CHAPTER 1
GENERAL INTRODUCTION

GENERAL BACKGROUND TO THE DISEASE

Crown rot is one of a number of diseases of small grain cereals caused by *Fusarium* species (Parry *et al.* 1994, Paulitz *et al.* 2002). Its characteristic feature is infection of the crowns, or stem tissues at or near the soil surface, by soil or residue borne inoculum. In Australia, crown rot is the accepted common name for the disease caused by the fungus *Fusarium pseudograminearum* (Fp). This fungus was earlier reported as *F. graminearum* Group 1 or its perfect stage *Gibberella zeae*, but was later described and illustrated as *F. pseudograminearum* by Aoki and O'Donnell (1999). The Group 1 population cause crown rot of temperate cereals and many grasses, while Group 2 cause head scab of temperate cereals, stalk and cob rot of maize and stub rot of carnations (Burgess *et al.* 2001).

Crown rot caused by Fp was first detected in Queensland in 1951 and northern New South Wales in 1955 (Magee 1957). It has now been reported from all wheat growing regions in Australia (Backhouse and Burgess 2002, Backhouse *et al.* 2004). It is considered to be one of the most important diseases limiting yield of winter cereals in the central and northern regions of the eastern Australia grain belt (Burgess *et al.* 1981, Murray and Brown 1987). Crown rot can cause yield losses of up to 89% in wheat (Klein *et al.* 1991).

The host range of Fp includes all winter cereals and many grass genera including *Avena, Agropyron, Bromus, Danthonia, Dicanthium, Hordeum*, *Panicum* and *Phalaris* (Burgess *et al.* 2001). Fp extensively colonizes wheat and barley among the winter cereals. Cereal oats and the grass weed wild oats or black oats (*Avena fatua* and *A. ludoviciana*) are symptomless hosts but the extent of colonization is less than wheat and barley (Purss 1966). The symptoms of this disease are the presence of small necrotic lesions on the coleoptile,

subcrown internode or leaf sheaths. Uniform browning of the stem base is the first visual indication of crown rot seen usually after flowering (Burgess *et al.* 2001).

In October, 1983 Fp was found to be responsible for head blight in the northern wheat belt of New South Wales (Burgess *et al.* 1987). The occurrence of perithecia (sexual stage) of *Gibberella corniculata* T. Aoki & O'Donnell on wheat residues in the Moree district of New South Wales was reported by Burgess *et al.* (2001). The fungus is also capable of producing mycotoxins such as zearalenone, deoxynivalenol and nivalenol in infected grains (Blaney *et al.* 1987, Blaney and Dodman 2002).

DISEASE CYCLE

F. pseudograminearum survives in infested stubble and during the growing season the fungus grows from the stubble and infects plants through their crowns, and tiller and stem bases. From infested stubble, Fp penetrates through the scutellum, subcrown internode and lower crown regions and stems can be parasitically colonized up to the fourth internode or higher (Summerrell *et al.* 1990). The fungus persists between host crops mainly as hyphae in stubble (stem and crown tissues) residues (Wearing and Burgess 1977). In this form the fungus can survive up to 2 years, although only a minor proportion of the inoculum survives longer than 12 months (Burgess and Griffin 1968, Summerrell and Burgess 1988b).

THE ROLE OF BIOCONTROL

Management practices currently used are not so successful in controlling crown rot which depends upon manipulation of agronomic practices. The use of tolerant cultivars is a common practice but there is a limited range of tolerance against Fp. As stubble retention is a preferred management practice, it is essential that more research be undertaken to reduce Fp inoculum even when we want to keep stubble on the surface. Rotation is a major tool for disease

management through biocontrol which works by giving time for mortality of Fp, which could be enhanced by the use of biocontrol or antagonist agents. This requires novel ways to tackle such problems.

Biocontrol is the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man (Cook and Baker 1983). Biocontrol is an approach which can be used as an alternative to hazardous chemicals and pesticide use. It is also ecologically harmless to plants and vertebrates. In stubble or soil there are populations of antagonists but these may be too low in amount to actually suppress disease under field conditions. There is potential for manipulating microbial populations in the stubble of wheat crops after harvest, or to search for candidates that could displace, or increase mortality of Fp populations. Studies have been done by researchers on *Fusarium* head blight (Pereyra *et al.* 2004, Luongo *et al.* 2005) but little has been done so far on crown rot in wheat. This study looked at some of the factors like temperature, water potential, rainfall and nutrients which affect antagonism and also determined which characteristics successful antagonists should have to displace Fp from stubble pieces.

RESEARCH OBJECTIVES

The overall aim of this study was to determine the effect of abiotic and nutritional effects on displacement of Fp from stubble by potential antagonists with the following objectives:

1) To study the occurrence of fungal species in stubble to find antagonists that can displace Fp from stubble.
2) To examine the effects of temperature and water potential on growth and displacement of Fp from straw by different antagonists.
3) To investigate the effects of wetting and drying periods on displacement of Fp from stubble.

4) To explore carbon source utilization patterns by antagonists and the target fungus Fp as well as the ability of each fungus to grow on carbohydrates typical of those found in cereal straw.
5) To understand the effects of application of different N fertilizers to straw to control crown rot disease.

THESIS OUTLINE

A literature review of the general background and management of crown rot disease followed by a critical review of biological control of Fp and related species such as *F. culmorum* and *F. graminearum* is presented in Chapter 2. Topics covered included fungi in wheat residues, and the effects of nutrition, temperature and water potential on interactions between fungal populations in residues.

Basic experimental work to build up a strong basis for further studies is described in Chapter 3. It included collection of wheat stubble, identification of different fungal species by morphological and molecular tools and basic experiments to test displacement of Fp by antagonists at different water potentials and time periods. An inoculum potential bioassay to test the pathogenicity of fungus was evaluated.

The outcome of a comprehensive investigation of the effect of temperature and water potential on interactions between Fp and other stubble fungi is presented in Chapter 4. The effects of these abiotic factors were tested on the growth rates of different fungi, and their interactions in dual culture. Displacement assays from straw pieces were also tested with different antagonists for their ability to displace Fp from straw.

Studies of the effect of different wetting and drying schedules, temperature and humidity on the displacement of the target fungus Fp in straw pieces by different antagonists including *Trichoderma harzianum* and some non-pathogenic species of *Fusarium* like *F. equiseti* and *F. nygamai* are presented in Chapter 5.

Studies described in Chapter 6 investigated carbon source utilization patterns by antagonists and the target fungus Fp using Biolog MicroPlates as well as the ability of each fungus to grow on carbohydrates typical of those found in cereal straw. The effect of resource quality on the antagonism on straw medium supplemented with selected sugars in dual culture interactions of antagonists with Fp in Petri plate assays is also explored.

The effects of different N sources on growth in a straw system and on dual culture interactions between antagonist and pathogen were investigated and are reported in Chapter 7, together with experiments on the effects of application of different N fertilizers in straw. Finally Chapter 8 contains a General Discussion which describes the key findings from each chapter and relates these for potential use of antagonistic fungi as biocontrol agents against Fp.

CHAPTER 2
LITERATURE REVIEW

Crown rot is caused by Fp which is a residue-borne pathogen and is the most important *Fusarium* disease of wheat in Australia and in a number of other regions. In Australia, the practice of retaining stubble after harvest on the soil surface has led to a significant increase in prevalence of crown rot over time (Burgess *et al.* 2001). The literature on Crown rot has been reviewed by Burgess *et al.* (2001) and this chapter will focus on aspects directly relevant to the project.

FACTORS AFFECTING EPIDEMIOLOGY

The most important aspect of epidemiology is the relationship between inoculum potential and disease incidence determined by environmental factors such as rainfall and temperature. For crown rot, research has been done to define the relationship between incidence (proportion of plants infected) or severity (degree of symptom expression) of disease and quantity of infested stubble to forecast crown rot in the next growing season. Backhouse (2006) analysed long-term weather data and disease from Moree, New South Wales and Billa Billa, Queensland to determine the factors that influence the incidence of crown rot in successive stubble-retained, no-till wheat crops. He found that the rate of infection was negatively correlated with rainfall during summer fallows because the conditions favoured residue decomposition. In-crop rainfall during winter however, stored soil moisture and temperature were not significantly correlated with infection rates. Backhouse also emphasized the usefulness of a simple formula of square root of the product of incidence and either yield or in-crop rainfall for on-farm forecasting of crown rot incidence in the next season.

The incidence and severity of crown rot are usually higher in fields where wheat and barley are grown in monoculture on heavy soils and where infested stubble residues were retained. Within fields, the incidence and severity of the disease are higher in low lying areas of the field. (Klein *et al.* 1988). Other researchers have found similar results showing that removing stubble reduces disease caused by Fp and related species (Swan *et al.* 2000, Pereyra *et al.* 2004).

The influence of soil moisture potential on infection of wheat by Fp has been studied in detail in laboratory and greenhouse conditions. These studies determined that water potential in the range of 0.3 to 0.7 MPa is optimum for infection and colonization of wheat seedlings by Fp (Liddell and Burgess 1985, Liddell and Burgess 1987; Liddell and Burgess 1988). Further studies indicated that surface soil moisture is likely to be a critical factor which determines the rate of infection of winter cereals by Fp under field condition (Liddell and Burgess (1985), Liddell and Burgess (1987), Liddell and Burgess (1988), and Swan *et al.* (2000). So the incidence, or number of plants infected, is likely to be greatest in wet seasons.

The influence of plant water stress on infection and colonization of wheat seedlings by Fp was studied by Beddis and Burgess (1992) who recorded that a decrease in soil water potential of 0.32 to -2.63 MPa (cv. Suneca) and 0.43 to -2.85 (Sunkota) at seedling stage corresponded with an increase in colonization of wheat seedlings by Fp. These results suggested that low water potential predisposed wheat seedlings to colonization and further damage by the fungus.

MANAGEMENT OF CROWN ROT DISEASE

Role of stubble management

The long term effects of stubble management regimes (e.g. burning after harvest, incorporation with a disc plough, retention on the surface) on the incidence of infection of wheat with Fp have been studied in New South Wales. There were no differences in the

incidence of infection between retained and incorporated treatments (Burgess *et al.* 1993). While retaining stubble on the surface would be expected to favor maximum carryover of the fungus, this treatment leads to the slowest decomposition of stubble; in contrast, incorporating stubble accelerates decomposition (Summerrell and Burgess 1988b). In most years during this and previous studies (Summerell and Burgess 1989), stubble weight was reduced by half in incorporated plots compared with retained plots. However, incorporation does not necessarily reduce the survival of Fp within individual stubble pieces compared with retention on the surface (Summerrell and Burgess 1988b).

Burning the infested stubble reduces the inoculum of Fp (Klein *et al.* 1988) and controls the disease substantially (Burgess *et al.* 1993). However stubble burning reduced the disease in only 2 of 5 years at one site (Burgess *et al.* 1993) and failure in other years was attributed to susceptible weed hosts (Purss 1969) and poor burns (relatively sparse stubble). Burning stubble is no longer an accepted practice and stubble is now retained for other reasons such as surface moisture retention.

Role of crop rotation

The report by Burgess *et al.* (1996) indicated that the incidence of infection was significantly lower in wheat rotated with sorghum than in continuous wheat, over three complete rotation cycles, although the disease was not eliminated as might have been expected from the data of Summerrell and Burgess (1988b). Wheat crops grown on long fallow after sorghum are high-yielding (Croft *et al.* 1988), and consequently would be expected to produce relatively large quantities of stubble. Higher stubble loads were found by Summerrell and Burgess (1988b) to increase the survival of the pathogen, and this may account for the continuing presence of the disease despite the 30 month break between harvesting one wheat crop and sowing the next.

The studies of Kirkegaard *et al.* (2004) compared the effectiveness of brassica break crops canola (*Brassica napus* L.) and mustard (*B. juncea* L.) with chickpea (*Cicer arietinum*

L.) on the reduction of crown rot in subsequent wheat crops. They found that yield of the Fp susceptible durum wheat was generally higher after brassicas than after chickpea. Possible explanations are that cereal residues decomposed more rapidly under dense brassica canopies thus reducing Fp inoculum, or that Fp severity was increased following chickpea due to higher soil N status, or that brassica resulted in soil or residue biology that was less conducive to Fp inoculum survival.

The effect of rotation with barley and oats on crown rot in wheat was studied by Nelson and Burgess (1995) in the northern wheat belt of New South Wales. They observed that incidence of the crown rot of wheat was greatest after 2 prior wheat crops and lowest after 1 or 2 years of oats.

Role of crop nutrition

Crown rot incidence (percentage of whiteheads) increased with rate of applied nitrogen from 0 to 176 kg N/ha (Smiley *et al.* 1996). They found that the percentage of whiteheads formed was inversely proportional to soil pH and directly proportional to organic N and carbon. Crown rot was increased by surface residues from previous crops which were directly correlated with soil organic nitrogen and carbon. Their explanation was that with increase in soil organic N and carbon content more nitrogen becomes available proportionately through the mineralization process.

The management of disease by *Fusarium* foot rot pathogens (Fp and *F. culmorum*) in the USA had been heavily dependent on practices like balanced use of nitrogen fertilizers and irrigation late in the growing season (Paulitz *et al.* 2002). Paulitz (2002) discussed that if a wheat crop is sown into summer fallow and high rates of nitrogen applied, it could result in the plants prematurely outstripping their water supply, and the resulting plant water stress can enhance the disease caused by *Fusarium* spp.

From the literature reviewed, management of crown rot under field conditions with crop rotation had been very effective but some farmers choose to grow continuous wheat which leads to high crown rot incidence. More research is required to develop other approaches such as managing the disease through displacement of Fp from wheat stubble with the help of potential fungal antagonists. Since this approach is ecofriendly and excludes the use of toxic chemicals, it is worth exploring as an option for integrated disease management.

BIOLOGICAL CONTROL OF CROWN ROT

Baker and Cook (1974) defined biological control as "the reduction of inoculum density or disease producing activities of a pathogen or parasite or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists." Biological control (biocontrol) agents can include virulent or hypovirulent individuals or populations within the pathogenic species. It can be achieved through cultural practices (habitat management) that create an environment favorable to antagonists, host plant resistance, or both; through plant breeding to improve resistance to the pathogen or suitability of the host plant to the activities of antagonists and through the mass introduction of antagonists, nonpathogenic strains, or other beneficial organisms.

The recovery of Fp from wheat straw was found to be adversely affected when the straw was exposed to conditions favoring increased microbial activity and subsequent straw decomposition (Burgess and Griffin 1968). They found the recovery of Fp was greatest when straw was exposed at low temperatures at all relative humidities. In another experiment the recovery was usually reduced by more frequent wetting of the soil. These results were associated with the increased activity of the soil microflora as estimated by the degree of straw decomposition and the number of species isolated from the straws (Burgess and Griffin 1968).

An isolate of *Burkholderia* (*Pseudomonas*) *cepacia* (A3R) was found to be effective in reducing crown rot infection in wheat in glasshouse and field experiments (Huang and Wong 1998). The bacterium applied as a soil drench (2.5×10^9 cfu/g soil) was more effective than a coating on wheat seed (3.4×10^7 cfu/seed) under glasshouse conditions. In field experiments, the bacteria were applied as a soil drench (1.8×10^{10} cfu/m row) and disease severity in the bacteria inoculated treatments was significantly less in a silt loam than in a sandy loam under both greenhouse and field conditions.

Wong *et al.* (2002) did some controlled environment experiments on the displacement of Fp by *Trichoderma* species. They found that survival of Fp in wheat straw infested with the pathogen was significantly reduced when the straw was sprayed with *Trichoderma koningii*, Tk (isolate BM1) spores and buried for four months in moist (-0.3MPa) or wet (-0.03MPa) soil at a temperature of 15°C or 25°C. After 6 months at 25°C in the moist soil Fp was completely eliminated from straw pieces and replaced by Tk. In this study Wong *et al.* (2002) compared seven antagonistic isolates of *Trichoderma* spp. sprayed on infested straw and found that Tk isolate (TW3) and a *T. harzianum* isolate (TW7) reduced the survival of the pathogen to low levels (3-30%) after 3 months at 30°C. They suggested that it may be possible at least in the summer-dominant rainfall wheat areas of Australia, for *Trichoderma* spp. to reduce considerably the inoculum of Fp during the 6 month fallow period.

Studies by Donovan *et al.* (2006) explored the effects of stubble management practices and crop rotation on suppression of soil borne pathogens in various trials in northern New South Wales in wheat cropping soils. They found greater displacement of Fp from straw pieces buried in soil from zero-till treatments at one site out of two studied. This gives evidence for enhanced displacement from buried straws in at least some sites where residue retention had been practiced. It is thus concluded that limited work has been done on biological control of the crown rot fungus but there are some indications of promise.

BIOLOGICAL CONTROL OF RELATED SPECIES

Biological control of *Fusarium graminearum*, which causes head blight in wheat and barley, was reviewed by Luz *et al.* (2003). They summarized the outcomes of several studies in which microorganisms (bacteria and yeasts) were screened through antibiosis for their inhibitory effects against *F. graminearum* in glasshouse and field conditions. The aim was to intervene in the fungal life cycle at spikelet infection, seedling blight, survival stage (ascospores in cereal debris) and the systemic movement of *Fusarium* within the rachis of wheat spikes. In most studies, biocontrol agents were sprayed on spikes of wheat and barley at flowering to reduce infection by *F. graminearum*. Significant levels of control were found by their use against head blight disease. However, because of the differences in the infection process, this work is not directly relevant to crown rot.

Knudsen *et al.* (1999) investigated the relationship between soil suppressiveness and C:N content of the soil microbial biomass and microbial activity on suppression of brown foot rot of cereals by *F. culmorum*. Their findings indicate that the competitive saprophytic ability of this fungus is poor, but that high microbial biomass and activity were not always correlated with high disease suppression. They suggested that specific organic amendments, such as mulching with straw and the practice of using lucerne as a breakcrop in cereal cultivation may influence inoculum potential of *F. culmorum*.

Studies by Rasmussen *et al.* (2002) explored the relationship between soil suppressiveness to seedling blight of barley caused by *F. culmorum* and the soil cellulolytic activities of β-glucosidase, cellobiohydrolases and cellobiohydrolase. A significant correlation was found, where soils representing the highest disease suppression had the highest activity. They proposed that soil cellulolytic activity can be used as an enzymatic approach to study the microbial turnover in soils and as an indicator of suppressiveness to seedling blight of barley caused by *F. culmorum*.

Saprophytic fungi, obtained from cereal tissues or necrotic tissues of other crops, were screened by Luongo *et al.* (2005) for their ability to colonize wheat straw and maize stalks to suppress sporulation of pathogenic *Fusarium* spp, normally *F. culmorum*, *F. graminearum*, *F. proliferatum*, and *F. verticillioides*. Results of bioassays conducted under controlled conditions were variable among *Fusarium* spp. and host substrates for most antagonists tested, such as yeasts, *Trichoderma* spp. and nonpathogenic *Fusarium* spp. (*F. equiseti*). Non-pathogenic *F. equiseti* applied to pieces of maize stalks or flowering ears under field conditions, showed strong antagonism against pathogenic *Fusarium* spp. Such isolates may have ecological characteristics, e.g., in substrate utilization and temperature requirements, which are very similar to those of pathogenic *Fusarium* spp. Isolates of *T. harzianum* and *T. viride* had only moderate effects on sporulation of *Fusarium* spp., and the effects on the various *Fusarium* spp. varied on maize stubble.

El-Naggar *et al.* (2003) conducted bioassays with six different isolates (no. 01, 02, 03, 09, 10 and 11) of saprophytic antagonistic fungi to suppress sporulation by *F. culmorum* and *F. graminearum* on cereal debris. Isolates 10 and 11 were more effective at low temperature of 5°C, while isolate 02 worked well at the higher temperature of 15°C but failed at 5°C. On the other hand, antagonism varied little when antagonists were tested on three different cereal substrates at 15°C. In their studies they did not comment on what their antagonistic organisms actually were.

A study by Kollmorgen (1974) investigated the effects of the bacterial antagonist *Streptomyces griseus* (2-A 24) on the survival of *F. avenaceum* in oat straw. The percentage of straws in which *F. avenaceum* was detected after burial in irradiated soil either not recontaminated or recontaminated with soil microflora for 17 and 34 weeks was reduced by *S. griseus* in both soil types.

Fernandez (1992) found *Trichoderma harzianum* inoculum effective in colonizing infested wheat and black oat straw and reducing the incidence of *Fusarium graminearum* and

other *Fusarium* spp. in wheat. However, it had no significant effects on decomposition (dry weight loss) of either type of residues.

Interesting work has been done by Pereyra *et al.* (2004) on the replacement of *Gibberella zeae* (*F. graminearum*) in wheat residues by other species of *Fusarium* in the field. They found that a reduction in the colonization of residues by *G. zeae* over a 2 year study was positively correlated with the reduction in the amount of residue by decomposition. They suggested that burial of residue contributes to greater microbial activity which accelerates decomposition. Recovery of *G. zeae* declined over time in all residue placement treatments, whereas the proportion of other *Fusarium* spp. increased. Their studies suggested that species of *Fusarium* such as *F. poae* which had already colonized the residue or other *Fusarium* spp. such as *F. oxysporum* and *F. solani*, which were present in adjacent soil, could be effective colonizers of partially decomposed wheat residues. They suggested that *G. zeae* had the ability to colonize wheat residue as a pathogen before the arrival of saprophytes and species such as *F. equiseti*, *F. oxysporum*, *F. solani* and *F. sporotrichioides*, which increased over time in their study. They could overcome *G. zeae* populations in stubble over longer time periods because of the greater competitive saprophytic ability of the other *Fusarium* spp.

The above experiments have shown that a number of potential antagonists are capable of suppressing the sporulation of potentially toxigenic *Fusarium* spp. on debris from cereal crops. In this way their intentional application might limit the carry-over of *Fusarium* from preceding cereal crops to new crops (Kohl *et al.* 2003). There is no evidence from the literature that spores are important in the epidemiology of crown rot so suppression of sporulation is not likely to be important. The infection court of head blight is different from crown rot, so studies on biocontrol on the heads of wheat have limited relevance to it. Therefore the survival stage of the crown rot fungus (residues of wheat and barley) is the major target area to be given emphasis for further studies.

BIOLOGICAL CONTROL OF OTHER PATHOGENS IN CEREAL RESIDUES

Microorganisms can be applied to crop residue to suppress inoculum production of residue-borne phytopathogens. Studies on biological control to reduce inoculum of the tan spot pathogen *Pyrenophora tritici-repentis* in wheat residues have been undertaken by Pfender *et al.* (1988, 1991b, 1993b). In their investigations they used the basidiomycete *Limonomyces roseipellis* (Pfender *et al.* 1991b, Pfender *et al.* 1993b) and other naturally occurring fungi such as *Laetisaria arvallis*, *Epicoccum nigrum* and *Sterile* spp. (Pfender *et al.* 1993b) as biological control agents for suppression of ascocarp production in *Pyrenophora tritici-repentis*. Their studies with wheat straw showed that the antagonist *L. roseipellis* could displace the prior colonist *P. tritici-repentis* from the straw pieces and they suggested that such an antagonist may be able to change the fungal community of straw or other natural substrates to which it is applied (Pfender *et al.* 1996).

Clarkson and Lucas (1993) devised tests to identify fungal antagonists which were capable of competing with the pathogen *Pseudocercosporella herpotrichoides* (the cause of eye spot of cereals) on straw, and which could also suppress inoculum production of the target fungus. They isolated 228 fungal species from wheat seedlings, plants or straw which were screened for antagonistic activity against other fungal pathogens. Potential antagonists were selected on the basis of inhibition or overgrowth of *P. herpotrichoides* on several contrasting media. They found that co-inoculating straw with the pathogen and test antagonists reduced disease severity in pot trials using straw as an inoculum source. When straw, preinoculated with the pathogen was used, fewer antagonists proved effective in suppressing eye spot disease. However, one isolate of *Trichoderma* spp. gave positive results both *in vitro* and in infection trials using different inoculation procedures, which indicated that it could be an effective antagonist of *P. herpotrichoides* during the saprophytic survival phase of the pathogen life cycle.

Research has been done on other crop residues such as chickpea residue by Dugan *et al.* (2005). They worked on biological control of *Didymella rabiei* (conidial state = *Aschochyta rabiei*) by exploring and identifying other fungi residing in chickpea debris. They tested several potential primary colonizers against *D. rabiei* and found that *Aureobasidium pullulans* consistently grew faster than *A. rabiei*, and excluded it from the substrate. Their results suggested that antagonists like *A. pullulans* and *Clonostachys rosea* can inhibit *A. rabiei* and its sexual stage D. *rabiei* in chickpea residues.

FUNGI IN WHEAT RESIDUES

Wheat crops after being harvested leave straw which consists of dead leaves, leaf sheaths and stems on the soil surface. With the course of time and environmental conditions, the retained stubble will become a habitat for colonization for the microbial community. The reason behind leaving stubble on the surface is "conservation tillage". It is an agricultural practice in which substantial amounts of crop residue are left on the soil surface, rather than being plowed into the soil during the intercropping period. This practice has several benefits for farmers like reduced fuel usage and increased retention of moisture and soil. However, some problems are more severe like an increase in plant diseases, including crown rot, whose pathogens survive well in surface-borne crop residues.

Pfender and Wootke (1988) studied the community structure of different fungi resident in wheat straw naturally infested with the fungi *Pyrenopora tritici-repentis* and *Septoria nodorum* relative to placement. They found pioneer colonizer fungal communities on above-soil straw, secondary saprophytes in the on-soil straws and actinomycetes and bacteria in buried straws. A similar trend of hierarchy of various organisms on wheat residues was found by Magan (1988).

Among the colonizers of crop residues, the earliest are parasitic fungi associated with living tissues and as the tissue senesces, the stubble residue is colonized in a general trend of

fungal succession first by a group of primary saprophytes with a weak parasitic ability like *Alternaria*, *Cladosporium*, *Epicoccum* followed by secondary saprophytes (*Fusarium*, *Acremonium, Verticillium*) (Magan 1988). The fungi most commonly associated with mycoflora of cereal (wheat and barley) straw consisted of yeasts, *Aureobasidium pullulans* and filamentous fungi such as *Alternaria alternata*, *Epicoccum nigrum*, *Fusarium* sp. and *Verticillium* sp.

The major components of straw are cellulose and hemicelluloses, which contribute 75-80% of the dry weight, 5-10% uncharacterized water solubles and 10% lignin (Harper and Lynch 1985). Thus the fungi which are able to rapidly utilize the solutes and cellulose would gain an ecological advantage in straw colonization and decomposition.

The major abiotic factors which determine both primary and secondary colonization and rate of decomposition are the water potential of the substrate, temperature and possibly pH and gaseous compounds (Magan and Lynch 1986). The decomposition of straw studied by Magan (1988) showed that higher temperature (25°) had greater dry matter loss than at 15° at -0.7 MPa, but the loss was only 7-10% over 12 weeks. Thus different factors such as water potential, temperature, and cellulolytic ability of different fungi could be involved in decomposition of cereal residues.

A range of fungi isolated from decomposing straw were compared for their ability to grow on straw and its components by Harper and Bowen (1985). Activity of fungal isolates was measured by clearing of cellulose and weight loss from straw. In all tests *Trichoderma harzianum* showed significantly high growth and activity. The studies of Harper and Bowen (1988) on decay of cereal straw in soil concentrated on the identity and activity of cellulolytic organisms. Only 25% at most of the cellulose and hemicelluloses in straw are available to cellulases; the reminder is made unavailable because of the presence of lignin. Lignin-degrading basidiomycetes have been isolated from straw. In laboratory tests some of the basidiomycetes degrade much more of the straw than the cellulolytic isolates (Harper and

Bowen 1988). The extent of increase in decay probably depends on the natural basidiomycete population in soil.

Magan and Lynch (1986) determined the effect of water potential (WP) and temperature on growth and cellulolysis of different soil fungi which colonized cereal straw *in vitro*. They observed that *F. culmorum* and *T. harzianum* colonized straw pieces best at high potential (-0.7 MPa) while only *F. culmorum* and *Penicillium* spp. grew at low potential (-7.0MPa). Magan (1988) determined the dominant colonizers and rate of deterioration of straw held at different WP and temperatures (15 & 25°C) over a period of 12 weeks. He found that at 15°C and -7.0 MPa *Penicillium* spp. were predominant and at -2.8 MPa *Mucor*, *Rhizopus* and *Fusarium* spp. were present. Saturated straw was colonized rapidly by *Mucor*, *Rhizopus*, *Fusarium* and sometimes *Trichoderma* spp. The percentage dry matter lost over 12 weeks varied with both temperature and water availability.

However, little work has been done in terms of displacing one fungus by an other competitive or antagonistic fungus present in cereal straw for managing residue borne pathogens like Fp. One possible approach could be manipulation of the microbial community associated with residue in such a way as to displace the target fungus Fp by fungal antagonists thus finding an ecologically safe way to manage residue-borne fungi with the help of antagonists.

EFFECT OF ENVIRONMENTAL FACTORS ON FUNGAL INTERACTIONS

From the literature reviewed, few studies have been done on the effects of temperature and water potential on interactions of Fp with other fungal species. The most relevant work presented here showed the effect of these interactions on *Aspergillus* and other spoilage fungi.

The effect of water availability (a_w) 0.995-0.90 and temperature (18-30°C) on *in vitro* interactions between an ochratoxin producing strain of *Aspergillus ochraceus* and six other spoilage fungi was assessed in dual culture experiments on a maize meal-based agar medium (Magan and Lacey 1984, Lee and Magan 1999b). It was observed that with freely available water (0.995 a_w) *A. alternata* and *A. niger* were dominant, with mutual antagonism between *A. ochraceus* and *A. flavus* at 25-30°C. In the driest condition (0.90 a_w) there was mutual antagonism between *A. ochraceus* and the two *Eurotium* spp. Under all conditions tested Index of Dominance for *A. ochraceus* was much higher than for other competing species combined suggesting that *A. ochraceus* was a good competitive colonist able to replace a number of other species.

Lee and Magan (1999a) did experiments on environmental factors (water availability and temperature) and nutritional utilization patterns affecting the niche overlap indices between *A. ochraceus* and other spoilage fungi. They found that with freely available water (0.995 a_w) there were ecological similarities between all *Alternaria* species studied in terms of exploiting the same C sources at both 18 and 30°C. However, under water stressed conditions their performance was different. Marin *et al.* (1998a) studied the effects of temperature (15, 25°C) and water activity (a_w, 0.995-0.85) on growth and interactions between fumonisin producing isolates of *Fusarium moniliforme* and *F. proliferatum* with some other fungi from maize grain. *Fusarium* species appeared to be dominant only at high water availability while *Eurotium* species dominated at lower.levels. Magan and Lacey (1984) did similar experiments on the effects of water activity, temperature and substrate on interactions of field and storage fungi.

Zhang and Pfender (1993b) studied the effects of alternate wetting (6, 12, 24, or 48 hours) and drying durations on ascocarp suppression by selected antagonistic fungi in wheat straw infested naturally or artificially with *P. tritici-repentis*. They observed that ascocarp production was reduced by several antagonists under 24 and 48 h wetting periods but not under 6 and 12 h compared to uninoculated control. This showed that ascocarp suppression in wheat straw by selected antagonistic fungi occurred only under relatively long (>12 h) wetting periods. Pfender *et al.* (1991b) examined the effect of water potentials (-0.1 to -7.0 MPa) on microbial antagonism to *P. tritici-repentis* in wheat residues and found that inhibition of pseudothecia occurred only at the wettest level tested, -0.1MPa.

From the literature suryed little been done about the effects of parameters like temperature and water potential on interaction patterns of *Fusarium pseudograminearum* and other non-pathogenic species. of *Fusarium*, i.e., *Fusarium equiseti* and *F. nygamai* and antagonists like *Trichoderma harzianum*. More work is required for studying such factors so as to predict environmental factors which favour activity of Fp or antagonists.

EFFECT OF NUTRITION ON FUNGAL INTERACTIONS

Yi *et al.* (2002) conducted greenhouse tests to determine *Fusarium graminearum* (*Fusarium* head blight) populations on crop residues affected by nitrogen and fungicides application. They found that fertilization with calcium ammonium nitrate enhanced the population levels of *F. graminearum* and sometimes reduced residue decomposition. In contrast, nitrolime (Calcium Cynamide) reduced the population level of *F. graminearum* without affecting the decomposition process.

Celar (2003) investigated the role of competition for nitrogen in interactions of pathogenic fungi (*Fusarium solani*, *F. sambucinum* and *F. moniliforme*) and antagonists (*Trichoderma viride*, *T. harzianum*, *T. koningii* and *T. viride*). Antagonistic fungi used nitrogen as ammonium at statistically higher rates during the first six days of cultivation.

After first using nitrogen as ammonium they switched to the nitrate form of nitrogen as ammonium was nearly used up. In comparison, the *Fusarium* spp. used nitrate significantly faster than the antagonistic fungi and used the two sources of nitrogen simultaneously. Even when there was ample ammonium in the beginning, they used nitrite at the same time. Celar (2003) concluded that using nitrogen as one variable; *Fusarium* spp. had a relative advantage over antagonistic fungi by the fact that they were able to use both forms of nitrogen simultaneously.

Pereyra *et al* (2004) suggested that N applications could reduce survival of *Fusarium* species in straw apart from the demonstration by Kollmorgen (1974) that urea reduced survival of *F. avenaceum* in non-sterile soil.

CONCLUSION

Researchers have used different microorganisms as biocontrol agents in several residue-borne diseases in wheat and chickpea by controlling either pathogen spore production or displacement of the pathogen by other potential antagonists. Studies by plant pathologists on crown rot disease have not explored the biocontrol agents present in stubble pieces which could be able to displace Fp from stubble. Antagonists such as *Trichoderma* spp. and other competitors such as related species of *Fusarium* are worth exploring for their abilities against Fp. A critical review of the literature suggested that there are not many records of work done on the use of potential antagonists for Fp displacement from wheat residues. So there is a need to take further steps for screening biocontrol agents for crown rot management and to test the efficacy of antagonists under different abiotic factors like temperature and water potential. Nutrient requirements of Fp and antagonists should also be investigated, since competition could lead to survival of either pathogen or antagonist. My research project is based on these research gaps that have been identified.

CHAPTER 3
SELECTION OF FUNGI AND PROTOCOLS FOR EXPERIMENTS

INTRODUCTION

The aim of this chapter was to identify potential antagonists and to establish the basic experimental protocols for studying antagonism. Wheat stubble was collected from commercial fields to determine the occurrence of different fungal species. Unidentified Fungal species were identified using molecular techniques.

Tests were done with the most common fungi in dual culture interactions to select potential (typical) antagonists against Fp. In these experiments, the effects of factors such as water potential, and temperature were examined to determine the effectiveness of various antagonists.

A plant bioassay was developed to test the effect of displacement on decline in inoculum potential. Quantitative detection of Fp from straw inoculated with antagonists grown in Petri plates on SNA medium was attempted using a real time-PCR technique. Studies were conducted to investigate the effects of culture filtrates of different fungi on themselves and on growth of other fungi tested in Petri plates.

MATERIAL AND METHODS

Collection of field samples of stubble

Standing wheat stubble was collected on 19 May 2005 from 5 fields in the Liverpool Plains, between Breeza (31.27°S, 150.46°E, 286m) and Gunnedah, (30.98°S, 150.25°E, 285m) New South Wales. From each field, one tiller was selected from the stubble of each of 10 plants. These were thoroughly washed under tap water to remove soil particles. Roots arising from each piece were trimmed and soil cleaned off. Segments were cut from the basal 4-5 cm and from above the second node, 8-10 cm above the base. The stubble segments were surface-sterilized with 1% NaOCl in 10% ethanol for 1 min, and 3 small pieces from each segment

were plated onto 1/4 strength potato dextrose agar (1/4 PDA) medium containing powdered PDA (Merck) 9.8 g and agar 11.3 g per litre supplemented with streptomycin sulphate (0.1g/L) and penicillin G (0.03g/L) and incubated in the dark at 25°C. Colonies emerging from the stubble pieces were subcultured onto 1/4 PDA 4-5 days later. Fungi were identified using morphological criteria, with selected isolates being identified with molecular criteria.

Dual culture test for screening antagonists

Out of the different fungi isolated, 14 of the most common fungi were tested in dual culture tests on ¼ PDA for antagonism towards an isolate of *F. pseudograminearum* from wheat from Tamworth, NSW. The fungi tested were representative isolates of *Nigrospora sphaerica* (Ns), *Epicoccum nigrum* (En), *Fusarium oxysporum* (Fo), *Bipolaris sorokiniana* (Bs), *Fusarium equiseti* (Fe), Sterile 1 and Sterile 2 (S1, S2), *Alternaria sp.* (Alt), *Fusarium nygamai* (Fn), *Acremonium* sp (Acr), *Trichoderma* sp. 1 and Trichoderma sp. 2 (Tri 1, Tri 2), *Rhizoctonia solani* (Rs) *and Fusarium chlamydosporum* (Fc).

The target fungus Fp and the test fungus were placed 2.5 cm apart on ¼ PDA in 9cm Petri plates and incubated in the dark at 25°C. After 3-4 days Petri plates were observed for any signs of antagonism including zone of inhibition, inhibition of Fp growth by the other fungus, or changes in fungus colony colour at the point of contact of the two fungi.

Molecular identification of fungi

Based on the results of the dual culture assay, four fungi were selected for further experiments. These were *F. equiseti*, *F. nygamai*, *Alternaria* sp. and *Trichoderma* sp. 1. The identity of these, as well as the isolate of *F. pseudograminearum* used in the experiments, was confirmed by gene sequencing. The fungi were grown on ¼ PDA for 5-6 days at 25°C. The fungal DNA was extracted by scraping 100 mg mycelium into a 2 ml microcentrifuge tube, which was then freeze-dried. A 3 mm tungsten carbide bead was added and the tubes were

shaken at 30 Hz for 1 minute in a Qiagen MM300 mixer mill, followed by extraction with Qiagen DNeasy Plant Mini kits according to the manufacturer's instructions. The resulting DNA was used as a template to amplify the ribosomal RNA ITS 1 and 2 regions of *Trichoderma* and *Alternaria* sp., and the partial translation elongation factor 1-α sequence for *Fusarium* spp. The primers used were ef1 and ef2 (Geiser *et al.* 2004) for the *Fusarium* spp. and ITS1 and ITS4 (White T.J. *et al.* 1990) for *Trichoderma* and *Alternaria* sp.

The polymerase chain reaction (PCR) was carried out using 1.25 U Taq polymerase (Qiagen), 0.2 μM of each primer, 200 μM dNTP and 1μL template DNA in QIAGEN PCR buffer in 50 μL reactions. The PCR was done in a Corbett PalmCycler with 30 cycles of denaturation at 95° for 30s, annealing at 50, 51 or 52° for partial elongation factor 1-α primers and 53° or 55°C for ITS primers for 1 min and extension at 72°C for 1 min and a final extension at 72° C for 10 min. For each species, the PCR product that gave the clearest band on 1.5% agarose gels was selected for sequencing. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN). The PCR-amplified fragments were sequenced in both directions using the PCR primers at SUPAMAC, University of Sydney. Forward and reverse sequences were aligned and edited manually. All sequences were subjected to BLASTn searches against the nucleotide databases at the National Centre for Biotechnology Information (NCBI). *Fusarium* sequences were compared with the FusariumID database (Geiser *et al.* 2004) and the *Trichoderma* sequence was compared with the *Trich*OKEY version 2.0 database (Druzhinina *et al.* 2006).

Displacement of *F. pseudograminearum* from straw pieces

Clean barley straw was chopped into pieces of about 5 cm length including one node. Straw pieces were soaked in distilled water in a 500 ml beaker overnight. The next day, when all the stubble pieces were moistened uniformly, water was drained from the container. The straw was placed into autoclavable plastic bags (polyester oven bags). Non-absorbent cotton wool

plugs were used to cover the mouth of plastic bags for air exchange, and mouths of these bags covered with aluminium foil. The bags were autoclaved for 15 minutes on each of 2 successive days.

Ten agar plugs were cut from cultures of Fp on ¼ PDA and placed on the straw pieces in these bags. These straw pieces were left for 2 weeks at 25° C with occasional mixing so that Fp inoculum could spread uniformly on all stubble pieces. Stubble pieces were then dried under laminar flow for 2 days.

From the previous experiments, 4 different antagonists were selected and one control treatment was used. The fungi used were *Trichoderma harzianum*, *Fusarium equiseti, Fusarium nygamai,* and *Alternaria infectoria.* These fungi were grown on 9 cm Petri plates containing SNA (Spezieller Nahrstoffarmer Agar; Leslie and Summerell (2006)). The composition of this medium was KH_2PO_4 1 g, KNO_3 1 g, $MgSO_4.7H_2O$ 0.5 g, KCl 0.5 g, glucose 0.2 g, sucrose 0.2 g and agar 20 g per litre. The fungi were allowed to grow until they had reached the edge of the plates. Eight pieces of Fp-colonized straw were placed into each Petri plate and the plates were sealed with Parafilm® to reduce evaporation. They were then incubated at 25°C in darkness.

At 2, 4 and 6 weeks, 5 replicate plates of each fungal treatment and the control were harvested. The straw pieces were removed from each plate, surface sterilized in 1% NaOCl in 10% ethanol for 1 min, blotted dry, and plated onto ¼ PDA. After 4 days incubation at 25° C in darkness, each straw piece was scored for displacement of Fp by noting the proportion of its length from which Fp (red colony) or the other fungus was growing. A 5-point scale (Table 3.1) was used. An index of displacement was calculated for each replicate using the formula:

$$DI = \Sigma\, n_i \times score_i$$

Data were analysed using SPSS software for the Kruskal-Wallis test.

Table 3.1. Five point scale for assessing displacement of *Fusarium pseudograminearum* from straw pieces by antagonistic fungi

Score	Description
0	Completely *F. pseudograminearum*
1	Predominantly *F. pseudograminearum*
2	Approximately even *F. pseudograminearum* and the antagonist
3	Predominantly the antagonist
4	Completely the antagonist

Effect of water potential on displacement of Fp from straw by antagonists

This experiment was based on the previous experimental methods except that the medium SNA was supplemented with different salt concentrations to lower the water potential to nominal values of -1, -2 and -5 MPa. The calculated osmotic potential of SNA was similar to PDA, so unamended SNA was assumed to have a water potential of about -0.3 MPa. To get the lower water potentials, the following amounts of NaCl were added to the basal SNA: -1 MPa, 8.28 g/L; -2 MPa, 20.32 g/L; and -5 MPa; 59.96 g/L.

The plates were incubated in sealed plastic boxes with containers holding 500 ml of NaCl solutions to buffer the relative humidity. Concentrations of these solutions were calculated as described by Liddell (1992).

The Petri plates were incubated at 25°C for four weeks, then displacement was recorded as described in Table 3.1.

An identical experiment was done on stubble decomposition (weight loss) after 4 weeks. Weighing of 8 straw pieces were done at the time of set up and after 4 weeks straw pieces were taken out from Petri plates, put in paper bags and dried in an oven at 80° for two days. The difference in weight was calculated for each treatment.

The experimental design for both experiments was a completely randomised design with five replicates. Data were analysed in SPSS by ANOVA.

Plant bioassay for inoculum potential in relation to displacement

Barley straw pieces pre-colonized with Fp were incubated on SNA cultures of *T. harzianum*, *F. equiseti*, *F. nygamai* and *A. infectoria* at 25° for 4 weeks as described above. Two control treatments were used: pre-colonized barley straw incubated on SNA alone, and pre-colonized barley straw that was not incubated on SNA.

Plastic crates with internal dimensions of 518×335 mm and a mesh base (Nally IH025, Viscount Plastics, Melbourne) were filled to a depth of 10 cm with peat:sand:loam (1:1:1 v/v) potting mix. Six rows of 20 seeds of wheat cv. Sunco were placed across the short dimension of the trays on top of the potting mix, with the rows 50 mm apart. The seeds were covered with a layer of air-dried and sieved (2 mm) black clay soil. The soil came from a University of New England trial site at Laureldale Farm, Armidale, where crown rot previously occurred in wheat and triticale crops, but was obtained from locations within the site where susceptible crops had not been grown. The crates were placed in a controlled environmental chamber at 25°C day/15°C night with a 12 h photoperiod at 600 $\mu M/m^2/sec$ photosynthetically active radiation, and 65% humidity. Crates were watered as required with a gentle spray from above.

Trays were inoculated 2 weeks after sowing by placing a row of barley straw pieces from each treatment end-to-end in contact with each row of seedlings. There were 5 replicated crates in each experiment and it was conducted twice on separate occasions, using independently prepared batches of infested barley straw pieces.

Plants were harvested 4 weeks after inoculation. The base of the plant, including the subcrown internode and the basal 2 cm of the shoot, were dissected out and surface sterilized with 1% w/v sodium hypochlorite in 10% alcohol for 1 minute, then plated onto ¼ PDA supplemented with 0.1 g/L streptomycin sulphate, 0.03 g/L penicillin G and 0.075 g/L

pentachloronitrobenzene (as Terraclor). Cultures were incubated at room temperature under mixed white and near-ultraviolet light with a 12 h photoperiod for 5-7 days. Identification of Fp and other antagonists was done by their distinctive colony morphology, or by microscopic examination of conidia for doubtful colonies. The incidence of infection was tested by ANOVA using a randomised complete block design.

Quantitative detection of *Fusarium pseudograminearum* from stubble pieces

Precolonized barley straw pieces were incubated for 4 weeks on SNA cultures of antagonist as described above, with a non-antagonist control, and a control consisting of straw pieces that had not been incubated on SNA. The experiment was replicated five times. The stubble pieces with different antagonistic fungal treatments were freeze-dried, then cut into very fine pieces with a sterile scalpel wearing clean gloves to avoid contamination. The finely cut stubble pieces were placed into 2 ml locking microcentrifuge tubes with a 3 mm tungsten carbide bead and shaken at 30 Hz for 1 minute in a Qiagen MM300 mixer mill, followed by extraction of 0.02 g plant material from each sample with Qiagen DNeasy Plant Mini kits according to the manufacturer's instructions. Real time PCR was done in a Corbett Rotor-Gene 3000 Thermal Cycler using a Qiagen Quantitect SYBR Green kit. Mastermix for RT-PCR was prepared using 2×Quantitect Syber Green PCR 12.5 µL, 0.3 µM each primer, template DNA 1 µL and RNase-free water 10.75 µL with total volume of 25 µL. The species-specific primers cgctcgacgactcgacac and caggcgtacttgaaggaacc amplified a 227 bp region of the translation elongation factor 1α gene (D. Backhouse, unpublished). RT - PCR was programmed for initial activation step for 15 min at 95° followed by 3 step cycle which included denaturation for 15s at 95°, annealing for 20s at 55° and extension for 30s at 72° for 40 cycles.

Culture filtrate studies

Culture filtrates of Fn, Fe, Ai, Fp and Th were used for testing inhibitory effects on the radial growth of other fungi. Spores of all fungi were washed with sterile water from colonies on ¼ PDA. Spore concentrations of each fungus was adjusted to 1×10^5 spores/ml and added into PDB flasks. The medium used was 100 ml potato dextrose broth (24 g/L) in 250 ml conical flasks. Flasks were incubated on a rotary shaker for 2 weeks at 120rpm at 20°C. Culture filtrate was extracted by vacuum pressure through 9 cm Whatman no. 1 filter paper. ¼ PDA medium was used for inhibition activity on 9 cm Petri plate with varying concentrations of Th culture filtrate. Different volumes (1 and 4 ml) of filtrate were passed through sterile 0.22 µm filters into 20 ml ¼ PDA. Fungal plugs of all fungi were placed onto the Petri plates and radial growth rates of all fungi were measured to check differences in growth rates. The experiment was replicated three times with each fungal treatment. Data were analysed by ANOVA separately for each test fungus, followed by mean separation using Tukey's HSD.

RESULTS

Isolation of fungi from field samples of straw

A diverse range of fungi were recovered from straw from the field sites of the Liverpool Plains area (Table 3.2). The saprophytic fungus *Alternaria* sp. was the most frequently isolated species mainly from stem parts of stubble followed by a Sterile sp. 1 from basal parts of the stubble. The crown rot fungus, Fp was the most frequently isolated species of *Fusarium*, occurring on over one third of stubble pieces. The other *Fusarium* spp. including Fn, Fo, Fe and Fc were found mostly on the basal parts of the stubble. There were two *Trichoderma* spp. found which were designated as *Trichoderma* 1 (Tri1) and *Trichoderma* 2 (Tri2). Tri2 was more abundant than Tri1 and both were found only from the basal part of the wheat stubble.

Table 3.2 Frequency of isolation of fungi from two sections of cereal stubble averaged across 5 sites

Species	Percent isolation	
	Basal	Stem
Acremonium sp.	12	2
Alternaria sp.	16	64
Basidiomycete	2	2
Bipolaris sorokiniana	14	14
Epicoccum nigrum	0	6
Fusarium chlamydosporum	2	0
F. equiseti	14	0
F. nygamai	8	4
F. oxysporum	8	0
F. pseudograminearum	38	34
Melanospora sp.	2	0
Nigrospora sphaerica	0	4
Rhizoctonia solani	16	8
Sterile sp. 1	50	30
Sterile sp. 2	10	4
Sterile sp. 3	12	8
Trichoderma sp. 1	2	0
Trichoderma sp. 2	8	0

Dual culture interactions

After 3-4 days of incubating different antagonists with the target fungus Fp, there were variations in signs of antagonism. On the 3rd day of observation a definite zone of inhibition of Fp by Tri 1 and Tri 2 was seen. The colour of the Fp colony had changed, possibly due to lysis of its hyphae. However, other combinations like Bs, En, Ns, S1, S2, Rs and Fc against Fp did not show any change in growth pattern of Fp. In these combinations the fungal colonies grew towards each other, met and overlapped without showing any clear cut zonation or lysis of hyphal tips. *Alternaria* sp. growth was inhibited by Fp and thus it was not a strong antagonist. On the 6th day of observation Fe and Fn showed strong competitive antagonism of Fp and no further growth of the Fp colony occurred after contact. Of the two species of *Trichoderma*, on the 6th day it was noticed that the Fp colony was still growing towards Tri2 but not towards Tri 1, suggesting it was better antagonist.

Three species were selected to represent the range of antagonistic interactions seen, i.e., Fe, Fn, and Tri 1. *Alternaria* sp. was chosen as a non-antagonistic control, representing the most abundant fungus on wheat stubble.

Molecular identification of fungi

Good quality sequences were obtained for the ITS regions for *Alternaria* in forward and reverse directions and for *Trichoderma* (forward only) and for the partial TEF 1α sequence for *F. equiseti* (forward only), *F. nygamai* and *F. pseudograminearum* (both forward and reverse). High-quality sequence lengths obtained were *Alternaria* ITS 600 bases, *Trichoderma* ITS 549 bases, *F. equiseti* EF 634 bases, *F. nygamai* EF 656 bases, and *F. pseudograminearum* 675 bases.

The most similar sequences to the ITS sequence of the *Alternaria* isolate were several from *Lewia* (*Alternaria*) *infectoria*, which were more than 99% identical. The most similar sequence was AY154692, which was identical at 597 of 598 bases. Among these highly

identical sequences were some from fungi placed in the *Alternaria infectoria* (Ai) species group by (Serdani *et al.* 2002).

For the isolate of *Trichoderma*, a search on the '*Trich*OKEY' database identified it as *T. harzianum* with high reliability.

When the TEF 1α sequence of the *F. equiseti* isolate was searched against the Fusarium ID database, it showed 98% similarity to *F.* sp. cf. *equiseti* NRRL 25795. A BLASTn search at NCBI showed that the most similar sequence was EF453007 from *Fusarium* sp. NRRL 43680 (O'Donnell and Sarver 2007) which was more than 99% identical. This isolate presumably represents an unnamed segregate species from *F. equiseti*, but will be referred to as *F. equiseti* (Fe) in this thesis.

The TEF 1α sequence of the *F. nygamai* (Fn) isolate was more than 99% identical to the sequence from the ex-holotype isolate NRRL 13448, and that of the *F. pseudograminearum* isolate was 100% identical to the sequence from NRRL 28062, the type strain of *F. pseudograminearum*, when compared with the FusariumID database.

The displacement assay

The effect of fungal treatment on displacement was highly significant ($P < 0.01$) at all sampling dates. The 4 different antagonists selected differed considerably in their efficacy in displacing Fp from straw pieces compared with the control in which Fp was yielded from all straw pieces at all times (Fig. 3.1). Th was highly effective in displacement of Fp at 2, 4 and 6 week intervals followed by Fn and Fe. At 4 and 6 weeks Fn displaced Fp significantly more than Fe did. The effect of non-antagonistic Ai. was negligible.

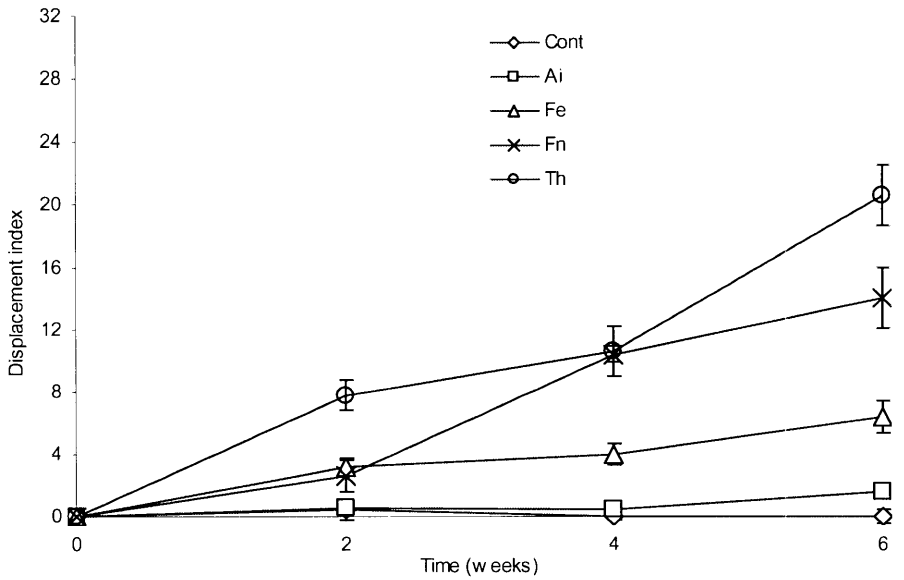


Fig. 3.1 Effect of fungal antagonists on displacement of Fp from barley straw pieces at 2, 4 and 6 weeks. Cont, control; Ai, *A. infectoria*; Fe, *F. equiseti*; Fn, *F. nygmai* and Th, *T. harzianum* (vertical bars are the standard errors of the means).

Effect of water potential on displacement

Effect of fungal treatment on displacement was highly significant ($P < 0.01$) at the four weeks sampling date with four different water potentials. The four antagonists differed considerably in their efficacy in displacing Fp from straw pieces compared with the control in which Fp was isolated from all straw pieces at all water potentials. *Trichoderma harzianum* displaced Fp most at -2 MPa but was ineffective at the driest condition (Fig. 3.2). Fn displaced Fp from straw a little better than Fe did at -0.3, -1, and -2 MPa water potentials but both decreased their activity at -5 MPa. *Alternaria infectoria* was poorest among all antagonistic fungi in terms of displacing Fp from straw pieces as compared to control.

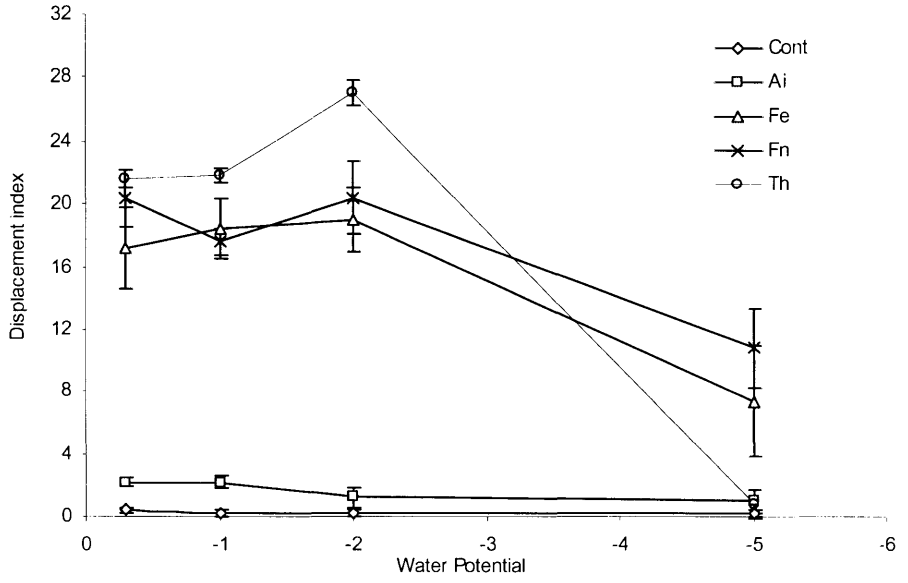


Fig. 3.2 Displacement of Fp from cereal residues by fungal antagonists at different water potentials Cont, control; Ai, *A. infectoria*; Fe, *F. equiseti*; Fn, *F. nygmai* and Th, *T. harzianum* (vertical bars are the standard errors of the means).

There was no significant difference in weight loss of straw between fungal treatments at each level of water potential. Weight loss declined as water potential decreased (Fig. 3.3).

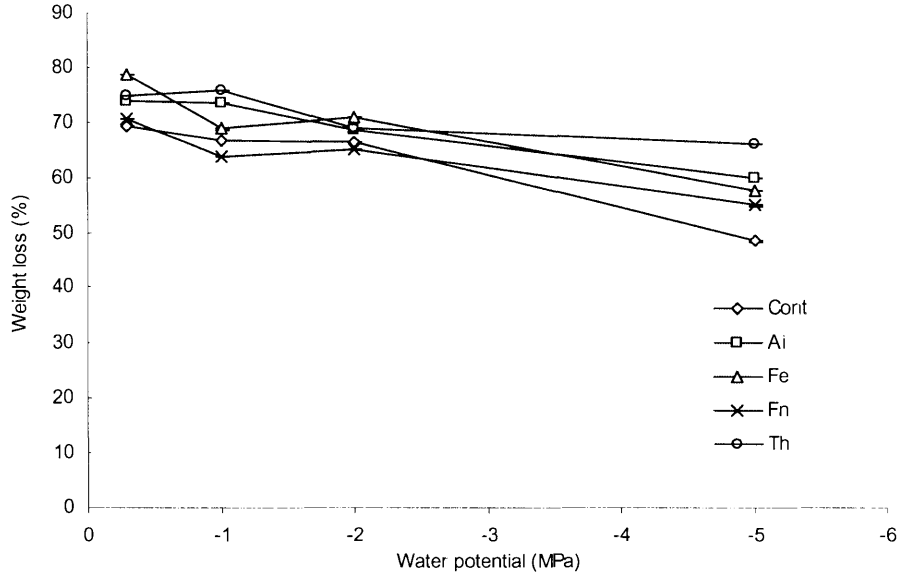


Fig. 3.3 Weight loss (%) of infected Fp cereal residues by fungal antagonists at different water potentials Cont, control; Ai, *A. infectoria*; Fe, *F. equiseti*; Fn, *F. nygmai* and Th, *T. harzianum*.

Plant bioassay for inoculum potential in relation to displacement

There was no significant difference in incidence of infection of plants between straw treatments. Approximately 50% of the plants were infected (Fig. 3.4).

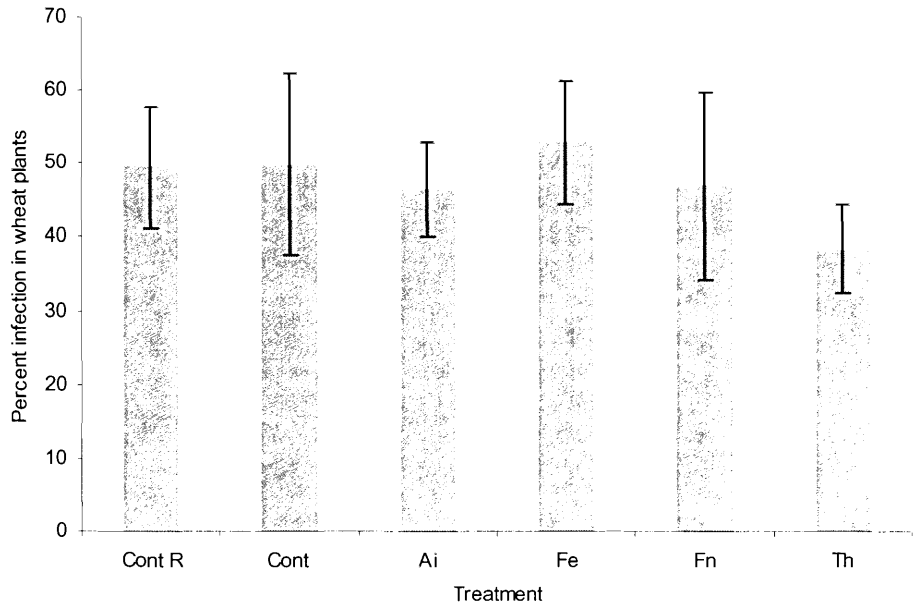


Fig. 3.4 Incidence of Fp in wheat plants inoculated with straw treated with antagonists. ContR, non-incubated control; Cont, incubated control; Ai, *A. infectoria*; Fe, *F. equiseti*; Fn, *F. nygmai* and Th, *T. harzianum* (vertical bars are the standard errors of the means).

Quantitative detection of Fp from stubble pieces

A significant effect of treatments was found on the relative concentrations of Fp DNA using real-time PCR. DNA concentration was higher in the SNA control, Fe, Fn and Th treatments than in the non-incubated control or Ai treatments (Fig. 3.5).

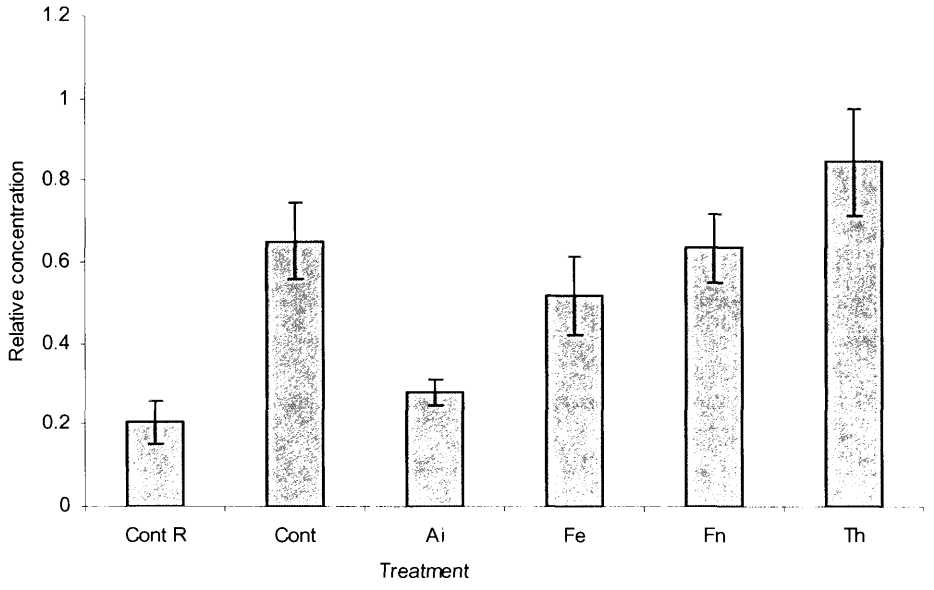


Fig. 3.5 Effect of antagonists on relative concentration of Fp DNA in straw pieces. ContR, non-incubated control; Cont, incubated control; Ai, *A. infectoria*; Fe, *F. equiseti*; Fn, *F. nygmai* and Th, *T. harzianum* (vertical bars are the standard errors of the means).

Culture filtrate effects on relative growth of all fungi

Fp grew best on Ai extract, least on Fe and on 4 ml of Th filtrate (Fig. 3.6 A).

Ai was most affected by Th at 4 ml (Fig. 3.6 B). Fe was inhibited by all extracts at 4 ml, especially by filter extracts of the same strain. Ai and Fp extracts at 1 ml inhibited Fe compared with its own extract (Fig. 3.6 C). Fn tended to grew more slowly with Ai filtrate. There was little difference between concentrations (Fig. 3.6 D). Th growth was significantly reduced by Fe filtrate compared to other fungi (Th, Fn, Fp and Ai) effect of 1 and 4 ml concentrations in all treatments was not significant (Fig. 3.6 E).

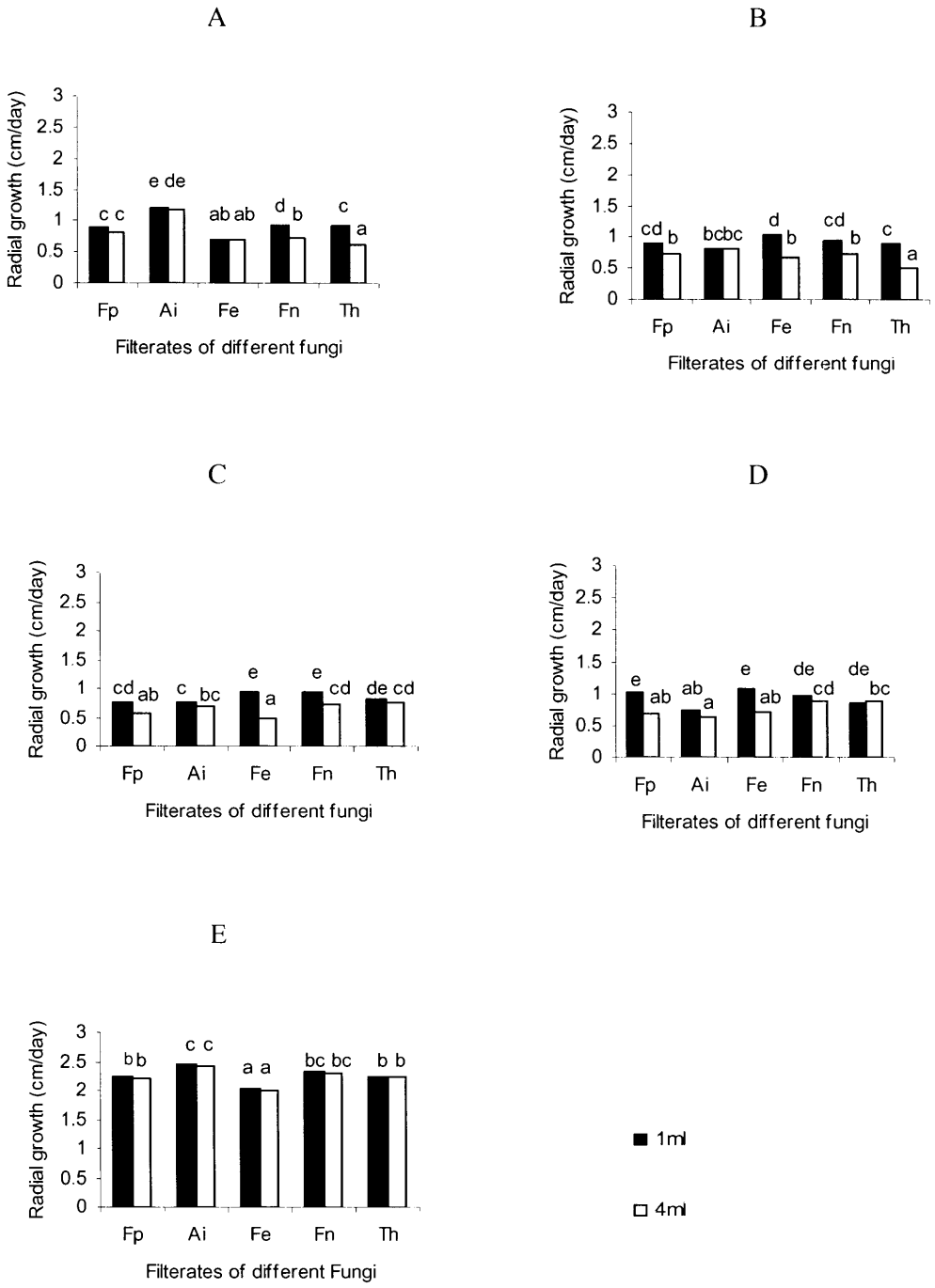


Fig. 3.6 Effect of different concentration of other filtrates of fungi on radial growth of *F. pseudograminearum* (A), *A. infectoria* (B), *F. equiseti* (C), *F. nygamai* (D) and *T. harzianum* (E). (Columns labeled with the same letter within a figure are not significantly different).

DISCUSSION

The general pattern of fungal isolated from wheat stubble is in agreement with work done by Pfender *et al.* (1991b, 1993b) and Pereyra *et al.* (2004). In this study the range of fungi recovered is typical of the fungal flora of wheat residues. The presence of pathogens such as Fp and *Bipolaris sorokiniana*, which were prior colonists, *Alternaria infectoria* and *Epicoccum nigrum* as pioneer colonists and other *Fusarium* spp like *F.chlamydosporum*, *F.equiseti*, *F. nygamai* and *F. oxysporum* as secondary saprophytes were in accordance with studies done by Pfender and Wootke (1988).

Selection of antagonists based on dual culture is a common tool used by researchers for screening pathogens and antagonists under controlled laboratory conditions. These tests however are not necessarily realistic when compared with field situations under fluctuating climatic conditions. A method for measuring Fp displacement from straw was determined for further experiments.

The displacement of *Fusarium* spp. has been studied using different methods (Summerrell and Burgess 1988b, Wong *et al.* 2002, Pereyra *et al.* 2004). These researchers studied the proportion of stubble pieces from which *Fusarium* spp. could be isolated, while others measured spores produced on residues of wheat or other crops (Yi *et al.* 2002, Gilbert and Fernando 2004, Luongo *et al.* 2005, Inch and Gilbert 2007). They had used these studies to look at *Fusarium* head blight management where inoculum is air-borne but not related to crown rot since the latter is not an air-borne disease and spores do not make much difference to disease severity. The displacement assays used in our studies measured Fp displaced from length of stubble based on 0-4 scale gave a more sensitive response than just measuring presence or absence of Fp from straw pieces. So it could be used as a convenient tool for measuring Fp displacement from straw.

The selected antagonists in this study such as *Trichoderma* spp. are known for their antagonistic abilities (Kubicek-Pranz 1998, Kredics *et al.* 2003). A study by Pereyra *et al*

(2004) showed that *F. equiseti* and *F. nygamai* had better saprophytic competitive abilities than *F.graminearum* in terms of displacing it from residues over time. *Alternaria* sp. which had not been reported as an antagonist of Fp was included in our studies as a non-antagonistic control since it was very commonly found in wheat stubble.

Water potential affects the rate of displacement, particularly low water potential. So it was very important to further detailed studies (see Chapter 4). Studies by Cook and Christen (1976) on the influence of water potential and temperature on *Fusarium* spp. showed that at lower water potential, the *Fusarium* spp. grew best at higher temperature. Marin *et al.* (1998a) and Lee and Magan (1999b) tested the effects of temperature and water potential on interactions between different maize spoilage fungi and observed a strong interaction between temperature and water availability in their effects on the competitive ability of fungi, and on the types of interactions between fungi. Therefore, interactions between temperature and water potential need to be studied.

Stubble weight loss and displacement effects were studied by Pereyra *et al.* (2004) and Summerrell and Burgess (1988b) who found that reduction in stubble weight during incubation time was correlated with mortality of *Fusarium* spp. Therefore particular fungi can have a bigger effect on mortality than just their ability to decompose / cause weight loss of stubble. In our studies, displacement and weight loss were not correlated and this may be due to additional antagonistic abilities of different fungi on stubble pieces and not on the weight loss alone.

The inoculum potential experiment did not yield significant differences between treatments, although *T. harzianum* had the lowest infection levels. It could be due to carry over of inoculum between treatments. We did not include healthy wheat plants without any inoculation which may have served as a control for this.

Results for Quantitative-PCR were not what was expected; presumably due to differences in the efficiency of extraction because of different effects of the antagonists on

straw structure and chemistry. There was no way to have an internal control or standard for extraction. So for quantifying the concentration of Fp from straw pieces, Q-PCR has not been a successful method in our studies.

Studies on culture filtrates found that Fp only showed a dose response effect for Th and Fn, the most effective antagonists against Fp. The effect of these filtrates may be due to antibiosis (toxins produced by fungus which inhibit other species) and staling (accumulation of its own waste products to inhibit itself) and possibly nutrient availability. Further studies are required to develop an understanding of the underlying processes of antagonism. This did not seem to be useful type of experiment to do further work on in this project.

Based on these experiments three antagonists (Th, Fn and Fe) and a non-antagonistic control (Ai) were chosen for further experiments. The displacement assay from straw was selected as the basic experimental tool for this next study.

CHAPTER 4

INTERACTIONS OF TEMPERATURE AND WATER POTENTIAL ON DISPLACEMENT OF *Fusarium pseudograminearum* FROM CEREAL RESIDUES BY FUNGAL ANTAGONISTS

INTRODUCTION

Wong *et al* (2002) did laboratory studies on the effects of temperature and moisture on the inoculum reduction of Fp following application of *Trichoderma* spp. to infested wheat straw buried in soil. Isolates of *Trichoderma* varied in their effectiveness, but displacement was generally greatest at high water potential and warmer temperatures. This was consistent with the findings of Burgess and Griffin (1968) that survival of Fp in straw is reduced under warm, wet conditions that were likely to be conducive to microbial activity. No work has been done on the displacement of Fp from cereal straw by other antagonists and their interactions with temperature and water potential.

Luongo et al. (2005) reported that the effects of antagonists on *Fusarium* species in cereal residues were not consistent between sampling dates or experiments. However, the factors that influence the effectiveness of antagonists in this type of system have not been characterised. Water availability has a major influence on fungal activity, and the activity is also influenced by other abiotic factors such as temperature which influences the colonization of straw by different antagonists. Several researchers have tested the effects of temperature and water potential on interactions between different maize spoilage fungi Marin *et al.* (1998a,b; Lee and Magan 1999b). They showed that there was a strong interaction between temperature and water availability in their effects on the competitive ability of fungi, and on the types of interactions between fungi. Temperature and water potential therefore need to be considered together.

The effect of temperature and water potential (WP) on interactions between Fp and other stubble fungi was investigated. The effects of these abiotic factors were tested on

growth rates of different fungi, and their interactions in dual culture. Displacement assays on straw pieces were also used to test the different antagonists for their ability to displace Fp from straw. The aim of the study was to determine the effect of interactions of temperature and WP on the ability of antagonists to displace Fp from residues.

MATERIALS AND METHODS

Effect of water potential and temperature on growth

The fungal species used in this experiment were Fp, Th, Ai, Fe and Fn. The basic medium used was 1/4 PDA. The WP of the basic medium was -0.3 MPa, and this was modified to -1, -2 and -5 MPa using NaCl. The experiment was set up at temperatures of 5°C, 10°C, 15°C, 20°C, 25°C, 30°C and 35°C.

For measuring the growth rates of the fungi, a mycelial plug (5mm) of the respective fungus was taken from an actively growing culture on 1/4 PDA and placed in the centre of a 9 cm Petri plate containing 20 ml of the amended or unamended 1/4 PDA medium. There were 2 replicate plates for each treatment. After inoculation, treatments at the same WP and temperature were sealed in polythene bags and incubated at different temperatures (5°C to 35°C). The diameter of the growing colonies were measured daily for 7 days. Measurements of all colonies were made in two directions at right angles to each other, and daily growth rate was calculated. For each fungus, the relative growth at each temperature and water potential combination was calculated as the proportion of the maximum growth rate recorded for that fungus. The relative growth rates were then plotted against water potential and temperature as contour plots. The experiment was conducted twice, with temperatures being randomly reassigned to incubators.

Effect of water potential and temperature on dual culture interactions

For dual cultures Fp plugs were placed 2.5cm from an inoculum plug of the antagonist to be tested on 1/4 PDA media amended as above, and incubated at the various temperatures used above. Measurements were made of how far Fp and each antagonist had grown towards each other when their mycelia made contact. The expected distance that each fungus should have grown at contact was calculated based on their growth rates in single culture. The expected and actual growth rates were plotted on contour graphs for comparison.

Following contact, the interactions between mycelia of dual cultures were examined and each species given a score based on mutual intermingling (1-1), mutual antagonism on contact (2-2), mutual antagonism at a distance (3-3), dominance of one species on contact (4-0) and dominance at a distance (5-0). These scores for each species were added up for each WP × temperature condition to obtain an overall Index of Dominance (I_D) (Lee and Magan 1999b). Dual-culture experiments were carried out with two replicates per treatment and were done twice.

Effect of water potential and temperature on displacement of Fp from straw

The barley straw substrate inoculum of Fp was prepared as described in Chapter 3. The antagonists Th, Ai, Fn, Fe were allowed to grow until they covered 9 cm Petri plates of Spezieller Nährstoffarmer Agar (SNA); (Leslie and Summerrell 2006) medium amended to -0.3, -1, -2, -5 MPa with NaCl. Eight Fp inoculated straw pieces were placed on top of the antagonist culture and the Petri dishes sealed with Parafilm. The plates were incubated for four weeks at different temperatures (5 to 35°C). The straw pieces were removed from the Petri dishes after incubation, surface sterilized for 1 min in 1% NaOCl in 10% ethanol, and plated onto ¼ PDA. The degree of displacement was recorded on a 0-4 scale based on the proportion of the length of each straw piece from which either Fp or the antagonist grew, where 0 = Fp only; 1 = predominantly Fp; 2 = equally Fp and the antagonist; 3 = predominantly the antagonist; and 4 = antagonist only. The scores for each straw piece were

summed for each Petri dish. There were three replicates of each treatment, and the experiment was repeated once.

RESULTS

Effects of temperature and water potential on growth rates

The relative growth rates of different fungal species were plotted as contour graphs. Results presented here are the average values of two repetitions. Fp had its greatest growth across a fairly broad range of temperatures (10°C-30°C) under drier conditions (-1MPa and lower). It did not grow at 35° (Fig. 4.1 A).

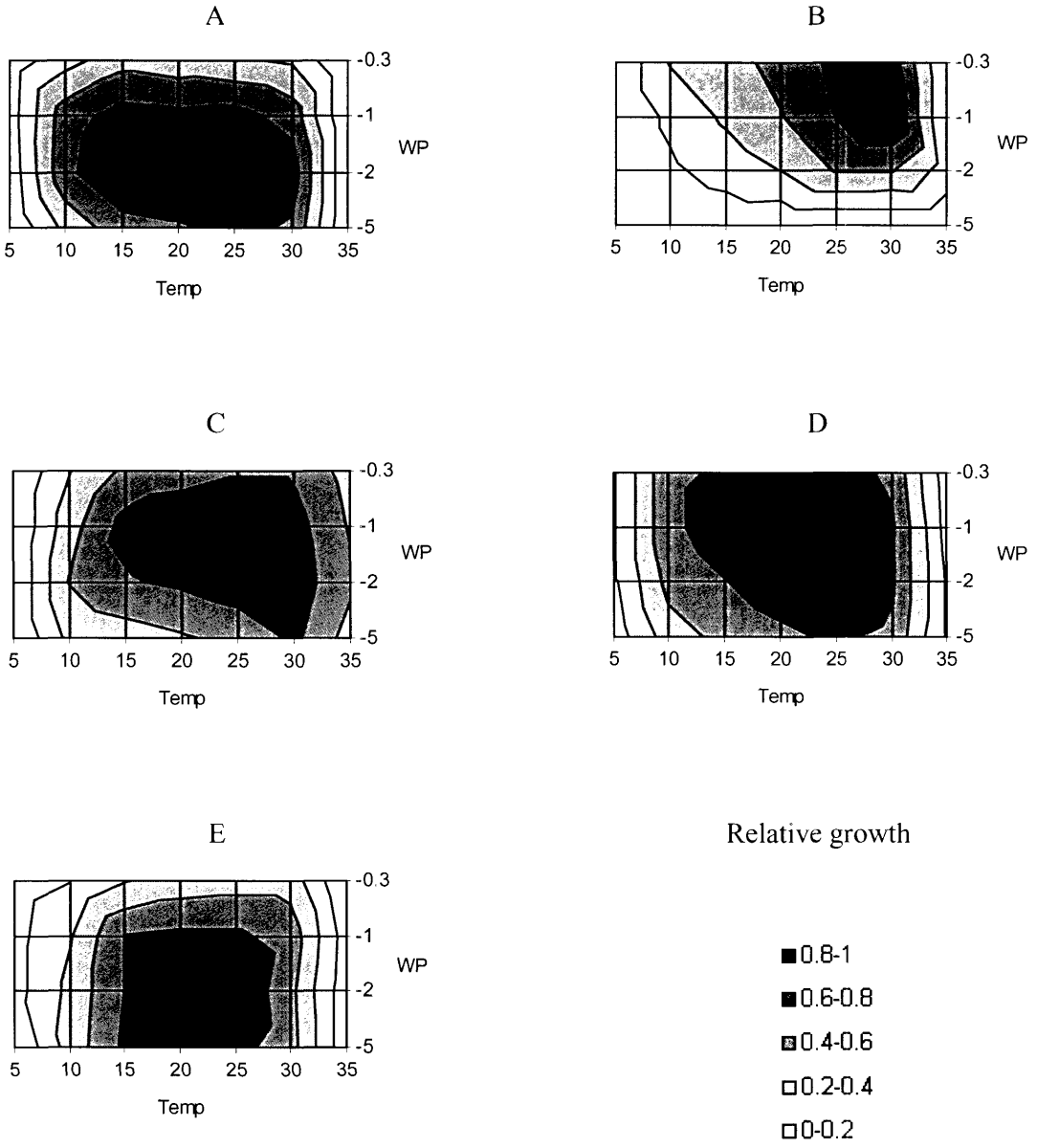


Fig. 4.1 The effect of temperature (Temp, °C) and water potential (WP, MPa) on the relative growth rate of (A) *Fusarium pseudograminearum*, (B) *Trichoderma harzianum*, (C) *Fusarium nygamai*, (D) *Alternaria infectoria* and (E) *Fusarium equiseti*. Relative growth is expressed as the proportion of the highest observed growth rate for each fungus.

Th grew fastest at wet (-0.3 to -1 MPa), warmer (25-30°C) conditions and grew poorly at low temperatures and low water potentials (Fig. 4.1 B). Th was unable to grow at 5°C.

Fn grew fastest at warm temperatures between 15-30°C and at moderate water potential (-1 MPa) (Fig. 4.1 C). It grew well over a broader range of water potentials at higher temperatures (25-30°). It did not grow at 5°C.

Ai was able to grow at all combinations of temperature and water potential tested (Fig. 4.1 D). It showed near-maximum growth rates at all water potentials at 25°, but showed reduced growth at lower water potentials at cooler temperatures.

Fe grew best between 15 - 30° at -1 to -5MPa (Fig. 4.1 E). No growth was observed under dry cold conditions and under wet hot conditions.

Effects of antagonism against Fp in dual culture at different temperatures and water potentials

All fungi studied were inoculated 2.5 cm apart in 9cm Petri plates. The distance that Fp grew in dual culture before being stopped on contact with the other fungus was measured. It was also calculated that what this distance should have been, based on growth rates, if there were no interactions between the fungi prior to contact. This was expressed in terms of the growth of Fp. Based on this, contour graphs of expected (left) and actual distances (right) were plotted in Fig. 2 for Th, Fn, Ai and Fe respectively. So, the darker the zone, the further Fp grew, and the lighter the zone, the further the other fungus grew. At 5° and 35°C, all combinations in the dual culture interactions could not be measured as either one of the fungi did not grow or their relative growth was too slow to be measured, within the period of the studies.

Th grew further than expected under warm dry conditions while Fp grew further than expected against Th under cool wet conditions Fig. 4.2 (A). It was concluded that temperature had an effect on the antagonism of Fp by Th in addition to its effect on growth. The

combinations under which Fp is most likely to escape antagonism by Th would be at low temperatures, possibly more so than at low water potentials.

The actual growth of Fp and Fn in dual culture was similar to that expected under most conditions Fig. 4.2 (B). Fp grew less than expected before contact with Fn at -2 and -5 MPa at 30°, suggesting antagonism by Fn prior to contact under these conditions.

Fp dominated Ai at all temperatures and water potential. It grew further than expected before contact at 2-30° at -0.3MPa Fig. 4.2 (C).

Fe reduced growth of Fp prior to contact at all temperatures and water potentials, compared with expectations based on individual growth rates Fig. 4.2 (D).

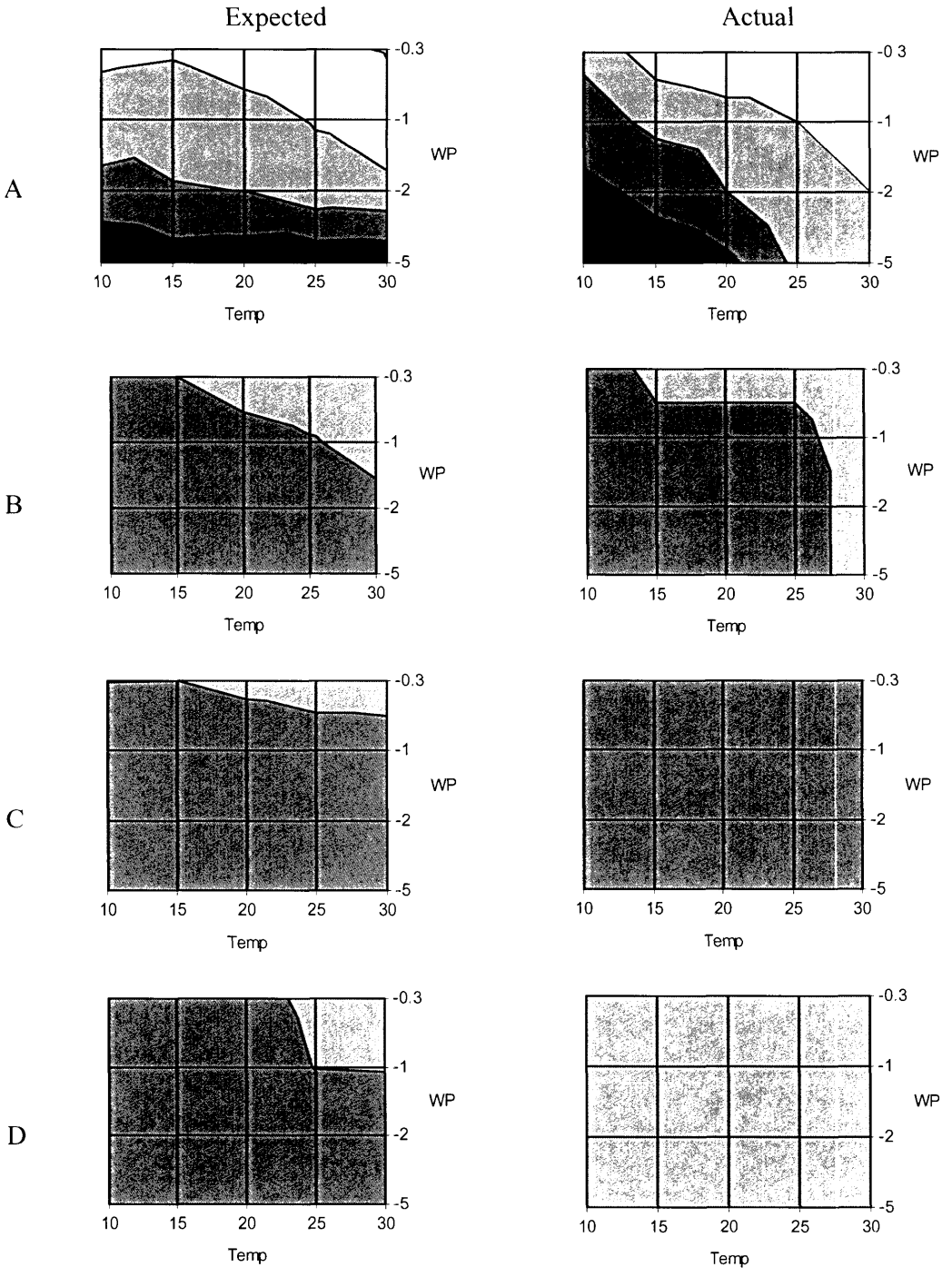


Fig. 4.2 Expected (left) and actual (right) growth rates of Fp with Th (A), Fn (B), Ai (C) and Fe (D) at different temperature (°C) and water potential (MPa) Fp, *Fusarium pseudograminearum*; Th, *Trichoderma harzianum*; Fn, *Fusarium nygamai*; Ai, *Alternaria infectoria*; and Fe, *Fusarium equiseti*.

In vitro interaction patterns and Index of Dominance

The interaction patterns and I_D between Fp and other interacting species varied considerably for paired species depending on water potential and temperature (Table 4.1).

From -0.3 to -2 MPa at 5°, Th showed mutual intermingling of hyphae with Fp and at 10° it showed mutual antagonism on contact. However, Th dominated Fp at temperatures of 20-35° at these water potentials. At -5 MPa no growth of Th was observed at 5 or 10° but there was complete dominance by Fp at 15 and 20° with mutual antagonism on contact from 25-35°.

At water potentials of -0.3 to -5 MPa, no growth of Fn was observed at 5° but mutual intermingling and mutual antagonism on contact with Fp was found at 10 and 15° respectively. At -0.3 to -1 MPa mutual antagonism at a distance was observed at 20-25°, while Fn dominated Fp on contact at 30-35° at -0.3 to -2 MPa. At -5 MPa the interactions were mutual antagonism on contact at 20 - 25° but mutual antagonism at a distance at 30 - 35°.

Fp dominated Ai completely at 5 - 15° and 35° at all water potentials except at -5 MPa, 15° where the interaction was just mutual intermingling of hyphae. At all other temperature and WP conditions there was mutual intermingling of Fp and Ai hyphae, although Fp dominated at -0.5 MPa at 30°C.

At all water potentials and temperatures from 5-15° Fe had mutual intermingling with Fp. However, at -0.3 to -2 MPa at 20-25° mutual antagonism on contact was found but it shifted to mutual antagonism at a distance at 30-35°C, whereas at -0.5 MPa it was just mutual intermingling of hyphae at 20-25 but mutual antagonism on contact at 30-35°C.

Table: 4.1 Effect of WP and temperature on numerical *in vitro* interaction scores and Index of dominance (I_D) for Fp and paired species

Antagonist	WP	Temperature (°C)							I_D
	MPa	5	10	15	20	25	30	35	
T. harzianum	-0.3	1/1 [B]	2/2	3/3	0/4	0/4	0/4	0/4	6/22
	-1	1/1	2/2	3/3	0/4	0/4	0/4	0/4	6/22
	-2	1/1	2/2	3/3	0/4	0/4	0/4	0/4	6/22
	-5	NG[A]	NG	4/0	4/0	2/2	2/2	2/2	14/6
	I_D	3/3	6/6	13/9	4/12	2/14	2/14	2/14	32/72
F. nygamai	-0.3	NG	1/1	2/2	3/3	3/3	0/4	0/4	9/17
	-1	NG	1/1	2/2	3/3	3/3	0/4	0/4	9/17
	-2	NG	1/1	2/2	2/2	3/3	0/4	0/4	8/16
	-5	NG	1/1	2/2	2/2	2/2	3/3	3/3	13/13
	I_D	NG	4/4	8/8	10/10	11/11	3/15	3/15	39/63
A. infectoria.	-0.3	4/0	4/0	4/0	1/1	1/1	1/1	4/0	19/3
	-1	4/0	4/0	4/0	1/1	1/1	1/1	4/0	19/3
	-2	4/0	4/0	4/0	1/1	1/1	1/1	4/0	19/3
	-5	4/0	4/0	1/1	1/1	1/1	4/0	4/0	19/3
	I_D	16/0	16/0	13/1	4/4	4/4	7/3	16/0	76/12
F. equiseti	-0.3	1/1	1/1	1/1	2/2	2/2	3/3	3/3	13/13
	-1	1/1	1/1	1/1	2/2	2/2	3/3	3/3	13/13
	-2	1/1	1/1	1/1	2/2	2/2	3/3	3/3	13/13
	-5	1/1	1/1	1/1	1/1	1/1	2/2	2/2	9/9
	I_D	4/4	4/4	4/4	7/7	7/7	11/11	11/11	48/48

[A]NG, no growth observed either by Fp (*Fusarium pseudograminearum*) or respective fungi

[B]Fp score/antagonist score 1/1, mutual intermingling of hyphae; 2/2, mutual antagonism on contact; 3/3, mutual antagonism at a distance; 4/0, antagonism by Fp on contact and 0/4, antagonism of Fp by antagonist on contact.

Effect of temperature and water potential on displacement of Fp from stubble pieces by other antagonists

Fp was almost completely displaced by Th from stubble pieces at higher temperatures ranging from 20-35°C at all water potentials except at -5 MPa, giving a very high displacement index (DI) of 25.6-32 (Fig. 4.3 A). Activity of Th was less effective at the lowest WP (-5 MPa) at all temperatures studied. Displacement by Th was intermediate at 15-20°C in wet regimes (-0.3 - -2MPa). At -5MPa there was no displacement by Th at temperatures of 5-10°C and it was negligible at 15°C.

Fn strongly displaced Fp from stubble at -0.3 to -2MPa between 30-35°C. DI decreased with a decrease in both temperature and water potential. At 5°C, there was very little displacement by Fn at all water potentials. Between 10-15°C, displacement was moderate (Fig. 4.3 B).

Ai showed very low levels of displacement of Fp under all conditions (Fig. 4.3 C). In the control treatment, Fp was retrieved completely from all stubble pieces at all temperatures and water potentials.

Temperatures ranging from 25-35°C were most favorable for Fe to displace Fp from straw pieces at all water potentials except -5 MPa. From 15-25°C, the displacement was moderate but decreased at the lowest water potential (Fig. 4.3 D). Fe showed the highest displacement among the antagonists at 5-10° at -2 to -5 MPa.

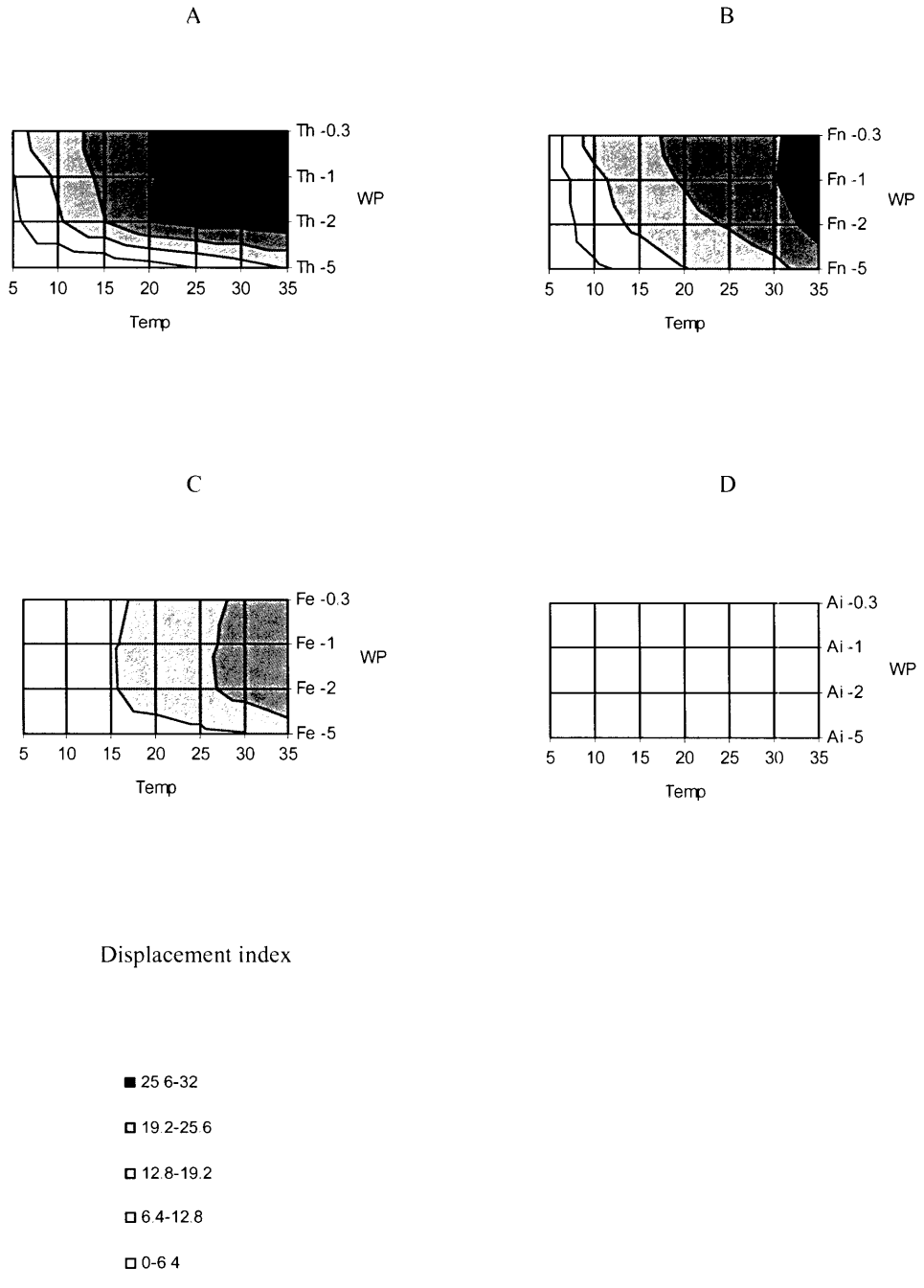


Fig. 4.3 Displacement of Fp from stubble pieces by antagonists Th (A), Fn (B), Fe (C)) and Ai (D) at different temperature (°C) and water potential (MPa) Fp, *Fusarium pseudograminearum*; Th, *Trichoderma harzianum*; Fn, *Fusarium nygamai*; Ai, *Alternaria infectoria*; and Fe, *Fusarium equiseti*.

Association between the displacement index and the dominance pattern

Box and whisker plots of displacement indices from straw for each category of interaction in dual culture showed a strong association between displacement index and pattern of dominance. Reaction category 0/4, representing dominance of the antagonist (Fn, Fe, Th, Ai) over Fp on contact corresponded to high levels of displacement (Figure 4.4) irrespective of changes in temperature and water potential. Reaction category 3/3, representing mutual antagonism at distance corresponded to displacement indices between 14.8 and highest 27.8. Within this category, cases with highest displacement tended to be at warmer temperatures. Reaction category 2/2, representing mutual antagonism on contact, corresponded to displacement indices of 6.5-23.2. Within this reaction type the strongest influence seemed to be of water potential, with low displacement found at -5 MPa and the highest displacement scores tending to be at higher water potential. Reaction category 1/1 was mutual intermingling of hyphae of fungi, corresponding to displacement scores of less than 19.5. In this category lower displacement was typical of Ai and the higher ranges of displacement were typical of Fe, Fn & Th. The highest displacement in the 1/1 category was with Fn and Th at -0.3 MPa at the lowest temperature at which they grew, with more displacement than might be expected from dual culture interactions. The last reaction category 4/0, which meant dominance of Fp on contact with other fungi, corresponded to low values of displacement index (Fig. 4.4).

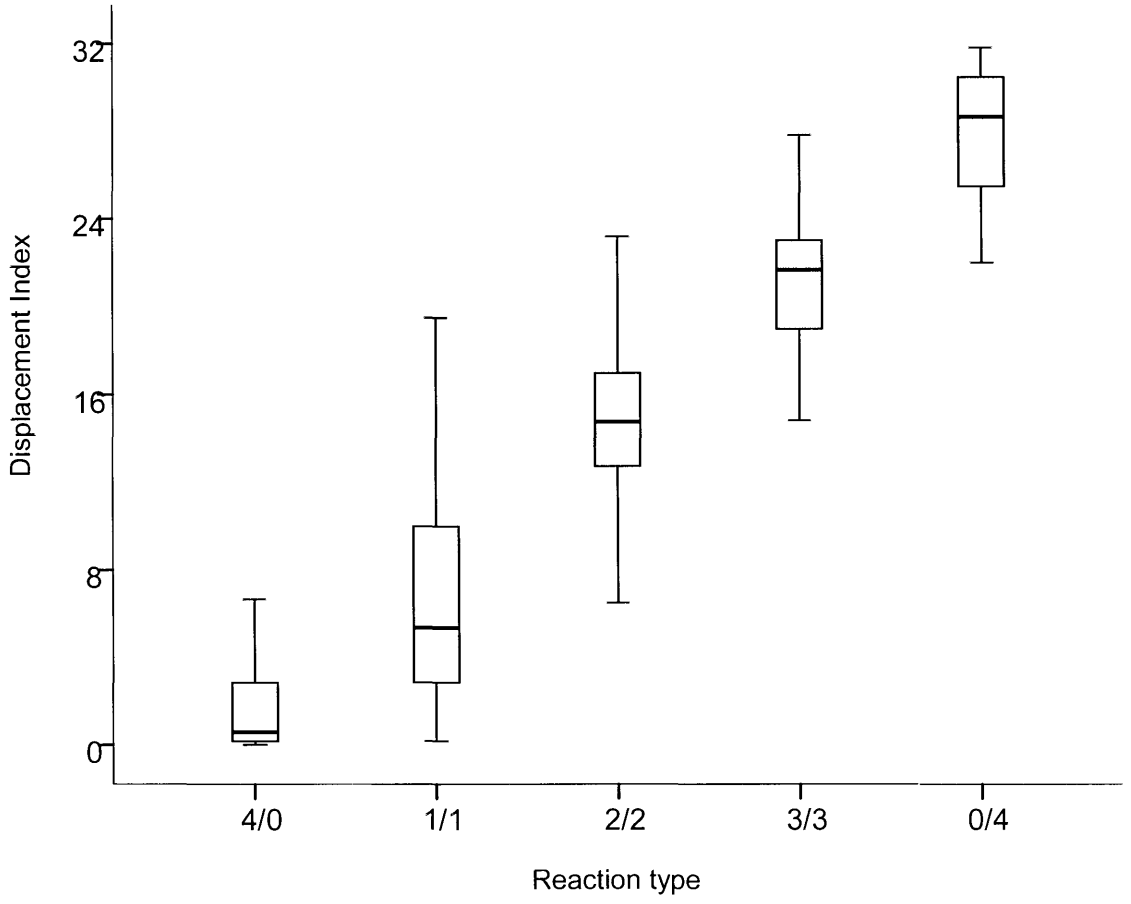


Fig. 4.4 Relationship between index of displacement from straw, and category of interaction in dual culture, for all combinations of antagonist, temperatures and water potentials. Boxes show quartiles and median, with whiskers showing full range.

DISCUSSION

Fusarium pseudograminearum growth was favoured at an intermediate temperature range (10 - 30°C) and water potentials (-1 to -2 MPa). This is consistent with the findings of Wearing and Burgess (1979) for growth of *F. pseudograminearum* on osmotically-adjusted media. There was a slight shift to a lower optimum water potential at higher temperature, which is also consistent with earlier findings (Cook and Christen 1976, Wearing and Burgess 1979). This confirms that the temperature and water relations of the isolate used here were typical of the species. Fp growth also followed a similar pattern to that reported by Liddell

and Burgess (1988) where the optimum water potential for infection was between -0.3 and -0.7 MPa. Although infection and growth rate are different phenomena there could still be some relationship as Fp grew moderately well at -0.3 to -1MPa at all temperatures except extreme cold and hot conditions.

F. nygamai and *T. harzianum* grew best under hot, wet conditions compared with Fp. However, *F. nygamai* showed significant overlap in relative growth with Fp under a broad range of conditions. The conditions where Fp is most likely to escape antagonism by Th would be at low temperatures, possibly more so than at low water potentials.

The effects of temperature and water potential on antagonism prior to contact in dual culture could be predicted reasonably well from their effects on growth of each fungus. Deviations from expected growth indicate antagonism at a distance by one or other of the fungi. Antagonism by Th seemed to be affected by temperature, because it reduced growth of Fp more than expected at high temperature and less than expected at low temperature. This could be because the effects of temperature on production of enzymes or volatile inhibitors involved in antagonism by Th differ somewhat from the effects on growth (Tronsmo and Dennis 1978). Fp grew less than expected towards Fe under all conditions, indicating antagonism by Fe irrespective of temperature or water potential.

The pattern of dominance matched the response before contact of fungal mycelium but differed from what might be expected based on individual relative growth rates. Index of Dominance was highly sensitive to changes in water potential or temperature with different antagonists (Th, Fn, Fe and Ai). Fn and Th dominated Fp at the hottest and wettest regimes on contact but at -5 MPa with similar temperature conditions the interaction pattern changed to mutual antagonism at a distance. At 20°, the interaction between Fp and Th changed from dominance by Th at -2 MPa to dominance by Fp at -5 MPa, which illustrated the key role of low water potential as a limiting factor in antagonism by *Trichoderma* species (Tronsmo and Dennis 1978, Kredics *et al.* 2000, 2003). Ai was completely dominated by Fp on contact at

high and low temperatures whereas at other regimes closer to its optimum for growth it showed intermingling of hyphae of both fungi.

Displacement of Fp from straw depended on specific antagonism by other fungi, since Ai, which was the most common fungus isolated from wheat stubble in our studies, had no activity against Fp. The antagonists that were most effective in displacing Fp from preinoculated stubble pieces were Th and Fn whereas Fe was only a moderate displacer.

El-Naggar *et al.* (2003) studied temperature effects on antagonism of spore reduction in cereal straw by *Fusarium* spp. They looked only at temperature, but our studies found strong interactions between temperature and water potential that need to be taken into consideration. Since temperature interacts with water potential, effects of temperature alone on antagonism could be misleading. Antagonism by Th was affected most by temperature in dual culture, but water potential had the greatest effect on displacement of Fp from straw.

Although *Trichoderma* species have shown potential for biocontrol of Fp (Wong *et al.* 2002), fungi with moderate potential for antagonism, such as other *Fusarium* species, may also be significant for mortality of Fp in straw under drier and cooler conditions. The results of Wong *et al* (2002) showed greater displacement of Fp by *Trichoderma* spp. at higher temperature, which is consistent with our findings. However, they also found greater displacement at -0.3 MPa than at -0.03 MPa (wet soil), which appears contrary to the results here. However, our studies looked at lower water potentials than they did and it could be possible that bacterial activity in wet soil may have inhibited Th growth or activity. Wong et al. (2002) acknowledged that stubble borne fungi like Fp in standing stubble would not be as effectively controlled by *Trichoderma* species as they would in buried straw, because the stubble can dry out. Th was the strongest antagonist under wet conditions at most temperatures, but Fn and Fe were also effective in displacement under warm dry conditions, and were more antagonistic than Th under dry conditions.

The box and whisker plot of displacement indices for each category of displacement indicate that there is a strong association between the displacement index and the dominance patterns. The methodology of dominance patterns of Lee and Magan (1999) could be correlated with the displacement of Fp by antagonists at different temperatures and water potentials. It could be said that if the dominance pattern is 0/4, where the antagonist dominates on contact, then displacement will always be high. If the dominance pattern is 3/3, with mutual antagonism at a distance, then displacement will be high especially at high temperature. If the displacement pattern is 2/2, with mutual antagonism on contact, then displacement will be moderate at high water potential. This type of interaction will most likely be limited by low water potential. If the displacement pattern is 1/1 and 4/0 then the antagonist will generally be ineffective at displacing Fp. Because of the interactions between water potential and temperature, the Index of Dominance numbers that are summed for main effects (Lee and Magan 1999b) really do not provide an indication of the responses observed in dual culture interactions.

The environmental factors that need to be taken into account while selecting biocontrol agents for this system should be temperature and water potential. The best antagonists need to have the ability to displace Fp under harsh conditions i.e. at cold and dry conditions.

CHAPTER 5
EFFECT OF WETTING-PERIOD DURATION ON DISPLACEMENT OF *Fusarium pseudograminearum* IN STRAW BY DIFFERENT ANTAGONISTS

INTRODUCTION

Residues of crops after harvest are exposed to different climatic conditions, including wet and dry periods of weather before the next crop to be sown. These variations in temperature and water availability may have an effect on the degree of competition among fungal species and the displacement of one fungus by other species. Various researchers have looked at interactions of temperature and water potential on competition between fungal species and residue borne *Pyrenophora tritici-repentis* (Ptr) causing tan spot in wheat. Summerell and Burgess (1989) found that at water potential greater than -39 MPa, Ptr was displaced by *Pythium oligandrum* at 10°C, and *Aspergillus terreus* at 20 and 30°C, succeeded by *Trichoderma harzianum* and finally by *Penicillium* sp. and *Fusarium solani*. Pfender *et al.* (1991b) explored different antagonists from wheat straw at several water potentials and found that antagonists from the soil contact area of the mulch layer (moist) were effective in inhibiting pseudothecia production.

Research by Zhang and Pfender (1992, 1993b) on wheat residues indicated the importance of how long straw remains wet for antagonistic activity against Ptr. They observed that some antagonists like *Laetisaria arvallis*, *Laetisaria roseipellis* and Sterile sp. suppressed the production of ascospores in wheat straw only when straw remained wet longer than 12 hours.

The epidemiology of a disease is better understood by correlation with weather parameters such as temperature, rainfall and humidity. Summerrell and Burgess (1988b) and Pereyra *et al*., (2004) showed that survival of *Fusarium* spp. in wheat residues was correlated with decomposition of the residues. Quemada (2004) compared the ability of different time

scales based on heat sums to predict biomass decomposition of wheat straw and to account for the effect of weather conditions on biomass decomposition. They found that only when time was calculated using methologies that included both temperature and moisture, the model was able to account for the effects of weather conditions on residue decomposition.

The aim of this study was to investigate the effects of wetting and drying schedules on displacement of Fp from stubble by antagonists and also to determine the effects of weather conditions on Fp displacement under greenhouse and field conditions.

MATERIALS AND METHODS

Barley straw substrate

Barley straw pieces were precolonized with Fp as described in Chapter 3.

Inoculum of test fungi

From the previous experiments four different antagonists were selected and one control treatment was used. The antagonists used were *Trichoderma harzianum*, *Fusarium equiseti, Fusarium nygamai* and *Alternaria infectoria.* Inoculum was prepared by growing each of the four fungi on wheat bran. Fresh wheat bran (15 ml) was combined with 12 ml of water in a glass Petri plate and autoclaved twice on successive days at 121°C for 20 min (Zhang and Pfender 1993b). After sterilization, 3 ml antibiotic solution (streptomycin sulphate 0.1g/l and penicillin-G 0.03 g/l) was added. The moistened bran in each plate was inoculated with four agar plugs of one of the four fungal cultures from ¼ PDA plates and incubated at room temperature (24 ± 2°C). After 5-10 days of fungal growth in bran, the colonized bran was air-dried overnight and ground in a sterile mortar with a pestle. For the control, bran was not inoculated with any of the fungal plugs and was ground similarly.

Inoculation of straw by potential antagonists

Straw infested with Fp was inoculated with bran inoculum of one of the four fungi at one end of the straw. A drop of autoclaved 1.3% methylcellulose solution was applied first in a narrow band to one end of the straw to serve as adhesive for the infested bran. A small amount of bran inoculum was immediately applied to the ring of methylcellulose on the end of the straw (Zhang and Pfender 1993b). The straw pieces were then placed vertically in racks with the bran inoculum uppermost. During the first experiment microcentrifuge tube racks with drainage holes drilled in them were used. Since they were retaining water, the next time the inoculated straws were placed on a thin spiked metal base having 32 teeth (Pryda SN5 Strapnail, Pryda Australia, Melbourne) so that each straw stood erect. Racks of inoculated straw pieces were supported 5 cm above the base of a rectangular plastic bin (597×362×381 mm) with holes drilled at one end to drain excess water. For the control, straw pieces were placed in the same way as other treatments.

Misting treatments under greenhouse conditions

The bins containing the racks of inoculated straw were placed on a bench in a greenhouse, and the inoculated straw was exposed to alternating wet and dry treatments to simulate rain and drying events under actual field conditions. The irrigation system was controlled electronically (Mist Master Sterling-M controller). Tap water was run through an in-line cartridge filter and then through splitters connected to solenoids (13mm). Pipes were used to make the connection to each plastic bin using elbow and tee joiners (13mm) and the ends of the pipes were closed using end sleeves (13mm). Fan spray nozzles (Moss Company G360-Q) capable of supplying 0.3 litre water per minute was fitted 15 cm above the straw pieces.

In the first experiment misting duration periods were 8, 16 and 24 hr. The straw pieces were kept in plastic microcentrifuge racks with holes drilled to drain water, supported by wick towels to absorb excess water from the holes to make the straw dry quickly. However, this did not work well, and displacement of Fp by antagonists was high at all misting schedules. Two

subsequent experiments used shorter wetting periods of 4, 7 and 10 hr misting with straw on spiked metal plates.

A misting frequency of 3s/10min was used within the misting period. The misting period was scheduled to end near midday, when the straw would dry in several hours. There were 3 replicates of each misting period in each experiment laid out in a randomized complete block design.

The displacement of Fp was recorded by measuring the length from which Fp had been removed by the antagonists, using the 0-4 scale. At several intervals during each experiment, 8 straw pieces per treatment were removed, surface sterilized and placed on ¼ PDA for scoring displacement after 4-5 days. Data were analysed by ANOVA in SPSS software, with mean separated by Tukey's HSD method.

Field experiment

A field experiment was set up in natural conditions of rainfall, dew and sunlight for recording displacement of Fp by antagonists. River sand was used as a base in rectangular plastic boxes (15×10cm) to absorb moisture effects through rain. 8 Fp inoculated straw pieces were coated with different antagonists and control (as explained earlier) and were kept in small bags made of wire mesh to keep them intact. The experiment was run 4 times between 20th April, 2007 and on 29th October, 2007 at UNE, Armidale campus. In each experiment, straw was exposed for one month starting from April to June with the last experimental set of observations recorded in October. Displacement was measured as for the greenhouse experiment. During the experimental period data was collected hourly for temperature, rainfall and wetness using a thermistor air temperature sensor, a tipping-bucket rain gauge and a MonitorSensors leaf wetness sensor. A completely randomised experimental design was used with five replications and five treatments. The results were statistically analysed using ANOVA in SPSS software.

RESULTS

Wetness duration effect on Fp displacement under greenhouse conditions

In the first experiment there were no statistically significant differences between Fn, Th or Fe at all misting durations but these were significantly greater than Ai or the control. Time only had an effect in the control, where displacement in the 24 h misting treatment was significantly greater than in the 16 h treatment (Fig. 5.1).

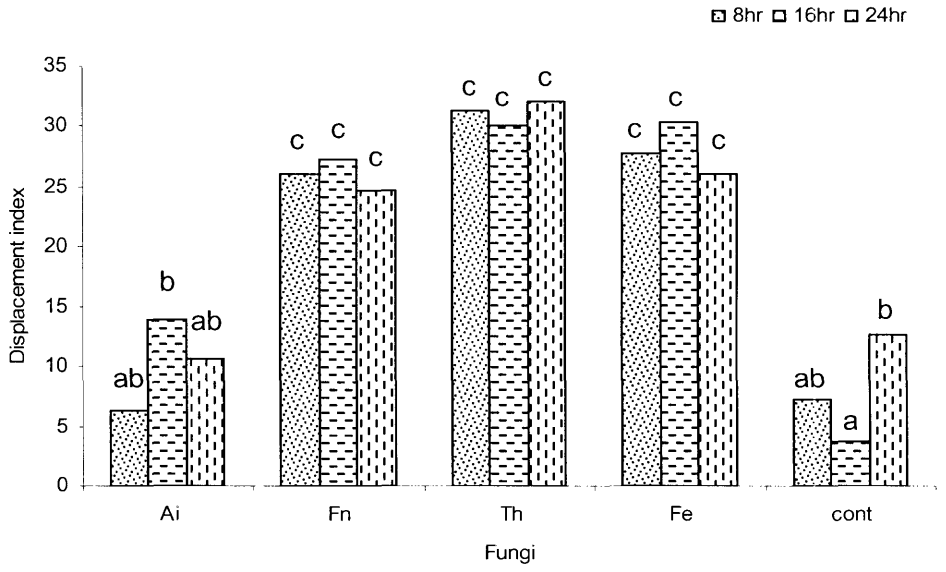


Fig. 5.1 Effect of 8, 16 and 24 hr misting cycle on Fp displacement by different antagonists after 2 weeks Ai, *A. infectoria*; Fn, *F. equiseti*; Th, *T. harzianum*; and Fe, *F. equiseti*; cont, control.

Displacement of Fp in stubble by Ai was found to be very low at all the misting durations of 4, 7and 10 hr. in both experimental sets. No difference between misting durations was observed in either experiment (Fig. 5.2 A).

Fn had a moderate effect on Fp displacement. Displacement of Fp did not vary significantly as the total wetness increased (Fig. 5.2 B).

Th displaced Fp from straw pieces significantly more than the other antagonists in all misting cycles in the first week of observations (Fig. 5.2 C). For a given total misting duration, displacement tended to be higher at shorter misting cycles (Fig. 5.2 C).

The displacement of Fp by Fe did not vary much with increase in wetness hours (Fig. 5.2 D).

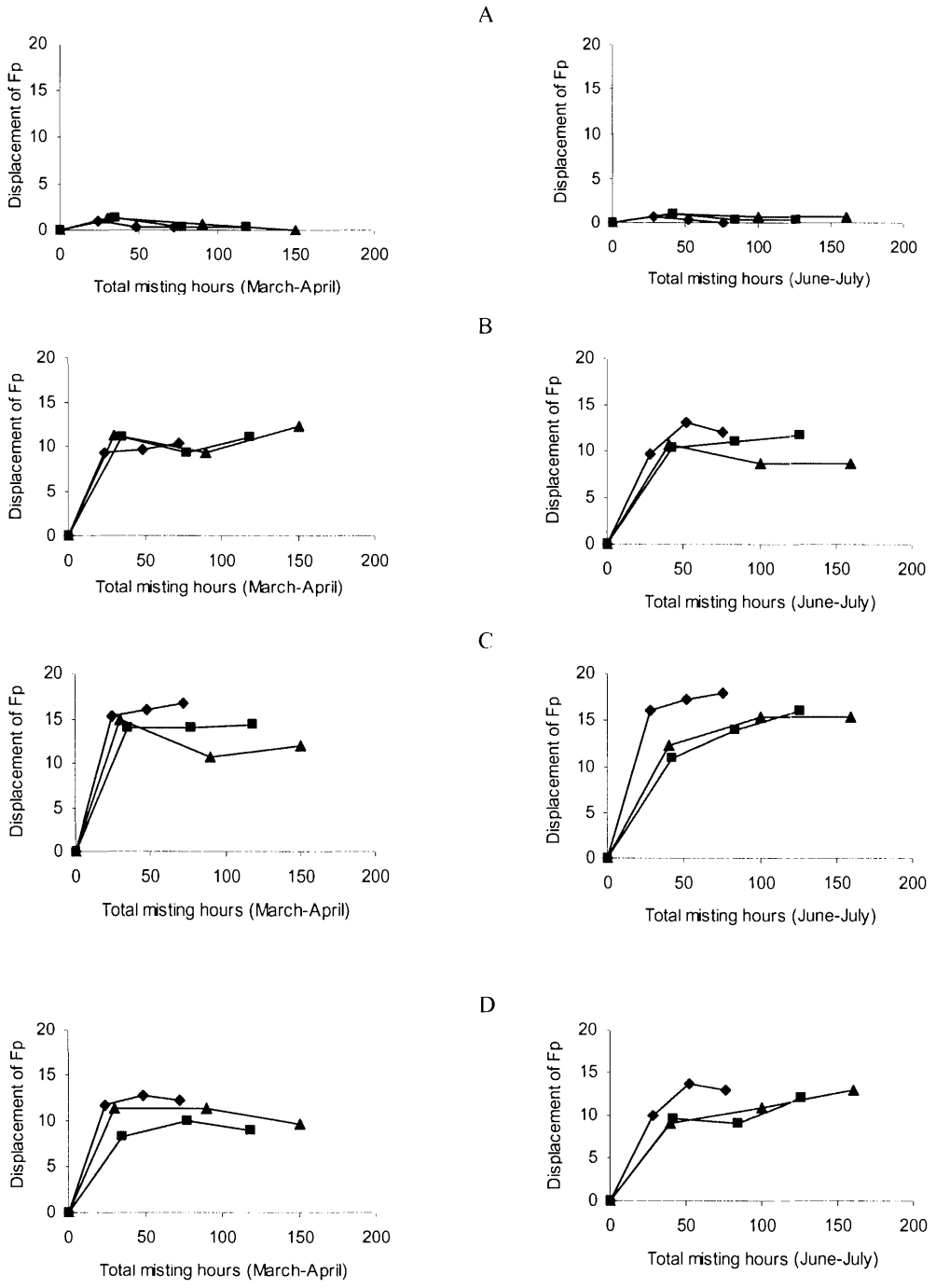


Fig. 5.2 Effect of total misting durations on *Fusarium pseudograminearum* displacement by *A. infectoria* (A), *F. nygamai* (B), *T. harzianum* (C) and *F. equiseti* (D) at 4 (◆), 7 (■) and 10 (▲) hour misting periods.

Since the 4 hour misting period appeared to have greater displacement, it is possible that the number of days of sprays had more effect than total misting hours. When displacement of Fp in the July experiment by Th (Fig. 5.3 A) and Fe (Fig. 5.3 B) was plotted against days, there were no differences between misting periods. This suggests that the straw was remaining wet for long periods.

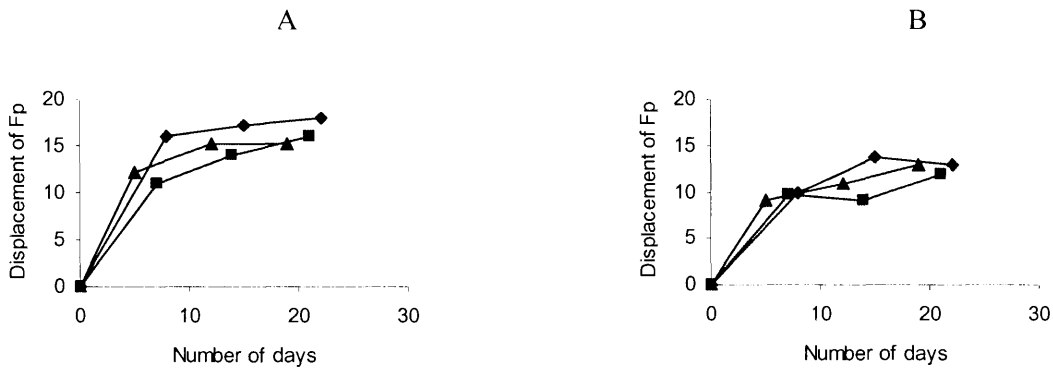


Fig. 5.3 Effect of durations on *Fusarium pseudograminearum* displacement by *T. harzianum* (A) and *F. equiseti* (B) at 4 (◆), 7 (■) and 10 (▲) hour misting durations.

Displacement of Fp from straw under field conditions

ANOVA tests revealed highly significant effects of experiment and antagonist on displacement and a highly significant interaction between antagonist and experiment. During the first set of observations in April-May, Th displaced Fp from straw pieces significantly more than all other antagonists, followed by Fe and Fn. Ai displaced Fp least from straw. The pattern was similar at other observation times but displacement declined during winter and increased in spring. The control yielded complete Fp recovery from all observation times (Fig. 5.4).

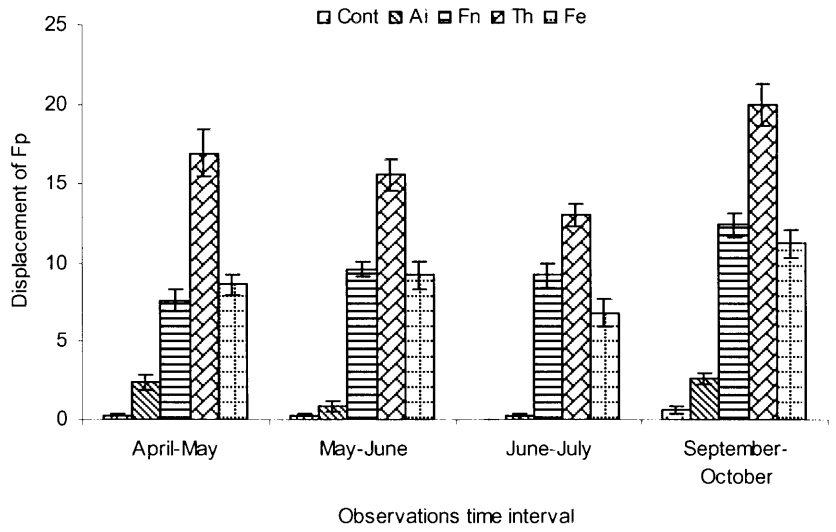


Fig. 5.4 Effect of time interval on displacement of Fp from straw pieces by different antagonists in field conditions Cont, control; Ai, *A. infectoria*; Fn, *F. nygamai*; Th, *T. harzianum*; and Fe, *F. equiseti* (vertical bars are the standard errors of the means).

Relationship between weather and displacement under field conditions

No relationship was found between sum of hourly temperatures when the wetness sensor was wet (value >30%) and displacement of Fp from straw pieces (Fig. 5.5). This is probably due to dew, which may not have wetted the straw sufficiently for fungal activity to occur.

The relationship between mean temperature during each experiment (equivalent to degree-days) and displacement was explored (Fig. 5.6). The fits of regression equations were not always very good and the intercepts of the regression lines were a long way from the origin.

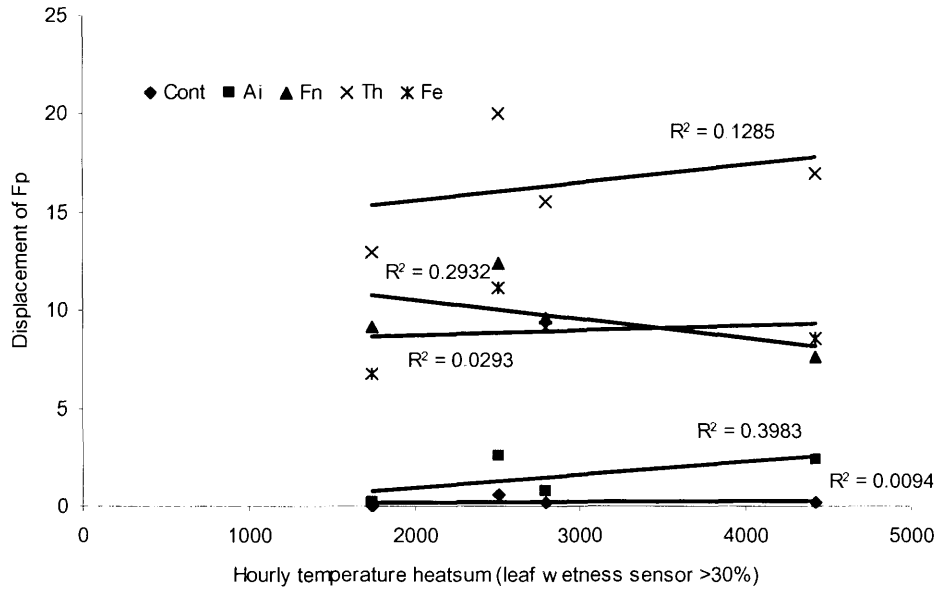


Fig. 5.5 Effect of hourly temperature heatsums if wetness sensor was greater than 30% with displacement of Fp from straw Cont, control; Ai, *A. infectoria*; Fn, *F. nygamai*; Th, *T. harzianum*; and Fe, *F. equiseti*.

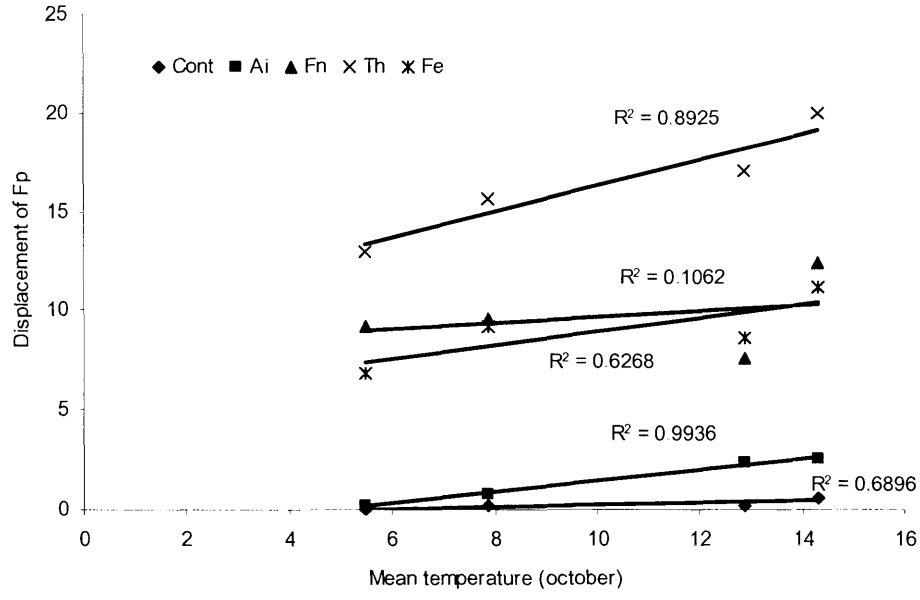


Fig. 5.6 Effect of mean daily temperature on Fp displacement from straw. Cont, control; Ai, *A. infectoria*; Fn, *F. nygamai*; Th, *T. harzianum*; and Fe, *F. equiseti*.

The influence of rain was explored by summing the mean temperature for every 24 hour period during which rain fell. Mean temperature was estimated as the average of the maximum and minimum temperatures.

Displacement was plotted against rainday heatsums for each antagonist and control (Fig. 5.7). The scatter plots suggested a linear relationship, so regression lines were fitted. None of these regressions was significant because of the small number of data points, although they did account for a high proportion of the variance in the data.

Regression lines fitted were:

$$\text{Control: } y = 0.0127x - 0.925;\ R^2 = 0.8651;\ p = 0.07$$

$$\text{Ai: } y = 0.0528x - 3.3965;\ R^2 = 0.6797;\ p = 0.176$$

$$\text{Fn: } y = 0.0774x + 2.5288;\ R^2 = 0.512;\ p = 0.284$$

$$\text{Th: } y = 0.1438x + 3.0743;\ R^2 = 0.8285;\ p = 0.09$$

$$\text{Fe: } y = 0.0806x + 1.4847;\ R^2 = 0.6723;\ p = 0.18$$

The regression equations gave good fits, with intercepts of lines close to the origin (Fig. 5.7). This suggests that a simple relationship based on temperature during rain days should give a good idea of relative rates of displacement.

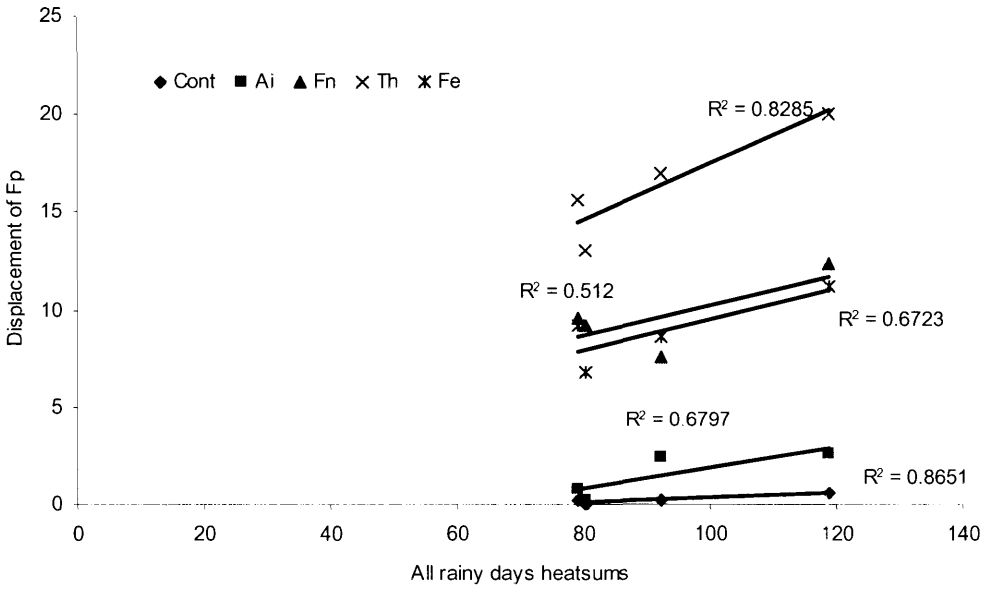


Fig. 5.7 Effect of sum of mean temperature of raindays on rate of displacement of Fp from stubble. Cont, control; Ai, *A. infectoria*; Fn, *F. nygamai*; Th, *T. harzianum*; and Fe, *F. equiseti*.

Tests on the data of Summerell and Burgess (1988b) were done. The sums of mean temperature of rainy days for Moree for the period of the experiment were calculated and plotted against the mean of stubble weight and recovery of Fp from stem and crown portions of wheat stubble for the heavy retained treatment. A very good fit was found with weight loss against rainday heatsums ($R^2 = 0.92$, $p = 0.003$). Fit for the displacement from the stem was $R^2 = 0.40$, $p = 0.174$ and from the crown $R^2 = 0.71$, $p = 0.036$ which was also good, confirming that the temperature of rain days is a useful and simple tool for estimating effects of weather on displacement of Fp from straw pieces (Fig. 5.8).

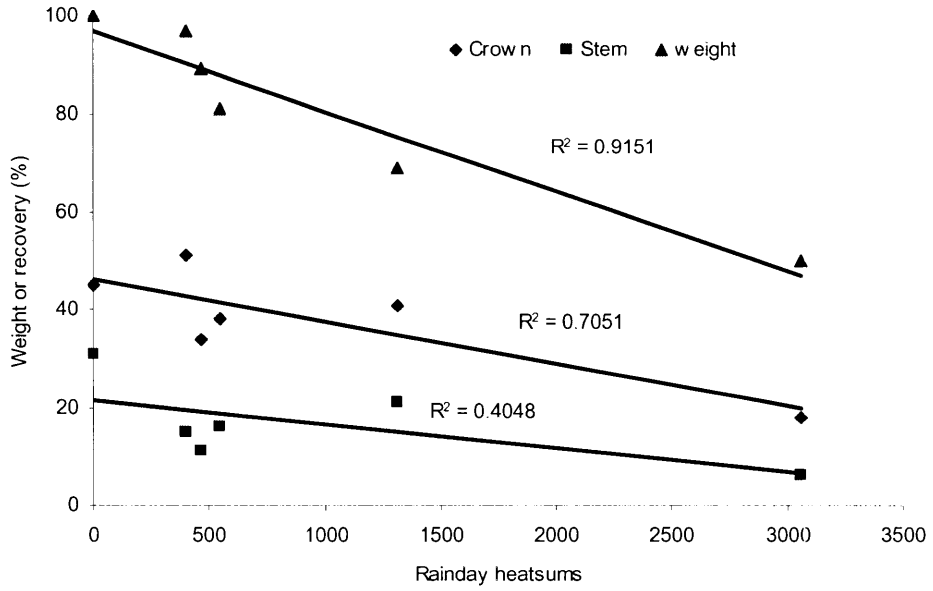


Fig. 5.8 Effect of rainday heatsums against stubble weight and recovery of Fp from stem and crown parts of stubble (data of Summerell and Burgess, 1988). Daily weather data for 1986-87 were obtained from the Bureau of Meteorology.

DISCUSSION

The initial experiment used 8, 16 and 24 hr misting periods based on the experiments of Zhang and Pfender (1993) to determine the range of time that would be critical for straw wetness and antagonistic activities to occur. However, high displacement by all antagonists was found at all misting cycles of 8, 16 and 24 hr. wetting and drying durations. So, later studies used shorter times of 4, 7 and 10 hr misting cycles.

At 4, 7 and 10 hr misting duration, observations from experiments could not determine the minimum wetness duration for displacement since straw remained wet longer than expected and thus reduced the differences between treatments of 4, 7 and 10 hr. This is in agreement with the findings of Zhang and Pfender (1992) that straw stayed wet for up to 3 hours after misting stopped. Also the lag period for Fp displacement may be shorter than for the *P. tritici-repentis* system used in their studies. The lag phase can be explained as the

period which compares straw wetness with water potential, i.e. when straw is first wet by misting the water potential does not change for approximately three hours. The water potential at this point shows a great change compared with the wetness reading three hours previously (Zhang and Pfender 1992).

Misting in the greenhouse had potential limitations especially in drying down the stubble pieces after a particular misting cycle stopped. This meant that it will not provide much information about what might happen under field conditions. Field experiments on Fp displacement from straw by antagonists relied more on natural temperature, rainfall, and humidity.

There was a problem in measuring wetness by sensors since it measured dew as well as rain. Dew may not be as effective because it only wets the surface so rain is a more useful measurement.

Mean temperature of raindays gave a good fit to *Fusarium* displacement. It also gave a good fit to the decomposition and displacement data of Summerrell and Burgess (1988b). This practice of using rainday heatsums is a very useful tool for predicting effects of seasons on displacement of *Fusarium* spp. form straw pieces since data is quite easy to get and calculate. Straw decomposition rates correlated for temperature and moisture follow an exponential decay curve (Quemada 2004). However, the degree of non-linearity is only over short periods (Quemada 2004). A linear fit may be good for comparing seasonal effects on Fp displacement.

Straw wets and dries fairly rapidly so it can be considered either wet or dry when it is on the surface. Water potential also declines quickly making it difficult for antagonists to remain active in these conditions. However, the case is different for buried straw, where water potential will decline more slowly. The activity of micro-organisms within the soil is largely determined by water potential which is typically above -2 MPa, so interactions such as competition or displacement of one fungus by an other will occur. However, the straw which

is lying above the ground will be wet for only short and could be for a long period in some years. Environmental fluctuation in temperature and water potential will limit the ability of most fungi particularly antagonists on above ground straw. So, low temperature tolerance will be more important in selection of antagonists above ground and low water potential below ground.

CHAPTER 6

NICHE OVERLAP OF *Fusarium pseudograminearum* AND ANTAGONISTS IN WHEAT STUBBLE RELATIVE TO CARBON SOURCE UTILISATION PATTERNS

INTRODUCTION

The coexistence of pathogen and antagonist in residues of crops after harvest is influenced by several abiotic factors such as the availability of nutrients, temperature and water in the substrate. Under field conditions rapid fluctuations in these parameters lead to the development of diverse microbial populations and competition for nutrients among the species present. Very few studies have been done on carbon source nutrition and utilisation by *Fusarium* spp. (Lilly and Barnett 1953, Ross 1960, Lopez and Fergus 1965), and none on *F. pseudograminearum* (Fp). Carbon source nutrition of *Trichoderma* spp. has been studied by Danielson and Davey (1973), Manczinger and Polner (1987) and Aube and Gagnon (1969). Mandels and Reese (1957) found cellulase induction in *Trichoderma* spp. was influenced by carbon sources.

The major carbohydrate in wheat straw is cellulose, a polymer of glucose molecules (Matulova *et al.* 2005). The second major carbon component present in wheat straw is hemicellulose, which is a collective name for polymers of a wide range of sugars, primarily D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose, D-glucuronic acid, D-galacturonic acid and secondarily L-rhamnose, and to a lesser extent L-fucose (Lawther *et al.* 1995). These are the main sugars that are released during the microbial decomposition of straw (Matulova *et al.* 2005).

Ji and Wilson (2002) tested the hypothesis that antagonists with a similar carbon source utilization profile to the pathogen would be most successful for biologically controlling bacterial speck disease (*Pseudomonas syringae* pv. *tomato*) of tomato. They found evidence that bacteria with high niche overlap with the target pathogen were effective in the

exclusion of the pathogen because they utilised carbon sources than would otherwise be available to the pathogen. Based on the findings of Ji and Wilson (2002), a similar hypothesis, that fungi with similar carbon source utilisation patterns would be effective in excluding Fp from straw.

The nutritional utilization behavior of the crown rot fungus and the antagonistic species is poorly understood, although some work had been done to test the ability of related *Fusarium* spp. to utilize carbon compounds (Lopez and Fergus 1965). *Fusarium roseum* from maize was able to use a wide range of sugars and polysaccharides, including many found in cereal straw, as sole carbon sources. The production of cell wall degrading enzymes, especially cellulases, xylanases and pectinases, has been shown to be important during infection of wheat heads by *F. culmorum* and *F. graminearum* (Kang and Buchenauer 2002, Wanjiru *et al.* 2002, Aleandri *et al.* 2007). It is likely that *F. pseudograminearum* can use a similar range of sugars and cell wall components as these species. *Trichoderma* spp. are well known for their ability to degrade a number of polysaccharides (Kubicek-Pranz 1998) and are capable of utilizing a wide variety of compounds as a sole source of carbon (Danielson and Davey 1973).

This chapter is a report of an investigation into carbon source utilisation by several antagonists and the target fungus Fp using Biolog MicroPlates 95 carbon substrate, as well as the ability of each fungus to grow on carbohydrates typical of those found in cereal straw. The effect of resource quality on antagonism was tested on straw medium supplemented with selected sugars in dual culture interactions of antagonists with Fp in Petri plate assays.

MATERIALS AND METHODS

Biolog plates

The utilization of different carbon sources by fungi (both antagonists and target fungi) was tested in Biolog FF MicroPlates (Biolog, Inc., CA). The 95 substrates present in a Biolog

plate include carbohydrate, carboxylic acids, amino acids, amines and amides and miscellaneous carbon compounds. The fungi Fp, Th, Fe, Fn and Ai were grown on ¼ strength PDA until conidia were present. To prepare a suspension, conidia were swabbed from the surface of the agar plate, and suspended at 4×10^4 spores/ml in FF Inoculating Fluid. Then 100 µL of suspension was pipetted into each well of the FF Microplate. These were incubated at 25°C and were examined daily for 5 days. Because there was a positive colour change in the negative (no C source) well in every plate, data were recorded as the relative growth of the fungi in the wells using a 0-3 scale (0, very poor; 1, low; 2, intermediate; 3, high fungal growth). To allow for inherent differences in growth rate between the fungi, the rating was done relative to the highest growth of each fungus. The experiment was done twice. Similarities between carbon utilization patterns were calculated as correlation coefficients (R^2), and these were used for unweighted pair group method with averages (UPGMA) clustering in SPSS.

C sources in minimal medium

Quantitative comparison of growth of Fp and four antagonists on different carbon sources (Table 6.1) was examined by measuring the growth rates of all fungi on carbon-free medium supplemented with the carbon sources to be tested. The experiment was replicated 3 times. The minimal medium of Asiegbu (2000) was used. The medium contained (g/L): NH_4NO_3 0.5; KH_2PO_4 0.6; K_2HPO_4 0.4; $MgSO_4.7H_2O$ 0.5; $CaCl_2$ 0.05 and micronutrients (mg/L): $FeCl_2$ 5; $ZnSO_4.7H_2O$ 4.4; $MnSO_4.7H_2O$ 5.0; $CoCl_2.6H_2O$ 1. Sugars and polysaccharides were added at 0.5% (5g/L) to measure radial growth. The pH of the media containing glucuronic acid and galacturonic acid was adjusted to between 6 and 6.5 using NaOH. The plates were inoculated with 5 mm plugs taken from cultures on ¼ PDA and were incubated at 25° in the dark. The growth of all five fungi was measured diametrically up to 7-8 days.

Relative growth was determined as the ratio of radial growth rate on a particular C source to the mean growth of that fungus on all C sources.

Table: 6.1 Mono and polysaccharides tested as sole carbon sources

Monosaccharides	Polysaccharides
d(-) Arabinose (Ara)	Cellulose powder (Cel)
d(+) Glucose (Glu)	Carboxymethyl cellulose (CMC)
d(+) Galactose (Gal)	Pectin (Pec)
d(+) Mannose (Man)	Polygalacturonic acid (PGA)
d(+) Xylose (Xyl)	Xylan
d - Glucuronic acid (Gln)	Starch
d - Galacturonic acid (Galn)	
l(-) Fucose (Fuc)	

Straw medium supplemented with C sources (radial growth and dual culture interactions)

Barley straw was ground through a 2 mm sieve and used as the nutrient base for an agar medium supplemented with different carbon sources. The recipe for straw media supplemented with different sugars (Ara, Xyl, Glu, Galn) was 12.5 g straw, 5 ml agar and 2.5 g sugars in 500 ml. A no-sugar control was also used. The individual growth rates and dual culture interactions with Fp were tested for the antagonists. Petri plates were inoculated with 5 mm plugs taken from cultures on ¼ PDA and were incubated at 25° in the dark. Growth of Fp with antagonists (Th, Fn, Fe and Ai) on straw medium with C sources in dual culture was done (method described in chapter 4). Growth was measured at the time when colonies first came into contact. Based on this and individual growth rates of different fungi on the straw medium, actual and expected growth of dual culture fungi was explored. Because growth was sparse, antagonistic interactions after contact could not be assessed. For all treatments 3 replicates were used. Data for each fungus were analyzed separately using ANOVA followed by Tukey's test for mean separation.

RESULTS

The utilisation patterns of 95 different carbon compounds by all three *Fusarium* spp. (Fp, Fn, Fn) were similar with correlations between species ranging from 46-57%. The patterns for the *Fusarium* spp. formed a tight cluster while the patterns for Th and Ai were more distant from the *Fusarium* species and from each other (Figure 6.1).

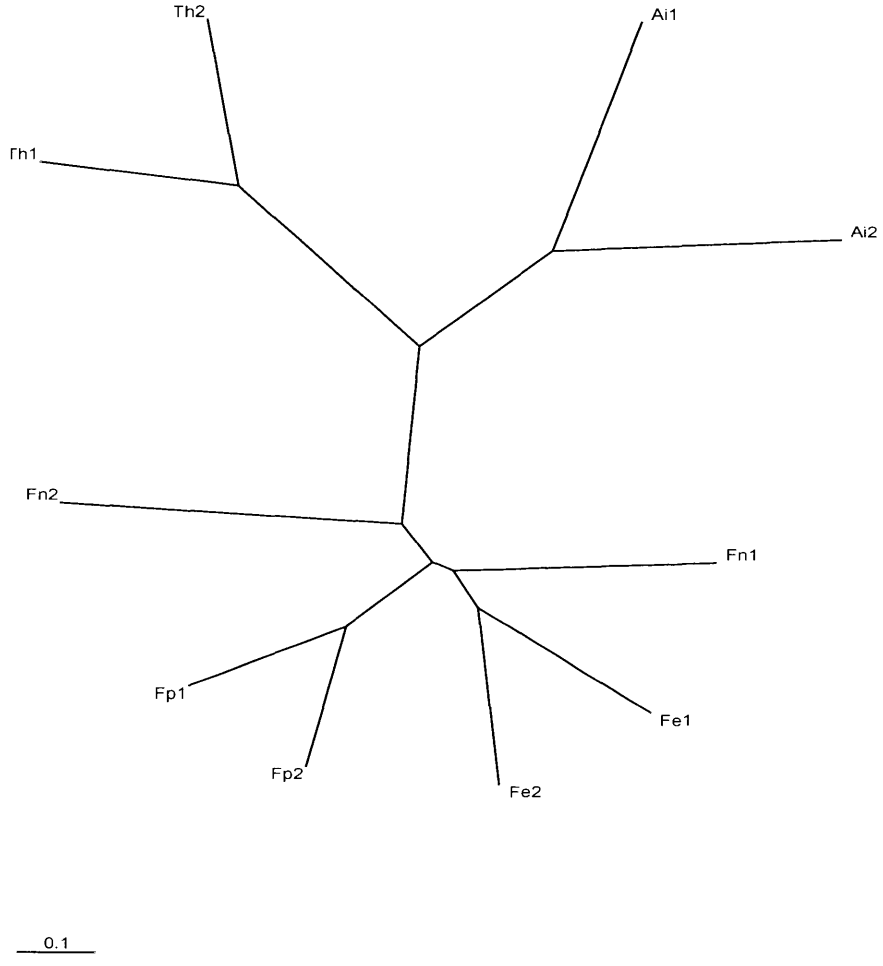


Fig. 6.1 Unrooted unweighted pair group method with averages (UPGMA) tree showing the relationship between carbon source utilization patterns of *F. pseudograminearum* (Fp), *F. equiseti* (Fe), *F. nygamai* (Fn), *A. infectoria* (Ai) and *T. harzianum* (Th) in 2 repeated experiments. Scale bar shows the correlation coefficient.

The relative growth rate of each fungus in Biolog plates on the substrates that occur in cereal straw is given in Table 6.2. Generally, all fungi grew well on most substrates, although no growth was recorded for Fp on l-Fucose in either experiment, and *F. equiseti* grew poorly on d-Arabinose.

Table: 6.2 Growth of fungi on Biolog plates on major sugars and sugar acids found in cereal straw on a 0-3 scale. Values are the mean of two repeat experiments.

Carbon Sources in FF MicroPlate	*F. pseudo-graminearum*	*F. equiseti*	*F. nygamai*	*T. harzianum*	*A. infectoria*
d(-) Arabinose	1.5	0.5	2.0	3.0	2.0
l(-) Fucose	0.0	1.5	1.5	2.5	2.0
d(+) Galactose	2.5	3.0	3.0	3.0	3.0
d-Galacturonic acid	2.0	1.5	2.0	3.0	1.5
α-d Glucose	1.0	2.0	3.0	3.0	2.5
d-Glucuronic acid	2.5	3.0	3.0	1.5	1.5
d(+) Mannose	1.5	3.0	3.0	3.0	3.0
d- Ribose	3.0	3.0	2.0	3.0	1.0
d-Xylose	2.0	3.0	2.5	3.0	3.0
l- Rhamnose	2.0	3.0	2.5	2.5	2.5

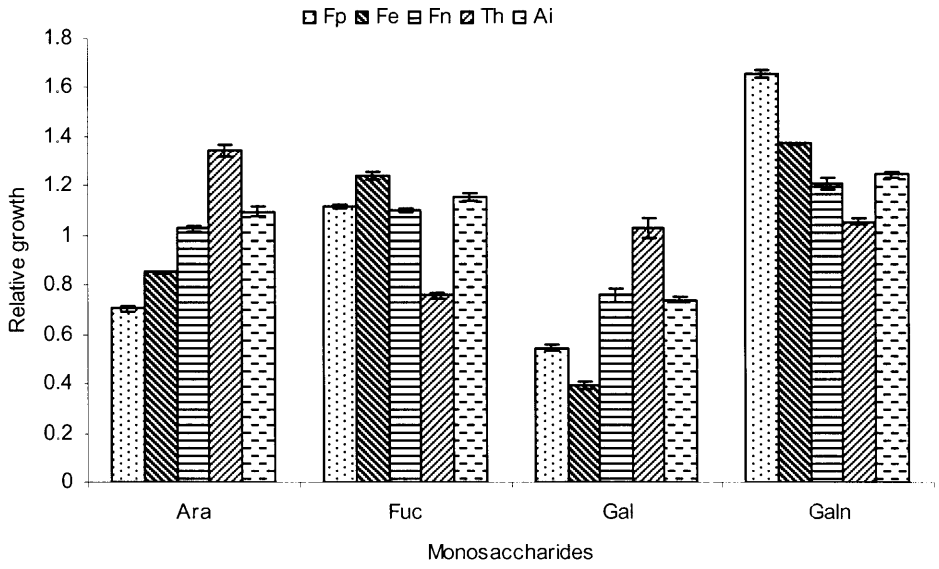
Relative growth rates of Fp and antagonists on sole carbon sources

The type of fungal growth varied on different carbon sources, with some promoting flat, rapidly spreading colonies and some promoting dense aerial hyphae. Comparisons were therefore made of relative growth among fungi within a carbon source, rather than between sources.

Arabinose gave strong growth for Th but was less favourable for Fp. Th grew less well on fucose than the other fungi. Th grew better than the other fungi on galactose, while Fp and Fe grew relatively poorly on this substrate. Growth of Fp was stimulated by galacturonic acid (Fig. 6.2 A).

Glucuronic acid favored the relative growth rate of Fe but supported slower growth by Th. Relative growth on glucose and mannose was greater in Fp and Th than the other fungi. Xylose supported faster growth of Th than the other fungi (Fig. 6.2 B).

A



B

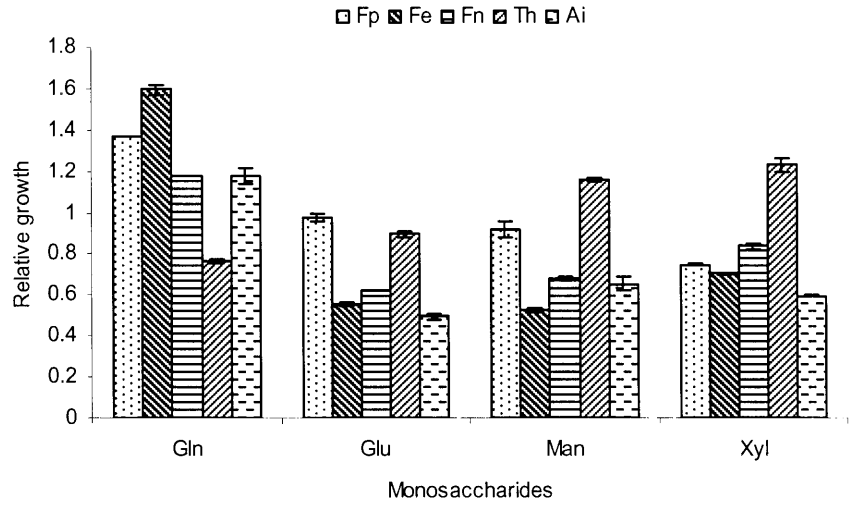


Fig. 6.2 Relative growth of *Fusarium pseudograminearum* (Fp) and antagonists Fe, *F. equiseti*; Fn, *F. equiseti*; Th, *T. harzianum*; Ai, *A. infectoria* on different monosaccharides as a sole carbon source (A and B) (vertical bars are the standard errors).

There was little difference in growth rates on cellulose and carboxymethylcellulose among fungi although Fp grew slightly faster than the other fungi on cellulose and slightly slower on carboxymethylcellulose. Pectin was less favorable for the growth of Fp and Ai than it was for the other fungi (Fig. 6.3 A).

Fe and Ai grew relatively well on polygalacturonic acid and Ai on starch. Fp grew relatively poorly on starch. Xylan was slightly favorable to Fp and unfavorable to Th (Fig. 6.3 B).

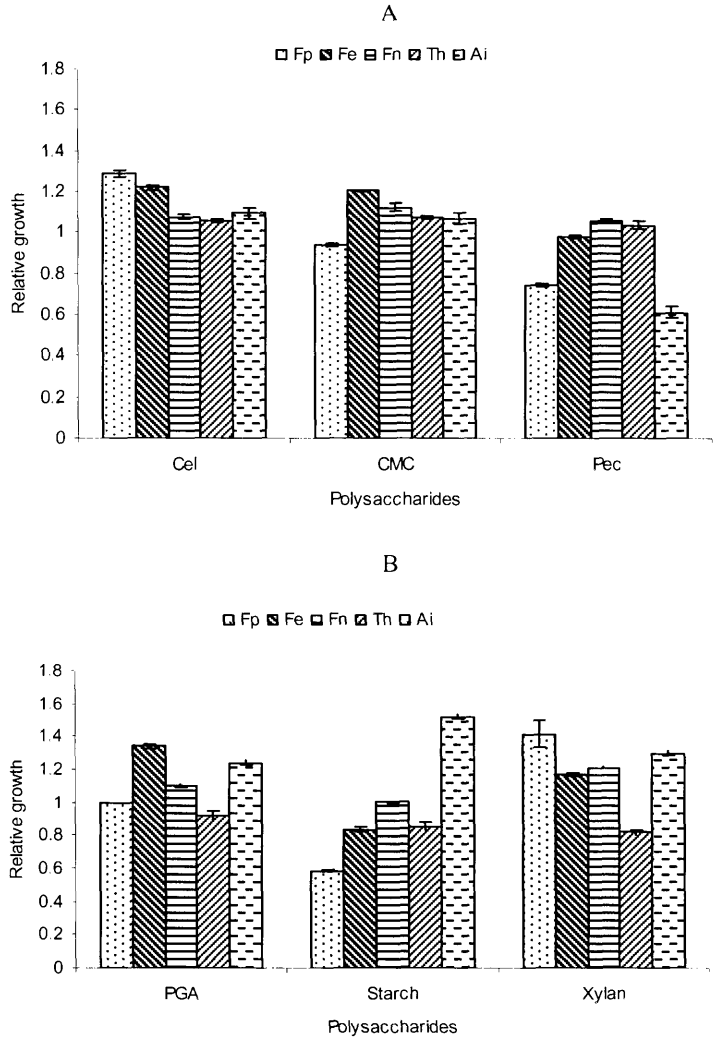


Fig. 6.3 Relative growth of *Fusarium pseudograminearum* (Fp) and antagonists Fe, *F. equiseti*; Fn, *F. equiseti*; Th, *T. harzianum*; Ai, *A. infectoria* on different polysaccharides as a sole carbon source (A and B) (vertical bars are the standard errors).

Radial growth rates, dual culture interactions of Fp and antagonists on straw medium supplemented with C sources

There was a significant effect of different monosaccharides on radial growth of fungi tested. All fungi grew relatively faster without any carbon substrates added to them. Growth of Fp was faster on galacturonic acid than on the other sugars. Fe, Fn and Ai grew faster on arabinose and glucuronic acid than they did on glucose or xylose. Th grew fastest on arabinose and most slowly on glucose (Fig. 6.4).

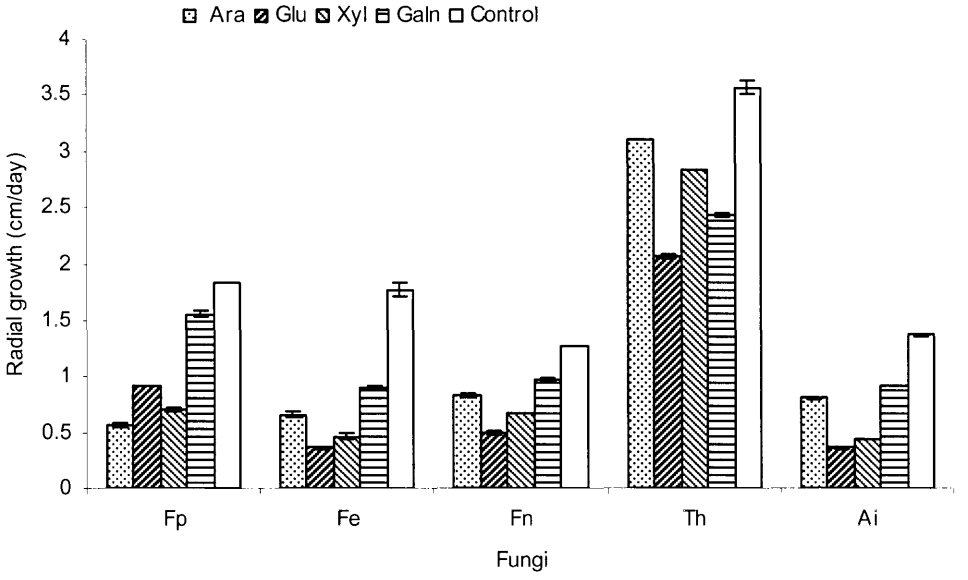


Fig. 6.4 Actual growth rates of different fungi (Fp, *Fusarium pseudograminearum* and antagonists Fe, *F. equiseti*; Fn, *F. equiseti*; Th, *T. harzianum*; Ai, *A. infectoria*) with selected sugars (Ara, Arabinose; Glu, Glucose; Xyl, Xylose and Galn, Galacturonic acid) on straw medium (vertical bars are the standard errors).

In dual culture, Fe grew better than expected with glucose and xylose and in the control, indicating antagonism prior to contact. Arabinose and glucuronic acid favored both fungi more or less equally (Fig. 6.5A).

Fn grew slightly further than expected when opposed to Fp with glucose and galacturonic acid added to the straw medium. In the control Fn grew more than expected (Fig. 6.5 B).

Fp grew further than expected against Th with each of the sugars added to the straw medium. Th grew further than expected in the control. This indicates that Fp inhibited Th prior to contact when sugars were added, but Th inhibited Fp when no sugars were added (Fig. 6.5 C).

Fp grew better than expected against Ai when supplied with arabinose, glucose, xylose and in the control whereas Ai grew better than expected when galacturonic acid was added to straw medium (Fig. 6.5 D).

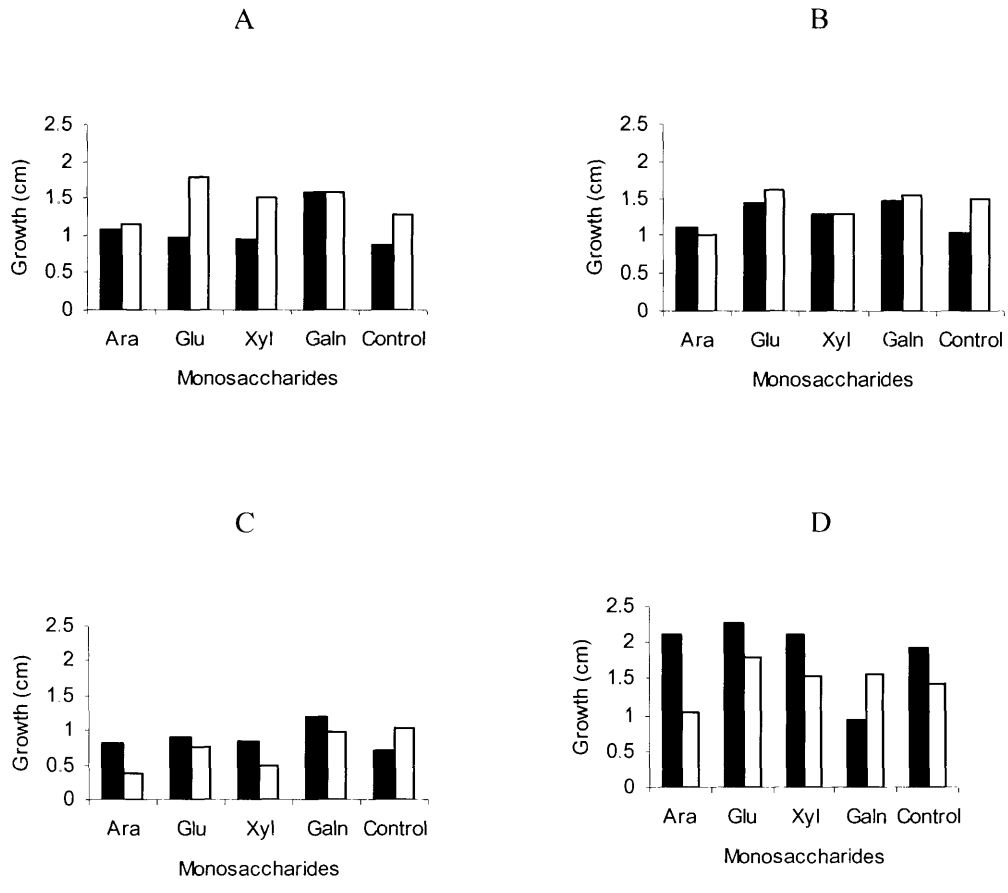


Fig. 6.5 Actual (■) and expected (□) growth of *Fusarium pseudograminearum* (Fp) in dual culture with Fe, *F. equiseti*; Fn, *F. equiseti*; Th, *T. harzianum*; Ai, *A. infectoria* Fe (A), Fn (B), Th (C) and Ai (D) on straw medium amended with monosaccharides at the time when colonies first came into contact Ara, Arabinose; Glu, Glucose; Xyl, Xylose and Galn, Galacturonic acid.

DISCUSSION

All *Fusarium* spp. had similar carbon sources utilisation patterns for the 95 substrates in the Biolog plates. In general competition for substrates would be expected to be greater between Fp and Fn and Fe than with other antagonists such as Th and Ai. This would be consistent with the hypothesis that competition for carbon sources was more important as a component of antagonism between Fe/Fp and Fn/Fp than it is for the *T harzianum*/Fp antagonism. However, Biolog tests may not be realistic as they included a large range of substrates that do not occur in straw. When the fungi were grown on the typical straw sugars as sole carbon sources, all of them grew with only small differences between sugars. It is possible that the differences detected in the Biolog test were not all that relevant to competition for C in straw. It could be said that niche overlap made a difference in some interactions but not in others. Ai and Th were both more different from Fp than were Fe and Fn, but Ai was the poorest displacer and Th the strongest. This suggests that specific antagonism (e.g. antibiosis) was more important than niche overlap. However, competition could still be a factor in the antagonism between *Fusarium* spp.

Some monosaccharides or sugar acids like galacturonic acid favored Fp whereas other *Fusarium* spp. preferred arabinose, fucose, galactose, glucuronic acid and xylose. Our results are in accordance with the findings of Lopez and Fergus (1965) on the broad abilities of *F. roseum* isolates to utilize different carbon compounds. Th grew well in presence of glucose, mannose and xylose and less on other monosaccharides, which is supported by the findings of Danielson and Davey (1973) for *Trichoderma* spp.

Among polysaccharides, there was not a lot of difference between fungal species. *Trichoderma* spp. are renowned for their ability to produce enzymes to degrade a variety of polysaccharides (cellulose, hemicellulose and carboxy-methylcellulose) and polymers such as chitin (Domsch and Gams 1969, Kubicek-Pranz 1998). *Fusarium* spp. (Fp, Fn and Fe) also grew fairly well on cellulose, carboxy-methylcellulose, pectin and xylan but less well on PGA and starch. This suggests their ability to degrade polysaccharides. *Fusarium graminearum* and

F. culmorum, which are closely related to Fp, possess the ability to produce cell wall degrading enzymes (Kang and Buchenauer 2002, Wanjiru *et al.* 2002, Aleandri *et al.* 2007). This could also apply to the *Fusarium* spp. included in this study, which were able to grow on the major substrates found in cell walls.

The dual culture experiment on straw medium indicated that the sugars present in the straw could influence the outcome of interactions between fungi. However, the effect of sugars on the interaction could not be predicted from their effects on growth. For example, Fp had grown much further than expected when it came into contact with Th and Ai in straw medium supplemented with arabinose. However, the relative growth rate of Fp on arabinose was lower than for the other fungi, suggesting that it was less favorable as a substrate.

The isolated fungi were all obtained from straw pieces but it could be expected that competition for nutrients among two *Fusarium* spp. (Fe and Fn) with Fp would be possible because of similarities in carbon source utilisation. There was evidence in dual culture interactions that Fe grew better than expected with glucose and xylose and Fn grew slightly further than expected with glucose and galacturonic acid, indicating antagonism prior to contact. Adding sugars to straw made Fp more competitive against Th in dual culture, signifying that Th is more specialized as a polysaccharide decomposer and not monosaccharides (Domsch and Gams 1969, Kubicek-Pranz 1998). Carbohydrate limitations or changes during decomposition are not likely to be significant in affecting displacement since straw commonly has ample cellulose and hemicellulose, which all fungi could use. (Summerell and Burgess (1989) showed that these polysaccharides were still present in significant quantities in two year old straw although their percentage decreased as the straw decomposed.

The greater growth on straw medium without added sugars could be because of the obvious reason that with low nutrients the hyphae explore the medium further trying to find

nutrients and when sugars are present they branch more and use the nutrients that are available (Ritz and Crawford 1999).

Pfender *et al.* (1996) suggested that carbon availability (such as glucose) limited microbial activity in straw. However, this was largely due to its effects on bacteria. Carbon availability does not appear to be an important factor limiting the activity of fungal antagonists against Fp.

CHAPTER 7

EFFECT OF DIFFERENT NITROGEN SOURCES ON *Fusarium pseudograminearum* DISPLACEMENT FROM STRAW PIECES

INTRODUCTION

Wheat straw has a high C:N ratio of 80:1 and there has been research interest in adding nitrogen to straw and wheat residues to enhance decomposition and death of *Fusarium* populations (Warren and Kommedahl 1972, Pereyra *et al.* 2004, Gaind *et al.* 2005). There is evidence that the form of N may be important in reducing *Fusarium* populations. Kollmorgen (1974) showed that urea reduced survival of *F. avenaceum* in oat straw, and studies by Cuilin (2002) found that ammonium nitrate increased populations of *F. graminearum* on wheat residues, while nitrolime (calcium cyanamide) reduced populations. Khonga and Sutton (1991) assessed the effects of urea on pieces of maize and wheat stems colonized by *Gibberella zeae* for its ability to produce perithecia or macroconidia and found that at 5 % concentration urea achieved a 97 % reduction of perithecial production on maize stems, while a lower concentration stimulated perithecial production. There has been no work on nitrogen nutrition of *F. pseudograminearum* and very little on related species. Studies on nitrogen source and nutrition of *Fusarium roseum* (*F. graminearum*) by Lopez and Fergus (1965) found that organic N sources gave the best growth of *Fusarium* sp.; ammonium compounds next, and nitrates the least.

Nitrogen source utilization by *Trichoderma* spp. has been studied by Danielson and Davey (1973), who observed that growth on ammonium N was superior to growth on nitrate N in a buffered medium. However, the reverse pattern was found in non buffered medium. Another study by Wakelin *et al.* (1999) on saprophytic growth of *Trichoderma* spp. recorded that N added as the ammonium form in sterile soil increased the saprophytic growth of *T.*

koningii whereas nitrate suppressed its growth. Celar (2003) reported that *Trichoderma* species preferentially used ammonium N, rather than nitrate N, when both are available.

The form of nitrogen also has been found to have a major influence on the interactions between antagonists and pathogens. Khattabi *et al.* (2004) found that suppression of *Sclerotium rolfsii* in soil by *T. harzianum* tended to be greater when nitrate was used as an N source compared with urea or ammonium. Suppression of *G. graminis* var. *tritici* by *T. koningii* was correlated with nitrate N, but not with ammonium N, in 28 soils (Duffy *et al.* 1997). This appears to be inconsistent with the reported preference of *Trichoderma* species for ammonium N for growth (Danielson and Davey 1973; Wakelin et al. 1999; Celar 2003).

No work has been done on *Fusarium* spp. (Fp, Fn and Fe), *Alternaria infectoria* and *T. harzianum* N utilisation and consequent effects on antagonistic abilities. Therefore a series of experiments were conducted to investigate the effects of different N sources on growth on a straw medium and on dual culture interactions between antagonist and pathogen. Further tests were done with different nitrogen sources for Fp displacement from straw in a Petri plate bioassay, and under more natural conditions when different N sources were sprayed on infested straw on field soil in a growth chamber. The objective of the study was to understand the effects of application of different N fertilizers on inoculum levels of the crown rot fungus in straw to control crown rot disease.

MATERIALS AND METHODS

Straw medium supplemented with N sources (radial growth and dual culture interactions)

The straw medium described in Chapter 6, supplemented with three different nitrogen sources along with a control was used to test radial growth and dual culture interactions. The N sources added were 0.48 g ammonium chloride (ammonium N), 0.76 g sodium nitrate, (nitrate N) and 0.27 g urea in 500 ml medium. This was equivalent to 1g N for every 40 g C in the straw. Petri plates were inoculated with 5 mm plugs taken from fresh cultures of different fungi (Fp, Fe, Fn, Ai and Th). Radial growth rates of different antagonists and fungi were assessed on straw medium. The dual culture assay was conducted as described in Chapter 6 but the medium used in this chapter was supplemented with N sources instead of C sources. There were 3 replicates for all treatments. Data for each fungus were analyzed separately using ANOVA followed by Tukey's test for mean separation in SPSS software.

Displacement assay

Displacement of Fp from precolonized straws pieces on SNA medium was done as described in Chapter 3, with the composition of the SNA modified to supply different nitrogen sources. To the basal SNA medium (KH_2PO_4 1 g, Mg $SO_4.7H_2O$ 0.5 g, KCl 0.5 g, glucose 0.2 g, sucrose 0.2 g and agar 20 g per litre) was added either 0.96 g ammonium chloride, 1.52 g sodium nitrate, or 0.54 g urea instead of KNO_3. The antagonists (Fe, Fn, Th, Ai) were allowed to grow on the modified SNA medium at 25° C until colonies covered the 9 cm Petri plate. Then 8 preinoculated Fp straw pieces were placed on top of the antagonist culture and the plates sealed with Parafilm. After 4 weeks incubation at 25° C in the dark, Fp displacement from the length of straw was recorded on a 0–4 scale. There were 4 replicates of each treatment. Data were analyzed in SPSS by factorial ANOVA.

Growth chamber Assay

The effect of N sources on displacement of Fp from straw placed on field soil was studied in a growth chamber. A black clay soil from Laureldale Farm, Armidale, NSW, Australia was used. This soil came from a field where crown rot caused by *F. pseudograminearum* and *F. culmorum* occurred in wheat and triticale, but was selected from a site which had not been used for growing cereals for at least 3 years. The soil was air-dried and passed through a 2 mm sieve. A sand-peat-loam potting mix was placed in rectangular plastic trays (29 cm × 33.5 cm) and layer of black soil 5 mm thick placed on top. Straw pieces precolonized with Fp were scattered on top of the soil. Solutions of urea, sodium nitrate and ammonium chloride were applied at rates equivalent to 100 kg N/ha by spraying on to the trays in 200 ml of water. Control trays were sprayed with water only. All trays were placed in a growth cabinet at 25°C day and 15°C night with a 12 h photoperiod, and watered at 4-5 days intervals to moisten the soil surface. At 2, 4, 8 and 16 weeks, 8 straw pieces were removed from each tray to test displacement of Fp from straw pieces. The experiment was replicated five times for each treatment.

Another experiment was done in the growth chamber with Fp inoculated straw pieces with Th bran inoculum on their surface (Chapter 5). These were sprayed with the same N sources as in trays, but in this experiment pots (radius 9.25 cm) were used and the straw was placed on top of the potting mix only. There were five replications and observations of Fp displacement were done after 4 weeks only. This experiment was carried out twice. Pots were watered once a week with 250 ml water for keeping the soil surface wet for microbial activity. Data were analyzed by ANOVA in SPSS with mean separations by Tukey's method.

RESULTS

Radial growth rates on straw medium

The nitrogen treatments had a significant effect on the growth of all fungi Growth of Th on straw media was promoted by urea and nitrate sources relative to the controls, whereas growth of Fp was favored by ammonium and inhibited by nitrate. Fe growth was promoted by nitrate. There were relatively small effects of added N on growth of Fn or Ai on straw medium. The growth of Fn was promoted by urea and ammonium, and growth of Ai inhibited by urea, relative to the controls (Fig. 7.1).

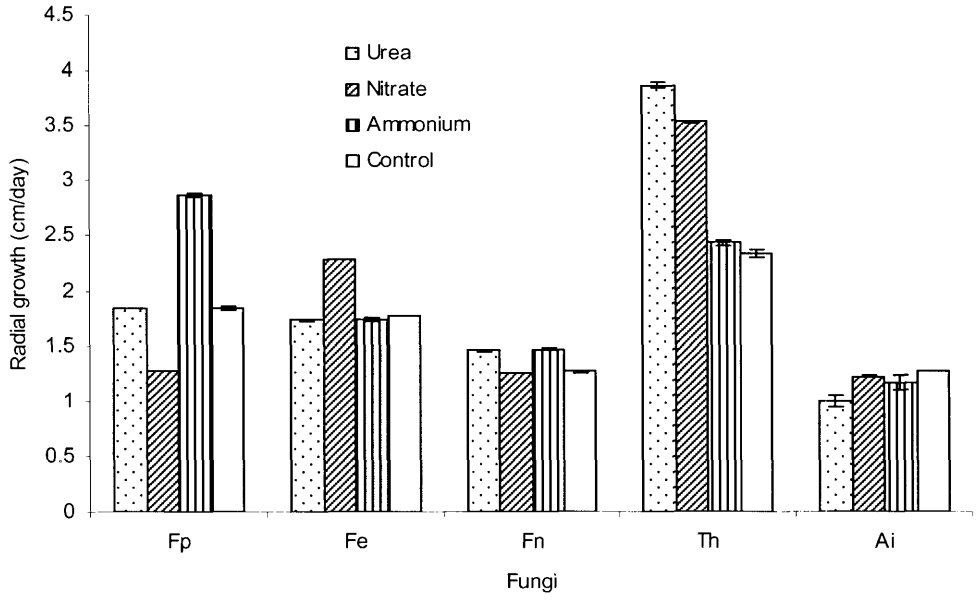


Fig. 7.1 Effect of N sources on radial growth rates of different fungi with N sources on straw medium (Fp, *Fusarium pseudograminearum*; Fe, *F. equiseti*; Fn, *F. nygamai*; Th, *Trichoderma harzianum* and Ai, *Alternaria infectoria*) Vertical bars are the standard errors.

Dual culture interactions on straw medium

Fp grew less than expected before contact with Fe with all N sources and the control. Inhibition of Fp by Fe was influenced most by ammonium (Fig. 7.2 A).

Fp also grew less than expected before contact with Fn with all N sources and the control. However, the greatest inhibition of Fp was seen with ammonium (Fig. 7.2 B).

On straw medium Fp grew less than expected against Th in urea, nitrate and control before coming in contact. Ammonium favored Fp, so it grew slightly further than was expected (Fig. 7.2 C).

Fp grew further than expected against Ai with all N sources and control. The difference between actual and expected growth was greatest for nitrate (Fig. 7.2 D).

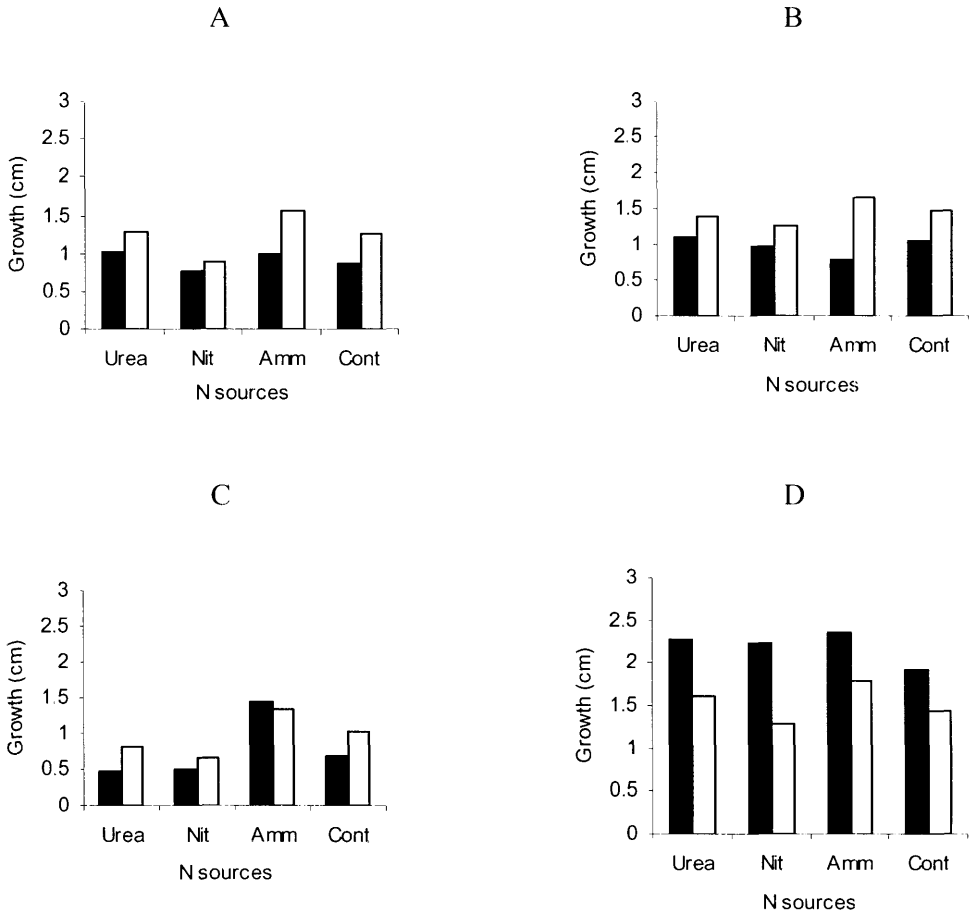


Fig. 7.2 Dual culture actual (■) and expected (□)interactions of *Fusarium pseudograminearum* (Fp) in dual culture with Fe, *F. equiseti*; Fn, *F. equiseti*; Th, *T. harzianum*; Ai, *A. infectoria* with Fe (A), Fn (B), Th (C) and Ai (D) Nit, Nitrate N; Amm, Ammonium N and Cont, Control

Effect of Nitrogen sources on displacement assay (Petri plates)

There was a significant effect of both antagonist and nitrogen source, and a significant interaction between the effects of these factors, on displacement of Fp by different antagonists. In general, displacement was least when ammonium was used (Fig. 7.3). Displacement by *T. harzianum* was significantly favored by urea followed by nitrate. The two *Fusarium* spp. (Fn, Fe) had a similar pattern of N sources behavior on Fp displacement with nitrate giving significantly more displacement than ammonium. Displacement by Ai was significantly greater with urea than with ammonium. In control treatments displacement of Fp was very low with all N sources.

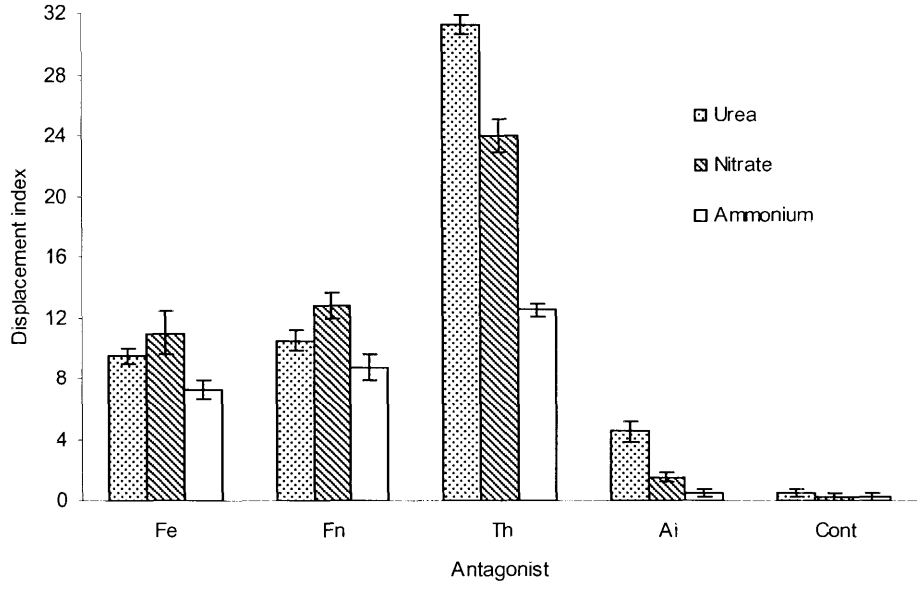


Fig. 7.3 Effect of nitrogen source on displacement of *Fusarium pseudograminearum* (Fp) from stubble pieces on SNA in Petri plates Fe, *F. equiseti*; Fn, *F. equiseti*; Th, *T. harzianum*; Ai, *A. infectoria* (vertical bars are the standard errors).

Nitrogen sources with Th inoculum displacement assay (growth chamber)

A significant effect of N sprays on displacement of Fp straw pieces coated with Th bran inoculum was found (Fig. 7.4). In both experiments, all N source additions gave significantly greater displacement than the control. In the first experiment, the highest level of displacement occurred with ammonium, followed by nitrate and then urea (Fig. 7.4 A). In the second experiment, ammonium gave significantly less displacement than either urea or nitrate, which was not significantly different from each other (Fig. 7.4 B).

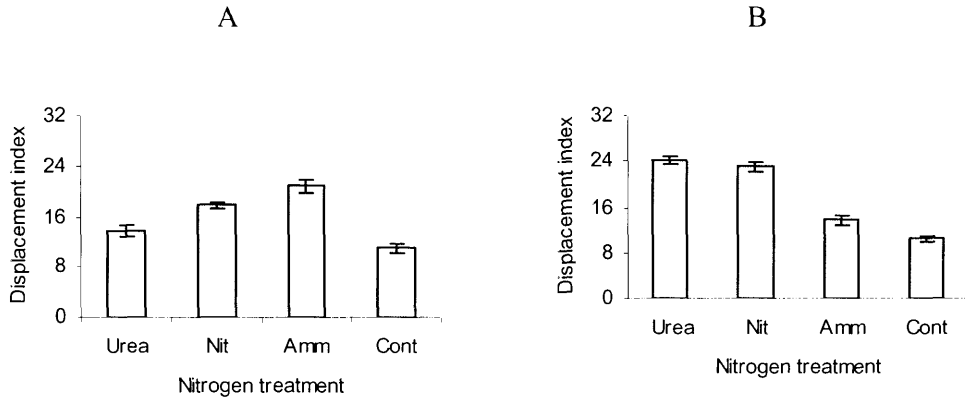


Fig. 7.4 Effect of nitrogenous fertilizers on displacement of *Fusarium pseudograminearum* (Fp) by *T. harzianum* (Th) from stubble pieces in growth chamber (A and B) Nit, Nitrate N; Amm Ammonium N and Cont, Control (vertical bars are the standard errors).

Nitrogen sources displacement assay (growth chamber)

Displacement of Fp from straw placed on black soil was significantly increased by nitrogen sprays at all time intervals. There were no significant differences between N sources at 2, 4 and 8 weeks. At 16 weeks displacement in the ammonium treatment was significantly less than in the urea or nitrate treatments (Fig. 7.5).

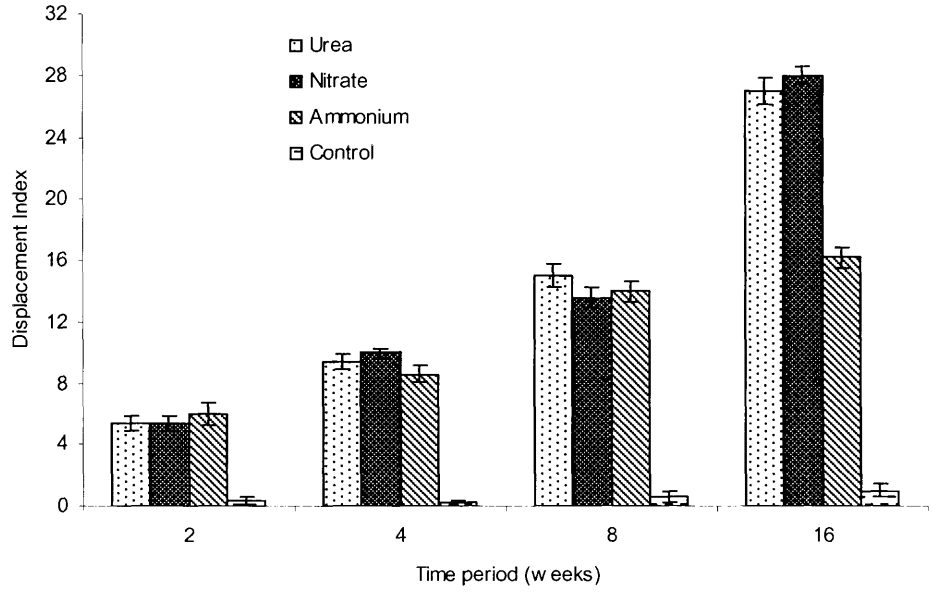


Fig. 7.5 Effect of nitrogenous sprays on displacement of Fp from straw pieces by native soil microflora at different time intervals (vertical bars are the standard errors).

DISCUSSION

Relative growth of Fp and antagonists were significantly favored by three N sources added to the straw medium. Fp was favored significantly by ammonium, Fe by nitrate, and Fn equally by ammonium and urea. Our results for *Fusarium* spp. (Fp, Fn) are consistent with the findings of Lopez and Fergus (1965) that *Fusarium roseum* isolates had best growth with ammonium and poorest growth with nitrate compounds. However, other organic compounds (glycine, valine, leucine etc.) tested by Lopez and Fergus (1965) were not included in this

study. In this study Fe had slightly more growth with nitrate addition than with ammonium, indicating preference of growth on two different N sources.

Ai was found to be favored by nitrate and ammonium whereas Th was significantly favored by urea and nitrate. These results are contrary to those of Danielson and Davey (1973) who reported that growth of *Trichoderma* spp. on ammonium was superior to that on nitrate N and that *Trichoderma* spp. mostly grew well on urea. The reason for this could be that they used non buffered media and the production of inhibitory H-ion concentrations in culture with ammonium may have changed the N utilisation. However, earlier studies by Hacskaylo *et al.* (1954) in non buffered medium indicated that nitrate was a better source of N for *Trichoderma* spp. than ammonium. This was consistent with the displacement assay in Petri dishes and suggests that using un buffered media gives a more realistic approach. Our results for *T. harzianum* are contrary to findings by Wakelin *et al.* (1999) saprophytic growth. of *T. koningii.* They found growth was increased when ammonium sulphate was applied and suppressed with calcium nitrate. This is probably due to differences in N sources tested; therefore it is necessary to test each situation to work out which N source is best.

In dual culture interactions on straw medium, inhibition of Fp by antagonists Fe and Fn was found to be greatest under the influence of ammonium N, which is inconsistent with the least displacement of Fp when ammonium N was added in the displacement bioassay. This suggests that the dual culture test was not a good predictor of displacement.

Nitrogen applications in combination with Th were found to increase Fp displacement from straw pieces. This is consistent with the findings of Kollmorgen (1974) that urea reduced survival of *F. avenaceum* in straw pieces whereas Yi *et al.* (2002) found that ammonium nitrate sometimes increased populations of *F. graminearum* on residues, while nitrolime (cyanamide) reduced populations. Hence N can increase displacement and mortality of *Fusarium* spp. on wheat residues, but it depends on the form in which it is applied. The effect of nitrogen form was not consistent between repeats of the experiment where Th was

inoculated on to straws. The reasons for this could not be determined within these experiments.

Pereyra *et al.* (2004) and Warren and Kommedahl (1972) discussed the importance of adding N fertilizers to accelerate the decomposition of wheat residues, thereby reducing *Fusarium* spp. populations. The slower decomposition of wheat residues could be explained by higher C:N ratio of wheat at harvest and is closely correlated with the relative mineralization potential of organic substrates added to soils (Yi *et al.* 2002). Adding N increased the displacement of Fp from straw pieces, both when a specific antagonist was applied and when displacement was by the background soil microflora. There was evidence that the form of nitrogen was important, but this was inconsistent between experiments. Field trials need to be done to determine which is the best form of nitrogen to use in practice.

CHAPTER 8
GENERAL DISCUSSION

The aim of this thesis was to determine the effects of abiotic (temperature and water potential) and nutritional factors (nitrogen and carbon sources) on displacement of *Fusarium pseudograminearum* (Fp) from stubble by potential antagonists. This major aim was successfully achieved through experimental work done in Chapters 3 to 7.

The dual culture techniques used in this project provided a reasonable prediction of temperature and water potential interactions on displacement of Fp from straw. (I_D Index of Dominance) in higher categories was fairly consistent with displacement patterns (Chapter 4). Dual culture studies in the carbon and nitrogen utilisation experiments (Chapter 6 and 7) measured growth at contact only. In the above experiments I_D could not be used because displacement was not determined on low nutrient media and growth prior to contact was not consistent with displacement. So the dual culture technique of I_D is preferred for use since it gave a good relationship with displacement. I_D patterns (Lee and Magan 1999b) were highly sensitive to changes in water potential or temperature with different antagonists (Chapter 4). The key role of low water potential as a limiting factor in antagonism by *Trichoderma* species could be illustrated well from studies by various researchers (Tronsmo and Dennis 1978, Kredics *et al.* 2000, Kredics *et al.* 2003).

The displacement assay used in this thesis is a sensitive tool and could detect changes over a shorter time than assay that measured complete displacement (Wong *et al* 2002). The time taken to complete displacement assay experiments was within 4 to 6 weeks (Chapter 3, 4) which is relatively shorter than experiments which were done in several months. However, the displacement assay could not demonstrate a reduction in inoculum potential. Over 4 weeks, presumably because there was still enough Fp mycelium to infect plants. Real time-PCR could not be used as an independent method to measure inoculum remaining in straw pieces (Chapter 3). It may be that the displacement assay had possibly measured antagonism

on agar after fungi emerged from the straw. However, I had added Terraclor in my experiments which is semi–selective and allows *Fusarium* species to grow but is inhibitory to other fungi. The use of the displacement assay tells us that the antagonist is becoming more established in straw as the displacement index increased over time (Chapter 3) suggesting that it gives an insight into the real processes occurring in straw.

The displacement of *Fusarium* spp. has been studied by other workers in different ways (Summerrell and Burgess 1988b, Wong *et al.* 2002, Pereyra *et al.* 2004). These researchers studied the proportion of stubble pieces from which *Fusarium* spp. could be isolated. Several other researchers measured spores produced on residues of wheat or other crops (Yi *et al.* 2002, Gilbert and Fernando 2004, Luongo *et al.* 2005, Inch and Gilbert 2007). The displacement assays used in this study measured proportion of length of straw from which Fp was displaced based on 0-4 scale. This scale gave a better and sensitive measure than just measuring presence or absence of Fp from straw which would not have detected all treatment effects in most experiments. Hence it was used as a convenient tool from measuring Fp displacement form straw in this thesis.

Antagonists were originally chosen since *Trichoderma harzianum* (Th) was a strong antagonist. *Fusarium* spp. chosen as antagonists were *Fusarium nygamai* (Fn) and *F. equiseti* (Fe) which were good competitors with Fp and were found to be reasonably abundant in stubble. *Alternaria infectoria* (Ai) was also included in all studies since it was the most common fungus in straw but only a weak antagonist against Fp.

Th was strongly antagonistic. *Trichoderma* spp. are known for their antagonistic abilities (Kubicek-Pranz 1998, Kredics *et al.* 2003). However, Fn and Fe were moderately antagonistic and possibly more direct competitors of Fp (Pereyra *et al*, 2004).

Water potential obviously does affect the rate of displacement, particularly low water potential. Studies by Cook and Christen (1976) showed the influence of water potential and temperature on growth of *Fusarium* spp. and found that at lower water potential, the

Fusarium spp. grew best at the higher temperature. Various researchers tested the effects of temperature and water potential on interactions between maize spoilage fungi and observed a strong interaction between temperature and water availability in their effects on the competitive ability of fungi, and on the types of interactions between fungi (Marin *et al.* 1998a,b; Lee and Magan 1999b). Therefore temperature and water potential were considered together in Chapter 4.

Temperature had a greater effect than water potential on displacement of Fp from straw by the antagonists *Fusarium nygamai* (Fn), *F. equiseti* (Fe), *Trichoderma harzianum* (Th) and *Alternaria infectoria* (Ai). Higher temperature around 25°C, which is the standard temperature for growth of fungi in culture, was better for displacement of Fp from stubble by antagonists such as Th (Chapter 4). The studies of Wong *et al.* (2002) showed greater displacement of Fp by *Trichoderma* spp. at 25°C. They acknowledged that stubble borne fungi like Fp in standing stubble would not be as effectively controlled by *Trichoderma* species as they would in buried straw, because the stubble can dry out quickly. Antagonism in dual culture by Th seemed to be affected by temperature, because it reduced growth of Fp more than expected at high temperature and less than expected at low temperature (Chapter 4). This may be due to the effects of temperature on production of enzymes or volatile inhibitors involved in antagonism by Th (Tronsmo and Dennis 1978). All antagonists were less effective at low temperatures.

Environmental effects like temperature and rainfall play an important role in fungal survival and competition in stubble pieces. Long-term average monthly temperatures and rainfall from Gunnedah Research Station, the nearest official weather station to the collection sites in the Liverpool Plains area used in this project, are plotted in Figs. 8.1 and 8.2. It could be noticed that there are significant periods of time above 25°C for only a few months in summer, and that for 6 months of the year the temperature is always below 25°. Average annual temperature is 18.2°, so half of the time the temperature will be below this. Average

annual rainfall was 632 mm and it could also be noticed that monthly rainfall was about 40-45 mm during most parts of the year, whereas in summer months rainfall was highest (Fig. 8.2).

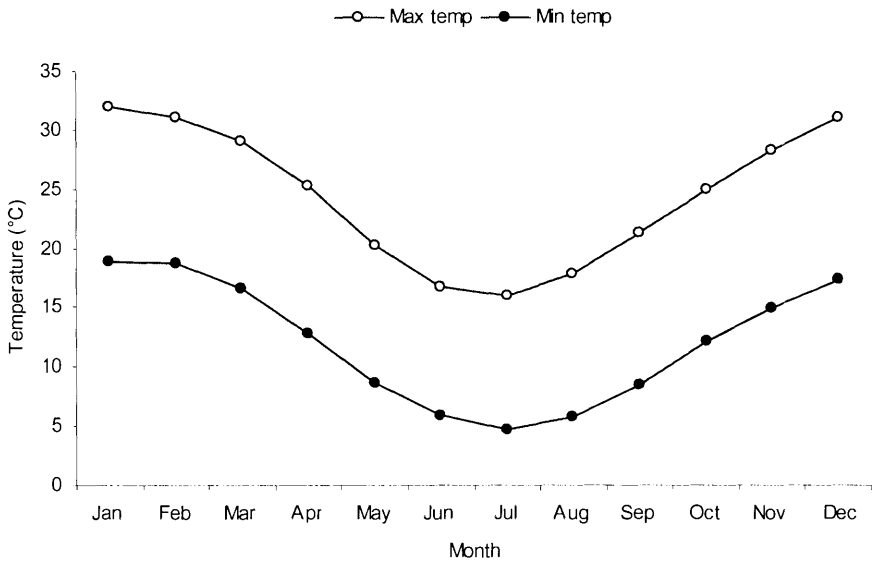


Fig. 8.1 Long-term average monthly temperatures for Gunnedah Research Station, NSW, Australia.

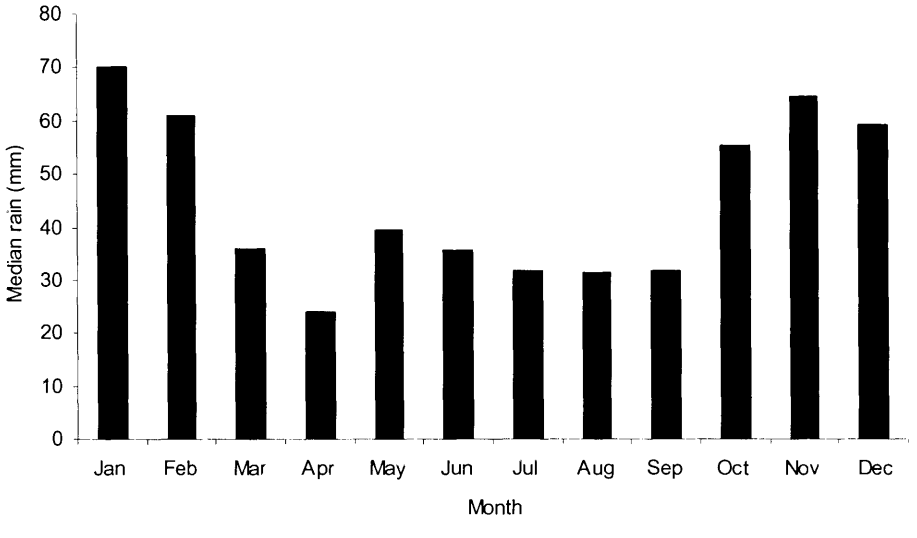


Fig. 8.2 Long term average monthly rainfall for Gunnedah Research Station, NSW, Australia.

The warm, wet conditions which are favorable for displacement of Fp from stubble by other fungi (Burgess and Griffin 1968) only occur for a short period each year. The ability to displace Fp at 15°C or below will be very important for large parts of the year.

Summing the mean temperature of raindays gave a good fit to stubble decomposition and to *Fusarium* displacement. The practice of using rainday heatsums is a simple but useful tool for predicting effects of seasons on displacement of *Fusarium* spp. from straw since data are quite easy to obtain and calculate (Chapter 5). Straw wets and dries fairly rapidly on the surface and water potential also declines faster making it difficult for antagonists to remain active for long after rainfall in these conditions. However, the case is different for buried straw, which will decline in water potential slowly. The activity of micro-organisms within the soil is largely determined by water potential which is typically greater than -2 MPa. All of the antagonistic fungi could displace Fp at this water potential. So interactions such as competition or displacement of one fungus by another will happen. However the straw which is lying above ground will have more environmental fluctuation in temperature and water potential and this will limit the ability of most fungi particularly antagonists more on above ground than below ground straw.

Competition for carbon sources occurred among different *Fusarium* spp. (Fn, Fe and Fp) (Chapter 6) since all *Fusarium* spp. grew with only small differences on typical straw sugars as sole carbon sources. Competition could also be linked with temperature and water potential requirements for growth which were more similar for Fp and Fe than for Fn, which preferred wet conditions rather than warm dry conditions preferred by Fp and Fn. Th grew best in wet hot regimes whereas Ai grew broadly under all of the conditions examined (Chapter 4). In dual culture studies inhibition of Fp by Th prior to contact was found which could be due to antibiosis rather than nutrient competition (Chapter 3). Ai, which was the most commonly occurring fungus on straw, could utilise all resources in straw (Chapter 6). However, it could not displace Fp from straw to a significant extent.

Nitrogen applications in combination with Th were found to increase Fp displacement from straw pieces (Chapter 7). N can increase displacement and mortality of *Fusarium* spp. on wheat residues, but it depends on the form in which it is applied. The effect of nitrogen form was not consistent where Th was inoculated on to straws (Chapter 7). It is hard to explain these differences based on small set of data. Field trials need to be done to determine the best form of nitrogen to use in practice to manage Fp in stubble.

OVERALL CONCLUSIONS

The best antagonists to be applied in the field need to have the ability to displace Fp under harsh conditions, i.e. under cold dry conditions.

It is practically not feasible to alter the environmental conditions such as temperature or rainfall. However, manipulation of the field system could be done by adding antagonists which otherwise have low populations (Chapter 3). It is not that useful to add other antagonists such as Fe and Fn which are already in reasonable populations (Chapter 3) in straw. Pereyra (2004) also showed that populations of Fe and other *Fusarium* spp. increased over time in buried residues. Th is potentially a strong antagonist but has infrequent occurrence under field situations. So addition of Th antagonists is possible for managing crown rot fungus Fp in residue.

Techniques such as the displacement assay or Index of Dominance should be preferably used for assessing antagonists for Fp displacement from straw. The conditions for testing displacement should include temperatures below 25°C to test the ability of antagonists under cool conditions typical of those occur in the field.

From the work done so far it is important to conduct field trials on the need to apply antagonists such as *Trichoderma harzianum* to test their effects under field conditions on Fp displacement from stubble. There is also a need to try different N sources applied to stubble to determine the best form of nitrogen application practice to manage Fp.

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APPENDIX 1

Frequency of isolation of fungi from two sections of cereal stubble averaged across 5 sites

Species	Site 1		Site 2		Site 3		Site 4		Site 5	
	Basal	Stem	Basal	Stem	Basal	Stem	Basal	Stem	Basal	Stem
Acremonium sp.	4	0	1	0	1	1	0	0	0	0
Alternaria sp.	1	10	2	5	2	2	1	7	2	8
Basidiomycete	0	0	1	0	0	0	0	1	0	0
Bipolaris sorokiniana	0	1	3	2	0	0	3	3	1	1
Epicoccum nigrum	0	3	0	0	0	0	0	0	0	0
Fusarium chlamydosporum	0	0	0	0	0	0	1	0	0	0
F. equiseti	5	0	0	0	0	0	2	0	0	0
F. nygamai	4	0	0	0	0	0	0	0	0	2
F. oxysporum	4	0	0	0	0	0	0	0	0	0
F. pseudograminearum	0	0	4	4	7	9	1	1	7	3
Melanospora sp.	0	0	1	0	0	0	0	0	0	0
Nigrospora sphaerica	0	2	0	0	0	0	0	0	0	0
Rhizoctonia solani	0	0	0	0	3	0	3	4	2	0
Sterile sp. 1	8	5	2	4	5	2	6	4	4	0
Sterile sp. 2	0	1	1	0	3	1	0	0	1	0
Sterile sp. 3	0	0	1	2	0	1	4	1	1	0
Trichoderma sp.1	0	0	1	0	0	0	0	0	0	0
Trichoderma sp.2	0	0	0	0	2	0	0	0	2	0

APPENDIX 2

Fungi identification test panel (Average of relative growths of different fungi)

Carbon Sources in FF MicroPlate	*F.pseudograminearum*	*F. equiseti*	*Trichoderma harzianum*	*F. nygamai*	*A. infectoria*
Water	0.0	0.0	0.0	0.0	0.0
Tween 80	1.5	2.0	0.5	1.5	2.0
N-Acetyl-D-Galactosamine	0.0	0.5	0.0	0.5	1.5
N-Acetyl-D-Glucosamine	2.5	3.0	3.0	3.0	1.0
Adonitol	2.5	2.0	2.0	1.5	3.0
Amygdalin	2.5	3.0	3.0	3.0	3.0
D-Arabinose	1.5	0.5	3.0	2.0	2.0
L-Arabinose	2.5	2.5	3.0	2.5	2.0
D-Arabitol	3.0	2.0	3.0	3.0	2.0
Arbutin	1.0	1.5	1.5	3.0	2.0
D-Cellobiose	3.0	2.5	3.0	3.0	1.5
α-Cyclodextrin	0.0	0.5	0.0	0.5	1.0
β-Cyclodextrin	0.0	0.0	1.0	0.5	1.0
Dextrin	2.5	3.0	3.0	2.5	3.0
i-Erythritol	0.0	1.0	3.0	1.0	3.0
D-Fructose	3.0	2.5	3.0	3.0	3.0
L-Fucose	0.0	1.5	2.5	1.5	2.0
D-Galactose	2.5	3.0	3.0	3.0	3.0
D-Galacturonic acid	2.0	1.5	3.0	2.0	1.5
Gentiobiose	3.0	2.5	3.0	3.0	3.0
D-Gluconic Acid	2.0	3.0	1.5	2.5	2.0
D-Glusoamine	3.0	3.0	1.5	3.0	1.0
α-D-Glucose	1.0	2.0	3.0	3.0	2.5
Glucose-1-Phosphate	0.5	0.0	.0.	0.5	0.5
Glucuronamide	0.0	0.0	0.0	0.5	0.0
D-Glucuronic Acid	2.5	3.0	1.5	3.0	1.5
Glycerol	2.5	3.0	3.0	3.0	1.5
Glycogen	3.0	1.5	2.5	2.5	1.5
m-Inositol	1.0	2.0	2.0	3.0	2.5
2-Keto-D-Gluconic Acid	2.0	3.0	3.0	2.0	3.0
α-D-Lactcose	2.0	2.0	3.0	2.0	3.0
Lactulose	0.0	0.5	3.0	2.0	3.0
Maltitol	0.5	3.0	1.0	2.0	2.0
Maltose	2.0	3.0	3.0	3.0	2.5
Maltotriose	1.5	2.0	3.0	2.5	2.5
D-Mannitol	3.0	3.0	3.0	2.5	3.0
D-Mannose	1.5	3.0	3.0	3.0	3.0
D-Melezitose	2.5	3.0	0.0	3.0	3.0

D-Melibiose	3.0	3.0	3.0	3.0	3.0
α-Methyl-D-Galactoside	3.0	2.5	2.5	3.0	3.0
β-Methyl-D-Galactoside	0.5	1.0	1.5	2.5	3.0
α-Methyl-D-Glucoside	1.5	3.0	1.0	2.0	2.5
β-Methyl-D-Glucoside	3.0	3.0	3.0	3.0	3.0
Palatinose	3.0	3.0	1.5	3.0	3.0
D-Psicose	0.0	1.5	1.5	1.5	2.0
D-Raffinose	3.0	3.0	3.0	3.0	3.0
L-Rhamnose	2.0	3.0	2.5	2.5	2.5
D-Ribose	3.0	3.0	3.0	2.0	1.0
Salicin	1.0	2.5	2.5	2.5	2.0
Sedoheptulosan	0.0	1.5	0.0	2.0	0.5
D-Sorbitol	3.0	3.0	3.0	2.5	3.0
L-Sorbose	1.5	2.5	1.5	3.0	2.0
Stachyose	3.0	3.0	2.5	3.0	3.0
Sucrose	2.5	3.0	3.0	3.0	3.0
D-Tagatose	0.0	1.0	2.5	2.0	2.0
D-Trehalose	3.0	2.5	3.0	3.0	3.0
Turanose	3.0	3.0	1.0	3.0	3.0
Xylitol	2.5	1.5	3.0	2.0	3.0
D-Xylose	2.0	3.0	3.0	2.5	3.0
γ-Amino-butyric Acid	2.0	3.0	3.0	2.0	1.5
Bromosuccinic Acid	0.5	1.5	2.0	2.5	1.0
Fumaric Acid	1.5	3.0	1.0	3.0	2.5
β-Hydroxy-butyric Acid	1.5	2.5	1.5	3.0	1.0
γ-Hydroxy-butyric Acid	0.5	2.0	1.0	2.5	1.0
ρ-Hydroxyphenyl-acetic Acid	2.0	2.0	0.5	2.5	0.0
α-Keto-glutaric Acid	1.5	2.0	0.5	2.0	0.5
D-Lactic Acid Methyl Ester	1.0	1.0	0.5	1.5	0.5
L-Lactic Acid	2.0	2.5	0.5	2.0	1.0
D-Malic Acid	1.5	3.0	1.0	3.0	2.5
L-Malic Acid	1.5	3.0	1.0	2.0	3.0
Quinic Acid	2.0	3.0	3.0	2.0	1.5
D-Saccharic Acid	1.5	2.5	2.0	1.5	1.5
Sebacic Acid	0.5	1.0	1.5	0.0	1.5
Succinamic Acid	0.5	0.5	1.5	2.5	2.5
Succinic Acid	1.5	3.0	1.5	2.5	2.0
Succinic Acid Mono-Methyl	0.0	1.5	2.0	1.5	2.5

Ester					
N-Acetyl-L-Glutamic Acid	0.0	0.5	1.5	1.0	0.5
Alaninamide	1.0	1.0	0.5	0.5	0.5
L-Alanine	2.0	3.0	1.5	3.0	0.5
L-Alanyl-Glycine	2.0	3.0	1.0	2.5	2.5
L-Asparagine	2.0	3.0	1.0	3.0	2.5
L-Aspartic Acid	2.0	3.0	1.5	2.5	2.5
L-Glutamic Acid	2.0	2.5	2.5	1.5	2.5
Glycyl-L-Glutamic Acid	0.5	1.0	1.5	0.0	1.0
L-Ornithine	2.0	3.0	2.0	3.0	1.0
L-Phenylalanine	1.5	2.0	2.0	2.0	0.5
L-Proline	2.0	3.0	1.0	3.0	2.5
L-Proglutamic Acid	2.0	3.0	3.0	2.5	0.5
L-Serine	2.0	1.5	1.0	2.5	2.0
L-Threonine	0.0	1.5	1.0	2.5	1.0
2-Amino Ethanol	1.5	3.0	0.5	2.0	0.0
Putrescine	1.5	2.0	0.5	2.0	1.0
Adenosine	0.0	0.5	1.0	0.5	1.0
Uridine	0.5	0.0	0.0	0.5	1.0
Adenosine-5′-monophosphate	0.5	0.0	2.0	1.0	1.5

0 Very poor fungal growth; 1 low; 2 Intermediate; 3 Extreme fungal growth