Jones and Clifford, 1983). The shank or ear stalk may also be infected. Ullstrup (1977) reported that infection may spread upwards from the shank and Burgess *et al.* (1981) reported that rotting may start from the butt. Kruger (1989) states that "*F. graminearum*, *F. culmorum* and *F. poae* infect either from the tip or from the bottom of the cob growing from the point of entry over the whole surface forming a red coat but when *F. poae* is involved, a whitish coat is produced". The red colour of mycelium distinguishes cob rot due to Fusaria from that due to Diplodia (Jones and Clifford, 1983). Sutton *et al.* (1980b) and Sutton (1982) state that cob rot caused by *F. graminearum* is often severe in the basal half of the ear in which husks have been shredded or opened by birds. Cob rot also develops around channels and tunnels made in the ears by various insects, including the European corn borer (*Ostrinia umbilalis* Hbn) and the corn worm (*Heliothis zeae* Boddie) (Christensen and Scheider, 1950; Koehler, 1959).

Perithecia of *Gibberella zeae* (Schw.) Petch may develop superficially on infested husks, kernels or stalks, especially during prolonged wet weather (Shurtleff,

1980; Sutton, 1982) but only in severe cob rot cases (Edwards, 1936). Early invasion of the shank by *F. graminearum* may result in premature ear/cob drop and poorly filled grain (Sutton, 1982).

2.3.4.5 Symptoms of cob rot caused by *F. moniliforme* and *F. subglutinans*

The symptoms of the cob rot caused by *F. moniliforme* and *F. subglutinans* are reported to be similar (Shurtleff, 1980; McGee, 1988; Kruger, 1989). This type of cob rot is called Fusarium kernel rot or ear rot (as opposed to Gibberella cob rot (Shurtleff, 1980). Dickson (1956) states that Fusarium kernel rot is "typically a rot of individual or groups of kernels" and Jones and Clifford (1983) refer to the symptoms as randomly scattered groups of kernels. McGee (1988) states that a cotton-pink mould forms on areas of the ears or on scattered seeds causing kernel rotting. However, Chelkowski (1989) reported that *F. subglutinans* and *F. culmorum* were isolated from cobs displaying *F. graminearum* symptoms. Also, Burgess *et al.* (1981) state that *F. subglutinans* and *F. moniliforme* may cause a generalised ear rot. Chelkowski (1989) reported a generalised cob tip rot due to *F. subglutinans*.

The colour of the rotted kernel is pink to reddish brown or grey depending upon the general prevalence of mycelium of the fungus, weather conditions (Dickson, 1956) and host genotype (Jones and Clifford, 1983). Chelkowski (1989) describes the disease as pink ear rot or pink fusariosis, whereas Kruger (1989) describes it as pale pink or reddish and, Jones and Clifford (1983) as whitish pink lavender or reddish brown. Shurtleff (1980) states that a salmon-pink discolouration appears on the caps of individual seeds or groups of seeds scattered over the ear. As the disease progresses, a powdery or cottony-pink growth develops on the infected seeds. Some seeds infected by *F. moniliforme* may have white streaks (Koehler, 1942, 1959; McGee, 1988), especially those infected late in the season (Shurtleff, 1980). The fungus commonly establishes around channels made by insects such as corn worm or borers or popped seeds with growth cracks (Koehler, 1959).

2.3.4.6 Multiple cob rot infections

Difficulties may occur in identifying pathogens by cob symptoms. Chelkowski (1989) reported that *F. subglutinans* caused damage to the ear similar to that by *F. graminearum* and/or *F. culmorum.* Chelkowski (1989) also reported that differentiation of red fusariosis caused by *F. graminearum*, *F. crookwellense*, *F. avenaceum* and *F. culmorum* "was possible sometimes before harvest, looking to the colour of the infected area of the cob and character of mycelium covering damaged seeds. Seeds infected by *F. graminearum*, *F. culmorum*, *F. crookwellense* and *F. sporotrichoides* were shrunken, covered by mycelium and were coloured red but those infected by *F. subglutinans*, *F. moniliforme*, *F. proliferatum* were white to pink. Chelkowski (1989) warned against the importance of prevalence of *F. graminearum* in old literature because:

a) *F. graminearum* and *F. crookwellense* both occurred in red coloured cobs.

b) *F. graminearum* looks almost like *F. crookwellense* in microscopic examination of morphological characters and mycelium on PDA.

c) *F. crookwellense* was only described/appeared in manuals on Fusaria taxonomy in 1983 (Nelson *et al.*, 1983) and probably previously was misidentified as *F. graminearum* or *F. culmorum*; after all, perithecia of *Gibberella* were not observed in Poland.

2.3.5 Seed infection and pathogen establishment in seed

The establishment of seed infection is governed by factors relating to the following conditions (Neergaard, 1979):

1. The growth stages of the seed crop, particularly those pertaining to the seed primordium (ovulum) from the beginning of the flowering and the moment of fertilisation until maturing of the seed.

2. The weather conditions which may be critical as to whether or how a given pathogen gets into contact with the seed primordium, spreads and gains foothold within its tissues.

3. The time of infection, as related to weather conditions in the period during which the coincidence of pathogen and host may happen to occur.

4. The paths of infection through which a given pathogen may enter the seed.

5. The affected parts of the seed or fruit, ie localisation of the infection being dependent on the time of infection and the weather conditions during the development of infection.

2.3.6 Time of harvest and fungal invasion : factors influencing the effects of *Fusarium* spp. on maize seed infection and quality

2.3.6.1 Stage of seed maturity, cob infection and seed penetration

Factors associated with the onset of seed development (ie silking and pollination) are associated with the onset of seed infection by *Fusarium* spp., especially *F. graminearum*, *F. moniliforme* and *F. subglutinans* (Edwards, 1936; Koehler, 1942; Sutton, 1982). Koehler (1942) and Hesseltine and Bothast (1977) studied the progression of natural infection of corn seeds by field fungi including *Fusarium* spp. and found that invasion of seeds increased as the seeds matured.

Neergaard (1979) states that the time of invasion of fungi at different stages in the development of the seed primordium and maturing seed determines the fate of the seed and the ultimate location of inoculum within the tissue of the mature seed. Neergaard (1979) also states that certain parasitic fungi belonging to the Fungi Imperfecti are pathogenic to flowers and ovules and young seed primordia but loose their infectivity as seed approaches maturity. *F. graminearum* and *F. moniliforme* in maize were given as some of the examples. Neergaard (1979) explains that if invasion takes place at the early stages, the seed is killed (seed rot diseases) but if

invasion takes place when the seed is nearing full maturity, only the seed coat or pericarp is invaded; often only the epidermis layer being invaded. At later stages of the development of the seed primordium or caryopsis, Neergaard (1979) and McGee (1986) explained that the "morphological barriers" within the seed may hinder penetration of the hyphae into deeper layers; for example in wheat caryopses such barriers are the endocarp, and the still deeper layer of the integuments, which may protect the seed from *F. culmorum.*

Edwards (1941) found in studies to determine establishment of internal infection in nearly mature ears of maize and to determine the relationship between time of inoculation and incidence of internal seed-borne infection that there was little, if any, internal grain infection with *G. fujikuroi* (*F. moniliforme*) or *G. fujikuroi* var *subglutinans* (*F. subglutinans*) in mature ears of maize, even under conditions highly favourable for fungus growth.

Field infection experiments showed that a high incidence of internal grain infection due to *G. fujikuroi* and *G. fujikuroi* var *subglutinans* could be established by inoculating the ears at all stages of maturity from the time of pollination up to the nearly mature stages but the highest incidence of infection was obtained by inoculating the ears between pollination and the dent stage of development (Edwards, 1941). Kernel rot infection rarely developed unless the grain had been injured.

2.3.6.2 Infection spread in the ear and seed penetration

Koehler (1942) found that nearly all fungi in maize ears entered by the channel through the husks at the tip of the ear and that *F. graminearum* infection originated from the tip of the ear and progressed down most rapidly in the region of the silks and it was half way down the ear before it caused visible rot. Ears found with infection on the butt half were extensively damaged on the tip half, although in a few of them infection apparently started from the butt. Edwards (1936) found that although *G. saubinetii* (*F. graminearum*) infection occurred at the tip of the ear, only about one third of the grains on the cob were rotten. With *F. moniliforme*, the seeds became contaminated at the point of contact with the silks and the infection spread

to all parts of the cob including the shank, but internal infection in sound appearing kernels was not established until the ears were approaching maturity. However, Koehler (1942) found that as the season advanced, the incidence of *F. graminearum*, *F. moniliforme*, *Cephalosporium acremonium*, *Nigrospora* spp., *Diplodia zeae* and *Penicillium* spp. progressively increased from the butt tissue of the cob, suggesting entry of these fungi, especially *Diplodia zeae* and *Nigrospora* spp., through the butt end of the cob.

Koehler (1942) stated that environmental conditions and stage of development of the ears are important factors determining the ability of fungi to overrun ears and penetrate into kernels. In classifying the fungi entering at the tip of the ears to their relative ability to penetrate into seeds, so that the fungus persists after surface sterilisation, the ranking, in descending order would be: *Diplodia zeae*, *G. zeae*, *Nigrospora* spp., *F. moniliforme*, *Cephalosporium acremoniunm* and *Penicillium* spp. The same order was said to apply for ability to cause rot, except that in this case *C. acremonium* would come last.

Koehler (1942) found that pericarp infection takes place before complete maturity and observed that while entry into very young seeds may apparently be effected anywhere through the pericarp and testa by *Gibberella zeae* as the seeds develop, the testa offers increasing resistance and finally the fungus is limited to entry via the tip cap. He also found that pericarp damage was necessary for *F. moniliforme* which only penetrated immature or damaged seeds when growth cracks (silk cuts) had occurred.

When entry is via the tip cap after denting has occurred, the fungus may pass into the pericarp and the internal tissues but apparently cannot pass through the testa (under the pericarp) (Koehler, 1942). Pericarp infection by *F. moniliforme*, *C. acremonium* and *Nigrospora* spp. can usually be diagnosed by the presence of white, pink or dark streaks (see Koehler, 1959) and it is found most often when ears dry or mature slowly, ie in lodged corn when the outer husks are in contact with the ground or in standing corn when protracted wet weather occurs during the final maturing

period (Koehler, 1942). *F. subglutinans* also forms streaks (Edwards, 1936). Koehler (1942) explained that the white colour of streaks is due to the disintegration of cells (by the infection) which causes them to lose their transparency and take on a chalky (starch) appearance. Fissures in the pericarp coincided with streaks (Koehler, 1942).

Manns and Adams (1923) and Koehler (1942) found that inoculum in the pericarp can be easily "killed" by ordinary surface disinfection, but inoculation in the tip cap and internal parts persists. Manns and Adams (1923) found that surface disinfection of seeds was not effective in removing the inoculum from the tip cap because the tissues of the cap could not soften materially even with prolonged presoaking.

2.3.6.3 Site of internal seed inoculum and seed germination

Investigating the manner in which inoculum is carried in seeds showing no external symptoms, Manns and Adams (1923) found that normal appearing seeds and ears may be infected. They found the mycelium of *G. saubinetii* (*F. graminearum*), *F. moniliforme*, *Cephalosporium sacchari* Butler (*F. subglutinans* - Booth, 1971) and *Diplodia zeae* established in the tissues of the seed tip cap and in the cavity between the black layer and the dorsal point of the scutellum where *F. moniliforme* and *F. subglutinans* formed abundant spores. Manns and Adams (1923) concluded that the development of the fungi from the cap into the germ and endosperm apparently is restricted by the black layer. Johann (1935) found that the black layer was rather impervious to *Diplodia* penetration once it had formed but that in the earlier stages of seed development this protection was lacking. Branstetter (1927) working with surface disinfected "sound-appearing" seeds, also found that in 31% of the kernels, infection occurred in this area. Koehler (1942) examined surface disinfected ears of Reid yellow dent maize, with or without external symptoms on the seeds for *F. moniliforme*, *D. zeae*, *Nigrospora* spp., *Cephalosporium acremonium* and *Penicillium* spp.. They were most common in the tip cap, the next sites in decreasing order of infection prevalence being tissues of the embryo, flour endosperm and the horny endosperm.

Koehler (1942) found that after entering the interior of the seed, *F. moniliforme*, *D. zeae*, *C. acremonium* and *Nigrospora* were all found more often in the embryo than in the soft or horny endosperm. Manns and Adams (1923) found that in some instances *G. saubinetii*, *F. moniliforme* and *C. sacchari* (*F. subglutinans*) grew upwards underneath the pericarp and under favourable conditions during germination, the fungi killed the embryo or attacked the seedlings and that the roots ("feeders"), scutellum, and mesocotyl ("epicotyl") were the first to show the symptoms. By germinating maize seeds in a rag doll, Manns (1923) found that cap mycelial growth appeared in 5 days and retarded or inhibited germination but when the cap was removed germination was increased and seedlings were not infected and it was further established that after surface disinfection of seeds and removal of the cap, perfect germination occurred in many instances.

Seeds from the tip of the ear are often rotted (Manns and Adams, 1923; Sutton, 1982) and the embryo of seeds from such ears is already destroyed (Edwards, 1936).

Kruger (1989) found that if one third of a cob (ear) was covered with mycelium, the adjacent one third was badly damaged and only the last one third showed slight infection, adding that seed from some distance away from the mycelium covered part can be dead. However, Manns and Adams (1923) found that maize seeds showing no external disease symptoms of *G. saubinetii* (*F. graminearum*) and *C. sacchari* (*F. subglutinans*) have high germination. A sample of kernels with 100% internal infection of *F. moniliforme* and 100% *C. sacchari* (*F. subglutinans*) gave 100% germination and produced very strong seedlings using the rag doll germination method. Similarly, a sample with 100% *C. sacchari* (*F. subglutinans*), 40% *G. saubinetii* (*F. graminearum*) had 100% germination.

2.4 THE LIFE CYCLE OF *F. graminearum*

2.4.1 Seed to plant transmission, establishment of infection and course of disease by fungi

Transmission of seed-borne organisms and disease establishment in the next crop has been discussed by Neergaard (1979). An organism (fungus or bacterium) or virus, may be seed-borne and yet not seed transmitted. The establishment of a pathogen in or with the seed implies that the pathogen is seed-borne; the transference of the seed-borne pathogen and establishment of infection in plant(s) from the seed implies that the pathogen is seed transmitted.

Seed-borne fungi may be carried with the seed in principally two different ways: the seed may be contaminated (infested), ie the pathogen may be carried adhering to the surface of the seed, as spores or the pathogen may be mixed with the seed, for instance as sclerotia or as fruiting bodies inside or on the surface of plant debris; or the seed may be infected, the pathogen having penetrated into the tissues of the seed, and often established in a resting stage as dormant mycelium.

Seed contamination is in most cases brought about by mechanical dissemination of the inoculum, such as during harvesting and processing operations, during transportation or may have taken place during seed maturation by wind, water or insects. However, factors for the establishment into seeds include physiological conditions pertaining to the pathogen, host and weather, all which must "work together" to bring about the infection (Neergaard, 1979).

2.4.2 Transmission of *F. graminearum*

2.4.2.1 Source of inoculum

Host debris is the principal source of inoculum, other sources being soil, seeds and debris of various susceptible plants such as wheat and barley (Sutton, 1982; Khonga and Sutton, 1988). *F. graminearum* survives longest on the soil surface

(Khonga and Sutton, 1988). When stems and ears of maize, and stems, spikelets and grains of wheat infested with *Gibberella zeae* were buried or placed on the soil surface, or suspended above the soil surface in the field, Khonga and Sutton (1988) found that the inoculum survived for up to 3 years in residues kept above or on the soil surface, but was not recovered after this time from residue which was buried. Burgess and Griffin (1968) recovered *F. graminearum* from colonised wheat straws up to 104 weeks after burying them in the field in Australia and observed that frequent wetting reduced the survival period to about 52 weeks or less. Burgess and Griffin (1968) also observed that *F. graminearum* survives longer in tissues that resist breakdown, eg in nodal than in internodal tissue of wheat.

In Minnesota, Warren and Kommedahl (1973) noted that *F. graminearum* overwintered on wheat straw but the proportion of straws that was colonised declined from 40 to 24% during winter.

F. graminearum was not detected in several hundred soil samples from cereal fields in Canada (Gordon, 1954, 1956, as cited by Sutton, 1982) and was found in only 30% of samples from maize fields in Minnesota (Windels and Kommedahl, 1974) indicating that soil *per se* probably is not a major inoculum source (Sutton, 1982).

Infected maize or wheat seed may give rise to diseased seedlings when used as seed (Atanoff, 1920; Gordon, 1944; McKay and Loughnane, 1945; Halfon-Meiri *et al.*, 1979). In some regions, particularly South Ontario, infected wheat or barley crops at heading stage have been considered potential sources of inoculum of *F. graminearum* for maize in nearby fields (Sutton, 1982). However, Teich (1989) noted that although soil, seeds and infected crops have been suspected of being inoculum sources, adequate evidence is not yet available to support this supposition. McGee (1988) noted that transmission of *F. graminearum* from seed to seedlings has not yet been clearly demonstrated.

2.4.2.2 Inoculum types and their dispersal

According to Sutton (1982), the inoculum includes ascospores, macroconidia, chlamydospores and hyphal fragments. However, ascospores and macroconidia are considered to be the main inoculum (Shurtleff, 1980; Sutton, 1982). Warm humid conditions favour formation of perithecia (Tschanz *et al.*, 1976). Christensen and Wilcoxson (1966) stated that perithecia form on maize stalks and stubble usually in summer and early autumn but that they develop on plant refuse virtually throughout the growing season and, in the northern regions of the USA, perithecia usually do not mature until moist cool weather prevails in the spring. Ascospores and macroconidia may infect wheat at anthesis and maize ears at the silking stage (Anderson, 1948; Sutton, 1982).

Field observations have revealed that ascospore discharge is at night (Ye, 1980; Sutton, 1982) when relative humidities of 95-100% and temperatures of 13-22°C prevail (Ayers *et al.*, 1975). Studies in controlled environments have confirmed that cool to moderate temperatures (about 11-23°C) with an optimum near 16°C favour ascospore discharge (Tschanz *et al.*, 1975b, 1976). Wind has been considered the primary transport mechanism for ascospore dispersal (Atanoff, 1920), although splashing or wind-driven rain has been regarded as the principal dispersal mechanism for macroconidia of *F. graminearum* in view of their sticky and hydrophillic nature (Sutton, 1982). However, this supposition appears to lack support by critical experimentation (Sutton, 1982).

Sutton (1982) has proposed the life cycle for cob rot caused by *F. graminearum* to be as the model described in Fig. 2.3

2.4.2.3 Infection and colonisation of maize ears

Susceptibility of maize to infection by *F. graminearum* is greatest shortly after silks have emerged and declines thereafter (Sutton and Baliko, 1981; Reid *et al.*, 1992b). Ear rotting epidemics are usually completed in one cycle because maize ears are highly receptive for only 10-20 days following silking (Sutton, 1982).

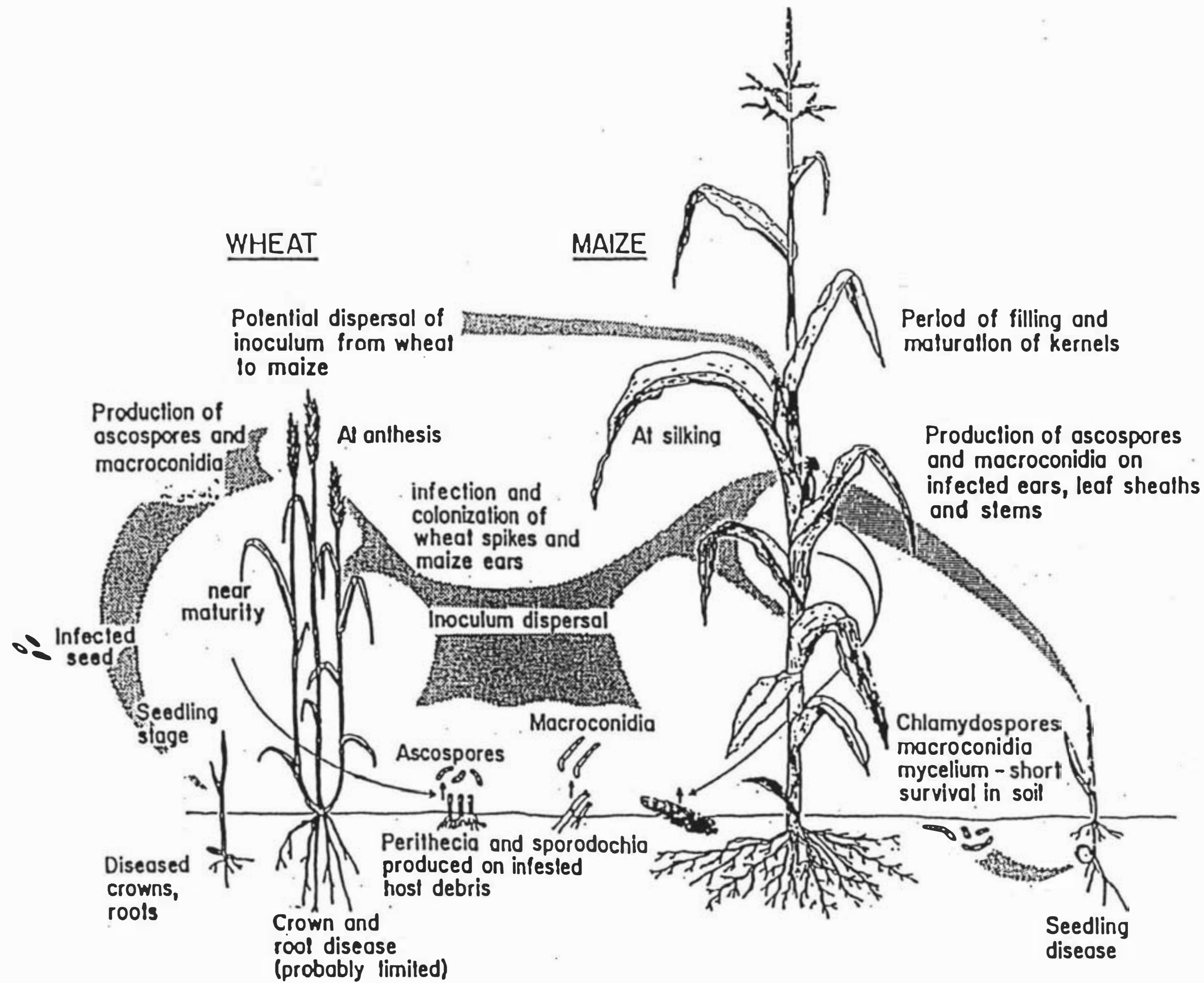


Figure 2.3 Disease cycle of *F. graminearum* in maize (Sutton, 1982).

Silks and anthers, maize pollen and dead senescent floral bracts are potential substrates for the establishment of *F. graminearum*. Several authors have suggested that infection of ears occurs through and around the silks when they senesce after pollination (Dickson, 1956; Naik and Busch, 1978; Reid *et al.*, 1992a, 1992b). Infected silks are thought to provide a source of energy for growth of *F. graminearum* and invasion of kernels and cobs. Reid *et al.* (1992b) found that silk degradation (susceptibility) decreases as silks age. Naik and Busch (1978) examined the effects of pollen on *F. graminearum* and observed that pollen stimulated conidial germination, tube growth and colonisation of detached silks. Pollen also stimulated infection of leaf tissues and stem nodes. It is thought that choline and related compounds in pollen promote infection in maize as has been found in wheat (Naik and Busch, 1978). Insects and birds are vectors (Sutton, 1982). Cob rot infection caused by *F. graminearum* is reported to be epidemic and destructive but occurs sporadically (Suttton and Baliko, 1981; Sutton, 1982; Hunter *et al.*, 1986).

Rainfall is a key factor in the outbreak of ear rot, but the relationship of rainfall and the infection are not entirely clear (Sutton, 1982). The rot is considerable in humid areas, especially when rainfall is above normal from silking to harvest (Shurtleff, 1980). Koehler (1959) in Illinois, noted that ear rot intensity correlated strongly with combined rainfall of August and September. In Yugoslavia, Maric *et al.* (1969) and Milic *et al.* (1969) concluded that heavy rainfall in August was a key factor in ear rot epidemics. Tuite *et al.* (1974) in Indiana observed that colonisation of maize ears was associated with rainfall for at least 7 days during the 21-day silking period. In Northern Ohio, high September rainfall was thought to favour *Fusarium* growth because persistent wetness for at least 48 hours favoured ear rot development (Teich, 1989). In Ontario, Sutton *et al.* (1980a) found that the incidence of zearalenone in maize kernels correlated strongly with rainfall during August when silking generally occurs, but not with rainfall in July, September or October. Zearalenone was infrequent in years when August rainfall totalled < 60 mm and fell on 10-16 days, but frequent when a total of > 70-80 mm rain occurred on 6 to 9 days in August. Conclusions were that rainfall in August favoured Zearalenone production indirectly by favouring epidemic development of *F. graminearum*. Sutton

(1982) summed up the data from various inoculation experiments in Ontario by suggesting that at least 60 mm of rain in August favours the development of ear rot caused by *F. graminearum*. Sutton *et al.* (1980) also observed that rainfall provides the persistent wetness required for the growth of the fungus and colonisation of kernels, but also delays the drying of the kernels, thus prolonging the period of colonisation.

Warm temperatures during the period of greatest host receptivity are also critical for infection and development of ear rot, and optimum temperatures of between 25-32°C have been reported (Hunter and Sutton, 1985; Teich, 1989). Previously, Maric *et al.* (1969) and Tuite *et al.* (1974) indicated that moderately low temperatures of < 22-24°C during August, but not higher are favourable for outbreaks of ear rot. Sutton (1982) states that periods of warm weather with persistent wetness are key conditions for epidemics of head blight and ear rot. Progress of almost all stages of the infection cycle require temperatures in the general range of 15-35°C, and disease progress probably ceases at less than 15°C. Delayed harvest may increase intensity of ear rot, probably by increasing the colonisation period (Sutton, 1982).

Colonisation of maize ears progresses during 8 weeks or more in the field (Sutton and Baliko, 1981). The pathogen invades various tissues of the cob and sometimes the husks and on the farm, ear rot is often not noticed until the crop approaches maturity when the husks loosen and expose mouldy kernels near the tips (Sutton, 1982).

Several characteristics of maize ears and kernels may favour or hinder infection. Upright ears and ears with tight husk coverage at harvest are more susceptible than pendant ears and those with loose husks (Koehler, 1959; Enerson and Hunter, 1980b). Ears that are rudimentary or with unfertilised tips are more susceptible to ear rot (Wimmer, 1978), as are ears with poorly covered tips (Cassini, 1981). Late maturing cultivars in which moisture content decreases slowly below 30% are most susceptible (Manninger, 1979). In addition, there is a wide variation

in susceptibility to ear rot among various inbreds and hybrids (Enerson and Hunter, 1980b).

2.5 LIFE CYCLE OF *F. moniliforme/F. subglutinans*

These fungi survive on crop residues in or on the soil surface. Under favourable conditions they infect maize stalks either directly or through wounds caused by insects or hail (Shurtleff, 1980). *F. moniliforme* can penetrate stalks at the base of the leaf sheath and progresses down in the lower internodes (Shurtleff, 1980). Seed as a source of inoculum is believed to be less important than air-borne or soil-borne inoculum (Shurtleff, 1980). Ear rot inoculum is thought to be wind-borne conidia which infect the immature seeds via the silk channel (Jones and Clifford, 1983). *F. moniliforme* and *F. moniliforme* var. *subglutinans* (*F. subglutinans*) rarely form perfect states (at least in the USA) (Shurtleff, 1980).

Disease development and spread are favoured by dry warm weather (Shurtleff, 1980). Rainfall during August to October is the most important factor for ear rot infection (Koehler, 1959) in addition to picnic beetles which carry propagules from buried previous ears to the new crop (Windels *et al.*, 1976). Ears are most susceptible to *F. moniliforme* 2 days after silking and to a lesser extent for an additional 40 days (Koehler, 1959), although Warren (1978) found infection was greatest 4-14 or 21 days after pollination, and infection was found to increase with time after pollination (Hesseltine and Bothast, 1977). Seed damage by birds and insects promote seed infection (Shurtleff, 1980).

Because the fungus has been found associated with nodes and internodal stalk tissue, it has been inconclusively suggested that seed infection may result from systemic vascular infections (Jones and Clifford, 1983). Koehler (1942, 1959) has suggested that *F. moniliforme* enters the cob through the silk, grows over the pericarp and then grows into the seed at the hilar end. The hyphae then grow into the cavity between the pedicel and the black layer into the embryo, floury and horny endosperm. Edwards (1936) suggested that internal infection occurs through the silks or from adjacent cob tissues.

F. moniliforme and *F. subglutinans* have a wide host range (Booth, 1971) including sorghum, sugarcane and maize. Sweet corn types of *Zea mays* are relatively more susceptible to seed rot disease because of their higher sugar content and perhaps because of a thinner seed coat than for normal maize. Lines with a relatively high fraction of lysine in the endosperm amino acids are considered to be more susceptible to the disease (Jones and Clifford, 1983). However, pathogenicity of *F. moniliforme* is questionable but it may be considered only weakly parasitic, occurring mainly on injured, senescent or decaying tissue (Koehler, 1942; Jones and Clifford, 1983; McGee, 1988; Nelson, 1992).

2.6 EFFECT OF *FUSARIUM* INFECTION ON SEED QUALITY

2.6.1 Time of harvest and seed deterioration

Austin (1972) stated that the decision when to harvest a seed crop is usually a compromise which takes into account past and expected weather, the availability of machinery and labour as well as the state of maturity of the crop. However, climatic conditions during the post-maturation and pre-harvest periods also have a great influence on seed quality. Deterioration in the field is attributed mainly to seed exposure to inclemental weather conditions, a situation which has been described as "weathering" (Delouche, 1980, 1992). Delouche (1975) suggested that seeds in the field after physiological maturity should be regarded as being stored in the field thus undergoing deterioration or aging (which is widely regarded as the main cause of seed vigour decline (Hampton, 1990)). Stresses set up by cycles of wetting and drying causing alternating swelling and contracting of seed tissues (weathering and damage) may occur after maturation (Moore, 1965; Delouche 1980). This may cause physiological (eg membrane, respiratory) damage (Coolbear, 1990). In some cases, especially in cereals with reduced sensitivity or low levels of ABA, imbibition (wetting) may initiate the gearing up of the seed's metabolic machinery for growth. Hydrolytic enzymes (eg α-amylase, protease) will be released in response to gibberelic acid (GA) and/or calcium ions stimulus and start the process of

mobilisation of food reserves while the seeds are still on the mother plant (Black, 1972; Bewley and Black, 1978). This may initiate germination on the ear itself (sprouting damage) (Gordon *et al.*, 1977; Matthews and Powell, 1986).

Williams and McDonald (1983) stated that related factors that promote or contribute to grain weathering (resulting in grain moulding) include prolonged rainfall during grain development and after maturity, and bird and insect damage. Delouche (1980) added that although these factors increase the incidence and severity of fungal invasion of seeds, the severity of weathering generally increases from cool to warm areas; the worst situation being in the humid subtropics and tropics. Delouche (1980) and McGee (1986) wondered whether under field weathering conditions fungal invasion of seed is the primary cause of seed deterioration.

Maize ears are usually tightly enclosed in husks, keeping the relative humidity high within the ears during the drying period, which favours fungal growth (Thomson, 1979), although husks offer protection against direct aerial fungal spore infection (Koehler, 1942). Delayed harvest increases the period of seed colonisation by *F. graminearum* (Caldwell and Tuite, 1974; Hesseltine and Bothast, 1977; Neish *et al.*, 1983; Watson, 1987) and *F. moniliforme* (Neish *et al.*, 1983).

2.6.2 Seed Health: occurrence of *Fusarium* spp. in seeds

The literature on the occurrence of *Fusarium* spp. in maize seeds is extensive. McGee (1988) and Richardson (1990) have listed the seed-borne *Fusarium* spp. and their occurrence in maize. Neergaard (1979) and Agarwal and Sinclair (1987) have discussed detection procedures (also see Chapter 4). Marasas *et al.* (1979), Kommedahl and Windels (1986) and Richardson (1990) reported that in the USA, Europe and Australia *F. moniliforme*, *F. subglutinans* and *F. graminearum* are the most frequently isolated *Fusarium* spp. from maize seed but when rating these species *F. moniliforme* is the most frequently isolated. Marasas *et al.* (1979) found in South Africa that *F. subglutinans* was the most prevalent species, followed by *F. moniliforme* and *F. graminearum* and the occurrence of each species was maximal in

a specific geographic area; *F. moniliforme* predominating in a subtropical area, *F. subglutinans* in the most temperate area, while *F. graminearum* was most commonly detected from the area with an intermediate climate. *F. moniliforme* is known to be the most prevalent *Fusarium* sp. in the tropics (Burgess *et al.*, 1988). Jones and Clifford (1983) state that *F. moniliforme* (apparently including *F. moniliforme* var *subglutinans* (*F. subglutinans*) is the most prevalent *Fusarium* sp. in the warmer, drier maize growing areas of the USA, such as California and the South Eastern states. In the literature, *F. subglutinans* is commonly referred to as a close relative of *F. moniliforme* (Edwards, 1936; Ullstrup, 1977) and frequently *F. subglutinans* has been misidentified and lumped with *F. moniliforme* (Koehler, 1959; Booth, 1971; Neergaard, 1979; Marasas *et al.*, 1979; Neish *et al.*, 1983; Bottalico *et al.*, 1989a; Nirenberg, 1989; Richardson, 1990; Nelson, 1992; Logrieco *et al.*, 1993), while Manns and Adams (1923) referred to *F. subglutinans* as *Cephalosporium sacchari* Butler (Booth, 1971) (see also Table 2.2). Therefore, the significance of *F. subglutinans* is not clear.

Fusarium spp., especially *F. graminearum*, *F. crookwellense*, *F. subglutinans*, *F. semitectum* and *F. poae* have been detected in maize seed/grain grown in New Zealand (Hussein and Baxter, 1985; Sayer and Lauren, 1991; Sayer, 1991; Mashauri, 1991; also see Chapter 1, section 1.3). Cox (1994) found *Fusarium* spp. present in all 53 samples with an average infection rate of 62.1% of grain. *Fusarium* infection was significantly positively but weakly correlated with the delay of harvest and was negatively correlated with grain moisture content. Significant (at 5%) hybrid difference in *Fusarium* infection were evident.

2.6.3 Effects of *Fusarium* spp. on seed germination and seedling emergence: seedling blights

Edwards (1936) and Jones and Clifford (1983) state that *F. moniliforme*, *F. moniliforme* var *subglutinans* and *F. graminearum* are weak pathogens, and similar remarks were made by Dickson (1923) and Pearson (1931) regarding the pathogenicity of *F. graminearum* (*G. saubinetti*). They also noted that these fungi

cause damage to the germinating seed only under unfavourable cold, wet soil conditions. They cause seed rot, pre- and post-emergence damping off, and seedling blight resulting in poor field stands (Pearson, 1931; Edwards, 1936; Christensen and Wilcoxson, 1966). Pearson (1931) states that the infection of the mesocotyl from the surrounding vicinity (seed or soil) is facilitated by the wounds produced in the mesocotyl as the result of the rupture of the pericycle of the stele and disorganisation of the cells in the cortex by the rapidly growing adventitious (secondary) roots which originate from the stele. The rupture of the pericarp by the growing main root would also form an excellent channel for mycelial entry into the mesocotyl.

F. graminearum, *F. moniliforme* and *F. subglutinans* cause cob rot and thus the rotting of seeds (refer to section 2.3) and because of this, these fungi are known to cause seed rot and seedling blight (Kruger, 1989). Ullstrup (1977) stated that sed rot and seedling blight are not often of economic importance. Kommedahl and Windels (1986) state that *F. moniliforme*, *F. subglutinans* and *F. graminearum* are the most frequently involved species in seedling blight. However, Jones and Clifford (1983) state that seedling blight is more common with *G. zeae* (*F. graminearum*) than *G. moniliforme* Sheld. Hans = *G. fujikuroi* (Saw) Wr. (*F. moniliforme*) or *G. fujikuroi* (Saw) Wr. var *subglutinans*, but Rheeder *et al.* (1990) found little influence on germination due to *F. graminearum*, *F. moniliforme* and *F. subglutinans*. Although these fungi were negatively correlated with germination at some locations it was only one of the two seasons of the study, and germination was positively correlated with *F. subglutinans* at only one location. Moreover, the effects of *F. moniliforme* on seed germination and as a cause of seedling blight has been controversial (see review by Van Wyk *et al.*, 1988).

For emergence, maize seeds need 5-6 days at 21°C, 10 days at 15.5-18°C, and 18-20 days at 10-15°C (Kruger, 1989). Using disease free mature maize seeds, Dickson (1923) found that low temperature alone has adverse effects on seed germination as no seedlings emerged at 8°C but vigorous seedling growth occurred at 24-28°C. In New Zealand, Hardacre *et al.* (1989) noted that under optimum field

germination conditions, seedling emergence should begin 12-16 days after planting and that planting when soil temperatures are at or below 8°C should be avoided.

Shurtleff (1980), Fowler (1985), Kommedahl and Windels (1986) and Kruger (1989) reported that geminating maize seeds may be attacked by a number of soil-borne or seed-borne fungi that cause seed rots, failure of seedlings to emerge, and seedling blight. Severe infection may kill the embryo before germination (seed rot) or destroy the seedling before or after emergence (seedling blight) (Shurtleff, 1980). The term pre-emergence damping off describes a situation where seedlings are killed before emergence, compared with post-emergence damping off where seedlings are killed after they have emerged above the soil surface (Wheeler, 1969; Shurtleff, 1980). Symptoms of post-emergence damping off are yellowing, wilting and death of the leaves, and discolouration and water soaking (soft rot) of the mesocotyl near the ground level (Shurtleff, 1980).

Fungi responsible for maize seedling blights include species of *Fusarium*, *Pythium*, *Diplodia*, *Helminthosporium* and *Penicillium oxalicum* (McKeen, 1952; Koehler, 1957; Shurtleff, 1980). The species reported as being able to attack germinating seeds, and seedlings, are *F. moniliforme* Sheld., *Diplodia maydis* (Berk) Sace, *F. graminearum* Shwabe, *Helminthosporium maydis* Nisik., *H. pedicellatum* Henry (Shurtleff, 1980). Also associated with seedling blights are *Aspergillus* spp., *Nigrospora oryzae* (Berk. Br) Petch., *Rhizoctonia zeae* Vorrhees, *Macrophomina phaseolina* (Tassi) G. Goid and *Cephalosporium maydis* Samra, Sabet & Hangorani, *Phymatotrichum omnivorum* (Shear) Duggar and *Colletotrichum graminicola* (Ces.) G.W. Wils (Shurtleff, 1980).

Fullerton (1987) reported that in New Zealand there is little information on the prevalence of those seedling diseases that destroy the seedlings at early stages of growth of maize, but that a wide range of seed-borne fungi, especially *Fusarium* spp., storage moulds, namely *Aspergillus* spp. and *Penicillium* spp., singly or together reduce seed viability and seedling emergence. Earlier, Fowler (1985) reported that fungi associated with seedling root rots of maize in New Zealand included

Chaetomium spp., *F. avenaceum* (Corda:Fries) Saccardo, *F. culmorum* (W.G. Smith) Saccardo, *F. moniliforme* Sheldon var *subglutinans* Wollenweber & Reinking, *F. oxysporum* Schlechtendal:Fries, *F. solani* (Martius) Saccardo, *Pythium afertile* Kanouse & Humphrey, *P. monospermum* Pringsheim, and *Trichoderma koningii* Oudemans. Although these pathogens were reported (Fowler, 1985) to be widely distributed, their incidence was usually low and rarely of economic importance.

Maize seedling diseases have been attributed to many factors but soil temperature and moisture have been emphasised as the key factor. Fowler (1985) reported that maize seedling infection occurs particularly when there is cold wet weather and waterlogging after planting. Shurtleff (1980) reported that seedling diseases are prevalent in poorly drained, excessively compacted and wet or cold soil situations (less than 10-13 °C soils). Fowler (1985) commonly isolated *F. oxysporum* and *Trichoderma koningii* from maize seedlings and found that seedlings were more susceptible to these fungi at 15°C than at higher temperatures. Crosier (1957) reported that *Gibberella zeae* and *Pythium* spp. are injurious to maize at temperatures of 7.5-12.5°C.

Isley (1950), referring to the mechanism of the cold test, and Kruger (1989) noted that high susceptibility of maize seeds and seedlings to attack by soil-borne fungi and their pathogenicity seems to be a function of the host rather than the parasite. The important factors are probably the extra length of time which seeds must lie in the soil in imbibed conditions and probably the low temperature mechanism behaviour of seed which renders it peculiarly susceptible. For example, low soil temperature is not optimum for vegetative growth of *Pythium* (the optimum for vegetative growth being 28-31 °C; Isely, 1950). Referring to *G. saubinetti* (*F. graminearum*) as a causal agent of maize seedling blight, Dickson (1923) observed that the soil-inhibiting organisms which cause seedling blight of maize and wheat usually become aggressive parasites only when the seedling or plant is weakened through its inability to react favourably to the environment.

Maize seedling disease severity is affected by planting depth, heavy soil, age and quality of seed, mechanical injury to the pericarp and genetic resistance, and sweet corn maize is more susceptible to seedling diseases than dent maize (Kommedahl and Windels, 1986; Fiala, 1987). Kruger (1989) states that poor seedling emergence usually occurs as a result of seed infection or mechanical damage and imbibed seeds with damaged pericarps liberate starch, thus providing an excellent nutritional source for proliferation of fungi (Harman and Stasz, 1986). Pericarp injury also facilitates penetration of seeds by soil-borne fungi (Koehler, 1957).

2.6.4 Maize seedling blight caused by *F. graminearum* (= *G. saubinetti*)

Dickson (1956) stated that the seedling blight caused by *G. saubinetii* occurs before emergence or when the seedlings are in the first to third leaf stage and rarely later, although with weather conditions favourable for disease development, new root lesions may develop after the 4th leaf stage and reduce the vigour of the plant but rarely develop sufficient to kill it.

Dickson (1923) reported that the maize seedling blight symptoms caused by *G. saubinetii* are similar to those caused on wheat, and that they vary depending on the severity of attack, the age of the seedling, and soil conditions. The lesions usually extend from the seed into the tap root and lateral roots as well as into the mesocotyl. The invaded areas are at first light brown, water-soaked regions, soon turning reddish brown. The seed turns reddish brown to carmine red, depending upon soil moisture conditions. The badly infected seedlings become yellow and wilt, and finally fall over and become overgrown by the fungus. Many of the plants partially recover by sending out new roots and make a fair growth, although a rather high percentage of such plants fail to produce marketable ears. Dickson (1956) and Pennypacker (1981) explained that the water-soaking or soft rot of the seedlings affects only the cortical tissue of the roots or mesocotyls.

Soil temperature is known to be an important factor in the development of maize seedling blight caused by *G. saubinetii.* Christensen and Wilcoxson (1966)

reported that seedling blight of maize caused by *Gibberella zeae* and *Fusarium* spp. is frequently prevalent during periods of cool weather when plants grow slowly and these plants may be weakened thus being pre-disposed to root rot, crown rot and stalk rot. As reported by Neergaard (1979) the influence of soil temperature on development of seedling blight caused by *F. graminearum* on maize, wheat and other cereals has been extensively investigated by Dickson (1923) who found that under field and greenhouse conditions maize infection takes place and blight progresses at all temperatures below 24°C, but does not occur above that. The most rapid development of the disease and the maximum blighting occurs at 12-16°C but below these temperatures, especially at 8°C, maize succumbs entirely to *G. saubinetii.* The upper limit soil temperature for the seedling blight of maize ranges between 20-24°C (Fig. 2.4). Dickson (1923) emphasised that mean soil temperatures over periods of considerable duration are more influential than brief extremes in soil temperature. Warren and Kommedahl (1973) observed that *F. graminearum* caused seedling blight at 18, 24 and 29°C but damage was greater at lower temperatures, although Covey (1959) found some isolates of *F. graminearum* which reduced seedling stand at 25°C than at 15°C or 20°C.

Pearson (1931) reported different reactions to seedling blight by different inbred lines; resistant inbred lines do not develop seedling blight at temperatures as low as 12°C, the intermediate ones are susceptible at temperatures below 20 and 24°C, while the susceptible inbred lines are blighted at all temperatures at which *G. saubinetii* (*F. graminearum*) grows. Chemical studies of seedlings of various cultivars of maize grown at different temperatures led to conclusions that resistance and susceptibility are strictly correlated to metabolic activities of the seedlings grown at these temperatures (Dickson, 1925; Dickson and Holbert, 1926, 1928). Also cultivation of *G. saubinetii* on artificial media showed that it makes its best growth on pectin-like and other pentose yielding substances (Dickson and Holbert, 1926).

Dickson (1925), Dickson and Holbert (1926, 1928) and Pearson (1931) explained how seedling infection takes place. At low temperatures the seedling cell walls are built up largely of pectin-like substances and the food reserves are mainly

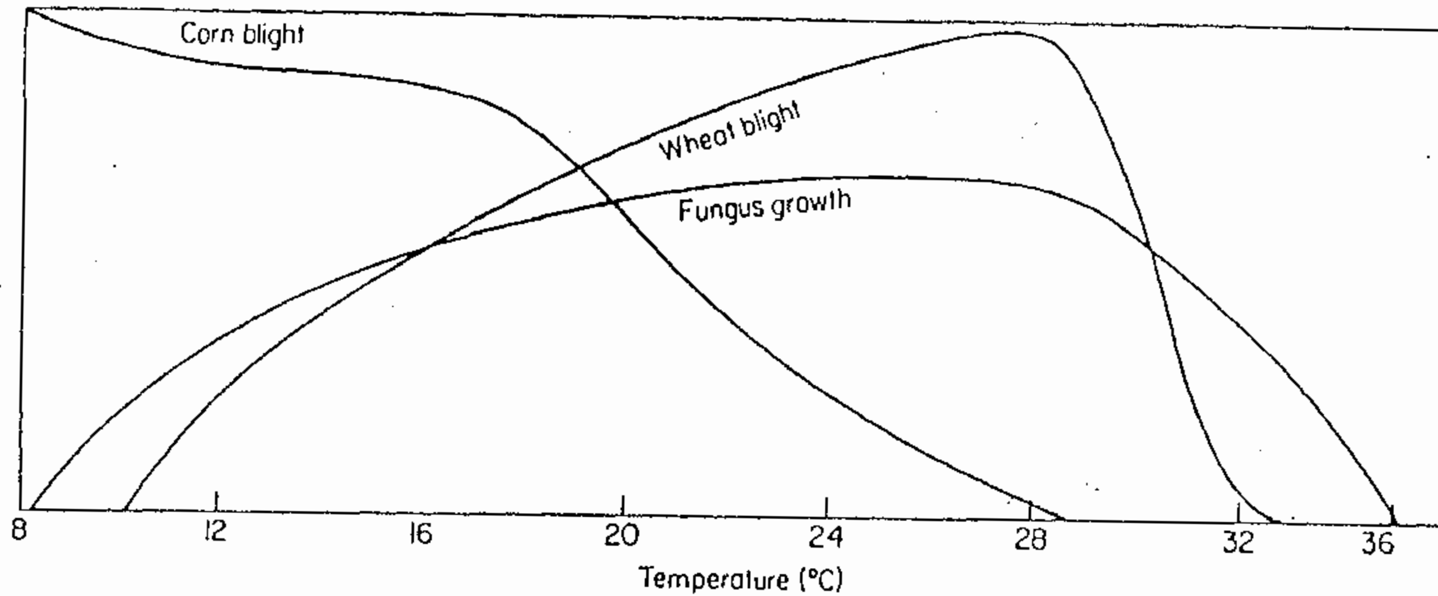


Figure 2.4 Influence of soil temperature on percentage of infection by *Fusarium graminearum* on seedlings of maize and wheat, compared with the growth rate of fungus on agar medium. Source: Neergaard (1979).

pentosans (Fig. 2.5). The cell walls are scantly suberised and lack of protective suberisation allows the invasion of the plant. After the fungus has entered the seedling it is able to grow and cause disease because sufficient hydrolysed food of a pectin-like nature is available in the cell walls and in the reserve substances.

Pearson (1931) state that maize is a high temperature plant, making its best growth at 24°C. Dickson (1925) and Dickson and Holbert (1926, 1928) have explained that the cell walls of the maize seedling grown at, and above 24°C, are chiefly of cellulose, and the epidermis, endodermis and cells surrounding wounds (caused by root emergence - Pearson (1931)) are well suberised, and the food reserves are largely hexose in nature. The suberised walls serve at least as partial barriers against fungus penetration and in case invasion is accomplished, the cellulose walls, hexose reserves and low nitrogen content offer little food for the fungus, thus lower the chances of disease development.

Pearson (1931) suggested that *G. saubinetii* exists as a saprophyte on crop refuse in the soil and is capable of becoming parasitic when it enters the host through wounds or when unfavourable environmental conditions have so altered the plan that it is incapable of resisting penetration. He reported that *G. saubinetii* enters the maize seedlings through ruptures in the cortex produced by the emergence of adventitious roots or by the pulling apart of cells in consequence of rapid growth. Pennypacker (1981) similarly reported that soil-borne pathogenic Fusaria, including *G. saubinetii*, which cause seedling blight attack the cortex of the roots or hypocotyl of the host by penetrating the stomata, wounds or directly through the root tip or meristematic region of the roots. The fungus initially grows intercellularly and intracellularly at a later stage (Pearson, 1931; Pennypacker, 1981).

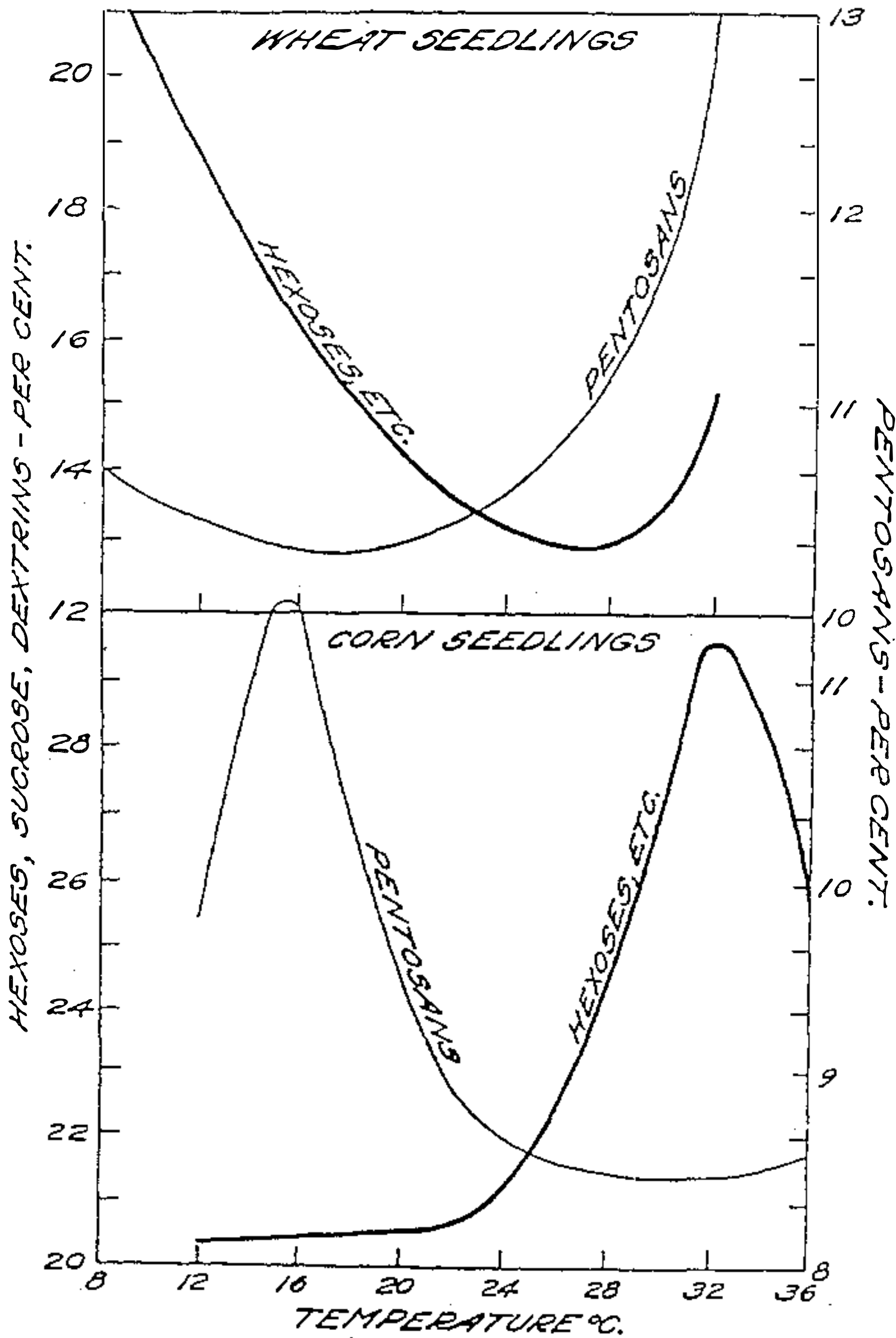


Figure 2.5 Curves showing the influence of temperature upon the composition of wheat and maize seedlings. The heavy lines represent such carbohydrate building-substances as hexoses, sucrose and dextrins in the seedling with the endosperm removed. The light lines represent total pentosans, contained largely in the cell walls. Note the difference in the trend of these curves with the rise in temperature. Wheat blights badly at temperatures above 12°C and maize at temperatures below 24°C.
Source: Dickson and Holbert (1926).

2.6.5 Mycotoxin production

One of the major consequences of *Fusarium* spp. colonisation of maize crops is contamination of the grain by mycotoxins (Sutton, 1982; Wilson and Abramson, 1992). Mycotoxins are fungal secondary metabolites that are toxic to animals including man, and mycotoxicoses are diseases resulting from ingestion of toxic fungal metabolites contaminating the food or feed supply (Wilson and Abramson, 1992). The principle naturally occurring *Fusarium* toxins are the trichothecenes, Zearalenone (F-2 toxin), moniliformin and Fusarin c, and Fumonisins (Wilson and Abramson, 1992). The *Fusarium* mycotoxins frequently encountered in maize and other cereals are often implicated in natural cases of mycotoxicoses and include Zearalenone and zearalenols, trichothecene derivatives, and moniliformin (World Health Organisation, 1979; Wyllie and Morehouse, 1977, 1978; Marasas and Nelson, 1987; Gilbert, 1989). Mycotoxins resulting from maize infection by *Fusarium* were discussed by Kruger (1989), Bottalico *et al.* (1989), Nelson (1992) and Lamprecht *et al.* (1994). The *Fusarium* species listed in Table 2.7 have been confirmed (Marasas *et al.*, 1984) as producers of the mycotoxins listed. In addition, while moniliformin is produced by *F. moniliforme* and *F. subglutinans*, *F. moniliforme* produces Fumonisins and Fusarin c, and *F. crookwellense* is able to produce Nivalenol, Zearalenone and Fusarenone (Visconti *et al.*, 1990; Wilson and Abramson, 1992; Logrieco *et al.*, 1993).

The natural occurrence of *Fusarium* toxins in New Zealand maize was first reported by Hussein *et al.* (1989). In a screening study for Deoxynivalenol, Diacetoxyscirpenol, T-2 toxin and Zearalenone from maize samples collected from healthy crops in Manawatu, Hussein *et al.* (1989) detected concentrations ranging from 0-9 ppm for all mycotoxins from maize direct from the field and from harvested samples, or stored maize, except for Zearalenone (0.2-16 ppm) from samples collected at harvest. The maize grain is frequently contaminated by Nivalenol, Doexynivalenol and Zearalenone (Lauren *et al.*, 1991; Lauren, 1944) but, in addition, the occurrence of α and ß Zearalenol was reported by Cox (1994), while Hussein *et al.* (1989) reported the natural occurrence of Diacetoxyscirpenol and T-2 toxin.

Table 2.7 ***Fusarium* mycotoxins and their producers.**

Mycotoxin	*Fusarium* species
Deoxynivalenol (vomitoxin) (DON)	*F. graminearum* *F. nivale*
Nivalenol (NIV)	*F. graminearum* *F. equiseti* *F. sporotrichioides*
3-Acetyldeoxynivalenol (3AcDON)	*F. graminearum* *F. culmorum*
Diacetoxyscripenol (DAS)	*F. graminearum* *F. sambucinum* *F. equiseti* *F. acuminatum* *F. semitectum* *F. moniliforme* *F. oxysporum* *F. poae* *F. sporotrichioides*
T-2 toxin	*F. graminearum* *F. equiseti* *F. acuminatum* *F. semitectum* *F. poae* *F. sporotrichioides*
HT-2 toxin	*F. graminearum* *F. acuminatum* *F. sporotrichioides*
Zearalenone (F-2 toxin) (ZEA)	*F. graminearum* *F. culmorum* *F sambucinum* *F. avenaceum* *F. equiseti* *F. semitectum* *F. moniliforme* *F. oxysporum* *F. solani* *F. sporotrichioides*

Source: Constructed from Wilson and Abramson (1992).

Zearalenone, together with Deoxynivalenol, are the best known *Fusarium* mycotoxins in maize and wheat (Funnell, 1979; Vesonder *et al.*, 1976; Vesonder and Ciegler, 1979; Neish and Cohen, 1981). Zearalenone was first isolated and characterised in 1962 (Stob *et al.*, 1962) and is known to be the principle contaminant of maize (Gilbert, 1989). Zearalenone, an oestrogenic mycotoxin, in commonly associated with maize moulded by *Gibberella zeae* (Schw.) Petch (anomorph *F. graminearum* Schwabe) (Caldwell *et al.*, 1970) and maize products (Gilbert, 1989). Zearalenone induces hyperestrogenism, particularly in swine (Sundlof and Strickland, 1985). Other problems associated with Zearalenone in livestock include foetal mummification, ovarian cyst formation and small litter size in pigs. Cattle are much less affected and the affects are minimal in poultry (Wilson and Abramson, 1992).

According to Bottalico *et al.* (1985), Zearalenol, an oestrogenic derivative of Zearalenone (Halger *et al.*, 1979), occurs as two diaestereomeric derivatives, namely, αZearalenol (low melting point 168-169°C) and ßZearalenol (melting point 174-176°C) (Hidy *et al.*, 1977; Shipchandler, 1975). αZearalenol isomer found occurring naturally in culture (Halgler *et al.*, 1979) and in animal feed (Mirocha *et al.*, 1979) was found to be 3-4 times more active than Zearalenone during a rat uterus bioassay whereas ßZearalenol isomer was equal to that of Zearalenone.

The naturally-occurring trichothecenes that are thought to be of major importance and the ones commonly found as contaminants of cereals, including maize, are Deoxynivalenol (vomitoxin), Nivalenol, T-2 toxin, HT-2 toxin, and Diacetoxyscirpenol (Gilbert, 1989; Wilson and Abramson, 1992). The other trichothecene derivatives include 3-Acetyldeoxynivalent, 15-Acetyldeoxynivalenol, T-2 triol, T-2 tetraol, Fusarenone (Fusarenone-x) (Bottalico *et al.*, 1989b). There is no one target organ for lethal doses of any of the trichothecenes and the toxic symptoms observed are varied. At sub-lethal doses there are characteristic effects on the central nervous system typified by vomiting and feed refusal in animals. Pigs are particularly sensitive to these effects and vomiting has been observed at doses as low of 0.05 mg/kg (Forsyth *et al.*, 1977). Trichothecenes including Deoxynivalenol are immunosuppressive (Rosenstein *et al.*, 1979; Atkinson and Miller, 1984). In

chronical exposures to low levels of Deoxynivalenol, this effect may be manifest in the form of a greater susceptibility of the animal to other infections, making it difficult to attribute the effects to Deoxynivalenol (Gilbert, 1989).

Deoxynivalenol has not been directly associated with cases of human intoxication but has been detected in mouldy maize intended for human consumption in South Africa (Marasas *et al.*, 1979).

Nivalenol is a highly toxic trichothecene metabolite (Ehrlich, 1989; Lauren, 1994), being dangerous to stock when fed at 0.4-0.5 mg/kg of feed (Cox, 1994). It commonly co-occurs naturally with Deoxynivalenol in many countries, eg Japan (Yoshizawa and Hosokawa, 1983), in Europe (Blass *et al.*, 1984) and in New Zealand (Lauren *et al.*, 1991). Ehrlich (1989) suggested that Nivalenol could be formed in nature as a result of oxidation of 7-deoxynivalenol. Lauren *et al.* (1991) noted that the prevalence of the Nivalenol-type of trichothecenes as a general, and as the major, mycotoxin contaminant (up to 3.60 mg/kg) was unusual and of great concern.

2.6.6 Factors affecting the production of mycotoxins

Wicklow *et al.* (1990) noted that "mycotoxicoses associated with red ear rot of maize caused by *Gibberella zeae* (Schw) Petch (anamorph *F. graminearum* Schw.) occur in temperate regions of the world when: 1) above average rainfall occurs at silking and ears become infected with *G. zeae*; 2) harvest is delayed or absent and infected maize is exposed to cool, wet conditions; or 3) infected ears are stored in open cribs or grain is left uncovered in piles and exposed to rainfall and low temperatures. Another factor affecting the formation of mycotoxins, including those produced by some *Fusarium* species, is the presence of antifungal agents, including biocides which cause stress to the fungi (Moss, 1991). In addition, different fungi growing in or on the same substrate can influence each other in various ways and combinations of mycotoxins produced by one or more fungi present can act additively, synergetically or depressively (Marasas and Nelson, 1987); bacteria, yeast or host plant metabolic products and enzymes are thought to affect mycotoxin

production (Miller *et al.*, 1983). Miller *et al.* (1983) and Scott *et al.* (1984) found a decline of Deoxynivalenol in later parts of the season, in maize and wheat, respectively, and attributed it to this phenomenon. Also there is evidence that Zearalenone may be translocated to the ears from infected maize stalks but that the mycotoxin is diluted considerably when this occurs (Sutton *et al.*, 1976).

In the field, rainfall promoting persistent wetness in maize favours epidemic disease development caused by *F. graminearum* , and indirectly increases ear rot and accumulation of Zearalenone (Sutton *et al.*, 1980a). Rainfall and warm temperatures during the period of greatest host receptivity are critical for infection and development of ear rot and Zearalenone accumulation (Koehler, 1959; Tuite *et al.*, 1974; Caldwell *et al.*, 1974).

In Canada, the incidence of maize samples contaminated with Zearalenone is correlated strongly with August (mid tasselling/silking period) rainfall but only moderately or weakly with rainfall in July (early silking) or September (seed maturation) (Sutton *et al.*, 1980a). Sutton *et al.* (1980a) reported concentrations of Zearalenone of 0.5-4.0 μg/g in heavily colonised maize seeds collected from the field in South Ontario during October and November but not earlier. However, the mycotoxin was found mainly in ears of highly susceptible hybrids, ears injured by birds, and ears artificially inoculated with *F. graminearum*.

Hart *et al.* (1984) reviewed the effect of maize genotypes on Zearalenone production in fermentation studies, and found significant differences among inbred lines but not between cultivars and pathogen isolates. Cullen *et al.* (1983) reported that inbred lines were more susceptible to *G. zeae*; that isolates differed in virulence and Zearalenone production; and that the toxin was positively correlated with increasing severity.

Hart *et al.* (1982) reported that Deoxynivalenol production increased as disease severity increased and that Deoxynivalenol and Zearalenone levels varied with isolates of *G. zeae*. Hart *et al.* (1984) found significant differences among genotypes in their

reaction to inoculation by *G. zeae* and the analysis of infected grains from these inbred lines indicated a positive but low correlation (r = 0.47) between concentration of Deoxynivalenol and disease ratings, the concentration being low at low rations but varying at higher rations. Zearalenone concentrations varied and no trends were observed.

Neish *et al.* (1983) reported that in Ontario, Canada, Fusaria were isolated from mouldy maize harvested in late autumn or in early spring after overwintering in the field and *F. graminearum*, *F. moniliforme*, *F. morniforme* var. *subglutinans* and *F. sporotrichioides* var. *sporotrichioides* accounted for 84% of the total isolates. Deoxynivalenol (0.05-6.5 ppm) and Zearalenone (0.002-0.11 ppm) were detected from samples of mouldy maize taken directly from the field.

In New Zealand Lauren (1994) reported that early (mid-May or mid-June) sampled maize grain showed lower levels of Nivalenol and Deoxynivalenol while Cox (1994) found that harvest date was correlated with total Nivalenol, Deoxynivalenol, Zearalenone and ßZearalenol.

2.6.7 Assessment of grain and maize parts for possible mycotoxin contamination

The amount of mycotoxin in a given lot of maize grain depends on the percentage of *Fusarium* damaged seeds in the lot and the seeds from the tip of the cob have been reported to have higher levels of mycotoxins than those from other parts (Caldwell and Tuite, 1974; Perkowski *et al.*, 1991). According to Tuite *et al.* (1974), grain containing 5% or more of *F. graminearum* visually damaged seeds should be considered toxic to pigs and those with as low as 3% damage were also found toxic and associated with decreased consumption and poor weight gain.

External cob or seed symptoms may be used to assess dangers of mycotoxin in maize. Tuite *et al.* (1974) described Zearalenone and emetic factor (Deoxynivalenol) containing seeds as having visible external mycelium and irregular red or pink colouration; lightly damaged seeds were partly discoloured on all sides,

usually light brown or grey in colour, sometimes resembling "a water mark" and with little or no surface mycelium. Perkowski *et al.* (1991) and Chelkowski (1991) found that *Fusarium* damaged seeds containing Zearalenone and trichothecenes had carmine red discolouration, were shrivelled and light in weight, with mycelium on the surface. Healthy looking seeds without symptoms were also found to be contaminated by Zearalenone and Deoxynivalenone but in small amounts. The amount of Deoxynivalenol in the total cobs was found to highly correlate ($r = 0.94$) with the percentage of damaged seeds in a given ear (Perkowski *et al.*, 1991).

Moniliform was detected from cobs with "pink ear rot" symptoms (Chelkowski, 1989) and trichothecenes of group B-(8-ketotrichthecenes) from cobs with "red ear rot" symptoms when *F. graminearum* and *F. culmorum* were the cob colonisers but when red ear cobs were infected with *F. sporotrichioides*, trichothecenes of group A (T-2 toxin derivatives) were detected (Chelkowski, 1989). Besides Deoxynivalenol and Zearalenone, red ear cobs infected by *F. crookwellense* and *F. graminearum* produced Nivalenol (33-56 mg/kg) and Fusarenone (0.6-20 mg/kg) (Visconti *et al.*, 1990). Mycotoxins have also been detected in other parts of the plant, eg axial stems, husks and stalks (Perkowski *et al.*, 1991). The occurrence of mycotoxins in the axial stem is of significant importance in the animal industry because axial stems are used together with seeds to produce maize/corn cob mixture ("CCM") as feedstuff for pigs (Chelkowski, 1989). Significant contamination of "CCM" silage with vomitoxin (Deoxynivalenol) and Zearalenone and associated pig performance problems have been reported by Chelkowski (1989).

2.7 EFFECT OF SEED STORAGE ON *FUSARIUM* SPP.

2.7.1 Survival of *Fusarium* during storage in dry seeds

There is a profound change in the grain ecosystem at harvest, from a field environment controlled by diurnal fluctuations in weather conditions to a more stable

environment of the grain store in which conditions are governed chiefly by the availability of water to the grain (Lacey and Magan, 1991). Once the seeds have been dried to their normal harvest moisture content in the field, or, in the case of maize, have been dried artificially soon after harvest, the field fungi no longer grow (Christensen, 1987). Most field fungi (those which infect plants and/or seeds in the field, including *Fusarium* spp. - Christensen and Kaufmann, 1965, 1969), die rapidly in seeds held at moisture contents in equilibrium with relative humidities of 75-90% or moisture contents of 14.8-19.9% for maize at 25°C (Sauer *et al.*, 1992).

The survival of seed-borne inoculum has been extensively reviewed by Neergaard (1979), and also Agarwal and Sinclair (1987) who outlined and discussed the factors influencing the longevity of seed-borne pathogens. These include: host genotype, inoculum (amount per seed, location in the seed and type of survival propogule), seed storage containers, storage environment, storage period and the presence of antagonistic microflora. In general, pathogens, including *Fusarium* spp., survive longer in seeds under cool, dry conditions than higher temperatures and relative humidities (Agarwal and Sinclair, 1987). Lacey and Magan (1991) stated that field fungi generally survive better at 4-5°C than at ambient temperatures.

However, species of *Fusarium* present on maize cobs as a result of field infection may continue to grow and even produce mycotoxins at high moisture contents (30-50%; Pelhate, 1988; Lacey and Magan, 1991). Colonisation of maize cobs may continue in storage if conditions are favourable for the growth of *F. graminearum*, and moisture content of the maize seed is the major factor (Sutton, 1982; Teich, 1989). In Ontario, extensive growth of *F. graminearum* was observed on husked ears in cribs, especially wide ones (more than 1.7 m) in which seed drying was slow, and in those without cover against precipitation (Hunter and Enerson, 1979). *F. graminearum* grows on maize seeds with moisture content ranging from as low as 20-22% and is most vigorous at 35% (Sutton, 1982; Teich, 1989). Harvested grain with moisture content greater than 18% may be infected by *F. graminearum*, at temperatures from 12-18°C (Teich, 1989), but visible *F.*

graminearum growth was not observed on cobs at 12 or 16°C and 18% seed moisture (Wicklow *et al.*, 1990).

Christensen and Kaufmann (1969) stated that *Fusarium* dies out rapidly in grain stored at moisture content of 12-13% at a temperature of 21°C. Shands (1937) found that *F. graminearum* could live in barley up to 27 months but Christensen (1963b) recorded 20 months of survival and Shands (1937) found that *F. culmorum* and *F. avenaceum* were viable in barley seeds for less than 2 months. Ponchet (1966) as cited by Neergaard (1979) recorded survival of *F. nivale* in wheat after 42 months in storage. Dungan and Koehler (1944) recorded that *F. graminearum* died out of maize seeds completely in 2 years, most of the seeds being free of the pathogen after 15 months, but the same seeds still harboured viable *F. moniliforme* after 8 years. *F. moniliforme* survived (5%) for 13 years at 4°C storage while *F. graminearum* survived (60%) for 13 years in frozen (-8°C) maize seeds (Abbas *et al.*, 1986). Lutey and Christensen (1963) found that when barley seeds were stored at 14% MC at 20°C for 24 weeks, *Fusarium* died out even though the germination capacity of the seeds remained over 90%. When the moisture content was reduced to 12%, *Fusarium* spp. were all present in low percentages after 53 weeks storage at 20°C. When the original seed lot seed moisture content was at 16% MC and was stored at 20°C and 30°C, they were invaded by storage fungi before field fungi, including *Fusarium* spp. died out, and the seed germination percentage decreased rapidly.

With the exception of *F. moniliforme*, *Fusarium* spp. are usually short lived (Neergaard, 1979; Russell *et al.*, 1982; Russell and Berjak, 1983; McLean and Berjak, 1987). Since *F. moniliforme* invades its host systemically (Foley, 1962), Neergaard (1979) suggested that the location and condition of this fungus may account for its protracted (8 years) longevity. According to Koehler (1942), *F. moniliforme* infects all parts of maize seeds. *F. moniliforme* survived anoxic conditions at 40°C for 35 days (Russell and Berjak, 1983).

2.7.2 Mycotoxin production during storage

Abramson (1991) reviewed the occurrence of Zearalenone in cereals and concluded that the high levels of Zearalenone found in cereals and their products arise from contamination during storage and not from pre-harvest development. The works of Miller *et al.* (1983) and Wicklow *et al.* (1990) support this observation. Caldwell and Tuite (1974) found that maize as it comes from the field even in years of heavy infection by *Fusarium*, does not generally contain Zearalenone at high levels. The most common site of Zearalenone formation in maize is in ears stored in cribs, where *Fusarium* spp. which have caused ear rot in the field can continue to develop slowly (Diener, 1976; Abramson, 1991; Wilson and Abramson, 1992). Diener (1976) also states that maize may be dry when harvested but exposure to weathering in open cribs results in increased seed moistures up to 22-30%, resulting in *F. graminearum* invasion of the grain, and decreasing autumn temperatures are optimal for biosynthesis of Zearalenone. In a regional survey Hunter and Enerson (1979) reported that maize stored in cribs was a frequent source of Zearalenone.

In a study on the effects of cool temperature storage on levels of Zearalenone and trichothecenes in diseased maize ears, Wicklow *et al.* (1990) found substantial levels of Deoxynivalenol (3,799-4,139 ppb), of 15-Acetyldeoxynivalenol (746-818 ppb), or Zearalenone (870-1,500 ppb) in severely rotted seeds which had been wound inoculated with *G. zeae*. Storage of the remainder of the ear with 15-16% MC at 12 to 16°C for 10 weeks resulted in the disappearance of 15-Acetyldeoxynivalenol, but an increase of Zearalenone to 1.7-2.3 fold. Sound seeds originally with trace amounts of Zearalenone had 64-132 ppm after storage, but no change occurred in levels of Deoxynivalenol.

The *Fusarium* spp. may die out after drying or in storage, but mycotoxins are stable and persistent (Mills, 1982). While Joffe (1963) showed that the toxins producing alimentary toxic aleukia persisted for 6 years in storage, Abbas *et al.* (1986) found that Deoxynivalenol, 15-Acetyldeoxynivalenol, Zearalenone and αZearalenol persisted for 13 years at 0°C or at 4°C.

Abramson (1991) advised that since infected maize from the field contains small amounts of Zearalenone, which are not enough to cause feed problems, the best protective measure against further contamination appears to be prompt shelling and drying, and storage of maize seeds at seed moisture content (SMC) of less than 15%. Kruger (1989) emphasised prompt drying of maize to SMC of 12-14% to stop continuation of *Fusarium* spp. growth after harvest and prevent grain contamination by mycotoxins. Similar recommendations were given by Shurtleff (1980), Cassini (1981) and Seaman (1982). However, Tuite *et al.* (1974) observed that although commercial seed drying eliminated almost all the fungi (*Gibberella zeae*) from maize seeds it did not eliminate the feed refusal or emetic principal (vomitoxin). Scott (1991) has extensively reviewed reduction or elimination of mycotoxins present in cereal grains, including maize.

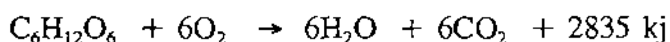
2.7.3 Airtight or sealed grain storage for control of micro-organisms

The high moisture conditions which favour the continued growth of fungi in grain and their products or new invasions have been taken advantage of to develop airtight or sealed storage systems to control micro-organisms.

Sealed storage is a term used to describe the process whereby grain is held in a supposedly airtight container in which depletion of the oxygen (O_2) from its normal level of approximately 21% results in the suppression of micro-organisms which would otherwise cause grain deterioration (McLean, 1989). This technique is most often used in the UK for feed grain with a moisture content of 18-24% (McLean, 1989; Bell and Armitage, 1992; Busta *et al.*, 1980) and airtight storage has also been developed to control insects in dry grain (Bell and Armitage, 1992).

The control of micro-organisms (as well as insects) in an airtight structure is due to changes in the atmospheric levels of oxygen around the stored grain as a result of respiration of grain, insects and micro-organisms. The principle of oxygen depletion within a sealed store or airtight structure depends on the fact that living organisms obtain energy by breaking down carbohydrates upon absorption of O_2

resulting in CO_2 and energy (heat) as well as water during the respiration process (Hyde, 1974; Busta *et al.*, 1980; McLean, 1989) as indicated in the equation (Lacey and Magan, 1991; Pomeranz, 1992):



$$C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2 + 2835 \text{ kj}$$

In airtight grain storage, after the O_2 has been used up by fungi, grain and insects, anaerobic respiration occurs. Little energy but more CO_2 is produced, to levels as high as 95% of the intergramular atmosphere - the amount depending on the grain moisture content (Lacey and Magan, 1991; Bell and Armitage, 1992). The requirement for respiration increases with temperature to a maximum of 40°C (Lacey and Magan, 1991).

For high moisture grain storage, Wilson and Abramson (1992) stated that *Fusarium* spp. are aerobic and Zearalenone cannot be formed in maize under anaerobic conditions, for example in silage. It can, however, develop when an air interface is formed (Escoula, 1979). In the laboratory T-2 toxin was controlled (reduced) using controlled atmosphere with carbon dioxide (Paster *et al.*, 1986).

2.8 EFFECTS OF SEED STORAGE ON SEED QUALITY

Seed quality components fall into three categories: accurate description; hygiene, viability and potential performance, including potential storability (Coolbear and Hill, 1987).

The most important factors which influence seed storage and longevity are moisture content of the stored seeds (or in non-sealed storage the relative humidity of the environment) and the temperature at which the seeds are stored (Justice and Bass, 1979; Copeland and McDonald, 1985).

Generally, the problem of maintaining seed quality increases with seed moisture contents as follows (Harrington, 1972):

Seed moisture content 8-9% insects become active and reproduce
Seed moisture above 12-14% fungi grow on and in seeds
Seed moisture above 18-25% heating may occur
Seed moisture above 40-60% germination may occur.

The "Rule of Thumb" for moisture indicates a doubling effect on seed life for every 1% decrease in seed moisture content within the range of 5-14% moisture content (Harrington, 1972). Douglas (1975) and Harrington (1972) have both suggested that maize seed should not be stored at 14% moisture content or higher. Kennedy (1979) stated that the safe storage moisture content for a starchy seed should be not more than 12%. Copeland and McDonald (1985) stated that maize seed will maintain satisfactory germination and vigour for about one year at SMC of 12-13% under normal warehouse temperatures and if longer term storage is needed, the seed moisture should not exceed 11% with temperature not above 20°C. Although storage of orthodox seeds with as low as 5-6% moisture content prolongs seed storage life, further drying to lower than 4-5% moisture content can cause damage (Harrington, 1973). Overdrying results in desiccation injury.

Temperature is the second most important factor that affects seed storage life. The "Rule of Thumb" for temperature indicates a doubling of the life of the seed for every 5°C reduction in temperature down to at least 0°C (Harrington, 1972). Seed moisture content and high storage temperature result in seed deterioration, ie reduction in seed vigour, seed germination capacity and finally loss of seed viability (these aspects of seed quality have been reviewed extensively by Roberts, 1972; Justice and Bass, 1979; AOSA, 1983).

Most seeds lose their viability at relative humidities of about 80% and temperatures of 20-30°C but can be kept for 10 years or longer at relative humidities of 50% or less at temperatures of 5°C or less (Toole, 1950). Although effects of

high seed moisture content and high temperature are interrelated, Copeland and McDonald (1985) explained that high seed moisture content results in biochemical events such as increased hydrolytic enzyme activity, respiration, and increase in free fatty acids, whereas high temperatures serve to enhance the rate at which these metabolic activities and seed deterioration occur.

Deterioration of seeds in storage is compounded by associated problems of invasion of seeds by storage fungi, ie species of *Aspergillus* and *Penicillium* (Christensen and Kaufmann, 1969). These fungi have the ability to grow in seeds whose moisture content is in equilibrium with relative humidity of 65-70 to 90% (ie moisture content of about 12.5-20% - Christensen and Kaufmann, 1974; Sauer *et al.*, 1992) and temperatures of -5 to 55°C (depending on species - Christensen and Kaufmann, 1974) and are capable of damaging stored seeds, initially reducing seed vigour and germination (Christensen and Kaufmann, 1969).

Although seed deterioration is known to begin as soon as physiological maturity occurs (Delouche, 1980), seed quality post-harvest is also largely determined by the extent of damage caused during mechanical seed harvesting, drying and processing (Hampton, 1992), processes which greatly affect seed cellular membrane integrity (major cause of seed deterioration) and enhance fungal invasion of seeds (Christensen and Kaufmann, 1969; Powell, 1986; Hampton, 1992). Escasinas (1986) and Mashauri (1991) studied and reviewed problems associated with interactions of seed moisture content, temperature and seed processing, and subsequent seed quality/seed deterioration during storage.

Packaging of seeds in moisture resistant or hermetically sealed containers (also see section 2.6.3 on air tight or sealed storage to control micro-organisms) for storage and marketing is in use (Harrington, 1972; Delouche *et al.*, 1973; Justice and Bass, 1979; Copeland and McDonald, 1985). Seeds stored in sealed containers will remain of good quality up to 10 years (Copeland and McDonald, 1985) provided the moisture content is low. Starchy seeds put in sealed storage at moisture contents above 12% will deteriorate faster than in open storage because the seeds are believed to come

into equilibrium at about 65% relative humidity (12.5-13.5% seed moisture content suitable for growth of *A. restrictus*). Justice and Bass (1979) have discussed the problems and values of sealed storage of maize seeds at different seed moisture contents, temperatures and in different atmospheres (air, carbon dioxide, oxygen and nitrogen).

Using airtight plastic humidity chambers, Gill and Delouche (1973) found that the storage environment of 30°C and 55% relative humidity (10-12.5% SMC) and 30°C and 75% relative humidity (about 14.5% SMC) were not suitable for maize seed storage. Significant deterioration occurred after 2 months of storage and progressed very rapidly until 10 months under 30°C and 75% relative humidity and 18 months at 30°C and 55% relative humidity. Under 30°C and 32% relative humidity (8.5-10.5% SMC) deterioration was not advanced enough to significantly affect performance until 18 months.

CHAPTER THREE

THE EFFECTS OF HARVEST TIMING ON THE OCCURRENCE OF *FUSARIUM* SPP. AND THEIR EFFECTS ON SEED QUALITY IN MAIZE

3.1 INTRODUCTION

Maize crops are attacked by several pathogenic *Fusarium* spp. viz., *F. graminearum*, *F. moniliforme*, *F. subglutinans* and *F. culmorum*, all of which can cause cob rot disease (Shurtleff, 1980; Fowler, 1985; McGee, 1988; Teich, 1989). However, of the four, *F. graminearum* is considered to be the major pathogen of maize (Sutton and Baliko, 1981; Sutton, 1982). Maize cob rot disease is a worldwide problem (Marasas *et al.*, 1979; Cook, 1981; Burgess *et al.*, 1981; Kommedahl and Windels, 1981; Cassini, 1981; Maric, 1981; Fowler, 1985; Teich, 1989; Kruger, 1989).

Cob rot caused by *F. graminearum* is favoured by warm wet weather, and in particular persistent wetness during silking. Thus rainfall is considered a key factor in the infection and colonisation of maize cobs (Sutton, 1982). The New Zealand climate has been described by Lauren *et al.* (1991) as being generally favourable for the growth of *Fusarium* spp., including *F. graminearum*

Infection of maize cobs by *Fusarium* spp. occurs through the silks (Koehler, 1959; Sutton and Baliko, 1981; Sutton, 1982). Susceptibility of maize ears to infection due to *F. graminearum* is greatest shortly after the silks emerge, and declines thereafter (Sutton, 1982). However, infection, which usually starts from the tip of the cob (Koehler, 1942, 1959; Sutton, 1982) continues during seed development, and under favourable conditions of seed moisture content and temperature also during seed maturity and drydown (Sutton and Baliko, 1981; Sutton,

1982), and in storage (Hunter and Enerson, 1979; Sutton, 1982; Pelhate, 1988; Lacey and Magan, 1991). Hardacre *et al.* (1991) recommended the use of early maturing cultivars, which attain a seed moisture content of around 25% in the month of May (late autumn) in order to reduce cob moulding and yield losses. However, there is a wide variation in the susceptibility to cob rot among various maize cultivars, and cultivars with certain characteristics such as upright ears, ears with tight husk coverage at harvest or loose husks in early stages of development, or those ears that are rudimentary and have unfertilised tips, thin pericarp and soft kernels, have a high lysine content, are most susceptible to cob rot disease (Koehler, 1959; Tuite *et al.*, 1974; Georgieve *et al.*, 1977; Wimmer, 1978; Palaversic *et al.*, 1979; Teich, 1989).

Fusarium infected maize grains usually contain mycotoxins, especially Zearalenone and trichothecenes (Caldwell and Tuite, 1974; Marasas *et al.*, 1979; Sutton *et al.*, 1980b; Sutton, 1982; Neish *et al.*, 1983; Hussein *et al.*, 1989; Kruger, 1989; Chelkowski, 1989; Bottalico *et al.*, 1989; Wicklow *et al.*, 1990; Lauren *et al.*, 1991). Delayed harvest increases seed invasion and intensity of cob rot due to *Fusarium* spp. (Koehler, 1942; Hesseltine and Bothast, 1977; Sutton, 1982) which also increases mycotoxin levels (Caldwell and Tuite, 1974; Tuite *et al.*, 1974; Sutton, 1982; Neish *et al.*, 1983).

Maize seed left in the field after physiological maturity undergoes weathering, a process which is known to cause seed deterioration or ageing, resulting in reduction in seed quality, especially loss or reduction in seed germination capacity and vigour (Harrington, 1972; Delouche, 1980, 1992; Powell, 1986; Hampton and Coolbear, 1990). Fungal invasion is also known to contribute to seed weathering in the field (Williams and McDonald, 1983; McGee, 1986). In New Zealand wet weather often delays harvest timing by delaying seed dry down. This, combined with problems of access to crops under wet ground conditions, often results in delays in harvesting maize until July or August (Bansal and Eagles, 1985; Hardacre *et al.*, 1989, 1991).

3.1.1 OBJECTIVES

Prior to 1990, little information was available in New Zealand on the effects of delayed harvest on *Fusarium* infection of maize grain, its associated mycotoxin contamination, and on seed quality.

The study described in this chapter was carried out over two main harvest seasons (1990 and 1991) with the following objectives:

1. to examine the effect of progressively delayed harvest dates on the incidence and identity of *Fusarium* spp. in maize seeds,

2. to determine the significance of seed infection by *Fusarium* spp. on seed germination and vigour,

3. to evaluate the effect of delayed harvest on grain contamination with mycotoxins, and

4. to assess the influence of cultivar differences on the incidence of *Fusarium* spp. in maize seed.

3.2 MATERIALS AND METHODS

Four commercial yellow dent Pioneer cultivars (P3551, 3591, 3709 and 3475), all of 'medium' maturity (Hardacre, A.K., pers. comm., Appendix 3.1) were used in this study. Plots were sown at Massey University in November 1989 and 1990 for seed harvests in 1990 and 1991. The soil type for both trials was an Ohakea silt loam and the previous crops were ryegrass/white clover pastures.

The seeds had been treated (prior to commercial sale) with Vitaflo 200 (Carboxin + Thiram; Genetic Technologies Ltd, New Zealand) a systemic fungicide,

but a seed health test prior to sowing showed the presence of *F. subglutinans* (1-5%) in all cultivars and *F. graminearum* (7%) in cultivar 3709.

Plot size in 1989/90 was 12 x 10 m. Each plot consisted of 12 rows, 75 cm apart, and seeds were precision sown at 15 cm spacing within the row. In 1990/91 plot size was 8 x 10 m, so that there were only 8 rows/plot, at the same seed spacing as for 1989/90, but unlike in the 1989/90 crop, each plot was separated by a 1 x 10 m gap. In both years cultivars were not replicated.

No fertiliser application was made for the 1990/91 experiment, but for the 1989/90 crop urea (46% N) was applied (92 kg N/ha) as a band application approximately 3 weeks after sowing (McGill, C., pers. comm.). In both years the herbicides dicamba (2 litres/ha) and atrazine (1.5 kg/ha), both 1-200 litres water/ha were applied 3 weeks after emergence between rows (without direct application to plants) and the insecticide esfenvalerate (Hallmark 5EC (250 ml/ha) was applied approximately one week after emergence to protect the seedlings from damage by cutworms (*Agrotis ipsilon*).

Crop development was followed by observation of the occurrence of silking and black layer formation (physiological maturity; Daynard and Duncan, 1969). Under the Manawatu environment, 'medium' maturity maize cultivars would normally be expected to reach harvest maturity in mid May (Hardacre *et al.*, 1989, 1991); for this reason harvest dates in April, May, June and July were chosen to represent an early harvest (April), normal harvest (May), and delayed harvests (June and July). Actual harvest dates were April 25, May 28, June 28 and July 30 1990, and April 24, May 30, June 30 and July 31 1991.

At each harvest, 50 cobs were randomly selected from each row of each cultivar, excluding the outside plot rows and the 3 plants at each end of the row. Care was taken to ensure that bird damaged cobs were not included. In the laboratory cobs were dehusked by hand and the number of cobs showing visual symptoms of *Fusarium* mycelial growth (moulding) (Plate 3.1) was recorded.

Plate 3.1 Maize cobs (parts) showing visual symptoms of *Fusarium* infection, ie white mycelial growth/mould. A, B & C, pieces of maize cobs, without mould, with tip mould and butt mould, respectively; D, a close up on mould showing the pinkish tinge.

For each cultivar at each harvest, 5 cobs were selected at random to provide a seed sample for determining seed moisture content (SMC). Seeds from 3-5 whole rows/cob were hand shelled, mixed and two replicates of 25 g seeds were drawn (using a soil divider, ISTA, 1993) for a two-stage (pre-drying) moisture content determination (because SMC was > 25%, ISTA, 1993). Each weighed replicate was placed in a pre-weighed aluminium tin with a lid, placed on the top shelf of a heated air (130°C) oven and left for 15 h to dry. The next day seeds were further dried at 70°C for 2-5 hours depending on harvest date. The loss in weight recorded was the water dried off (= S_1, ISTA, 1993). For the second stage, the partly dried seeds were ground, the ground material dried at 130°C for 4 h, cooled in a desiccator and reweighed to determine the water lost in the second stage (= S_2). The total seed moisture content was calculated using the formula:

$$(S_1 + S_2) - \frac{S_1 \times S_2}{100} \qquad \text{(ISTA, 1993)}$$

The remaining cobs were dried on separate trays in a forced air drier (Thomson, 1979) at a maximum air temperature of 32°C (Hill, M.J., pers. comm.; Arvier, 1983), until SMC had reached 11-14% (1990) or 13-15% (1991). This took 4-7 days depending on harvest time. Seed moisture levels were checked during this drying period by the use of an 'Agromatic' portable moisture meter (Agromatic Soby A/S, Denmark). Once the desired SMC had been attained, cobs were hand shelled, and a representative seed sample drawn to determine final SMC using the 130°C oven method (ISTA, 1993). Dried seed was then placed in paper bags, which were sealed in double layer polyethylene bags, which in turn were placed in plastic buckets with airtight covers and stored at 5°C.

For seed quality assessments, a sample of between 150-200 g was drawn from each cultivar/harvest seed bulk using either a conical divider or soil divider (ISTA, 1993). Two hundred seeds were used for germination (4 x 50), 200 seeds for seed health testing, and 61-63 g for mycotoxin determination.

Germination was assessed using the BP (roll towel) method (ISTA, 1993) at 25°C for 7 days. Seeds were dusted with thiram (1% w/w) (0.03 g per 50 seeds) before testing. Seedlings were classified as normal or abnormal using the definitions provided by international agreement (ISTA, 1993). Non-germinated seeds were subjected to a tetrazolium test (ISTA, 1993) to determine viability. In 1990 seeds were only germinated after drying, but in 1991, germination was also determined for seeds before drying.

For seed health testing, 200 seeds of each cultivar/harvest were wrapped in nylon net bags and surface disinfected with 1% sodium hypochlorite (Janola, 31.5 g/l w/v) for 2 minutes before being rinsed for 5 minutes under running tap water. Bags were immediately aseptically transferred to a Laminar Flow cabinet, where seeds in the bags were allowed to dry on sterile paper towels under a sterile air flow for 1 h prior to plating.

One hundred seeds (5 seeds/plate) were aseptically plated onto Malt Agar (MA) (Difco, 30 g malt extract, 15 g agar, 1 litre distilled water and sterilised at 121°C for 20 minutes) with the embryo side down to ensure early imbibition (McDonald *et al.*, 1994) and to allow embryo infecting fungi to grow onto the agar plate. The remaining 100 seeds were similarly plated onto Malt Salt Agar (MSA) (MA plus 7.5 g NaCl, 1 litre distilled water, sterilised as for MA) for the detection of storage fungi (*Aspergillus* and *Penicillium* spp.). The plated seeds were then incubated in a germinator in diffuse light at 25°C for 5 days, after which cultures were examined visually or by stereobinocular microscope (x 40) to identify *Fusarium* spp, other field fungi and storage fungi. Because the initial series of tests in both years showed a very low (< 1%) incidence of field and storage fungi (data not presented), the use of MSA was discontinued, and this study therefore concentrates solely on *Fusarium* spp.

In this study, the term "seed-culture colony" relates to *Fusarium* spp. cultures on an agar medium as a result of the fungal growth from naturally infected seeds.

This is in contrast to "pure cultures" obtained by inoculating an agar medium with fungal mycelium, conidia or other fungal structures.

Often after 5 days it was found that *Fusarium* colonies had coalesced, and in some cases the mycelium overran the seedlings, thus making it impossible to identify individual colonies and the seeds which had not shown *Fusarium* seed infection. This was particularly so with seeds harvested in June or July which were heavily infected with *Fusarium* spp. Consequently, the exercise was repeated by plating only one seed per plate. This was found to be entirely satisfactory for determination of the seed-culture colony morphology.

The *Fusarium* spp. colonies that grew out of the seeds plated on MA (seed-culture colonies) were identified visually by first characterising them and then categorising them according to colour (red, pink, purple, cream and white) or by colony morphological features such as form, texture of mycelium and in some cases sporulation features. Representative seed-culture colonies were then subcultured onto MA and 27 pure cultures were sent to the International Mycological Institute (IMI), Kew, London, for identification. Duplicate MA culture slants were retained and stored at 5°C for future description and use as reference for the identification of *Fusarium* to species level. Results of the identification reports by IMI were then matched against the seed-culture colony categories for proper *Fusarium* spp. recording.

The vigour of harvested seeds was determined by conductivity testing using the modification of the method of Matthews and Powell (1987). Four replicates of 50 seeds per lot were each weighed, placed in 500 ml Erlenmeyer flasks containing 250 ml of distilled water which had been equilibrated at 25°C for 24 h, then each flask was covered with para-film to prevent contamination. Flasks containing seeds, plus two control (water only) flasks, were then left at 25°C for 24 h, after which the seed leachate conductivity readings were taken using a CDM 83 (Cell Type CDC 304 [Radiometer FRR, Copenhagen]) conductivity meter. Distilled water control

conductivity readings were subtracted from the seed flask readings before conductivity/g was calculated.

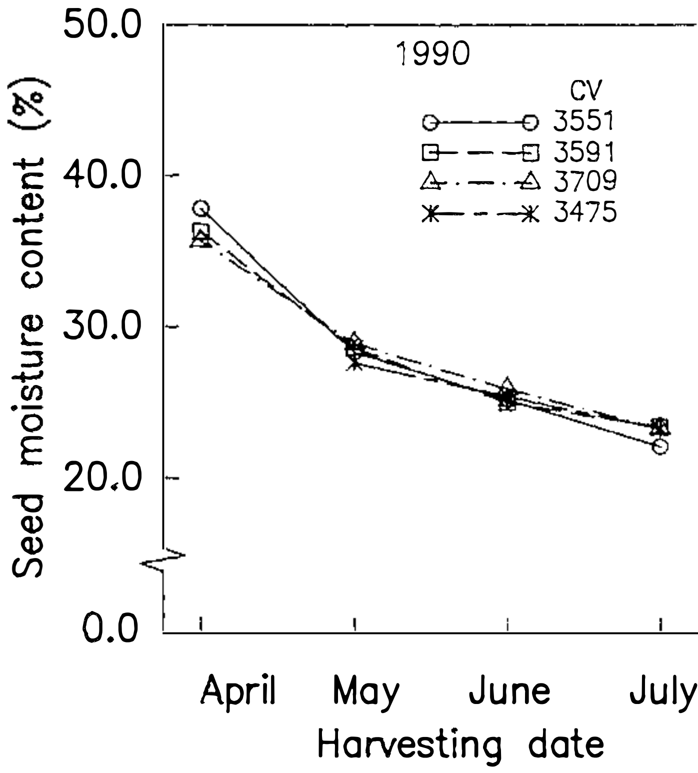
The 61-63 g seed samples destined for mycotoxin testing had been stored in a deep freeze (Smith and Moss, 1985) rather than 5°C as for all other seed bulks. Samples were analysed at Ruakura Research Centre, Hamilton, New Zealand by Dr Denis Lauren, for the presence of mycotoxins, namely Nivalenol (Niv), Deoxynivalenol (DON), Zearalenone (ZEA) and αZearalenol (αZEA).

Samples were extracted, the extracts were cleaned up and hydrolysed to produce parent alcohols prior to analysis by High Performance Liquid Chromatography (HPLC) with ultraviolet light (UV) detection at 245 nm for Nivalenol and Deoxynivalenol. Zearalenone analysis was by HPLC of extracts using fluorescence detection. Detection limits were < 0.05 mg/kg for Nivalenol and Deoxynivalenol and 0.2 mg/kg for Zearalenone. The mycotoxin analyses were made on single samples without replication and therefore data were not statistically analysed.

3.3 RESULTS

3.3.1 Factors influencing *Fusarium* spp. seed infection and seed quality

While seed development (in terms of periodic determination of seed moisture content) was not monitored in either 1990 or 1991, developmental stages were recorded by observation. Peak silking occurred during the last week of January in both seasons, so that pollination and fertilisation were completed by early February. Physiological maturity, as determined by the presence of the black layer was reached in late March/early April (from 60-70 days after pollination) and while harvest maturity (as determined by data supplied by the seed company - Appendix 3.1) should have been reached during May (106-108 days after pollination), seed moisture contents at the harvests on May 28 1990 and May 30 1991 were 27-29% (1990) and



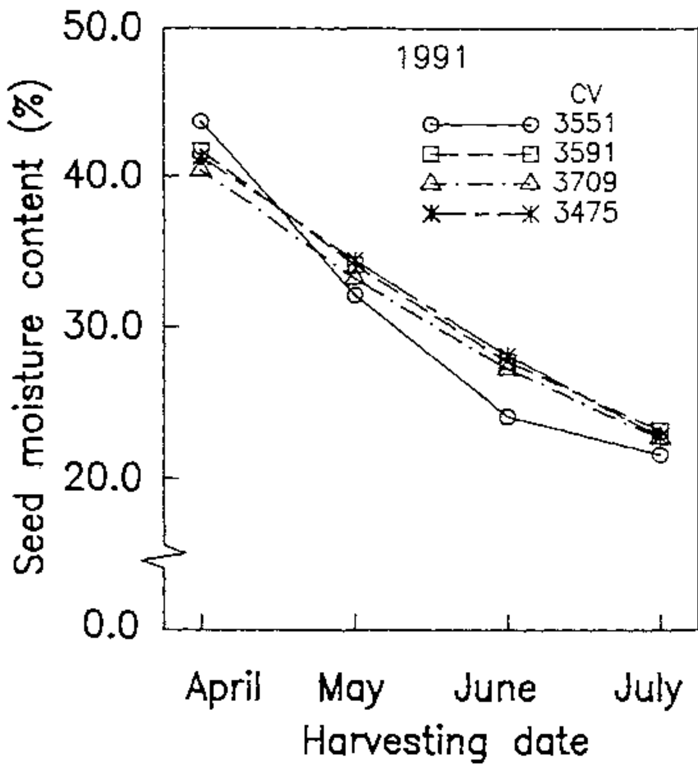


Figure 3.1 Seed moisture content (%) on different harvest dates in 1990 and 1991.

33-35% (1991). Harvest maturity seed moisture contents of approximately 25% (Hardacre *et al.*, 1991) were not reached until the end of June in 1990 and mid July in 1991 (Fig. 3.1), but there were indications that cultivar 3551 dried fastest betweenApril and May 1990 and 1991 and that the 1991 crop dried faster than the 1990 crop (Appendix 3.2).

Total rainfall for the period January to July did not differ markedly between seasons (672 mm in 1990 cf 700 mm in 1991). However, the distribution varied between years and months (Table 3.1), with both February and April being drier in 1990 than 1991, while the converse occurred in March 1991. February 1990 was 3°C warmer than February 1991 (Table 3.1), and in general air temperatures were warmer in 1990 than 1991. The first ground frosts occurred in April in both years, but the number of frosts did not differ between years (Table 3.1).

Table 3.1 Monthly weather data, 1990 and 1991[1].

	Rainfall (mm)		Mean air temp. (°C)		Ground frost (No. below °C)	
	1990	1991	1990	1991	1990	1991
January	104.2	120.2	17.7	17.3	0	0
February	17.5	131.5	20.0	17.0	0	0
March	182.1	28.7	18.0	16.8	0	0
April	66.3	162.7	14.3	13.1	1.0	0.7
May	92.1	80.3	11.9	11.4	1.3	1.3
June	124.8	83.1	9.3	8.6	2.3	3.0
July	85.0	93.5	8.9	7.8	2.3	3.5

[1] Data recorded 2 km from trial site at AgResearch Grasslands, Palmerston North. Relative humidity was ranged from 72-86% from late summer through to winter.

As the cobs lost moisture they declined from an upright position; this began in May and by June around 75% of the cobs had declined (Plate 3.2). Around 10% of cobs developed loosening of the husk and became exposed at the tips (particularly cultivars 3591 and 3709), and this began in late June/July. Exposed seeds were bleached but not visually moulded (Plate 3.3), despite the fact that husks carried field fungi, especially *Cladosporium*, *Alternaria* and *Epicoccum* spp. (Plate 3.4). Kernels with streaks (symptoms of *F. moniliforme* or *F. subglutinans* -Koehler, 1942, 1959) were not observed.

No symptoms of stalk rot, or signs of *Fusarium* growth on stems, leaves or cob husks were detected in the field except that late in July exposed shanks began rotting, resulting in cob butt mould (Plate 3.4). However, when bundles of stems (including leaves) were left outside until the following summer (December), mycelium and sporodochia of *Fusarium* spp. were observed, and subsequently many stalks showed perithecia of *G. zeae* (Plate 3.5) and one stalk showed perithecia of *G. subglutinans* on the nodes of the outer stems only of all four cultivars (Plate 3.6). After maturing, ascospores were discharged in January 1994 (Plates 3.5 and 3.6).

3.3.2 Seed germination and vigour

Seeds were not germination tested before drying in 1990, but in 1991, seeds from all four cultivars harvested in April, May, June and July had a germination of over 90% (Table 3.2). Germination for the April harvest was 51% or lower for the four cultivars (Table 3.2), because of the presence of a high percentage of fresh ungerminated seeds. After drying in both years, germination was between 86-99% for all cultivars at all four harvest dates (Table 3.3). Most of the dead seeds were covered by mycelium of *F. graminearum* (Plate 3.7 - p. 100).

Conductivity did not differ significantly among cultivars in either season or among harvest dates (Table 3.4).

Plate 3.2 Maize cobs in a declined position.

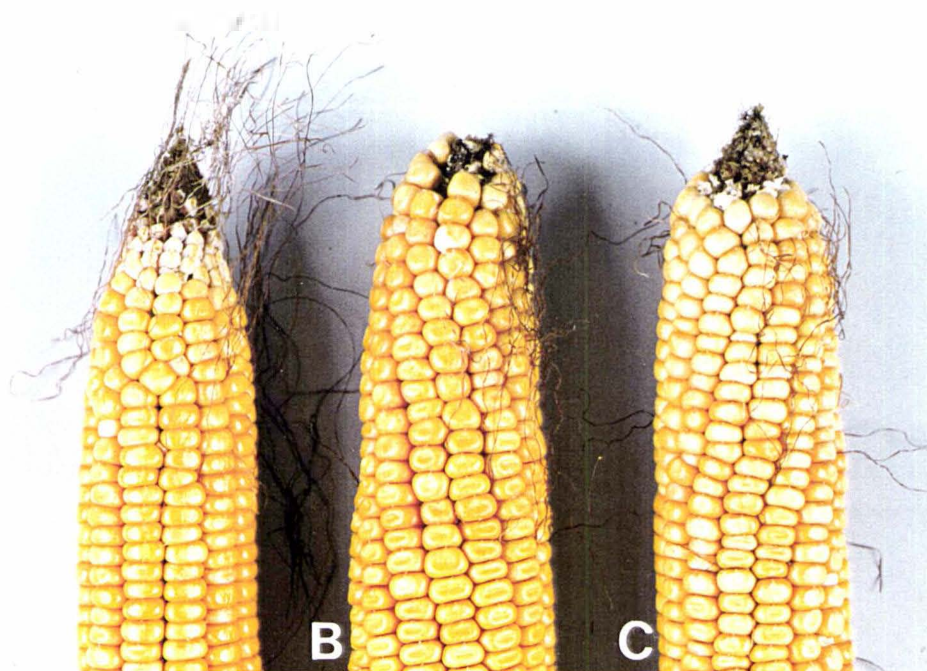


Plate 3.3 Dehusked maize cobs showing bleached tip kernels without *Fusarium* mycelial growth (B & C); that which had tip cover showing a mouldy tip (A).

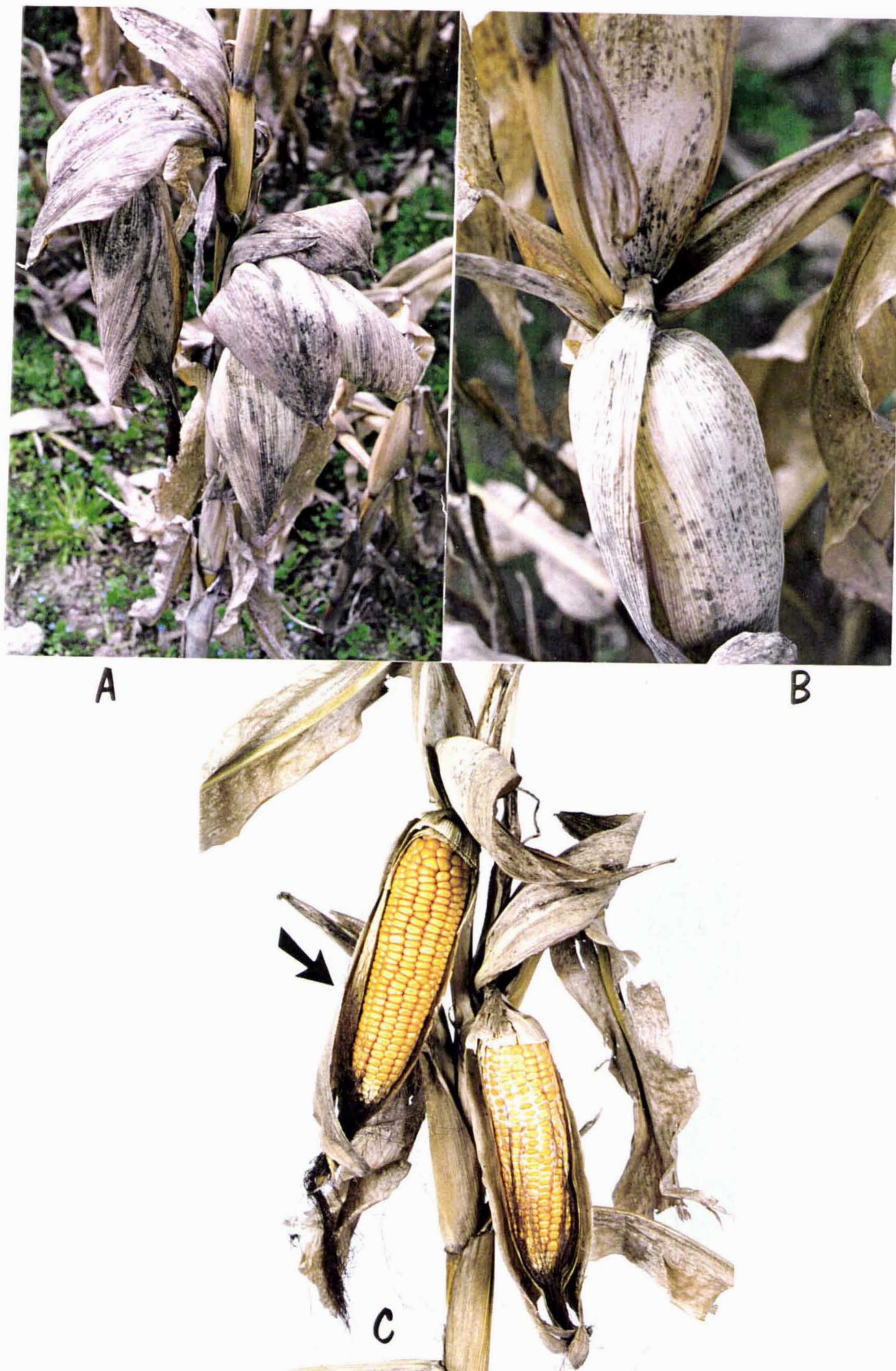


Plate 3.4 Maize cobs showing field fungal growth on husks (A & B); cobs with exposed shank (B & C); cob with mainly butt moulding (C), mould-free cob (C, see arrow).

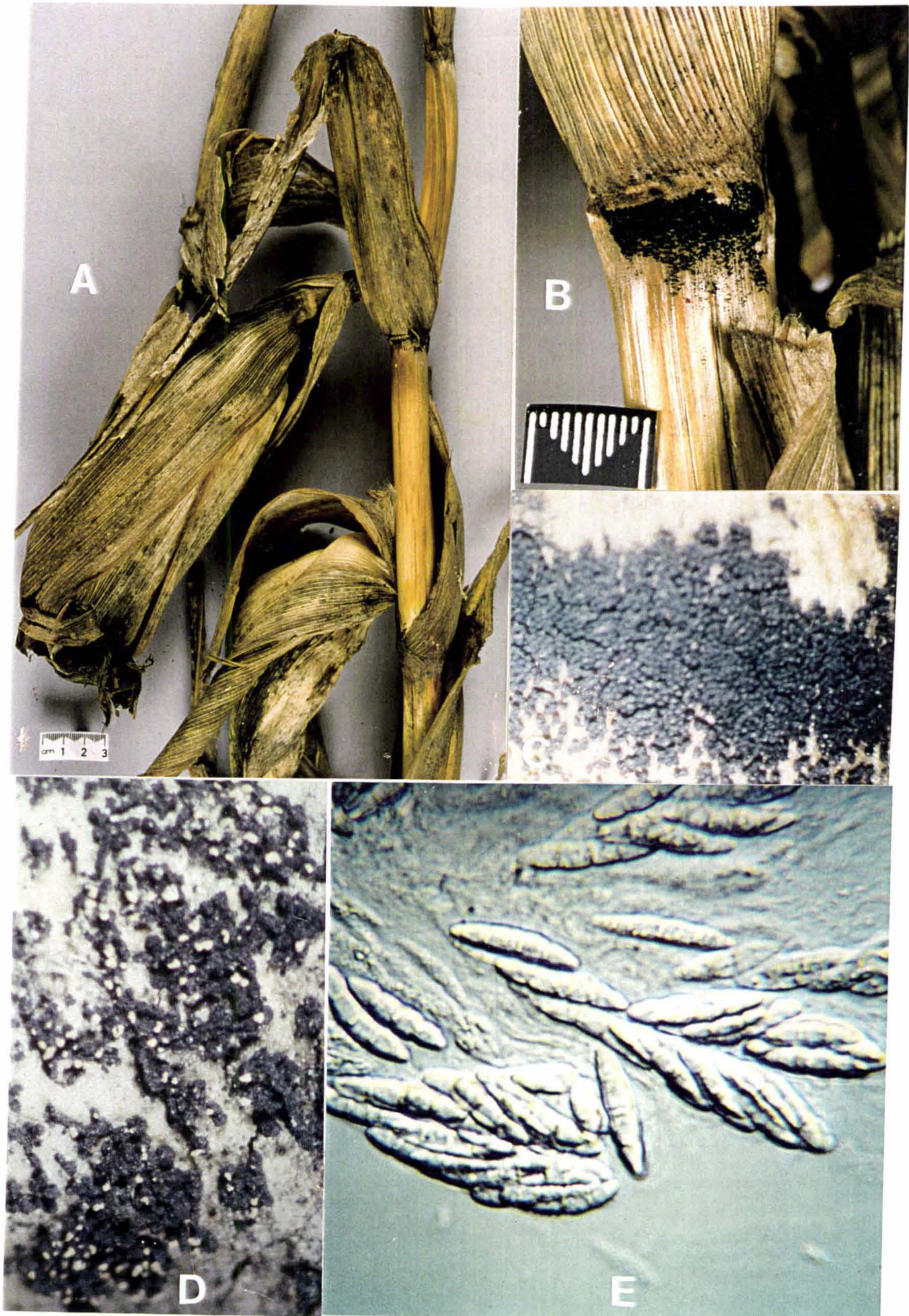


Plate 3.5 Perithecia, asci and ascospores of *G. zeae*: perithecia on a maize stalk nodes (A, B & C); perithecia discharging ascospores (D); asci and ascospores (E); (C and D x 10; E x 750).

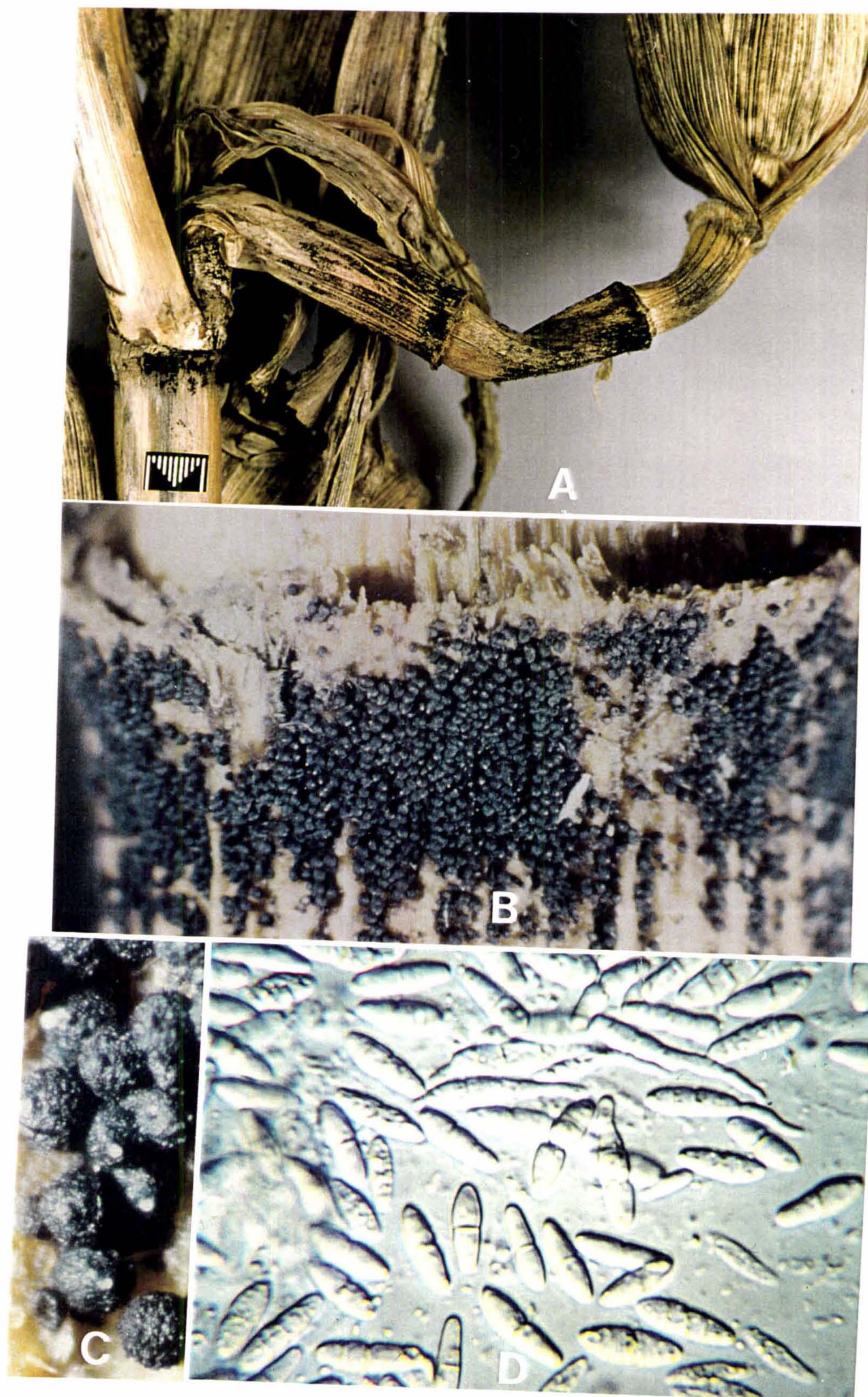


Plate 3.6 **Perithecia and ascospores of *G. subglutinans*: perithecia on a maize stalk (A & B); perithecia discharging ascospores (C); ascospores (D); (B x 10; C x 40; D x 750).**

Table 3.2 Normal, abnormal and ungerminated seeds after a germination test before drying of seeds harvested in 1991 (%).

Cultivar	Date of harvest	Seedlings		Ungerminated seeds	
		Normal	Abnormal	Dead	Fresh ungerminated
3551	April	17	2	7	74
	May	100	0	0	0
	June	96	1	3	0
	July	97	2	1	0
3591	April	37	1	5	57
	May	100	0	0	0
	June	93	3	4	0
	July	98	2	0	0
3709	April	51	1	2	46
	May	97	2	1	0
	June	93	7	0	0
	July	96	2	2	0
3475	April	50	0	2	48
	May	97	1	2	0
	June	97	3	0	0
	July	96	2	2	0

Table 3.3 Normal, abnormal and dead seeds from germination tests of seeds harvested in 1990 and 1991 after drying (%).

Cultivar	Date of harvest	1990			1991		
		Normal	Abnormal	Dead	Normal	Abnormal	Dead
3551	April	99	1	0	99	1	0
	May	96	3	1	99	1	0
	June	93	2	5	96	3	1
	July	86	6	8	95	1	4
3591	April	97	3	0	98	2	0
	May	96	2	2	99	1	0
	June	93	3	4	93	4	3
	July	92	1	7	94	3	3
3709	April	98	1	1	99	1	0
	May	95	1	4	99	1	0
	June	92	2	6	96	3	1
	July	93	1	6	96	1	3
3475	April	97	1	2	99	1	0
	May	98	0	2	98	2	0
	June	95	1	4	94	5	1
	July	96	1	3	94	3	3

Table 3.4 Conductivity of leachates (mean reading) from maize seeds harvested in 1990 and 1991 from April to July ($\mu S\ cm^{-1}g^{-1}$).

Date of harvest	1990				1991			
	3551	3591	3709	3475	3551	3591	3709	3475
April	1.9	2.3	2.6	2.0	3.3	3.0	3.0	2.8
May	2.1	2.2	2.8	2.0	2.3	2.4	2.3	2.1
June	2.8	3.0	2.8	1.9	2.1	2.4	1.8	1.8
July	3.6	3.2	3.2	3.1	2.0	1.9	1.6	1.7
LSD $P < 0.05$	NS	NS	NS	NS	NS	NS	NS	NS

3.3.3 Occurrence of *Fusarium* spp. and seed infection

The percentage of dehusked cobs showing visual symptoms of *Fusarium* mycelial growth (mould) increased as the harvest was delayed and was greater in 1990 (range 0-80%) than in 1991 (range 0-26%) (Table 3.5). In 1990 cultivar 3551 had low levels of cobs showing *Fusarium* mycelial growth at the April and May harvests, but this rapidly increased for the latter two harvests. Nearly one third of cobs were infected for the other cultivars at the May harvest (Table 3.5), but while there was little further increase for cultivar 3475, cultivars 3591 and 3709 had around 80% of cobs infected by the July harvest. The percentage of cobs infected was much lower in 1991 (Table 3.5), and did not differ greatly among the four cultivars. Individual cob moulding was also less severe in 1991 than in 1990.

Table 3.5 Percentage of cobs showing visual symptoms of *Fusarium* mycelial growth (mould).

Date of harvest	1990				1991			
	3551	3591	3709	3475	3551	3591	3709	3475
April	0	38	27	33	0	0	5	0
May	6	50	50	14	6	26	16	26
June	44	60	68	40	9	7	1	14
July	68	76	80	40	8	20	10	20

The symptoms of this mycelial growth were commonly a thick, white growth with a pinkish tinge, in the top third of the cobs, usually not extending beyond the middle of the cob (Plate 3.1), although in the July harvest there was an increase in butt infection, and cultivar 3475 often had sparse mycelium (apparently not of *F. graminearum*) covering the whole cob. Seed rotting (decay or discolouration) was not common, but around 2-6% of cobs had rotted seeds at the cob tip (Plate 3.8).

Plate 3.7 Germinating seeds and a dead seed in a germination test showing *F. graminearum* infection (dead seed) and secondary infection (seedlings covered by mycelium).

Plate 3.8 Cobs showing *Fusarium* mould and rotten or discoloured seeds at the tips (A & B); cob without rotten or discoloured seeds (C).

For each cultivar and in both years, the percentage of seeds carrying *Fusarium* spp. increased as harvest was delayed (Table 3.6). In 1990 the mean percentage of seeds carrying *Fusarium* spp. for all four cultivars was 26%, 39%, 70% and 82% for the April, May, June and July harvests respectively, while the corresponding levels for 1991 were 1%, 9%, 31% and 40% respectively. With the possible exception of cultivar 3551 at the April 1990 harvest, there appeared to be few major differences among cultivars for the percentage of seeds carrying *Fusarium* spp. (Table 3.6).

Fusarium graminearum was the species detected most often in all cultivars in both season (Table 3.6). The mean percentage of seeds carrying *F. graminearum* was 16%, 31%, 63% and 72% for April-July in 1990 respectively, and 0%, 6%, 23% and 30% for the same harvest months in 1991. *F. subglutinans*, *F. poae* and other *Fusarium* spp. were less commonly detected (Table 3.6). While initially all these identifications were tentative, especially for the *F. graminearum* types, isolates were later confirmed by IMI (see section 3.3.5), and thus the data presented in Table 3.6 are confirmed as belonging to those species.

3.3.4 Description of *Fusarium* spp. seed-culture colonies

From the seeds of all four cultivars harvested in 1990 and 1991, a number of different seed-culture colony types (5 days old) were detailed. These included colonies described as 'red and fluffy', 'red centre', 'red and lobed', 'cream and fluffy', 'pinks and strands', and purple and powdery/white and powdery, etc. The first four colony types were all subsequently identified as being *F. graminearum*, although some of the 'red centre' and 'red and lobed' group were *F. crookwellense* (Table 3.7). A brief description of these types of seed-culture colonies is given below.

Table 3.6 **Percentage of seeds carrying *Fusarium* species, 1990 and 1991.**

Cultivar	Date of harvest	Year of Harvest									
		1990					1991				
		F.g*	F.s	F.p	Other	Total	F.g	F.s	F.p	Other	Total
3551	April	5	3	2	0	10	0	0	1	0	1
	May	18	0	8	1	27	5	1	0	1	7
	June	59	0	3	1	63	27	6	2	0	35
	July	66	2	3	2	73	31	5	5	0	41
3591	April	16	0	2	2	20	0	0	0	0	0
	May	35	0	1	0	36	5	4	0	0	9
	June	72	2	1	1	76	22	4	1	0	27
	July	88	1	1	0	90	29	11	2	0	42
3709	April	35	2	6	0	43	0	1	0	0	1
	May	45	1	2	1	49	5	2	1	0	8
	June	71	3	3	0	77	20	7	1	0	28
	July	88	0	5	5	98	38	2	2	0	42
3475	April	7	0	18	1	29	0	0	1	0	1
	May	26	6	11	3	44	8	0	4	0	12
	June	50	2	12	0	62	21	7	4	0	32
	July	44	8	15	0	67	23	12	1	0	36

* F.g = *F. graminearum*; F.s = *F. subglutinans*; F.p = *F. poae*;
Other = *F. equiseti*, *F. decemcellulare*, *F. sambucinum* and non-sporulating mycelium thought to be of *Fusarium* spp.

Table 3.7 Relationship between cultures identified by IMI and category of seed-culture colonies from which the cultures were obtained.

Colony colour	Categories of seed-culture colonies	IMI identification number	IMI culture identification
Red	Red and fluffy	347824	*F. graminearum*
	Red and fluffy	350280	*F. graminearum*
	Red and fluffy	350279	*F. graminearum*
	Red and fluffy	350281	*F. graminearum*
	Red and fluffy	350282	*F. graminearum*
Red	Red centre	350370	*F. graminearum*
	Red centre	350385	*F. crookwellense*
	Red centre	350373	*F. crookwellense*
Red	Red and lobed	350278	*F. graminearum*
	Red and lobed	347823	*F. graminearum*
	Red and lobed	350374	*F. graminearum*
	Red and lobed	347831	*F. graminearum*
	Red and lobed	347832	*F. graminearum*
	Red and lobed	350369	*F. crookwellense*
Cream	Cream and fluffy	350371	*F. graminearum*
	Cream and fluffy	- *	*F. graminearum*
Pink	Pink and strands	350283	*F. subglutinans***
	Pink and strands	347833	*F. subglutinans*
	Pink and strands	350284	*F. subglutinans*
Purple	Purple and powdery	347825	*F. poae*
	Purple and powdery	347826	*F. poae*
	White and powdery	347827	*F. poae*
	Creamish and powdery	350372	*F. poae*
	White and autolysed mycelium	347829	*F. poae*
White	Deep centre	347830	*F. equiseti*
	White and crusty	350286	*F. equiseti*

* Identified in our laboratory following breakage of culture bottle in transit
** Identified as *F. moniliforme* var *subglutinans*

1. *F. graminearum*: 'red and fluffy' type (Plate 3.9)

A red, large colony with dense floccose, fast growing, fluffy mycelium with an even edge. The mycelium typically was brownish orange around the seed, had a purple tinge, and covered the whole seed. This type of mycelium was associated with dead seed. The petri dish was covered in 5-6 days but was not usually sporulating. On the reverse side of the petri dish the pigment was deep red.

2. *F. graminearum*: 'red centre' type (Plate 3.10)

A colony with a conspicuous red centre but this centre generally devoid of mycelium cover. Mycelium was moderately fast growing and formed a medium to large colony with a generally big edge zone (area of mycelium between the colony edge and around the seed) and an even edge. Mycelial hyphae were red in the centre but purplish pink at the periphery. By 5 days usually there was no sporulation apparent although some sporulation could be found in some colonies when a stereobinocular microscope was used. Reverse side was deep red in the centre and pink to pale pink in the periphery.

3. *F. graminearum*: 'red and lobed' type (Plate 3.11)

Colony usually small to medium and slow growing; red to pale red, or pink to very pale pinkish cream. Some colonies were red, orange and brown with pigments irregularly intermixed. Usually the colony had lobed edges, lobing ranging from compact inconspicuous overlapping to regular obvious fronts or to irregular spreading ("scattered") formless fronts. Some lobed colonies had zonation of growth, other had edges visually similar to typical bacterial growth. Mycelial growth varied from short to scanty or almost nil. In some cases when no mycelial growth occurred, the surface of the colony was shiny with or without sporulation, while a few colonies were covered in sheets of sporodochia with or without forming a ring as a response to light

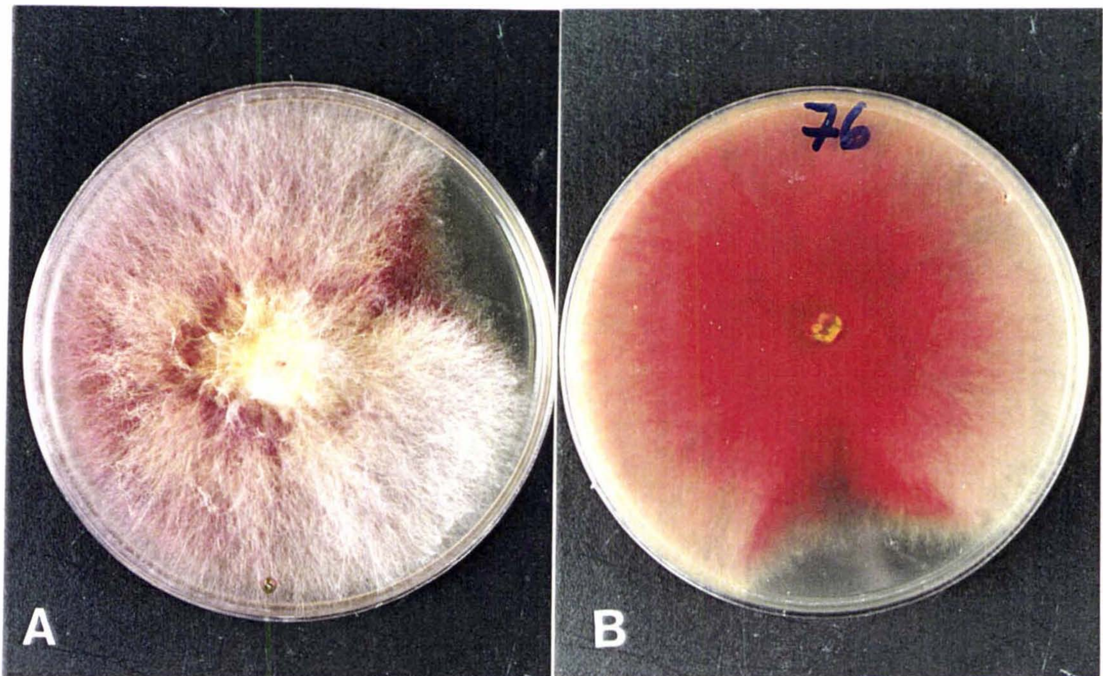


Plate 3.9 *F. graminearum*; 'red and fluffy' type. A, top view; B, reverse side.

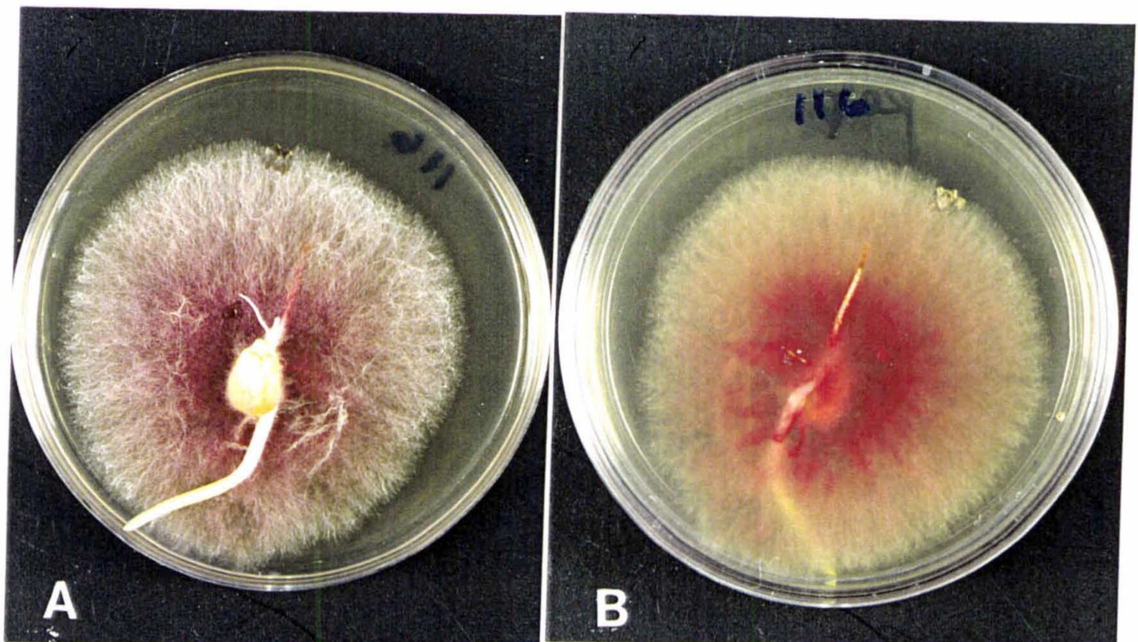


Plate 3.10 *F. graminearum*; 'red centre' type; A, top view; B, reverse side.

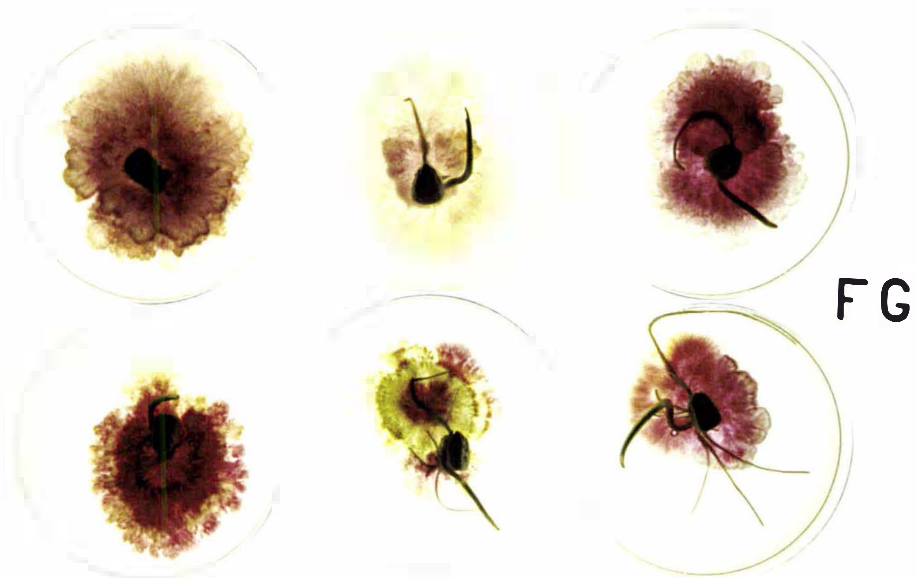


Plate 3.11 *F. graminearum*: 'red and lobed' type; Various morphological shapes as seen over transmitted light.

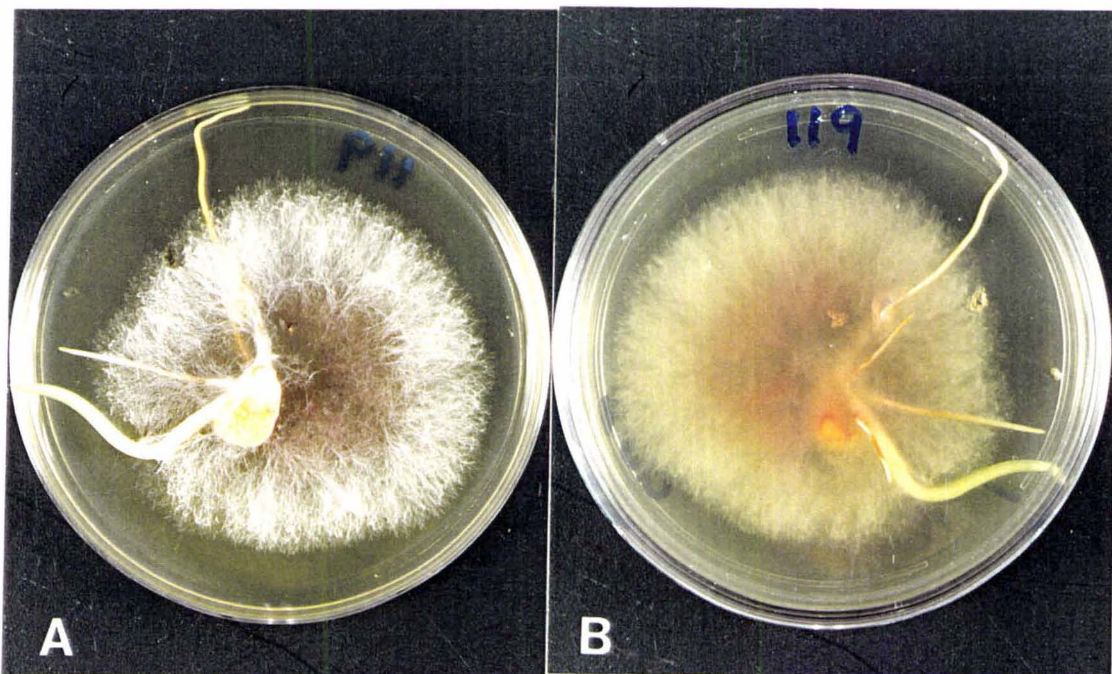


Plate 3.12 *F. graminearum*: 'cream and fluffy' type; A, top view; B, reverse side.

during examination. In some colonies "sectoring" occurred, seen as though the mycelium was streaming out of part of the colony. Reverse side was also red or red, orange and brown irregularly intermixed, the intensity of red depending on that seen from the top of the colony.

4. *F. graminearum*: 'cream and fluffy' type (Plate 3.12)

The general colony appearance was cream but the centre was pale pink to reddish pink (salmon to flesh - Rayner, 1970) while the periphery of the colony was white to cream. The colony was usually large with fast growing, dense floccose and fluffy mycelium which formed a large edge zone, ending in an even edge but the centre of the colony had scanty or no mycelium. The culture covered the petri dish in 6-7 days. The colony did not sporulate. On the reverse of the petri dish the pigment produced in the agar was salmon to pale red, although some colonies showed irregular mixtures of red in a yellow to orange background.

5. *F. moniliforme* var *subglutinans* (*F. subglutinans*): 'pink and strands' type (Plates 3.13a, 3.13b and 3.13c)

The colony had scant to almost nil mycelium, and was powdery. Mycelium was pale pinkish or whitish in colour. Seen under magnification (x 20-40) most mycelium was autolysed, leaving a few loosely hanging hyphae bearing translucent heads (false heads) which were responsible for the powdery colony appearance. The hyphal strands hung over a wet agar surface, wetness being due to a dark cream butter-like substance (sporodochial spore mass of macroconidia) radiating from the seed. Seen against the light the spore mass appeared to be running along "subterranean" hyphae radiating from the seed. On the reverse side the colony was cream to cream brown with hyphae radiating from the seed. Some colonies formed a violet to dark purple (almost black) pigment in the agar and this could be seen on both sides of the plate.

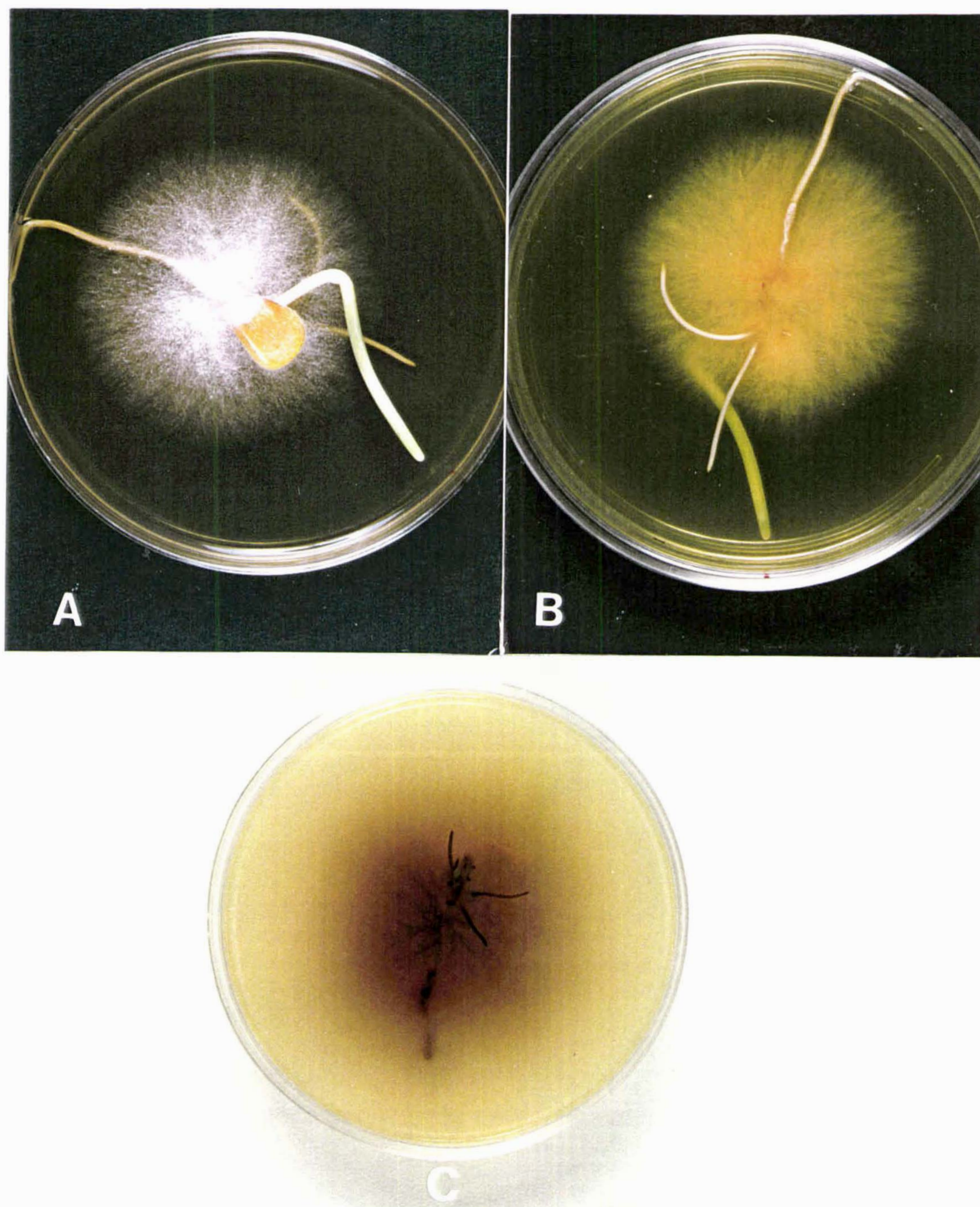


Plate 3.13a *F. moniliforme* var *subglutinans* (*F. subglutinans*); 'pink and strands' type; A, top view; B, reverse side; C, reverse side of another culture showing dark purple pigmentation.

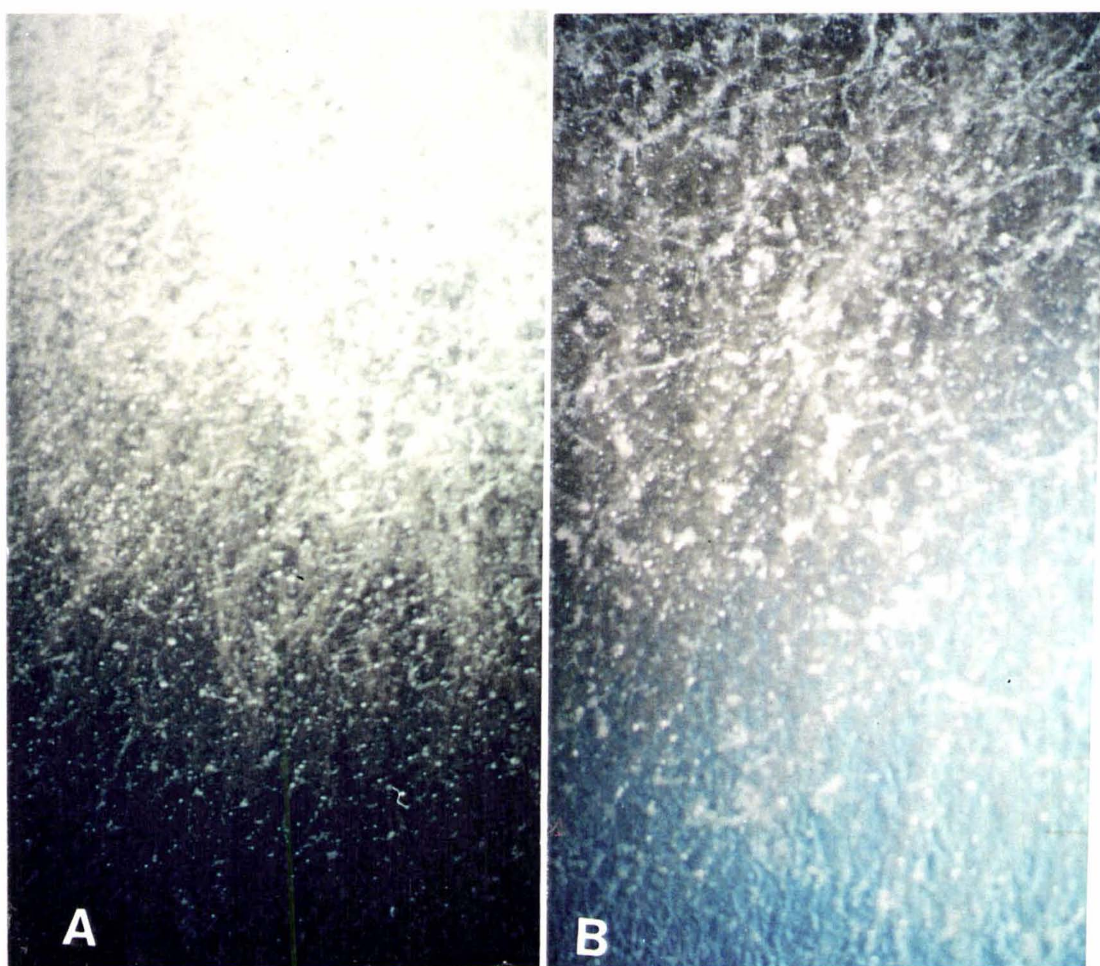


Plate 3.13b *F. moniliforme* var *subglutinans* (*F. subglutinans*); 'pink and strands' type; A, autolysed mycelium with radiating sporodochial spore mass; B, hanging hyphae, false heads (not prominent) and butter-like spore mass; (A x 15, B x 25).

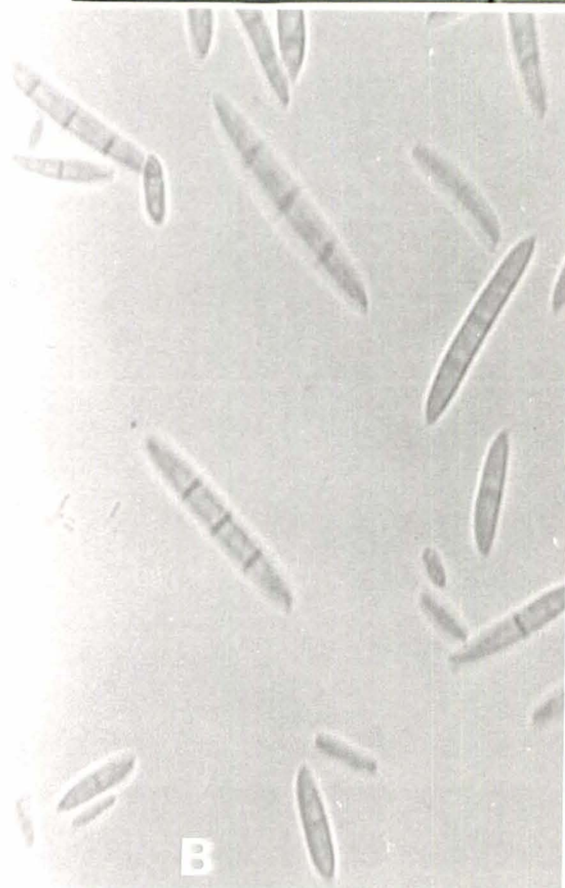


Plate 3.13c *F. moniliforme* var *subglutinans* (*F. subglutinans*); 'pink and strands' type; A, macroconidia and microconidia; B, microconidia and spindle-shaped microconidia; C, polyphialides; (A x 470; B and C x 750).

6. *F. poae*: 'purple and powdery', 'white and powdery', etc (Plates 3.14a and 3.14b)

Other colonies were described variously as 'purple and powdery', 'white and powdery', 'cream and powdery' and 'white with autolysed mycelium' (Table 3.7). These were identified as *F. poae* (Plate 3.14) and a brief description is given below.

Colony was usually puffed and generally circular. The mycelium was dense floccose and often appeared shaggy (roughened). Mycelial growth over and around the seed was scanty and more so at the position of the maize kernel root protrusion.

The colony was usually pale purplish pink or white with a purplish pink centre, the pink centre sometimes being inconspicuous unless the plate was seen on the reverse where it had a red colouration. Often reddish purple/reddish pink colonies occurred and these tended to be flat and irregular in shape, with generally sparse mycelial growth. On the reverse a red colour with a tinge of purple was irregularly diffused in a yellow orange colour, which was more to the periphery of the colony. White or creamish white colony types occurred and these were yellow or becoming deep yellow (vivid to deep yellow - Kornerup and Wanscher, 1978) with age. Some white types of colonies were flat while other showed mycelial autolysis which usually started from the edge of the colony, the flat types and those showing mycelial autolysis usually not showing colouration on the reverse side.

The most outstanding characteristic was the powdery appearance in the sparse mycelium around and over the seed. Under a low magnification (x 20) these powdery areas were found to be an aggregation of clusters of microconidia hanging on the hyphae. Under a stereobinocular microscope (x 20 - x 40) *F. poae* mycelium could be seen to occur in curls and loops, especially in the colony just behind the edge. In the white and cream types

A

B

Plate 3.14a *F. poae*: 'purple and powdery', 'white and powdery' types; A, the purple, white and cream seed-culture colonies (upper row, top view; lower row, reverse sides, respectively); B, mycelial type (left to right: woolly, curled, autolysed).

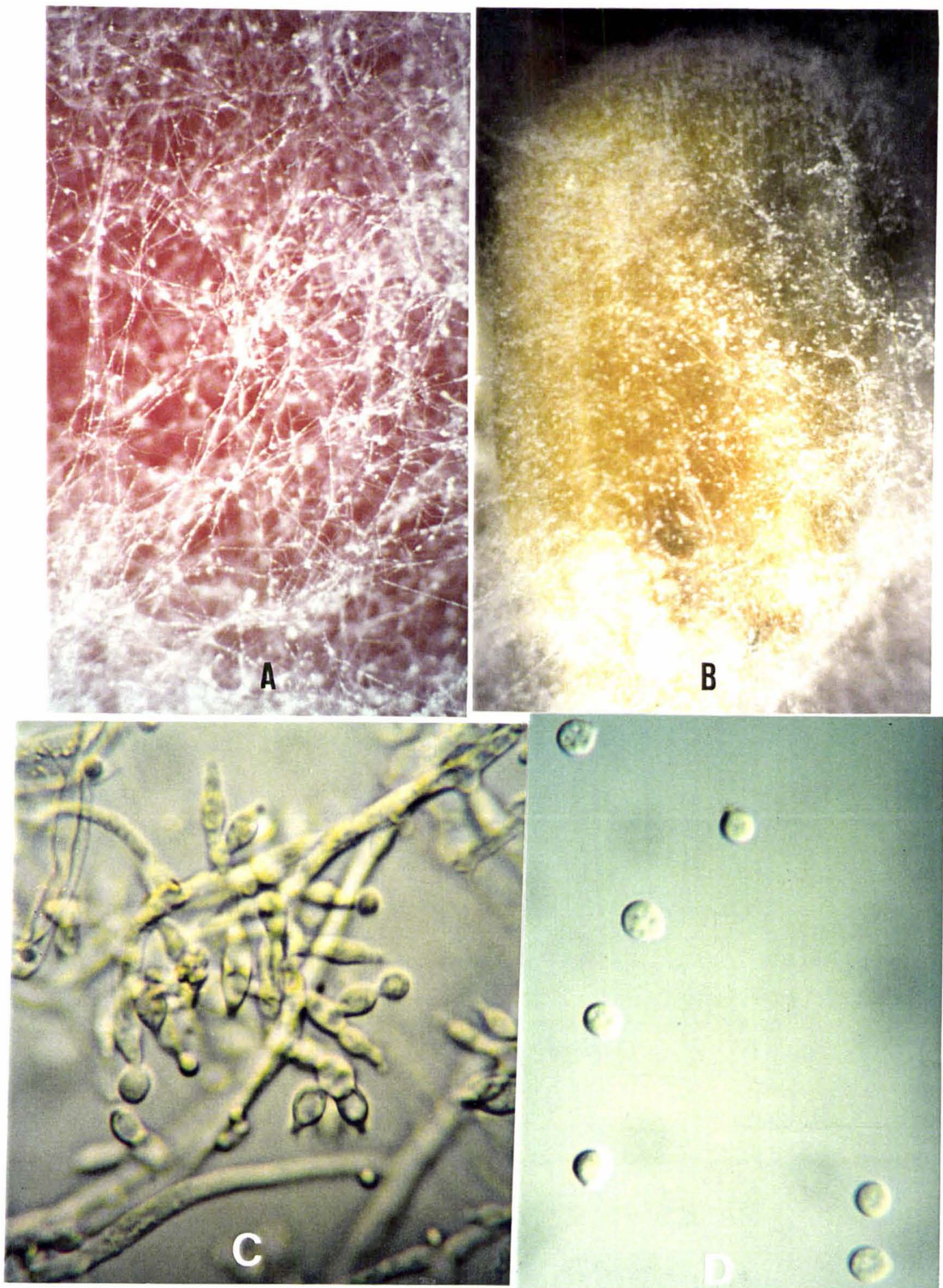


Plate 3.14b *F. poae*: 'purple and powdery', 'white and powdery' types; A & B, clusters of microconidia hanging on hyphae; C, short fat (urn-shaped) monophialides; D, globose to almost lemon shaped microconidia; (A x 20; B x 10; C and D x 940).

of colonies, the mycelium looked white and glistened like white sisal. A mouldy smell (of coriander - Pitt & Hocking, 1985) was easily detected from the colony.

A microscope slide preparation from the clusters of microconidia easily showed the characteristic globose microconidia and the urn shaped (Burgess *et al.*, 1988) conidiophores. *F. poae* macroconidia were not easily found.

Note: Identification of *F. subglutinans* and *F. poae* was confirmed in pure cultures on carnation leaf agar (see Chapter 4). A description of the colonies and conidia is produced in Appendix 3.4.

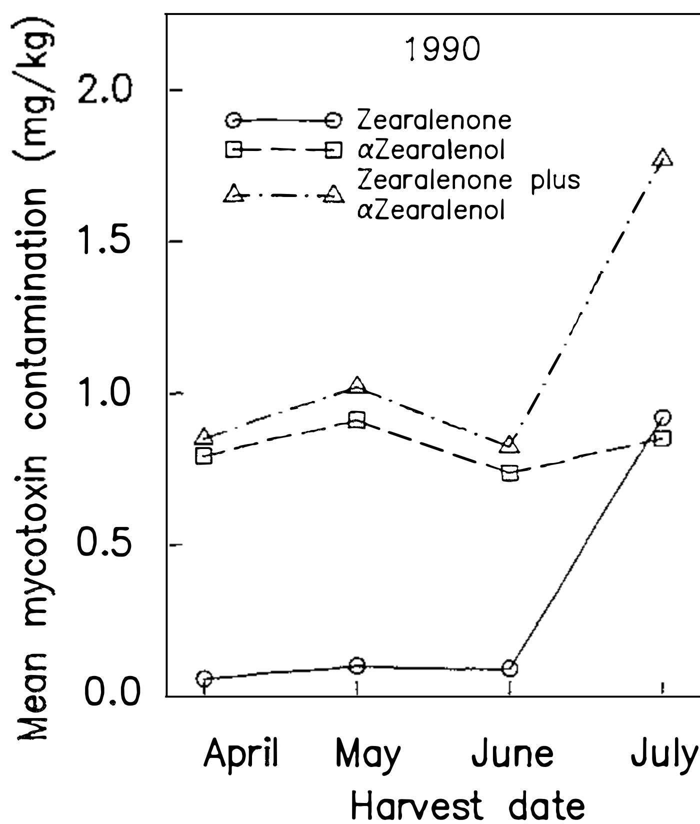
3.3.5 Identification of seed-culture colonies in pure cultures by the IMI

The summary of the report of the IMI identification of 26 *Fusarium* species isolates representing different seed-culture colony categories/types is presented in Table 3.7. Of the 14 red colonies, 11 were of *F. graminearum* and 3 were of *F. crookwellense* (Table 3.7). It was also found that all the 'red and fluffy' colonies were of *F. graminearum* and 5 out of 6 of the 'red and lobed' colonies were of *F. graminearum* but the remaining one was of *F. crookwellense*. 'Red centre' colonies were either *F. graminearum* or *F. crookwellense* (Table 3.7). All 'cream and fluffy' colonies were of *F. graminearum*. All the cultures from the 'pink and strands' type of colonies were of *F. subglutinans* while those from the 'purple and powdery' category, and from the 'white or cream and powdery' types of colonies were of *F. poae*. White colonies showing autolysis were also found to be of *F. poae*. White colonies with depressed centres (deep centre) or with a crusty appearance were found to be *F. equiseti* (not described).

3.3.6 Mycotoxin contamination of seeds

Data for types and levels of mycotoxins detected in seeds of the four cultivars for the four harvests in both years are presented in Appendix 3.3. As samples tested were not replicated and therefore no statistical analysis was possible, pooled cultivar means are prescribed in Fig. 3.2 and 3.3. Seeds harvested in 1990 were more heavily contaminated by Zearalenone, αZearalenol, Nivalenol and Deoxynivalenol (means ranging from 0.06 - 1.42 mg/kg) than seeds harvested in 1991 (means ranging from 0.0 - 0.54 mg/kg) (Appendix 3.3). There were variations in contamination levels in seeds of different cultivars harvested on the same or different harvesting dates in both years (Appendix 3.3). Except for harvested seeds of cultivar 3709, Zearalenone was almost absent in seeds harvested in 1991 and seeds of cultivar 3551 harvested in 1990 were also almost free of Zearalenone, whereas seeds of all cultivars harvested in April 1991 were free of Deoxynivalenol (Appendix 3.3). With the exception of seeds of cultivar 3709 harvested in June 1990 and seeds of cultivar 3591 harvested in July 1990, all the mycotoxin levels increased as harvest was delayed (Fig. 3.2 and 3.3). The highest increases occurred in the 1990 harvest due to Zearalenone in cultivar 3591 (0.31 mg/kg in June to 3.04 mg/kg in July), and to Deoxynivalenol in cultivar 3709 (0.33 mg/kg in June to 1.59 mg/kg in July) (Appendix 3.3). There was an obvious increase in individual mycotoxin levels or in combinations (Zearalenone plus αZearalenol and Nivalenol plus Deoxynivalenol) as the harvest was delayed in both 1990 and 1991 (Fig. 3.2 and 3.3).

In all cultivars and at most harvests in both years, levels of α Zearalenol and of Nivalenol increased earlier than those of Zearalenone and Deoxynivalenol, respectively (Fig. 3.2 and 3.3, Appendix 3.3). αZearalenol was the only mycotoxin with higher than 1.00 mg/kg (especially in cultivars 3591 and 3709) in April (1990) harvested seeds (Appendix 3.3).



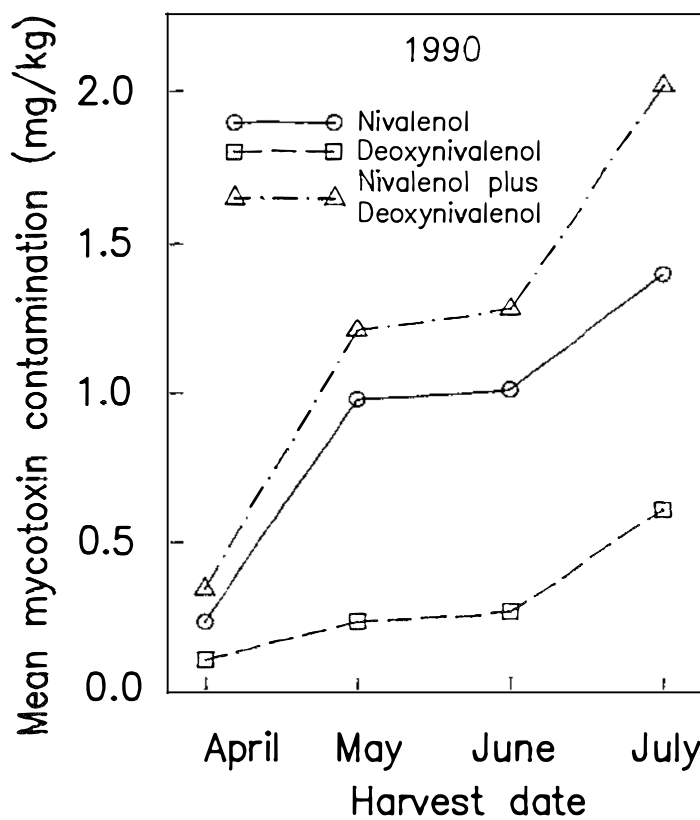
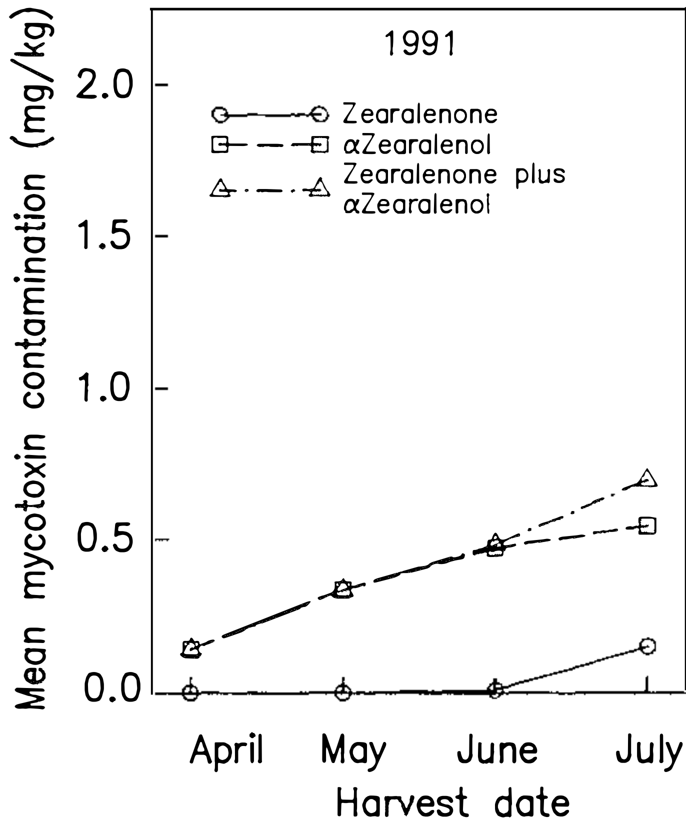


Figure 3.2 Mean level of contamination (mg/kg) by different mycotoxins for pooled cultivar data



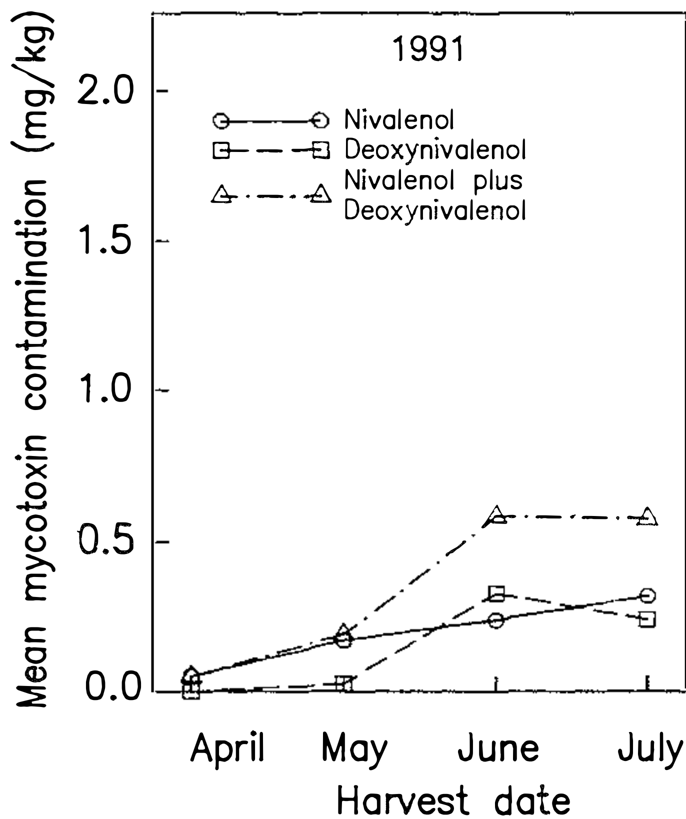


Figure 3.3 Mean level of contamination (mg/kg) by different mycotoxins for pooled cultivar data

Based on mean level contamination for all harvests for each cultivar, the data suggest that in 1990 cultivars 3591 and 3709 had higher levels of contamination by all mycotoxin (Zearalenone, αZearalenone, Nivalenol and Deoxynivalenol) while cultivars 3551 and 3475 generally had similar levels of contamination (Appendix 3.3). In 1991 contamination levels were much lower (0 - 0.45 mg/kg) and there appeared to be no differences between cultivars.

3.4 DISCUSSION

F. graminearum, *F. subglutinans* and *F. poae* were the usually detected Fusaria but *F. graminearum* was the most commonly detected species in seeds from the 1990 and 1991 harvests. These results agree with other reports from New Zealand (Hussein and Baxter, 1985; Sayer and Lauren, 1991; Sayer, 1991) and from other parts of the world; for example in Australia (Edwards, 1936, 1940, 1941; Blaney, 1986); South Africa (Marasas *et al.*, 1979, 1981; Rheeder *et al.*, 1990); USA (Kommedahl and Windels, 1981); Canada (Neish *et al.*, 1983; Teich, 1989); Peru (Logrieco, 1993); Western Europe (Maric, 1981; Bottalico *et al.*, 1989a; Kruger, 1989) and Poland (Chelkowski, 1989). However, *F. graminearum*, *F. moniliforme* and *F. subglutinans* are often the most commonly isolated *Fusarium* spp. from maize kernels (Kommedahl and Windels, 1986; Richardson, 1990), but *F. moniliforme* was not detected in seed from either season. Although Sayer (1991) detected *F. moniliforme* (at low levels) in maize seeds from Waikato and Hussein (1989) in maize from the Manawatu (1%), the Manawatu climate may be too cool for *F. moniliforme* to develop (Booth, 1971; Marasas *et al.*, 1979).

The dominance of *F. graminearum* in New Zealand maize seed lots has been previously reported (Hussein and Baxter, 1985; Sayer and Lauren, 1991; Sayer, 1991). By comparison, *F. subglutinans* and *F. poae* were of relatively minor importance; *F. subglutinans* has been suggested as being of significance in New Zealand maize crops (Fowler, 1985; Hussein and Baxter, 1985), but infection levels of < 10% which did not increase as harvest was delayed suggest that at least in this

climate and in the presence of *F. graminearum*, *F. subglutinans* is not of major concern. Sayer (1991) isolated *F. poae* from New Zealand maize seed (1-21%, depending on season) and similarly low levels of this pathogen have been reported overseas (Marasas *et al.*, 1981; Neish *et al.*, 1983; Chelkowski, 1989). It is unlikely therefore that *F. poae* is a serious pathogen of maize crops (McGee, 1988). The subsequent identification of *F. crookwellense* (from isolates originally thought to be *F. graminearum*) emphasises the difficulty in separating these two species, and raises questions about the accuracy of identification in previous studies (Burgess *et al.*, 1982; Chelkowski, 1989). However, the occurrence of *F. crookwellense* was extremely low. *F. culmorum* was apparently not detected in seed from the 1990 and 1991 harvests, which was somewhat surprising considering this species has previously been reported from New Zealand maize seed (Hussein, 1987; Sayer and Lauren, 1991; Sayer, 1991). However, Sayer (1991) recorded an incidence of 0-8% for this pathogen, and in a further unreported study of the 1990 harvested seeds and another seed lot produced at Massey University in the 1994 season, *F. culmorum* was detected, but at a level of < 5% (see Chapter 4).

The infection and colonisation of maize ears by *F. graminearum* is known to be favoured by high rainfall, high humidity and high temperatures during silking (Tuite *et al.*, 1974; Sutton *et al.*, 1980a; Sutton, 1982). The plant is most susceptible shortly after silk emergence but infection can continue during the entire silking period of up to 21 days (Tuite *et al.*, 1974), and often for 10-20 days post silking, after which the maize ears become resistant (Sutton, 1982). Rainfall of > 70-80 mm in one week during silking favours *F. graminearum* infection, while > 60 mm rain over a 10-16 day period favours epidemic development (Sutton, 1982). Infection progresses at temperatures between 15-35°C, with optimum temperatures of 25-32°C (Sutton, 1982) and it is believed that temperatures below 15°C limit further spread of the pathogen. Seed moisture contents of > 35% also allow vigorous growth of the pathogen (Sutton, 1982; Teich, 1989). It should be noted, however, that in culture *F. graminearum* is capable of slow growth at 3°C (Dickson, 1923).

Because climate data were not recorded directly at the trial site in either season, it is difficult to relate the climatic data available to the infection levels recorded, so that any discussion is therefore speculative. However, *Fusarium* infection levels were greater in 1990 than in 1991. Mid-silking occurred in late January in both season, and rainfall over the last 10 days of January was 63 mm in 1990 and 69 mm in 1991, with relative humidities of 77% and 81% respectively. Temperature during this week was around 20°C in both seasons. However, in the first three weeks of February 1990 only 11.6 mm rain fell, whereas for the same time period in 1991 108 mm rain was recorded. If rainfall was the major environmental component influencing intensity of infection, more *Fusarium* should have been recorded in 1991 than in 1990. The fact that the reverse occurred suggests that perhaps temperature is the important factor. The first three weeks of February 1990 averaged 20.9°C (mean of 20°C for the month) while in 1991 the comparable data were 17.6°C for the first three weeks (mean 17°C for the month). Temperature was 1.2°C, 0.5°C, 0.7°C and 1.1°C high for the months of April through July in 1990 than in 1991. It is likely that the critical period was during the second and third weeks of March when temperature was 20.4 and 18.4°C in 1990 cf. 16.4 and 15.4°C in 1991. Although frost occurred from April in both years, the seeds were already mature and this therefore did not affect seed quality (Rossman, 1949; Justice and Bass, 1979).

Fusarium colonisation of the maize ears starts from the tip of the cob (Manns and Adams, 1923; Koehler, 1942, 1959; Sutton, 1982), and during seed development eg starchy endosperm formation (March), fungal invasion and kernel damage can be rapid (Manns and Adams, 1923; Koehler, 1942). Once pericarp development has been completed (40-50 days after fertilisation - Aldrich *et al.*, 1976; Jugenheimer, 1976), fungal penetration becomes restricted, and the formation of the epicarp (a waxy cutin layer; Bruggink *et al.*, 1991) provides a barrier to further penetration of *F. graminearum* and other cob rotting fungi (Manns and Adams, 1923; Johann, 1935; Koehler, 1942).

While infection from external (of the kernel) inoculum may cease at this time, the pathogens are obviously capable of continued growth within the cob (ie infection levels increased as harvest was delayed), despite the fact that temperatures after late March were below 15°C (Sutton, 1982). However, as seed moisture contents were still over 35%, seed colonisation continued, possibly through invasion of the pedicel (Manns and Adams, 1923; Koehler, 1942). Koehler (1942) suggested that under these conditions, *F. graminearum* is not able to penetrate and damage seeds, but is capable of vigorous growth over and between seeds (Sutton, 1982). However, seed invasion did occur, as if the inoculum had been only surface borne, surface sterilising of seed prior to plating would have removed this inoculum source; the seed health test results show clearly that the pathogens were carried internally. This situation was explained by Neergaard (1979), who stated that if *F. graminearum* and *F. moniliforme* invasion of developing seeds takes place early in seed development, the seed is killed, resulting in seed rot. Conversely, if infection occurs when the seed is nearing maturity, only the pericarp is invaded. Unfortunately the site of the inoculum within the seed was not determined in this study, but the small number of dead seeds suggests mostly pericarp, endosperm or tip cap invasion. Both Neergaard (1979) and McGee (1986) describe the seed defence mechanism as being due to "morphological barriers". Koehler (1942) noted that *F. graminearum* was more capable of penetrating the developing and maturing maize seeds than other species, particularly *F. moniliforme/F. subglutinans*. This may explain why the latter was usually recorded at a level much lower than the former from seed lots.

Several other factors are known to predispose maize cobs to *Fusarium* infection. These include upright ears, bird and insect damage and tight husk coverage (Sutton, 1982). Neither bird damage or insect damage were important features in this study. Some cobs had tip damage following bird attack at the milk stage of seed development, and rain water ingresses from these sites may have assisted fungal infection (Attwaters and Busch, 1983). However, there was no visual evidence that bird damaged cobs had a greater degree of *Fusarium* mycelial growth. However, as cobs with bird damaged tips were not included amongst those harvested for the seed quality study, no data for seed-borne *Fusarium* levels from such seeds are available.

Similarly, insect damage (by army worm; *Mythimna separata* Wallor) was restricted to the tips of a few cobs only, and was therefore unlikely to have been a factor affecting *Fusarium* infection levels. Maize cultivars with upright cobs and tight husks, or rudimentary cobs are considered more susceptible to *Fusarium* infection than cultivars with pendant cobs and cobs with loose husks (Koehler, 1959; Tuite *et al.*, 1974; Wimmer, 1978; Enerson and Hunter, 1980b). While the cultivars used in this study had cobs with tight husk covers, they were pendant, suggesting that seeds were well protected from rain, even when harvest was delayed. However, as already discussed, even when cobs did have exposed tips, there was little visual evidence of fungal growth; this was in contrast to the observations of Koehler (1959) who reported that open cob tips lead to greater cob rotting from *F. graminearum* and *F. moniliforme*. Nevertheless, delayed harvest increased butt cob moulding, most probably due to rotting shanks (Koehler, 1942, 1959). The lack of bright colouration (red or pink - Shurtleff, 1980; McGee, 1988) of the *Fusarium* mycelium was thought to have been due to the complete covering of cobs by husks and/or insufficient sunshine (light - Zacharia *et al.*, 1956) during autumn and winter.

While *F. graminearum* and *F. moniliforme*/*F. subglutinans* have been reported to form perithecia on infected plants and on the husks of cobs in the field (Edwards, 1936; Dickson, 1956; Christensen and Wilcoxson, 1966; Shurtleff, 1980; Burgess *et al.*, 1981), perithecia (or indeed external visual symptoms) were not detected in either 1990 or 1991. This was presumably because both relative humidity, temperature and near ultraviolet light were lower than those required for perithecial formation (Tschanz, 1976; Neergaard, 1979; Sutton, 1982; Burgess *et al.*, 1988). Lacey and Magan (1991) reported that *F. graminearum* requires a relative humidity of 89-99%, and Tschanz *et al.* (1976) stated that temperatures of 15-25°C were required for the formation of perithecia, but Ye (1980, cited by Sutton, 1982) reported *G. zeae* perithecia formation at temperatures ranging from 5-35°C. The formation of perithecia and the subsequent discharge of ascospores from maize stalks collected from the field and left outside until the following summer was presumably in response to the warmer temperatures (Christensen and Wilcoxson, 1966) and sufficient near ultraviolet light (Neergaard, 1979). This suggests that ascosporic inoculum may be

unimportant in the transmission of the pathogen, as in normal agronomic practice, most maize stubble would be removed or ploughed under early in the spring following harvest. However, incomplete/poor stubble burial will leave crop debris on the soil surface where *F. graminearum* survives well (Khonga and Sutton, 1988). The very low occurrence of *G. subglutinans* perithecia recorded was in agreement with similar observations by Dickson (1956) and Shurtleff (1980), the reason for this being that some strains are heterothallic (Booth, 1971; Francis and Burgess, 1975; Nelson *et al.*, 1983; Burgess *et al.*, 1988; Kedera *et al.*, 1994).

There were no major differences in seed-borne infection levels amongst the four cultivars in either season, with the possible exception of 3551 at the April harvest in 1990. This was also the situation for the percentage of cobs showing signs of *Fusarium* mycelial growth. The reason for this initial 3551 response to *Fusarium* infection is not clear, but may be associated with the fact that this cultivar is a true dent (100% dented seeds), whereas the other cultivars contained cobs with both dent and flint seeds. Dent maize has a less dense endosperm than flint maize (Watson, 1987), and denting appears to increase surface area for water loss during seed dry down. Cultivar 3551 is considered to have a relatively fast dry down (Pioneer 1991/92), and the high placement of the cobs on the plant also gives additional advantage for fast drying. However, any possible links between these characters and susceptibility to *Fusarium* infection are yet to be determined. Certainly by the time the cultivars had all reached the recommended seed harvest moisture content of 25% (Hardacre *et al.*, 1991), there was no real evidence of differences in susceptibility to *Fusarium* infection among the four cultivars. While in 1990 cultivar 3475 had a lower (50% cf 88%) incidence of *F. graminearum* than 3591 and 3709, this difference did not occur in 1991. In the USA, cultivar 3475 is considered to be resistant to *G. zeae* (Anon., 1991/92), but the data recorded from this work do not support that claim.

While seed-borne inoculum levels were high in 1990, *Fusarium* spp. had little effect on seed quality (germination and vigour) in either season. *F. graminearum* and *F. subglutinans* are considered to be weak pathogens under favourable (ie 24°C) seed

germination conditions (Dickson, 1923; Edwards, 1936; Shurtleff, 1980; Burgess *et al.*, 1981; Jones and Clifford, 1983; Kommedahl and Windels, 1986), but cause damage (seed rot and seedling blight) to the germinating seed under cold (8-20°C) wet soil conditions (Dickson, 1923, 1925; Dickson and Holert, 1926; Shurtleff, 1980). As with many seed-borne pathogens, germination of *Fusarium* infected seed is not usually seriously affected (Rheeder *et al.*, 1990) because besides unfavourable germination conditions, only those kernels seriously infected early in the stages of seed development are damaged (seed rot) as the embryos are likely to be infected (Edwards, 1941; Koehler, 1942; Neergaard, 1979). It is also likely that as with most other seed-borne pathogens *Fusarium* spp. do not kill seeds, so that their transmission process to the next crop is not impaired. In this study, the small percentage of seeds that did not germinate (ie were dead) were covered with red mycelial growth typical of *F. graminearum* (Plate 3.7), suggesting that these were seeds which had become infected at the cob tips during the early stages of seed development.

Similarly, the low (Bruggink *et al.*, 1991) and relatively constant (for cultivar x harvest date) conductivity data recorded indicate little effect of *Fusarium* infection on seed deterioration. Pericarp integrity is known to affect maize seed conductivity results (Herter and Burris, 1989); these data indicate no pericarp damage as a result of *Fusarium* infection, a result which agrees with data presented by Loeffler *et al.* (1988) for *Phomopsis* spp. and *Cercospora kichuchii* (Matsu and Tomoyasku) infection of soybean (*Glycine max*) seed.

The mycotoxins detected in the maize samples (ie Zearalenone, αZearalenol, Nivalenol and Deoxynivalenol) were those commonly associated with natural *F. graminearum* infection in maize (Caldwell and Tuite, 1974; Neish *et al.*, 1983; Hussein *et al.*, 1989; Bottalico *et al.*, 1989a; Visconti *et al.*, 1990; Perkowski *et al.*, 1991; Lauren *et al.*, 1991) or from *F. graminearum* inoculated maize crops (Miller *et al.*, 1983; Bennett *et al.*, 1988). All these mycotoxins have been implicated with mycotoxicoses in animals and humans (Wyllie and Morehouse, 1978; WHO, 1979; Hagler *et al.*, 1979; Marasas *et al.*, 1984; Joffe, 1986; Marasas and Nelson, 1987; Gilbert, 1989). The mycotoxin Zearalenone, Nivalenol and Deoxynivalenol have

been detected in maize grown in New Zealand (Hussein *et al.*, 1989; Lauren *et al.*, 1991; Lauren, 1994) and Cox (1994) reported the occurrence of αZearalenol in New Zealand grown maize in addition to these other mycotoxins. Although *F. crookwellense*, *F. culmorum* and *F. equiseti* are also known to produce these mycotoxins, *F. subglutinans* and *F. poae* have not been implicated in this regard (Marasas *et al.*, 1984; Bottalico *et al.*, 1989; Visconti, 1990) although Blaney (1986) indicated that *F. subglutinans* may produce Zearalenone.

Although statistical analyses were not made on the incidence of *Fusarium* spp. (especially *F. graminearum*) and of mycotoxin levels, it is clear in this study there was a relationship between *Fusarium* spp. occurrence and the mycotoxin levels in seed from the 1990 and 1991 crops. The tendency for mycotoxin levels to increase as time of harvest was delayed agrees with findings by Caldwell and Tuite (1974), Miller *et al.* (1983) and Neish *et al.* (1983) and also supports similar suggestions by Lauren (1994), who reported that in 1992 in New Zealand, maize samples showed lower contamination by Nivalenol plus Deoxynivalenol in crops harvested before mid-June, but that in 1993 contamination levels began increasing after mid-May. Lauren (1994) did find cultivar differences for mycotoxin contamination.

The detection of low levels of Zearalenone in seeds harvested in 1990 and 1991 is in conformity with the findings of Caldwell and Tuite (1974), Neish *et al.* (1983), Miller *et al.* (1983) and Bennett *et al.* (1988) that Zearalenone contaminations do not usually increase considerably under field conditions compared to, for example, Deoxynivalenol.

Bennett *et al.* (1988) demonstrated that "trichothecenes and Zearalenone occur almost exclusively in heavily damaged and moderately damaged corn kernels and underlying cobs" at the tip of the cobs. Caldwell and Tuite (1974) also concluded that mycotoxin production was greatest in kernels at the tip of the ears where *G. zeae* infection is most severe. In the present study late (June and July) harvests in 1990 showed a high percentage of mouldy cobs, including those with rotten seeds at the tip, and it was mostly during the June and July harvests that increases in mycotoxin

contamination was highest. Rotten seeds from the cobs correspond to those obtained from Zone I according to the cob part characterisation by Bennett *et al.* (1988), Wicklow *et al.* (1990), or damaged kernels (Blaney, 1986; Perkowski *et al.*, 1991), while mouldy cobs appear to correspond to cob Zone II (Bennett *et al.*, 1988; Wicklow *et al.*, 1990), and since seeds from Zones I and II produce Deoxynivalenol or Zearalenone up to 100 or 4000 times (μg/g) higher, respectively, than those from the butt (Bennett *et al.*, 1988; Perkowski *et al.*, 1991), the possible effects of seed from rotted or mouldy cobs are potentially dangerous. Also, since Bennett *et al.* (1988), Wicklow *et al.* (1990) or Perkowski *et al.* (1991) found nil to insignificant levels of Zearalenone and Deoxynivalenol in seeds from the butt end of the cobs, it is suggested that perhaps most of the mycotoxins in this study resulted from the mouldy seed, and therefore variation in the number of rotten seeds could have been responsible for the variations in mycotoxins recorded in different cultivars.

Maize contamination by Nivalenol and αZearalenol is significant with regards to the utilisation of maize based products. Nivalenol is considered to be about ten times more toxic than Deoxynivalenol (Ueno, 1984; Agnew *et al.*, 1986). This potential health hazard aspect has implications in the feeding of maize grain to animals (sows are particularly susceptible) and in the human consumption of corn based breakfast foods and snacks. If it is accepted that 0.4 mg/kg of combined Nivalenol and Deoxynivalenol is the maximum mycotoxin contamination level in animals (especially pigs) and human health as suggested by Lauren (1994), 35 of the 43 samples tested for these mycotoxins in the present study were a potential health hazard. Since αZearalenol has been found to be about three to four times more oestrogenic than Zearalenone (Hagler *et al.*, 1979), by the same argument, if a Zearalenone plus αZearalenol level of 1 mg/kg of seed is potentially damaging to animals, especially sows, by causing an oestrogenic response (Agnew *et al.*, 1986, citing Tanaka, 1985) or with as little as 0.02 mg/kg Zearalenone having been shown to exhibit oestrogenic effects in pigs as suggested by Gilbert (1989), then at least 24 of the 43 samples tested had total Zearalenone plus αZearalenol in excess of these reported for overseas guidelines.

The problem of mycotoxins is not new in New Zealand. It has, however, come to prominence since 1987 as a result of the defined extent of contamination in New Zealand produced grains by Lauren (1994) and his co-workers at HortResearch, Ruakura Research Centre in Hamilton, New Zealand. Their work, which is supported by the present results, suggests there is a need to build an awareness of possible mycotoxin contamination into production and cultural practices. This may impact on aspects such as site selection, cultivar selection, site preparation, planting date, plant population and harvesting date of maize crops grown for grain consumption purposes. To date, little attention has been given to eliminating the source of mycotoxin contamination by strategies designed to minimise it in maize prior to harvest.

3.4.1 Identification of *Fusarium* spp.

From the culture identification report from IMI, it became apparent that it was possible to visually identify certain seed-culture colony categories/types of *F. graminearum*, namely the 'red and fluffy' and the 'cream and fluffy' types, but that it was difficult to differentiate the 'red centre' type of colonies and 'red and lobed' type colonies of *F. graminearum* from those of *F. crookwellense*. It was surprising that one culture out of 6 from the 'red and lobed' seed-culture colonies was identified as *F. crookwellense* because in a later study (see Chapter 4) all cultures from the 'red and lobed' seed culture colonies were identified as *F. graminearum* since they formed perithecia of *G. zeae* on carnation leaf agar (see Chapter 4). It was, however, not surprising that cultures from the 'red centre' type of seed-culture colonies were not differentiated since the macroconidia of *F. crookwellense* are easily confused with those of *F. graminearum* and the pure cultures of both on PDA are almost identical (Nelson *et al.*, 1983; Burgess *et al.*, 1988). In later studies (see Chapter 4) *F. graminearum* 'red centre' type of seed-culture colonies still could not be differentiated from *F. crookwellense* colonies on MA. However, it is possible to identify seed culture colonies of *F. subglutinans* from those of *F. poae*, and colonies of these two species were visibly different from those of *F. graminearum*, or *F. graminearum*/*F. crookwellense*.

The identification of *F. subglutinans* seed-culture colonies did not present difficulties on MA. According to Nelson *et al.* (1983) some of the variants of *F. subglutinans* in pure culture are "pionnotal forms in which the aerial mycelium is suppressed and replaced by sheets of macroconidia that give the culture a yellowish wet appearance". This description fits the types of seed-culture colonies of *F. subglutinans* observed in this study. This characteristic, together with the formation of "subterranean" radial strands in the agar, particularly as seen against light, as well as the powdery nature of the aerial mycelium, made it possible to readily identify *F. subglutinans* seed-culture colonies. Another diagnostic feature was that *F. subglutinans* seed-culture colonies were distinct. In seed-culture colony plates in which many seeds were infected with the fast growing species such as *F. graminearum*, *F. crookwellense*, the colony of *F. subglutinans* was often conspicuous since it did not intermix with these other species or with *F. poae*. Microscope slide preparations made directly from seed culture colonies showed a mixture of macroconidia and microconidia and polyphialide conidiophores, with the later two being satisfactorily typical of *F. subglutinans*.

F. poae seed-culture colonies were generally easy to identify, particularly the purplish pink colonies which were distinct. This was due to the cultural morphology described earlier and the easily identifiable microconidia and conidiophores. *F. poae* is known to be stable in culture (Nelson *et al.*, 1983) and this could account for the relatively low variations in seed-culture colonies. The red pigment in the agar (reverse side) was not found to be helpful in the identification of *F. poae* when seed-culture plates had many seeds infected by red coloured Fusaria, eg *F. graminearum*, because the pigment from all the infected seeds looked identical and tended to mix. However, in the colonies that appeared white in early stages, 6-7 days seem to be appropriate for sporulation and for red or yellow pigment colour to increase in intensity.

3.4.2 Need for verification of identification of *F. graminearum*

Because of the difficulty in visually differentiating the seed-culture colony categories/types of *F. graminearum* from those of *F. crookwellense*, it was considered necessary to carry out a separate study for the identification of *F. graminearum* and its related *Fusarium* spp., namely *F. crookwellense*, *F. culmorum* and *F. sambucinum* (Nelson *et al.*, 1983; Burgess *et al.*, 1988) (see Chapter 4).

CHAPTER FOUR

IDENTIFICATION AND DESCRIPTION OF SEED-BORNE FUSARIUM SPECIES

4.1 INTRODUCTION

A fungus is said to be seed-borne if it is established and can be detected in, on, or with the seed (Neergaard, 1979; Agarwal and Sinclair, 1987; McGee, 1988). Pathogens are associated with seed in two ways: as infection or infestation. Infection implies that the pathogen is carried internally, embedded in seed tissue. When the pathogen is carried externally and passively by the seed, it is an infestation or contaminant (Agarwal and Sinclair, 1987).

The assessment of incidence (presence or absence) of a seed-borne organism is called seed-health testing (Neergaard, 1979; Agarwal and Sinclair, 1987; ISTA, 1993). The object of a seed health test is to determine the health status of the seed sample, and by inference that of the seed lot, thus gaining information that can be used to compare the value of different seed lots (ISTA, 1993).

Agarwal and Sinclair (1987) stated that the selection of a method for detection of seed-borne pathogens depends on the purpose of the test results, and that in general, the method must be simple and quick, results must be reproducible, and identifying characteristics of a pathogen should be recognisable with ease and certainty.

Examination of seeds after incubation is one of the methods used in seed-health testing (ISTA, 1993), and two methods, ie the blotter and the agar plate method are recommended (Neergaard, 1979; ISTA, 1993). Seed pre-treatment or

surface disinfection is recommended before testing of seeds, especially by the agar plate method, to avoid spread of secondary organisms or saprophytes, which usually occur on the surface of the seeds as natural contaminants, and to detect pathogens and not contaminants (Neergaard, 1979; Agarwal and Sinclair, 1987).

In the blotter method, the basic principle is to provide a high level of relative humidity and optimum light and temperature conditions conducive for fungal development on the seeds. Seeds are usually examined within 7-8 days using a stereobinocular microscope with up to 50 or 60 x magnification (Neergaard, 1979). Fungal identification is based on mycelial growth, length, colour and arrangement of conidiophores; colour, size, septation and arrangement of conidia on conidiophores; and the production and appearance of other fruiting structures such as acervuli, pycnidia, sporodochia and perithecia (Nath *et al.*, 1970; Neergaard, 1979; Agarwal and Sinclair, 1987). A stereo-microscope enables observation of the fungi as developed on the host *in situ*, undisturbed, in a condition of natural growth (Neergaard, 1979). Nath *et al.* (1970) made an attempt to establish workable diagnostic colony characters of different *Fusarium* spp. on the seed using the blotter method, and identified a range of seed-borne *Fusarium* spp. from various types of seeds, including maize. The *Fusarium* species identified included *F. avenaceum*, *F. chlamydosporum*, *F. culmorum*, *F. dimerum*, *F. equiseti*, *F. graminearum*, *F. longipes*, *F. moniliforme*, *F. nivale*, *F. poae*, *F. semitectum* and *F. solani* (Nath *et al.*, 1970).

The blotter method is still recommended for identification of *Fusarium* species (Mathur, S.B., pers. comm.). However, the blotter method has some disadvantages. Slow growing fungi may be overgrown by more vigorous ones; also when fungi fail to sporulate they have to be subcultured onto an agar medium for later identification.

With the agar plate method, two nutrient agar media are recommended, namely, Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) (Neergaard, 1979; ISTA, 1993). In the agar plate method, identification of fungi is based on growth and colony characteristics on the agar medium. The principal method of

recording is macroscopic examination of fungal colonies, which should be identified and counted visually while checking both sides of the plate. MEA has been used to detect seed-borne fungi of cereals and flax (Musket and Malone, 1941; Neergaard, 1979; Agarwal and Sinclair, 1987). According to Neergaard (1979) the following *Fusarium* spp. can be detected by the agar or the blotter method: *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. moniliforme*, *F. nivale* (*Microdochium nivale*), *F. oxysporium*, *F. semitectum* and *F. solani*.

There is, however, a paucity of information regarding characters to be used for identifying *Fusarium* to species level when the agar plate seed health test method is used, and especially for Malt Extract Agar or Malt Agar. Burgess, L.W. (pers. comm.) observed "I am not aware of any reliable work on the identification of seed-borne *Fusarium* on Malt Agar".

Conventionally, especially in taxonomic studies, *Fusarium* species are identified in pure cultures, each started from a single germinated spore or conidium (Booth, 1971; Nelson *et al.*, 1983; Pitt & Hocking, 1985; Burgess *et al.*, 1988) after a long procedural "protocol" of culturing and incubation (Fig. 2.2. Chapter 2) which involves isolation of the species from infected plant material, the "cultivation" of the isolated species on low nutrient media, culturing on PDA and Carnation Leaf Agar (CLA), and incubation under NUV lights at 25°C/20°C day and night, respectively, with a 12 hr photoperiod, in readiness for description and identification (Nelson *et al.*, 1983; Burgess *et al.*, 1988), using characters summarised in Appendix 4.1. Nelson *et al.* (1983) and Burgess *et al.* (1988) described the cultural characters on 10-14 day old cultures grown on PDA slants but Pitt and Hocking (1985) described PDA and MEA cultures in petri dishes after incubation at 25°C for 7 days under NUV light with alternating dark and light under a 12 hr photoperiod. Burgess *et al.* (1988) assessed culture growth rate (colony diameter) at 25°C and 30°C after incubation in the dark for 72 hrs. Burgess *et al.* (1988) give key characters for identification of *Fusarium* species found in Australasia on PDA and on CLA (Table 2.4, Chapter 2) but not on MA. Nevertheless, Nelson *et al.* (1983) warned of the great difficulties

in identifying *Fusarium* species in the taxonomic section Discolor (Wollenweber and Reinking, 1935; Nelson *et al.*, 1983; Burgess *et al.*, 1988).

4.2 OBJECTIVES

The International Mycological Institute (IMI) identified *F. graminearum* and *F. crookwellense* from similar looking seed-culture colonies grown on MA from maize seeds harvested in 1990 (see Chapter 3). *F. culmorum* was not detected in these seeds, despite the fact that this species has been commonly associated with maize seeds grown in the Manawatu region (Hussein, 1987; Sayer and Lauren, 1991). The objectives of this study were therefore to find a method (or methods) which would allow the differentiation of *F. graminearum* seed-culture colonies from those of *F. crookwellense* and to detect *F. culmorum* and to differentiate its seed-culture colonies from those of *F. graminearum* and *F. crookwellense.*

4.3 MATERIALS AND METHODS

4.3.1 Identification of *F. graminearum* in pure cultures

4.3.1.1 Colony morphology on PDA and MEA

Ten duplicate isolate cultures of *F. graminearum* previously identified by IMI (see Chapter 3) and stored on slants on Malt Agar (MA) Difco (containing 30 g malt extract, 15 g agar) were used in this study. These isolates represented different seed-culture colony types of *F. graminearum* (see Chapter 3). Isolates designated 4R, 7R, 9R, 10R represented the 'red and fluffy'; 1R, 2R, 26R, 27R, the 'red and lobed'; 21R, the 'red centre'; and 20R, the 'cream and fluffy' types of *F. graminearum.* Cultures on slants were subcultured onto Malt Extract Agar (MEA) Gibco BRL (containing 30 g malt extract, 15 g agar, 2.5 g peptone 100 and 2.5 g peptone 140) (Gibco BRL, 1993) by mass transfer of mycelium at the tip of a sterile dissecting needle. One plate was used for each culture and the plates were incubated in a seed

germinator, in diffuse light at 25°C for 10 days. From each plate, macroconidia were lifted from sporodochia with the tip of a sterile needle to make a spore suspension in 10 ml of distilled water in 25 ml sterile McCartney bottles (Fig. 4.1). The spore concentration of the suspension was checked by examination of a loopfull of the suspension under a x10 objective of the compound microscope. A suspension containing up to 10 macroconidia in a field view was considered to have the correct concentration for seeding water agar plates (Hussein, H.M., pers. comm.).

To each bottle of suspension one drop of 25% lactic acid was added as an antibacterial agent and the bottle was allowed to stand for 10 minutes before being poured onto water agar plates prepared seven days earlier (Nelson *et al.*, 1983; Burgess *et al.*, 1988). After a thorough draining of the water from the agar, the seeded water agar plates were incubated in the dark at 25°C for 20-24 h and individual single germinating spores were transferred onto home-made PDA (Nelson *et al.*, 1983; Burgess *et al.*, 1988). After 20-24 h, each young single spore culture on PDA was subcultured by the hyphal tip technique (Nelson *et al.*, 1983; Burgess *et al.*, 1988) onto MEA. These plates, together with the PDA plates, were incubated in the dark at 25°C for 72 h after which comparative culture growth rate was determined by the measurement of colony diameter (Burgess *et al.*, 1988). Colony diameters of > 4 cm in 72 h were classed as a fast growth rate and those of < 4 cm in the same time period as a slow growth rate (Burgess *et al.*, 1988). Finally the cultures on PDA and on MEA were incubated under near ultra-violet (NUV) light illumination (2 x 18 W black light fluorescent tubes, ie 36 W [Phillips: TLD 18W/08, made in Holland] and 2 x 20 W cool white fluorescent tubes, ie 40 W [Phillips TL 20W 33 RS white, made in Australia], positioned 41 cm above the cultures) with a 12 h photoperiod and alternating temperature of 25°C day/20°C night (Neergaard, 1979; Nelson *et al.*, 1983, 1986; Burgess *et al.*, 1988).

Nine days after inoculating the PDA and MEA plates, the colony morphology was described. Characters used were mycelium texture, mycelium colour, culture sporulation and pigmentation in agar (Nelson *et al.*, 1983; Pitt and Hocking, 1985; Burgess *et al.*, 1988). Culture pigmentation was determined using the "Methuen

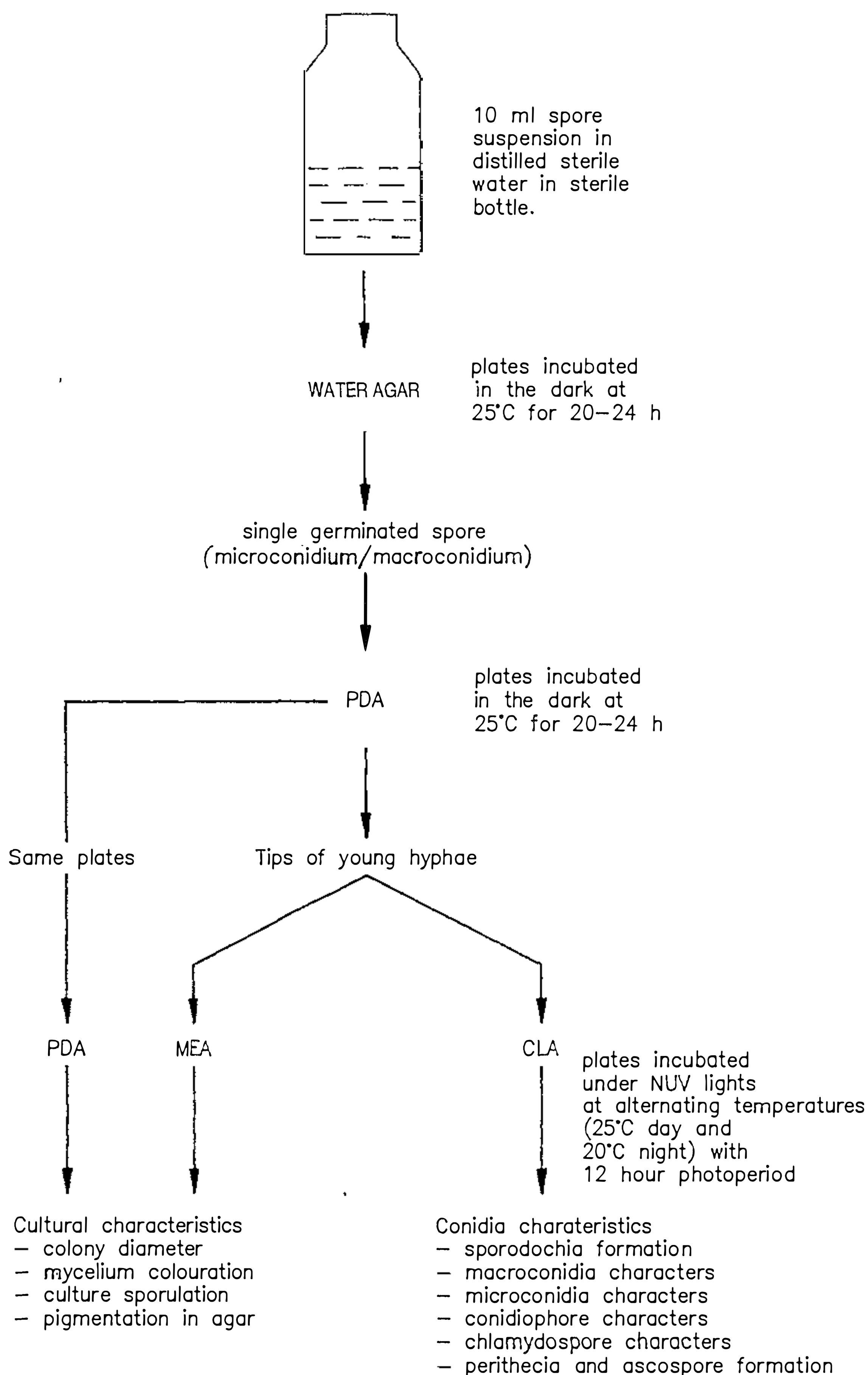


Figure 4.1 Diagram showing procedures followed in preparing and studying pure cultures initiated by single germinated spores (conidia)

Handbook of Colour" (Kornerup and Wanscher, 1978), and occasionally "A mycological colour chart" (Rayner, 1970) was consulted (Burgess *et al.*, 1988) when the appropriate colour for the culture was not found in the former book.

4.3.1.2 Conidial and perithecial formation on CLA

Conidial and perithecial formation on carnation leaf agar (CLA), consisting of water agar (20 g agar in 1 l tap water) containing dry sterile carnation leaf pieces (approximately 1 piece/2 ml agar), which had been sterilised by gamma-irradiation (2.5 megarads - Burgess *et al.*, 1988) was studied in pure cultures initiated from single germinated spores (see section 4.3.1.1). These cultures were prepared concurrently with the PDA and MEA cultures for the colony morphology study (see section 4.3.1.1 and Fig. 4.1) and the incubation procedure was the same as described in section 4.3.1. However, this type of illumination (2 x 18 W black light) was found to be suboptimal for perithecial formation, so that consequently, the preparation of cultures was repeated and the new cultures were incubated under NUV light illumination consisting of 2 x 36 W (72 W) cool white fluorescent tubes and one 40W black light tube [Phillips TL 40/08] (40 W black light) positioned at 33.5 cm above the cultures, with a 12 h photoperiod and alternating temperature of 25°C day/20°C night (Neergaard, 1979; Nelson *et al.*, 1983, 1986; Burgess *et al.*, 1988).

After 6-7 days incubation, the cultures were examined for perithecial initials and also sporodochia and microconidia. After 10-14 days incubation, the same cultures were re-examined visually or using a stereo-microscope for the presence of developed *G. zeae* perithecia (Tschanz *et al.*, 1975a). Macroconidia, microconidia and phialides were studied under the compound microscope using a 0.15% solution of gelatin as mounting fluid (Nelson *et al.*, 1983). They were identified according to Nelson *et al.* (1983) and Burgess *et al.* (1988), then photographed under the light microscope. Those cultures in which perithecia were not present after 10-14 days were periodically re-examined over a further 30 days of incubation (Booth, 1981) after which they were finally re-examined for the presence of perithecia. For cultures of *F. crookwellense* and *F. graminearum*, microscope slide preparations of macroconidia from PDA plates were examined under the compound microscope (x

40 magnification) and the general macroconidial morphology (shape and size) was determined and compared (Hussein, H.M., pers. comm.). No particular attention was paid to the prescence of chlamydospores as they are not reliable identification characters (Burgess *et al.*, 1988).

4.3.2 Identification of *F. crookwellense* and *F. culmorum*

4.3.2.1 Colony morphology on PDA and MEA

The methods and procedures used in the preparation of pure cultures initiated by single germinated spores for identification of *F. graminearum* were followed (see Section 4.3.1.1 and Fig. 4.1). For *F. crookwellense*, isolates designated 3R and 19R representing the 'red and lobed', and the 'red centre', respectively, were used. For *F. culmorum* the macroconidia used for the preparation of single germinated spores (Fig. 4.1) were obtained from sporodochia in seed-culture colonies designated SM1, SM2, SM3 and SM4, on MEA (section 4.3.4).

4.3.2.2 Conidial development on CLA

The methods and procedures used in the preparation of pure cultures for the identification of *F. graminearum* were followed (see section 4.3.1.2).

4.3.3 Verification of identification of *Fusarium* spp. from seed-culture colonies on MA

4.3.3.1 Seed-culture colony categorisation and description

A composite sample of 100 seeds of cultivars 3551 and 3591 harvested in July 1990 (see Chapter 3) and 50 seeds from cultivar Pac 42 harvested in September 1994 were surface disinfected, plated on MA and incubated in a seed germinator in diffuse light at 25°C, as described earlier (see Chapter 3). After 5-6 days all 78 seed-culture colonies (excluding those of *F. subglutinans* and *F. poae*) were categorised and described (as per Chapter 3) and the presence of other fungi was noted. In order to check whether an extended period of incubation would cause changes in colony morphology which would facilitate identification of seed-culture colonies, the plates

were returned to the germinator for a further 5 days after which the colonies were described.

4.3.3.2 Seed-culture colony identification

The 5 day old seed-culture colonies were subcultured directly on to CLA, by mass transfer of inoculum which consisted of mycelium from the edge of the colonies and of mycelium together with macroconidia, if present, from the area around the seed (see Plates 4.10, 4.11 and 4.13). This positional subculturing was used to check whether the fungal growth consisted of only one *Fusarium* species. The pure cultures so prepared were incubated under NUV light illumination with a 40 W black light tube and 2 x 40 W white cool fluorescent tubes (see section 4.3.1.2) with a 12 h photoperiod and alternating temperature of 25°C day/20°C night. After 6-8 days the cultures were examined using a stereo-microscope to detect the formation of perithecial initials, and were then re-incubated. After 11-12 days the cultures were re-examined visually or with the stereo-microscope to detect the presence of mature perithecia of *G. zeae* or perithecial initials and sporodochia.

Microscope slides were prepared from the sporodochia and were studied using the compound microscope to determine macroconidial, microconidial and conidiophore characteristics (Nelson *et al.*, 1983; Burgess *et al.*, 1988). Cultures which failed to produce perithecia of *G. zeae* were incubated until 30 days after subculturing (Booth, 1981), after which they were re-examined for perithecia or perithecial initials formation. After approximately 40 days incubation those cultures of *F. graminearum* in which perithecia had failed to form were aseptically washed with sterile distilled water in a Laminar Flow Cabinet, and re-incubated under NUV light illumination (40 W black light tube) for a further 30 days, after which they were finally re-examined for perithecia formation.

4.3.4 Effects of medium on colony appearance: Malt Extract Agar

4.3.4.1 Seed-culture colony categorisation

100 maize seeds of cultivars 3591 harvested in July 1990 and Pac 42 harvested in September 1994 were plated on MEA using one seed per plate. The seeds were incubated in a seed germinator in diffuse light at 25°C. After 5 days of incubation, the plates were examined visually and an attempt was made to categorise the colonies as earlier described (Chapter 3) and to describe them. Representative *Fusarium* spp. colonies were selected, labelled and were returned to the germinator at 25°C in diffuse light to allow time for sporulation and any changes in the colony morphology which would facilitate colony identification. After 10 days of incubation, the seed-culture colony morphology was described again and cultures which were not sporulating were re-incubated under NUV light illumination using 2 x 18 W black and 2 x 36 W white cool light as described earlier (see section 4.3.1.2) to induce sporulation (Nelson *et al.*, 1983; Burgess *et al.*, 1988).

4.3.4.2 Seed-culture colony identification

Sporulating 10 day old seed-culture colonies on MEA designated numbers 2, 3, 4, 5, 7, 8, 9, 11, 12, SM7 and SM9 were subcultured onto PDA, MEA and CLA by the single germinated spore technique as described earlier (see section 4.3.1.1 and Fig. 4.1) while those which failed to sporulate were directly subcultured on CLA by mass transfer of mycelium using a sterile dissecting needle (see section 4.3.3.2). The pure cultures on PDA and MEA were then described (see Section 4.3.1.1) and those on CLA were studied for sporodochia, and conidial and perithecial formation as described earlier (see Section 4.3.1.2).

4.4 RESULTS

4.4.1 Colony morphology of *F. graminearum* on PDA and on MEA

F. graminearum colony morphology (Plates 4.1 and 4.2) on PDA was different from that on MEA (Tables 4.1a and 4.1b). On PDA, colony morphology was generally similar to that described by Burgess *et al.* (1988) (Table 4.1a and Plate 4.1). All the isolates produced a 'rapid' (4.6 - 6.2 cm) colony growth rate. All the cultures showed a floccose type of mycelium. Mycelial growth was thickest in the centre. The edge of the mycelium (as seen before it reached the edge of the plate) was generally even and not lobed. Mycelium colour varied from greyish red, pale red to pastel red, greyish red to pale red, pinkish white and pink to pale red. Only one culture (7R) had a greyish red centre, indicating sporulation. All the other cultures did not sporulate readily on PDA. The colour of the pigment produced in PDA as seen on the reverse side of the plate varied from brownish red to greyish red, to pastel red in the periphery of the petri dish with the centres of the cultures being violet brown, dark ruby, greyish red or dark red to reddish brown. Some cultures were greyish red throughout the agar.

On MEA (Table 4.1b and Plate 4.2) colony morphology was different from that described by Pitt and Hocking (1985) and Burgess *et al.* (1988). All the cultures (except one) had a 'slow' growth rate, the colony diameter being 2.9 - 3.6 cm. The texture of the mycelium varied from velvet to almost velvet, with very short mycelium to sparse or scanty mycelial growth. Mycelium colour varied from orange white to pinkish white, light orange to pale orange. Only culture 4R had almost the same mycelial colour as observed on PDA, being reddish brown, while culture 7R almost had no mycelium and the culture surface was covered by a sheet of pionnotes. Only cultures 4R and 7R sporulated on MEA. Cultures 2R, 7R, 26R and 27R had lobed edges and grew very poorly on MEA (Table 4.1b). The colour of the pigment produced in agar as seen on the reverse side of the plate varied from orange, light orange, light orange to pale orange, and pale orange to light brown in the periphery

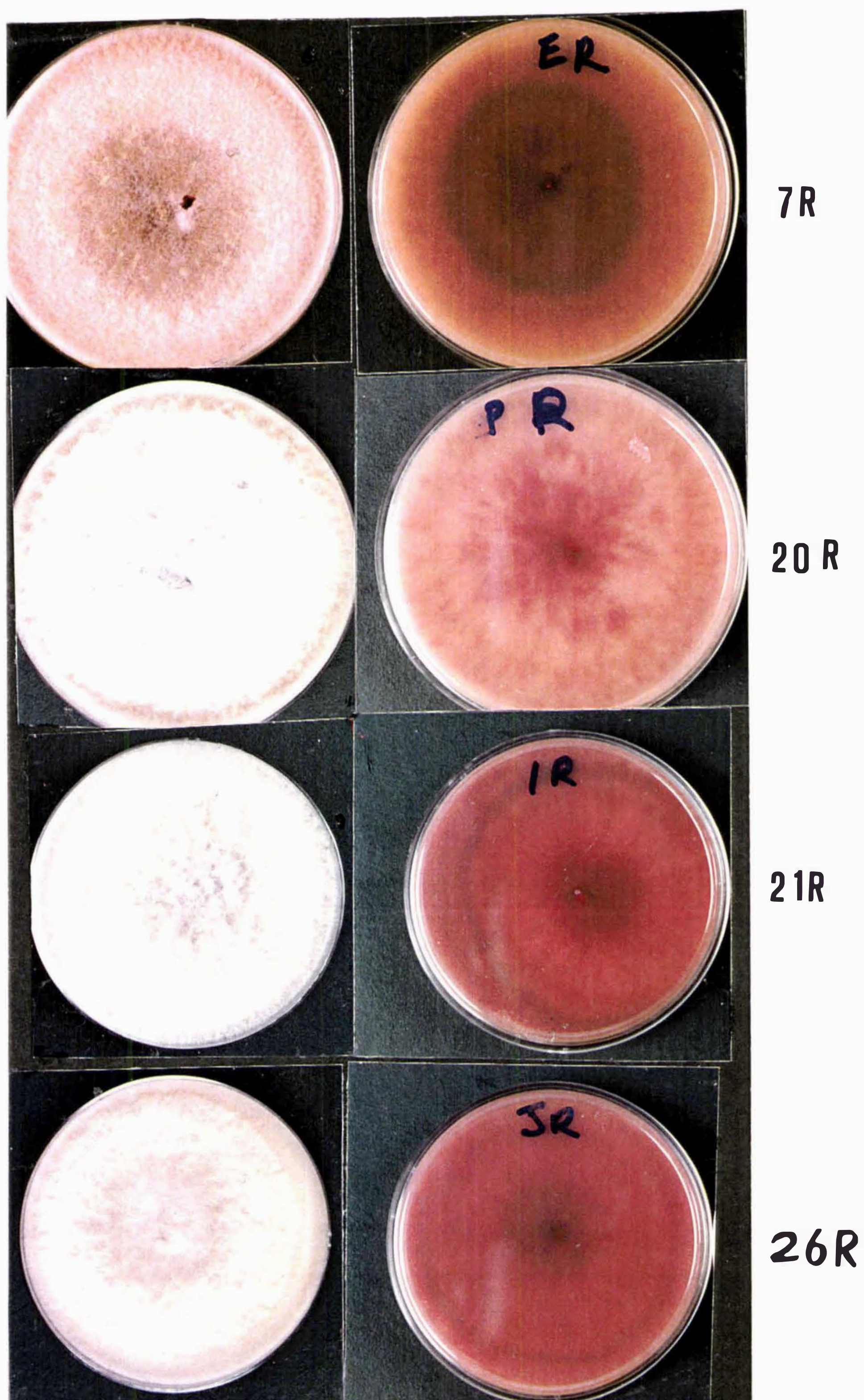


Plate 4.1 Colony morphology of *F. graminearum* on PDA, with cultures 7R, 20R, 21R and 26R representing the 'red and fluffy', 'cream and fluffy', 'red centre' and 'red and lobed' types.

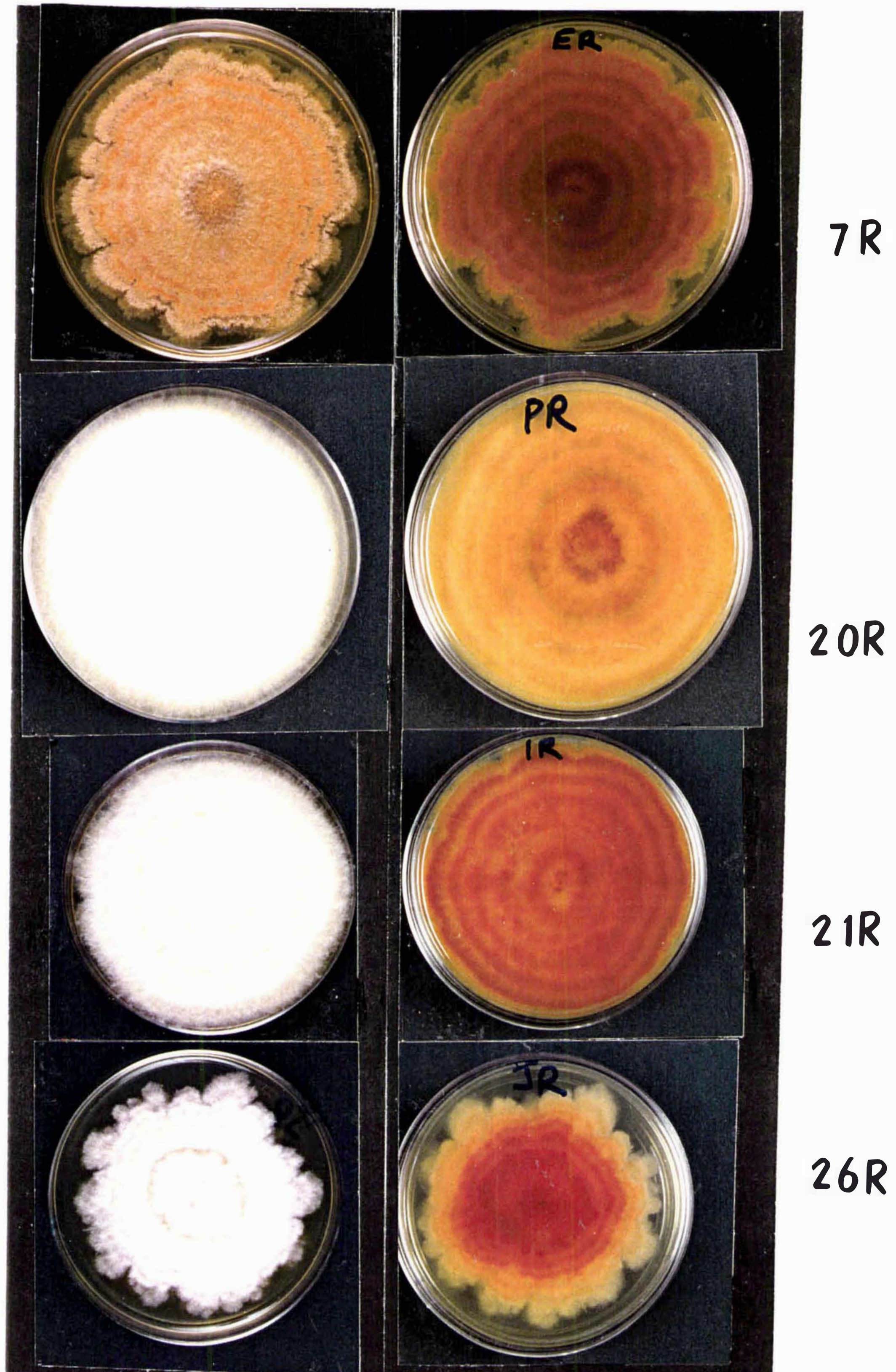


Plate 4.2 Colony morphology of *F. graminearum* on MEA, with cultures 7R, 20R, 21R and 26R representing the 'red and fluffy', 'cream and fluffy', 'red centre' and 'red and lobed' types.

Table 4.1a Colony morphology of *F. graminearum* on PDA.

Seed-culture colony type	Red and fluffy				Red and lobed				Red centre	Cream and fluffy
Isolate	**4R**	**7R**	**9R**	**10R**	**1R**	**2R**	**26R**	**27R**	**21R**	**20R**
Growth rate	rapid	rapid	rapid	rapid	rapid	rapid	rapid	rapid	rapid	rapid
Colony diameter: cm	4.6	5.2	5.0	5.6	6.2	5.7	6.2	6.2	5.4	6.1
Mycelium texture	Floccose	Floccose	Floccose	Floccose	Floccose	Floccose	Floccose	Floccose	Floccose	Floccose
Mycelium colour										
- periphery	White to pinkish white	Pale red to Greyish red	Pale red	Greyish red to pale red	Greyish red	Greyish red	Greyish red to pale red	Pink to pale red	Pinkish white	Greyish red to pale red
- centre	Same	Greyish red	Same	Same	Same	Same	Same	Same	Same	Same
Sporulation	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Reverse side										
- periphery	brownish red	pastel red	greyish red	brownish red	brownish red	greyish red	greyish red	greyish red	greyish red	pastel red
- centre	Same	greyish red	Same	violet brown	violet brown	dark ruby	Same	dark ruby	dark red to reddish brown	greyish red

(+) = positive; (-) = negative.

Table 4.1b Colony morphology of *F. graminearum* on MEA.

Seed-culture colony type	Red and fluffy				Red and lobed				Red centre	Cream and fluffy
Isolate	**4R**	**7R**	**9R**	**10R**	**1R**	**2R**	**26R**	**27R**	**21R**	**20R**
Growth rate	slow	slow	-	slow	slow	slow	slow	slow	slow	rapid
Colony diamter: cm	3.1	-*	-	3.2	3.6	3.2	3.3	2.9	3.5	4.0
Mycelium texture	Thick cottony mycelium	Hardly any mycelium	Scanty mycelium, some occurring tufts of mycelium	Velvet (short fine mycelium)	Almost velvet with central tufts mycelium	Very short and sparse with zonation and heavily lobed	Very short and sparse with zonation and heavily lobed	Very short and sparse with zonation, hardly any growth	Almost velvet with central tufts of mycelium	Short and sparse, almost velvet with central tufts of mycelium
Mycelium colour - periphery	white to pinkish white	Light orange	pale orange	Orange white to pinkish white with some orange zonations	Orange white to pinkish white with zones of yellow	Orange white to pinkish white	Orange white	Orange white	Orange white to pinkish white with orange zonations	Orange white to pinkish white
- centre	Reddish brown	Same	Pale orange and zonate	Same	Same	with zones of yellow to orange	Orange zonation	Same	Same	Same
Sporulation	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Pigment in agar (Reverse) - periphery	Cinnamon	Brown (burnt sienna to brick/tile red)	Brownish orange	Light orange	Orange (chrome yellow)	Light orange to pale orange	Light orange to pale orange	Pale orange	Brownish red	Light orange
- centre	Same	Same	Same	Brownish red	Brownish red	Brownish red	Brownish red	Light brown to brownish red	Same	Brownish red

* - not determined; (+) = positive; (-) = negative.

of the petri dish, but some cultures had brownish red centres, while others were cinnamon, brown or brown orange throughout the plate.

4.4.2 Conidial and perithecial formation by *F. graminearum* on CLA

4.4.2.1 Sporodochial formation

By 5 days after inoculation, some *F. graminearum* cultures initiated either from a single germinated conidium or by mass transfer of inoculum had formed profuse pale orange sporodochia, mainly on the carnation leaf pieces while others formed sparce sporodochia. The sporodochia were initially pale orange but turned brownish orange with age (Plates 4.3 A and B). The macroconidia formed in sporodochia on carnation leaf pieces were regular in shape and size and within the expected ranges (Nelson *et al.*, 1983; Burgess *et al.*, 1988) (Plate 4.3 C). Cultures from some red centre type of colonies of *F. graminearum* initiated by the mass transfer of inoculum formed dark red pigmentation in agar and some formed prominent annular zones (Plate 4.3b). Cultures which formed abundant sporodochia did not form perithecia.

4.4.2.2 Perithecial formation

Perithecia were formed in cultures with sparse sporulation regardless of whether cultures were initiated by single spore or mass transferred inoculum (Plate 4.4a). Perithecia initials appeared after 5-6 days and developed to full size by 10-14 days. Under a 40 W black light tube, cultures developed very sparse aerial mycelium and abundant perithecia. Perithecia formed and matured on most carnation leaf pieces. However, under 2 x 18 W black light tubes usually only perithecia initials formed mainly in the periphery of the cultures, and very few matured. The lower wattage tubes 2 x 18 W black light) promoted dense mycelial growth and dense and tall 'perithecial initial hairs' (bristle like hyphae that surrounded perithecial initials) (Plate 4.4b). The dense mycelial growth usually submerged the few perithecia that developed to full size.

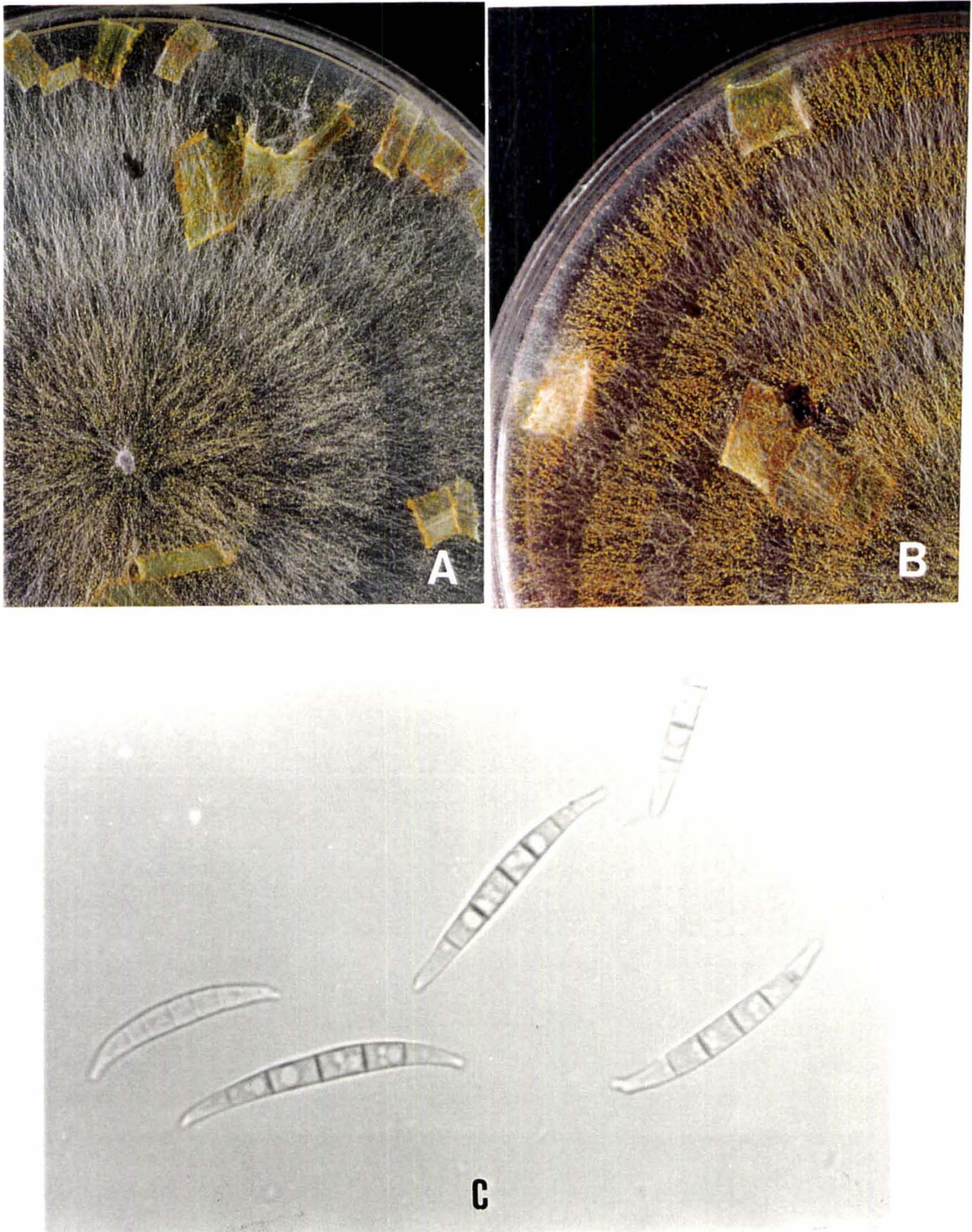


Plate 4.3 *F. graminearum*: sporodochia (A & B) formed on CLA; macroconidia from CLA culture (C); (B; from 'red centre' type of seed-culture colony; (C, x 750).

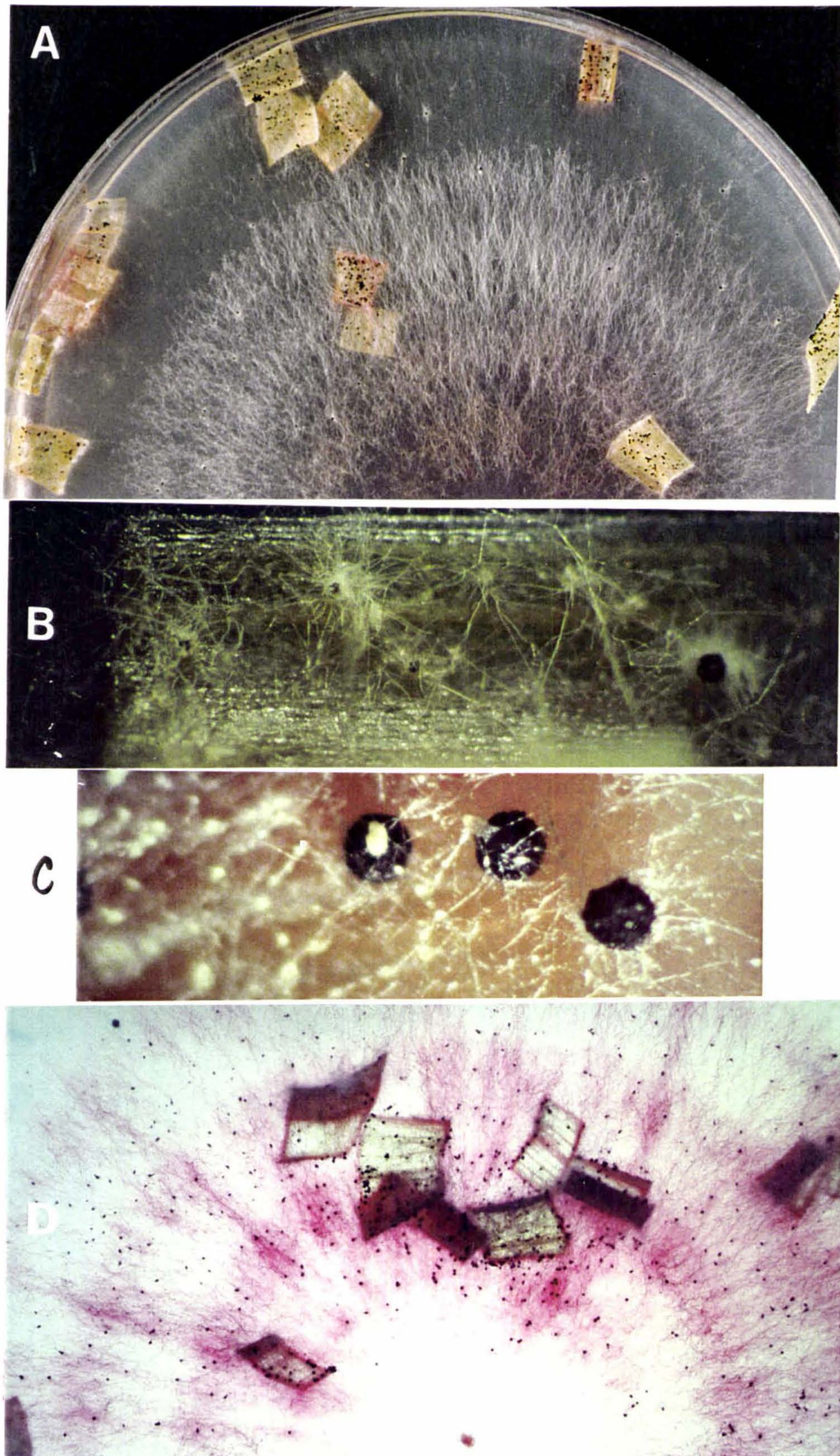


Plate 4.4a Perithecia initials and mature perithecia of *G. zeae* on CLA formed under 40W NUV light conditions: A, abundant perithecia formed on carnation leaf pieces; B, perithecia initials; C, mature perithecia; D, inoculum from 'red centre' type of seed-culture colony; (B x 50; C x 30).

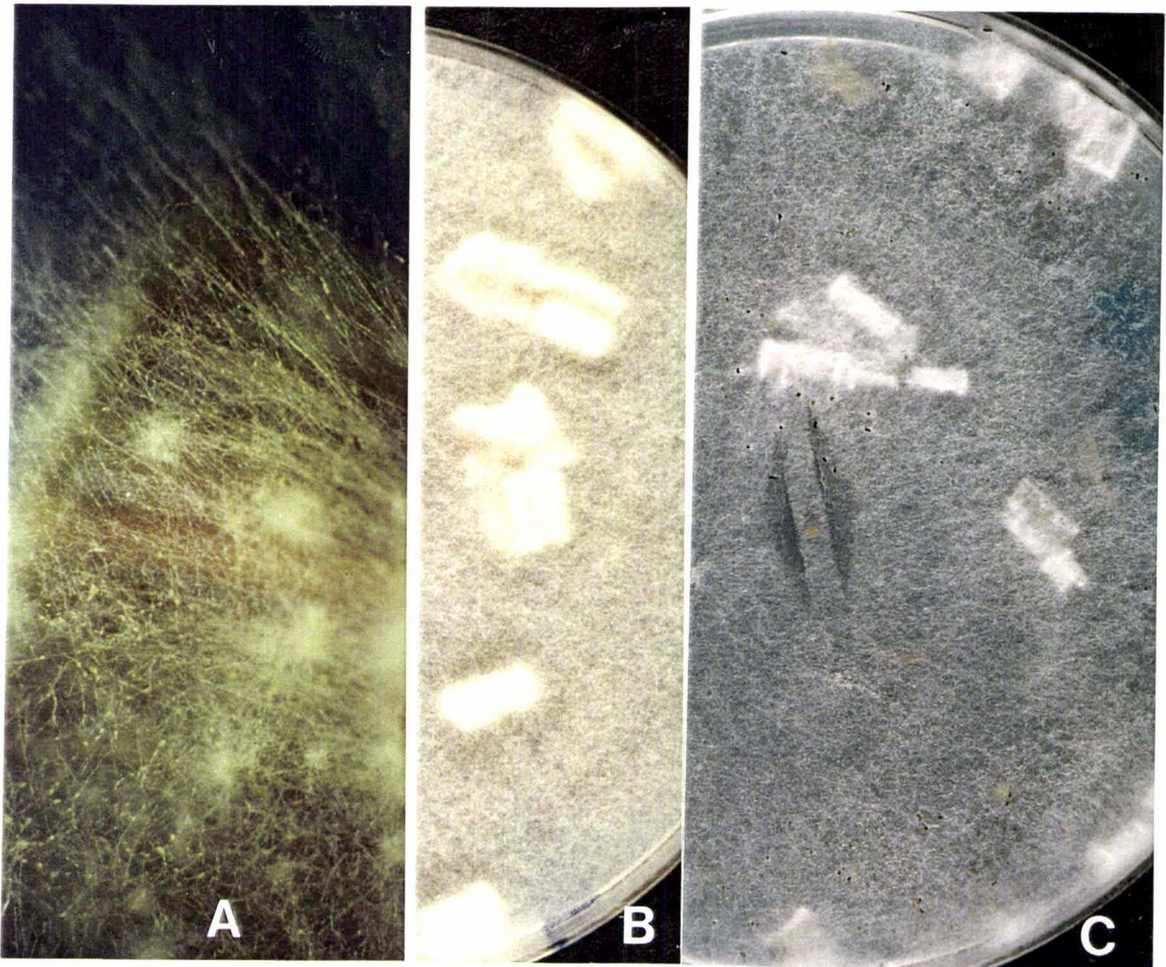


Plate 4.4b Perithecia initials and perithecia formation on CLA under 2 x 18 W NUV light. A, tall initial perithecial hairs; B, dense mycelium and tall perithecial hairs interfere with perithecia formation; C, plates previously under 2 x 18 W NUV light formed mature perithecia under 40 W NUV lights.

After incubation under the 2 x 18 W tube illumination, if the plates were transferred to 40 W tube illumination, new perithecia were formed in the mycelium both over the carnation leaf pieces and on agar (Plate 4.4b).

4.4.2.3 Description of macroconidia, perithecia and ascospores

The macroconidia from the sporodochia were relatively slender and gently curved or almost straight, the curvature being mainly at the apical cell. They were usually 5 septate but occasionally 4 or 6 septate with a tapered apical cell which ended in a 'snout' and a distinct foot-shaped basal cell (Plate 4.3c). The macroconidia were produced from monophialides on branched conidiophores in sporodochia. Microconidia were absent in cultures of *F. graminearum*.

Perithecia development started with formation of perithecia initials which were marked by points surrounded initially by translucent to white perithecial hairs (Plate 4.4b). The perithecia were dark blue to black when viewed under a dissecting microscope but blue when seen in slide preparation (Plate 4.4c). They were spherical, with an inconspicuous short beak and an ostiole, had a rough outer wall and were superficially borne on the carnation leaf pieces. The asci were clavate, with short stipe and contained 8 obliquely arranged ascospores which were hyaline, straight or curved, mostly being 3 septate (Plate 4.4c).

4.4.3 Colony morphology of *F. crookwellense* and *F. culmorum* on PDA and MEA

4.4.3.1 Colony morphology of *F. crookwellense*

The description of the colony morphology of the two isolates of *F. crookwellense* on PDA and MEA studied is given in Table 4.2 and Plate 4.5 A-D. The colony morphology on either medium was relatively the same as for *F. graminearum* (Table 4.1a and 4.1b, and Plate 4.1). On PDA (Table 4.2 and Plate 4.5 A and B) isolates 3R and 19R showed rapid growth rates (4.8 and 5.4 cm, respectively) in 3 days. The mycelium texture of the two isolates was floccose. The colour of the

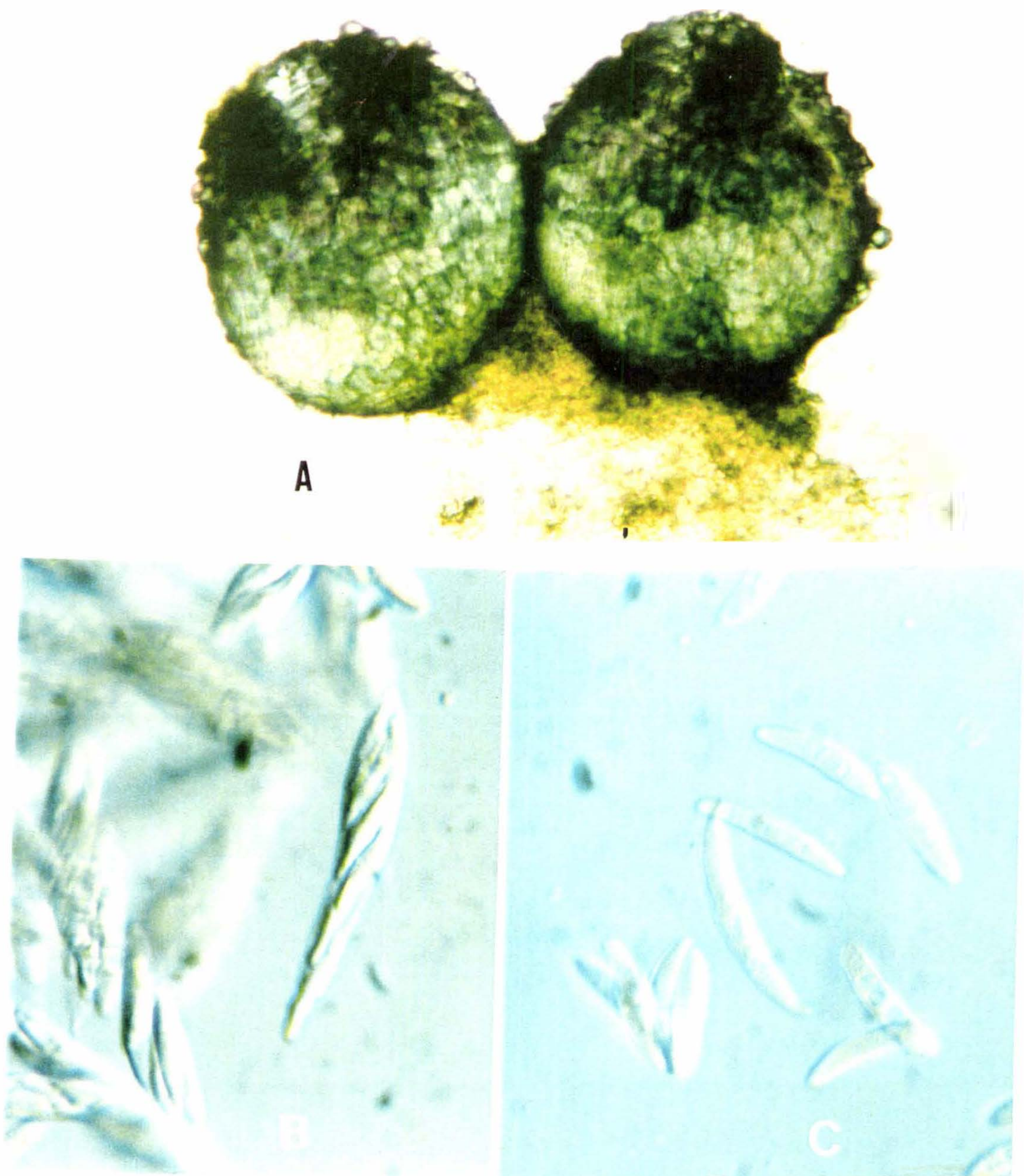


Plate 4.4c A, *G. zeae* perithecia; B, asci; C, ascospores; (A x 150; B and C x 500)

Table 4.2 Colony morphology of *F. crookwellense* on PDA and MEA.

Medium	PDA		MEA	
Seed-culture colony type	**Red and lobed**	**Red centre**	**Red and lobed**	**Red centre**
Isolate	**3R**	**19R**	**3R**	**19R**
Growth rate	rapid	rapid	slow	slow
Colony diameter	4.8	5.4	3.4	3.8
Mycelium texture	Floccose	Floccose and fluffy	Short, sparse mycelium in the centre, periphery of mostly pionnotes; colony lobed and zonate	Short mycelium, edge devoid of aerial mycelium; colony lobed and zonate
Mycelium colour - periphery - centre	 Pale red Greyish red	 Pastel red Dull red	 Pale red to pastel red Same	 Pale orange Same
Sporodochia	(+)	(+)	(+)	(+)
Pigment in agar (Reverse) - periphery - centre	 Pastel red to pale red Red (Cock's comb red)	 Pastel red Greyish red	 Brownish orange to reddish golden Same	 Brownish orange to reddish golden Same

(+) = positive; (-) = negative

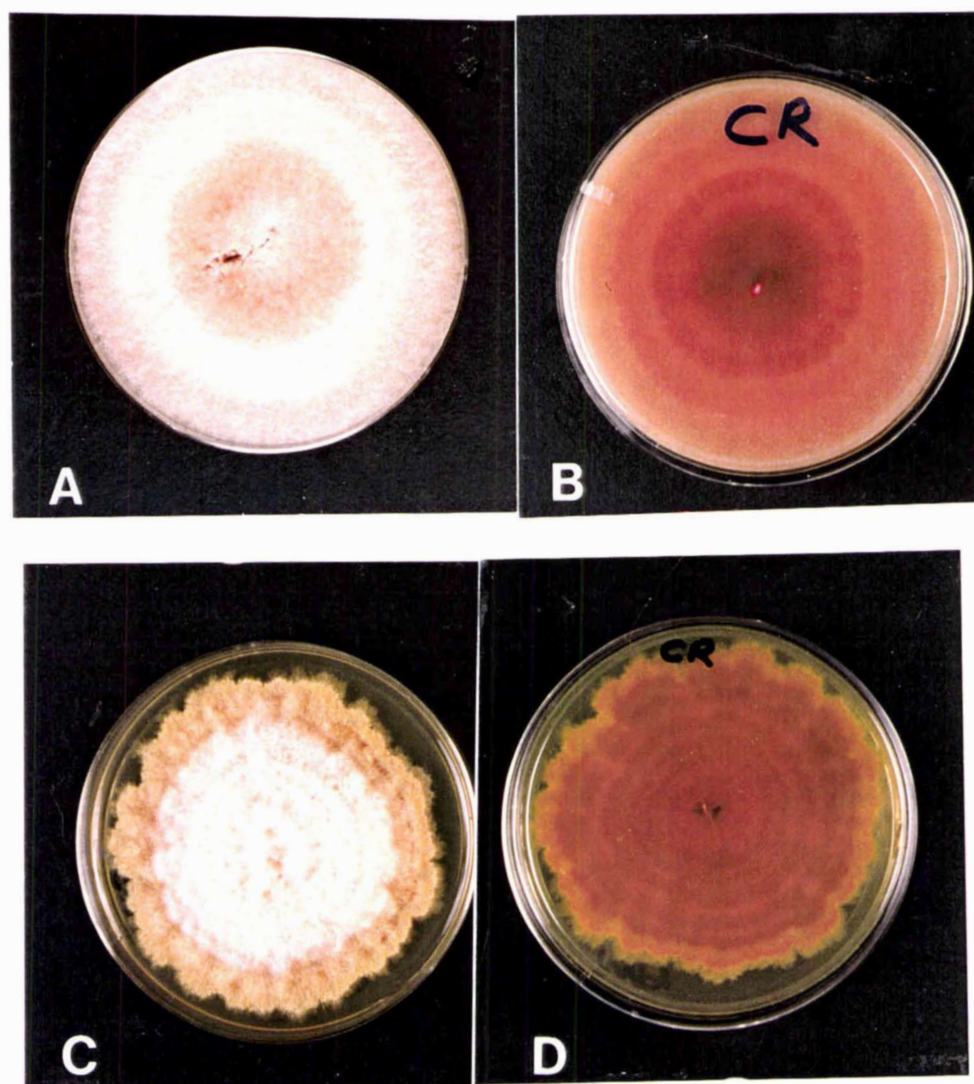


Plate 4.5 *F. crookwellense* colony morphology on PDA (A & B) and on MEA (C & D). A & C, top view; B & D, reverse side on PDA and MEA, respectively.

mycelium was pale red in the periphery for both isolates but was greyish red in the centre of theculture for isolate 3R and dull red in isolate 19R. Both isolates sporulated on PDA. On the reverse of the plates the pigment produced in PDA was pastel red to pale red in the periphery but at the centre of the plates was either red or greyish red.

On MEA (Table 4.2 and Plate 4.5 C and D), isolates 3R and 19R had a slow growth rate (3.4 and 3.8 cm, respectively). Both isolates had short to sparse mycelium with lobed edges and annular zonation. The two isolates sporulated on MEA. On the reverse side of the petri dishes the pigment was brownish orange to reddish golden for both isolates.

4.4.3.2 Conidial development by *F. crookwellense*

F. crookwellense formed abundant sporodochia on CLA. The sporodochia were pale orange and turned brownish orange with age (Plate 4.6 A). The macroconidia were regular in shape and size and within the expected range (Nelson *et al.*, 1983; Burgess *et al.*, 1988) (Plate 4.6 C). The cultures did not form perithecia or microconidia. The macroconidia produced in the sporodochia were intermediate in length (when compared with those of *F. graminearum*), falcate with the dorsal side slightly more curved than the ventral one. They were usually 5 septate and widest at the mid point but closer to the upper half of the macroconidium. The macroconidia of *F. crookwellense* were similar to those of *F. graminearum*, except they tended to be broader and generally more curved than those of *F. graminearum*.

4.4.3.3 Colony morphology of *F. culmorum*

The colony morphology data for four different isolates of *F. culmorum* on PDA and on MEA are given in Table 4.3. As for *F. graminearum*, the pure culture colony morphology on PDA was different from that on MEA but generally did not differ from those of *F. graminearum* (Table 4.1a and 4.1b) in respect to growth rate, aerial mycelium texture and colour and pigmentation in the agar. On PDA (Table 4.3 and Plate 4.7 A and B), all isolates had rapid (5.6 - 6.6 cm) growth rate. The mycelium texture was floccose and the cultures had regular edges. The mycelium colour was pale red to dull red in three of the four cultures while that of the other culture was greyish

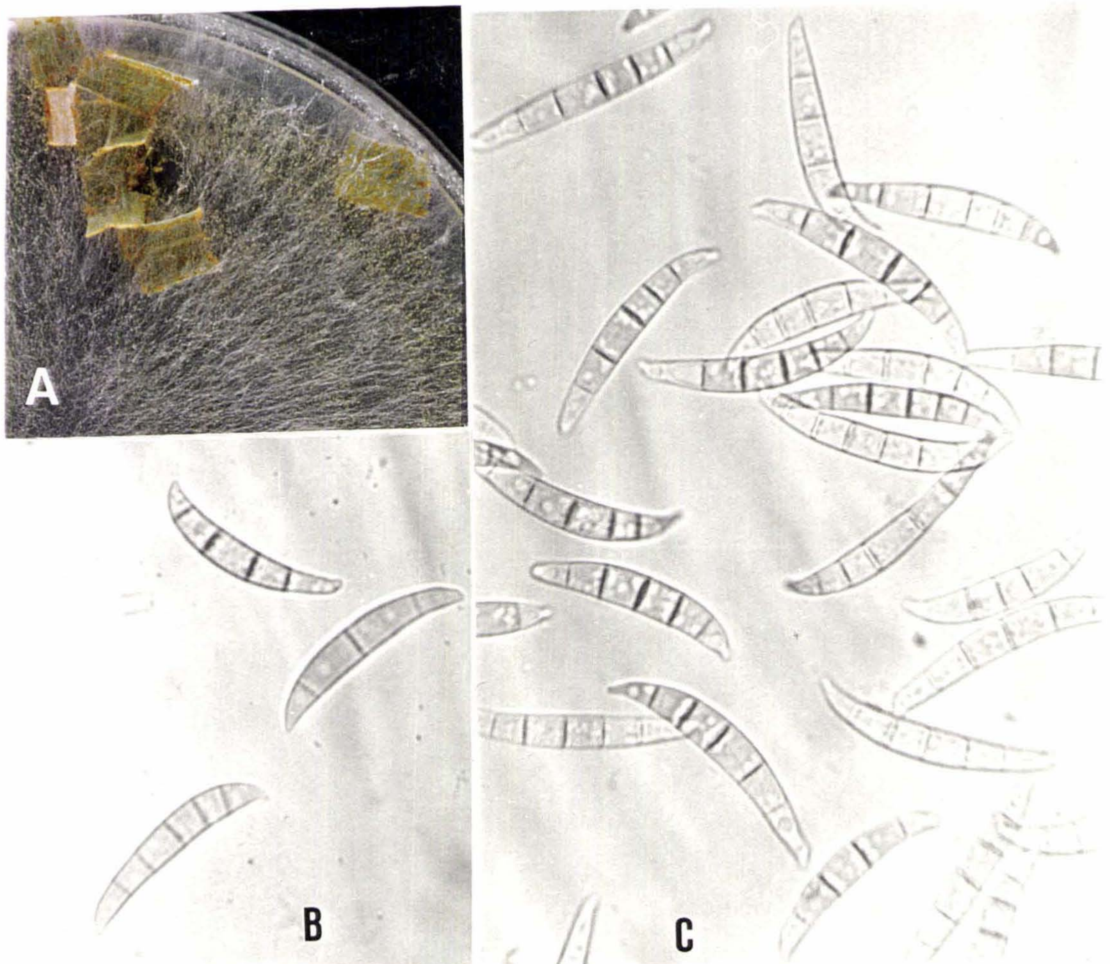


Plate 4.6 Sporodochia (A) and macroconidia (B) and (C) of *F. crookwellense*. (B & C, from CLA and PDA, respectively, x 750).

Table 4.3 Colony morphology of *F. culmorum* on PDA and MEA.

Medium	Isolate	SM1	SM2	SM3	SM4
PDA	Growth rate	rapid	rapid	rapid	rapid
	Colony diameter: cm	6.5	5.6	6.4	6.6
	Mycelium texture	Dense floccose	Dense floccose	Dense floccose	Dense floccose
	Mycelium colour - periphery	Greyish rose to dull red	Pale red to dull red	Pale red to dull red	Pale red to dull red
	- centre	Reddish brown (rosewood)	Dull red	Dull red	Dull red
	Sporulation	(+)	(-)	(-)	(-)
	Pigment in agar (Reverse) - periphery	Ruby	Greyish red	Pastel red	Greyish red
	- centre	Dark ruby	Dark ruby	Dark ruby	Dark ruby
MEA	Growth rate	rapid	slow	rapid	rapid
	Colony diameter: cm	4.2	3.9	4.3	4.2
	Mycelium texture	Sparse mycelium to almost velvet	Velvet and gently zonate	Velvet and gently zonate	Velvet and gently zonate
	Mycelium colour - periphery	Pinkish white to pale orange	Pinkish white to pale orange	Pinkish white to pale orange	Pinkish white to pale orange
	- centre	Same	Same	Same	Same
	Sporulation	(-)	(-)	(-)	(-)
	Pigment in agar (Reverse) - periphery	Yellow to orange	Brownish orange	Brown orange	Brown orange
	- centre	Same	Same	Same	Same

(+) = positive; (-) = negative

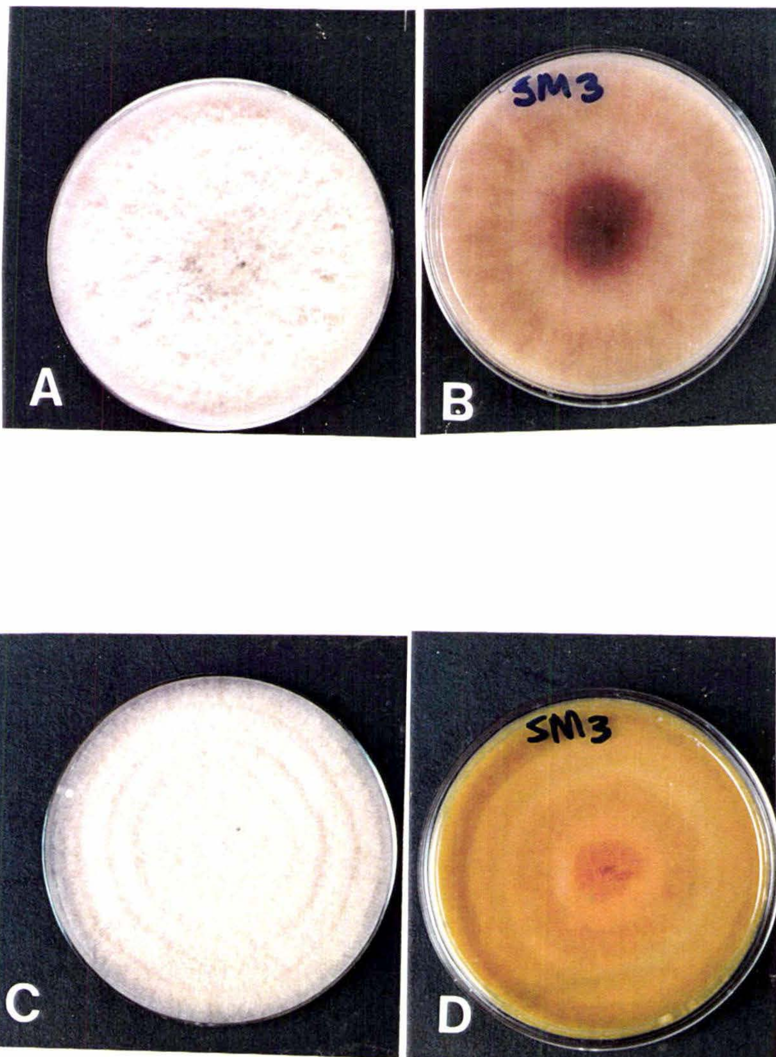


Plate 4.7 *F. culmorum* colony morphology on PDA (A & B) and on MEA (C & D); A & C, top view; B & D, reverse side, respectively.

rose to dull red with the centre being reddish brown. On the reverse side of the plate the pigment was ruby or pastel red or greyish red in the periphery but dark ruby in the centre.

On MEA (Table 4.3 and Plate 4.7 C and D), three of the four isolates had a rapid growth rate (4.2 - 4.3 cm) but this was slower than on PDA, while that of isolate SM2 was slower (3.9 cm). The mycelium texture was velvet to almost velvet, the culture edges being nearly smooth and annular zonations occurred in all cultures. The colour of the mycelium was pinkish white to pale orange and that of the pigment produced in the agar as seen from the reverse side of the petri dish was yellow to orange in isolate SM1 while the plates of the other three isolates it was brown orange.

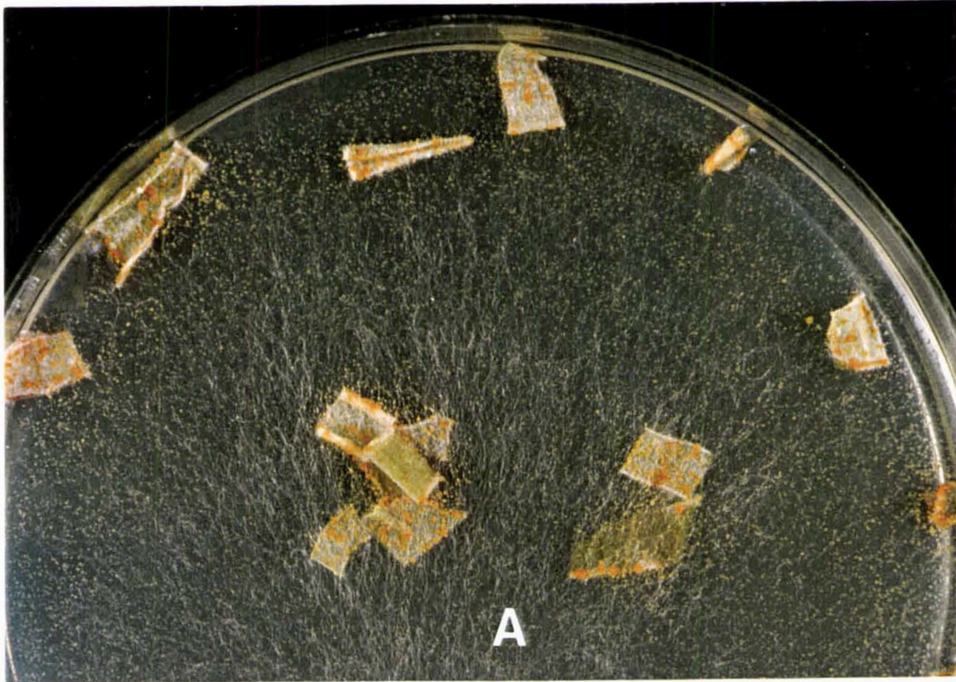
4.4.3.4 Conidial development by *F. culmorum* on CLA

F. culmorum formed abundant sporodochia on CLA which were orange to golden yellow and turned brownish orange with age (Plate 4.8 A). The macroconidia were regular in shape and size and within the expected range (Nelson *et al.*, 1983; Burgess *et al.*, 1988) (Plate 4.8 B). The macroconidia were short and stout, varying in septation, but 3 to 5 septate macroconidia were commonly observed. Some macroconidia had rounded apical cells while others had apical cells slightly pointed or papillate. The base of the foot-cell was usually notched but a few macroconidia had almost foot-shaped bases of the foot-cells. The macroconidia were formed from monophialides on branched conidiophores in the sporodochia. Microconidia and perithecia were absent in the cultures of *F. culmorum*.

4.5 VERIFICATION OF SEED-CULTURE COLONY IDENTIFICATION

4.5.1 Seed-culture colony categorisation on MA

The seed-culture colony categories described earlier (Chapter 3) which were believed to be of *F. graminearum*; viz 'red and fluffy', 'red centre', 'red and lobed' types, were found in both the 1990 and the 1994 seed lots, but the 'cream and fluffy'



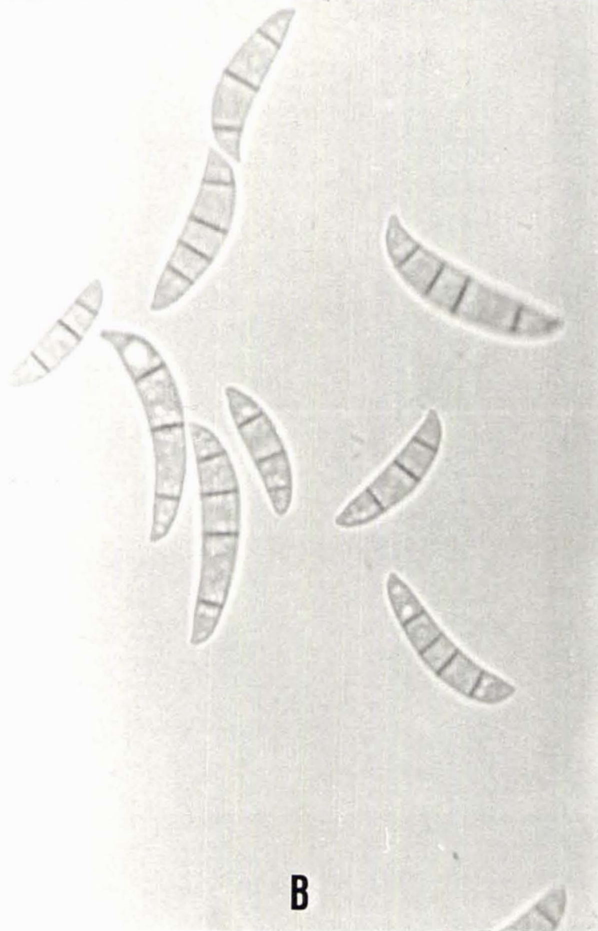


Plate 4.8 Sporodochia (A) and macroconidia (B) of *F. culmorum* from CLA; (B x 750).

type was not detected in the 1994 seed lot (Table 4.4). Also detected were the 'pinkish red tufts and lobed' and the 'red with milky centre' types, which occasionally occurred in the 1990 seed lot, together with *F. subglutinans* and *F. poae*. Two apparently new types of seed-culture colonies, ie 'cream and flat' and 'cream and lobed' were detected, the former being observed both in the 1990 and the 1994 seed lots, but the latter being detected only in the 1990 seed lot.

4.5.2 Seed-culture colony identification on CLA

It was confirmed that different seed-culture colony types were characteristic of different *Fusarium* spp (Table 4.4). *Fusarium* species identified by IMI, and others not found in cultures identified by IMI, ie *F. culmorum*, *F. decemcellulare* and *F. sambucinum* were detected in the seeds.

Of the 78 seed-culture colonies which consisted of 51 red and 27 cream types (Table 4.4), all 5 colonies of the 'red and fluffy' type (see Chapter 3) were *F. graminearum* but none of the these cultures formed perithecia of *G. zeae* (Table 4.4). Of the 26 colonies of the 'red centre' type, 21 were identified as *F. graminearum*, but only 4 formed perithecia of *G. zeae*, while the remaining 17 formed abundant sporodochia on carnation leaf pieces; the remaining 5 colonies of the red centre type also formed abundant sporodochia on carnation leaf pieces and were identified as *F. crookwellense* (Table 4.4), confirming that *F. crookwellense* seed-culture colonies have the same appearance as those of *F. graminearum*, viz, of the 'red centre' type (see Chapter 3).

Of the 16 red colonies with lobed edges, 11 of the 'red and lobed' type of seed-culture colonies were identified as *F. graminearum* and all formed perithecia on CLA (Table 4.4), while the one colony which had pinkish red tufts of mycelium and was lobed was identified as being possibly *F. sambucinum* Fuckel (Nelson *et al.*, 1983; Burgess *et al.*, 1988) (Table 4.4 and Plate 4.9 A and B). Red and lobed colonies contaminated by *Acremonium* spp. were not identified to species level (Table 4.4),

Table 4.4 Listing of seed-culture colony 'types' on MA, number of colonies in each type, and identification of *Fusarium* species.

			Seed lots			
	Seed-culture colonies on MA	Number of colonies on MA	1990	1994	Perithecia (of *G. zeae*) formation on CLA	Pure culture identification of species on CLA
	<u>'Red' colonies</u>					
(i)	Red and fluffy	5	3	2	No	*F. graminearum*
(ii)	Red centre	4 17 5	4 16 3	0 1 2	Yes No No	*F. graminearum* *F. graminearum* *F. crookwellense*
(iii)	Red and lobed	11	9	2	Yes	*F. graminearum*
(iv)	Pinkish red tufts and lobed	1	1	0	No	*F. sambucinum*
(v)	Red and lobed but contaminated	4	4	0	No	*Fusarium* spp.
(vi)	Red with milky centre	4	4	0	No	*F. decemcellulare*
	<u>'Cream' colonies</u>					
(i)	Cream and fluffy	13	13	0	Yes	*F. graminearum*
(ii)	Cream and flat	9	5	4	No	*F. culmorum*
(iii)	Cream and lobed	5	5	0	Yes	*F. graminearum*

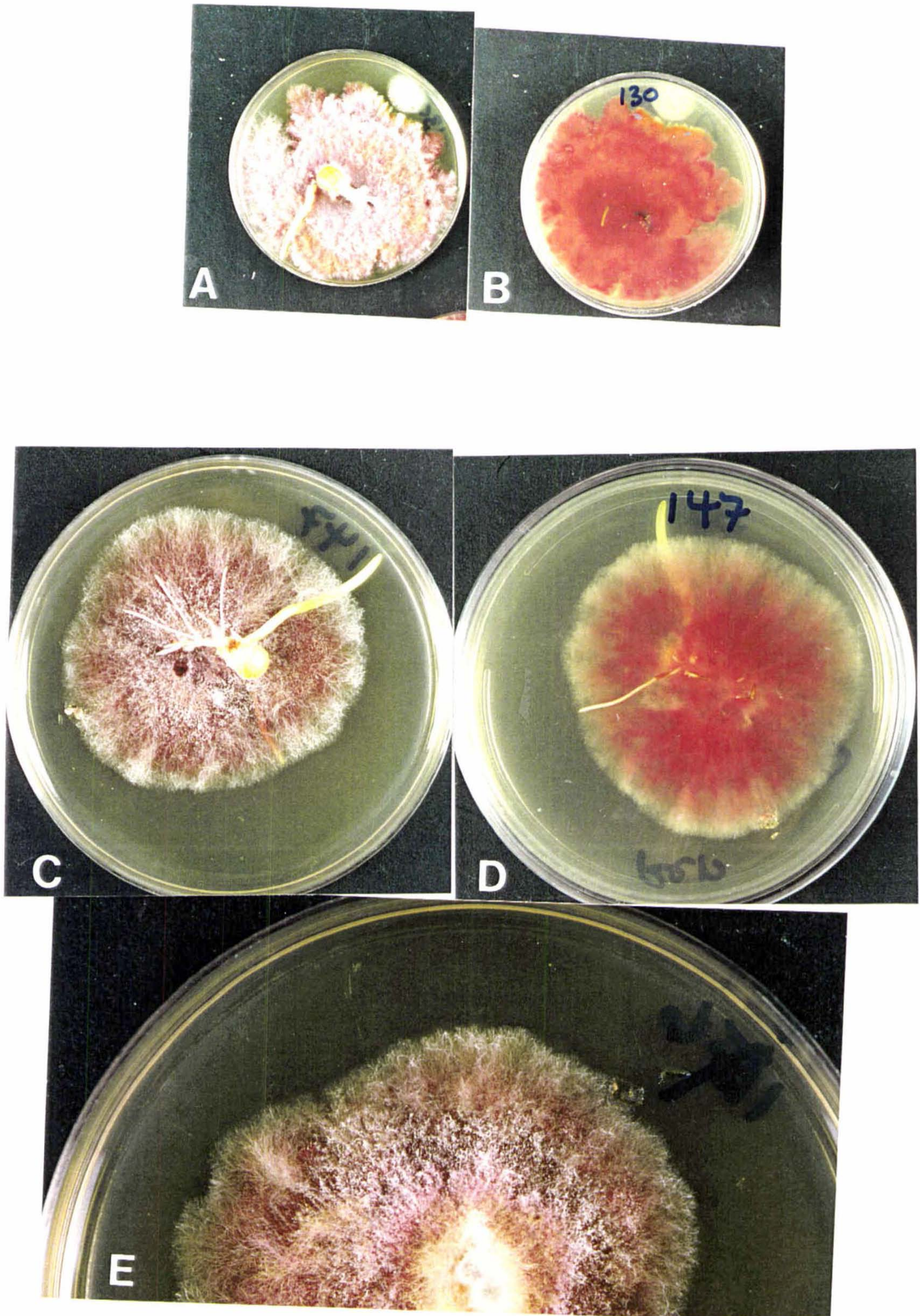


Plate 4.9 Seed-culture colony morphology of *F. sambucinum* (A & B) and *F. decemcellulare* (C-E) on MA: A, C & E, top view; B & D, reverse side; E, close up of *F. decemcellulare* showing the white/milky substance.

but the 4 red and lobed colonies with milky sporodochia-like structures in the centre of the colony and chains of microconidia were identified, possibly as *F. decemcellulare* Brick (Nelson *et al.*, 1983; Burgess *et al.*, 1988) (Plate 4.9 C - E)

Of the 22 colonies which were creamish in appearance, 13 (the cream and fluffy type) were identified as *F. graminearum* and all formed mature perithecia (Table 4.4), but the remaining colonies of the 'cream and flat' type were identified as *F. culmorum* (Table 4.4). All 5 colonies of the 'cream and lobed' type were identified as *F. graminearum*, and all formed mature perithecia.

In all cases of identification, cultures obtained by subculturing from either the edge or the centre of the seed-culture colonies gave similar *Fusarium* species. The seed-culture colony morphology types (5 days old) of *Fusarium* spp. verified on MA are described in Plate 4.10-4.12 (also see Plate 4.9).

4.5.2.1 Description of *F. graminearum* seed-culture colonies: 'cream and lobed' type (Plate 4.10)

After 5 days incubation the colonies were usually of retarded growth and creamish yellow in colour. They had irregular edges with semi-circular 'fronts' or lobes which sometimes overlapped, while lobing varied from heavy to mild and showed annular growth zonations. The colonies had scanty mycelium which sometimes occurred as a variegation of pinkish red or yellow orange on a creamy background - the general combination of these colours being salmon to flesh (Rayner, 1970). The pink or red colouration was more pronounced in the annular growth zonations, both on the top and reverse of the colony. After 10 days incubation the colony became red both on the top and reverse sides of the plate and lobing became prominent (Plate 4.10 C and D), with scanty mycelium still present but in shades of greyish rose or orange, or brownish orange. There was no sporulation in the seed-culture colonies. On the reverse side of the petri dish, the pigment in the agar was deep red and some brownish red colouration was seen around zonations of the lobes.

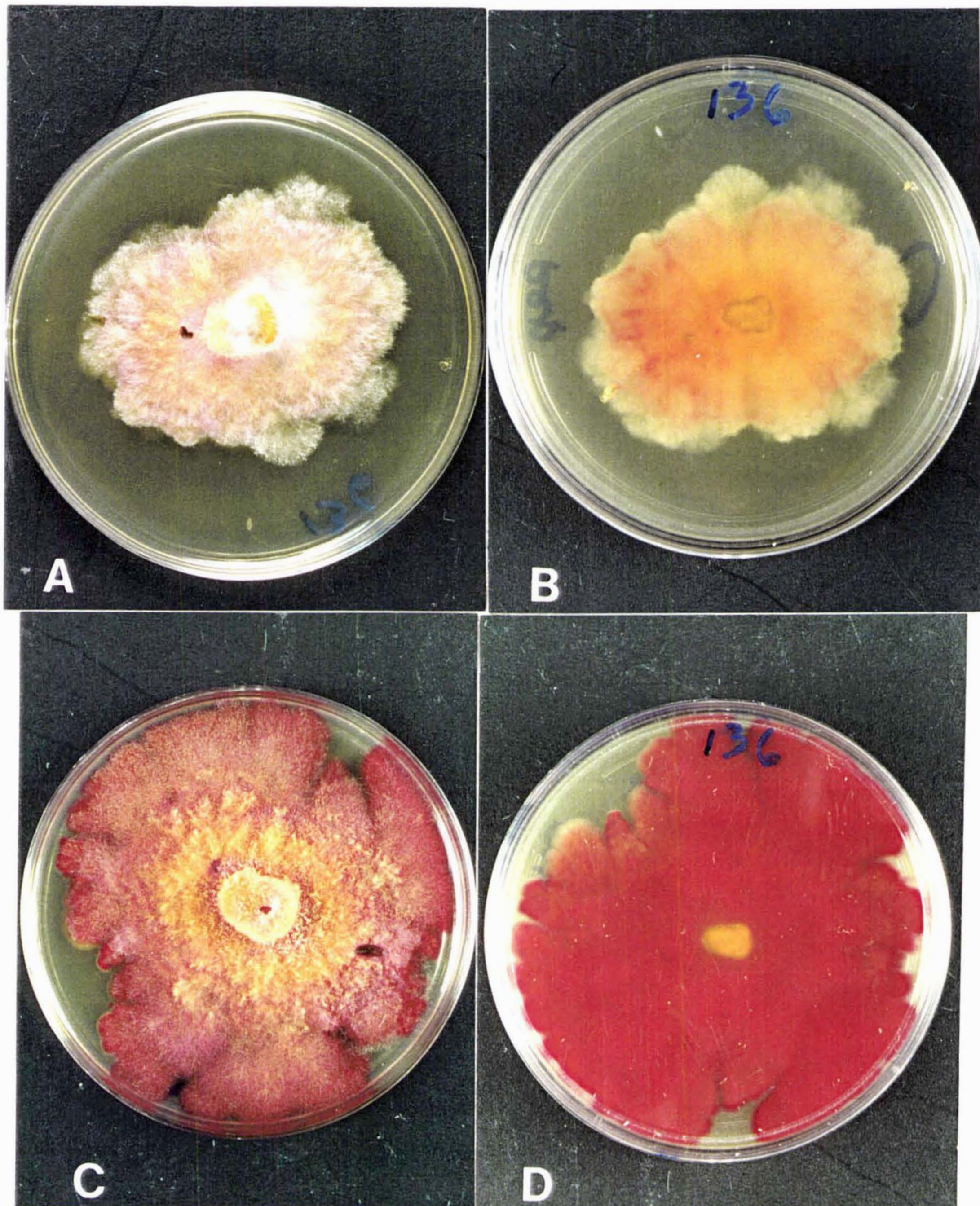


Plate 4.10 *F. graminearum* ('cream and lobed' type) on MA: A & B, 5 days old; C & D, 10 days old; A & C, top view; B & D, reverse side.

4.5.2.2 Description of *F. crookwellense* seed-culture colonies: 'red centre' type (Plate 4.11)

The appearance of the seed-culture colony morphology of *F. crookwellense* was the same as that of *F. graminearum* ('red centre' type) (Plate 4.11 A and B, also see Plate 3.10). However, on dead seeds, *F. crookwellense* colony was relatively similar to that of the 'red and fluffy' *F. graminearum* type (Plate 4.11 C and D, also see Plate 3.9).

4.5.2.3 Description of *F. culmorum* seed-culture colonies: 'cream and flat' type (Plate 4.12)

Colonies of *F. culmorum* were very large with the mycelium covering a petri dish in 6 days. The mycelium grew smooth and flat on the agar surface in a thin layer, forming a very large edge zone and ending in an even edge, but was almost absent in the centre of the colony. The colour of the mycelium was whitish cream but the centre of the colony was faintly purplish red (Plate 4.12 A and B) to red (Plate 4.12 C and D) with a brownish tinge. In some colonies sporulation was present after 5 days incubation. On the reverse side of the petri dish the pigment in the agar was faint pink to pale red or in shades of pastel red but was yellowish cream in the periphery. Because the mycelium occurred thinly on the agar, groups of hyphal strands were seen (through the reverse side) radiating from the centre of the plate (Plate 4.12 B and D).

After 10 days the mycelium was overflowing the petri dish but was still somewhat floccose and pastel red in colour with brownish orange patches or around the seed remains (almost as in *F. graminearum* 'red and fluffy' type). Sporulation was obvious in most colonies, commonly around the seed, but sometimes occurred in a ring around the area of first exposure to direct light. On the reverse side, the pigment in agar was vivid red (in red-brown-orange shades).

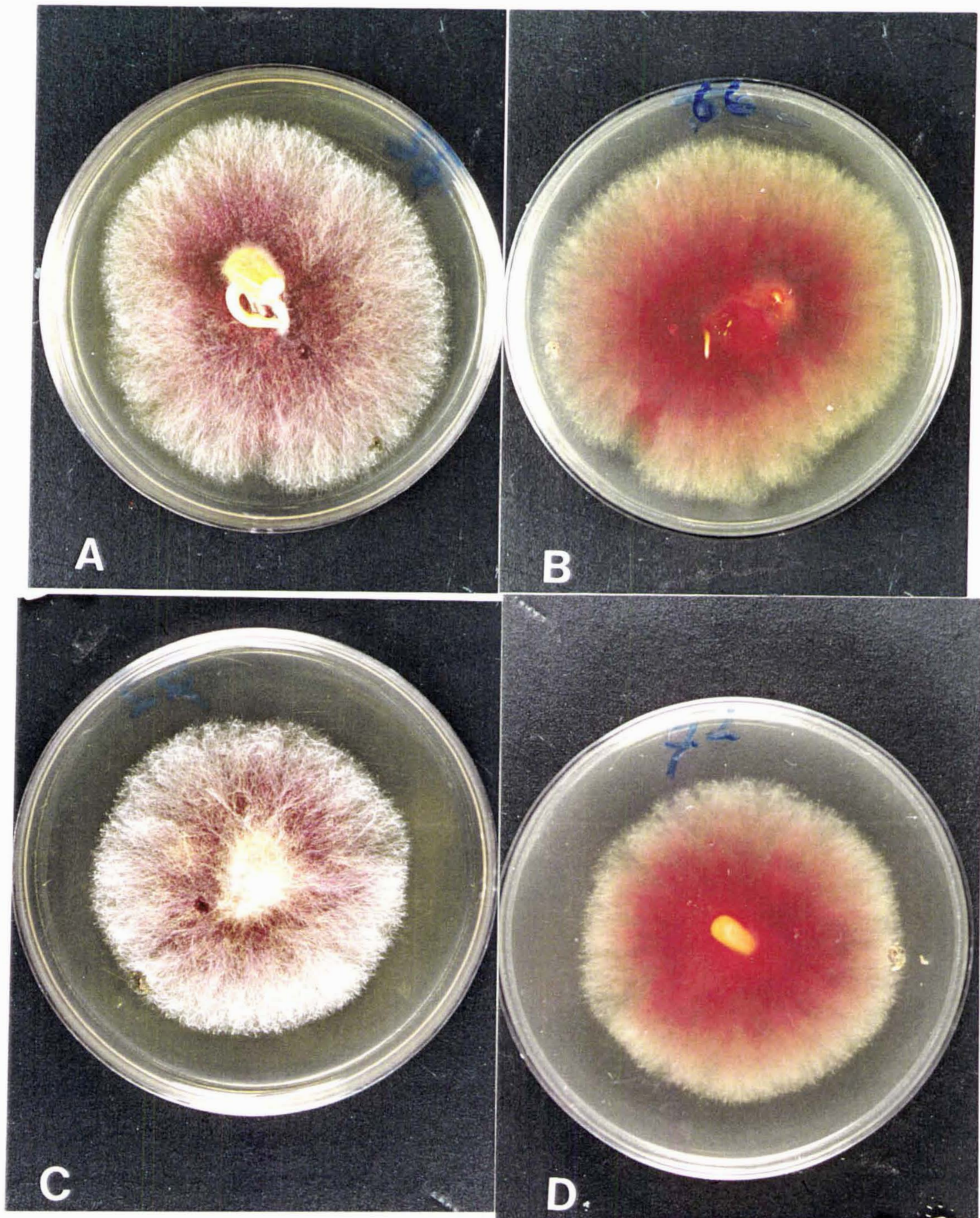


Plate 4.11 *F. crookwellense* **on MA: A & C, top view; B & D, reverse side.**

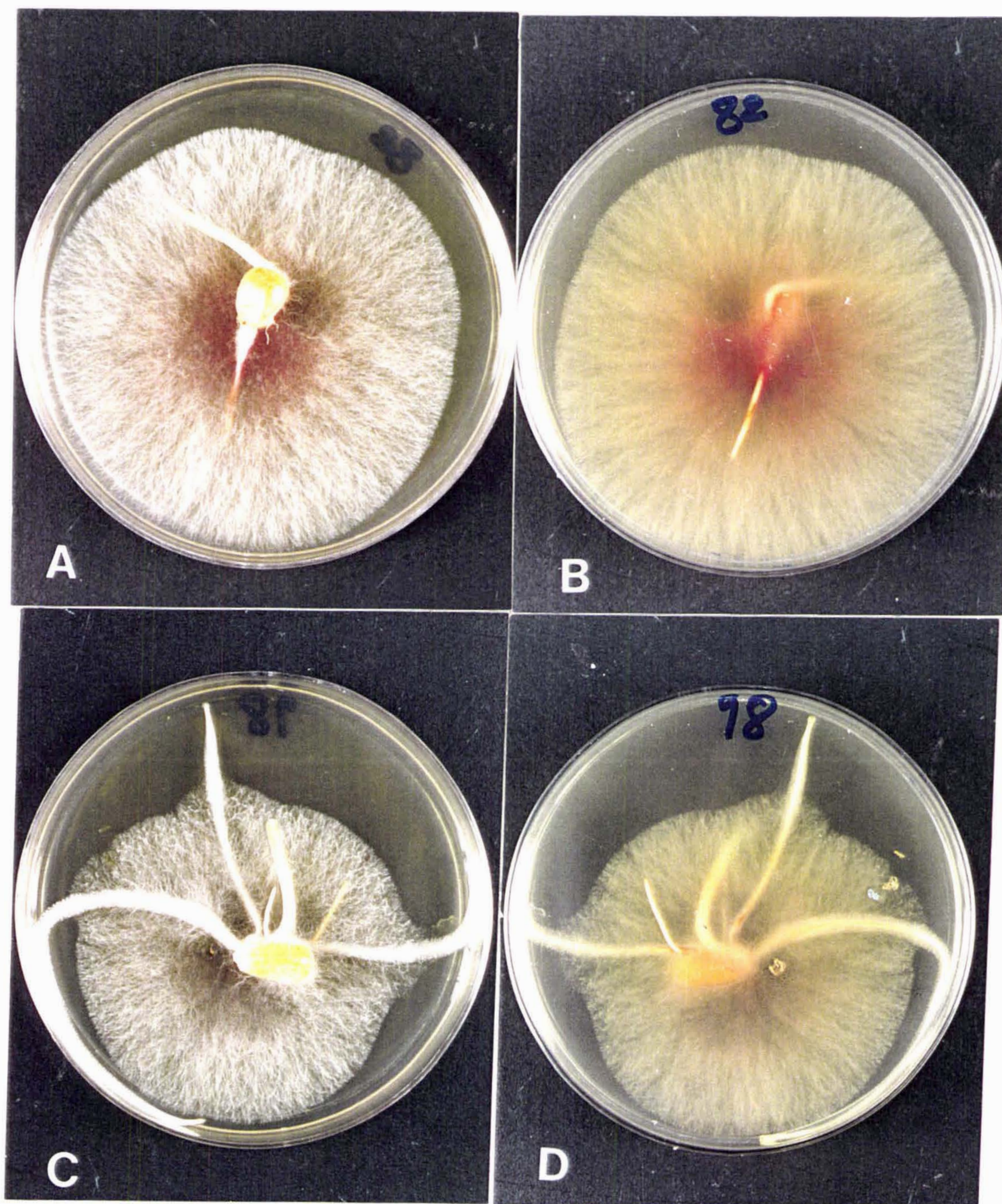


Plate 4.12 *F. culmorum* on MA: A & B, colony with a red-brown centre; C & D, with pale purplish brown centre ; A & C, top view; B & D, reverse side.

4.5.3 Effects of medium on colony appearance : Malt Extract Agar

4.5.3.1 Seed-culture colony categorisation

When seeds were plated on MEA, the *F. graminearum* seed-culture colony morphology (Table 4.5) differed from that observed on MA (Table 4.4), except for the 'cream and lobed' type. The red colouration of the colonies which were dominant on MA, both on the top (including mycelium) of the cultures and as seen from the reverse of the petri dish was replaced by a pink colouration, although the red pigment produced in agar (as seen on the reverse side of the plates) was not completely altered (Plates 4.13, 4.14 and 4.15). In addition, unlike on MA, some of the *F. graminearum* pink type of colonies sporulated abundantly (Plates 4.13 and 4.14). However, the morphology of the *F. graminearum* pink type of colonies had some features resembling those found on MA. For example, the red and fluffy type of colony (see Plate 3.9, Chapter 3) maintained the fluffy brownish orange mycelium around the dead seed and in one sector of the plate (while sporulation occurred in the other sector) (Plate 4.13); and some 'pink and lobed' type colonies were not sporulating. *F. culmorum* seed culture colonies ('cream and flat' type) were easily identified on MEA indicating that the colony appearance of this species (*F. culmorum*) was not affected by MEA medium.

4.5.4 Seed-culture colony identification on CLA

A study of the macroconidia on CLA from the 19 seed-culture colonies (14 pink and 5 cream types) (Table 4.5) showed that all the pink colonies were *F. graminearum*, but 7 of those which were sporulating abundantly only formed perithecia initials on CLA (Table 4.5). All non-sporulating 'pink and lobed', and the 'cream and lobed' types of colonies were also identified as *F. graminearum*, and all formed mature perithecia (Table 4.5). The cream and flat types of colonies were identified as *F. culmorum* (Table 4.5) and they neither formed perithecia initials nor mature perithecia.

Table 4.5 **Listing of seed-culture colony 'types' and their sporulation tendency on MEA, number of colonies in each type, and identification of *Fusarium* species.**

				Seed lots			
	Seed-culture colonies on MEA	**Seed-culture colony sporulation on MEA**	**Number of colonies on MEA**	**1990**	**1994**	**Perithecia (of *G. zeae*) formation on CLA**	**Pure culture identification of species on CLA**
	<u>'Pink' colonies</u>						
(i)	Pink and fluffy	Sporulating	1	1	0	Yes (initials)	*F. graminearum*
(ii)	Pink and lobed	Sporulating	7	7	0	Yes (initials)	*F. graminearum*
			2	2	0	No	*F. graminearum*
		Not sporulating	4	0	4	Yes (mature)	*F. graminearum*
	<u>'Cream' colonies</u>						
(i)	Cream and flat	Not sporulating	4	0	4	No	*F. culmorum*
(ii)	Cream and lobed	Not sporulating	1	1	0	Yes (mature)	*F. graminearum*

4.5.5 Description of seed-culture colonies on MEA

4.5.5.1 *F. graminearum*: 'pink and fluffy' type (Plate 4.13)

On MEA, the pink and fluffy *F. graminearum* type (Plate 4.13 A and B) was observed only on dead seeds. After 5 days incubation, the colony displayed sectoring (Nelson *et al.*, 1983), ie on one side the colony had floccose (fluffy), rapidly growing brownish orange mycelium as was observed on MA, but the other side was covered in a sheet of pinkish red sporodochia and had sparse mycelium which formed lobed edges. However, as on MA, the pigment produced in agar was dark red to brownish red and this had not changed after 10 days. Apart from the sporodochia which became red brown as they matured, the colony morphology also had not changed after 10 days incubation.

4.5.5.2 *F. graminearum*: 'pink, lobed and sporulating' type (Plate 4.14)

On MEA, a new morphological type for *F. graminearum*, characterised by heavy sporulation (Plate 4.14 A and B) was observed. After 5 days incubation, colonies demonstrated slow to medium growth and were almost devoid of mycelium or had only scanty to short mycelium with irregular edges. Their surfaces were covered in sheets of sporodochia or had rings of sporodochia giving the colonies pale red to greyish red colouration. On the reverse side of the petri dishes, the periphery of the colonies were light orange or pastel red while the centre was brownish red.

After 10 days incubation, in some cultures the mycelium showed increased lobing although it had grown up to the edge of the petri dish. Due to the lobed nature of the sparse or patchy mycelium and the maturation of the sporodochia (becoming red brown or red orange in colour), the colonies looked dry. On the reverse side the colouration of the pigment in the agar had intensified, becoming brownish red to deep red or reddish brown but in some colonies the periphery was light orange.

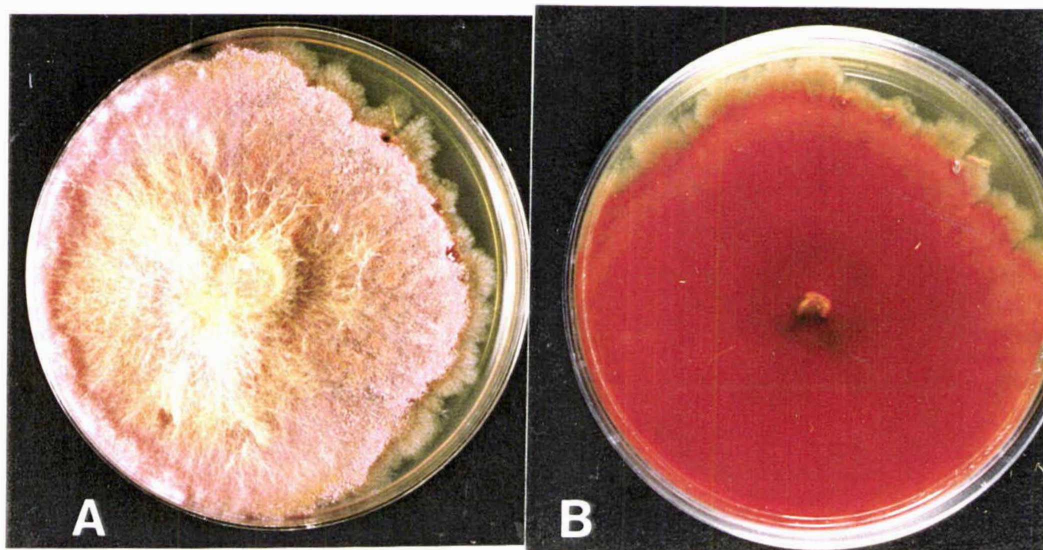


Plate 4.13 *F. graminearum*: 'pink and fluffy' type on MEA: A, top view; B, reverse side.

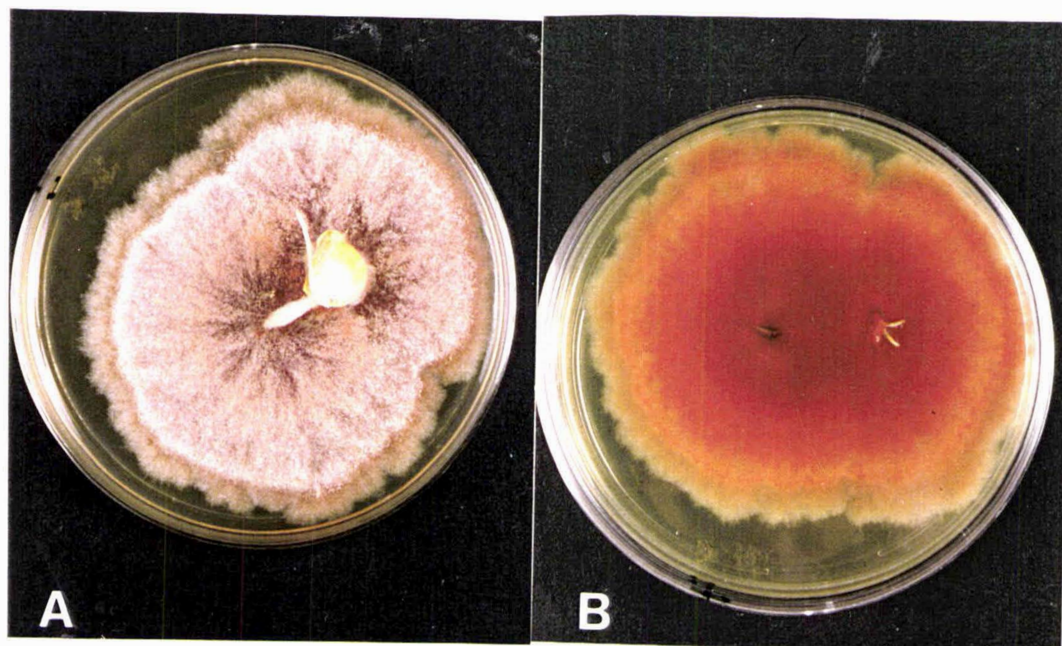


Plate 4.14 *F. graminearum* 'pink, lobed and sporulating' on MEA: A, top view; B, reverse side.

4.5.5.3 *F. graminearum*: 'pink and lobed, non-sporulating' type (Plate 4.15)

After 5 days of incubation, seed culture colonies of the pink and lobed morphological type of *F. graminearum* (Plate 4.15) exhibited slow to moderately rapid growth, with the mycelium texture ranging from short to almost floccose, being gently lobed at the edges but absent in the centre of the colonies. The mycelium colour was pinkish white. The colonies did not sporulate. The pigment produced in the agar was orange in the periphery, but pastel red in the centre.

After 10 days incubation the mycelium had not changed in texture although it had filled the petri dish. The mycelium colour had turned to greyish rose but colonies had still not sporulated. The pigment produced in the agar (on the reverse side) was pastel red but the red and orange colour mixture occurred in a variegated manner.

4.5.5.4 *F. graminearum*: 'cream and lobed' type (Plate 4.16)

Although on MEA only one colony (Plate 4.16) occurred in the seeds studied, the 'cream and lobed' *F. graminearum* type looked very similar to the same colony on MA (Plate 4.10). However, after 10 days incubation the colour of the mycelium and the pigment in the agar (reverse side of plate) were not red as on MA. Instead, the mycelium was pinkish white in the periphery and greyish red to dull red in the centre, while the pigment in the agar was brownish red, the red colour being poorly mixed (variegated) in a light orange background.

4.5.5.5 *F. culmorum*: 'cream and flat' type (Plate 4.17)

After 5 days incubation *F. culmorum* seed-culture colony morphology on MEA (Plate 4.17) differed little from that on MA (Plate 4.12), but after 10 days incubation differences were observed. On MEA the mycelium colour became pinkish rather than reddish orange as observed on MA; that is, on MEA *F. culmorum* mycelium was pale red, greyish rose to pastel red. While, in some colonies the mycelium had undergone autolysis (the dissolution of the hyphae by enzymes produced by the mycelium; self digestion - CAB, 1983), it was more or less unaffected in some colonies. The colouration of the pigment produced in agar on MEA (reverse side) was pastel red

Plate 4.15 *F. graminearum*: 'pink and lobed' non-sporulating on MEA: A & B, top views; C & D, reverse sides, respectively.

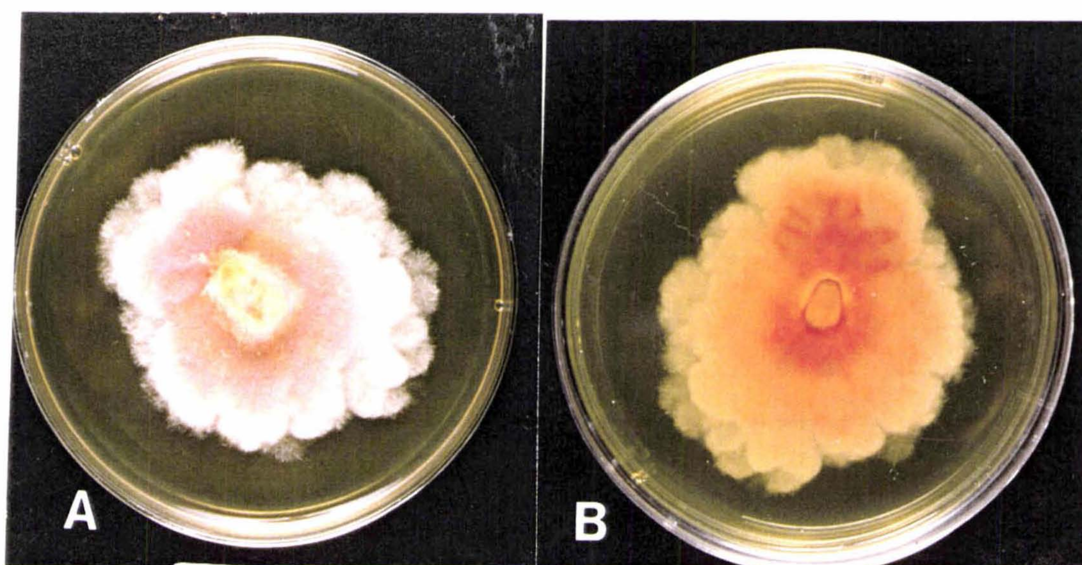


Plate 4.16 *F. graminearum*: 'cream and lobed' on MEA: A, top view; B, reverse side.

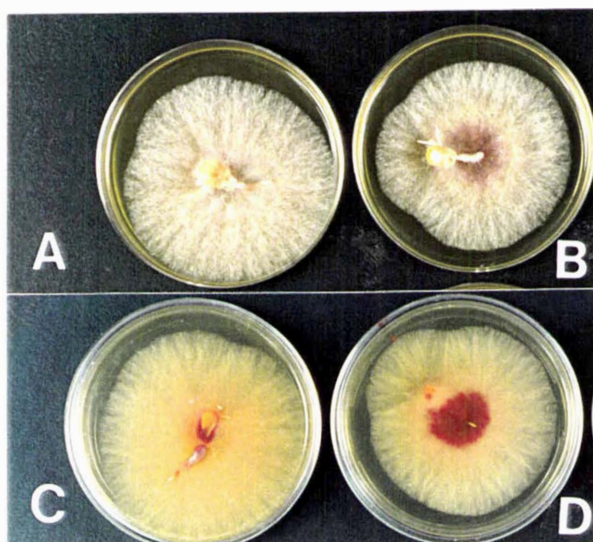


Plate 4.17 *F. culmorum* on MEA: A & B, top views; C & D, reverse sides.

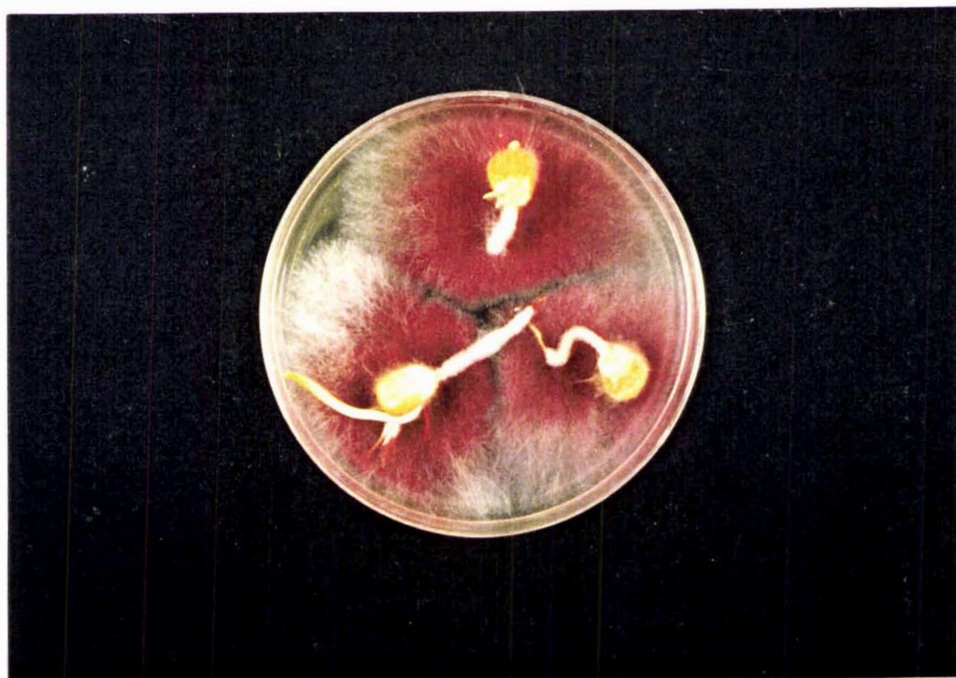


Plate 4.18 *F. graminearum/F. crookwellense* aerial mycelium absent at the colony junctions due to inter-colony effect.

to light orange being slightly less dark than on MA. All cultures sporulated after 10 days incubation.

4.5.6 Colony morphology of *F. graminearum* on PDA and on MEA following subculturing from seed-culture colonies growing on MEA

The data on colony morphology of *F. graminearum* on PDA and MEA following subculturing from seed-culture colonies growing on MEA are given in Appendices 4.2a and 4.2b. The colony morphology on PDA was standard and similar to that obtained when pure culture on MA slants were subcultured onto PDA (see Section 4.4.1 and Table 4.1a) and similar to that described by Burgess *et al.* (1988). On MEA, again, the colony morphology was poor and similar to that described in Section 4.4.1 and Table 4.1b which was different from that described by Pitt and Hocking (1985) and Burgess *et al.* (1988). These results suggested that MEA had a nutritional adverse effect on *F. graminearum.*

4.6 DISCUSSION

4.6.1 Differentiation of *F. graminearum* from *F. crookwellense* and *F. culmorum* in pure cultures on PDA

PDA is a recommended medium for the identification of *Fusarium* spp. (Nelson *et al.*, 1983; Burgess *et al.*, 1988) but it is only a valuable medium for determining gross morphological appearance, colouration and growth rate (Nelson *et al.*, 1983; Burgess *et al.*, 1988). The shape of the macroconidium is generally the most important character in the definition and identification of *Fusarium* spp. (Nelson *et al.*, 1983; Burgess *et al.*, 1988). Because of its high available carbohydrate content, PDA generally emphasises mycelial growth to the detriment of sporulation, with cultures sporulating poorly and frequently taking more than a month to do so (Nelson *et al.*, 1983). When sporulation finally occurs, the macroconidia are usually

variable in shape and size, are misshapen and atypical, and not suitable for use in identification of cultures (Nelson *et al.*, 1983; Burgess *et al.*, 1988).

The use of PDA to differentiate *F. graminearum* from *F. crookwellense* as well as from *F. culmorum* in pure cultures was not successful because the colony morphology of the three species was similar. These results were not surprising since Burgess *et al.* (1988) clearly state "*F. graminearum* can easily be confused with *F. crookwellense* and *F. culmorum.*" However, Nelson *et al.* (1983) stated that on PDA, *F. culmorum* resembles *F. graminearum*, *F. crookwellense* and certain clones of *F. sambucinum*, but that on this medium the abundant sporulation around the point of inoculation separates *F. culmorum* from *F. graminearum* but not from *F. crookwellense*. In this study only one of four isolates of *F. culmorum* produced sporodochia and this agrees with observations by Pitt and Hocking (1985) who indicated that production of abundant sporodochia by *F. culmorum* does not always occur.

The failure to clearly differentiate *F. graminearum* from *F. crookwellense* or *F. culmorum* on PDA agrees with the observation by Burgess *et al.* (1988) that colony characteristics are only useful secondary criteria for identification of *Fusarium* spp., while the description of *Fusarium* spp. is based on the morphology (shape) of macroconidia formed on CLA. Nelson *et al.* (1983) stated that on PDA *Fusarium* spp. cultures superficially look alike. This is particularly likely because *F. graminearum*, *F. crookwellense* and *F. culmorum* are all in the taxonomic section Discolor of Wollenweber and Reinking (1935); (Booth, 1971; Nelson *et al.*, 1983; Burgess *et al.*, 1988).

4.6.2 Identification and differentiation of *Fusarium* spp. seed-culture colonies on MA: *F. graminearum*, *F. crookwellense* and *F. culmorum*

There is a paucity of information on identification of *F. graminearum*, *F. crookwellense* and *F. culmorum* on MA. Burgess, L.W. (pers. comm.) stated that there is no reliable work on identification of seed-borne *Fusarium* spp. on MA, and

that this medium is not being used at the Fusarium Research Laboratory, University of Sydney, Australia. In this study no attempt was made to examine macroconidia of *F. graminearum* and *F. crookwellense* direct from seed culture colonies on MA because some colonies were not sporulating, and from a small earlier trial it was found that macroconidia from those colonies which did sporulate were variable, atypical and misshapen. But seed culture colonies of *F. culmorum* were relatively easy to identify from the macroconidia obtained from the sporodochia on MA after 6-10 days because of their short and stout distinct shape (Burgess *et al.*, 1988).

F. graminearum, *F. crookwellense* and *F. culmorum* seed-culture colonies have not been previously described on MA (Mathur, S.B., pers. comm.). In this study it was only possible to distinguish *F. graminearum* colony types 'red and fluffy', 'red and lobed', 'cream and fluffy', and 'cream and lobed' (Plates 3.9-3.12, see Chapter 3) from those of *F. crookwellense* (Plate 4.11) because their colony morphology and colours were different. However, it was not possible to distinguish the 'red centre' type of *F. graminearum* seed culture colonies from those of *F. crookwellense* because their morphology and colour looked similar (Plate 3.10 and 4.11). These results conform with the identifications by IMI (see Chapter 3). This was not surprising since *F. graminearum* and *F. crookwellense* are difficult to differentiate on PDA (Nelson *et al.*, 1983; Burgess *et al.*, 1988) and MA is also a rich medium like PDA (Smith and Onions, 1983). Moreover, *F. crookwellense* was described as a new species only in 1983 (Nelson *et al.*, 1983). Before that its identification was confused with that of *F. graminearum*, with isolates being referred to as "Graminearum-like isolates", or "Soil graminearum" (Burgess *et al.*, 1982). IMI identified *F. crookwellense* (isolate No 3R from a 'red and lobed' seed-culture colony (see Chapter 3)). In this study none of the red and lobed type of seed-culture colonies were identified as *F. crookwellense*. It appears therefore that the identification of culture/isolate No 3R by IMI may have been due to a miscategorisation of the particular seed-culture colony from which the isolate was obtained. The likely cause for this miscategorisation could be due to the fact that the colony had its aerial mycelium reduced or adversely affected by effects of the adjacent seed-culture colonies, as was observed when more than one *Fusarium* spp. (especially *F.*

graminearum/F. crookwellense) infected seeds were plated per petri dish (Plate 4.18, see p. 173). It is recommended that for accurate categorisation and identification of seed-culture colonies, not more than one or two seeds be plated per petri dish to allow full expression of colony morphology, especially aerial mycelial growth.

While visually and through macroconidial shape it was possible to distinguish *F. culmorum* seed-culture colonies from those of *F. graminearum* on MA, the difference between the appearance (morphology) of *F. culmorum* ('cream and flat' type) (Plate 4.12) and *F. graminearum* ('cream and fluffy' type) (Plate 3.12 - see Chapter 3) was small; the only difference between the morphology of the colonies of the two species being the flat, thinly growing mycelium of *F. culmorum* on the agar (Plate 4.12). These colony colours and morphology types may not initially be different, unless when examined on the reverse side after 5 days incubation on MA under diffuse light. However, on MA *F. culmorum* seed-culture colonies could not be confused with those of *F. crookwellense* because the colony morphology and colour of the two species were different.

It can be concluded therefore that MA is a useful medium for differentiating *F. culmorum* from *F. graminearum* and *F. crookwellense* seed-culture colonies, and for distinguishing some (four) types of *F. graminearum* seed-culture colonies, viz, 'red and fluffy', 'red and lobed', 'cream and fluffy' and 'cream and lobed' from those of *F. crookewellense* which were the red centre type of colonies. On MA it was not possible to distinguish *F. crookwellense* from *F. graminearum* (red centre type of colony) because seed-culture colony appearance was similar.

4.6.3 Effects of MEA on pure cultures and on seed-culture colony morphology of *F. graminearum*, *F. crookwellense* and *F. culmorum*; and perithecia formation by *F. graminearum*

Although Pitt and Hocking (1985) described *F. graminearum* and *F. culmorum* in pure cultures on MEA, there is a paucity of information on the description of *F. crookwellense* on this medium. Pure culture growth performance of *F. graminearum*,

F. crookwellense and *F. culmorum* was poor, ie slow growing colonies with reduced aerial mycelium and some with lobed edges, unlike that observed on PDA by the same isolates. Similar growth behaviour was observed in seed-culture colonies of *F. graminearum*, but all colonies were pink in colour, most colonies showed heavy sporulation and lobed edges (lobing also occurring in those colonies which did not sporulate), and consequently these characteristics caused difficulties in categorising different colonies on MA. The dramatic change in the morphology of pure cultures of *F. graminearum*, *F. crookwellense* and *F. culmorum* and of seed-culture colonies of *F. graminearum* on MEA was not expected, particularly the performance of *F. graminearum* seed-culture colonies, since MEA and MA contain malt extract, agar and water (Difco Laboratories, 1972; Commonwealth Mycological Institute - CAB, 1983). The poor performance of these *Fusarium* species on MEA, was attributed to the presence of peptone in this medium (Gibco BRL, 1993). CAB (1983) recommends that peptone be omitted from fungus media. While peptone in the medium, eg Nash medium (Peptone PCNB agar - Nash and Snyder, 1962) has been recommended for the stimulation of sporulation in *Fusarium* spp., especially for *F. graminearum*, it may be toxic to fungi (Toussoun and Nelson, 1976). The metabolism of peptone from Nash medium leads to the accumulation of toxic ammonia which kills fungi (including *Fusarium* spp.) if left on the agar for 20-30 days (Toussoun and Nelson, 1976; Burgess *et al.*, 1988). Peptone medium or Peptone PCNB agar are also acidic (Toussoun and Nelson, 1976; Burgess *et al.*, 1988) and so is MEA (CAB, 1983; Pitt and Hocking, 1985). It is likely therefore that the low pH (3-4 - CAB, 1983; or near 5 - Pitt and Hocking, 1985) and perhaps the presence of some ammonia in the agar resulted in the heavy sporulation and reduced growth rate of *F. graminearum* on MEA.

The failure to form perithecia on CLA by cultures initiated by single germinated spores from heavy sporulating seed-colonies on MEA after incubation under NUV black light illumination (40W) appears to have some relationship with the results of Ross *et al.* (1971) who found that perithecia of *Venturia inaequalis* did not form in culture media in which ammonium salts were added as the nitrogen source because the cultures became acidic with age. However, these formed perithecia when

calcium carbonate was added to the media to control pH. Since the macroconidia used in this study were obtained from a peptone containing medium it was thought that they were physiologically/genetically adversely affected. However, since the ability to form perithecia was not affected in *F. graminearum* seed-culture colonies which had not sporulated, it seems that the factor (possibly peptone) that caused heavy sporulation of seed-culture colonies did not affect all strains of *F. graminearum*.

By the fact that when pure cultures of *F. graminearum* initiated by single germinated spores from morphologically poor and MEA heavily sporulating seed-culture colonies were grown on PDA the expected normal colony morphology (floccose aerial mycelium, red colouration and sporodochia formation - Burgess *et al.*, 1988) was recovered made the understanding of the real effects of peptone in MEA elusive.

F. culmorum seed-culture colony morphology on MEA did not differ greatly from that on MA, although after 5 days incubation the aerial mycelium was pinkish and not red as on MA. These results suggest that the effects of MEA may be species specific.

Low concentrations of 1.5% MEA (Marasas *et al.*, 1979, 1981; Rheeder *et al.*, 1990) have been used in *Fusarium* spp. seed infection studies and ISTA (1993) recommends use of 2% MEA in fungal seed health studies. Perhaps if these concentrations of MEA had been used, rather than the 5% used in this study, adverse cultural morphology effects may not have occurred.

It is therefore concluded that MEA is not a suitable medium for differentiating *F. graminearum* from *F. crookwellense* or these species from *F. culmorum* in pure cultures, but may be used to differentiate *F. graminearum* from *F. culmorum* seed-culture colonies. It is also suggested that further study on the effects of MEA on growth and reproduction of *F. graminearum*, *F. crookwellense* and *F. culmorum* is necessary to explain these results.

4.6.4 Incubation period for seed-culture colonies

Continued incubation of seed-culture colonies for up to 10 days on MA was not found to be of advantage in categorising seed-culture colonies of *F. graminearum*, or in differentiating seed-culture colonies of *F. graminearum* from those of *F. crookwellense* or *F. culmorum*, because in most of the colonies the aerial mycelium had undergone autolysis. In addition, the aerial mycelium of the *F. graminearum* 'cream and fluffy' type and of *F. culmorum* looked similar, with both also resembling the 'red and fluffy' type of *F. graminearum* after 5 days incubation. Also, by 10 days incubation, the mycelium overflowed the petri dishes. Therefore, the seed-culture colony categorisation for the identification of *F. graminearum*, *F. graminearum/F. crookwellense* or *F. culmorum* on MA should be done after 5 days incubation, since by that time any morphological characters useful for colony characterisation are already sufficiently developed.

4.6.5 Mass transfer of inoculum direct onto CLA versus single spore culture initiation for identification of *F. graminearum*, *F. crookwellense* and *F. culmorum* from maize seed-culture colonies on MA

Use of a single germinated spore/conidium for the preparation of cultures for the identification of *Fusarium* spp has been emphasised (Booth, 1971, 1977; Toussoun and Nelson, 1976; Nelson *et al.*, 1983, 1986; Pitt and Hocking, 1985; Burgess and Liddell, 1983; Burgess *et al.*, 1988), mainly to be able to observe and discard mutant cultures which are avirulent in pathogeneicity studies, and to ensure purity of cultures by allowing separation of the component species of a mixed culture (Burgess and Liddell, 1983). In this study, in most cases, it was possible to get pure cultures of *F. graminearum*, *F. crookwellense* and *F. culmorum* by mass transfer of inoculum from seed-culture colonies, and the pure cultures of *F. graminearum* prepared in this way formed mature perithecia of *G. zeae*, both in cultures initiated using inoculum from the edge or from the centre of the seed-culture colonies. The exception to this was in cultures which sporulated abundantly. It appeared that success in obtaining pure cultures by mass transfer was due to the observation that commonly when a

maize seed was infected by *F. graminearum*, *F. graminearum/F. crookwellense* (red centre type of colony) or *F. culmorum*, other Fusaria were not observed in the same seed-culture colony (although occasionally *F. subglutinans* was detected from the same maize seed that yielded *F. graminearum*). This observation could be due to the fact that *Fusarium* spp. are known to be antagonistic (Rheeder *et al.*, 1990; Lacey and Magan, 1991). For example, Rheeder *et al.* (1990) found negative association between *F. moniliforme* and *F. graminearum*.

Although some pure cultures of *F. graminearum* on CLA initiated by mass transfer of inoculum from 'red and fluffy' and some 'red centre' types of colonies from MA did not form perithecia, the cause of this did not appear to be related to the method of inoculum transfer, but perhaps was a strain related occurrence or to an unidentified factor referred to by Booth (1981) that occurs in some *F. graminearum* pure cultures ("artificial cultures").

Mass transfer of *Fusarium* species mycelium inoculum was used by Cullen *et al.* (1982) in a study of morphological stability and Zearalenone production by *G. zeae* isolates, while Tuite *et al.* (1974) used the same procedure in the identification of *G. zeae*. Considering the lengthy "protocol" (Fig. 2.2 - see Chapter 2) for isolation and preparation of *Fusarium* pure cultures for identification, it appears that the use of mass inoculum transfer of *F. graminearum*, *F. crookewellense* and *F. culmorum* directly from seed-culture colonies onto CLA offers an alternative but quick method of preparing pure cultures during seed-health testing, during which many seeds are usually examined and each seed needs to be screened/assessed individually for seed-borne *Fusarium* infection.

4.6.6 Identification of *Fusarium* spp. on CLA

4.6.6.1 Identification based on macroconidia

The use of CLA for identification of *Fusarium* spp. is based on the principle that Fusaria sporulate readily (in 5-10 days) on this medium forming sporodochia in which macroconidia are produced, and that these macroconidia are uniform in shape

and size making it possible for correct identification of different Fusaria (Fisher *et al.*, 1982; Nelson *et al.*, 1983, 1986; Burgess *et al.*, 1988).

Sporulation is stimulated by light but is favoured by alternating light and darkness and fluctuating temperature (Zacharia *et al.*, 1956; Toussoun and Nelson, 1976; Nelson *et al.*, 1983; Burgess *et al.*, 1988). Leach (1962a, 1963a, 1964, 1967a, 1967b) found that radiation in the near ultraviolet (NUV) region of the spectrum (320-420 nm) is efficient in inducing sporulation. Black fluorescent light (Black light) tubes which emit NUV light at wavelengths of 320-420 nm (maximum at 360 nm) and cool white fluorescent tubes which emit some NUV light at wavelengths of 300-750 nm have become standard equipment in seed health testing (Leach, 1967a, 1971b, 1979a; Neergaard, 1979), and in *Fusarium* species identification studies (Booth, 1971, 1977; Fisher *et al.*, 1982; Burgess and Liddell, 1983; Nelson *et al.*, 1983; Nelson *et al.*, 1986; Burgess *et al.*, 1988). *Fusarium* spp. cultures for identification are incubated under NUV Black Light at alternating temperatures of 25°C day/20°C night with a 12 h photoperiod, and depending on the space available, using one 40W Black Light tube and one, two or four 40W cool white fluorescent tube (Booth, 1971, 1977; Nelson *et al.*, 1983; Burgess and Liddell, 1983; Burgess *et al.*, 1988); in all cases the tubes must be 40-45 cm (Nelson *et al.*, 1983) or 40 cm (Neergaard, 1979; Burgess and Liddell, 1983; Burgess *et al.*, 1988) above the culture. Two NUV black light tubes give better results (Leach, 1967b; Neergaard, 1979).

In this study, the identification of macroconidia of *F. graminearum*, *F. crookwellense* and *F. culmorum* or of *F. sambucinum* and *F. decemcellulare* on CLA cultures from pure cultures on MA or from seed-culture colonies on MA, initiated by a single germinated spore or by mass transferred inoculum was possible because the macroconidia were uniform and their shape was as has been described by Nelson *et al*, (1983) and Burgess *et al.* (1988). Thus, the results were in conformity with the observations by Nelson *et al.* (1983, 1986), Burgess and Liddell (1983), Burgess *et al.* (1988) and Nelson (1991), ie that CLA is an ideal medium for the identification of *Fusarium* species. Incubation conditions of 2 x 18W (36 W) black light and 2 x 20W (40W) white cool fluorescent light were as good as 1 x 40W black light and 2

x 36W (72W) white cool fluorescent light for the sporulation of the *Fusarium* species. Because *F. sambucinum* and *F. demcellulare* occurred rarely, their morphology was not sufficiently studied and therefore the two species were not fully described.

4.6.6.2 Identification of *F. graminearum* based on perithecia formation

Francis and Burgess (1977) describe two populations of *F. graminearum*, viz *F. graminearum* Group 1 and *F. graminearum* Group 2, and Nelson *et al.* (1983) and Burgess *et al.* (1988) state that the two populations cannot be differentiated on the basis of the morphology of their macroconidia or conidiophores but can be distinguished on the basis of perithecia formation, which is a characteristic of *F. graminearum* Group 2. CLA is recommended for the formation of perithecia of *G. zeae* (Tschanz *et al.*, 1975a; Fisher *et al.*, 1982; Nelson *et al.*, 1983; Burgess *et al.*, 1988), but incubation under NUV black light (40W) and white cool fluorescent light (40W) and alternating temperatures and light, ie 25°C day/20°C darkness with a 12 h photoperiod (with light tubes held at 40-45 cm) is necessary (Tschanz *et al.*, 1976; Fisher *et al.*, 1982; Nelson *et al.*, 1983, 1986; Burgess *et al.*, 1988). Under these conditions the perithecia of *G. zeae* form readily on CLA (Nelson *et al.*, 1983; Burgess *et al.*, 1988). In this study, most cultures of *F. graminearum* on CLA, either initiated by a single germinated spore, or by mass transferred inoculum, readily formed perithecia of *G. zeae* when incubated under NUV light using 1 x 40W black light tube with 2 x 36W white cool fluorescent tubes held 33.5 cm above the culture plates under laboratory temperature conditions 25°C day/20°C night or 20°C day/16°C night with a 12 h photoperiod. Although this seemed to suggest that the recommended (Nelson *et al.*, 1983; Burgess *et al.*, 1988) incubation conditions can be adjusted, it appears that the 40W black light is still essential since incubation under black light with a lower wattage did not stimulate perithecia maturation.

The cause of the failure to get full size development of *G. zeae* perithecia when cultures of *F. graminearum* on CLA were incubated under light conditions with light source of 2 x 18W (36W) NUV and 2 x 20W (40W) white cool fluorescent light tubes was unclear. There is little information on the effects of this type of wattage

combination on the development and maturation of *G. zeae* perithecia, although it does not seem to differ much from the recommended (Nelson *et al.*, 1983) 1 x 40W NUV black light and 1 x 40W cool white fluorescent light tubes. However, the cause of failure for perithecia to mature may have been due to any of the following problems or a combination of them: viz, lamp (tube) types used, lamp power supply, ie voltage fluctuation, or temperature within the petri dishes (Leach, 1967a, 1971b, 1979a). Unfortunately none of these factors were specifically examined at the start of the course of this study as suggested by Leach (1979a).

Tschanz *et al.* (1976) found that the number of perithecia produced on CLA medium increased with increasing temperature to about 29°C but then decreased sharply at temperatures higher than 29°C and temperatures between 27° and 31 °C appeared to increase the percentage of small, collapsed, immature or aborted perithecia. In this study, the temperature of 27°C occasionally observed is close to 27°-31°C which caused abortion in perithecia (Tschanz *et al.*, 1976). Leach (1971a) found that protoperithecia of *Pleospora herbarum* (Per) Rabenhorst were influenced by the temperature during NUV irradiation, ie at 24-27°C and apparently maturation was prevented by a temperature of 30°C. Since Leach (1967a) reported that due to heat produced by fluorescent lamps there is a likelihood that the temperature within cultures may be higher than the outside if lamps are too close to the cultures, it is possible that even when the culture plates were 41 cm below the light tubes, the temperature inside the petri dishes was higher than 20-25°C/20-27°C which was measured on the outside of the petri dishes, and this therefore may have caused the failure of perithecia development to full size.

Incubation in darkness favours dense mycelial growth (Zacharia *et al.*, 1956). It was observed that incubation under the light conditions of 2 x 18W (36W) NUV and 2 x 20W (40W) white cool fluorescent light tubes favoured dense mycelial growth and tall "perithecial initial hairs" (Plate 4.4b), unlike cultures incubated under 1 x 40W NUV black light and 2 x 36W (72W) white cool fluorescent tubes under which mature perithecia formed readily. This suggests that illumination light intensity was insufficient under the conditions of 2 x 18W NUV and 2 x 20W white light. Sparse

aerial mycelium grew in the cultures under 1 x 40 W black light and 2 x 36 W white light suggesting stress conditions in the culture. It is possible that under 2 x 18 W NUV and 2 x 20 W white light the aerial mycelial growth and perithecial initial hairs interfered with the NUV light transmission into the lower layers of the cultures (especially onto the carnation leaf pieces) thus causing additional reduction in light intensity, resulting in perithecia abortion.

Although *G. zeae* is known to form perithecia readily on CLA under favourable incubation conditions of light and temperature (Nelson *et al.*, 1983; Burgess *et al.*, 1988), the failure to get the perithecia to mature under conditions of this study seem to conform to the observation by Burgess and Liddell (1983), who stated that "getting *Fusarium* to form perithecia can be a most frustrating experience."

4.6.7 Relationships among *F. graminearum* seed-culture colony types, population Groups 1 and 2 of Francis and Burgess (1977) and morphological types A and B of Cullen *et al* (1982)

The observation that CLA cultures from *F. graminearum* seed-culture colonies which did not sporulate on MA (or on MEA) readily formed perithecia of *G. zeae* on CLA, but those which sporulated on these media only formed abundant sporodochia on CLA, suggests a relationship between the different seed-culture colonies with *F. graminearum* Group 1 and Group 2 populations as described by Francis and Burgess (1977). Group 1 is a pathogen of wheat, barley and other small cereals and grasses, while Group 2 is a pathogen of maize as well as these other crops (Sutton, 1982; Burgess *et al.*, 1988). Group 2 readily forms perithecia of *G. zeae* on CLA, while Group 1 forms abundant sporodochia and not perithecia (Nelson *et al.*, 1983; Burgess *et al.*, 1988). It therefore appears that seed-culture colonies of the 'red and fluffy' and the majority of the 'red centre' types of *F. graminearum* which failed to form perithecia of *G. zeae* were Group 1; but since they were isolated from surface disinfected maize seeds they were expected to be of *F. graminearum* Group 2 (*G. zeae*).

Windels (1991), however, stated that isolates of Group 2 mistakenly may be classified as Group 1 if they have lost the ability to form perithecia by frequent transfer and/or mutations, or if they have been incubated under inappropriate conditions. In this study since cultures from 'red and fluffy' and 'red centre' types of colonies were incubated under illumination with 40W black light tubes and still failed to form perithecia of *G. zeae*, it was considered that inappropriate incubation conditions were not applicable. The question then remained as to how these colonies may have lost their ability to form perithecia since subculturing was kept to a minimum (usually one or two subcultures). A possible option was that mutation caused the failure of these cultures to form perithecia. Nelson *et al.* (1983) have stated that mutations are enhanced when *Fusarium* species are grown on PDA and similar carbohydrate rich media including seeds. In this study the 'red and fluffy' seed-culture colonies were associated with dead seeds which are expected to have a high available carbohydrate content (Nelson *et al.*, 1983). These authors discouraged use of seeds as an identification medium for *Fusarium* species as sporulation would not readily occur. It is therefore suggested that perhaps the fluffy nature of the colony is attributable to the rich substrate (the dead seed). If this is true, there is strong reason to consider mutation as a possible cause of the failure to form perithecia by the 'red and fluffy' type of *F. graminearum*. Moreover, mechanism(s) of mutation in *Fusarium* is not well understood (Burgess *et al.*, 1988). The other aspect to consider for the failure to form perithecia by the 'red and fluffy' types of *F. graminearum* is the possibility of the causes being related to production of the mycotoxin Zearalenone, a sex metabolite (Eugenio, 1968).

Cullen *et al.* (1982) isolated on PDA two distinct morphological forms (type A and B) of *G. zeae* from naturally infected corn kernels from the north central United States. The typical (type A) *G. zeae* isolates were fast growing, produced abundant white aerial mycelium and circular colonies on freshly prepared PDA, and red pigment that darkened with culture age. In contrast, type B isolates were slower growing, produced irregularly shaped appressed colonies on PDA and were pigmented brownish yellow. Type A isolates produced varying amounts of Zearalenone but were lower than those of type B; while type A was pathogenic (causing cob rot), type

B was non-pathogenic and reproduced morphologically in PDA cultures. However, both type produced fertile perithecia on carnation leaf disks.

In this study, although Zearalenone production and pathogenicity studies on ear rot disease were not made from isolates from individual seed-culture colony types, Zearalenone production and seedling pathogenicity were detected in the seed-lots used for seed-culture colony studies (see Chapters 3 and 5).

The' red or cream' lobed types of colonies of *F. graminearum* seem to correspond to the type B of Cullen *et al.* (1982), while the 'red and fluffy', the 'cream and fluffy', and the 'red centre' all seem to correspond to type A of Cullen *et al.* (1982). The only difference between the seed-culture colonies and their findings is that while all the Cullen *et al.* (1982) isolates produced perithecia of *G. zeae*, the 'red and fluffy' types of colonies in this study did not. Cullen *et al.* (1982) considered their type A isolates to be analogous to Group 2 of Francis and Burgess (1977) because of the abundant aerial mycelium, the red pigmentation in agar and the formation of perithecia on carnation leaf disks, but could not relate type B to Group 1 of Francis and Burgess (1977) because type B formed perithecia while Group 1 did not. The main relationships of types A and B of Cullen *et al.* (1982) with Group 1 and Group 2 of Francis and Burgess (1977) and the seed-culture colonies observed in this study are presented in Fig 4.2.

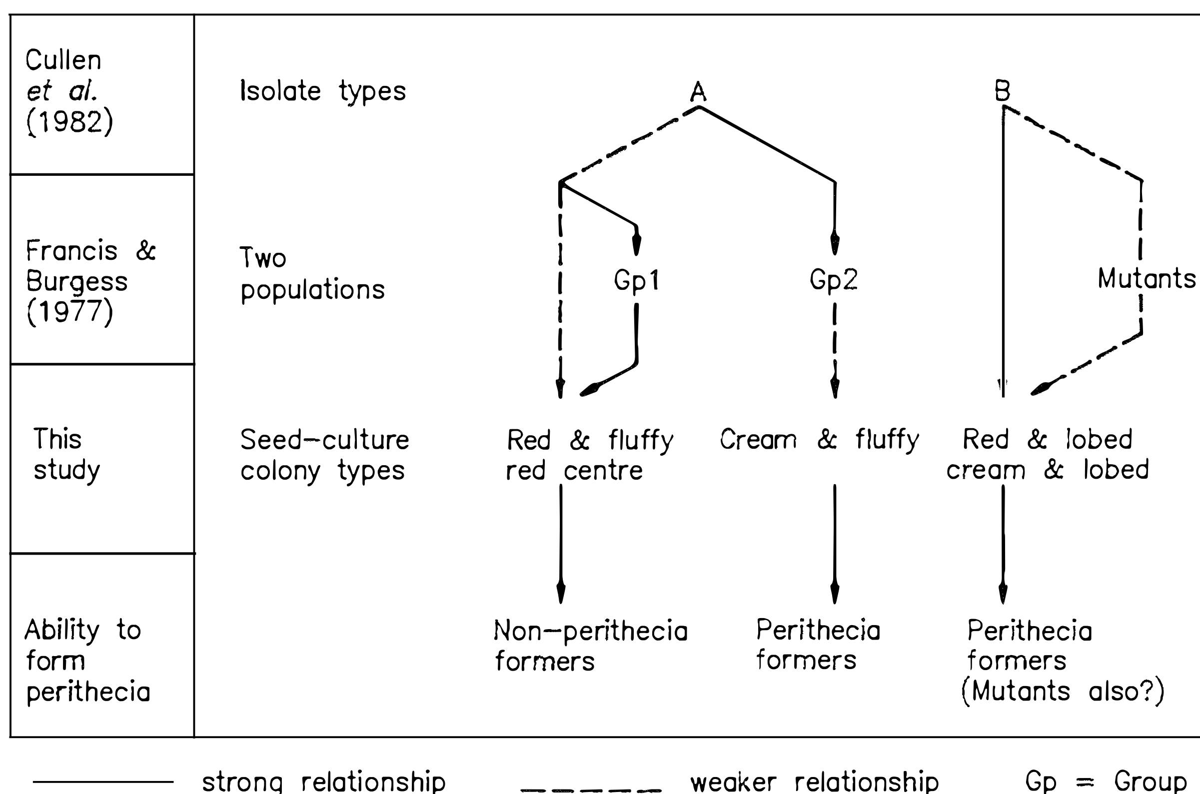


Figure 4.2 Relationships for *F. graminearum* isolates A and B of Cullen *et al.* (1982), Group 1 and Group 2 of Francis and Burgess (1977) and seed-culture colony types.

Whereas the descriptions of the 'red and fluffy' and some of the 'red centre' types of colonies agree with the Cullen *et al.* (1982) description of isolate A, they did not form perithecia, and because of this, they have a relationship with Group 1 of Francis and Burgess (1977) (Fig. 4.2). However, the occurrence of the 'red and fluffy' type on maize seeds dead because of cob rot disease caused by *G. zeae* and that of 'red centre' types from surface disinfected maize seeds relates them to pathogenic *F. graminearum* isolates - which agrees with the behaviour of type A of Cullen *et al.* (1982) and *F. graminearum* Group 2 (Fig. 4.2).

While the 'cream and fluffy' types of colonies relate to type A of Cullen *et al.* (1982) in respect to abundant mycelium growth and formation of perithecia, they differ from Group 2 of Francis and Burgess (1977) since they did not form significant red pigment, but perhaps pigmentation was minimal because of poor illumination

(Zacharia *et al.*, 1956) during incubation in the seed germinator. If this is true, then the 'cream and fluffy' colonies are analogous to type A of Cullen *et al.* (1982) and Group 2 of Francis and Burgess (1977) (Fig. 4.2).

Cullen *et al.* (1982) found that type B isolates produced higher levels of Zearalenone than type A, and Zearalenone is a sex metabolite (Eugenio, 1968; Eugenio *et al.*, 1970; Wolf and Mirocha, 1973, 1977; Vesonder and Hesseltine, 1981) which promotes or inhibits perithecia production (Eugenio 1968; Eugenio *et al.*, 1970; Wolf and Mirocha, 1973). Eugenio *et al.* (1970) found that an isolate of *Fusarium roseum* (*F. graminearum*/*G. zeae*) that produced the greatest amount of F-2 (Zearalenone) commonly formed mature perithecia in culture, although it has been found by Eugenio (1968) and Wolf and Mirocha (1973) that low amounts of 1 to 10 ng/g enhance whereas high amounts of 10 to 100 μg/g inhibit sexual reproduction. Since the 'cream and fluffy' and the 'lobed' (red or cream) types readily formed perithecia, then perhaps they had the right levels of Zearalenone produced in the CLA cultures, thus confirming the apparent relationship between the lobed types and type B of Cullen *et al.* (1982). However, this disagrees with the observations by Caldwell and Tuite (1970) who found that isolates of *G. zeae* (*F. graminearum*) with appressed growth habit did not form Zearalenone. (It is not known/reported whether mutants form perithecia though they are known to be non-pathogenic - Nelson *et al.*, 1983; Burgess *et al.*, 1988; Windels, 1991). Since the 'cream and lobed' types were usually associated with dead seeds and are therefore considered to be pathogenic, this disagrees with observations by Cullen *et al.* (1982), Nelson *et al.* (1983), Burgess *et al.* (1988) and Windels *et al.* (1991)). Cullen *et al.* (1982) refuted the suggestion that mutants occur due to natural spontaneous mutation and suggested that the lack of prominence of type B isolates was due to their low occurrence (5%) but it appears this is because it is recommended (Nelson *et al.*, 1983; Burgess *et al.*, 1988) that mutants should be discarded whenever they occur in pure cultures.

4.7 CONCLUSION

While it was possible to identify five of the *F. graminearum* seed-culture colonies on MA, differentiation of *F. graminearum* ('red centre' type) from *F. crookwellense* ('red centre' type) remained difficult. However, on MA, *F. graminearum/F. crookwellense*, *F. culmorum* and to a grerat extent *F. sambucinum* were distinctly different from the four other *F. graminearum* seed-culture colony types. Since *F. graminearum/F. crookwellense* seed-culture colonies ('red centre') had a tendency to sporulate, further studies, including effects of light (for sporulation) on seed-culture colonies on MA, might give more information on differentiation of colony morphology of these two species.

It was also difficult to acertain the identity of *F. graminearum* Group 1 (Francis and Burgess, 1977) among isolates from surface disinfected maize seeds, growing on CLA. Since different levels of mycotoxin Zearalenone are reported to have either inhibitive or promotive effects on cultures' ability to form perithecia of *G. zeae*, perhaps further study would establish why some of these isolates failed to form perithecia when other factors (substrate, light, inoculum type and frequency of subculturing) did not appear to be hinderances.

From the results of the study of seed-culture colonies on MA, and the work of Cullen *et al.* (1982), it is clear to expect variations in culture morphology in any study of *F. graminearum* from maize seeds other than one, ie Group 2 of Francis and Burgess (1977).

CHAPTER FIVE

EFFECTS OF SEED MOISTURE CONTENT AND STORAGE TEMPERATURE ON SURVIVAL OF *FUSARIUM* SPP. AND ON SEED QUALITY

5.1 INTRODUCTION

While considerable research has been conducted to develop practical methods for retaining seed viability and vigour in stored seeds (Christensen and Kaufmann, 1969, 1974; Harrington, 1972, 1973a, 1973b; Roberts, 1972; Abdul-Baki and Anderson, 1972; Delouche and Baskin, 1973; Bass, 1973; Delouche *et al.*, 1973; Brooker *et al.*, 1974; Justice and Bass, 1979; AOSA, 1983; McDonald and Nelson, 1986; Powell, 1986; Roos, 1986; Fiala, 1987; Hampton and Coolbear, 1991; Bruce and Rynieck, 1991; Delouche, 1992; Hampton, 1992) comparatively little information is available on management methods which can be used to reduce or eliminate seed-borne fungi (storage fungi and field fungi) in storage. Attention to the latter aspect has more commonly been given to seed disinfection methods which control seed-borne fungi by chemical dusts, slurries, steeps or mist sprays (Christensen, 1973; Neergaard, 1979; Jones and Clifford, 1983; Moreno-Martinez and Vidal-Gaona, 1981; Moreno-Martinez and Mandugano, 1985; Moreno-Martinez and Ramirez, 1985; Jeffs, 1986; Kommedahl and Windels, 1986; Agarwal and Sinclair, 1987; Close and Harvey, 1987) or by heat treatment such as aerated steam or hot water (Baker, 1962; Neergaard, 1979; Daniel, 1983; Russell and Berjak, 1983; Agarwal and Sinclair, 1987; Van Wyk *et al.*, 1988; Berjak, 1989); microwave heating (Dewan, 1988; Cavalcante and Muchovej, 1993) and gamma irradiation (Russell and Berjak, 1983). Disinfection can be an effective control method, particularly when the pathogen is present as externally borne spores or mycelium (Jones and Clifford, 1983; Jeffs, 1986). Systemic fungicides have no deleterious effects on seed viability when used as seed treatments and are effective in controlling internally seed-borne fungi

(Jeffs, 1986). However, internally seed-borne pathogens are often difficult to eliminate without affecting seed viability when heat disinfection methods are used, because in many fungal/seed associations the thermal death point of the pathogen may be similar to, or higher than, the temperature which destroys seed viability (Baker, 1962; Russell and Berjak, 1983; Van Wyk *et al.*, 1988). Such heat treatments are often either only partially effective in terms of pathogen control or have a subsequent deleterious effect on seed quality (Russell and Berjak, 1983; Dewan, 1988; Van Wyk *et al.*, 1988).

Christensen and Kaufmann (1969) and Sauer *et al.* (1992) have stated that the field fungi (including *Fusarium* spp.) present in seeds at harvest gradually die, but that the length of time they survive depends mainly on the moisture content and temperature of the stored seeds. They have also stated that field fungi (including *Fusarium* spp.) die fairly rapidly in seed held at moisture contents above 14%, and at temperatures above 21°C, and suggested that with the right combination of moisture and temperature and time of storage it is possible to rid barley seeds completely of field fungi, with no invasion of seeds by storage fungi and little or no reduction in germination of seeds. Christensen and Kaufmann (1969) also wondered whether this treatment of seeds might be of practical value.

Results of a preliminary experiment in the present study (Appendix 5.1) also confirmed that simply storing maize seeds infected with *Fusarium* spp. at different temperatures had a different effect on fungal survival without causing major deterioration in seed quality (germination, seedling dry weight and conductivity). This study involved the storage of maize seed with maximum pre-storage *Fusarium* infection levels of 65-98% at either 5°C in sealed plastic containers or at 25°C in paper bags. At 5°C, levels of *Fusarium* spp. were retained almost at pre-storage values, while at 25°C the incidence of *Fusarium* fell to around 10% after 6 months storage and to between 3-6% after 12 months storage (Appendix 5.1). At both storage temperatures the initial high seed germination levels (> 90%) were maintained throughout the storage period.

5.2 OBJECTIVE

The objective of the present storage experiment was to more critically examine the effects of six different storage temperatures (between 5-30°C) and two different seed moisture contents (10% and 14%) on *Fusarium* spp. survival and seed quality (germination and vigour) over time (0-9 months).

5.3 MATERIALS AND METHODS

5.3.1 Seed lots and seed lot preparation

Two seed lots of each of two maize cultivars (3591 and 3475) (see Chapter 3) were used in this study. Within each cultivar, lots from two harvest months (May and June) were used. Seed lots (3591 May, 3591 June, 3475 May and 3475 June) were homogenised using a soil divider (riffle type) (ISTA, 1993) and were then subsampled and tested for seed moisture content (ISTA, 1993). Each seed lot was then divided into two sublots of 1.8-2 kg each for subsequent adjustment of seed moisture content. The new fresh weight (FWT) then required to adjust the seed to the desired SMC (ie 10% and 14%) was then calculated as follows:

$$W2 = \frac{(100 - A)}{(100 - B)} \times W1) - W1$$

where A = initial % SMC

B = desired % SMC

W1 = weight of seed sample (g)

W2 = weight (g) of water to be added or reduced

Seeds were then either dehydrated over silica gel (10% SMC) or hydrated over water (14% SMC) until the required FWT was achieved (as determined by weighing seed at regular intervals).

When the required seed moisture contents had been attained, each seed lot was divided into six sublots of approximately 300 g each using a soil divider. Each sublot was placed in a sealed glass jar and all the sublots (jars) were held at 5°C for 48 h to allow within-kernel moisture equilibration before being put into storage. One jar of each seed lot (with 10% or 14% moisture content) was placed in each of the 6 storage temperatures, either in an incubator (10°C, 15°C, 30°C) or in a temperature controlled room (5°C, 20°C, 25°C) for 9 months. All storage temperatures were controlled to ± 0.5°C.

5.3.2 Pre-storage seed quality assessment

5.3.2.1 Seed moisture content determination

Seed moisture content tests immediately prior to storage (ie post-moisture adjustment) were conducted using the 130°C air oven method for 4 h (ISTA, 1993) in duplicate 10 g ground samples.

5.3.2.2 Seed germination and seedling growth

A 100-seed representative sample from each moisture adjusted seed lot was used for a seed germination/seedling growth test by the Between Paper (roll towel) method (ISTA, 1993). Seeds were dusted with thiram fungicide prior to germination and tested in four replicates of 25 seeds at 25°C for 7 days prior to evaluation and categorisation into normal and abnormal seedlings and dead seeds (ISTA, 1993). The normal seedlings were oven dried at 65°C for 4 days to determine seedling dry weight. The normal seedling dry weight data were analysed using the SAS (Release 6.09) software package PROG GLM (General Linear Models) procedure since the experiment was a nested (hierarchial) design using a fixed effects model.

5.3.2.3 Seed health tests

One hundred seeds from each moisture adjusted seed lot were surface disinfected (1% sodium hypochlorite) for two minutes and were plated (embryo side down) onto Malt Extract Agar, five seeds per plate. After five days incubation in a seed germinator under diffuse light at 25°C, plates were examined visually or under

a microscope to identify *Fusarium* spp. (for details see Chapters 3 and 4). Re-incubation for a further 1-2 d allowed more accurate identification of younger or slower growing colonies. The presence of other fungal species, particularly *Aspergillus* and *Penicillium* spp. was also recorded.

5.3.3 Seed quality changes during storage

During storage, seed samples were obtained at 3-month intervals from the bulk samples stored in sealed glass jars from all six storage temperatures (5, 10, 15, 20, 25 and 30°C). This was done by briefly opening and closing each jar after stirring the seeds and scooping out a pre-estimated seed sample size (approximately 210 seeds) sufficient for a germination/seedling growth and seed health test. The seeds were then tested as described above (section 5.3.2.2 and 5.3.2.3). At the end of 9 months storage, in addition to these tests, a seed moisture content determination was conducted using the oven test method (ISTA, 1993).

5.4 RESULTS

5.4.1 Seed Moisture Content

Initial (pre-storage) and equilibrium seed moisture content levels (in storage) are shown in Table 5.1. Prior to storage, SMC was within 0.2% of the desired target SMC % (Table 5.1), the standard errors ranging from ± 0.1 to ± 0.3%. At the end of the 9 month storage period, the seed moisture content had virtually remained unchanged, although small equilibrium levels of ± 0.1 to 0.4% had occurred in different seed lots stored at different temperatures compared to pre-storage moisture (Table 5.1). The only exception occurred in seed lot 3591 May stored at 30°C and 14% moisture content, in which the moisture content dropped from 13.8% to 9.8% (Table 5.1). This reduction was unexplained but was thought to be due to container seal malfunction.

Table 5.1 Seed moisture content before and after 9 months storage at six temperatures.

Seed lot	Targetted SMC (%)	Actual pre-storage SMC (%)	SMC recorded after 9 months storage at temperatures of:					
			5°C	10°C	15°C	20°C	25°C	30°C
3591 May	10 14	9.9 13.8	10.1 13.8	9.8 13.6	10.0 13.8	10.0 13.5	9.8 13.5	9.9 9.8
3591 June	10 14	10.0 13.8	10.4 13.9	9.9 13.5	10.0 13.7	9.9 13.6	10.2 13.6	10.0 13.7
3475 May	10 14	10.1 13.9	10.3 13.9	10.2 13.8	10.3 13.9	10.2 13.9	9.9 13.7	10.3 13.9
3475 June	10 14	10.0 13.9	10.2 14.1	10.1 13.8	10.2 13.9	10.1 13.8	10.0 13.8	10.2 13.9

5.4.2 *Fusarium* spp. incidence and decline

Seed moisture equilibration had no effect on the incidence of *Fusarium* spp. in the seed lots (Table 5.2). *Fusarium* spp. incidence varied with cultivar and time of harvest; the early (May) harvested seed lots having lower (20-26%) levels than later (June) harvested seed lots (51-58%). All seed lots were infected by *F. graminearum*, *F. subglutinans* and *F. poae* but *F. graminearum* predominated in seed lot 3591 May, 3591 June and 3475 June, and formed 75-90%, 81-94% and 58-87%, respectively, of the total *Fusarium* spp. in these seed lots. However, seed lot 3475 May had almost equally low levels of *F. graminearum* (8-9%), *F. subglutinans* (3-5%) and *F. poae* (7-8%), while seed lot 3475 June had higher levels (11%) of *F. poae* than the other seed lots.

Table 5.2. **Incidence of *Fusarium* species prior to storage.**

Seed lot	SMC (%)	% seeds carrying *Fusarium* species			
		F. graminearum	*F. subglutinans*	*F. poae*	Other *Fusarium* spp.
(a) Before moisture equilibration					
3591 May	13.9	20	1	3	0
3591 June	13.1	44	3	1	2
3475 May	14.1	8	4	7	2
3475 June	12.3	46	1	6	0
(b) After moisture equilibration and immediately prior to storage					
3591 May	10	23	1	1	1
	14	18	4	1	1
3591 June	10	48	1	1	1
	14	43	6	3	1
3475 May	10	8	5	7	0
	14	9	3	8	2
3475 June	10	38	2	11	1
	14	32	6	11	9

During storage, levels of seed-borne *Fusarium* spp. declined (Table 5.3), starting at or before 3 months storage both in seeds with 10% or 14% moisture content, but this decline was greatest in seeds stored at 14% moisture at temperatures of 30°C and 25°C (Table 5.3). Seed storage at 20°C had an intermediate effect while temperatures lower than 20°C had a minimal or nil effect on the survival of *Fusarium* spp. The rate of *Fusarium* spp. decline was greatest at 30°C both in seeds with 10% or 14% moisture and appeared not to be related to initial infection level (Table 5.3). After 3 months seed storage at 30 and 25°C, *Fusarium* spp. levels in seeds with 14% moisture were only 1-2% and 2-14%, respectively and after 9 months storage all *Fusarium* spp. had died. In contrast, by 9 months of storage the seeds stored at 10% moisture content at 30°C and 25°C still contained *Fusarium* spp. at 1-8% and 6-12%, respectively.

Table 5.3 Incidence (%) of *Fusarium* spp. during 9 months storage at six temperatures and two seed moisture contents.

			Seed moisture content 10%					Seed moisture content 14%				
Seed lot	Storage temperature (°C)	Storage period (months)	*F. gram.*	*F. subglut.*	*F. poae*	Other *Fusarium* spp.	Total	*F. gram.*	*F. subglut.*	*F. poae*	Other *Fusarium* spp.	Total
3591 May	5	0	23	1	1	1	26	18	4	1	1	24
		3	22	2	0	0	24	13	4	0	0	17
		6	13	7	1	0	21	7	3	3	1	14
		9	20	1	4	2	27	20	0	1	1	22
	10	0	23	1	1	1	26	18	4	1	1	24
		3	21	1	1	0	23	18	0	0	1	19
		6	8	0	1	0	9	9	1	1	0	11
		9	20	2	1	0	23	9	0	2	1	12
	15	0	23	1	1	1	26	18	4	1	1	24
		3	18	2	0	0	20	8	1	1	0	10
		6	15	0	1	0	16	5	0	1	1	7
		9	19	2	1	1	23	6	2	2	0	10
	20	0	23	1	1	1	26	18	4	1	1	24
		3	11	1	2	0	14	6	2	0	0	8
		6	11	1	2	0	14	3	1	0	0	4
		9	9	0	1	1	11	5	0	0	0	5
	25	0	23	1	1	1	26	18	4	1	1	24
		3	9	2	1	1	13	2	0	0	0	2
		6	4	2	0	0	6	0	0	0	0	0
		9	5	2	1	0	8	0	0	0	0	0
	30	0	23	1	1	1	26	18	4	1	1	24
		3	8	1	0	0	9	0	1	1	0	2
		6	5	0	0	0	5	0	0	0	0	0
		9	0	1	0	0	1	0	0	0	0	0

Table 5.3 cont'd

Seed lot	Storage temperature (°C)	Storage period (months)	Seed moisture content 10%					Seed moisture content 14%				
			F. gram.	*F. subglut.*	*F. poae*	Other *Fusarium* spp.	Total	*F. gram.*	*F. subglut.*	*F. poae*	Other *Fusarium* spp.	Total
3591 June	5	0	48	1	1	1	51	43	6	3	1	53
		3	44	5	2	1	52	36	3	2	1	42
		6	41	4	2	2	49	24	5	0	0	29
		9	42	2	3	1	48	19	2	4	3	28
	10	0	48	1	1	1	51	43	6	3	1	53
		3	51	6	3	0	60	30	0	2	0	32
		6	39	6	1	1	47	28	0	1	1	30
		9	39	4	4	0	47	15	3	1	0	18
	15	0	48	1	1	1	51	43	6	3	1	53
		3	41	2	1	1	45	18	0	3	0	21
		6	28	3	1	0	32	12	1	1	0	14
		9	34	1	0	0	35	5	4	3	1	13
	20	0	48	1	1	1	51	43	6	3	1	53
		3	13	5	1	0	19	17	1	0	1	19
		6	24	3	1	1	29	3	1	1	0	5
		9	20	6	2	0	28	6	0	1	0	7
	25	0	48	1	1	1	51	43	6	3	1	53
		3	23	2	1	0	26	4	5	2	1	12
		6	12	3	1	2	18	0	1	1	0	2
		9	4	2	0	0	6	0	1	0	0	1
	30	0	48	1	1	1	51	43	6	3	1	53
		3	3	6	3	0	12	0	2	0	0	2
		6	4	7	0	0	11	0	1	0	0	1
		9	2	2	0	0	4	0	0	0	0	0

Table 5.3 cont'd

Seed lot	Storage temperature (°C)	Storage period (months)	Seed moisture content 10%					Seed moisture content 14%				
			F. gram.	*F. subglut.*	*F. poae*	Other *Fusarium* spp.	Total	*F. gram.*	*F. subglut.*	*F. poae*	Other *Fusarium* spp.	Total
3475 May	5	0	8	5	7	0	20	9	3	8	2	22
		3	2	3	5	2	12	6	6	12	1	25
		6	7	0	8	1	16	3	1	10	2	16
		9	1	6	10	0	17	4	2	14	0	20
	10	0	8	5	7	0	20	9	3	8	2	22
		3	2	8	7	3	20	7	5	12	1	25
		6	6	5	11	1	23	1	3	12	0	16
		9	6	5	11	1	23	2	5	6	2	15
	15	0	8	5	7	0	20	9	3	8	2	22
		3	6	4	4	0	14	3	5	8	0	16
		6	8	3	6	2	19	2	3	10	0	15
		9	4	3	10	1	18	1	3	4	0	8
	20	0	8	5	7	0	20	9	3	8	2	22
		3	3	8	10	2	23	1	7	8	0	16
		6	4	1	7	0	12	0	5	4	0	9
		9	3	4	9	0	16	0	2	7	0	9
	25	0	8	5	7	0	20	9	3	8	2	22
		3	6	2	9	0	17	1	0	7	0	8
		6	2	5	10	1	18	0	0	0	0	0
		9	2	4	6	0	12	0	0	0	0	0
	30	0	8	5	7	0	20	9	3	8	2	22
		3	2	6	7	0	15	0	0	1	0	1
		6	0	2	4	1	7	0	0	0	0	0
		9	0	2	1	0	3	0	0	0	0	0

Table 5.3 cont'd

Seed lot	Storage temperature (°C)	Storage period (months)	Seed moisture content 10%					Seed moisture content 14%				
			F. gram.	*F. subglut.*	*F. poae*	Other *Fusarium* spp.	Total	*F. gram.*	*F. subglut.*	*F. poae*	Other *Fusarium* spp.	Total
3475 June	5	0	38	2	11	1	52	32	6	11	9	58
		3	35	8	6	4	53	40	5	6	0	51
		6	43	5	8	0	56	24	4	7	0	35
		9	43	5	4	1	53	17	1	11	1	30
	10	0	38	2	11	1	52	32	6	11	9	58
		3	43	6	4	4	57	27	3	6	0	36
		6	32	5	5	0	42	16	3	4	0	23
		9	36	5	7	0	48	15	2	5	0	22
	15	0	38	2	11	1	52	32	6	11	9	58
		3	32	1	8	0	41	30	5	10	0	45
		6	31	7	8	0	46	11	2	10	0	23
		9	28	3	6	0	37	5	4	6	0	15
	20	0	38	2	11	1	52	32	6	11	9	58
		3	42	3	5	1	51	12	1	7	0	20
		6	30	5	9	0	44	5	2	2	0	9
		9	18	4	5	2	29	1	4	3	0	8
	25	0	38	2	11	1	52	32	6	11	9	58
		3	15	3	7	4	29	11	1	2	0	14
		6	7	1	5	0	13	0	0	0	0	0
		9	4	5	2	0	11	0	0	0	0	0
	30	0	38	2	11	1	52	32	6	11	9	58
		3	4	3	4	4	15	0	2	0	0	2
		6	3	3	3	0	9	0	1	0	0	1
		9	1	7	0	0	8	0	0	0	0	0

F. gram. = *F. graminearum*
F. subglut. = *F. subglutinans*

F. graminearum was eliminated from seeds at 14% moisture content stored at 30°C and 25°C after 3 or 6 months storage, respectively (Table 5.4). Although *F. graminearum* was eliminated from seed lot 3591 May and 3475 May stored with 10% moisture at 30°C after 6 or 9 months (Table 5.4), it still survived at low levels (1-5%) together with *F. subglutinans* (1-7%) and *F. poae* (1-2%) in seed lots 3591 June and 3475 June at 10% seed moisture content stored at 30°C and 25°C. The levels of survival of *F. graminearum* increased as storage temperatures decreased and at 5°C after 9 months storage *F. graminearum*, *F. subglutinans* and *F. poae* levels were similar to the initial values (Table 5.3, 5.4 and 5.5). The effect of storage temperature and seed moisture content on survival of *F. subglutinans* and *F. poae*, however, was inconclusive, since these fungi were present at very low levels in most samples compared to much higher initial levels of *F. graminearum*.

Incidence of storage fungi (*Penicillium* spp. and *Aspergillus* spp.) was variable with levels of *Penicillium* from 0-25% and of *Aspergillus* spp. from 0-14% (Appendix 5.2). However, while the occurrence of *Penicillium* spp. was found to be more prevalent than *Aspergillus* spp. there was no consistent association either with seed moisture content or storage temperature. It was observed, however, that consistently, *Penicillium* spp. was detected growing on and from the tip cap (pedicel) and in more than 50% of cases was not growing on agar. These observations suggest that storage fungi did not contribute appreciably to the deterioration of seeds even at storage conditions of 14% seed moisture content and 25 or 30°C.

Table 5.4. A comparison of *F. graminearum* survival levels (%) and retention of germination capacity (%) during storage at 5-30°C of seeds at 14% moisture content in sealed containers.

Storage Temp (°C)	Seed lot	Storage period (months)							
		0		3		6		9	
		Fg	Germ	*Fg*	Germ	*Fg*	Germ	*Fg*	Germ
30	3591 May*	18	92	0	84	0	89	0	93
	3591 June	43	92	0	92	0	40	0	6
	3475 May	9	95	0	91	0	49	0	17
	3475 June	32	94	0	80	0	42	0	2
25	3591 May	18	92	2	95	0	89	0	90
	3591 June	43	92	4	93	0	79	0	78
	3475 May	9	95	1	97	0	83	0	90
	3475 June	32	94	11	93	0	74	0	84
20	3591 May	18	92	6	94	3	89	5	94
	3591 June	43	92	17	92	3	79	6	95
	3475 May	9	95	1	98	0	88	0	94
	3475 June	32	94	12	92	5	79	1	95
15	3591 May	18	92	8	95	5	92	6	94
	3591 June	43	92	18	95	12	84	5	89
	3475 May	9	95	3	96	2	93	1	93
	3475 June	32	94	30	94	11	87	5	90
10	3591 May	18	92	18	94	9	88	9	98
	3591 June	43	92	30	91	28	92	15	89
	3475 May	9	95	7	99	1	87	2	94
	3475 June	32	94	27	96	16	85	15	97
5	3591 May	18	92	13	94	7	95	20	95
	3591 June	43	92	36	94	24	82	19	93
	3475 May	9	95	6	96	3	93	4	93
	3475 June	32	94	40	94	24	88	17	91

* Storage container lid must have malfunctioned as seed moisture content dropped from 13.7% to 9.8%

Fg = *F. graminearum*
Germ = Germination %

Table 5.5 A comparison of *F. graminearum* survival levels (%) and retention of germination capacity (%) during storage at 5-30°C of seeds with 10% moisture content in sealed containers.

Storage temp (°C)	Seed lot	Storage period (months)							
		0		3		6		9	
		Fg	Germ	*Fg*	Germ	*Fg*	Germ	*Fg*	Germ
30	3591 May	23	92	8	95	5	94	0	96
	3591 June	48	92	3	89	4	94	2	97
	3475 May	8	96	2	97	0	92	0	95
	3475 June	38	95	4	96	3	89	1	96
25	3591 May	23	92	9	92	4	93	5	94
	3591 June	48	92	23	93	12	92	5	88
	3475 May	8	96	6	96	2	96	2	95
	3475 June	38	96	15	95	7	95	4	93
20	3591 May	23	92	11	96	11	92	9	93
	3591 June	48	92	13	95	24	95	20	92
	3475 May	8	96	3	96	4	94	3	95
	3475 June	38	95	42	96	30	91	18	92
15	3591 May	23	92	18	94	15	94	19	96
	3591 June	48	92	41	92	28	95	34	91
	3475 May	8	96	6	93	8	97	4	97
	3475 June	38	95	32	95	31	91	28	91
10	3591 May	23	92	21	94	8	89	20	94
	3591 June	48	92	51	89	39	96	39	95
	3475 May	8	96	2	99	6	96	6	97
	3475 June	38	95	43	94	32	94	36	93
5	3591 May	23	92	22	97	13	94	20	91
	3591 June	48	92	44	94	41	94	42	91
	3475 May	8	96	2	98	7	97	1	96
	3475 June	38	95	35	96	43	93	43	96

Fg = *F. graminearum*
Germ = Germination

5.4.3 Seed germination

A summary of germination percentage data for seeds stored at 14% and 10% moisture content, storage temperatures and sampling times is shown (in comparison with *F. graminearum* survival data) in Tables 5.4 and 5.5, and also in Appendix 5.3.

The germination data (Table 5.4 and 5.5) compared with pre-storage, pre-moisture equilibration values (92%, 95%, 98% and 97% for seed lots 3591 May, 3591 June, 3475 May and 3475 June), respectively, and immediate pre-storage (after moisture adjustment) (Tables 5.4 and 5.5) values show no significant or consistent reduction in seed germination percentages at all storage temperatures at 10% moisture content (Table 5.5). However, a significant loss of seed germination began and continued at or after 3 months storage at 30°C in seeds stored at 14% seed moisture content (Table 5.4). Storage temperatures of 20°C or less had no effect on seed germination percentage during the 9 months storage period irrespective of seed moisture levels (Tables 5.4 and 5.5). The data also suggests that late (June) harvested seeds tended to be more sensitive to the deteriorative effects of high temperatures (30°C and 25°C) and high moisture contents (14%) after 9 months (Table 5.4). However, the cause of the lower germination percentages at the six months sampling (Table 5.4) is unclear, although it is considered to have been due to suboptimal conditions during seed germination at that time.

5.4.4 Seedling dry weight

Mean seedling dry weight data are shown in Table 5.6 and the Analysis of Variance (ANOVA) Pr > F values are shown in Table 5.7. The mean seedling dry weight data show that although there were some variations in individual seedling dry weight, generally the weights were similar irrespective of cultivar, seed moisture content, storage period or storage temperature, except for the June harvested seeds stored at 30°C and 14% moisture in which decreases in seedling dry weight (from 247 mg to 148 mg and from 231 mg to 150 mg for seed lots 3591 June and 3475 June, respectively) occurred after 9 months storage (Table 5.6). This was further revealed by the analysis of variation which showed no significant differences (P => 0.05) between cultivars; between harvest times within cultivars; between seed moisture contents within harvest times within cultivars; or among temperatures within seed moisture contents within harvest times within cultivars (Table 5.7). However, there were significant differences (P =< 0.05) between storage times within temperatures within seed moisture contents within harvest times within cultivars. A plot of residuals suggested the data were

Table 5.6. Mean seedling dry weight (mg/seedling) after seed storage at 10% and 14% SMC and temperatures of 5-30°C for 9 months in sealed containers.

Seed lot	SMC (%)	Storage period (months)	Storage temperature (°C)					
			5	10	15	20	25	30
3591 May	10	0	231	251	240	243	235	241
	10	3	226	237	225	221	234	237
	10	6	236	238	239	238	251	236
	10	9	239	245	244	248	240	247
3591 May	14	0	234	236	233	232	225	243
	14	3	234	229	234	230	235	247
	14	6	244	241	233	236	229	235
	14	9	243	248	244	247	253	244
3591 June	10	0	238	254	245	245	237	245
	10	3	238	230	234	213	244	235
	10	6	230	245	236	230	236	243
	10	9	250	242	232	240	249	247
3591 June	14	0	229	236	249	242	251	247
	14	3	235	237	228	232	244	246
	14	6	230	244	244	241	250	244
	14	9	243	244	250	241	257	148
3475 May	10	0	235	239	230	229	223	235
	10	3	223	219	225	225	227	217
	10	6	230	229	234	230	235	236
	10	9	217	232	237	236	234	245
3475 May	14	0	220	223	228	242	235	234
	14	3	239	227	234	228	236	230
	14	6	238	243	231	234	227	258
	14	9	237	250	235	236	241	264
3475 June	10	0	245	238	234	249	253	230
	10	3	231	217	224	225	236	234
	10	6	247	255	236	243	235	250
	10	9	238	235	235	232	231	236
3475 June	14	0	237	218	235	233	231	231
	14	3	230	235	244	237	239	252
	14	6	235	254	240	257	252	274
	14	9	232	250	238	253	253	150

normally distributed and did not require transformation. These results clearly show a time deterioration effect in storage which occurs differentially, particularly depending on temperature/seed moisture content relationships. This deteriorative influence of high temperatures (particularly 30°C) and higher moisture content (14%) was also seen in loss of germination towards the end (6-9 months) of the storage period (Table 5.4).

Table 5.7. Analysis of Variance table showing nested treatment combinations and probability (F) values for seedling dry weight data at 3[1] to 9 months storage at 5-30°C and 10% and 14% SMC.

Nested Treatments	Nesting	Pr > F Values
Cultivars		0.4840
Harvest[2]	(cv)	0.5191
Seed moisture content	(cv*Harv)	0.1861
Temperature	(cv*Harv*SMC)	0.9625
Time[3]	(cv*Harv*SMC*Temp)	0.0001

[1] Data for sampling at storage was not replicated and therefore was not statistically analysed
[2] Date of harvest
[3] Sampling time/storage period

SMC = Seed moisture content
CV = cultivar
Harv = Harvest
Temp = Temperature
Pr = Probability

5.4.5 Mycotoxins

For the preliminary storage temperature study (see Introduction to this chapter), samples stored at 5°C (sealed storage) or 25°C (open storage) were tested for mycotoxin contamination (see Chapter 3). Even after 12 months storage mycotoxin levels were generally similar to pre-storage levels, although there were some increases and decreases, particularly in Zearalenone levels (Appendix 5.4). This suggests mycotoxins

are temperature stable metabolites capable of remaining at pre-storage levels, despite generally low seed moisture contents prevailing under good storage conditions.

5.5 DISCUSSION

5.5.1 *Fusarium* species decline

The reducing incidence of *Fusarium* spp. in the seeds as the period of open storage at 25°C or sealed storage at 14% SMC at temperatures ≥15°C increased, was in agreement with the general observation that field fungi (including *Fusarium* spp.) will generally not survive in storage (Christensen and Kaufmann, 1969, 1974; Christensen and Sauer, 1982; Russell *et al.*, 1982; Russell and Berjak, 1983; Sauer *et al.*, 1992). However, the fact that *Fusarium* spp. did not completely die out after 12 months storage (open storage) is not surprising. Dungan and Koehler (1944) found that *F. graminearum* took 2 years to die out of maize seeds, although most seeds were free of the pathogen by 15 months. In sealed storage, *F. graminearum* died out at a faster rate than other Fusaria (*F. subglutinans* and *F. poae*). This agrees with the results of Christensen (1963) who found that *Fusarium* spp. (especially *F. graminearum* died out from barley seeds within 6-20 months storage at room temperature.

The rate of *F. graminearum* decline in seeds stored in paper bags (open storage) in the preliminary experiment, appeared to be dependent on the initial level of infection, and this is similar to observations by Christensen (1963) for barley stored at room temperature. However, in the main study, under sealed storage conditions, the rate of *F. graminearum* decline was the same for high or low infection levels in seeds stored at 14% moisture content. This apparent difference could be attributed to perhaps the fact that in sealed storage, anoxic conditions occurred (Hyde, 1974; Justice and Bass, 1979; Bell and Armitage, 1992), resulting in a continuous stress on *Fusarium* spp. respiration (Russell *et al.*, 1982; Bell and Armitage 1992). Conversely, in open storage (paper bags) where oxygen supply is continuous (Justice and Bass, 1979; Copeland and McDonald, 1985) the cause of death of *Fusarium* spp. may be more likely to have been

due to effects of seed moisture content particularly early in the storage period (at 2-4 months) when the moisture content was around 14% in some seed lots (data not shown). The similar rate of decline of *F. graminearum* in the four seed lots in sealed storage is considered beneficial since it suggests that *F. graminearum* can be controlled/eliminated from seed lots with different levels of infection under one set of storage conditions.

The seed lots used in this study showed similar decline in *Fusarium* levels when stored at the same temperature. This in itself was not surprising. However, the infection levels of each of the three main *Fusarium* species in these lots varied considerably. Although lots 3475 and 3591 May had similar infection totals (20-26%) the former had similar levels of *F. graminearum*, *F. subglutinans* and *F. poae* (8, 4 and 7% respectively), while the latter was dominated by *F. graminearum* (20%) with low levels of *F. subglutinans* (3%) and *F. poae* (1%). Similarly, the two high *Fusarium* lots (3475 June and 3591 June) both had total infection levels of approximately 50%, of which approximately 45% was contributed by *F. graminearum*. The response of these *Fusarium* species to storage temperature suggests there may be a differential resistance to high temperature and moisture conditions between *F. graminearum* and *F. subglutinans*. After 9 months storage at 30°C *F. graminearum* levels were very low (0-2%) compared to original levels (8-46%). Under the same conditions, however, *F. subglutinans* was still detected at approximately the original levels in the 10% SMC lots (1-4%), but was dead in 14% SMC seed.

5.5.2 Seed germination decline

The reduction in germination percentage of seeds stored at 30°C and 14% moisture is most likely explained in terms of the combined adverse effects of high seed moisture content and high temperature storage, and also perhaps accumulation of carbon dioxide in the storage containers, all which are known to affect seed longevity (Harrington, 1972; Roberts, 1972; Gill and Delouche, 1973; Copeland and McDonald, 1985). Harrington (1972) and Copeland and McDonald (1985) recommend that to store starchy seed successfully, it must be dried to a moisture content of less than 14% (12 to 13% - Copeland and McDonald, 1985) as higher seed moisture content results in

biochemical events such as increased hydrolytic enzyme activity, enhanced respiration and increase in free fatty acids, while high storage temperatures enhance the rate at which enzymatic and metabolic reactions occur in the seeds. Consequently, these processes all lead to rapid seed deterioration. Seeds stored at 10% seed moisture and high temperatures (30 or 25°C), however, retained their germination, presumably because of lower enzymatic activity, a situation previously recorded by Gill and Delouche (1973).

5.5.3 Effects of storage conditions on *Fusarium* spp. and seed quality

The seed lots used in this experiment showed different initial levels of *Fusarium* spp. - particularly *F. graminearum* (which ranged from 8-51%). Levels of *F. subglutinans* and *F. poae*, however, were generally low (0-11%), making judgements on the effect of seed moisture and temperature on these two species difficult during the 9 month storage period. In all lots, however, levels of *Fusarium* had no direct initial influence on seed quality, as shown by high germination before storage. This situation continued through the 9 month storage period at temperatures of 20°C or less and irrespective of seed moisture content. This is not surprising since both 10% and 14% seed moisture content are considered 'safe' levels for maize storage (Hill, M.J., pers. comm.; Chappell, 1985). Seeds also retained their vigour, as evidenced by maintained seedling dry weights during storage for at least 6 months.

All seed lots retained high germination in storage at 10% SMC irrespective of temperature. Despite this, a similar storage time also resulted in a significant drop in *Fusarium* levels (to 1-8%) at 30°C in seed stored at 10% SMC. Is this 'acceptable' control compared with nil *Fusarium* at 14% SMC but an accompanying loss of germination? The results suggest an interaction between temperature and seed moisture content in storage, but are also an indication of the greater thermal sensitivity of *Fusarium* compared to the thermal sensitivity of maize seed.

Mycotoxin analysis of samples from the preliminary experiment indicate that *F. graminearum* in seeds is unlikely to continue to produce mycotoxins once seeds have

been dehydrated to safe seed storage levels, a result noted by Mills (1982). This is also in agreement with reports that *Fusarium* mycotoxins (especially Zearalenone) are a problem when seeds (on cobs in cribs) are stored with moisture contents of over 18% (Naik *et al.*, 1978; Peir, 1981; Mills, 1982; Sutton, 1982; Wicklow *et al.*, 1990; Lacey and Magan, 1991). The high germination values obtained from mycotoxin contaminated seeds during storage especially at 10% moisture content also suggest that like their principal producer (*F. graminearum*) they have no effect on seed viability under storage at this seed moisture content.

5.5.4 Causes of *Fusarium* spp. decline

The rate of decline in *Fusarium* spp. was fastest at 30°C and in seeds at 14% moisture content, as opposed to seeds at 30°C and at 10% seed moisture content. This is thought to be due to activated hydrolysed enzymatic metabolic processes at 14% seed moisture in hyphae. Baker (1962) referring to the principles of thermotherapy., stated that the greater the moisture content of the living material the greater is its susceptibility to thermal destruction, and that denaturation of proteins was the main cause of this. Referring to seed storage at high moisture content, Copeland and McDonald (1985) stated that the deleterious effects of high seed moisture content (seed deterioration) result from increased hydrolytic enzymes, enhanced respiration and increased free fatty acids, and that high storage temperature serves to enhance these reactions. A 14% seed moisture content appears to be intermediate between high and low moisture content in this regard (Harrington, 1972). Cook (1981) states that *Fusarium* is subjected to the same water potential requirements in seeds as in any other medium and that *Fusarium* hyphae are in equilibrium with their environment, suggesting that the turgor pressure of the hyphae is related to the seed moisture content. Therefore, *Fusarium* spp., including *F. graminearum*, being living organisms like seed, and composed of apparently hydrated mycelium (Russell and Berjak, 1983) probably also undergo deterioration due to increased metabolic processes under high (30°C or even 25°C) temperature seed storage conditions (Langvad, 1972; Anderson and Smith, 1976; Russell and Berjak, 1983).

The ability for *Fusarium* spp. (especially *F. graminearum*) to survive at higher temperatures (30°C and 25°C) in seeds with low moisture content (10%) may be attributable to the greater osmoregulation (a process whereby cell osmotic potentials are raised or lowered as necessary to maintain cell turgor - Brown, 1976; Cook, 1981) by these fungi at these storage conditions than at 14% seed moisture content. Cook (1981) explained that by osmoregulation *Fusarium* spp. are able to change the optimum temperature for growth, and that the higher the temperature in the range of 15 to 35°C and the lower the water potential, the higher the temperature at which the *Fusarium* spp. can grow. For example, *F. roseum* 'Graminearum' (*F. graminearum*) is reported as being able to grow maximally at about -10 bars to -15 bars at 20°C and at -55 bars at 35°C (Cook and Christen, 1976; Cook, 1981). It is suggested (Cook, 1981) that the shift in optimal temperature with drier conditions may be an adaptive mechanism in these fungi since in general, dry conditions and higher temperatures occur simultaneously.

The limited or nil reduction in *Fusarium* spp. levels during seed storage at temperatures of 20°C or less for up to 9 months from seeds at 10% or 14% seed moisture content and the fact that this was accompanied by retention of high seed germination (over 90%) and seed vigour (seedling dry matter) is thought to be due to the slowing or inactivation of metabolic rates of *Fusarium* spp. and of the seeds. This result is not surprising since low temperature (up to 20°C) and low seed moisture (10-13%) are recommended for the preservation of seed longevity (Harrington, 1972; Justice and Bass, 1979; Copeland and McDonald, 1985; Roberts and Ellis, 1989); and field fungi (including *Fusarium* spp.) are preserved in seed at 10°C (in sealed containers) at the Danish Institute of Seed Pathology for Developing Countries, Copenhagen, Denmark (Mathur, S.B., pers. comm.). Moreover, *F. graminearum* and *F. moniliforme* have been reported to have survived for 13 years in maize seeds stored at 0°C and 4°C respectively (Abbas and Mirocha, 1986; Abbas *et al.*, 1986).

5.6 CONCLUSION

Fusarium spp., and especially *F. graminearum*, can be eliminated from seeds by 3 months storage at 30°C or 25°C at 14% seed moisture content, can be reduced to very

low levels at this SMC at storage temperatures of 20°C and 15°C, and even at 10% SMC, storage at 30°C and 25°C for 9 months can significantly reduce *Fusarium* levels.

Although it is recommended that for good storage seeds must be dried down to a "safe" seed moisture content and that 14% is acceptable for maize (Chappell, 1985), the results of this study clearly show that while *Fusarium* spp. are eliminated from seeds, there is an adverse effect on seed germination in storing seeds at 14% moisture content when this is accompanied by high temperature (30 or 25°C) conditions, and that while seed germination and vigour are retained by storing seeds at 10% seed moisture, *Fusarium* spp. survive at all storage temperature conditions between 5 and 25°C, but are virtually eliminated at 30°C.

The results in this experiment suggest that seed storage for 3 months at 30°C and 14% seed moisture content could be useful in providing pathogen-free seed for planting without any of the potential deleterious effects on seed viability or vigour which can occur following chemical or heat based seed treatments. What is not known is whether 3 months is critical for *Fusarium* spp. death, or whether a shorter period of storage may suffice. This requires investigation. Despite this, however, it is important to appreciate that this is only one aspect of *Fusarium* control, since other inoculum sources usually exist in the field. Such considerations as cropping history, presence of infected crop residues and alternative hosts, should also be considered in attempts to control *Fusarium* maize diseases under field conditions.

CHAPTER 6

A STUDY OF THE TRANSMISSION OF *FUSARIUM GRAMINEARUM* FROM MAIZE SEEDS TO SEEDLINGS

PART 1 : GLASSHOUSE STUDY

6.1 INTRODUCTION

In the delayed harvest study (Chapter 3), *F. graminearum* was the predominant species in seeds of four hybrids harvested from April through to July in 1990 and 1991. The incidence of *F. graminearum* was respectively 50-72% and 44-88% in June and July harvested seeds in 1990, and 20-27% and 23-38% in June and July harvested seeds in 1991. Despite these comparatively high infection levels, particularly in the seed harvested in 1990, all seed lots in both years had high germination (86-99%).

F. graminearum was the most common species isolated from maize kernels in a survey of crops grown in the Manawatu region (Hussein and Baxter, 1985). Similarly, Sayer and Lauren (1991) found *F. graminearum* to be the commonest *Fusarium* spp. isolated from maize grains in 1987-89, with infection levels of 22-65% in grain from the Waikato, the East Coast and the Manawatu regions. These findings suggested a further need to investigate the role played by *F. graminearum* seed-borne inoculum with regards to seed germination and seedling disease status.

Two populations of *F. graminearum* have been differentiated and designated as Group 1 and Group 2 (Francis and Burgess, 1977). Members of Group 1 are usually associated with diseases of the crowns of cereal plants such as crown rot of wheat, whereas Group 2 is usually associated with diseases of the aerial parts of

plants, such as stalk rot, ear rot of maize and scab of wheat (Burgess *et al.*, 1975, 1981, 1987a; Francis and Burgess, 1977). Members of *F. graminearum* Group 1 do not form perithecia in culture and only rarely in nature, whereas those in Group 2 readily form perithecia of *Gibberella zeae* in nature and culture (Burgess *et al.*, 1975, 1988; Francis and Burgess, 1977).

In maize and other cereals, host debris is considered to be the principal reservoir of *F. graminearum* inoculum. This inoculum includes ascospores, macroconidia, chlamydospores and hyphal fragments which survive on host debris such as old maize stalks, ears and cereal straw and stubble at or near the soil surface (Warren and Kommedahl, 1973; Sutton, 1982; Khonga and Sutton, 1988; Teich, 1989). Soil, seed and infected cereal crops such as wheat have long been suspected as being sources of inoculum (Sutton, 1982) but "adequate evidence is not yet available to support this supposition" (Teich, 1989).

McGee (1988) listed Gibberella ear rot and stalk rot among maize diseases which occur as a result of seed-borne infection, but stated that the transmission of the pathogen (*F. graminearum*) from seed to seedling has not been clearly demonstrated, although it is widely recognised as being seed-borne (Neergaard, 1979; McGee, 1988; Richardson, 1990).

Seed transmission of a pathogen implies that infected or contaminated seeds are the means by which the pathogen can be transmitted to subsequent plants (Agarwal and Sinclair, 1987; McGee, 1988). Establishment and development of the infection within a seedling or subsequent plant is the last decisive link in the process of seed transmission, and seed transmission has been established only if this completion of infection has been positively demonstrated to the exclusion of other means of transmission (Neergaard, 1979).

6.2 OBJECTIVES

The objectives of this study were to:

1. determine whether *F. graminearum* is seed transmitted, and if so, to determine how this transmission occurs, and which parts of the infected seedling carry the pathogen

2. determine the symptoms of *F. graminearum* on maize seedlings

3. to detect the presence of any other fungi associated with the seedlings and to determine whether they may also have a role in maize seedling diseases.

6.3 MATERIALS AND METHODS

Seeds of a Pioneer yellow dent hybrid, P3591 harvested in July 1990 were used for this experiment. Two sublots, one with a high infection (H) level (72%) and the other with a low infection (L) level (1%) of *F. graminearum* seed-borne inoculum were created by storing one sublot at 5°C from harvest (= high infection) and the other at 25°C for 12 months during 1991 which resulted in the death of *F. graminearum* (= low infection). Surface disinfected (SD) and non-surface disinfected (NSD) seeds of each seed lot were used to provide a total of four treatments. Disinfection was carried out using 1% sodium hypochlorite (Janola, 31.5 g/l w/v, Rickett & Colman NZ Limited) as previously described (Chapter 3).

6.3.1 Initial seed quality assessment

Initial seed quality was assessed using a standard germination test (see Chapter 3) and a seed health test (ISTA, 1993). For seed health assessment, surface disinfected seeds were tested on Malt Agar as described in Chapter 3, but non-surface

disinfected seeds were tested by the freezing blotter method (ISTA, 1993) to detect surface-borne fungi. Blotters used for top of paper (TP) seed germination (ISTA, 1993) were cut into approximately 9 cm circles. These were placed in 9 cm diameter plastic petri dishes after being dipped in sterile water and allowing excess water to drain off. One seed was tested per petri dish. The seeds were allowed to imbibe and initiate germination for 36 hours at 25°C. They were then placed in a freezer at -18°C for 24 hours after which they were incubated for up to 14 days at 25/20°C under NUV light with 12 hours light and 12 hours darkness, respectively. The seeds were finally examined using a dissecting stereo-microscope (x 40) and *Fusarium* spp. were identified macroscopically and microscopically (Nath *et al.*, 1970; descriptions in Chapters 3 and 4 of this study) and the other fungi were identified according to procedures described by Neergaard (1979). To confirm the occurrence of *F. graminearum* Group 2 in the high infection seed lots, surface disinfected seeds were plated on carnation leaf agar (Fisher *et al.*, 1982; Burgess *et al.*, 1988) using one seed per plate. The plates were incubated as described above and were examined after 10-14 days for the presence of *G. zeae* following the descriptions in Chapter 4 of this study.

6.3.2 Establishing seedlings in the glasshouse

Seedlings were established in sterile root trainers (20 cm deep; [East Coast Packaging Limited, Havelock North, New Zealand]) containing sterile sand which had been wetted to 100% water holding capacity. One seed was sown per trainer at a depth of 3-4 cm. For each of the four seed lot treatments, four replicates of 36 seeds were sown. Because of the numbers of seedlings to be examined, the experiment was divided into two sowings. Non-surface disinfected seeds were sown on 14 August and surface disinfected seeds were sown on 17 September. The root trainers for each replicate were supported in a wire frame which was then covered with a sterile polyethylene bag supported above the surface of the root trainers at a height of 42 cm (Plate 6.1). Treatments were arranged on a bench in a glasshouse using a completely randomised design. The glasshouse was not heated and temperature ranged from 3-25°C (mean 14-17°C) as recorded daily using a maximum/minimum thermometer

(Appendix 6.1). Seedling emergence was recorded daily and disease symptoms noted.

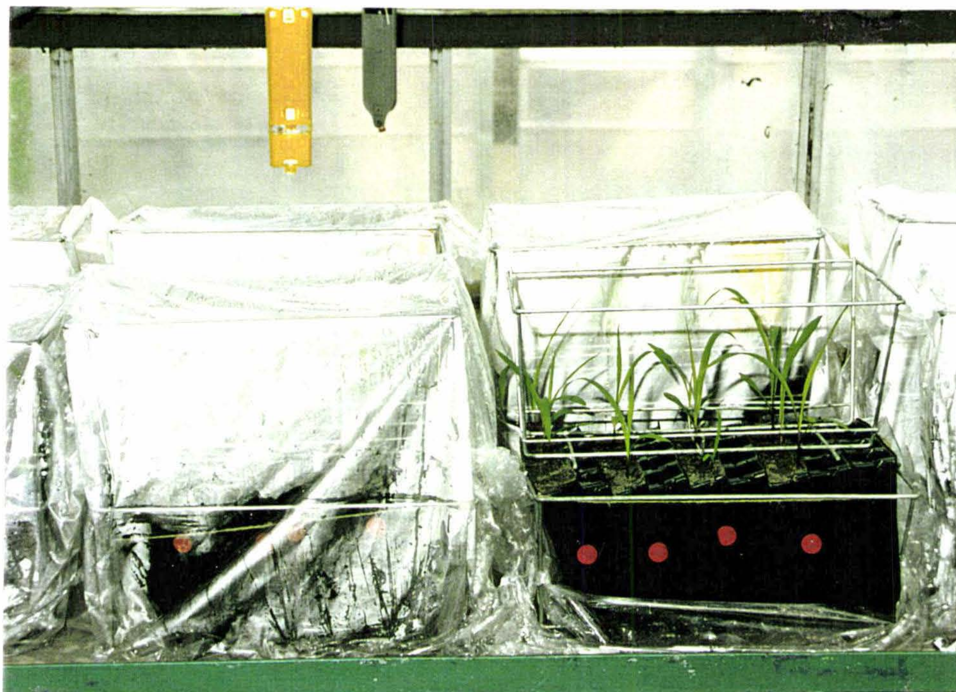


Plate 6.1 **Photograph showing seedling establishment conditions in the glasshouse: ie sand filled root-trainers with supporting metal frames; high relative humidity in polyethylene bags and thermometers for temperature recording.**

6.3.3 Seedling harvesting

Seedling harvests were carried out twice: at 10 and at 21 days after the first day of seedling emergence, ie when seedlings had 2-3 and 3-4 leaves, respectively. At the first harvest, five root trainers were randomly selected and aseptically lifted from each replicate after removal of the plastic cover. A new sterile cover was installed and the remaining seedlings were returned to the glasshouse. At the second harvest, seedlings from the remaining four root trainers in each replicate were harvested.

In the laboratory, seedlings were carefully lifted from the sand without breaking the roots and ungerminated (dead seeds) were also picked from the sand. The seedlings harvested included the live ones (those showing continuing growth) and those whose growth had been terminated or nearly so by pre- and post-emergence infection (damped off seedlings - Wheeler (1969)).

6.3.4 Dead seeds and pre- and post-emergence damped off seedlings; external fungal growth examination and pathogen isolation

Dead seeds and pre- and post-emergence damped off seedlings were counted and examined for external fungal growth by the use of a dissecting microscope (x 40). They were then surface disinfected with sodium hypochlorite (1% for one minute, rinsed for 5 minutes in 3 changes of sterile water in a Lamina Flow cabinet and were allowed to dry on sterile hand towels for at least one hour before being plated on to carnation leaf agar (CLA). Depending on the size, some seedlings had to be dissected into 0.5-1 cm pieces before being plated onto agar. The plates were incubated under NUV light (12 hours darkness and 12 hours light) at 20/25°C, respectively (see Chapter 4). After 10-14 days, the cultures were examined under a dissecting microscope for the presence of perithecia of *Gibberella zeae*. Other fungi growing on the plated pieces of seeds or seedlings were also recorded. The number of dead seeds and seedlings from which the different fungi were recovered was counted and the percentage recovery of each fungus was calculated based upon the 144 seeds sown.

6.3.5 Live seedlings disease symptom and external fungal growth examination

Live seedlings from each seed lot were counted, individually serially labelled and then visually, or by use of a dissecting microscope, examined for the presence of lesions/symptoms, as well as external fungal growth.

Disease lesions/symptoms on the seedlings were categorised as either those on plant parts above sand level or below sand level (Fig. 6.1). The below sand level

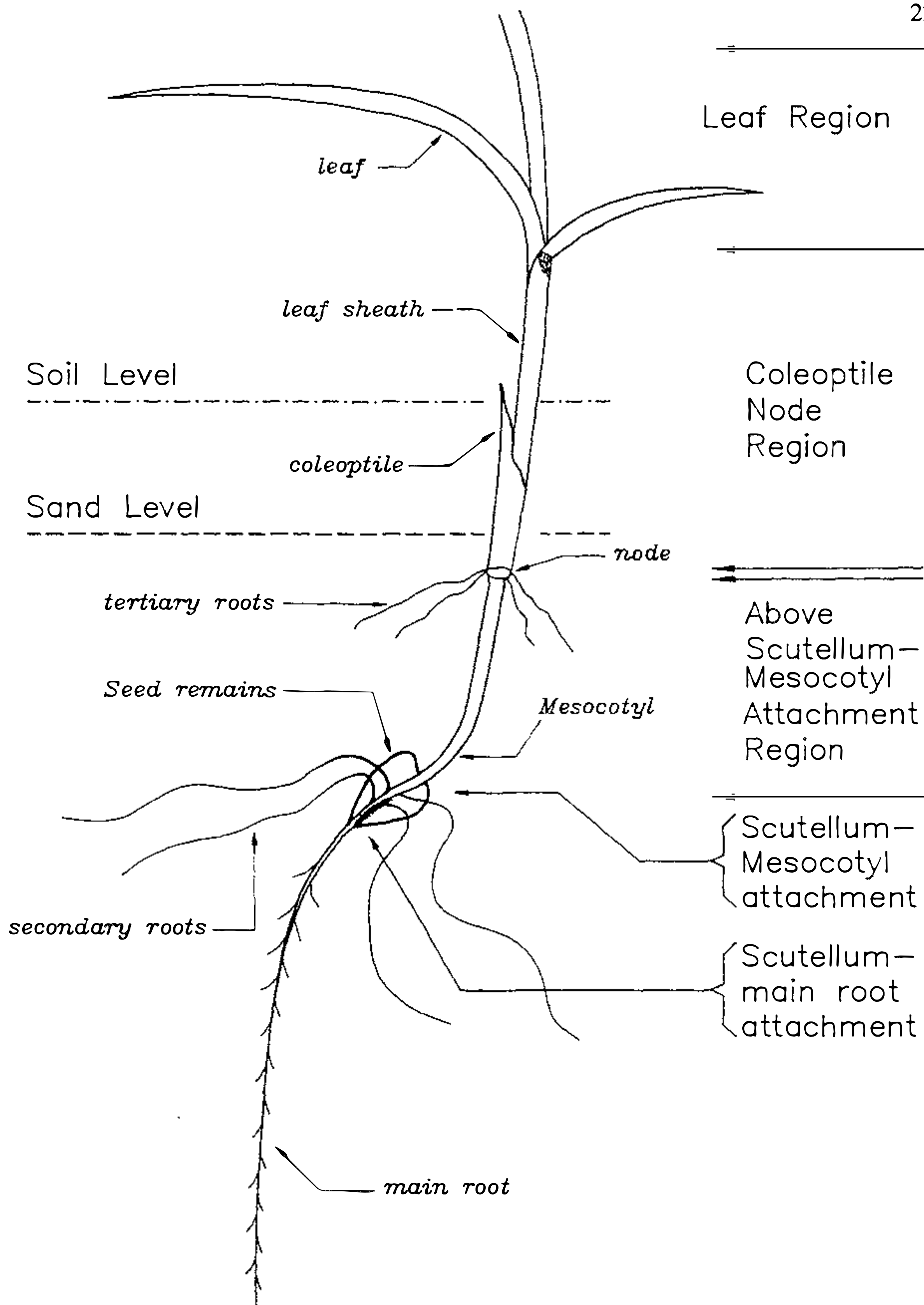


Figure 6.1 Diagram showing maize seedling regions, parts and orientation in the sand bed in the glasshouse and in the soil in the field.

parts were examined before and after washing the seedlings under running tap water. Washing the sand off the lower parts of the seedlings facilitated a clear study (usually with the help of the dissecting microscope) of seedling discolouration and serial labelling was to facilitate selection of the desired seedling categories during pathogen isolation at a later date.

6.3.5.1 Description of seedling parts (Fig. 6.1)

The above ground level parts included:

(a) Leaves

The true green leaves, the leaf bases and part of the leaf sheath closest to the leaf.

(b) Coleoptile-Node region

This included the coleoptile, node and tertiary roots (Fig. 6.1). Because the coleoptile was partly exposed above the sand level and yet attached to the node from which the tertiary roots arose, the coleoptile was regarded as an intermediate part between the above sand level and below the sand level regions of the seedlings. Tertiary roots were almost absent (undeveloped by the first harvest (10-12 days after seedling emergence)) but had developed significantly at the second harvest.

The below sand level parts included:

(a) Scutellum-mesocotyl attachment

That part of the seedling where the mesocotyl is in touch with the seed remains in the formerly embryo area, extending upwards to opposite the top of the seed remains. Because the secondary roots originate from this part of the mesocotyl, they were regarded as part of the scutellum-mesocotyl attachment area.

(b) Above scutellum-mesocotyl attachment

That part of the mesocotyl away from contact with the seed remains, extending up to the node.

(c) Scutellum-main root attachment

That part of the main root (3-5 cm) extending from the scutellum-mesocotyl attachment towards the main root tip.

(d) Below scutellum-main root attachment

That part of the main root extending from 3-5 cm to the tip of the main root.

6.3.6 Preservation of live seedlings

Live seedlings (seedlings not affected by pre- or post-emergence damping off infection) were spread on trays previously sterilised with 70% ethanol and allowed to dry at room temperature (20-25°C) for up to 7 days. The dry seedlings were then stored at 5°C in plastic bags for 1-2 months. The preservation and cool storage of seedlings was necessary as it was not possible to start pathogen isolation immediately.

6.3.7 Pathogen isolation from live seedlings

Seedlings were selected according to categories relating to the position of the lesions/symptoms on them as follows:

(a) Seedlings without visible lesions.

(b) Seedlings with lesions on tissue previously above or above and below sand level.

(c) Seedlings with lesions on those tissues which were below the sand level.

The objective of this categorisation was to consider a possible association between the fungi and the lesions/symptoms from which they were isolated and the part of the plant where lesions/symptoms were found.

Five plants were selected from each category in each treatment, were surface disinfected with sodium hypochlorite (1% for one minute) and rinsed for 5 minutes in 3 changes of sterile water in a Lamina Flow cabinet. They were left to dry on sterile hand towels in the Lamina Flow cabinet for at least one hour. In order to be able to detect which seedling parts *F. graminearum* was transmitted to, individual seedlings were aseptically dissected into the different parts described in Fig. 6.1, ie seed remains, main root, mesocotyl, coleoptile-node region and leaves. The parts were dissected into small pieces (0.5-1 cm) which were then plated on CLA, and were incubated under NUV light, 12 hours darkness and 12 hours light at 20/25°C, respectively. Cultures were examined after 10-14 days of incubation using a dissecting microscope to detect the presence of perithecia of *G. zeae*. Other fungi were also recorded. A compound microscope was also used to confirm fungal identification.

6.3.8 Determination of recovery of *F. graminearum* and other fungi from dead seeds and seedlings

Results from fungal isolations from dead seeds and from seedlings indicated that the date of harvest did not affect seedling infection level. Consequently results from the first and second harvests were combined to provide a 10 plant sample for each of the three seedling categories. Live seedling percentage recovery was based on 30 seedlings tested both for overall recovery and for recovery within each seedling category. In the case of dead seeds and pre- and post-emergence damped off seedlings, percentage recovery of individual fungi was calculated based on the number of seeds or seedlings infected out of the 144 seeds sown.

6.3.9 Calculation of transmission rate of *F. graminearum* from seed to seedlings

The number of live seedlings which yielded *F. graminearum* after plating was used to calculate total *F. graminearum* infected seedlings in each treatment. To this value was added the number of pre- and post-emergence killed seedlings which yielded *F. graminearum.* This total figure was expressed as a percentage of total seeds sown to provide a transmission rate in each treatment. The same procedure was used to calculate the transmission rate of *F. subglutinans.*

6.4 RESULTS

6.4.1 Initial seed quality

All seed lots had a high germination capacity (89-92%) which was not affected by *Fusarium* level or surface disinfection (data not presented). On Malt Agar 72% of the surface disinfected high infection seeds yielded *F. graminearum* cf. 1% for the low infection seed lot (Table 6.1). Low levels of *F. subglutinans*, *F. poae* and *Penicillium* spp. were recorded for both seed lots. In contrast, for the freezing blotter testing of non-surface disinfected seed lots, *F. subglutinans* and *Penicillium* spp. predominated (Table 6.1), while the percentage of seeds yielding *F. graminearum* was confirmed on CLA by the presence of *G. zeae* on 59% of seeds tested (Table 6.1).

6.4.2 Seedling emergence

For both sowings, emergence began 8 days after sowing (DAS) and no further increase in seedling number was recorded after 15 DAS (Fig. 6.2). Seedling emergence rate was greater for surface disinfected seeds than for non-surface disinfected seeds, and for the former did not differ between the high and low infection seed lots. However, for the latter emergence rate was initially higher for the high infection seed lot (Fig. 6.2).

Table 6.1 Initial seed quality: percentage of seeds yielding *F. graminearum* and other fungi.

Media	Blotter		Malt Agar		Carnation Leaf Agar
Seed lots	High NSD	Low NSD	High SD	Low SD	High SD
Fungi					
F. graminearum	42	12	72	1	59
F. subglutinans	51	96	1	2	NT
F. poae	6	0	5	0	NT
Penicillium spp.	57	63	1	1	NT

High = High infection seed lot
Low = Low infection seed lot
NSD = Non-surface disinfected
SD = Surface disinfected
NT = Not tested

Final emergence did not differ for the surface disinfected seed lots and the low infection non-surface disinfected seed lot (89-94%), but was lower (81%) for the high infection, non-surface disinfected seed lot (Fig. 6.2). For the low infection seed lot, all seedlings which emerged survived (Table 6.2), while non-emergence was explained by dead seeds. For the high infection seed lot, 5% of seedlings died pre-emergence (for both surface disinfected and non-surface disinfected seed lots), but post-emergence death (Plate 6.2) was slightly higher for the surface disinfected seed lot (Table 6.2). The non-surface disinfected seed lot had the greater percentage of dead seeds (Table 6.2).

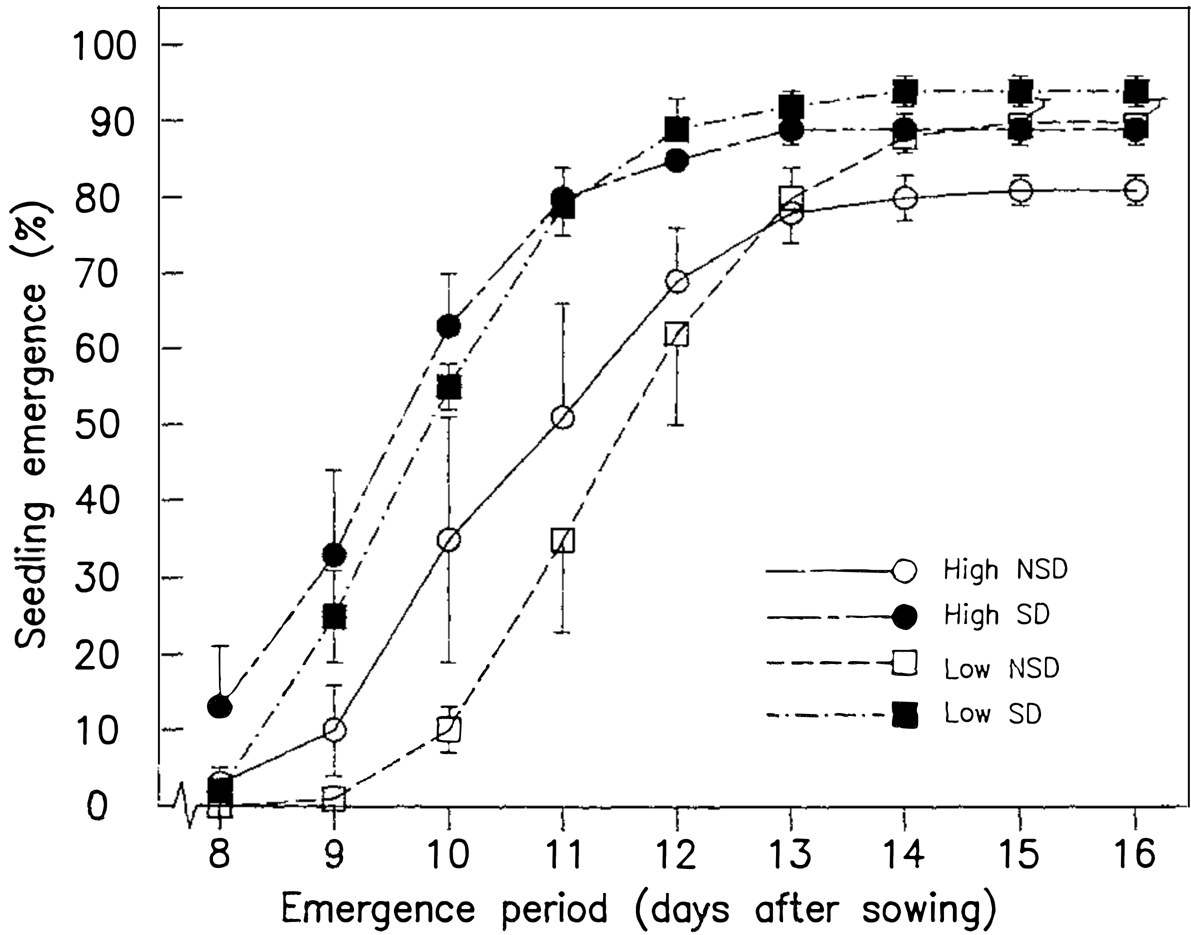


Figure 6.2 Seedling emergence under glasshouse conditions (%)

A

B

Plate 6.2 Seedling death post-emergence; A, death soon after emergence; B, death after 2-leaf stage.

Table 6.2 Seedling emergence and survival under glasshouse conditions (%).

Seeds and Seedlings	Seed Lot			
	High NSD	Low NSD	High SD	Low SD
Seedlings emerged	81	90	89	94
Seedlings survived	79	90	83	94
Post-emergence damping off seedlings	2	0	6	0
Pre-emergence damping off seedlings	5	0	5	0
Dead seeds	14	10	6	6

6.4.3 Disease symptoms on ungerminated seeds and on seedling parts

The most common 'above sand level' symptoms found in all four treatments was scalding/scorching of leaves. However, no fungi were isolated from this tissue, and the symptoms were considered an interaction between high relative humidity (within the polyethylene bags) and low temperatures and reflected sunlight (Sutic and Sinclair, 1991). Symptoms later associated with fungi (*F. subglutinans* - see Table 6.3) included:

(i) blighted seedlings (Plates 6.3a - A & D; 6.3b - A)

(ii) wilted seedlings with leaves with brown/orange oval to elongated spots with or without shot-holes (Plate 6.3a - B & E)

(iii) wilted seedlings with cream powdery mycelial growth on coleoptiles and/or first leaf (Plate 6.3a - C & F)

(iv) mycelial (cream/powdery) growth on the coleoptile at sand level (Plate 6.3b - B)

(v) stunted seedlings, usually devoid of red sheath pigment and with cream powdery mycelial growth (Plate 6.3b - D & E)

After 21-23 days after emergence (DAE), most of the seedlings had become yellow, particularly those from the high infection seed lots.

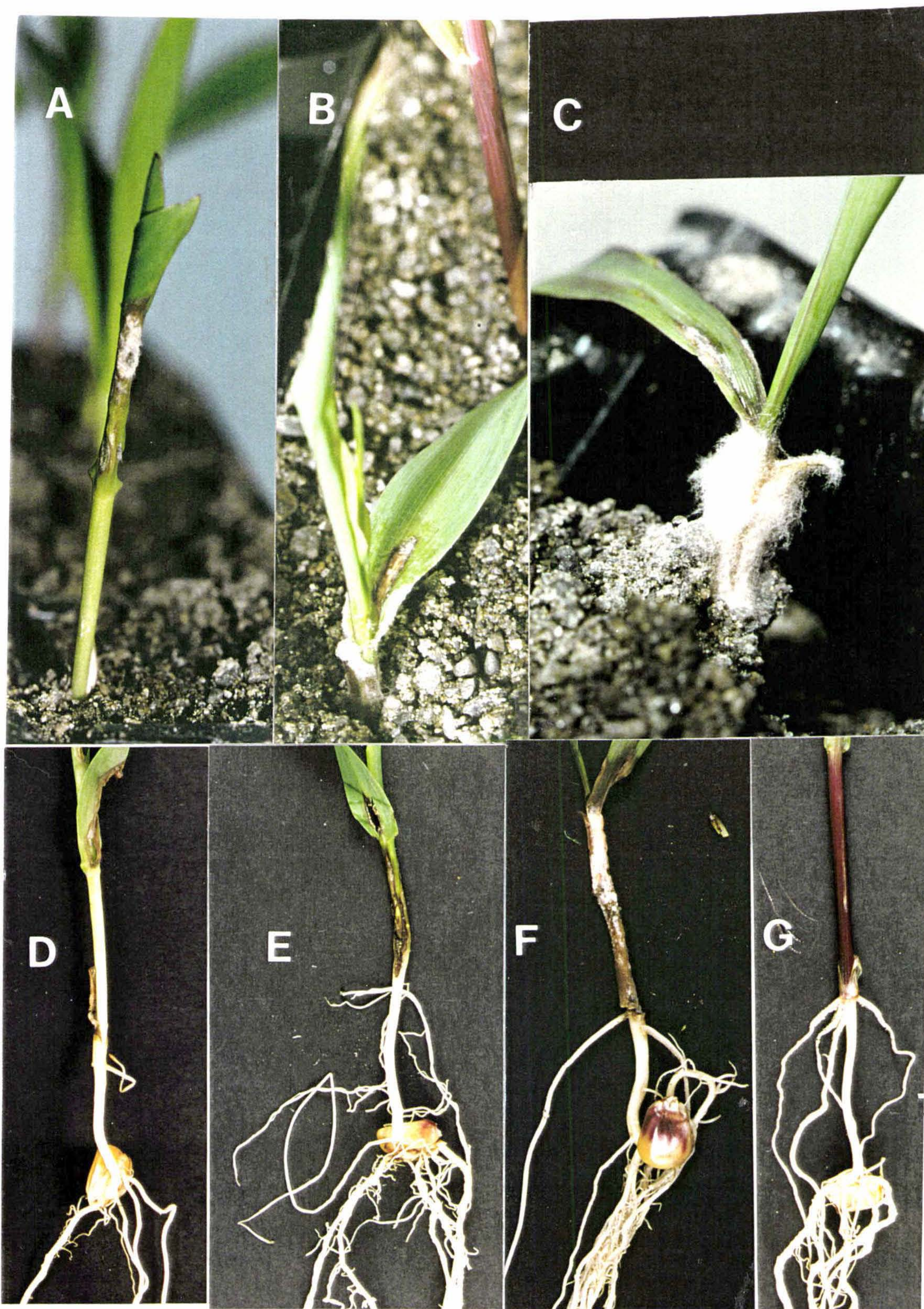


Plate 6.3a 'Above sand level' lesions/symptoms (A-C) and those found 'below sand level' (D-F) associated with *F. subglutinans*; G, undamaged seedling; A & D, B & E and C & F are same seedlings respectively.

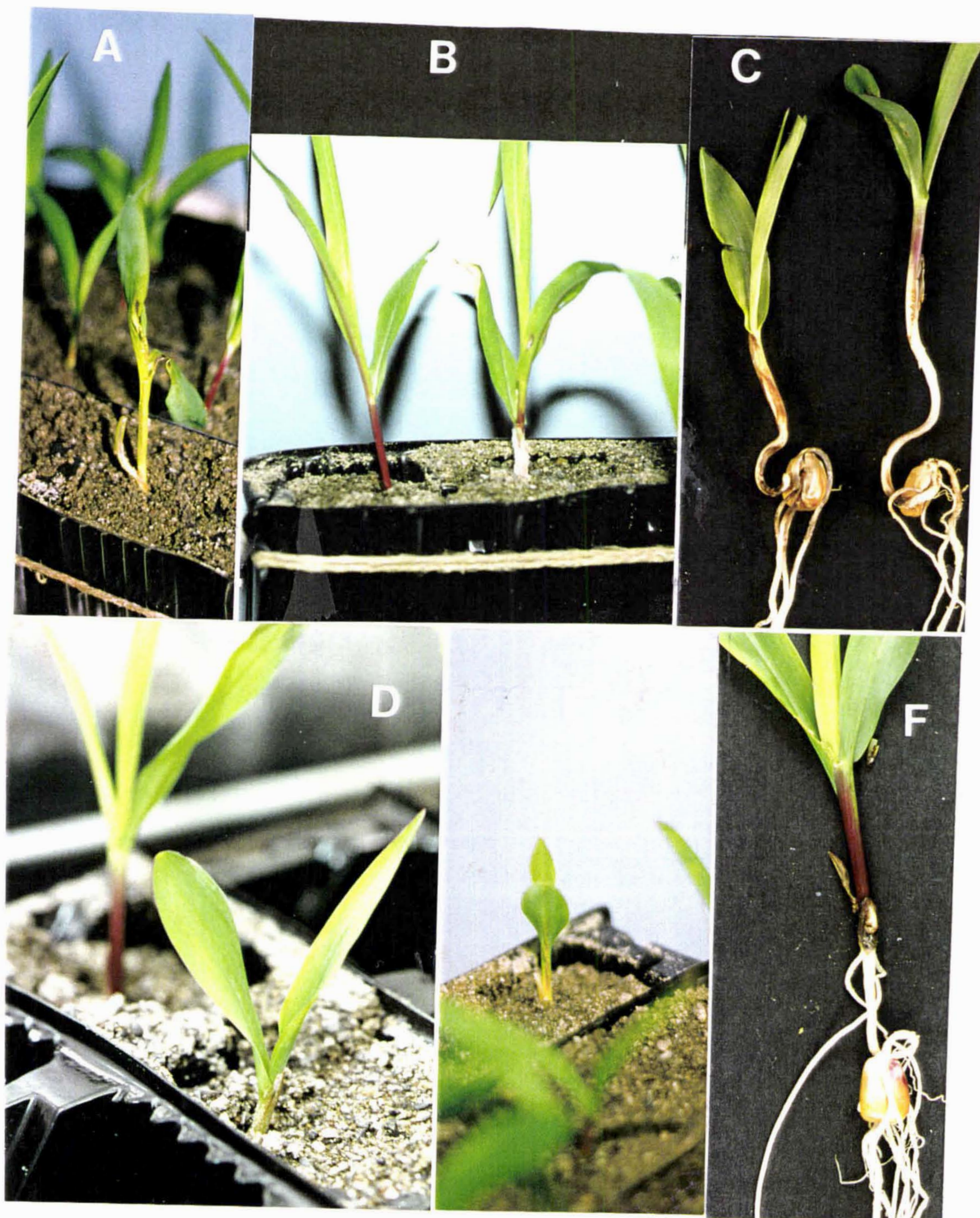


Plate 6.3b 'Above sand level' lesions/symptoms (A,B,D,E and those found 'below sand level' (C & F) associated with *F. subglutinans*.

'Below sand level' seedling symptoms included mainly discolouration (brown to reddish brown) and/or rotting of mesocotyls, roots and nodes (Plate 6.4). The scutellum-mesocotyl and scutellum-main root region (including secondary roots) were particularly damaged (Plate 6.4 A), but after 21 days the node and tertiary roots were also commonly damaged (Plate 6.4 B). In the early stage of infection the affected areas, especially on the mesocotyls, had a water-soaked orange-brown appearance with canoe-like sunken centres but later the area turned brown (Plate 6.4 C). The sunken centres tended to occur as streaks (Plate 6.4 C). These lesions were later associated with *F. graminearum* (Table 6.3; Dickson, 1923).

The percentage of seedlings with 'above sand level' symptoms was low and did not differ markedly with treatment. Less than 2% of seedlings were wilted, from 2-9% of seedlings were stunted, and 10% of seedlings had spotted/tattered leaves. However, 23% (range 15-32%) of seedlings had lesions/symptoms in the coleoptile region (Plate 6.3b - B), and from 30-70% of seedlings had lesions/symptoms on the scutellum-mesocotyl/scutellum-main root attachment (Plate 6.4). Initial inoculum levels did influence root symptoms, in that 14% of the seedlings from the low infection seed lot had main root lesions cf. 46% for the high infection seed lot. There were no differences between the disinfection treatments for the percentage of seedlings with any category of lesions/symptoms with the exception of the seed remains on live seedlings, where surface disinfected seeds had no evidence of 'red' seeds, cf. 15% of non-surface disinfected seed which were discoloured 'red', indicating the presence of *Fusarium* particularly *F. subglutinans* (Plates 6.3a and 6.3b).

Mycelial growth and/or sporulation on dead and living tissue was that of *F. graminearum*, *F. subglutinans* and *Penicillium* spp. *F. graminearum* occurred on dead seeds, seed remains/scutellum-mesocotyl/scutellum-main root attachment as a cream/brown/red, non-sporulating mycelium. The red mycelium being usually on dead seeds. This fungus also covered the pre- and post-emergence 'damped off' seedlings. On live seedlings *F. graminearum* was rarely found on above ground sand level seedling parts. 59% of the seedlings from the high infection surface disinfected

Plate 6.4 'Below sand level' seedling reddish-brown lesions/symptoms or damage due to *F. graminearum* infection(A, B & C); A, far right, undamaged seedling; B, node and tertiary root damage (left = undamaged seedling); C, characteristic orange-brown canoe-like young lesions.

seed lot with scutellum-mesocotyl attachment-main root region discolouration showed external mycelial growth of *F. graminearum* compared with 5% of seedlings which showed *F. subglutinans* external growth.

F. subglutinans was mainly observed on the coleoptiles, leaf sheaths and on the first leaf (Plates 6.3a and 6.3b). It occurred as white to cream fine mycelium with false heads which gave the fungal growth a powdery appearance. On the coleoptile, *F. subglutinans* growth looked characteristically powdery. On the leaf, it caused brown lesions which occurred at the base of the first leaf, affecting mainly the main vein and later making a hole through the leaf or "eating" away the lower part of the leaf resulting in leaf wilting (Plate 6.3a). Sometimes the leaf was covered by heavy growth of the fungus with orange sporodochia within the mycelium (Plate 6.3a - C), *F. subglutinans* was associated with stunted seedlings which lacked red pigmentation (Plate 6.3b - D and E) but did not often cause discolouration of the mesocotyl or scutellum-mesocotyl attachment, even in cases where seedlings showed severe blighting of the leaves (Plates 6.3a - D-F and 6.3b - F). However, occasionally it was associated with mesocotyl lesions which appeared in the form of long deep scoops or notches (cankers) (Plate 6.3b - C). Notched nodes with mycelial growth on the notches were also observed (Plate 6.3b - F). *F. subglutinans* was also observed on or around the seed remains as fine scanty webs of mycelium with the false heads giving the mycelium a powdery nature.

Penicillium was common on dead seeds and around the scutellum-mesocotyl region of seedlings but did not occur on leaves or coleoptiles. This fungus was noted on all four seed lots, a result which is contrary to that expected (ie seed surface disinfection removed most of the *Penicillium* contamination, Table 6.1). Therefore, the appearance of *Penicillium* on seeds and seedling tissue is presumed to be due to the rapid spread of this contaminant within the blocks of root trainers. As *Penicillium* spp. are usually not pathogens of maize (but see Discussion) any lesions which appeared to have been caused by this species was discarded and results for *Fusarium* spp. only are presented.

6.4.4 Isolation of *F. graminearum* and *F. subglutinans* from dead seeds/seedlings and lesioned/non-lesioned living seedlings

F. graminearum and *F. subglutinans* were isolated from dead seeds, pre- and post-emergence 'damped off' seedlings, and all the parts of living seedlings (Table 6.3 and Plates 6.5a and 6.5b). Both deep red and poorly coloured *F. graminearum* mycelium was observed. For *F. graminearum* seed surface disinfection did not alter the recovery, the differences in percentage infection being related to the initial seed-borne inoculum level. For the 'high infection' seed lot, 6-10% of dead seeds and 3-6% of 'damped off' seedlings yielded the pathogen, compared with 47-57% of the seed remains, main roots and mesocotyls of living seedlings (Table 6.3). Only 3% of the tested seedlings yielded *F. graminearum* from the leaves. For the "low infection" seed lot, *F. graminearum* was rarely found. *F. subglutinans* was not associated with dead seeds from surface disinfected seed lots and seed surface disinfection substantially reduced the recovery of this pathogen from other plant tissues (Table 6.3). The recovery percentages for *F. subglutinans* from the mesocotyls and main roots (ie ca. 90% for non-surface disinfected seeds) were higher than the percentage of seedlings with lesions, because this fungus was also isolated from seedlings which did not have symptoms on either the mesocotyl or main root.

6.4.5 Transmission rate

The isolation of one or both *Fusarium* spp. from any seedling tissue (dead or living) was taken as evidence of seed to seedling transmission. For *F. graminearum*, transmission rate for the 'high infection' seed lot was 44% for non-surface disinfected seeds and 52% for surface disinfected seeds. Corresponding data for *F. subglutinans* were 74% and 4% (Table 6.4). For the 'low infection' seed lot, there was a 2% transmission rate for *F. graminearum* for the low non-surface disinfected treatment, but 88% for *F. subglutinans* for the same treatment. The corresponding data for the two species in the surface disinfected treatment were 0 and 10%.

Table 6.3 Recovery (%) of *F. graminearum* and *F. subglutinans* from live, pre- and post-emergence damped off seedlings and from dead seed raised in the glasshouse.

Type of material plated/Part	Seed lot	*F. graminearum*	*F. subglutinans*
Dead seeds and Pre-/Post-emergence damped off seedlings			
Dead Seeds	HNSD	10	12
	HSD	6	0
	LNSD	0	7
	LSD	3	0
Pre-emergence damped off seedlings	HNSD	3	4
	HSD	2	1
	LNSD	0	0
	LSD	0	0
Post-emergence damped off seedlings	HNSD	2	2
	HSD	6	1
	LNSD	0	0
	LSD	0	0
Live seedlings*:			
Seed remains	HNSD	57	93
	HSD	43	10
	LNSD	0	93
	LSD	0	17
Main root	HNSD	47	87
	HSD	47	7
	LNSD	0	93
	LSD	0	10
Mesocotyl	HNSD	50	87
	HSD	53	3
	LNSD	3	97
	LSD	0	10
Coleoptile-Node region	HNSD	13	40
	HSD	17	20
	LNSD	3	73
	LSD	0	7
Leaves	HNSD	3	17
	HSD	3	17
	LNSD	0	53
	LSD	0	10

HNSD = High infection non-surface disinfected
HSD = High infection surface disinfected
LNSD = Low infection non-surface disinfected
LSD = Low infection surface disinfected
* 30 randomly selected seedlings

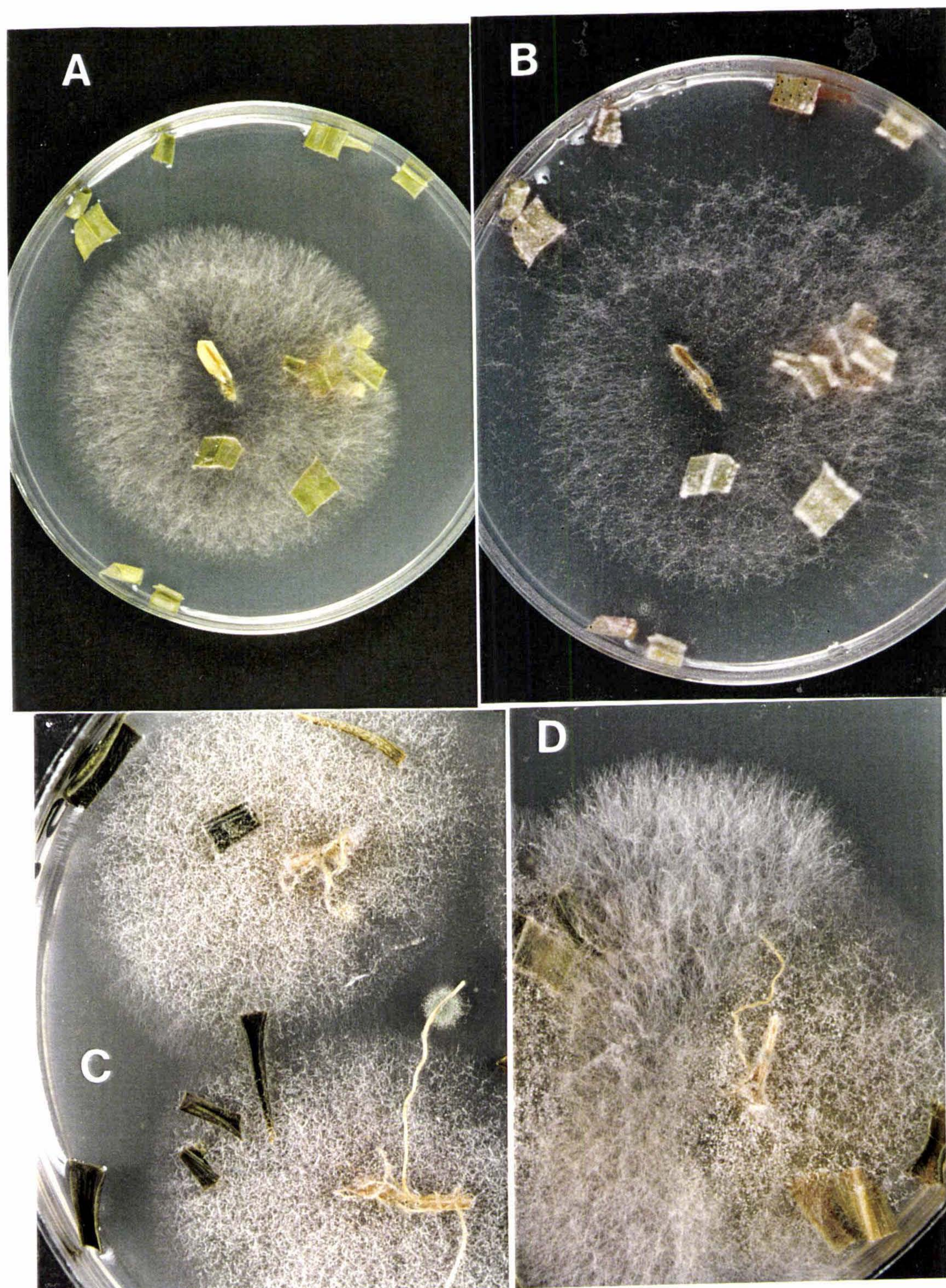


Plate 6.5a Cultural morphology of *F. graminearum* (A & B); and *F. subglutinans* (C): A, young *F. graminearum* culture; B, 14-day old culture with mature perithecia of *G. zeae*; D, *F. graminearum* and *F. subglutinans* growing from same tissue.

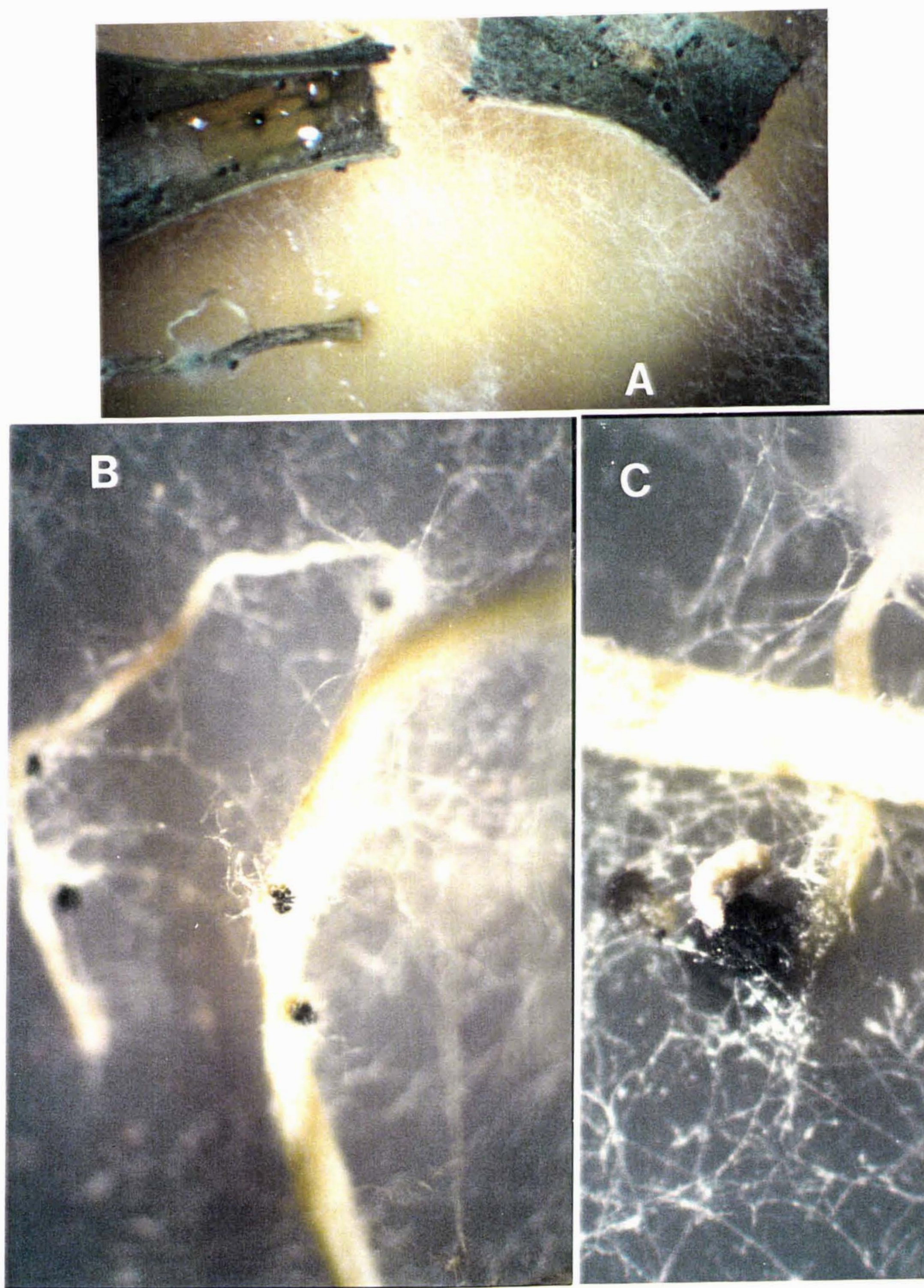


Plate 6.5b Perithecia of *G. zeae* on carnation leaf pieces (A, top) and on roots of a maize seedling on CLA (A, bottom, B & C); C, *G. zeae* perithecium discharging ascospores; (A x 10; B x 20; C x 40).

Table 6.4 Transmission rate of *F. graminearum* and *F. subglutinans* to seedlings under glasshouse conditions (%).

Fungi	Seed lot			
	HNSD	HSD	LNSD	LSD
F. graminearum	44	52	2	0
F. subglutinans	74	4	88	10

HNSD = High infection non-surface disinfected
HSD = High infection surface disinfected
LNSD = Low infection non-surface disinfected
LSD = Low infection surface disinfected

6.5 DISCUSSION

Fusarium graminearum was transmitted from maize seeds to seedlings, being consistently isolated from seedlings raised under aseptic conditions from seeds which carried the pathogen. The transmission rates were similar to the seed-borne inoculum levels, suggesting that under favourable environmental conditions, the pathogen will be transferred from the seed to the living seedling. It should be noted, however, that a small percentage of seeds were killed, and that some seedlings 'damped off' either pre- or post-emergence.

One apparent inconsistency in the relationship between initial seed-borne inoculum level and the transmission rate occurred for the 'high inoculum' surface disinfected seed lot, when health test (MA) results for the pathogen (72% of seeds infected) were 10% higher than the transmission rate. However, when tested on CLA, only 59% of the seeds produced cultures which formed perithecia of *G. zeae*, a figure much closer to the transmission rate. This seems to suggest that the CLA method is a more accurate detection method (ie that some of the colonies on MA were misidentified), agreeing with recommendations (Nelson *et al.*, 1983; Burgess *et al.*, 1988) for pure cultures. However, this requires confirmation. The remaining 10% discrepancy which occurred in comparing the initial seed-borne inoculum on MA

and the transmission rate may be attributed to the low levels of *F. crookwellense* and *F. culmorum* detected in the high infection surface disinfected seed lot (see Chapter 4).

While the deep freezing method (Limonard, 1966, 1968) has the advantage of improved observation of seed-borne fungi growing on the seed, it has been found to remove most surface-borne inoculum, eg *Alternaria* and *Mucor* spp. (Hampton, J.G, pers. comm.), and also certain pathogens such as *Nigrospora Colletotrichum* and *Corynospora* spp. (de Tempe, 1979). The reported fungi were not a problem in the seeds used in this study and therefore were not particularly investigated. However, the results of this study suggest that at least the level of *F. subglutinans* (96%) on the seeds may not have been affected. The likely effect on *F. graminearum*, *F. poae* and *Penicillium* may need to be checked.

While *F. graminearum* is known to cause seed rot (in the soil) and seedling blighting before emergence resulting in poor seedling emergence and blighting after emergence (Dickson, 1923, 1956; Pearson, 1931; Edwards, 1936; Covey, 1959; Christensen and Wilcoxson, 1966), the temperature of the soil is reported to be the most important single fact determining the extent of seed rot and seedling blight (Dickson, 1923). The most favourable soil temperatures for the blighting of maize range from 8-20°C, and above 24°C no blighting occurs (Dickson, 1923). This may therefore explain why in this experiment *F. graminearum* had no effect on germination of high infection seed lots at 25°C (in the laboratory), one of the internationally agreed germination temperatures for *Zea mays* (ISTA, 1993).

While the most favourable soil temperature for seedling blight ranges from 8-20°C, the most rapid disease development and maximum blighting (post emergence) occurs at 12-16°C and below this the seedlings succumb entirely (Dickson, 1923). This experiment was carried out at daily temperatures (mean) of 14-17°C which according to Dickson (1923) and Jones (1924) are close to the "edge" of the region of maximum seedling blighting by *F. graminearum* (see Fig. 2.4, Chapter 2). It is therefore not surprising that most seedlings which emerged survived and very few

seeds and seedlings from high infection seed lots died pre-emergence (Table 6.2). (It should be noted that the experimental study temperature (14-17°C) was selected on the basis of its being in the range of the late spring (mid November) soil sowing temperatures at Massey University and the surrounding areas (AgResearch Grasslands, Palmerston North, 1989, 1992; Hardacre *et al.*, 1989, 1991)

The fact that *F. graminearum* had little effect on seedling emergence and survival but was associated with high (up to 70%) seedlings with scutellum-mesocotyl and scutellum- main root lesioning (mainly discolouration) suggests seedling "resistance" to the pathogen, as explained by Dickson and Holbert (1926): that the development of maize seedling blight is primarily a host response (seedling metabolism) to different temperatures and that seedling metabolism has a direct correlation with the disease development. Maize seedlings grown at high temperatures (24°C and above) have a large reserve of soluble carbohydrate building substances (hexoses etc) which are poor food for *F. graminearum*, and at this temperature, seedling cell walls are well impregnated with suberin in the protective tissues (coleoptiles and the cortex). On the contrary, seedlings grown at low soil temperatures (8-20°C) are relatively low in soluble carbohydrate building substances, and have cell walls (especially in the protective tissues) composed largely of pentosan-yielding substances, eg pectin and xylan, which are excellent food for rapid vegetative growth of *F. graminearum* (see Fig. 2.5, Chapter 2).

The results of this study suggest that for demonstration of severe effects (rotting of seedlings - Dickson, 1923; Pearson, 1931) the study may require to be conducted at lower (12-16°C) temperatures than 14-17°C, but that 14-17°C appears most likely to have been sufficient for the demonstration of transmission rate (which was close to maximum) of *F. graminearum* from maize seed to seedlings.

F. graminearum did affect seed germination and seedling emergence, especially in the high infection surface disinfected seed lot where the fungus was isolated from 8% of the material (6% dead seeds and 2% pre-emergence seedling

death), compared to isolation of *F. subglutinans* from only 1% (due to pre-emergence death) of the same material (Table 6.3). These data also compare with that in Table 6.2 (seedling emergence and survival). In the high infection non-surface disinfected seed lot, however, both *F. graminearum* and *F. subglutinans* appear to have affected the seeds and seedlings as almost equal number of dead seeds and damped off seedlings carried these fungi (16% with *F. graminearum* and 18% with *F. subglutinans* - Table 6.3).

While it appears possible that *F. graminearum* could have been responsible for the death of the few seeds in the low infection surface disinfected seed lot, it was not clear whether *F. subglutinans* was responsible for the death of low infection non-surface disinfected seeds (7%, Table 6.3) as this fungus may have only survived in the seeds which were killed by *F. graminearum* before itself died in storage at 25°C (refer to Chapter 5 of this study).

F. graminearum also afected seedling survival, especially in the high infection surface disinfected seed lot where 6-8% of the post-emergence killed seedlings carried *F. graminearum*, compared to 1-3% of the same material (Table 6.3, also see Table 6.2) which carried *F. subglutinans*.

Although *F. graminearum* and perhaps *F. subglutinans* caused seed rot, the percentage of dead seeds recorded under glasshouse conditions did not differ greatly from that (6-8%) recorded in the standard germination test in the laboratory at 25°C, suggesting that seed rot occurred before harvest and confirming that glasshouse temperature conditions (14-17°C) had only a limited effect on seedling emergence. These results agree with the reports of Dickson (1923) and Edwards (1936). However, *F. graminearum* did cause severe lesioning of the mesocotyls and roots (Plate 6.4) which may have weakened the seedlings resulting in yellowing of the leaves, an observation which also agrees with the findings of Dickson (1923).

The presence of *Penicillium* spp. was a confounding issue because the characteristic blue/green sporulating colonies were seen on seed remains/scutellum-

mesocotyl/scutellum-main roots even in surface disinfected seed lots which did not carry this fungus (1%). The assumption that contamination was a problem was justified. *P. oxalicum* is known to cause seedling blight and leaf blight of maize (Koehler, 1957; Johann *et al.*, 1931; Shurtleff, 1980). Koehler (1957) described the symptoms as blue green areas at the tip of young leaves which develop into dead tissue streaks and later become dry and tan (or drab) in colour. These symptoms were not observed in this study. Although *Penicillium* spp. were associated with discoloured (lesioned) mesocotyl-scutellum area, this type of lesioning was different (no reddish brown lesioning and sunken centres were absent in lesions) from that of *F. graminearum* so that in most cases it was easy to differentiate the symptoms of both fungi.

The mesocotyl and root lesioning (discolouration) with affected tissues appearing water-soaked and later turning brown to reddish brown and showing sunken centres consistently recorded on seedlings in this experiment is characteristic of *F. graminearum* infection symptoms (Dickson, 1923, 1956; Pearson, 1931). These authors (Dickson, 1923, 1956; Pearson, 1931) have also stressed the confinement of the symptoms in the mesocotyl-root area (subcrown internode and roots), other symptoms of *F. graminearum* seedling blight being yellowing, wilting and stunting of seedlings (Dickson, 1923; Mohamed *et al.*, 1968) due to a weakened root system (Dickson, 1923) and leaf chlorosis (Mohamed *et al.* 1968). Of these, only the yellowing of plants was prominent 21 days after seedling emergence, and this coincided with the severe discolouration/rotting of roots. Leaf tip chlorosis due to *F. graminearum* reported by Mohamed *et al.* (1968) was not a problem since only 3% of the 30 seedlings studied yielded *F. graminearum* from the leaf tips.

While it might be that the absence of leaf infection by *F. graminearum* was due to the short study period, it is reported (Dickson, 1923, 1956) that the period of seedling infection is usually limited to the early seedling stage (ie before emergence) or when seedlings are in the first to third leaf stage, and rarely later.

While *F. graminearum* was recovered from all parts of the seedlings, it was most often recovered from those tissues closest to the seed (ie the scutellum-mesocotyl and scutellum-main root region (43-57%)), less often from the coleoptile-node region (13-17%) and rarely from the leaves (3%), suggesting that with time, seedling infection progresses upwards from the original site (seed) and under favourable conditions, in the absence of post-emergence blight, leaf infection is likely to be a result of inoculum from outside the seedling rather than the seed. However, infection of the coleoptile node region is likely to be of significant importance in the introduction of the seed-borne inoculum from the seed remains in the soil to the soil surface and perhaps to the young seedling, mature plant and consequently to cobs and kernels. First, the observation that in a few cases *F. graminearum* grew out of the sand bed along senescing coleoptiles suggests that such a situation is likely to occur under field conditions. Secondly, the nodal infection may extend to above the soil surface on the young plant. Certainly, with post-emergence blighting, the fungus is readily introduced to the soil surface. Consequently, with this availability of "above soil surface" inoculum, under favourable conditions in spring *F. graminearum* is likely to grow, sporulate and perhaps form perithecia of *G. zeae* on the senescing leaves from where the conidia and ascospores will be splashed onto the stems, possibly causing stalk rot disease, and later on to ears (silks), infecting cobs and seeds causing cob rot disease (Sutton, 1982- also see Fig. 2.3, Chapter 2).

The reason for the higher (74%) transmission rate of *F. subglutinans* than the recorded seed-borne inoculum level (51%) in the high infection non-surface disinfected seed lot was unclear. However, due to the powdery nature of the microconidia of *F. subglutinans*, this was attributed possibly to further seed to seed contamination and seed to sand contamination during sowing, and also perhaps later seedling to seedling contamination (leaf contact) and by moisture drop under the humid plastic bag conditions (Plate 6.1). Moisture was released each morning by tapping the bags in order to see the seedlings to make records on seedling status inside the bag. For the low infection surface disinfected seed lot, the higher transmission rate (10%, ie 8% higher than the recorded seed-borne inoculum) of *F. subglutinans* was attributed to undetected seed-borne inoculum, since erratic

occurrence of higher (sometimes more than ten times) incidences of this fungus were observed during the screening of seed samples on MA (Chapters 3 and 5 of this study). *F. subglutinans* is reproted to form microconidia in the maize seed tip cap and this part of the seed does not soak materially during surface disinfection as it is made of cork-like tissue (Manns and Adams, 1923). It was therefore thought that during the rinsing (5 minutes under running tap water) there was a release of the microconidia (either from damage/weakened tip caps or by small amounts soaking out) into the disinfected seeds thus causing seed surface contamination, a situation which would result in more seedlings being infected than was estiamted. All these aspects need further checking.

F. subglutinans was recovered form living seedlings (main root, mesocotyl, coleoptile/node region and leaf tissue), and from both dead seeds and 'damped off' seedlings. That the majority of this inoculum was surface-borne was illustrated by the significant reduction in recovery from infected seedlings when seeds were surface disinfected. *F. subglutinans* was associated with leaf spotting, seedling blight, shoot wilt and mesocotyl and node lesioning. However, *F. subglutinans* was also consistently associated with leaf and node infection (damage) but more so with coleoptiles, except that these structures do not serve much purpose after the leaves have emerged. However, from the fact that *F. subglutinans* appeared first on the coleoptiles and since this fungus was mainly surface-borne (Table 6.1), it appears the coleoptiles are contaminated by the pericarp as the embryonic axes breaks through the pericarp during germination, and perhaps also by the sand (substratum) in contact with the seed. Since *F. subglutinans* affects the coleoptiles and the leaves early in the seedling stage, this suggests that under favourable field conditions, in the presence of rainsplash, this fungus would readily be transmitted to maturing seedlings and plants, and thus seed-borne infection may become a source of stalk rot and leaf infection of maize plants, and subsequently, to ear (silks) and to cobs/kernels, thus infecting the seeds.

Since *F. moniliforme*, a close relative of *F. subglutinans* (*F. moniliforme* var. *subglutinans*) is generally associated with nodal stalk rot (Roane, 1950; Windels and

Kommedahl, 1984), it is possible that the observed consistent association of *F. subglutinans* with damaging of nodes of maize seedlings is likely to result in nodal stalk rot in the mature maize crop under favourable conditions for the disease. Since, at the seedling stage, the growing point of the seedling is still close to nodal tissue, infection of the seedling node is likely to lead to growing point area infection (Neergaard, 1979), perhaps leading to shoot wilting as was observed in this study (Plate 6.3a - B & E).

That *F. subglutinans* was found associated with seedling blight, and more so leaf blight, is in agreement with the pathogenicity studies conducted by Edwards (1935) who found that maize seedlings inoculated with a pure culture of *F. moniliforme* var *subglutinans* (*F. subglutinans*) were infected and collapsed, and seedling stunting occurred, but Edwards (1935) noted that low temperature (corresponding to those of early spring in New South Wales, Australia) and high relatively humidity conditions were necessary for seedling infection. Therefore, the prevalence of *F. subglutinans* leaf/shoot infection under the glasshouse conditions (plastic bag covering) was not surprising.

The leaf wilting and seedling stunting are in agreement with reports that *F. moniliforme* var *subglutinans* (*F. subglutinans*) causes wilt in sugar cane (Manns and Adams, 1923; Perez and Mauri, 1989) and in pineapples (Pissarra *et al.*, 1979) and maize shoot stunting (Dey *et al.*, 1989). Ethyl acetate extracts of *F. moniliforme* var *subglutinans* (*F. subglutinans*) have been found to cause curling and stunting of maize and pea roots (Kumar *et al.*, 1987). The occasional mesocotyl damage which occurred in the form of cankers was not surprising since *F. subglutinans* is known to cause pitch cankers in pine (McCain *et al.*, 1987; Correll *et al.*, 1992). However, the apparent failure for *F. subglutinans* to cause root and mesocotyl lesioning/rotting does not conform with observations by Edwards (1935) that *F. moniliforme* var *subglutinans* (*F. subglutinans*) caused brown water-soaked lesions on the mesocotyl after inoculation or under natural infection in the field from naturally infected seeds.

Although *F. subglutinans* was associated with above sand level tissue lesioning, (spotting, leaf blight and shoot wilting) this was commonest in the low infection non-surface disinfected seed lot. This corresponds well with the recovery of *F. subglutinans* from leaves of 53% of this seed lot compared to its recovery from 10% of seedlings from the surface disinfected seeds. In this seed lot, however, 20% of the symptomless seedlings ("no lesion" category) yielded *F. subglutinans* from the leaves. The isolation of *F. subglutinans* from symptomless seedlings agrees with the report by Maffia (1980) who re-isolated this fungus from the roots of symptomless maize plants one month after sowing seeds in infected soil. This occurrence in symptomless plants suggests that *F. subglutinans* spreads systemically in seedlings, in addition to local seedling infection. Whether systemic infection would contribute to infection of the mature plant and eventually to seed infection in the cobs under field conditions needs to be determined.

PART 2 : FIELD STUDY

6.6 INTRODUCTION

Contaminated or infected seeds sown in disinfected soil may lead to more severe development of diseases than the same seed sown in non-disinfected soil where antagonistic soil organisms have not been eliminated (Neergaard, 1979). This suggests that the composition of the soil microflora may, to a considerable extent, determine the development of pathogenic fungi introduced into the soil with seed because competition occurs between these organisms for nutritional material and the relationship between the microflora may be synergetic or antagonistic (Neergaard, 1979).

6.7 OBJECTIVES

The main objective of this study was to determine whether seed-borne *F. graminearum* could be transmitted from infected maize seeds, using the same four seed lots sown in sterile sand in the glasshouse, to seedlings under field conditions. The other objectives were to determine whether the effects of *F. graminearum* on germinating seeds and emerging and establishing seedlings in the field were comparable to those observed under glasshouse conditions, and to also detect the fungal microflora associated with the seedlings, so as to consider their role in disease establishment in the field.

6.8 MATERIALS AND METHODS

The four seed lots already described for the glasshouse study (Part 1) were also sown in the field on 13 November 1992. Prior to sowing, seeds were treated with an insecticide, Promet 300 EW (40 ml/kg seed + 25 g absorbent (inert powder)/kg seed). Ten days after seedling emergence, Hallmark 5EC, 53 g

esfanvalerate/ha (used against cutworms - *Agrotis ipsilon*) was applied. In addition, Musurol snail and slug bait was applied twice (7 days after sowing and then 10 days after seedling emergence at a rate of 5.9 kg methiocarb/ha). The site at Massey University was an Ohakea silt loam previously cropped with fodder radish (*Raphanus sativus* L.). After the final radish grazing, the stubble had been deep ploughed and the site rotary cultivated to prepare a seed bed.

Seeds were sown by hand, with 40 seeds (at 20 cm within row spacing) being a replicate for each seed lot. With four replicates of the four seed lots there were therefore 16 rows, each spaced one metre apart. Seed lots and replicates were arranged in a randomised complete block design. Seeds were sown at a depth of 5 cm using a jab planter; a wooden spatula peg was placed in the ground beside each seed sown, to assist in the harvest recovery and the recovery of non-emerged seeds/seedlings.

Soil temperature was recorded daily from four thermometers inserted randomly into the soil, to a depth of approximately 10 cm in the experimental field. Soil moisture samples were taken at random on alternate days from the experimental field using a soil core sampler, from a depth down to approximately 10 cm. The soil moisture content was then determined on a wet weight basis (Hampton, 1991) by drying a 25 g soil subsample in the oven at 105°C for 3 hours. The moisture content was then calculated using the formula:

$$\text{Soil moisture content (\%)} = \frac{\text{Loss in weight (g)} \times 100\%}{\text{Fresh weight (g)}}$$

Seedling harvesting was carried out twice, at 10 and 21 days after emergence. At the first harvest, half of each row was harvested. At each harvesting time, the ground was broken with a hoe and seedlings were individually lifted from the soil, care being taken not to damage the roots. As many as possible of the dead seeds and seedlings which did not emerge were also harvested. The harvested seedlings and

dead seeds were studied and treated in the laboratory in the same way as described for those harvested from the glasshouse experiment (Part 1).

Dead seeds and non-emerged seedlings were counted, examined for the presence of externally borne fungi, then surface disinfected and plated on CLA + 1% streptomycin sulphate. Incubation conditions and identification of *G. zeae* were as previously described (Part 1). The number of seeds and non-emerged seedlings yielding *G. zeae* and other fungi were recorded. *Fusarium* spp. (other than *G. zeae*) and other fungi were identified macroscopically (Neergaard, 1979) using a stereomicroscope (x 40 magnification) according to mycelium type and sporodochia formation (in case of *Fusarium* spp.) and conidia or other fruit body or sclerotia formation on CLA and/or on agar, but microscope preparations were also made and confirmation of identity was done according to Barnett and Hunter (1987).

The live seedlings from each seed lot were individually labelled and processed in the same way as previously described for Part 1, the only change being the addition of 1% streptomycin sulphate to the CLA.

Post-emergence damping off was not detected in the field. The calculations for transmission rate were not found applicable, since *F. graminearum* from soil-borne inoculum obviously infected seedlings in the field.

6.9 RESULTS

Over the 29 days from sowing to final seedling harvest, 10 cm soil temperature ranged from 13.8°C to 18°C, averaging around 16°C (Fig. 6.3). Mean air temperature (recorded at a site 2 km away) ranged from 11.5°C to 19.5°C over this same time period (Appendix 6.2). Soil moisture at 10 cm depth ranged from 22.1-26.7% (Fig. 6.3), and was generally lower (22-23%) during seed germination and early seedling emergence (0-11 days) than during seedling establishment (25-27%).

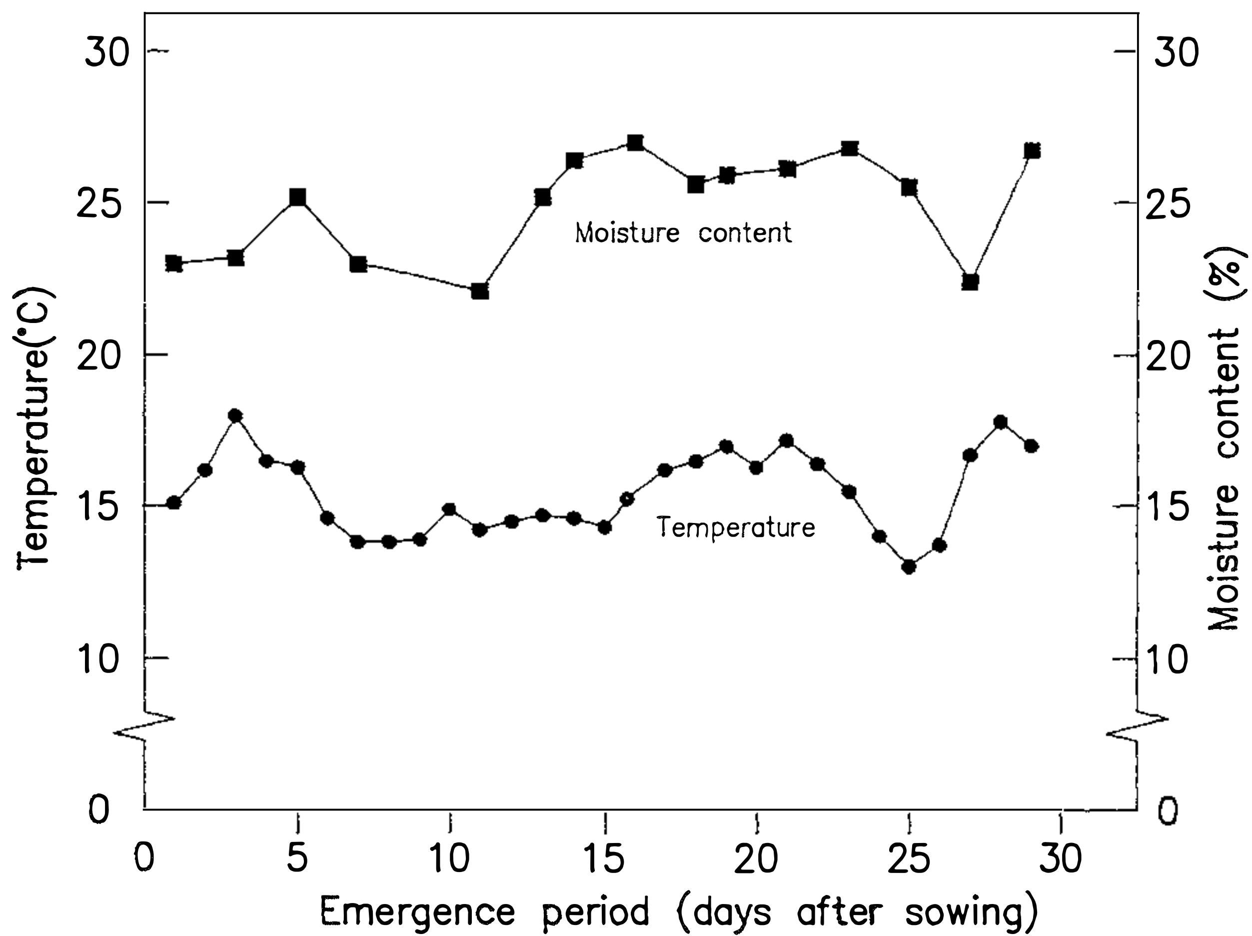


Figure 6.3 Mean soil moisture content (%) and soil temperature (°C) recorded during seed germination, seedling emergence and establishment under field conditions

Seedling emergence began 8 days after sowing and maximum emergence had occurred by 12-14 days after sowing (Fig. 6.4). There were initially some differences among seed lots in emergence rate, but final emergence percentages did not differ (Table 6.5). None of the emerged seedlings subsequently died, and some unemerged seedlings were lesioned but not dead. The number of dead seeds did no differ amongst the seed lots, and 2-3% of the seeds/seedlings for each seed lot were not recovered (Table 6.5).

Table 6.5 Seedling emergence and survival under field conditions (%).

Seed lot	High NSD	Low NSD	High SD	Low SD
Seedlings emerged	88	89	91	86
Unemerged seedlings	3	1	1	3
Ungerminated dead seeds	6	7	6	9
Missing	3	3	2	2

NSD = Non-surface disinfected
SD = Surface disinfected

Both the dead seeds and non-emerged seedlings were almost devoid of external fungal growth. Dead seeds were rotten, and some of the surviving but non-emerged seedlings appeared to have been restricted physically from emerging, as they were coiled. Occasionally *Trichoderma* spp. and some unidentified hyphae were observed on these seedlings.

Emerged seedlings were similar in size and apart from leaf lesioning, appeared to be robust (Plate 6.6 C). Only two stunted seedlings were found. Leaf damage consisted mainly of various inconspicuous tiny orange or brown spots, somewhat round to elongate in shape. Tiny shot-holes were common and were elongate in shape when they occurred on the main leaf vein. Occasionally some spots with white centres were observed with or without water-soaked edges or pycnidia. Some of the

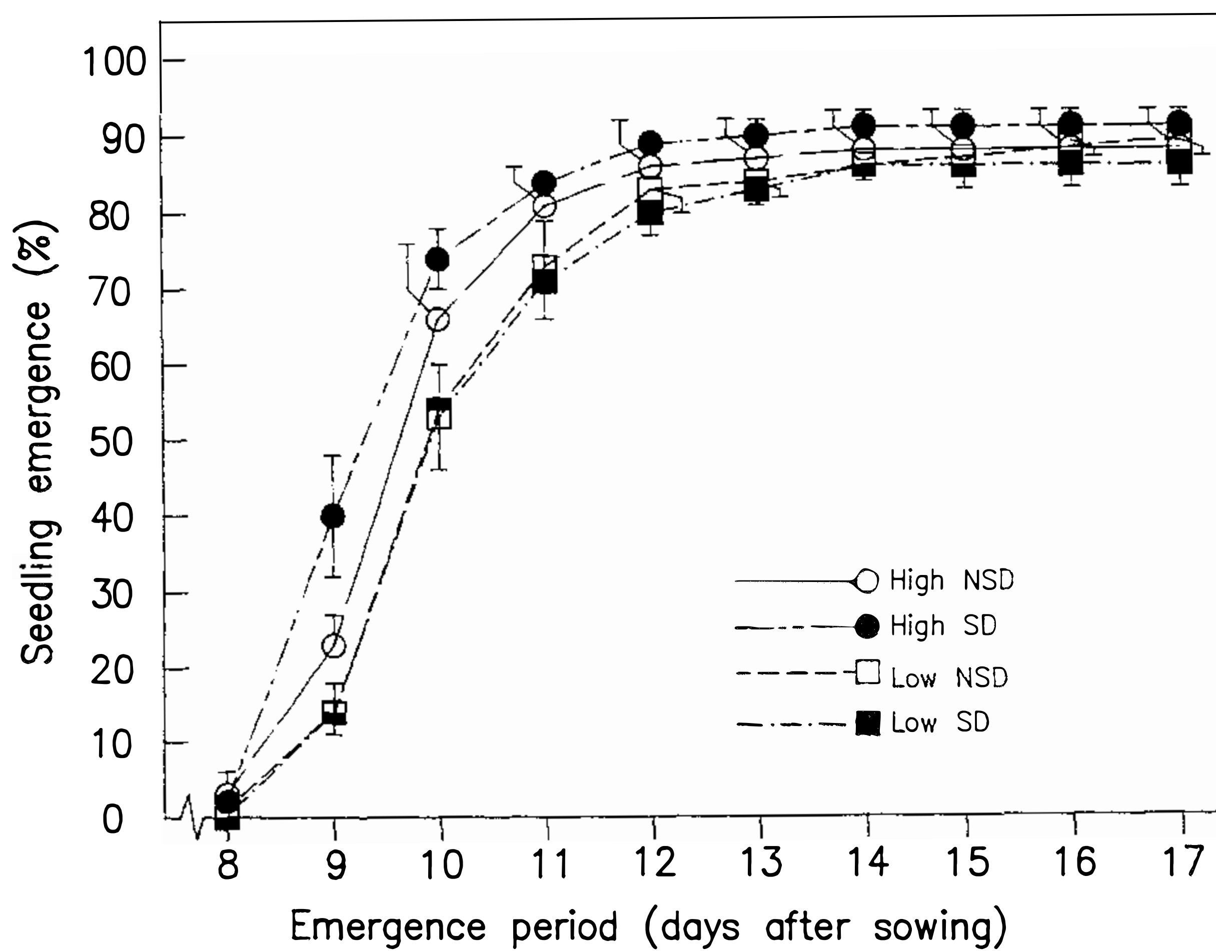


Figure 6.4 Seedling emergence under field conditions (%)

apparent leaf spots and shot holes were thought to be due to insect damage. In most cases spots occurred on the first and second leaves but occasionally on the third and fourth leaves. By 21 DAE many plants from all seed lots had developed a purple leaf colouration. Also, by 21 DAE the coleoptiles had senesced and decayed. Delaying seedling harvest increased the percentage of seedlings with leaf spotting from 12-25% after 10 d harvest to 26-45% after 21 d harvest (Table 6.6). With the exception of two seedlings (stunted) on which *F. subglutinans* (Plate 6.6 D) was found and pycnidia seen in some leaf spots, no obvious fungal growth was observed on the seedling parts above ground level. Unidentified hyphae were observed on decaying coleoptiles of some seedlings below ground level.

Seedlings from both the high and low infection seed lots had similar discolouration on the below soil level parts (Plate 6.6). The scutellum-mesocotyl attachment and the coleoptile-node region were the most commonly discoloured areas, while the main roots were least discoloured. Time of harvest had little effect on the extent of scutellum-mesocotyl area discolouration as the percentages of seedlings affected was 66-82% after the 10 d harvest and 85-96% after the 21 d harvest. However, delaying seedling harvest caused an increase in the number of seedlings with discoloured main roots, from 3-24% after the 10 d harvest to 26-37% after the 21 d harvest.

Disease symptoms (tissue discolouration and/or rotting) were found on the nodes, the tertiary roots, mesocotyls, scutellum-mesocotyl attachment area, including the secondary roots and on the main roots, especially the scutellum-main root attachment area. Dark brown to black discoloured lesions occurred in the scutellum-mesocotyl attachment region, also affecting the secondary roots and the mesocotyl-main root attachment region. Occasionally, lesions with sunken centres characteristic of *F. graminearum* infection symptoms as found under glasshouse conditions (Part 1) were observed, mainly in the scutellum-mesocotyl attachment region. However, commonly, this discolouration was restricted to the area closest to the seed remains, making it necessary to lift the pericarp under a dissecting microscope to confirm the

Plate 6.6 Seedling disease symptoms under field conditions: A & B, *F. graminearum* scutellum-mesocotyl, root and node lesions; C, healthy plants; D, stunted seedlings infected by *F. subglutinans* (B x 15).

occurrence of discolouration on the scutellum-mesocotyl attachment; thus the seedling infection was less severe in the field than it was under glasshouse conditions.

Table 6.6 Percentage of live seedlings* with visual disease symptoms at 10 d and 21 d harvests.

Seedling Part	Seed lot	% of seedlings with discolouration or lesions	
		10 d	21 d
Main Root	HNSD	24	29
	HSD	20	37
	LNSD	8	26
	LSD	3	26
Mesocotyl	HNSD	73	96
	HSD	82	85
	LNSD	66	87
	LSD	70	88
Coleoptile-Node region	HNSD	62	76
	HSD	61	86
	LNSD	50	83
	LSD	50	81
Leaves	HNSD	25	28
	HSD	13	26
	LNSD	12	45
	LSD	25	45

* 65-74 seedlings were examined
HNSD = High infection non-surface disinfected
HSD = High infection surface disinfected
LNSD = Low infection non-surface disinfected
LSD = Low infection surface disinfected
10 d and 21 d = days after first seedling emergence

In some cases, discolouration and lesioning around the node/tertiary root bases appeared to be independent of scutellum-mesocotyl discolouration as the above scutellum-mesocotyl area of the same seedling in many cases was more discoloured or damaged than the scutellum-mesocotyl area. Some seedlings exhibited decayed nodes and tertiary roots (Plate 6.6 A) as observed under glasshouse conditions, but

some showed this injury when minimum discolouration occurred on the same seedling in the scutellum-mesocotyl area, suggesting that the seed was not the source of infection.

The number of dead seeds and unemerged seedlings did not differ with seed lot (Table 6.7). *F. graminearum* was recorded from some but not all dead seeds and unemerged seedlings, but *F. subglutinans* was not detected. The other *Fusarium* spp. recorded were not identified to species level. *Gleocladium roseum* was detected from nearly all of the dead seeds and unemerged seedlings, while *Trichoderma* spp., *Arthrobotrys* spp. and *Trichothecium* spp. were also common. Members of the *Mucorales* and black sclerotia were also detected (Table 6.7). There was no apparent relationship between fungi detected and seed lot or disinfection treatment.

Irrespective of initial seed-borne inoculum level or disinfection treatment, most live seedlings carried lesions on either the leaves, coleoptile-nodes, mesocotyl or main root. The fungi isolated from these lesions (Tables 6.8 and 6.9) mostly differed according to the plant tissue examined. For example, of the mycopathogenic fungi (Vakili, 1992) (Table 6.8), *Gleocladium roseum* was most commonly recorded from seed remains, and occasionally the main root and mesocotyl, whereas the field fungi (Table 6.9), eg *Colletotrichum* spp., *Drechslera* spp. and *Epicoccum* spp. were recovered only from above ground tissues. While *Chaetomium* and *Trichoderma* spp. were consistently isolated (3-87%) from all tissues, *Chaetomium* spp. was almost absent from the seed remains but *Trichoderma* was mostly absent from the leaves (Table 6.8). No *Penicillium* spp. were detected.

Table 6.7 **Number of dead seeds and unemerged seedlings harvested and number from which *F. graminearum* and other fungi were isolated.**

Seed lot	Number of dead seeds and unemerged seedlings	*F. graminearum*	*Fusarium* spp.	*Gleocladium roseum*	*Trichoderma* spp.	*Arthrobotrys* spp.	*Trichothecium* spp.	*Mucorales*	Black sclerotia (*Macrophomina* spp)
Dead seeds									
HNSD	9	3	4	9	5	4	1	5	3
HSD	10	1	7	10	4	1	6	4	3
LNSD	11	0	7	10	8	1	2	5	1
LSD	14	3	7	13	8	1	4	5	0
Unemerged									
HNSD	4	3	1	2	2	2	1	1	1
HSD	2	0	2	2	2	1	0	2	0
LNSD	1	0	1	1	1	0	0	1	0
LSD	4	2	2	4	3	3	0	4	0

HNSD = High infection non-surface disinfected
HSD = High infection surface disinfected
LNSD = Low infection non-surface disinfected
LSD = Low infection surface disinfected

Table 6.8 Percentage of live* seedlings that yielded *F. graminearum*, *Fusarium* spp. and mycopathogenic fungi after harvest from the field.

Seedling part	Seed lot	*F. graminearum*	*Fusarium* spp.	*Chaetomium* spp.	*Gleocladium roseum*	*Trichoderma*
Seed remains	HNSD	17	17	0	27	77
	HSD	23	14	3	20	87
	LNSD	13	16	0	17	77
	LSD	17	17	0	23	87
Main root	HNSD	20	0	57	3	13
	HSD	20	2	37	0	3
	LNSD	10	4	17	3	20
	LSD	7	4	33	3	7
Mesocotyl	HNSD	30	9	27	3	53
	HSD	33	10	13	0	23
	LNSD	27	8	13	7	43
	LSD	37	6	17	0	33
Coleoptile-node region	HNSD	60	4	40	0	13
	HSD	53	3	43	0	10
	LNSD	33	2	33	0	13
	LSD	37	8	43	0	23
Leaves	HNSD	20	4	57	0	3
	HSD	13	7	57	0	3
	LNSD	7	6	60	0	3
	LSD	17	5	53	0	3

HNSD = High infection non-surface disinfected
HSD = High infection surface disinfected
LNSD = Low infection non-surface disinfected
LSD = Low infection surface disinfected
* out of 30 randomly selected seedlings

Table 6.9 Percentage of live seedlings* that yielded field fungi and mucorales after harvest from the field.

Seedling part	Seed lot	*Altern-aria*	*Epicoc-cum*	*Drech-slera*	*Muco-rales*	*Pycnidial/ Ascomy-cetous* fungi	*Colleto-trichum*
Seed remains	HNSD	0	0	10	27	7	0
	HSD	0	0	13	37	0	0
	LNSD	0	0	10	47	0	0
	LSD	0	0	0	43	3	0
Main root	HNSD	3	0	0	7	47	0
	HSD	3	0	0	7	27	0
	LNSD	3	0	0	13	30	0
	LSD	3	0	0	13	33	0
Mesocotyl	HNSD	0	0	7	33	10	0
	HSD	0	0	3	23	10	0
	LNSD	0	0	3	23	13	0
	LSD	0	7	0	17	3	0
Coleoptile-node region	HNSD	10	7	7	20	23	3
	HSD	7	0	0	23	40	0
	LNSD	13	0	0	23	20	3
	LSD	7	0	3	17	33	0
Leaves	HNSD	20	33	3	30	40	0
	HSD	57	30	0	37	77	0
	LNSD	50	33	13	20	43	10
	LSD	57	50	7	33	63	3

HNSD = High infection non-surface disinfected
HSD = High infection surface disinfected
LNSD = Low infection non-surface disinfected
LSD = Low infection surface disinfected
* out of 30 randomly selected seedlings

F. graminearum was isolated from the main roots, mesocotyl, coleoptile-node region and leaf tissue, as well as seed remains, with the highest recovery being from the coleoptile-node region (37-60%) (Table 6.8). However, the recovery percentage from most parts of the plant, including the seed remains for the low infection seed lot, was greater than the initial seed-borne inoculum recorded, suggesting the presence of soil-borne inoculum. Although other *Fusarium* spp. were consistently isolated (Table 6.8), apparently none of these were *F. subglutinans*, but *F. subglutinans* was isolated from the two stunted seedlings (Plate 6.6 D).

6.10 DISCUSSION

F. graminearum was presumably transmitted from maize seeds to seedlings under field conditions, having been isolated from seedlings from the high infection seed lots, with the characteristic symptoms on the scutellum-mesocotyl attachment, coleoptile-node and main root regions as recorded in the glasshouse seedlings. *F. graminearum* was also isolated from the seed remains of seedlings in the high infection seed lots, implicating the seeds as a source of inoculum. However, *F. graminearum* was also isolated from a higher (up to 37%) percentage of seedlings from the low infection surface disinfected seeds than was detected in seed prior to sowing (1%), and from seedlings raised under glasshouse conditions (3%). These data strongly suggest that an additional source of inoculum was present in the soil. Since the experiment was conducted in a field previously not under maize, this indicates that under natural cropping systems, it may be difficult to find a field that is free of *F. graminearum* inoculum.

In the high infection seed lots, under field conditions, the highest recovery of *F. graminearum* was obtained from the seedling parts (coleoptile-node region) away from the seed remains (ie a lower percentage of seedlings yielding *F. graminearum* was recorded from the seed remains (17-23%), than from the coleoptile-node region (53-60%)). This was contrary to the situation in seedlings from the glasshouse, where the highest recovery of *F. graminearum* was obtained from the scutellum-mesocotyl region (53-50%) in proximity to the seed remains (43-57%), while only 13-17% of the seedlings yielded *F. graminearum* from the coleoptile-node region. An explanation for this situation could be that since the coleoptile-node region was in proximity with the soil surface, with the coleoptile protruding above the soil surface (Fig. 6.1, Part 1), and since *F. graminearum* survives best on or above the soil surface (Khonga and Sutton, 1988), then it was likely for the senescing coleoptiles to be infected by soil-borne inoculum and in turn infect the coleoptile-node region, including the tertiary roots. Where the soil-borne inoculum of *F. graminearum* arose was unclear, but the crop prior to the radish was a perennial ryegrass/white clover pasture, and previously to that wheat had been grown.

The fact that fewer (17-33%) of the seedlings yielded *F graminearum* from below ground parts under field conditions than under glasshouse conditions (43-57%) is likely to be because field conditions and seed exudates may have promoted heavy competition for substrate (seed remains, seed exudates and lesioned seedling tissues) between seed-borne *F. graminearum* and soil-borne mycoflora including *Chaetomium* spp., *Gleocladium roseum*, *Trichoderma* spp., *Fusarium* spp. and bacteria (Harman, 1983; Harman and Stzas, 1986). It is also likely that the soil conditions may have provided antagonistic activity against *F. graminearum*, since *Chaetomium globusum* Kunze ex Fr., *Penicillium* spp. and *Bacillius subtillis* (Cohon) Prazmowski, are certainly known to be antagonistic to *F. graminearum* under maize seedling infection situations (Kommedahl and Brock, 1954; Chang and Kommedahl, 1968; Kommedahl and Chang, 1975; Chaube and Singh, 1991).

Overall, the good performance of the seed lots under field conditions was surprising but could be attributed, among many factors (Isley, 1950; Shurtleff, 1980; Fiala, 1987) to:

(a) the relatively dry (especially during seed germination) soil and air conditions compared to the cold (8-13°C) and water logged soil conditions (Dickson, 1923; Shurtleff, 1980; Kruger, 1989) which favour infection.

(b) the initial high vigour of the seed lots which enabled them to emerge quickly and to "out compete" the effects of soil microflora.

(c) the potentially beneficial effects of some soil micro-organisms to *Fusarium* infected maize seed: as mentioned earlier (in this section) antagonistic soil fungi may have contributed to the occurrence of fewer seedlings yielding *F. graminearum* from roots or mesocotyls and the less severe lesioning of these parts under field conditions than in the glasshouse. For example, coating maize kernels with *Bacillus subtilis* or *Chaetomium globosum* and sowing them in soil emended with inoculum of *F. roseum* f. sp. *cerealis* "Graminearum" (*F. graminearum*) resulted in control of seedling blight at 18-

20°C in the greenhouse and in the field, increased seedling emergence, better root vigour, greater fresh plant and root dry weights and better seedling stands (Chang and Kommedahl, 1968).

The effects of *Gleocladium roseum* (Vakili, 1984, 1992) might explain the almost non-existence of *F. subglutinans* (a species closely related to *F. moniliforme* [*F. moniliforme* var subglutinans] - Nelson *et al.*, 1983)) and *Penicillium* spp. from dead seeds and seedlings harvested from the field, considering that the two species occurred in up to 96% or 57% of the seeds prior to sowing and having predominated in seedlings harvested from the glasshouse. *G. roseum* inoculated maize kernels reduced *F. moniliforme* infection in seedlings germinated on agar (Vakili, 1984) and decreased (100-70%) colonisation of seedlings by seed-borne fungi, including *Penicillium oxalicum* and *F. moniliforme* in laboratory, glasshouse and field studies (Vikili, 1992). This suggests that *G. roseum* may have been antagonistic to both *F. subglutinans* and/or *Penicillium* spp. under field conditions. Alternatively, the relative humidity in the field may have been insufficient for infection, especially foliar infection. Edwards (1935) stressed the necessity for high relative humidity for *F. subglutinans* to produce necrosed maize seedling leaf tissue, a condition also noted by Ocfemia and Mendiola (1932-1933) and Ramos (1933) for heart rot of abaca (banana [*Musa textilis* Nee]). This aspect of the pathogenicity of *F. subglutinans* needs further study.

The role and reason for the consistent occurrence of *Trichoderma* spp. was not clear, but the recovery from seed remains (77-87%), the mesocotyl-coleoptile-node region (10-53%) and leaves (3%) suggested a vertical increase in seedling contamination/infection with time, and the possibility of a protective role for the seedlings. Although *Trichoderma* spp. are known to be pathogens of maize seedlings (McFadden and Sutton, 1975; Fowler, 1985; Lipps and Deep, 1991) and are involved in the stalk rot disease complex (Christensen and Wilcoxson, 1966; Dodd, 1980), *T. harzianum* Rifai, *T. viride* and *T. frequent* have been reported to give biological control of *Fusarium* spp. and *Pythium* spp. in tomato, beet and mustard seedlings (Wright, 1956; Liu and Vaughan, 1965; Sivan *et al.*, 1987), while *T. harzianum* and

T. koningii Ondem have been reported to cause increased growth of maize plants (Windham *et al.*, 1989).

F. graminearum did not affect seed germination or seedling emergence under field conditions. Seedling emergence figures (86-91%) was comparable to the laboratory seed germination percentages (89-92%) prior to sowing, and also to glasshouse seedling emergence (81-94%). High infection seed lots had a higher seedling emergence rate than the low infection seed lots (Fig. 6.4), whereas under glasshouse conditions, surface disinfected (high or low infection) seed lots performed better than non-surface disinfected seed lots (Fig. 6.2). However, low infection seed lots had final seedling emergence percentage figures (86-89%) comparable to those of high infection seed lots (81-89%), suggesting that emerged seedlings were not affected by resident soil micro-organisms even though soil microflora may have influenced seedling vigour in the early stages of seedling emergence.

F. graminearum caused below ground level seedling discolouration and sometimes rotting, since it was isolated from discoloured mesocotyls, secondary and main roots, and from the tertiary and coleoptile-node region. But, because in most cases the discolouration was often much darker than was observed under glasshouse (aseptic) conditions, soil-borne mycoflora may also have participated in the discolouration and rotting of these seedling parts (Shurtleff, 1980; Fowler, 1985).

More seedlings yielded *F. graminearum* from the coleoptile-node (including tertiary roots) (37-60%) than other parts (7-37%), supporting the fact that discoloured/rotten coleoptile-node (including tertiary roots) region was infected by *F. graminearum* (Tables 6.6 and 6.8) either from seed-borne or soil-borne inoculum or both types of inocula. Since most seedlings were robust, they would have been likely to grow into mature plants, as maize seedlings produce supporting roots above the soil surface while the mesocotyl and primary roots shrivel away. However, since *F. graminearum* is generally associated with basal or crown stalk rot of maize plants (Roane, 1950; Koehler, 1960; Windels and Kommedahl, 1984; Kruger, 1989) it can be assumed that under conditions which favour the fungus (cultivar, moisture and

temperature, or the presence of insect pests such as stem borers - Christensen and Wilcoxson, 1966; Dodd, 1980; Shurtleff, 1980) seed-borne *F. graminearum* inoculum (alone or in combination with soil-borne inoculum) transmitted to seedlings can result in basal or crown stalk and leaf infection or contamination, and finally become a source of silk infection (on young maize cobs) by means of rain splash and wind blown rain water (Sutton, 1982), subsequently causing cob and seed infection or cob rot disease. However, it should be noted that the below ground infected parts, including the seed remains, dead seeds and seedlings, may not be a potential source of inoculum for the next crop. *F. graminearum* inoculum on maize straw, cobs or seeds buried to a depth of 10 cm has been found to not survive for more than one year (Khonga and Sutton, 1988).

Although various inconspicuous lesions in the form of spots and shot-holes were observed on the leaves of seedlings under field conditions, they were not similar to those caused by *F. subglutinans* under glasshouse conditions. Only two stunted seedlings (Plate 6.6) yielded *F. subglutinans* and they resembled the stunted seedlings observed under glasshouse conditions. In the literature there is a lack of descriptions of *F. graminearum* disease symptoms on the leaves of maize seedlings, except for the yellowing of leaves due to poor root system (Dickson, 1923). As under glasshouse conditions, *F. graminearum* appears not to have caused any apparent leaf disease symptoms in the field. The spots and shot-holes observed did not cause serious damage to the leaves or seedlings and were thought to have been caused by other field fungi (Table 6.9), most of them being known (Shurtleff, 1980; Fowler, 1985; Kruger, 1989) to be associated with maize, and causing leaf spots/seedling blight/root rot. Although the pycnidial and ascomycetous fungi were not studied in detail, the pycnidial fungi may have included *Ascochyta sorghii* Saccardo and *Kabatiella zeae* (perfect state = *Aurobasidium zeae*) which have been reported to cause leaf sports in maize in New Zealand (Fowler, 1985). Similarly, *Colletotrichum graminicola* (Cesati) Wilson and *Dreschlera maydis* (Nisikado) Subrananian and Jain have been reported as causing leaf spots and leaf blight, respectively in New Zealand (Fowler, 1985). That the seedling leaf spots did not cause significant damage under field

conditions is not surprising since maize leaf spot disease in New Zealand is considered to be of minor importance (Fowler, 1985).

The reason for the heavy occurrence of mucorales (*Mucor* spp.) was not clear but *Mucor* spp. are prevalent in the soil (Domsch and Gams, 1972; Domsch *et al.*, 1980) and gain access into the seeds and seedling tissue saprophytically through damage caused by pathogenic fungi (eg *F. graminearum*) and insects. Similarly, the roles of *Fusarium* spp. were unclear. These fungi are also known to be resident in soil as saprophytes or pathogens of seedlings and plants, or saprophytes on debris (Domsch and Gams, 1972; Domsch *et al.*, 1980; Burgess, 1981). Further study of the role of soil-borne microflora in the presence of seed-borne *F. graminearum* and *F. subglutinans* is required.

PART 3 : SEED AND SEEDLING INOCULATION STUDY: KOCH'S POSTULATES VERIFICATION

6.11 INTRODUCTION

In the glasshouse study of the transmission of *F. graminearum* from maize seeds to seedlings (Part 1), *F. graminearum* and *F. subglutinans* were re-isolated from diseased maize seedlings raised from seeds infected with seed-borne fungi. *F. graminearum* caused pre- and post-emergence damping off, seed, root and mesocotyl rot while *F. subglutinans* was more consistently implicated as the causal agent of above-ground seedling blight. Since reports in the literature have not verified the seed transmission of these fungi, the present study was undertaken to more clearly determine this.

The ability of an organism to cause disease is designated as its pathogenicity (Parris, 1970; Jones and Clifford, 1983). Proof of pathogenicity is achieved by following the logical rules of proof which were enunciated by Robert Koch in 1882. These rules are called Koch's postulates or Koch's rules of proof of pathogenicity (Parris, 1970). The steps followed to verify Koch's postulates have been stated by various authors (Parris, 1970; Agrios, 1978; Chaube and Singh, 1991). According to Agrios (1978):

1. The pathogen must be found associated with the disease in all of the diseased plants examined.

2. The pathogen must be isolated and grown in pure culture on nutrient media and its characteristics described (non-obligate parasites).

3. The pathogen from pure cultures must be inoculated onto healthy plants of the same species or variety on which the disease appears, and must produce the same disease on inoculated plants.

4. The pathogen must be isolated in pure culture again and its characteristics must be exactly as observed in step 2 (above).

The principles of Koch's Postulates and steps taken to verify these postulates are summarised in Fig. 6.5.

6.12 OBJECTIVES

The objective of the study was to use Koch's Postulates to verify the pathogenicity of *F. graminearum* and *F. subglutinans* on maize seedlings.

Two related experiments, namely, inoculation of the above ground seedling parts (leaves and coleoptiles) and inoculation of disease free seeds were carried out. In both cases, fungal spore suspensions were used.

6.13 MATERIALS AND METHODS

Seeds of cultivar P3591 which were harvested in June 1990 and had subsequently been stored for 12 months at 25°C in 1991 with a resultant loss of viability of seed-borne fungal inoculum and had then been kept at 5°C were used. This seed was free from *F. graminearum* and *F. subglutinans* when tested on Malt Agar.

Seedlings were raised in sterile sand in 4-hole root-trainers as described in Part 1. The sand was saturated with sterile water and aseptically placed in root-trainers. A total of 88 surface disinfected (1% sodium hypochlorite for 2 minutes and rinsed for 5 minutes under running tap water) seeds were sown in root-trainer sand beds, one seed per hole, and each bed was placed in a large plastic bag to prevent contamination and sand drying. Sown sand beds were transferred to the glasshouse. Temperature and seedling emergence were recorded daily.

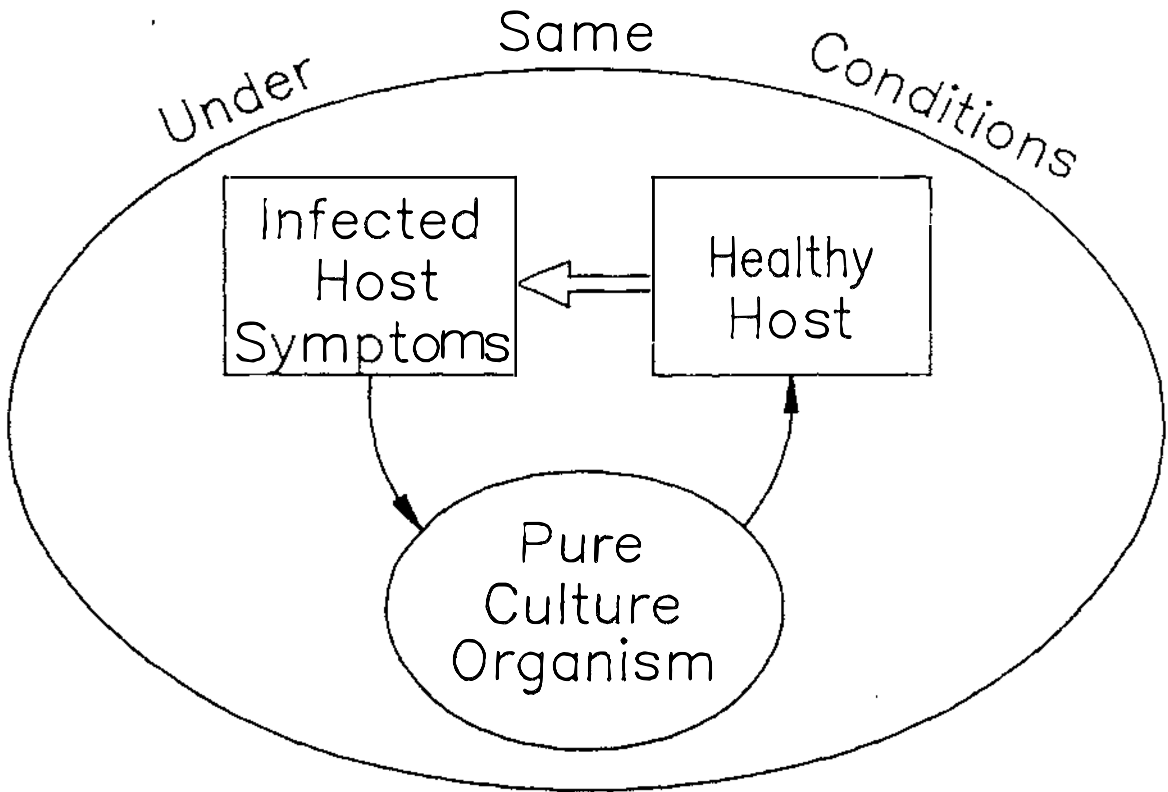


Figure 6.5 Koch's postulates: proof of pathogenicity – diagram showing the principles of/or steps taken to verify Koch's postulates

Seedlings with mesocotyl lesions obtained in the *F. graminearum* seed-borne inoculum transmission study (Part 1) were used as the inoculum source. Seedling parts with lesioned tissue were surface disinfected (1% sodium hypochlorite, 1 minute), aseptically air dried and plated on carnation leaf agar (CLA). Cultures showing the presence of *F. graminearum* and the absence of *F. subglutinans* were selected as the source of *F. graminearum*. Conversely, cultures in which *F. graminearum* was absent but having *F. subglutinans* was present were selected for the latter source.

F. graminearum and *F. subglutinans* were purified by the single germinated spore subculturing method as described by Burgess *et al.* (1988). Single germinated macroconidia were transferred to CLA and incubated under NUV light at 25°C/20°C day and night, respectively, for 14 days during which *F. graminearum* formed perithecia. Spore microscope slide preparations were also made to confirm identity. The conidial morphology (macroconidia for *F. graminearum*, and microconidia and polyphialides of *F. subglutinans*) (Plates 6.7 and 6.8) were compared with those of cultures identified by IMI (Chapter 3).

6.13.1 Inoculum preparation

Three to four carnation leaf pieces from four culture plates each of *F. graminearum* and *F. subglutinans* were used to prepare fungal spore suspensions. These leaf pieces were aseptically placed in 10 ml sterile water containing 0.1% Tween 20 surfactant in a sterile McCartney bottle. The bottle was shaken to make the suspension. Using a sterile pipette and avoiding the leaf pieces, the suspension was pipetted into another bottle and made up to 20 ml with sterile water containing 0.1% Tween 20 surfactant. The spore suspension concentration was estimated (with a haemocytometer) to contain approximately 5 x 10^4 spores per ml of water of macroconidia (Jones and Clifford, 1983), but for *F. subglutinans*, microconidia in the suspension concentration were not estimated. Both *F. graminearum* and *F. subglutinans* suspensions also contained some mycelial fragments.

Plate 6.7 *F. graminearum* pure culture (A) and macroconidia (B) before inoculation (B x 750).

Plate 6.8 *F. subglutinans* before inoculation. A, pure culture on CLA; B, macroconidia and polyphialides (see arrow); C, macroconidia and microconidia (see arrow); (B & C x 470).

6.13.2 Seedling inoculation

6.13.2.1 Above ground parts seedling inoculation

Three root-trainers each containing four 3-day old seedlings (first and second leaves still rolled up) and three more root-trainers containing only wet (100% WHC) sterile sand (to increase the relative humidity) were aseptically (under the Laminar Flow cabinet) transferred to a sterile metal frame. The above ground seedling parts (leaves and coleoptiles) were then inoculated with spore suspension of either *F. graminearum*, *F. subglutinans* or sterile water (control). In each treatment seedlings were inoculated using a 10 ml spore suspension by brushing the leaves, leaf sheaths and coleoptiles with a new, sterilised (70% ethanol) paint brush (No. 4 TVB Goldmaster Lettering 20381 Synthetic England) following dipping it into the spore suspension. A separate brush was used for each treatment. Some suspension was also dropped into the young leaf roll and between the leaf sheath and coleoptile. The same procedure was used to inoculate the control seedlings using sterile water. After inoculation, each treatment was enclosed in a large sterile plastic bag supported by a metal frame (as described in Part 1 of this study) and returned to the glasshouse where disease development was monitored and the temperature of the room recorded daily using a minimum and maximum thermometer.

6.13.2.2 Seed inoculation

For each treatment, 12 randomly selected surface disinfected (1% sodium hypochlorite) seeds were used for each fungus inoculation. Seeds were placed in a 50 ml sterile beaker. To each beaker 10 ml spore suspension or sterile water (control) was added until seeds were just covered and the beaker was swirled to ensure even coating with spores. After inoculation, each beaker was covered with sterile aluminium foil and placed in the dark in an incubator at 20°C to allow seed imbibition and spore germination and possibly fungal penetration. At intervals, the beakers were gently swirled to ensure aeration in the suspension. Approximately 24 hours after soaking, the seeds were aseptically sown in sterile wet (100% WHC) sand. The sand bed for each treatment was placed in a large sterile plastic bag supported with a metal frame as described in Part 1 of this study and transferred to

the glasshouse. Temperature and seedling emergence were recorded daily and disease development was monitored.

6.13.3 Seedling harvesting and pathogen re-isolation

Seedlings were harvested 21 days after inoculation (in the case of above ground seedling part inoculation) or emergence. The root-trainers were opened and the seedlings were carefully hand lifted out of the sand to avoid root damage. They were examined visually or under a dissecting microscope, on the leaves, coleoptiles, mesocotyls, seed remains and roots for the presence of fungal growth, were then washed under running tap water and re-examined for the presence of lesions or discolouration.

Ten seedlings from each fungus inoculation treatment and from the control were surface disinfected with sodium hypochlorite (1%) for 1 minute and rinsed in 3 changes of sterile water for 5 minutes. Excess water was allowed to dry under sterile air on hand towels for at least one hour. Seedlings were aseptically dissected into different parts (Fig. 6.1) but each leaf was studied separately. These parts were plated on CLA, and incubated at 20/25°C under NUV light, with 12 hours darkness and 12 hours light. Ten and fourteen days after plating the occurrence of *F. graminearum* and *F. subglutinans* was recorded. *F. graminearum* and *F. subglutinans* were subcultured by the single germinated spore technique (Burgess *et al.*, 1988) onto CLA. *F. graminearum* was identified by the formation of perithecia of *Gibberella zeae* and macroconidia morphology, while *F. subglutinans* was identified using colony morphology, macroconidia, microconidia and polyphialides.

6.14 RESULTS

6.14.1 Initial Seed Quality

Surface disinfection did not affect seed quality (seed germination capacity or vigour) of the seeds used in the study (Table 6.10). Laboratory seed germination was

high (93%). After surface disinfection, the seeds were free of *F. graminearum*, *F. subglutinans* and *F. poae.*

Table 6.10 Initial quality of seed used in the seed and seedling inoculation study.

Laboratory germination	93%
Seed vigour : Seedling shoot length	13.3 cm
Seedling dry weight	250 mg
Seed health - seed-borne infection:	
F. graminearum	0%
F. subglutinans	0%
F. poae	0%

6.14.2 Temperature conditions during the study

Glasshouse temperature ranged from 10-22.5°C with a daily mean ranging from 15-18°C (Appendix 6.3). This was considered optimum for *F. graminearum* seedling infection and disease development (Dickson, 1923) and was within the temperature range for growth of *F. subglutinans* (2-38°C; Lacey, 1989).

6.14.3 Seedling emergence

Seedling emergence occurred 6 days after sowing (7 days after inoculation) in seeds which were inoculated by soaking them in the spore suspension and after 7 days for those which were directly sown. Seedling emergence was high, being 100% for the seeds soaked in *F. graminearum* spore suspension and sterile water (control) and 90% for those soaked in the spore suspension of *F. subglutinans*. Seedling emergence was completed in 3-6 days, most of it occurring in two to four days. All seedlings which emerged survived.

6.14.4 Disease symptoms

Leaves of seedlings inoculated by brushing with spore suspension of *F. graminearum* or *F. subglutinans* developed mycelial growth in the upper half of the leaves (Plate 6.9). Mycelial growth was first visible 9 days after inoculation on the 1st leaf and progressed to the 2nd and 3rd leaf, but the 4th leaf (the youngest) was free of fungal growth (Table 6.11). *F. subglutinans* growth appeared on coleoptiles about 14 days after inoculation, and as on the leaves, had a whitish cream and powdery appearance (Plate 6.9 C). *F graminearum* appeared as a fluffy pale cream to white mycelium (Plate 6.9 B). About 18 days after emergence, seedlings became somewhat yellow, and some leaves were purple in colour, possibly as a sign of phosphate deficiency (Shurtleff, 1980). At the time of harvest all coleoptiles and most 1st leaves were senesced and dry. Seedlings from the control did not show fungal growth.

Seedlings inoculated by brushing the spore suspension onto the leaves and coleoptile did not develop discolouration on the parts below the sand level and fungal growth was not visually found on these parts. The mesocotyl, root and node tissues looked healthy. This was also true of seedlings from the control.

In seedlings from seeds inoculated by soaking in fungal spore suspension, general leaf yellowing occurred and increased by 14-15 days after seedling emergence. Seedlings from seeds inoculated with *F. subglutinans* appeared more yellow than those inoculated with *F. graminearum*. Around 10-14 days after emergence fungal growth appeared on the coleoptiles of seedlings from seeds inoculated with *F. graminearum* (2 seedlings) or *F. subglutinans* (8 seedlings) and leaves of seedlings infected with *F. subglutinans* were severely blighted (Plate 6.10a). Seedlings from the control showed no fungal growth. Generally all the seedlings appeared strong enough for continued growth.

Plate 6.9 *F. graminearum* (A & B) leaf infection and *F. subglutinans* (C) leaf and coleoptile infection following inoculation of these parts with respective spore suspensions; B, close up of A showing fluffy mycelial growth, compared to scanty powdery mycelial growth on coleoptile and with sporodochia on leaves (C).

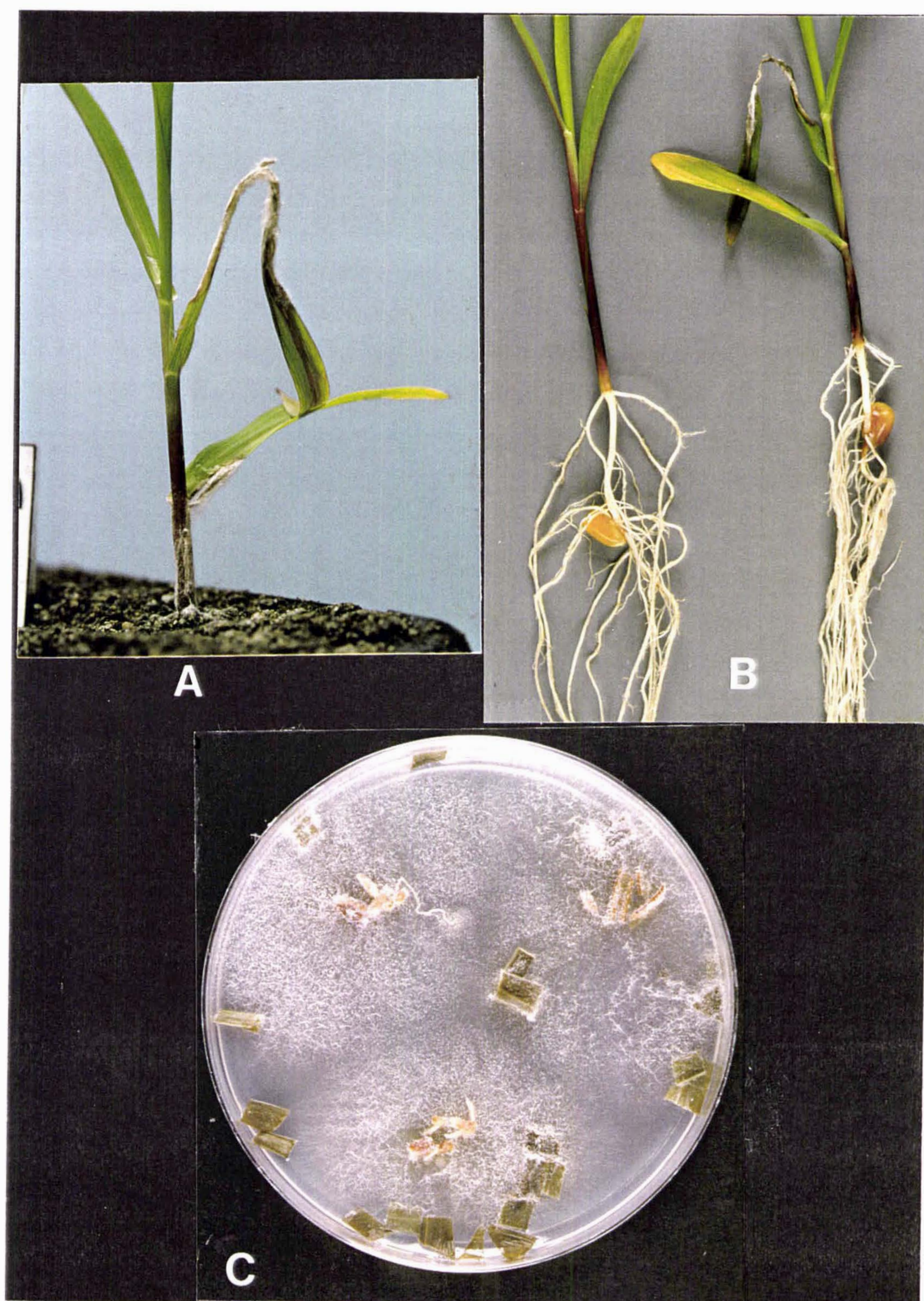


Plate 6.10a *F. subglutinans*: A & B, leaf and coleoptile infection following soaking (inoculation) seeds in a spore suspension; C, *F. subglutinans* growing out of the infected seedling tissue on CLA.

Only seedlings from seeds inoculated by soaking in *F. graminearum* spore suspension had discoloured and/or rotting in the scutellum-mesocotyl and scutellum-main root attachment region, including the secondary roots, and in the coleoptile-node area, including tertiary roots (Plate 6.10b, Table 6.11). Characteristic water soaked and brown to reddish brown lesions with sunken, canoe-like centres as signs of *F. graminearum* infection were observed, especially on the mesocotyl (Plate 6.10b). The characteristic tendency of these depressed spots to form in streaks on the mesocotyls was also observed (Plate 6.10b).

Table 6.11 Number of seedlings visually showing lesions and external *F. graminearum* and *F. subglutinans* growth and from which the species were re-isolated on CLA.

	F. graminearum		*F. subglutinans*	
	Visually showing lesions and external *F. graminearum* growth	From which *F. graminearum* was re-isolated on CLA*	Visually showing lesions and external *F. subglutinans* growth	From which *F. subglutinans* was re-isolated on CLA*
Seeds inoculated by brushing leaves with fungal spore suspension				
4th leaf	0	0	0	0
3rd leaf	2	5	0	3
2nd leaf	6	8	8	9
1st leaf	6	7	11	9
Coleoptile	12	7	12	10
Node	0	4	0	10
Scutellum-mesocotyl	0	0	0	8
Scutellum-main root	0	-**	0	-
Seed remains	0	-	0	-
Seeds inoculated by soaking seeds in spore suspension prior to sowing				
4th leaf	0	0	0	2
3rd leaf	0	0	0	1
2nd leaf	0	0	1	2
1st leaf	0	1	1	3
Coleoptile	2	9	8	10
Node	4	10	8	10
Scutellum-mesocotyl	9	8	10***	10
Scutellum-main root	7	10	10***	10
Seed remains	7	10	10	10

* = Only 10 seedlings tested
** = not tested on CLA
*** = Fungal growth only

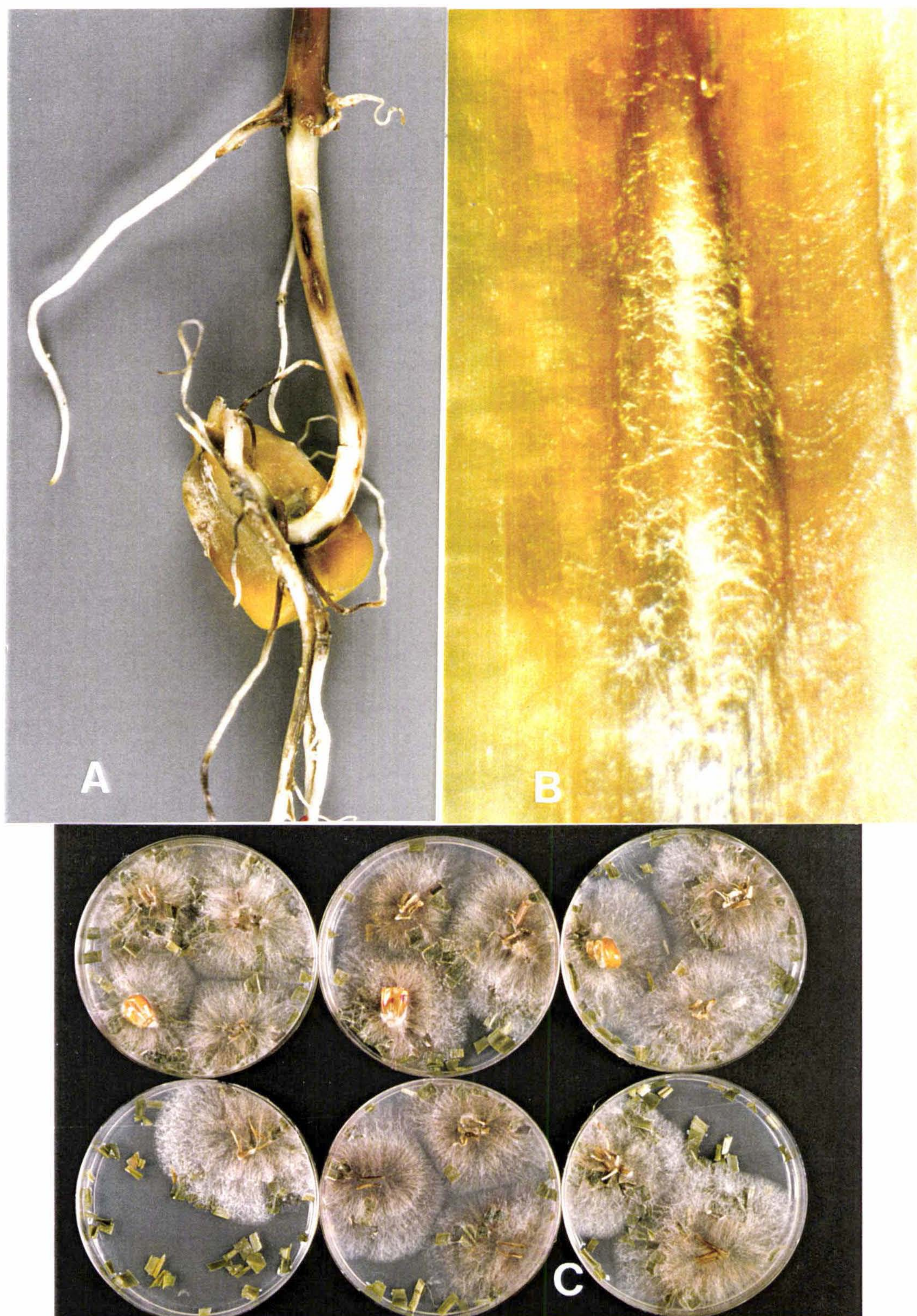


Plate 6.10b *F. graminearum*: A, characteristic mesocotyl, root and node lesioning/disease symptoms following soaking of seeds in spore suspension; B, close up of sunken centre; C, *F. graminearum* growing out of the infected seedling tissue on CLA; (B x 10).

6.14.5 Re-isolation of pathogens

F. graminearum and *F. subglutinans* were re-isolated from all the seedlings inoculated either by brushing the leaves and coleoptiles with fungal spore suspension or by soaking seeds in the spore suspension. These fungi were not re-isolated from the control. In seedlings inoculated by brushing seedlings with the inoculum, neither *F. graminearum* nor *F. subglutinans* were re-isolated from the 4th leaf, but in seedlings from seeds soaked in inoculum, *F. subglutinans* was isolated from all parts of the seedling, including the 1st, 2nd, 3rd and 4th leaves, while *F. graminearum* was restricted to the first leaf (Table 6.11).

The pure culture morphology of both *F. graminearum* (Plate 6.11) and *F. subglutinans* (Plate 6.12) from seedlings brushed with the inoculum or seeds soaked in the inoculum, on CLA, was identical to that before inoculation. The macroconidia of *F. graminearum* and of *F. subglutinans*, and the microconidia as well as the polyphialides of *F. subglutinans* were similar to those observed in the cultures used for inoculation (Plates 6.11 and 6.12). *F. graminearum* cultures formed perithecia of *G. zeae* (Plate 6.11).

6.15 CONCLUSION

The results of this study proved Koch's Postulates for the pathogenicity of *F. graminearum* and *F. subglutinans* isolated from maize seedlings. Since the seedlings from which the inocula of *F. graminearum* and of *F. sublgutinans* had been infected from seed-borne inoculum under aseptic conuditions in the glasshouse (Neergaard, 1979), it is therefore concluded that *F. graminearum* and *F. subglutinans* are seed transmited from maize seed to seedlings.

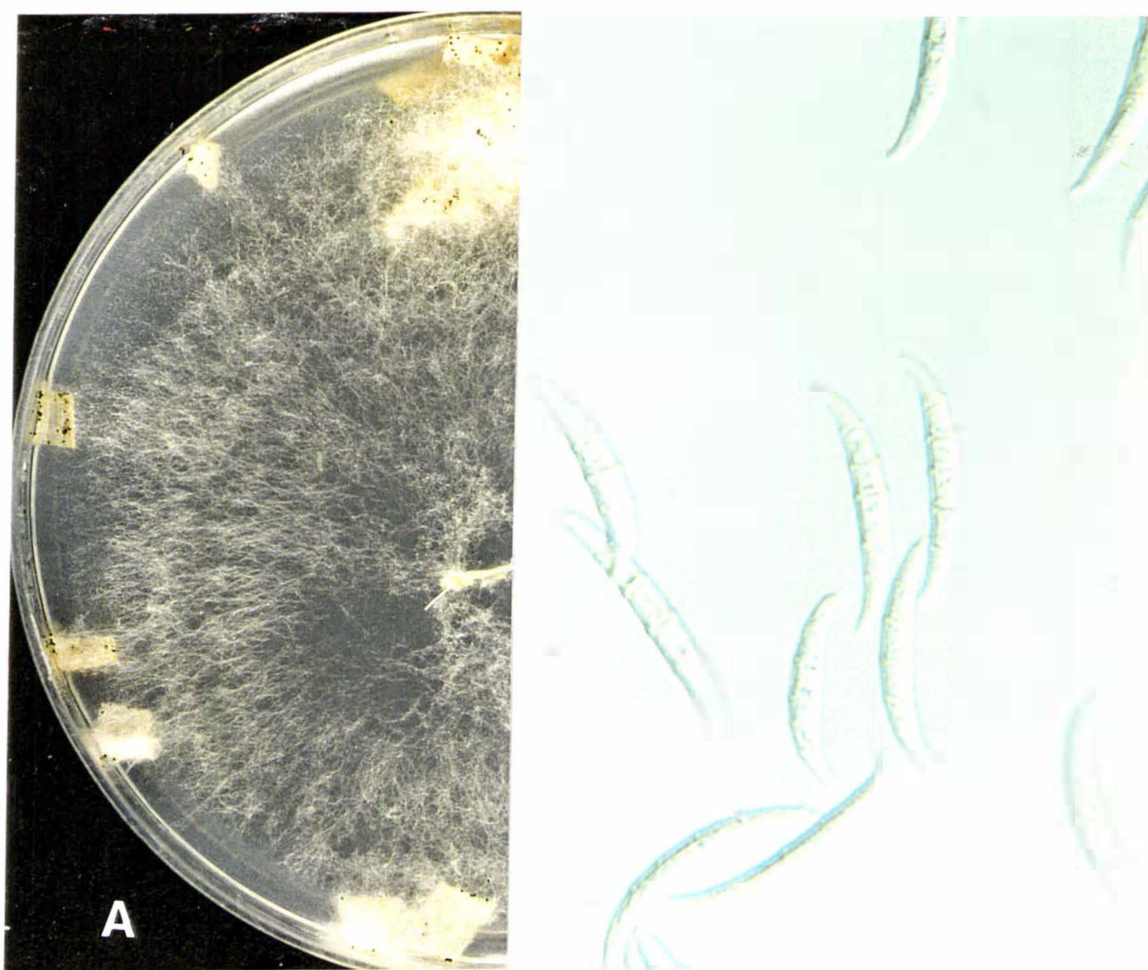


Plate 6.11 *F. graminearum*: A, pure culture on CLA after re-isolation from inoculated maize seedling; B, macroconidia from the pure culture (B x 470).

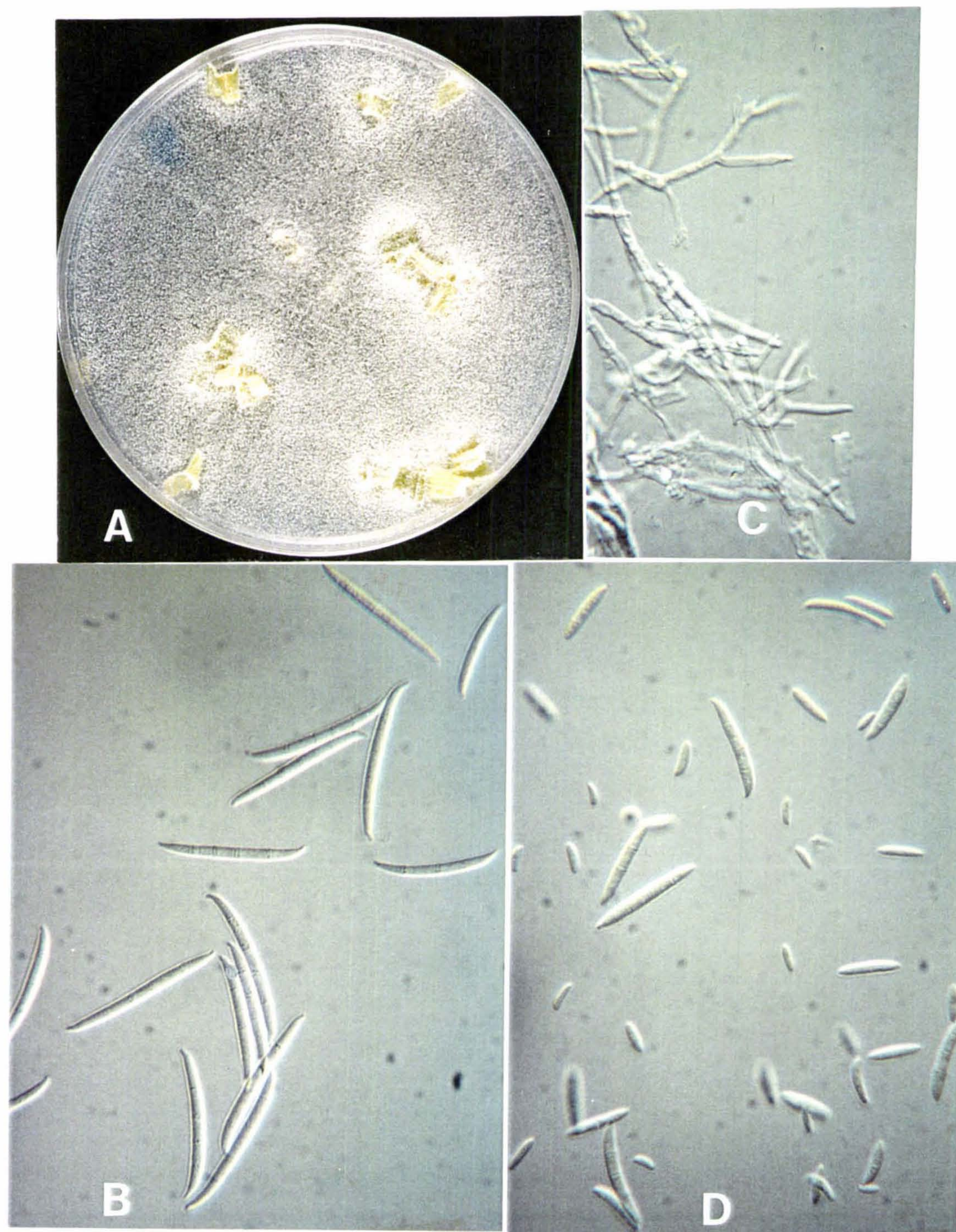


Plate 6.12 *F. subglutinans*: A, pure culture after re-isolation from inoculated maize seedling; B, macroconidia from pure culture; C, polyphialides; D, microconidia and macroconidia (B, C & D x 470).

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 SEED HARVEST TIMING

This study of maize grown in two consecutive seasons at Massey University in the Manawatu has shown that delaying harvest leads to an increasing incidence of *Fusarium* cob moulding and seed infection, and consequently an increase of mycotoxin contamination, particularly by αZearalenol and Nivalenol, which are known to be very toxic to animals at low levels (Halger *et al.*, 1979; Ehrlich, 1989; Lauren, 1994). *F. graminearum* was found to be the *Fusarium* species most commly recovered from seed (mean percentage of up to 72% or 30% in 1990 and 1991, respectively), while other species including *F. subglutinans*, *F. poae*, *F. crookwellense* and *F. culmorum* occurred at much lower levels (mean percentage of less than 10%). These results were similar to those subsequently reported by Cox *et al.* (1994) for the 1992/93 season, which was also characterised by cool temperatures and delayed harvesting. What is yet to be determined is the extent of this problem on a national scale - previous work has tended to concentrate on surveys of mycotoxin occurrence (eg Lauren *et al.*, 1991), and attempts to relate *Fusarium* infection and mycotoxin levels are yet to be successful (Cox *et al.*, 1994).

Growers are recommended to harvest maize at around 22-25% seed moisture content (Hardacre *et al.*, 1991; Sayer, 1991), but this can pose problems. Firstly, delaying harvest to allow grain to dry down naturally to this moisture content may take considerable time, particularly in a wet/cold season, with the result that *Fusarium* infection levels increase. Secondly, however, harvesting at higher seed moisture contents can result in increased drying costs, more seed damage, and difficulties with harvester access onto wet fields. It is evident that some form of control of *Fusarium* spp. on maturing maize is required. Whether fungicide application is possible (climate and ground conditions), effective (can all *Fusarium*

spp. be controlled) and importantly cost effective, requires investigating. It may be that fungicide spraying needs to become one aspect of a integrated control package which also involves seed treatment, crop rotation and hybrid resistance/tolerance and/or with early maturing characteristics. Since at harvest cobs often show obvious infection (mouldy cobs), one of the simplest forms of control would be hand sorting of mouldy cobs and breaking off the mouldy tips. Early harvesting just after physiological maturity would greatly reduce cob/seed infection. These measures however are not practical.

7.2 IDENTIFICATION OF SEED-BORNE *FUSARIUM* SPP.

The results of this study suggest that routine macroscopic (visual) identification of *Fusarium* spp. on Malt Agar (MA) is possible but the differentiation of *F. crookwellense* seed-culture colony morphology from the 'red centre' type of *F. graminearum* is difficult. These results provide for the first time, descriptions of seed-culture colonies of *F. graminearum*, *F. culmorum*, *F. subglutinans*, *F. poae* and of *F. graminearum*/*F. crookwellense* on MA, and of *F. graminearum* and *F. culmorum* on Malt Extract Agar (MEA). *F. decemcellulare* and *F. sambucinum* were also identified on the MA medium. Identification and description of *Fusarium* was made possible by plating from 3-5 seeds per agar plate, although one seed per plate was preferred for seed lots with high *F. graminearum*, *F. crookwellense* or *F. culmorum* infection levels. This is important because inter-colony interference was found to adversely affect aerial mycelium, an essential character in the macroscopic identification of *Fusarium* spp.

Descriptions of *Fusarium* spp. based on seed-culture colony colour and pigment formed in agar are often difficult due to lack of appropriate colour/shades despite the availability of colour charts (Rayner, 1970; Kornerup, 1978). Macroscopic identification of *F. graminearum* on Malt Extract Agar (MEA) was also difficult because, unlike on MA, seed-culture colony morphology was distorted, making it difficult to differentiate different cultural types. However, the occurrence

of different *F. graminearum* seed-culture colony types on MA creates an additional confusion in this species, since these types may include both Groups 1 and 2 of Francis and Burgess (1977). There is need to further examine the cause of the different morphological types of *F. graminearum* on MA. Further, it is important for manufacturers and literature to clearly differentiate Malt Agar from Malt Extract Agar (Difco Laboratories, 1972; CAB, 1983; Gibco BRL, 1993) as both contain malt extract, but latter has peptone as an additive.

The conventional (Nelson *et al.*, 1983; Burgess *et al.*, 1988) identification of *Fusarium* spp. in pure cultures initiated from single spores on PDA and/or CLA is an inappropriate technique for identification of seed-borne *Fusarium* spp. because of the amount of work involved in laboratory seed health testing which requires the examination of up to 400 seeds per lot (ISTA, 1993). Moreover, on PDA pure cultures, *F. graminearum*, *F. crookwellense* and *F. culmorum* cannot be differentiated.

CLA is a useful medium for the identification of all *Fusarium* spp. by slide preparation techniques (for conidia or conidiogenous cells). Burgess *et al.* (1988) described this medium, in combination with NUV light illumination, as being very convenient for identification of *F. graminearum* because of its ability to favour the formation of perithecia of *Gibberella zeae*. The use of CLA is important not only in correctly identifying *F. graminearum* but also in establishing the identify of the "Groups" (Francis and Burgess, 1977) of this species since *F. graminearum* Group 1 is a pathogen of small grain cereals (wheat and barley etc) but Group 2 (*Gibberella zeae*) is a pathogen of maize (Nelson *et al.*, 1983; Burgess *et al.*, 1988). For example, CLA was found to be a convenient and accurate medium for determining and comparing seed-borne inoculum levels in maize and for assessing seed to seedling transmission rate of *F. graminearum* by direct plating of seeds and seedling pieces on this agar after surface disinfection.

There is no prescribed method for identifying *Fusarium* spp. in seed health testing except the blotter/pentachloronitrobenzene (PCNB) procedure for *Fusarium*

moniliforme var *subglutinans* Wollenw. & Reink. (*F. subglutinans*) on *Pinus taeda* and *P. elliottii* (ISTA, 1993). It is therefore recommended that the use of carnation leaf agar (CLA) for screening seed lots for *Fusarium* spp., especially *F. graminearum*, in seed health testing, be considered.

7.3 *FUSARIUM* EFFECT ON SEED QUALITY AND *FUSARIUM* CONTROL

Although *F. graminearum* was the most common *Fusarium* spp. detected, it was the easiest to eliminate from seeds by storage at 30°C or 25°C and 14% seed moisture content, for 3 months in sealed containers, regardless of cultivar, harvest date or infection level. *F. subglutinans* and *F. poae*, both of which had low levels of infection (0-8% and 1-18%, respectively) were more difficult to eliminate, requiring up to 9 months at 30°C and 14% seed moisture content before dying out in the seed. The cause of this was thought to be microconidial resistance to desiccation (Liddell and Burgess, 1985) (microconidia being absent in *F. graminearum* - Burgess *et al.*, 1988). However, the cause of persistence of *F. subglutinans* and *F. poae* in storage needs further work, particularly since the loss of viability of *Fusarium* spp. was much slower in infected seed lots stored at 10% seed moisture content at high temperatures.

The survival of *Fusarium* spp. under low temperature storage (5-10°C) is in agreement with observations that field fungi (including *Fusarium* spp.) survive better at low temperatures (Mathur, S.B., pers. comm.; Neergaard, 1979; Sauer *et al.*, 1992) and especially at 4-5°C (Lacey and Magan, 1991). This poses a problem since seed storage for preservation of viability and vigour requires low temperature storage (Justice and Bass, 1979; Copeland and McDonald, 1985).

Sealed storage showed greater advantage for control of *Fusarium* spp., (especially *F. graminearum*) than open storage, presumably because of better control of seed moisture content and gaseous components, especially carbon dioxide, produced during respiration. Both seed moisture content and gas levels fluctuate in

open storage. The presence of carbon dioxide was considered an additional useful control factor for *Fusarium* spp. in sealed storage but this needs further work. Control of *F. graminearum* with seed storage at high temperatures and high moisture content appear promising as it is cheap, and unlike the use of fungicide, is environmentally friendly. However, there is a danger of hazards to seed quality, as germination in open storage can be relatively quickly lost though storage of seed at 14% seed moisture content under high temperatures.

Fusarium spp. did not affect the germination and vigour of maize seeds as both early (April and May) and late (June and July) harvest, dried seeds had high (86-99%) germination percentages and low (1.6-3.6 μS cm^{-1}/gm^{-1}) leachate electrical conductivity, despite high *Fusarium* infection levels in late harvested seeds. Most authors (Marasas *et al.*, 1979, 1981; Sutton, 1982; Atlin *et al.*, 1983; Hart *et al.*, 1984; Hunter *et al.*, 1986; Reid *et al.*, 1992b, 1993; Logrieco *et al.*, 1993) have emphasised mycotoxin production as the significant problem in maize seed infected by *F. graminearum* and *F. subglutinans*. The persistence of mycotoxins in seeds after 12 months storage at 25°C (open storage) emphasises their greater significance than *Fusarium* seed infection.

7.4 TRANSMISSION OF SEED-BORNE INOCULUM OF *F. graminearum* AND OF *F. subglutinans*

The transmission of seed-borne inoculum of *F. graminearum* and *F. subglutinans* from maize seeds to seedlings was demonstrated (including verification of pathogenicity of the inocula of these *Fusarium* species to maize seeds and to seedlings by fulfilling Koch's Postulates) under aseptic glasshouse conditions but was not demonstrated under field conditions. This is considered the first verified report of the seed transmission of these species.

Under glasshouse conditions, the transmission rate of *F. graminearum* was equal to the seed-borne inoculum level suggesting that under favourable environmental

conditions high infection seed lots would produce high percentages of infected seedlings and perhaps cause seedling blight (Leach, 1979b). But, for *F. subglutinans*, despite the fact that most of the inoculum was surface-borne, the transmission rate was higher than the originally detected seed-borne inoculum level. This was thought to be due to seed to seed or seed to sand contamination (in the case of non-surface disinfected seeds), or to ineffective seed disinfection of seed tip-cap derived inoculum. Other contributing factors could have been seedling to seedling (leaf contact) or water (condensation) drop/splash onto seedlings under the plastic bag cover in the glasshouse. Most of these problems are thought to have occurred due to the abundant sporulation, especially production of *F. subglutinans* microconidia, especially on the seed surface, on coleoptiles above the sand level shortly (about 7 days) after seedling emergence and on leaves (Plate 6.3). Since *F. subglutinans* readily sporulated on the above ground parts of the seedlings, especially on the coleoptiles, this suggests that under favourable environmental conditions in the field, especially water splash and/or insects, *F subglutinans* would readily be spread from seedling to seedling, but this was not demonstrated in the field.

Under glasshouse conditions, *F. graminearum* and *F. subglutinans* had little effect on seedling emergence and survival but *F. graminearum* was associated with a high percentage (up to 70%) of seedlings with scutellum-mesocotyl and scutellum-main root lesioning, in some cases lesioning being severe, resulting in weakened root system and perhaps yellowing of seedlings.

F. subglutinans had devastating effects on seedlings as it was associated with seedling and leaf blight, shoot wilt, seedling stunting, node damage and coleoptile infection, but unlike *F. graminearum*, the root/mesocotyl region was generally unaffected. Coleoptile infection possibly originated from seed-surface/seed-sand contamination. But since surface disinfection almost removed *F. subglutinans* from the seeds, one would tend not to consider its presence as significant. It is therefore recommended that detection of *F. subglutinans* in seed lots be carried out both on surface disinfected and non-surface disinfected. The deep freezing blotter method proved appropriate for its detection.

Results from the study of seed to seedling transmission of *F. graminearum* under field conditions was inconclusive presumably because of the presence of soil-borne inoculum. Nevertheless, seedlings raised from infected seed showed lesioning characteristics of *F. graminearum*, particularly on seedling parts closest to the seed remains. This suggests that although seed to seedling transmission of *F. graminearum* can be clearly demonstrated, there is likely to be an overriding effect from soil-borne inoculum surviving in the soil from previous crops of maize and other cereals. *F. subglutinans* seedling infection under field conditions was rare. As for the glasshouse, seedling emergence was high and all emerged seedlings survived. This high performance was attributed to several factors, namely warm temperature, relatively dry weather conditions, absence of seed damage (Isley, 1950; Fiala, 1987) and possibly antagonistic/suppressive effects of micro-organisms in the soil (Chang and Kommedahl, 1968; Vakili, 1984, 1992).

The significance and transmission of seed-borne *F. graminearum* and *F. subglutinans* under field conditions needs further study.

7.5 SOURCES OF *FUSARIUM* INOCULUM AND THEIR EFFECT ON DISEASE DEVELOPMENT

Disease free seed is desirable for sowing. *Fusarium* spp., especially *F. graminearum*, *F. subglutinans*, *F. crookwellense* and *F. culmorum* are all maize pathogens causing cob rot, stalk rot, seedling blight and contamination of seed/grain by mycotoxins (Shurtleff, 1980; McGee, 1988; Kruger, 1989). The results of this study are in agreement with those of other workers who have shown that delayed harvest increases the percentage of seeds infected with *Fusarium* spp., especially *F. graminearum* and that seeds harvested in May (normal harvest date) could also be heavily (18-45%) infected by *F. graminearum*. This creates a problem on source of seed for sowing. While chemical seed treatment may be effective in controlling seed-borne *Fusarium* spp. (Kommedahl and Windels, 1986), in New Zealand only two seed treatment fungicides, namely captan (Orthocide 80W) and carboxin plus thiram

(Vitaflo 200) are currently registered for maize (Walton and Walton, 1993) and only the latter provides some control of *Fusarium* spp. (Hampton *et al.*, 1994).

For epidemiology of maize cob rot caused by *Fusarium* spp, especially *F. graminearum*, *F. moniliforme* and *F. subglutinans* (Shurtleff, 1980; Kruger, 1989), soil, seed, infected crops, and crop residues are all thought to be a source of inoculum (Burgess, 1981; Sutton, 1982). In this study, seed to seedling transmission of *F. graminearum* and *F. subglutinans* was clearly demonstrated under glasshouse conditions, and strongly suspected under field conditions. Nevertheless, these results suggest that under favourable field conditions, when infected seed is sown, seedling infection and possibly mature plants and cob/seed infection (Sutton, 1982) may occur. Alternatively, any post-emergence killed seedlings or senescing coleoptile infection (in the case of *F. subglutinans*) would serve to establish the initial inoculum source which would be spread by rain splash, although the effectiveness of this inoculum would depend on the cultivar or hybrid sown.

Precepts for seed storage are that seeds should be stored under cool (10°C or less) and dry (11-13% SMC) conditions (Copeland and McDonald, 1985) to preserve seed viability and vigour. But results from this study have shown that under such conditions *Fusarium* spp. will survive for longer than one year. However, this study has also clearly demonstrated that *F. graminearum*-free seed for planting can be obtained following 3 months storage at high temperature (30°C) and 14% seed moisture content in sealed containers without reduction in planting quality, while *Fusarium* spp. (including *F. subglutinans* and *F. poae*) free seed may be obtained following storage at this seed moisture content but at 25°C for 6-9 months. The more serious difficulty is in obtaining *Fusarium* free crops in the field where the previous crop residue and soil-borne inoculum may infect the sown "clean" seeds. The observation of perithecia of *G. zeae* and *G. subglutinans* on maize stalks 6 months after harvest was a first reporting for New Zealand and has confirmed that infection of a new crop from such a source is therefore theoretically possible (Sutton, 1982). Since the stalks would have been cut down and the stubble ploughed under, a threat is still posed by its incomplete burial within or adjacent to a new sown maize

crop. This suggests that attention to previous cropping history, cultural practices and integration of maize production within a non-susceptible crop rotation will always be important adjuncts to the management systems needed to provide *Fusarium* free maize crops. This perhaps explains why the bulk of literature in this area has concentrated more on field management control measures against stubble-borne inoculum (Khonga and Sutton, 1988, 1991; Lipps and Deep, 1991) and plant breeding for resistance to *Fusarium* spp. (Mesterhazy, 1989; Reid *et al.*, 1992a) than on seed transmission of pathogens (*Fusarium* spp.).

A study programme which would look into the need to rid maize seeds of all *Fusarium* spp. seed-borne inoculum, the seed transmission of *F. graminearum* and *F. subglutinans* under field conditions, and the management of crop residue inoculum, all combined with hybrid selection for resistance against *Fusarium* maize infection would provide a comprehensive information package on the significance of *Fusarium* spp. and their association with seed quality for New Zealand crop and seed production conditions.

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APPENDICES

Appendix 3.1 Relative ratings for four Pioneer maize cultivars[1].

Cultivar	CGDU[2] to silk	CGDU to black layer formation	Days from pollination to harvest maturity	Grain dry down[3]
3551	792	1502	108	8
3591	760	1461	107	5
3709	750	1419	106	5
3475	766	1481	108	5

[1] Data from Pioneer Maize Diagnostic Guide and Silage Manual (1990 and 1991)

[2] Celsius Growing Degree Units - units of heat required for a cultivar to reach various stages of growth

[3] Grain drying rate after maturity. Rating scale: 9 = outstanding; 1 = poor.

Appendix 3.2 Drying rate (moisture content loss (%)) in 1990 and 1991.

Cultivar	Harvest period	Year of harvest			
		1990		1991	
		Drying rate (mean weekly % loss)	Drying rate (mean % loss over 5 months)	Drying rate (mean weekly % loss)	Drying rate (mean % loss over 5 months)
3551	April to May May to June June to July	1.9-2.4 1.3 0.8	1.2	2.3 2.0 0.6	1.6
3591	April to May May to June June to July	1.5-1.9 0.9 0.4	0.9	1.5 1.6 1.1	1.3
3709	April to May May to June June to July	1.4-1.7 0.8 0.8	0.9	1.4 1.5 1.6	1.3
3475	April to May May to June June to July	- 0.6 0.5	-	1.4 1.8 1.3	1.3

(-) seeds not available.

Appendix 3.3 **Types and levels (mg/kg) of mycotoxins detected in seeds of four cultivars harvested in 1990 and 1991.**

Year of harvest		1990					1991				
Cultivar		3551	3591	3709	3475	Mean	3551	3591	3709	3475	Mean
Mycotoxin	Date of harvest										
Zearalenone	April	nd	0.08	0.12	0.04	0.06	nd	nd	nd	nd	0.0
	May	nd	0.19	0.23	nd	0.10	nd	nd	nd	nd	0.0
	June	nd	0.31	0.04	nd	0.09	nd	nd	nd	0.04	0.01
	July	0.04	3.04	0.29	0.32	0.92	nd	nd	0.59	nd	0.15
	Mean	(0.01)	(0.92)	(0.17)	(0.09)		(0.0)	(0.0)	(0.15)	(0.01)	
αZearalenol	April	0.37	1.05	1.11	0.64	0.79	nd	0.23	0.09	0.23	0.14
	May	0.85	0.85	1.17	0.78	0.91	0.42	0.28	0.41	0.19	0.33
	June	0.87	0.99	0.37	0.70	0.73	0.67	0.50	0.35	0.39	0.47
	July	1.03	0.65	1.02	0.71	0.85	0.69	0.46	0.60	0.41	0.54
	Mean	(0.78)	(0.89)	(0.92)	(0.71)		(0.45)	(0.37)	(0.36)	(0.30)	
Nivalenol	April	0.05	0.23	0.45	0.23	0.24	0.02	nd	nd	0.17	0.05
	May	0.62	1.17	1.83	0.28	0.98	0.04	0.31	0.11	0.21	0.17
	June	0.73	2.33	0.46	0.50	1.01	0.28	0.20	0.01	0.48	0.24
	July	0.76	1.90	2.11	0.90	1.42	0.02	0.39	0.35	0.53	0.32
	Mean	(0.54)	(1.41)	(1.21)	(0.47)		(0.09)	(0.23)	(0.12)	(0.35)	
Deoxynivalenol	April	nd	0.25	0.20	nd	0.11	nd	nd	nd	nd	0.0
	May	0.04	0.36	0.48	0.06	0.24	0.03	0.03	0.05	nd	0.03
	June	0.14	0.49	0.33	0.13	0.27	0.83	0.24	nd	0.26	0.33
	July	0.43	0.29	1.59	0.11	0.61	0.02	nd	0.83	0.12	0.24
	Mean	(0.15)	(0.35)	(0.65)	(0.08)		(0.22)	(0.06)	(0.22)	(0.09)	
Zearalenone plus αZearalenol	April	0.37	1.13	1.22	0.68	0.85	nd	0.23	0.09	0.23	0.14
	May	0.85	1.04	1.40	0.78	1.02	0.42	0.28	0.41	0.19	0.33
	June	0.87	1..31	0.41	0.70	0.82	0.67	0.50	0.35	0.41	0.48
	July	1.07	3.69	1.30	1.03	1.77	0.69	0.46	1.19	0.41	0.69
	Mean	(0.80)	(1.79)	(1.08)	(0.80)		(0.45)	(0.37)	(0.51)	(0.31)	
Nivalenol plus Deoxynivalenol	April	0.05	0.48	0.65	0.23	0.35	0.02	nd	nd	0.17	0.05
	May	0.66	1.54	2.31	0.34	1.21	0.06	0.34	(0.16	0.21	0.19
	June	0.88	2.81	0.79	0.62	1.28	1.11	0.45	0.01	0.74	0.58
	July	1.19	2.19	3.70	1.01	2.02	0.04	0.39	0.18	0.65	0.57
	Mean	(0.70)	(1.76)	(1.86)	(0.55)		(0.31)	(0.30)	(0.34)	(0.44)	

nd = not detected

Appendix 3.4

Description of *F. subglutinans* and *F. poae* on carnation leaf agar (CLA).

***F. subglutinans*: sporodochia, macroconidia, microconidia and conidiophores**

On CLA *F. subglutinans* had a scanty mycelial growth with a powedery appearance, formed sparse pale orange sporodochia and the macroconidia were formed in the sporodochia on branched conidiophores from monophialides. Macroconidia were usually slender, falcate to almost straight and usually 3 to 5 septate, with a curved and tapering apical cell. The base of the basal cell was foot-shaped. The microconidia were produced in abundance in false heads, mainly from polyphialides but also from monophialides. The microconidia were of various shapes but usually oval, elliptical or allantoid, being 0 to 1 septate but occasionally up to 3 septate. Chlamydospores and perithecia were absent in cultures of *F. subglutinans*.

***F. poae*: sporodochia, macroconidia, microconidia and conidiophores**

Sporodochia were very sparsely produced on CLA. Macroconidia were formed in sporodochia on branched conidiophores and were falcate, usually 3 septate with short, curved and a pointed apical cell. The base of the basal cell was foot-shaped but some conidia had the basal cell notched. Microconidia were produced abundantly. They were globose but some were almost lemon shaped. They were 0 to 1 septate and had a papilla. The microconidia were formed from short and fat (urn-shaped - Burgess *et al.*, 1988) monophialides on compact conidiophores.

Appendix 4.1 **Description of some *Fusarium* species in pure cultures in petri dishes or test tubes.**

Species	Substrate and Description Characters	AUTHOR		
		Pitt & Hocking (1985) (Petri dish)	**Nelson *et al.* (1983) (Test tube)**	**Burgess *et al.* (1988) (Test tube)**
F. graminearum	**PDA**			
	Growth rate	Fills the petri dish	Rapid; fills the tube	Rapid; fills the tube: GP 1 = 3.9-5.1 cm at 25°C GP 2 = 4.7-6.1 cm at 25°C
	Mycelium - texture	Dense floccose	Dense aerial mycelium	Uniform dense floccose (GP 1) Uneven floccose (GP 2)
	- colour	Olive brown Yellowish brown Reddish brown Pale salmon or in combination of those colours Greyish rose to golden brown	Frequently yellow to tan Margins white to carmine red	GP 1: light orange; at periphery is predominantly light yellow to greyish rose GP 2: at periphery greyish rose but generally colour varies from white to pale orange, to apricot
	Sporulation	Some with a central mass of red brown to orange sporodochia	Red brown to orange sporodochia If present are often appearing only when the cultures are more than 30 days old	Sporodochia may form central spore mass but are normally overgrown by mycelium
	Pigment in agar (Reverse)	Ruby to dark ruby centrally Sometimes violet brown	Usually carmine red	Greyish rose to burgundy
	MEA			
	Mycelium - texture	Dense to open floccose	None	None
	- colour	In shades of greyish rose and greyish yellow to golden brown	None	None

Appendix 4.1 cont'd

	Sporulation	None	None	None
	Pigment in agar (Reverse)	Orange brown to yellowish brown Sometimes paler at the margins	None	None
F. crookwellense	**PDA**			
	Growth rate	Not given	Rapid	Rapid, 5.4-6.6 cm at 25°C
	Mycelium - texture - colour	None None	Dense aerial mycelium White in colour then tan	Floccose toward apex White towards apex Pale yellow below the spore mass Greyish rose at the periphery
	Sporulation	None	Orange to red brown sporodochia generally appear in the centre of the culture and in the other portion of the culture	Abundant sporodochia in a conspituous central spore mass Initially pale orange becoming reddish brown to dark brown with age. Annular zonation of spore mass occurs under alternating conditions of light and temperature
	Pigment in agar (Reverse)	None	Carmine red	Greyish rose to burgundy
	MEA			
	Mycelium - texture - colour	None None	None None	None None
	Sporulation	None	None	None

Appendix 4.1 cont'd

F. culmorum	**PDA**			
	Growth rate	Not given	Rapid	Rapid, 5.5-6.8 cm at 25°C
	Mycelium - texture - colour	Dense floccose As described for *F. graminearum*	Dense aerial mycelium Generally white Often yellow to tan towards the base	Floccose Usually light yellow around the spore mass At apex: white At periphery: greyish rose to pink May be: olive brown
	Sporulation	Sporodochia may be produced more abundantly than by *F. graminearum*, and are usually darker	Unlike for *F. graminearum* abundant orange to red-brown sporodochia appear (around the point of inoculation) as the culture ages	Abundany sporodochia form in a large central spore mass (1-2 cm in diameter), initially pale orange becoming violet brown to dark brown with age Annular zonations of spore mass are formed by some isolates under alternating light and temperature conditions
	Pigment in agar (Reverse)	As described for *F. graminearum*	Carmine red	Greyish rose to burgundy
	MEA			
	Mycelium - texture - colour	As for *F. graminearum* As for *F. graminearum*	None None	None None
	Sporulation	None	None	None
	Pigment in agar (Reverse)	As for *F. graminearum*	None	None

Appendix 4.1 cont'd

F. subglutinans	**PDA**			
	Growth rate	Not given	Rapid	Not given
	Mycelium - texture	Colonies low to moderately deep, of floccose to funiculose mycelium	Rapid growing aerial mycelium resembling that of *F. oxysporium*	Floccose
	- colour	White, pale salmon, pale pink or mauve	White aerial mycelium sometimes tinged with purple	White, but may become greyish violet or greyish magenta with age
	Sporulation	Sometimes powdery with microconidia	None	Sporodochia normally absent If present are pale orange Dark coloured sclertoia develop in some isolates
	Pigment in agar (Reverse)	Violet grey to deep violet centrally Paler at the margins or uniformly pale	Varies from colourless to dark purple	Quite variable, ranging from no pigmentation or greyish orange to violet gray, dark violet or dark magenta, almost black
	MEA			
	Mycelium - texture	Less dense than on CYA (Czapek Yeast Extract Agar)	None	None
	- colour	White to pale salmon, sometimes powdery	None	None
	Sporulation	Sometimes powdery with microconidia	None	None
	Pigment in agar (Reverse)	Pale yellow, salmon or violet	None	None

Appendix 4.1 cont'd

F. poae	**PDA**			
	Growth rate	Not given	Rapid	Not given
	Mycelium - texture	Colonies moderate to deep, of floccose mycelium	Dense aerial mycelium	Dense floccose
	- colour	Pale salmon to pale rose Darker centrally	White to pink	Initially white becoming pinkish white to brown orange with age
	Pigment in agar (Reverse)	Varying from pale salmon at the margins to ruby centrally or entirely dark ruby to dark magenta	Usually carmine	Greyish rose to burgundy
	MEA			
	Mycelium - texture	colonies filling the whole petri dish deep, or sparse, pale rose	None	None
	- colour	Pale rose	None	None
	Pigment in agar (Reverse)	Brownish orange or pale	None	None

Appendix 4.2

Description of pure culture colony morphology of *F. graminearum* on PDA and on MEA following subculturing from seed-culture colonies growing on MEA.

On PDA (Appendix 4.2a) all the isolates demonstrated rapid growth (5.2-6.0 cm). The mycelium texture was floccose to densely floccose with regular edges, and the mycelium colour in the periphery of the petri dish varied from pastel red, greyish red, greyish red to pastel red, greyish rose and greyish rose to dull red. In the centre of the cultures the mycelium colour was reddish brown or violet brown. The colour of the pigment produced in PDA, as seen on the reverse side of the petri dish, was either red brown, or ruby in the periphery of the dish but dark ruby in the centre of the culture.

On MEA (Appendix 4.2b) the colony diameter was 3.4-3.7 cm. The mycelium texture varied from velvet to short with tufts of aerial mycelium being observed in the petri dish. Some cultures had rugged edges while in others the edges were lobed, particularly culture No 4. The colour of the mycelium varied; being orange white, greyish orange or as greyish orange spots on the periphery of the plate, while in others the colour was brownish orange, to golden. The centre of the cultures varied from pale red, pale orange or pale red spots, brown orange or reddish golden. The colour of the pigment produced in MEA petri dishes, as seen on the reverse side, varied from lemon yellow to light orange or greyish orange and to brownish orange to reddish golden in the periphery, while some cultures showed brown pigmentation throughout the plate.

Appendix 4.2.1 **Colony morphology of *F. graminearum* isolates on PDA following subculturing from seed-culture colonies growing on MEA.**

Description Characters	Isolate 2	3	4	5	7	8	9	11	12	SM 7	SM 9
Growth rate - colony diameter	rapid 5.2	rapid 5.4	rapid 5.3	rapid 5.5	rapid 5.2	rapid 5.5	rapid 5.7	rapid 5.5	rapid 5.8	very rapid 6.0	very rapid 6.0
Mycelium texture	Dense floccose	Dense floccose	Dense floccose	Dense floccose	Dense floccose	Dense floccose	Dense floccose	Dense floccose	Dense floccose	Dense floccose	Dense floccose
Mycelium colour - periphery	Greyish red to pastel red	Pastel red	Greyish red to pastel red	Greyish red	Greyish red	Greyish red to pastel red	Greyish rose to dull red	Greyish red	Greyish rose to dull red	Greyish rose to dull red	Greyish rose to dull red
- centre	Reddish brown	Reddish brown	Reddish brown	Reddish brown	Reddish brown	Reddish brown	Greyish rose to dull red	Reddish brown	Violet brown	Violet brown	Violet brown
Sporulation	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)
Pigment in agar (Reverse) - periphery	Brownish red	Brownish red	Brownish red	Brownish red	Brownish red	Brownish red	Greyish red	Brownish red	Brownish red	Ruby	Ruby
- centre	Dark ruby	Dark ruby	Dark ruby	Dark ruby	Dark ruby	Dark ruby	Same	Dark ruby	Dark ruby	Dark ruby	Dark ruby

Appendix 4.2.2 **Colony morphology of *F. graminearum* isolates on MEA following subculturing from seed-culture colonies growing on MEA.**

Description Character	Isolate										
	2	3	4	5	7	8	9	11	12	SM 7	SM 9
Growth rate	slow	slow	slow	slow	slow	slow	slow	not tested	slow	slow	slow
- colony diameter	not tested	not tested	not tested	not tested	not tested	not tested	3.4	not tested	3.6	3.7	3.6
Mycelium texture	short mycelium (with loose tufts of mycelium), zonate	short mycelium dotted with tufts of mycelium, zonate	velvet; poorly zonate	short mycelium (with loose tufts of mycelium), quite lobed zonate	velvet and zonate	velvet and zonate	very short, sparce zonate and heavily lobed	velvet and zonate	short tufty mycelium and zonate	short tufty mycelium and zonate	velvet and zonate
Mycelium colour - periphery	greyish orange spots	greyish orange	greyish orange	greyish orange spots	greyish orange	greyish orange	orange white	greyish orange	orange white to pinkish white	brownish orange	brownish orange to reddish golden
- centre	pale orange	pale red	pale orange	pale red with brown centre	pale orange	pale orange with brown centre	orange white	pale orange	orange white to pinkish white	brownish orange	brownish orange to reddish golden
Sporulation	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)
Pigment in agar (Reverse) - periphery	Lemon yellow/light orange to greyish orange	Lemon yellow/ light orange to greyish orange	Lemon yellow/light orange to greyish orange	Brown (burnt sienna to brick/tile red)	Brownish orange	Brown (burnt sienna to brick/tile red)	Brownish orange to reddish golden	Brown (burnt sienna to brick/tile red)	Brownish orange to reddish golden	Brown (burnt sienna to brick/tile red)	Brown (burnt sienna to brick/tile red)
- centre	Brownish orange	Brownish orange	Brownish orange to reddish golden	Same	Same	Same	Same	Same	Same	Same	Same

Appendix 5.1 **Decline in the percentage of seeds carrying various *Fusarium* spp during 12-months seed storage at 25°C in single layered paper bags or 5°C in sealed containers.**

Fusarium spp.			*F. graminearum*				*F. subglutinans*				*F. poae*				Other *Fusarium* spp.			
Harvest Date			April	May	June	July	April	May	June	July	April	May	June	July	April	May	June	July
Cultivar	Storage temp. (°C)	Storage period (months)																
3551	25	0	5	18	59	66	3	0	0	2	2	8	3	3	0	1	1	2
		2	3	13	24	45	1	1	1	2	0	1	3	0	0	0	0	0
		4	1	3	9	16	4	1	3	1	0	0	1	2	0	0	0	0
		6	0	5	2	11	0	0	2	1	0	0	0	1	0	0	0	0
		8	-*	2	0	4	1	0	1	1	-	0	3	2	-	0	0	1
		10	-	0	0	3	-	0	1	1	-	2	0	0	-	0	0	0
		12	-	0	0	3	-	0	4	3	-	1	0	0	-	0	0	0
	5	12	9	24	55	74	1	1	2	0	1	0	3	3	1	0	0	1
3591	25	0	16	35	72	88	0	0	2	1	2	1	1	1	2	0	1	0
		2	4	24	27	54	0	0	2	2	0	0	0	0	0	0	0	0
		4	2	6	10	29	0	0	2	1	0	0	0	0	0	0	0	0
		6	0	4	3	18	0	0	4	4	0	0	2	3	0	0	1	0
		8	-	2	2	3	-	0	1	1	-	0	0	6	-	0	0	0
		10	-	2	3	1	-	0	0	1	-	0	0	3	-	0	0	0
		12	-	2	1	2	-	0	2	0	-	1	0	1	-	0	0	0
	5	12	32	23	64	75	1	0	3	0	1	1	0	2	0	1	1	1
3709	25	0	35	45	71	88	2	1	3	0	6	2	3	5	0	1	0	5
		2	2	18	28	51	1	0	3	3	0	3	1	7	1	0	0	0
		4	0	6	13	11	0	3	0	1	0	1	1	4	0	0	0	0
		6	1	5	2	11	2	0	5	1	0	1	0	1	0	0	0	0
		8	-	4	1	7	-	0	2	3	-	0	3	4	-	0	0	1
		10	-	0	1	3	-	0	5	0	-	0	1	2	-	0	0	0
		12	-	3	0	1	-	0	3	1	-	0	1	3	-	0	0	0
	5	12	14	33	49	71	0	2	2	2	0	0	3	2	0	0	3	3
3475	25	0	7	24	52	44	0	6	2	8	18	11	1	13	4	4	0	0
		2	8	13	25	38	0	5	4	6	12	2	10	6	1	0	0	1
		4	6	8	8	15	0	2	2	6	0	8	4	1	0	0	2	2
		6	1	2	5	15	0	3	3	3	4	1	3	3	5	0	0	0
		8	-	1	1	3	-	2	2	5	-	6	3	6	-	0	0	4
		10	-	0	1	4	-	5	1	5	-	1	3	3	-	0	1	3
		12	-	0	0	2	-	2	1	2	-	0	0	0	-	0	0	1
	5	12	18	10	56	44	0	4	10	6	6	16	7	20	5	0	1	0

* (-) insufficient seeds

Appendix 5.2 **Incidence of *Pencillium* and *Aspergillus* during storage for 9 months at seed moisture contents of 10% and 14% and six temperatures (5-30°C) in sealed containers (%).**

Seed lot	Seed moisture content	Storage period (months)	*Pencillium* spp						*Aspergillus* spp					
			5°C	10°C	15°C	20°C	25°C	30°C	5°C	10°C	15°C	20°C	25°C	30°C
3591 May	10	0	2	2	2	2	2	2	3	3	3	3	3	3
		3	0	0	1	0	18	16	0	0	0	0	6	3
		6	1	4	1	4	2	0	0	2	0	0	0	0
		9	4	1	3	0	0	5	0	0	0	0	0	0
3591 May	14	0	3	3	3	3	3	3	3	3	3	3	3	3
		3	0	0	1	1	10	3	0	0	0	0	1	10
		6	2	5	2	2	4	0	1	0	0	0	5	1
		9	3	1	3	2	0	1	0	0	0	0	0	0
3591 June	10	0	5	5	5	5	5	5	1	1	1	1	1	1
		3	0	0	3	2	6	14	0	0	3	2	6	14
		6	2	2	6	4	2	0	0	1	0	0	1	1
		9	8	4	4	3	2	3	0	0	1	0	0	1
3591 June	14	0	3	3	3	3	3	3	1	1	1	1	1	1
		3	0	0	1	3	0	12	0	0	1	3	0	12
		6	7	1	5	1	0	1	0	0	0	0	1	0
		9	5	4	2	2	0	3	0	0	1	0	0	0
3475 May	10	0	0	0	0	0	0	0	0	0	0	0	0	0
		3	0	0	1	6	7	14	0	0	3	0	0	1
		6	5	15	2	1	7	1	0	0	0	0	2	0
		9	10	10	6	8	2	2	0	1	0	0	1	1
3475 May	14	0	1	1	1	1	1	1	0	0	0	0	0	0
		3	0	0	1	3	4	25	0	0	0	0	1	5
		6	6	21	4	2	2	0	0	0	3	0	0	2
		9	6	6	3	2	0	4	0	1	0	1	0	0
3475 June	10	0	6	6	6	6	6	6	0	0	0	0	0	0
		3	0	0	11	6	17	18	0	0	0	0	4	2
		6	10	11	8	11	7	1	0	0	0	0	1	5
		9	10	15	14	8	0	3	0	1	0	0	0	1

Appendix 5.3 **Normal and abnormal seedlings and dead seeds after 3, 6 and 9 months storage at 10% and 14% seed moisture contents and temperatures of 5-30°C in sealed containers.**

Seed lot	Storage temp (°C)	3 months storage						6 months storage						9 months storage					
		SMC 10%			SMC 14%			SMC 10%			SMC 14%			SMC 10%			SMC 14%		
		Norm*	Abn	Dead	Norm	Abn	Dead	Norm	Abn	Dead	Norm	Abn	Dead	Norm	Abn	Dead	Norm	Abn	Dead
3591 May	5	97	2	1	94	3	3	94	5	1	95	4	1	91	5	4	95	3	2
	10	94	3	3	94	4	2	89	7	4	88	10	2	94	4	2	98	2	0
	15	94	2	4	95	2	3	94	2	4	92	6	2	96	2	2	94	1	5
	20	96	0	4	94	3	3	92	6	2	89	11	0	93	5	2	94	2	4
	25	92	7	1	95	1	4	93	6	1	89	5	6	94	5	1	90	6	4
	30	95	5	0	84	13	3	94	4	2	89	11	0	96	3	1	93	3	4
3591 June	5	94	3	3	94	2	4	94	2	4	82	9	9	91	2	7	93	3	4
	10	89	4	7	91	4	5	96	1	3	92	6	2	95	0	5	89	5	6
	15	92	6	2	95	5	0	95	2	3	84	12	4	91	4	5	89	3	8
	20	95	2	3	92	6	2	95	2	3	79	15	6	92	3	5	95	1	3
	25	93	3	4	93	5	2	92	4	4	79	13	8	88	5	7	78	11	11
	30	89	8	3	92	2	6	94	3	3	40	21	39	97	0	3	6	20	74
3475 May	5	98	2	0	96	1	3	97	2	1	93	6	1	96	4	0	93	4	3
	10	99	1	0	99	0	1	96	4	0	87	10	3	97	1	2	94	3	3
	15	93	3	4	96	3	1	97	1	2	93	6	1	97	3	0	93	3	4
	20	96	4	0	98	0	2	94	5	1	88	9	3	95	3	2	94	5	1
	25	96	3	1	97	3	0	96	2	2	83	17	0	95	4	1	90	5	5
	30	97	2	1	91	7	2	92	6	2	49	21	30	95	5	0	17	31	52
3475 June	5	96	3	1	94	5	1	93	5	2	88	9	3	96	4	0	91	4	5
	10	94	5	1	96	2	2	94	4	2	85	9	6	93	4	3	97	2	1
	15	95	2	3	94	2	6	91	8	1	87	11	2	91	8	1	90	10	0
	20	96	3	1	92	6	1	91	7	2	79	16	5	92	2	6	95	2	3
	25	95	4	1	93	4	3	95	2	3	74	21	5	93	5	2	84	8	8
	30	96	2	2	80	11	9	89	5	6	42	22	36	96	4	0	2	17	81

* Norm = Normal seedling
Abn = Abnormal seedling
Dead = Dead seeds

Appendix 5.4 **Levels (mg/kg) of mycotoxins after seed storage at 5°C and 25°C for 12 months.**

Mycotoxin		Zearalenone			αZearalenol			Nivalenol			Deoxynivalenol		
		S0	S12		S0	S12		S0	S12		S0	S12	
Cultivar	Date of harvest		5°C	25°c		5°C	25°C		5°C	25°C		5°C	25°C
3551	April	nd	nd	FIN	0.37	0.36	FIN	0.05	0.06	FIN	nd	nd	FIN
	May	nd	nd	nd	0.85	0.97	1.16	0.62	0.34	0.34	0.04	0.04	0.02
	June	nd	nd	nd	0.87	1.09	1.06	0.73	0.69	0.86	0.14	0.04	0.15
	July	0.04	0.67	3.64	1.04	0.94	1.18	0.76	1.55	1.52	0.43	0.47	0.24
3591	April	0.08	0.26	FIN	1.05	1.04	FIN	0.23	0.35	FIN	0.25	0.26	FIN
	May	0.19	0.26	0.13	0.85	0.95	0.91	1.17	0.75	0.73	0.36	0.88	0.48
	June	0.31	0.09	0.17	0.99	0.90	0.99	2.33	1.07	1.27	0.49	0.09	0.32
	July	3.04	0.39	0.25	0.65	0.70	0.64	1.90	2.14	1.67	0.29	0.25	0.50
3709	April	0.12	FIN	FIN	1.11	FIN	FIN	0.45	FIN	FIN	0.20	FIN	FIN
	May	0.23	0.09	0.34	1.17	1.37	1.00	1.83	1.11	0.81	0.48	0.35	0.93
	June	0.04	0.09	0.08	0.37	0.89	0.84	0.46	1.43	0.63	0.33	0.46	0.20
	July	0.29	1.39	0.70	1.02	0.86	1.11	2.11	2.17	1.99	1.59	0.58	0.63
3475	April	0.04	0.08	FIN	0.64	0.64	FIN	0.23	0.10	FIN	nd	nd	FIN
	May	nd	nd	nd	0.78	0.63	0.69	0.28	0.56	0.19	0.06	0.12	0.07
	June	nd	nd	nd	0.70	0.57	0.75	0.50	0.91	0.58	0.13	0.53	0.18
	July	0.32	0.50	0.08	0.71	0.97	1.11	0.90	1.20	0.93	0.11	0.13	0.29

FIN = No seeds available
nd = Not detected
S0 = At start of storage
S12 = Storage for 12 months

Appendix 6.1 **Minimum, maximum and mean daily temperatures recorded during germination of non-surface and surface disinfected seeds and seedlings emergence and establishment under glasshouse conditions.**

Non-surface Disinfected Seeds				Surface Disinfected Seeds			
Date (1992)	Temperature (°C)			Date (1992)	Temperature (°C)		
	Min.	Max.	Mean		Min.	Max.	Mean
August 15	13.0	21.0	17.0	Sept 17	10.0	19.5	14.8
16	10.0	19.5	14.8	18	12.0	19.0	15.5
17	3.0	18.5	10.8	19	12.0	19.5	15.8
18	13.0	19.5	16.3	20	11.8	19.0	15.4
19	13.9	20.0	16.5	21	11.0	22.8	16.0
20	12.0	19.5	15.8	22	10.5	25.0	17.8
21	6.0	21.0	13.5	23	10.5	22.5	16.5
22	10.5	22.5	16.5	24	9.0	23.5	16.3
23	12.5	19.5	16.0	25	10.0	16.5	13.3
24	10.0	20.5	15.3	26	9.0	20.5	14.8
25	12.5	19.5	16.0	27	11.0	20.5	15.8
26	12.5	19.5	16.0	28	9.5	17.5	13.5
27	11.0	11.5	15.3	29	12.5	19.0	15.8
28	11.0	21.5	16.3	30	11.3	20.0	15.7
29	10.0	20.5	15.3	Oct 1	11.5	22.5	17.0
30	7.5	21.0	14.3	2	10.0	23.0	16.0
31	10.5	22.0	16.3	3	13.0	19.0	15.0
Sept 1	10.5	22.0	16.3	4	9.5	20.0	14.8
2	10.5	22.5	16.5	5	8.0	19.5	13.8
3	10.5	19.5	15.0	6	11.5	23.0	17.3
4	11.0	22.0	16.5	7	14.0	19.0	16.5
5	13.0	23.5	18.3	8	14.5	21.0	17.8
6	12.5	18.5	15.5	9	13.0	20.5	16.8
7	12.0	18.5	15.3	10	11.0	20.0	15.5
8	12.5	21.0	16.8	11	12.5	29.0	20.8
9	13.0	25.0	19.0	12	14.5	25.0	19.8
10	11.0	20.0	15.5	13	12.5	29.0	21.0
11	12.5	21.0	16.8	14	9.5	19.5	14.5
				15	8.0	17.5	12.8
				16	8.0	18.5	13.3

Appendix 6.2 **Minimum, maximum and mean daily air temperatures recorded during seed germination, seedling emergence and establishment under field conditions.**

Date (1992)		Temperature (°C)		
Month	Day	Minimum	Maximum	Mean
November	13	10	23	16.5
	14	11	25	18.0
	15	16	19	17.5
	16	14	23	18.5
	17	14	20	17.0
	18	11	18	14.5
	19	11	19	15.0
	20	13	20	16.5
	21	12	18	15.0
	22	14	20	17.0
	23	14	18	16.0
	24	14	16	15.0
	25	15	20	17.5
	26	12	20	16.0
	27	13	21	17.0
	28	14	16	15.0
	29	15	21	18.0
	30	16	18	17.0
December	1	17	22	19.5
	2	16	22	19.0
	3	14	23	18.5
	4	13	16	14.5
	5	9	18	13.5
	6	11	17	14.0
	7	5	18	11.5
	8	6	21	13.5
	9	10	22	16.0
	10	14	18	16.0
	11	13	19	16.0
	12	13	21	17.0

Appendix 6.3 Minimum, maximum and mean daily air temperatures recorded during the Koch's Postulates study.

Date (1993)			Temperature (°C)		
Month	Day	Experimental stage	Min.	Max.	Mean
August	26	Sowing to raise inoculation : seedlings	11.5	20.0	15.8
	27		12.0	22.5	17.3
	28		11.5	21.0	16.0
	29		13.0	20.5	17.0
	30		12.5	20.5	16.5
	31		11.5	21.0	16.0
Sept	1		11.0	20.0	15.5
	2	Seedling emergence	14.5	22.5	18.0
	3		11.5	20.0	16.0
	4	Inoculation : Above ground parts Sowing : Soaking inoculated seeds	12.0	20.0	15.0
	5		11.0	18.5	15.0
	6		10.0	21.5	16.0
	7		12.5	21.0	17.0
	8		12.5	22.0	17.0
	9		13.5	21.5	17.0
	10	Seedling emergence : soaking inoculation	13.5	22.5	18.0
	11		13.0	21.0	17.0
	12		13.0	22.0	17.5
	13		13.5	21.5	17.5
	14		14.0	22.5	18.0
	15		13.5	21.0	17.0
	16		15.0	20.5	18.0
	17		13.0	22.0	17.5
	18		13.0	21.5	17.0
	19		13.0	21.0	17.0
	20		12.0	21.0	16.5
	21		13.0	21.5	17.0
	22	Harvesting : above ground inoculated seedlings	11.5	22.0	17.0
	23		13.0	21.5	17.0
	24		14.5	22.0	18.0
	25		12.5	21.5	17.0
	26		12.0	19.5	16.0
	27		14.0	21.5	18.0
	28	Harvesting : soaking inoculation seedlings	11.5	19.5	15.5
	29		12.0	22.0	17.0