Molecular techniques for studying *Fusarium* ear blight of wheat

Thesis

UNRESTRICTED

MOLECULAR TECHNIQUES FOR STUDYING *FUSARIUM* EAR BLIGHT OF WHEAT

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ABSTRACT

This work has compared polymerase chain reaction (PCR) assays and traditional visual disease assessment to evaluate the severity of *Fusarium* ear blight (FEB) of wheat under field and glasshouse conditions. In a field trial, PCR analysis highlighted the problem of diagnosis of FEB of wheat based on visual disease assessment where natural inoculum was present. PCR-based analysis detected *F. poae* predominantly in the glumes and *Microdochium nivale* sub-species were predominantly found in the rachis component of ears. *M. nivale* var. *majus* was more frequently observed than var. *nivale* (64 and 36 %, respectively).

Quantitative PCR analysis and conventional visual disease assessment were used to evaluate fungicide efficacy against FEB of wheat caused by *F. culmorum* and *F. poae* in three glasshouse trials (1994/5-1996/7). Prochloraz and tebuconazole significantly decreased both visual symptoms of FEB and fungal DNA content of *F. culmorum* and *F. poae* ear blight of wheat. Overall, both fungicides appeared equally effective, although the efficacy of these fungicides was consistently greater as measured by PCR analysis rather than by visual disease assessment. Inoculation with *F. culmorum* significantly reduced yield (1000 grain weight) whereas inoculation with *F. poae* had no significant effect on yield.

Fusarium culmorum was successfully transformed with the GUS reporter gene. GUS activity levels of transformants varied, but transformation did not affect pathogenicity on wheat seedlings. A GUS transformant was used to study the effectiveness of two fungicides against *F. culmorum* foot rot of wheat.

Primers to the *Tri5* gene were used to develop a PCR-based assay for the specific detection of potential trichothecene-producing *Fusarium* species. These primers were also used to develop an RT-PCR-based assay for the detection and semi-quantification of *F. culmorum Tri5* gene expression 'relative to β-tubulin gene expression' in RNA extracts from *F. culmorum*. This assay was used to show that time and fungicides can affect *Tri5* gene expression in liquid culture.

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ABBREVIATIONS

ANOVA	analysis of variance	NaCl	sodium chloride
approx.	approximately	ng	nanogram(s)
^{0}C	degrees celcius	nm	nanometre(s)
cm	centimetre(s)	nM	nanomole(s)
conc.	concentrated	No.	number
dATP	deoxyadenosine triphosphate	p.	page
dCTP	deoxycytidine triphosphate	PDA	potato dextrose agar
dGTP	deoxyguanidine triphosphate	PDB	potato dextrose broth
dil.	dilute	%	percent
DNA	deoxyribonucleic acid	pH	hydrogen ion concentration
DNase	deoxyribonuclease	psi	pounds per square inch
dNTP (s)	2'-deoxynucleoside 5'-triphosphates	RNase	ribonuclease
		sec	second(s)
dTTP	deoxythymidine triphosphate	sol.	soluble
EDTA	diaminoethanetetra - acetic acid	soln.	solution
g/l	grams per litre	S.D.	standard deviation
g a.i./l	grams active ingredient per litre	S.E.M.	standard error of the mean
expt (s).	experiment(s)	t	time
h	hours	TAE	tris - acetate buffer
Kb	kilobase(s)	TE	tris - EDTA buffer
KCl	potassium chloride	temp	temperature
kPa	kilopascal	µg	micrograms
l	litre	µl	microlitres
log	logarithm	µm	micrometres
m	metre	µM	micromoles
$MgCl_2$	magnesium chloride	U.V.	ultraviolet
min	minute(s)	V	volt (s)
min.	minimum	vol.	volume
mM	millimole(s)	v/v	volume per volume
mg	milligram(s)	wt.	weight
ml	millilitre(s)	w/v	weight per volume

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GENERAL INTRODUCTION

Fusarium are among the most common and widespread of fungal plant pathogens (Parry *et al.*, 1995a) and all members of the Gramineae suffer diseases caused by *Fusarium* (Cook, 1981a). Several species of *Fusarium* cause ear blight of cereals as does another pathogen, *Microdochium nivale* (teleomorph: *Monographella nivalis*), formerly known as *F. nivale* (Meuller, 1977). *Fusarium* ear blight of wheat (FEB) is regarded as being of great importance throughout the world, the potential consequences of such a disease outbreak including yield loss (Häni, 1991; Parry, 1991; Wong *et al.*, 1992; Miedaner *et al.*, 1993) and mycotoxin contamination (Snijders, 1990c; Desjardins & Hohn, 1997). Therefore, FEB is a cause for concern not only for cereal growers, but also with respect to the potential health risks involved in using contaminated wheat grains in food products and animal feed.

CAUSAL ORGANISMS AND GEOGRAPHICAL DISTRIBUTION

While a range of different species have been implicated in ear blight of wheat, most records of the disease have been associated with four species: *F. graminearum* (teleomorph: *Gibberella zeae*), *F. culmorum*, *F. avenaceum* (teleomorph: *G. avenacea*) and *F. poae* (Parry *et al.*, 1995a). Disease records have also been associated with *M. nivale*, which shall be considered under the general heading of *Fusarium* for the purposes of this thesis. The species implicated in FEB have been isolated from soil and plant material throughout the world, including Canada (Gordon, 1956; Clear & Abramson, 1986; Wong *et al.*, 1992), the United States of

America (Cook, 1981a; Inglis & Cook, 1981; Inglis & Maloy, 1983; Stack & Mullen, 1985; Wilcoxon *et al.*, 1988), Europe (Daamen *et al.*, 1991; Polley *et al.*, 1991; Maurin & Chenet, 1993; Nicholson *et al.*, 1993; Parry & Nicholson, 1996), Asia (Cook, 1981b; Brahma & Singh, 1985; Koizumi *et al.*, 1991) and Australia (Burgess *et al.*, 1987). The relative importance of the individual causal organisms appears to vary, depending on the country involved, although it should be borne in mind that some earlier attempts at taxonomy were questionable.

F. graminearum ear blight tends to occur in hotter regions of the world such as parts of the USA (Wilcoxon *et al.*, 1988), China (Cassini, 1967, 1969; Booth & Taylor, 1976), Australia (Wearing & Burgess, 1977) and Central Europe (Mesterhazy, 1974). *F. culmorum* is a widely distributed species and appears to survive greater extremes of drought and freezing temperatures than *F. graminearum* (Cook, 1981a). This pathogen tends to predominate over other FEB causal organisms in the cooler maritime regions of Northwest Europe (Ahrens & Fehrmann, 1984; Daamen *et al.*, 1991; Parry *et al.*, 1995a). *F. poae* also appears to be a significant pathogen of small grain cereals in cooler maritime regions of the world such as the UK (Polley *et al.*, 1991). However, the significance of *F. poae* as a pathogen of wheat and other small grain cereals is considered doubtful in hotter regions of the world such as North America (Cook, 1981a). *F. avenaceum* has also been isolated from wheat over a range of climatic zones, such as parts of the USA (Wong *et al.*, 1992), Europe (Zwatz, 1975; Maurin & Chenet, 1993; Daamen *et al.*, 1991; Polley *et al.*, 1991), China (Kelman & Cook, 1977) and Asia (Roy, 1974), although the frequency of detection of this pathogen is usually relatively low, compared with other

Fusarium species such as *F. culmorum* and/or *F. graminearum*. *M. nivale* is distributed worldwide, and like *F. poae*, this pathogen assumes a greater importance in cooler maritime regions of the world (Lees *et al.*, 1995; Parry & Nicholson, 1996).

Isolates of *F. graminearum* and isolates of *M. nivale* have been differentiated into two groups (Francis & Burgess, 1977; Cook, 1981a, Gerlach & Nirenberg, 1982; Lees *et al*, 1995). In the case of *F. graminearum*, isolates have been differentiated on the basis of their ability to produce perithecia; Group I isolates are heterothallic and generally found at the stem base, while Group II isolates are homothallic and tend to occur on cereal ears (Francis & Burgess, 1977). Francis & Burgess (1977) believed that the nature of the disease caused by *F. graminearum* in wheat and other susceptible hosts was determined both by the relative inoculum levels of the two groups and by the prevailing environmental conditions during the growing season. Using the same classification system, Cook (1981a) has divided a North American population of *F. graminearum* into Group I and Group II isolates. Benyon *et al.* (1995), used the molecular taxonomy to distinguished two sub-groups among isolates of *F. graminearum* and, because of the distant relationship between the two groups, even went as far as to suggest that the sub-groups represented separate species. In *M. nivale,* observations of spore morphology (Gerlach & Nirenberg, 1982) and molecular taxonomy (Lees *et al.*, 1995) have revealed two distinct sub-groups of this pathogen; *M. nivale* var. *majus* and *M. nivale* var. *nivale*.

CLASSIFICATION AND IDENTIFICATION OF THE COMMON CAUSAL ORGANISMS OF FEB OF WHEAT

Due to problems that arose in the classification and identification of *Fusarium* species, several classification systems, based on morphological and microscopic criteria, have been developed in the last century (Nelson, 1991). Taxonomically, *Fusarium* species are notorious for their variability, especially in culture, to the extent that different species may appear morphologically identical (Puhulla, 1981). More recently, attempts have been made to classify and identify species using analytical techniques involving physiology (Glynn & Reid, 1969; Abd-el-Rehim & Fadel, 1980; Ianelli *et al.*, 1983; Partridge *et al.*, 1984; Bosland & Williams, 1987) and molecular genetics (Puhalla, 1985; Bosland & Williams, 1987; Michelmore & Hulbert, 1987; Manicom *et al.*, 1990; Guthrie *et al.*, 1992; Nicholson *et al.*, 1993; Ouellet & Seifert, 1993). Though these techniques have helped to solve many problems in the classification and identification of *Fusarium* species, several gaps and problematic areas in *Fusarium* classification have not yet been addressed. These include the anamorph-teleomorph connection, section relationships, species delimitation, mutational variants and subgroup identification (Windels, 1991). In the case of the common causal organisms of FEB, it is generally recognised that *F. graminearum, F. culmorum*, *F. poae*, *F. avenaceum* and *M. nivale* represent distinct species.

Currently, there are several options available for identifying and classifying the common causal organisms of FEB of wheat. These include conventional morphological and microscopical taxonomy (Nelson, 1991), enzyme-linked immunosorbent assay (ELISA) (Beyer *et al.*, 1993) and polymerase chain reaction

(PCR) analysis (Ouellet & Seifert, 1993; Schilling *et al.*, 1996; Nicholson & Parry, 1996; Nicholson *et al.*, 1996, Parry & Nicholson, 1996; Nicholson *et al.*, unpublished). An ELISA-based test is available for the detection of *F. culmorum*, *F. graminearum* and *F. avenaceum* (Beyer *et al.*, 1993); however, this assay cannot differentiate between these species. A species-specific ELISA-based assay has been developed and used for the quantitative detection of *M. nivale* in rye (Höxter *et al.*, 1991). Species-specific PCR-based assays are available which enable identification and differentiation of *F. culmorum* (Schilling *et al.*, 1996; Nicholson *et al.*, unpublished), *F. graminearum* (Ouellet & Seifert, 1993; Schilling *et al.*, 1996, Nicholson *et al.*, unpublished), *F. poae* (Parry & Nicholson, 1996), *F. avenaceum* (Lees, 1995; Schilling *et al.*, 1996), *M. nivale* var. *majus* and *M. nivale* var. *nivale* (Nicholson & Parry, 1996; Nicholson *et al.*, 1996) and these assays do not require the use of pure axenic fungal cultures, enabling identification and classification of the pathogens within infected plant tissue.

SYMPTOMS

Mathre (1982) described early symptoms of FEB as consisting of small brown water-soaked spots at the base or middle of the glume or on the rachis. These symptoms then spread from the site of infection, and in severe infections, a salmon pink/red fungal growth may be seen along the edges of the glumes or at the base of the spikelet. Wiese (1987) described premature bleaching of spikelets as a common symptom of FEB and the proportion of bleached spikelets per infected head has been

used as a quantitative measure of FEB of wheat, (Snijders, 1990b; Miedaner, 1997) including *F. culmorum* ear blight of wheat (Fig. I.1a). During prolonged warm, moist weather, Mathre (1982) observed that spikelets on early-infected heads appeared speckled because of the development of perithecia. Such perithecia are commonly associated with *G. zeae* (anamorph: *F. graminearum*) and these symptoms give ears a `scabbed' appearance, and consequently, FEB is often referred to as cereal 'scab' (Parry *et al.*, 1995a). Parry *et al* (1995a) noted that in wheat ears with severe FEB, the peduncle may turn dark brown. Infected grains may appear shrivelled with a powdery, poorly filled interior and reports regarding the colour of infected grain vary from grey/brown (Parry *et al.*, 1995a) to pink (Martin & Johnson, 1982). Symptoms of FEB caused by *F. poae* appear to be distinct from those caused by other *Fusarium* species (Fig. I.1b). In UK surveys of stem base diseases of barley and wheat (1989-1991), Polley *et al.* (1991) and Polley & Turner (1995) reported *F. poae* disease, the symptoms including the development of glume spots that had bleached centres and dark brown margins. In 1990 glume spots detected on wheat were predominantly associated with *F. poae* rather than other *Fusarium* species or fungal pathogens. However, Parry *et al.* (1995a) observed these *F. poae*-like symptoms as the initial symptoms of FEB caused by *F. avenaceum*, *F. culmorum* and *M. nivale*. Likewise there has been controversy about the symptoms of FEB produced by *M. nivale*. Reports vary from the development of brown glume spots with dark brown margins (Rapilly *et al.*, 1973) to symptoms indistinguishable from those caused by *F. graminearum*, *F. culmorum* or *F. avenaceum* (Cassini, 1981; Inglis & Cook, 1981).

a

b

Fig. I.1 *Fusarium* ear blight visual disease symptoms observed at growth stage 80 following inoculation of winter wheat (cv. Avalon) with *F. culmorum* (a) and *F. poae* (b). Arrows indicate ears which were inoculated at mid-anthesis (growth stage 65) with conidia (5×10^5 conidia/ear) of the appropriate pathogen and then covered with polyethylene bags for one week to maintain high humidity.

EPIDEMIOLOGY

Epidemics of FEB have occurred worldwide during wet growing seasons (Parry *et al.*, 1995a) and such epidemics may not be independent from those of seedling blight and foot rot, as it is likely that all three play an integral part in the disease cycle (Fig. I.2) (Parry *et al.*, 1994). Sowing cereals into *Fusarium*-infested soil may result in the infection of plants and the development of both seedling blight and foot rot. Later in the growing season, air-borne inoculum, usually in the form of conidia or ascospores, may infect the ears of the plant, causing FEB and the resulting grain can then provide an important source of inoculum for the development of seedling blight, thus completing the disease cycle (Parry *et al.*, 1990).

Forms of inoculum include conidia, chlamydospores and hyphal fragments. The sexual reproduction of *G. zeae* (*F. graminearum*) and *M. nivalis* (*M. nivale*) also provides ascospores as a source of inoculum (Cook, 1981a). Early studies (Atansoff, 1920, 1923) showed that pathogens can overwinter on infected host tissue in the soil. Crop debris present in the soil may directly give rise to infectious mycelia, or it may serve as food base for survival and dissemination of the pathogens (Cook, 1981a). Sutton (1982) considered crop debris to be the principal reservoir of inoculum for the infection of wheat ears. According to Tusa *et al.* (1981) and Teich & Nelson (1984), continuous wheat cropping or wheat-maize rotations provide an increased source of inoculum for the development of an ear blight epidemic. Alternative sources of inoculum include grasses and broad-leaved weeds (Gordon, 1959; Jenkinson & Parry, 1994b). The mode of transmission of inoculum is unclear, but alternatives proposed include wind and rain splash dispersal (Tusa *et al.*, 1981; Strausbaugh & Maloy, 1986; Jenkinson & Parry, 1994a), arthropod vectors (Gordon, 1959; Hardison, 1961, Windels *et al.*, 1976) and

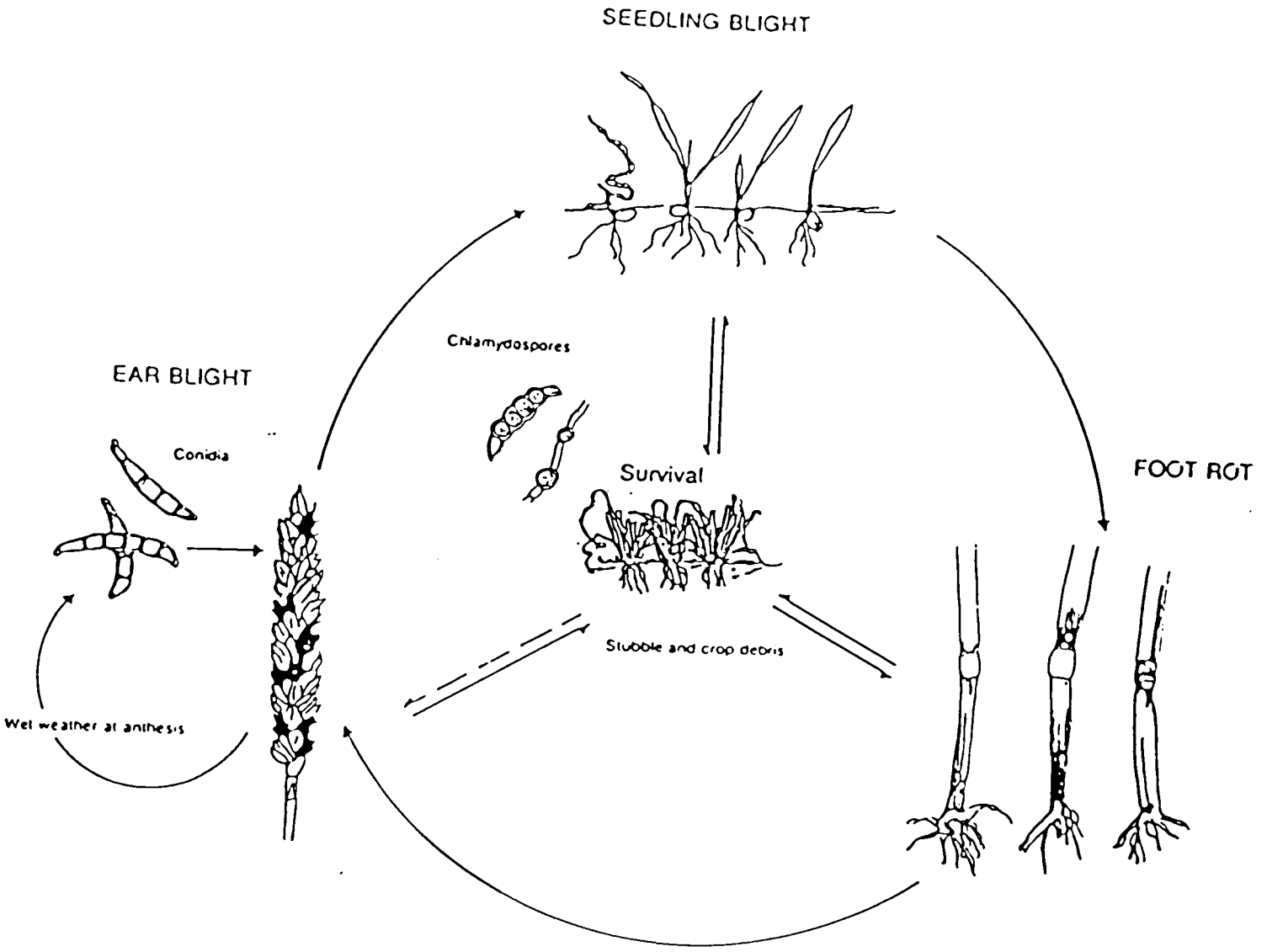


Fig. I.2 The *Fusarium* disease cycle
After: Parry *et al.* (1994)

systemic growth (Snijders, 1990a; Hutcheon & Jordon, 1992).

Anthesis appears to be a period of considerable importance in determining the incidence and severity of FEB. Studies carried out by various workers showed increased infection by *Fusarium* species during anthesis (Pugh *et al.*, 1933; Andersen; 1948; Strange & Smith; 1971). Strange & Smith (1971) showed that extracts of wheat anthers stimulated the growth of *F. graminearum*, and that emasculation of wheat ears reduced the incidence of florets infected with the pathogen. Subsequently, two compounds were isolated from wheat anther extracts, choline chloride and betaine hydrochloride, which stimulated *F. graminearum* hyphal extension but not spore germination (Strange *et al.*, 1974). Strange & Smith (1978) showed that choline and betaine were as effective as the anther extracts in stimulating infections.

Weather conditions during the growing season can also influence the severity of FEB, moisture and temperature both playing an important role in the development of FEB of wheat (Parry *et al.*, 1994, 1995a). High humidity is required for optimal infection of wheat ears by *Fusarium* species and the species differ in their respective temperature requirements (Cook, 1981a, Parry *et al.*, 1995a). Andersen (1948) found that infection by *F. graminearum* occurred most frequently at 25 ^{0}C; few spikelets developed symptoms unless wet periods exceeded 24 h, post-inoculation. At 20 ^{0}C, moderate infection occurred, but only after 60-72 h of exposure to wetness, and at 15 ^{0}C infection was negligible. Similar optimal moisture and temperature requirements have been observed for the development of *F. culmorum*, *F. avenaceum* and *F. poae* ear blight of wheat (Parry *et al.*, 1995a). In contrast, Parry *et al.* (1994) found that *M. nivale* infection was optimal at the lower temperature of 15 ^{0}C. In an earlier report, Polley *et al.* (1991) suggested that the higher incidence of *M. nivale* in Scottish seed

compared to other UK regions was attributable to ambient temperatures being generally lower in this region.

MYCOTOXINS

Several fungal species, including the common causal organisms of FEB of wheat (*F. culmorum*, *F. graminearum*, *F. poae*, *F. avenaceum* and *M. nivale*) have the potential to produce various secondary metabolites known as mycotoxins which pose a threat to both human and animal health (Desjardins & Hohn, 1997). Trichothecenes and zearalonones are the two major classes of mycotoxins produced by some common causal organisms of FEB of wheat. Other classes of mycotoxins produced by causal organisms of FEB include butenolide, moniliformins, fusarins and enniatins (Thrane, 1989; Desjardins & Hohn, 1997).

The trichothecene mycotoxins constitute a large family of sesquiterpene epoxides that inhibit eukaryotic protein synthesis and the major end-products of the trichothecene biosynthetic pathway are known as T-2 toxin, deoxynivalenol (DON) and 4-15 diacetoxyscirpenol (DAS) (Desjardins *et al.*, 1993) The ability of *F. culmorum*, *F. graminearum* and *F. poae* to produce trichothecene mycotoxins both in liquid culture medium (Miller *et al.*, 1983a; Marasas *et al.*, 1984; Thrane, 1989) and in cereal grains (Cook, 1980, 1981a; Miller *et al.*, 1983b; Visconti *et al.*, 1990; Ryu *et al.*, 1996; Schaafsma *et al.*, 1993) has been well documented. In a survey of mycotoxins contaminating cereals and feed in European countries during 1979-1988, Gareis *et al.* (1989) found that the trichothecene DON, along with the mycotoxin zearalenone, occurred most frequently and in some cases the concentrations of these mycotoxins may

have reached clinically significant levels. Visconti *et al.* (1990) reported the presence of the trichothecenes DON, 3- or 15-acetyl DON, nivalenol and fusarenone in the kernels and cobs of corn ears from Poland naturally infected with *F. graminearum* and another ear blight pathogen, *F. crookwellense*. Significant levels of DON (up to 40 mg/kg) were detected in Polish wheat kernels showing symptoms of damage (Perkowski *et al.*, 1990a, b, c, 1991). The results of work performed by Ryu *et al.* (1996) suggested that, in 1992, nivalenol and DON were the major contaminating trichothecene mycotoxins in Korean Barley. It is questionable whether *F. avenaceum* and *M. nivale* have the potential to produce trichothecene mycotoxins, as there are few reports of trichothecene production by isolates of these fungal species (Abbas *et al.*, 1984; Marasas *et al.*, 1984). Abbas *et al.* (1984) reported that a Norwegian strain of *M. nivale* produced trichothecene mycotoxins. However, the identity of this strain has never been confirmed. Similarly, the identity of isolates of *F. avenaceum* reported to produce trichothecene mycotoxins have not been verified (Thrane, 1989).

Trichothecene mycotoxins have been associated with acute and chronic mycotoxicosis in both animals and humans. In animals it has been shown that trichothecene mycotoxins produce a variety of toxic effects such as emesis, feed refusal (Vesonder *et al.*, 1979), and reduced body weight (Ryu *et al.*, 1987, 1988; Ohtsubo *et al.*, 1989). Exposure of animals to trichothecenes induced vomiting several hours post-administration, followed by intestinal congestion and haemorrhaging a day thereafter and leukopenia after repeated exposures (Sato *et al.*, 1978; Ryu *et al.*, 1987). Other toxic manifestations include cytoxicity (Ryu *et al.*, 1986), dermal toxicity (Hayes and Schiefer, 1979; Ryu *et al.*, 1986), haemostatic derangement (Cosgriff *et al.*, 1986) and severe immunosuppression (Lafarge-Frayssinet *et al.*, 1979; Rosentein *et al.*, 1981). In humans,

outbreaks of the fatal disease known as alimentary toxic aleukia (ATA), which has occurred in Russia since the nineteenth century, have been associated with the consumption of overwintered grains contaminated with *Fusarium* species that produce trichothecene mycotoxins (Desjardins & Hohn, 1997). More recently, in India and Japan trichothecenes were detected in toxic grain samples involved in human disease outbreaks (Marasas *et al.*, 1984; Beardall & Miller, 1994).

Another major class of mycotoxin known to be produced by *F. culmorum* and *F. graminearum* are the zearalenone mycotoxins, zearalenone and zearalenol (Thrane, 1989). Zearalenone was first isolated from metabolites of *G. zeae* (*F. graminearum*) as an estrogenic mycotoxin (Stob *et al.*, 1962). Ichinoe *et al.* (1983) reported the production of zearalenone mycotoxins by trichothecene-producing isolates of *Fusarium* that belonged to two distinct chemotaxonomic groups (nivalenol and fusarenone-X producers, and DON and 3-acetyl-DON producers). However, these workers could find no exclusive relationship between zearalenone production and chemotaxonomic grouping. Tanaka *et al* (1988) reported high incidences of zearalenone, along with the trichothecenes nivalenol and DON, in barley and maize samples collected from nineteen countries worldwide. Natural occurrence of zearalenone mycotoxins has also been reported in Korean cereals harvested in 1992, although this toxin was not a major contaminant compared to trichothecene mycotoxins (Ryu *et al.*, 1996).

Marasas *et al.* (1984) reported that consumption of feeds contaminated with these mycotoxins caused severe reproductive and fertility problems in animals. Similarly, Mirocha & Christensen (1974) reported an association between zearalenone and hyperoestrogenism in farm animals.

Other classes of mycotoxins known to be produced by the causal agents of FEB

include butenolide, fumonisins, fusarins and enniatins. Butenolide, a water soluble mycotoxin, has been reported to be produced by isolates of *F. graminearum*, *F. culmorum*, *F. poae* and *F. nivale* (*M. nivale*) (Wang & Miller, 1988; Vesonder & Golinski, 1989; Savard & Blackwell, 1994). There have been few reports regarding the production of fusarin mycotoxins by common causal agents of FEB, although Savard & Blackwell (1994) reported the production of fusarins by a range of species, including *F. graminearum* and *F. culmorum*. Six fusarin mycotoxins, designated A, B, C, D, E and F, have been identified (Savard & Miller, 1992).The fusarins are particularly notable because Fusarin C is a powerful mutagen, with reported activity comparable to that of aflatoxin B1 (AFB_1) and sterigmatocystin (Wiebe & Bjeldanes, 1981). Moniliformin is an unusual cyclobutane derivative (Cole *et al.*, 1973; Leslie *et al.*, 1996). There are reports of moniliformin production by isolates of *F. avenaceum* (Marasas *et al.*, 1984; Herrmann *et al.*, 1996b), although this mycotoxin is more commonly associated with *F. moniliforme* which is the causal organism of several important diseases, including Bakanae disease of rice (Cole *et al.*, 1973; Marasas *et al.*, 1986). Enniatin mycotoxins are cyclic dispeptides which are known to be produced by isolates of *F. avenaceum* (Herrmann *et al.*, 1996a, b).

The role of most of these mycotoxins in plant disease has not been well studied, but some studies which investigated the role of trichothecenes in various plant diseases indicate that trichothecene mycotoxins may play an important role in the aggressiveness of fungi on plant tissue (Desjardins *et al.*, 1989; Desjardins *et al.*, 1992; Proctor *et al.*, 1995a; Desjardins *et al.*, 1996). The *Tri5* gene codes for the enzyme trichodiene synthase, an enzyme which catalyses the first step in the trichothecene biosynthetic pathway, and Proctor *et al.* (1995a) used transformation-mediated disruption of the *Tri5*

gene to block trichothecene production in *G. zeae* (*F. graminearum*). These workers then showed that the aggressiveness of two trichothecene non-producing mutants was significantly reduced in seedling blight and ear blight tests, under growth chamber conditions. In field tests, ears infected with trichothecene non-producing mutants also showed less disease according to several parameters, including visual disease symptoms, grain weight, grain viability and trichothecene contamination (Desjardins *et al.*, 1996). Revertants of a *Tri5* disruption mutant (generated by allowing the *G. zeae* mutant to undergo sexual reproduction, *G. zeae* being homothallic) were indistinguishable from the wild-type parent in their ability to cause FEB of wheat. This showed that the reduced virulence of *Tri5*$^-$ mutants was due to *Tri5* disruption and not a result of non-target effects caused by the transformation process (Desjardins *et al* 1996). Similarly, Herrmann *et al.* (1996b) used transformation-mediated gene disruption to generate enniatin non-producing mutants of *F. avenaceum* and showed that these mutants were less virulent towards potato tubers than both the wild type or enniatin-producing hygromycin B-resistant ectopic transformants.

DISEASE CONTROL

The importance of cultural control techniques and genetic resistance of wheat cultivars has been recognised for many years (Arthur, 1891). Nevertheless, these disease control methods have, as yet, only achieved moderate success (Parry *et al.*, 1995a). There are comparatively few reports regarding successful fungicidal control of FEB of wheat and the study of biological control of the disease is only in its infancy.

The first observation of differences in FEB susceptibility between cultivars was

made by Arthur (1891), who noted that early-maturing cultivars tended to be more resistant to FEB than cultivars which matured later. The importance of resistance to FEB in cultivar choice was recognised in some earlier plant breeding work, with the cultivar Progress being particularly popular because it combined FEB resistance with higher yield (Parry *et al.*, 1995a). Durum wheats have been shown to be more susceptible than common wheats to FEB of wheat caused by *F. graminearum* and *F. culmorum* (Hanson *et al.*, 1950; Maurin & Chenet, 1993). Whether or not resistance to FEB is race-specific has been the subject of debate (Tu, 1930; Mesterhazy, 1984; Snijders & Van Eeuwijk, 1991), but at present there is no strong evidence for race-specific resistance in wheat to any of the species causing FEB.

Much effort has gone into identifying the components of resistance. Schroeder & Christiansen (1963) suggested two components of resistance; type I and type II. Type I resistance operates against initial infection and they assumed such resistance to be physiological and type II resistance operates against the spread of the pathogen within the host, with both types of resistance varying independently among cultivars. The host response to DON has been proposed as a basis for the determination of two further resistance types. Type III resistance is the ability to degrade DON (Miller & Arnison, 1986) and type IV resistance is tolerance of high DON concentrations (Wang & Miller, 1988). Snijders & Krechting (1992) believed that resistance associated with lower DON levels was one of the mechanisms behind type II resistance. Several workers have found that resistant cultivars have lower DON concentrations (Miller *et al.*, 1985; Teich *et al.*, 1987; Mesterhazy, 1989; Snijders & Perkowski, 1990; Liu & Wang, 1991; Snijders & Krechting, 1992). It is a matter of debate whether cultivar morphology, particularly of the ear, can influence FEB resistance. As pointed out earlier, when discussing the

epidemiology of the disease, there is evidence to suggest that cereal ears are more susceptible to FEB during anthesis and that initial infection takes place via extruded anthers (Pugh *et al.*, 1933; Strange & Smith, 1971). Parry *et al.* (1995a) concluded that regarding cultivar resistance, most authors would agree that no wheat cultivar is immune to FEB, most are susceptible, but a few are moderately resistant. Under high inoculation pressure resistance may not be sufficient to control the disease and fungicides may still be required if disease pressure is high.

For many years, it has been recognised that cultural control techniques provide a potential control measure for FEB of wheat (Arthur, 1891; Cook, 1980, 1981a; Teich & Nelson, 1984; Teich & Hamilton, 1985; Wilcoxon *et al.*, 1988). Cultural control techniques which have helped to combat FEB of wheat include crop rotation (Cook, 1981a Teich & Nelson, 1984; Teich & Hamilton, 1985), the removal or burial of crop debris (Teich & Nelson, 1984; Teich & Hamilton, 1985; Wilcoxon, 1988) and irrigation to avoid water stress (Cook, 1980), although overhead irrigation has been shown to increase the severity of FEB (Tusa *et al.*, 1981; Strausbaugh & Maloy, 1986; Teich, 1987). Jenkinson & Parry (1994b) suggested that effective weed control may reduce the incidence of FEB. However, the relationship between weed densities and FEB severity in wheat remains unclear and needs further investigation. Martin *et al.* (1991) suggested that increased water stress due to nitrogen fertilizers may increase the incidence of FEB. But, Teich & Hamilton (1985) and Fauzi & Paulitz (1994) failed to observe any relationship between the application of nitrogen fertilizers and the incidence and severity of FEB.

The earliest fungicides used to control FEB of wheat were the methyl benzimidazole (MBC) fungicides, first introduced in 1974 for use on wheat, and these

provided a cheap method for controlling diseases of cereals (Locke *et al.*, 1987). Resistance to MBC fungicides in populations of several plant pathogens, including *M. nivale* (Parry, 1990; Pettitt *et al.*, 1993), rapidly developed after their widespread adoption. Possible alternatives to MBC fungicides for control of FEB of wheat included the two groups of sterol biosynthesis inhibitors (SBI`s) which are widely used as fungicides in cereals. The first group are the demethylase inhibitors (DMI`s) (Sisler & Ragsdale, 1984) and the second the morpholines and 3-phenylpropylamines (Burden *et al.*, 1989; Debieu *et al.*, 1992). Members of both SBI fungicide groups have been investigated as potential control agents for FEB of wheat (Fehrmann & Ahrens, 1984; Polley *et al.*, 1991; Boyacioglu *et al.*, 1992; Hutcheon & Jordan, 1992; Suty *et al.*, 1996).

Sterol biosynthesis begins from acetic acid and involves about thirty steps and the two SBI fungicide groups differ with respect to the specific enzymes they inhibit in the post-squalene segment of the fungal sterol biosynthetic pathway (Mercer, 1991). A considerable amount of work has been done on the effect of SBI fungicides on yeast, namely *Saccharomyces cerevisiae*. This work has shown that, in *S. cerevisiae*, SBI fungicides inhibit a cytochrome P-450 which catalyses the three oxygen and NADPH-requiring steps of sterol 14-demethylation (Yoshida & Aoyama, 1984; Aoyama *et al.*, 1984). Mercer (1991) suggested that a similar cytochrome P-450 exists in filamentous fungi. The DMI fungicide tebuconazole, a broad spectrum fungicide (Reinecke *et al.*, 1986), is generally accepted as the industry standard for the control of FEB of wheat, its efficacy against FEB having been verified both in glasshouse trials (Hutcheon & Jordan, 1992) and in the field (Suty *et al.*, 1996). This fungicide also effectively controlled FEB of wheat when used as a mixture with a second DMI fungicide,

triadimenol (Hutcheon & Jordon, 1992). Another DMI fungicide, prochloraz, has also been shown to give good control FEB of wheat, again, both in glasshouse trials (Hutcheon & Jordan, 1992) and in the field (Fehrmann & Ahrens, 1984). Guazatine, a guanidine-based fungicide, is a widely used broad spectrum seed treatment which has been shown to give good control of FEB in the field (Cameron *et al.*, 1986). However, Olvang (1987) warned that if this fungicide became widely used as a foliar or ear spray, resistance to the active ingredient could increase, and this could jeopardize its effectiveness as a seed treatment.

It is important for human and animal health that fungicides used in the control of FEB of wheat also reduce the levels of mycotoxins produced in cereal grains. But, it has been shown that effective disease control is not always accompanied by significant reductions in mycotoxin concentration (Martin & Johnson, 1982, Moss & Frank, 1985). Also, the disparity between the effectiveness of fungicides in laboratory and field tests (Polley *et al.*, 1991) indicates that further work on the rate and timing of fungicide application is required to optimize the efficacy of fungicide sprays against FEB pathogens. Overall, chemical control of FEB appears at best inconsistent in the field. Use of techniques such as quantitative PCR analysis to study fungicide efficacy may provide a more objective measure of disease severity than traditional visual disease assessment, and so reduce some of the inconsistency associated with fungicidal control of FEB. An extensive review of control of FEB of wheat can be found in Parry *et al.* (1995a).

No detailed studies have been undertaken to examine the potential of using biological agents to control FEB of wheat. However, several biocontrol agents have shown potential for controlling FEB of wheat in the field, including *Chaetonium* species, *Idriella bolleyi* and *Gliocladium roseum* (Knudsen *et al.*, 1995).

TECHNIQUES FOR STUDYING FEB OF WHEAT

Studying FEB of wheat ideally requires sensitive, reliable, accurate and rapid techniques for the detection, differentiation and often quantification of the individual pathogens. This is true for most fields of study, from disease development to disease control. Traditionally, techniques such as visual assessment of disease severity, number of grains per ear and thousand grain weight, combined with isolation/cultural methods have been used for the detection, identification and quantification of FEB of wheat (Snijder, 1990b, c; Hutcheon & Jordan, 1992; Suty, 1997). Techniques based on the quantification of chemical constituents of fungal pathogens, e.g. chitin or ergosterol, have also been used to study fungal diseases of plants (Ride & Drysdale, 1972; Miller *et al.*, 1985; Snijders & Krechting, 1992). More recently, techniques such as ELISA-based assays (Höxter *et al.*, 1991; Beyer *et al.*, 1993) and polymerase chain reaction assays (Ouellet & Seifert, 1993; Lees, 1995; Nicholson & Parry, 1996; Nicholson *et al.*, 1996; Parry & Nicholson, 1996; Schilling *et al.*, 1996; Nicholson *et al.*, unpublished) have been developed and used to study FEB diseases of small grain cereals. As discussed earlier, PCR-based techniques can be used for the classification of *Fusarium* species, but they have also been adapted to enable quantification as well as identification of the common causal organisms of FEB of wheat. Although an ELISA-based assay has been developed which enables quantitative detection of *F. culmorum*, *F. graminearum* and *F. avenaceum*, it cannot discriminate between these pathogens (Beyer *et al.*, 1993). However, quantitative PCR analysis enables both differentiation and quantification of the common causal agents of FEB of wheat (Nicholson *et al.*, unpublished).

A number of pathogens have been genetically transformed ('tagged') with so called 'reporter genes' and these tagged isolates have been used to detect, monitor, and

quantify disease development within the host plants (Couteaudier *et al.*, 1993; Oliver *et al.*, 1993). This technique has been applied to the study of *F. oxysporum* (Couteaudier *et al.*, 1993), which is pathogenic on roots of flax (*Linum usitatissimum*). Since it has been shown that genetic transformation of *F. culmorum* (Curragh *et al.*, 1992), *F. graminearum* (Proctor *et al.*, 1995a) and *F. avenaceum* (Herrmann *et al.*, 1996b) is feasible, the development of 'tagged' isolates of these pathogens would prove useful in FEB studies. One of the earlier reporter genes used to transform filamentous fungi was the *Escherichia coli* β-galactosidase (*LacZ*) gene, which was used to transform successfully *Penicillium chrysogenum* (Van Gorcom *et al.*, 1985). However, as pointed out by Roberts *et al.* (1989), the use of this reporter system was limited due to the presence of endogenous β-galactosidase activity in other fungi. Jefferson *et al.* (1986, 1987a & b, 1989) developed the *E. coli* β-glucuronidase (*gusA*) reporter system (commonly known as the GUS gene reporter system) for use in the nematode *Caenorhabitus elegans* and this system has been adapted and used for the transformation of filamentous fungi such as *F. oxysporum* (Couteaudier *et al.*, 1993). Recently, the gene encoding the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* has been cloned and sequenced (Prasher *et al.*, 1992) and a number of recent articles have reviewed the use of GFP in a range of organisms (Stearns, 1995; Prasher, 1995). Although the GFP reporter gene has several advantages over the GUS reporter system, such as the simplicity of the methods used for the detection of transformed plant cells (Baulcombe *et al.*, 1995; Oparka *et al.*, 1995), to date there are no known reports of constitutive expression of this system in filamentous fungi, whereas transformation of plant pathogens with the GUS reporter system under the control of constitutive promotors has been well documentated (Roberts *et al.*, 1989; Bunkers, 1991; Ashby &

Johnstone, 1993; Oliver *et al.*, 1993; Couteaudier *et al.*, 1993; Monke & Schäfer, 1993; Wubben *et al.*, 1994).

As stated earlier, the role of mycotoxins in FEB of wheat has not been fully investigated and elucidation of the relationship between infection, fungal biomass and mycotoxin concentrations requires closer examination. Various different chemical methods have been used for the detection and quantification of mycotoxins in liquid culture and in cereal grains, including preparative thin layer chromatography (TLC) (Visconti *et al.*, 1990), high performance liquid chromatography (HPLC) (Miller *et al.*, 1983b; Tanaka *et al.*, 1988; Visconti *et al.*, 1990; Schaafsma *et al.*, 1993; Jimenez *et al.*, 1997) and gas chromatography/mass spectroscopy (GC-MS) (Ryu *et al.*, 1996; Roinestad *et al.*, 1993). GC-MS analysis provides an extremely sensitive technique for the analysis of *Fusarium* mycotoxins (Ryu *et al.*, 1996). Immunological assays have also been developed and used for the detection of *Fusarium* mycotoxins in cereals (Gendloff *et al.*, 1984; Goodbrand *et al.*, 1988; Lacey *et al.*, 1991; Barna-Vetro *et al.*, 1994; De Saeger & Van Peteghem, 1996; Yuan *et al.*, 1997).

Most studies of trichothecene mycotoxin accumulation in cereals have concentrated upon DON as the indicator of overall toxin biosynthesis. Detection and quantification of all trichothecenes is costly, time-consuming and generally impractical. An alternative approach would be to monitor the flow through the trichothecene biosynthetic pathway rather than accumulation of a single product. Monitoring of gene expression using northern blot analysis of RNA extracts offers such an opportunity. The *Tri5* gene encodes the enzyme trichodiene synthase which catalyses the first step of the trichothecene biosynthetic pathway (Desjardins *et al.*, 1993), and is therefore an ideal target for gene expression studies. Hohn *et al.* (1993) used northern blot analysis to

study the effect of time on the *Tri5* gene expression of *F. sporotrichoides* grown in liquid culture, using a culture medium known to induce trichothecene production by *Fusarium* species. More recently, reverse-transcriptase-PCR (RT-PCR) assays have been developed for to monitor expression of specific genes of interest in the plant pathogenic fungi *Phanerochaete chrysosporium* (Stewart *et al.*, 1992; Lamar *et al.*, 1995; Bogan *et al.*, 1996a,b) and *Cochliobolus carbonum* (Jones & Dunkle, 1995). RT-PCR has been shown to be more sensitive than northern blot analysis (Byrne *et al.*, 1988; Wang *et al.*, 1989; Mocharla *et al.*, 1990). The development of an RT-PCR assay specific for the *Tri5* genes would lead to more sensitive detection and quantification of gene expression, and hence trichothecene production, by *Fusarium* species.

1. THE USE OF SPECIES-SPECIFIC PCR-BASED ASSAYS TO ANALYSE *FUSARIUM* EAR BLIGHT OF WHEAT

1.1 INTRODUCTION

Fusarium ear blight (FEB) of wheat has been linked to at least 17 causal organisms, with most records of disease being associated with 5 species: *Fusarium culmorum, F. avenaceum* (*Gibberella avenacea*), *F. graminearum* (*G. zeae*, formerly known as *G. saubinetii*), *F. poae* and *Microdochium nivale* (*Monographella nivalis,* formerly known as *F. nivale*) (Parry *et al.,* 1995a). *F. graminearum* is a major pathogen worldwide, and in hotter regions of the world such as parts of the USA, Canada, Australia and Central Europe, this species is generally regarded as the most important causal agent of FEB of wheat (Burgess *et al.*, 1987; Clear & Abramson, 1986; Wilcoxon *et al.,* 1988). *F. culmorum*, *F. avenaceum, M. nivale* and *F. poae* are regarded as important causal agents of FEB in the cooler maritime regions of Northwest Europe (Ahrens & Fehrmann, 1984; Daamen *et al.*, 1991; Parry *et al.,* 1995a; Polley *et al.,* 1991). *F. poae* was the most frequently isolated species in a UK survey of affected ears carried out in 1989 and 1990 (Polley *et al.,* 1991).

The complex of causal organisms responsible for FEB of wheat greatly complicates the study of this disease using conventional disease assessment techniques, including visual disease assessment and isolation/culture methods. Visual disease assessment is based on the recognition of the classical symptoms of FEB, such as premature bleaching of spikelets (Wiese, 1987). However, there has been controversy regarding the symptoms of FEB produced by some of the causal agents (Rapilly *et al.*,

1973; Cassini, 1981; Inglis & Cook, 1981; Parry *et al.*, 1995a). For example, according to Polley *et al.* (1991), *F. poae* appears to cause symptoms distinct from those caused by other *Fusarium* species, resulting in lesions with a bleached centre and dark brown margin on the glumes. However, Parry *et al.* (1995a) observed these *F. poae*-like symptoms as the initial symptoms of FEB caused by *F. avenaceum, F. culmorum* and *M. nivale*. Likewise, reports regarding symptoms produced by *M. nivale* vary from brown glume spot with a dark brown margin (Rapilly *et al.*, 1973) to symptoms indistinguishable from those caused by *F. culmorum, F. avenaceum* or *F. graminearum* (Cassini, 1981; Inglis & Cook, 1981). Difficulties are also encountered in the taxonomy of *Fusarium* species so even when the causal organism has been isolated its identity may remain in doubt. The *Fusarium* species are notorious for their variability, especially in culture, to the extent that some species appear morphologically similar (Puhulla, 1981). Differing opinions regarding the classification and identification of *Fusarium* species has resulted in several taxonomic systems being developed in the last century (Wollenweber & Reinking, 1935; Snyder & Hansen, 1940; Gordon, 1956, 1959; Booth, 1971; Matuo, 1972; Gerlach & Nirenberg, 1982; Nelson *et al.* 1983).

There has been considerable interest in the development of alternative systems for the identification of *Fusarium* species. Attempts have been made to identify and classify *Fusarium* using soluble protein electrophoretic patterns (Glynn & Reid, 1969), zymograms (Scala *et al.*, 1981), and immunological techniques (Abd-el-Rehim & Fadel, 1980; Iannelli *et al.*, 1983; Höxter *et al.*, 1991; Beyer *et al.*, 1993) with partial success. For many of these studies, cross reactivity among *Fusarium* species and indeed with other fungal genera restricted the usefulness of these methods of identification and classification of *Fusarium* species. However, immunological studies have yielded a

species-specific enzyme linked immunoabsorbant assay (ELISA) for the detection of *M. nivale* in infected wheat (Hoxter *et al.*, 1991).

Molecular biology techniques, including restriction fragment length polymorphism (RFLP) analysis, random amplified polymorphic DNA (RAPD) analysis and the polymerase chain reaction (PCR) assay, have successfully been used for the identification of sub-populations of *Fusarium* species in infected plant material. Nicholson *et al.* (1993) investigated genetic variation of *Fusarium* species on wheat ears by RFLP analysis of rDNA (pTA 71), total genomic DNA and M13 genetic fingerprinting. The use of RAPD`s has revealed polymorphism among isolates of *M. nivale* from wheat, indicating the presence of a distinct sub-group (Lees *et al.*, 1995). RAPD analysis has also been used for the characterisation of *F. oxysporum* f.sp. *pisi* (Grajal-Martin *et al.*, 1993). Ouellet & Seifert (1993) characterised strains of *F. graminearum* using RAPD and restriction analysis of amplified fragments from the PCR reaction. Although they concluded that neither RAPD nor the PCR restriction assay method were superior, the PCR assay was less sensitive to assay conditions and was more easily reproduced and interpreted than the RAPD assay. Also, the RAPD assay required axenic cultures, whereas the PCR assay could be performed on fungal-infected plant tissues. This is of particular relevance because the isolation of fungi from infected plants reveals only what may be grown out of the plant and not necessarily what was in it. For example, Pettitt *et al.* (1993) demonstrated that, in the absence of selective media, the incidence of *M. nivale* was underestimated in winter wheat seedlings where *F. culmorum* was also present. Thus assays, such as PCR, which may be used directly on plant tissues may provide a rapid and sensitive means of detecting those fungi that are in the plant rather than those that may be isolated from the plant.

Several such PCR diagnostic assays have been developed for the detection, identification and differentiation of the major fungi involved in FEB of wheat, including diagnostic assays specific for *F. graminearum* (Ouellet & Siefert, 1993; Schilling *et al.*, 1996), *F. poae* (Parry & Nicholson, 1996) and *F. culmorum* and *F. avenaceum* (Schilling *et al.*, 1996). Two varieties are recognised within *M. nivale*, var. *majus* and var. *nivale* (Lees *et al.*, 1995) and species specific assays have been developed for each of these (Nicholson & Parry, 1996; Nicholson *et al.*, 1996). Using these assays, it is possible to identify and distinguish between the individual pathogens within the disease complex.

In the present work, samples of wheat, originating from a field trial in which the central region was inoculated with *F. culmorum* (field trial carried out by P. Jenkinson, Harper Adams Agricultural College, Shropshire, UK), were analysed by PCR and the results related to visual disease assessment.

1.2 MATERIALS & METHODS

1.2.1 Field trial

A field experiment, using the winter wheat cultivar Avalon, was conducted during the 1993/94 season at Harper Adams Agricultural College, Shropshire. The winter wheat cultivar Avalon was used due to its susceptibility to *Fusarium* ear blight pathogens (D. Parry, pers. comm.) and the field plot used for this experiment was 30 x 2 m, sub-divided into 15 sub-plots, each 2 x 2 m. Wheat was planted in early October and was kept free from weeds and foliar diseases by application of the appropriate agrochemicals, according to manufacturers' recommendations. At mid anthesis (GS 65) the central sub-plot (sub-plot 8) was inoculated with 167ml of a conidial suspension of *F. culmorum* (strain Fu 42) at a rate of 2.1×10^7 conidia m^{-2} using a pressurised hand sprayer. Following inoculation, the field trial was mist-irrigated until harvest. Visual disease assessment was performed by P. Jenkinson at GS 80 on a range of samples randomly chosen from each sub-plot and was based on the percentage infected spikelets/ear (Table 1.1). FEB symptoms scored were premature bleaching of ears, formation of small pink grains and, in severe cases, mycelial growth. At GS 90, those wheat ears examined at GS 80 were harvested, separated into grain, glume and rachis tissue, DNA was extracted and PCR analysis was used for comparison with visual diagnosis.

1.2.2 Origin and maintenance of fungal isolates and inoculum production

DNA from *Fusarium* species and *M. nivale* sub-species obtained from the John Innes Centre facultative pathogen culture collection (Table 1.2), were used as positive controls

for PCR analysis. The isolates were maintained on potato dextrose agar (PDA) (Difco) containing Penicillin G (50 μg ml^{-1}) and streptomycin sulphate (100 μg ml^{-1}). For DNA preparations, mycelium from 7-day-old colonies on PDA was used to inoculate aseptically 50 ml of potato dextrose broth (PDB), using a sterile scalpel. PDB cultures were incubated on an orbital shaker at 20-22 ^{0}C, 110 rpm, for 7 days.

Preparation of inoculum and inoculation of the central 2 x 2 m central sub-plot (sub-plot 8) was carried out by P. Jenkinson. To provide inoculum for the field trial, *F. culmorum* (strain Fu 42) was grown at 15 ^{0}C on agar (1 % w/v, Difco) containing milled wheat straw for the production of conidia. After 7 days conidia were washed from plates with sterile distilled water and adjusted to 5 x 10^5 conidia ml^{-1} for inoculation of the field trial.

1.2.3 DNA extraction

Wheat ears were harvested, separated into glume, grain and rachis tissue, placed in flat-bottomed tubes, freeze-dried and the dry weight recorded. Dried plant material was ground to a fine powder by milling, using 3 steel ballbearings (8 mm diameter) per tube, for 10 min in a Glen Creston mixer/mill 8000 (Glen Creston, UK). Rachis material was incubated at 65 ^{0}C for 2 h in 7 ml CTAB buffer (sorbitol 2.3 g, n-lauryl sarcosine 1.0 g, hexadecyl trimethyl-ammonium bromide 0.8 g, sodium chloride 4.7 g, polyvinylpolypyrolidone 1.0 g, water to 100 ml) together with 15 μl proteinase K (10 mg ml^{-1}) and 10 μl RNase (10 mg ml^{-1}). Following incubation, an equal volume of chloroform/isoamyl alcohol (24:1) was added to the tubes, mixed, and centrifuged at 2600 g for 15 min. The aqueous phase was removed to a fresh tube and two volumes

of ethanol (100 %) were added followed by centrifugation as above to precipitate the DNA. The pellet was washed in a 70 % solution of cold ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA).

The method used for DNA extraction from glume and grain samples was similar except 20 ml of CTAB buffer together with 50 µl of proteinase K and 30 µl of RNase were used. Due to the presence of substances which inhibited the PCR reaction, glume samples were also subjected to a phenol/chloroform (1:1) extraction step prior to chloroform/isoamyl alcohol extraction, using the same procedure. DNA from all tissues was diluted in TE buffer for use in PCR amplification reactions (0.4 mg plant dry weight equivalent μl^{-1}). DNA was extracted from fungal isolates using a method similar to that described by Nicholson & Parry (1996). Mycelium was harvested onto Whatman No.1 filter paper, and freeze dried, prior to extraction as for rachis material. Fungal DNA was diluted to 10 ng μl^{-1} in TE buffer for use in PCR reactions.

1.2.4 PCR amplification and agarose gel electrophoresis

Amplification conditions were similar to those described by Nicholson & Parry (1996). Reactions were performed in volumes of 50 µl and contained DNA from 0.8 mg dry weight of plant material or 10 ng of fungal DNA. The reaction buffer consisted of 100 μM each of dATP, dCTP, dGTP and dTTP, 100 pM each of the relevant forward and reverse primers, and 0.8 units of Taq polymerase (Boehringer Mannheim Ltd., Germany) in 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 µg ml^{-1} gelatine and 0.05 % (w/v) Tween 20 and Nonidet P-40. Primers used included those specific for *F. culmorum*/*F. graminearum* (Fc F/R: CAAAAGCTTCCCGAGTGTGTC/GGCGAAGG

TTCAAGGATGAC) (P. Nicholson, pers. comm.), *F. graminearum* (Fg11F/R: CTCC GGATATGTTGCGTCAA/GGTAGGTATCCGACATGGCAA) (Nicholson *et al.*, manuscript in preparation), *F. poae* (Fp82F/R: CAAGCAAACAGGTCTTCACC/TGTT CCACCTCAGTGACAGGTT) (Parry & Nicholson, 1996), *F. avenaceum* (AF/R: CAA GCATTGTCGCCACTCTC/GTTTGGCTCTACCGGGACTG) *M. nivale* var. *majus* (MnmF/R: TGCAACGTGCCAGAAGCT/AATCGGCGCTGTCTACTAAAAGC) (Nicholson & Parry, 1996) and *M. nivale* var. *nivale* (Y13NF/R: ACCAGCCGATTTGT GGTTATG/GGTCACGAGGCAGAGTTCG) (Nicholson *et al.*, 1996). Reaction mixtures were overlaid with mineral oil prior to PCR.

Amplification was performed in a Perkin-Elmer Cetus 480 DNA thermal cycler (Perkin Elmer, USA). The programme used to amplify fungal DNA from infected plant samples varied depending on the specific primers used. When using *F. culmorum/F. graminearum* or *F. avenaceum*-specific primers the cycler was programmed for 40 cycles of 30 s at 95 ^{0}C, 30 s at 60 ^{0}C and 40 s at 72 ^{0}C. Programmes used with *M. nivale* var. *nivale*, *M. nivale* var. *majus*, *F. graminearum* and *F. poae* primers were similar except that annealing temperatures of 61, 61, 62 and 62 ^{0}C respectively, were used. Aliquots (15 µl) of amplification products were electrophoresed through agarose gels (1.5 % w/v), prepared using TAE buffer (40 mM Tris base, 1 mM EDTA, 20 mM acetic acid) and containing 0.05 mg ethidium bromide per 100 ml TAE buffer.

1.2.5 Statistical analysis

The arcsine of the sub-plot frequency data (as measured by visual disease assessment or species-specific PCR analysis) was used to normalise the distribution of the data for the

purposes of statistical analysis (calculation of correlation coefficients). The correlation coefficients between visual disease assessment and PCR-based assays were determined using the Pearson Product Moment Correlation of arcsine-transformed sub-plot frequency data (Snedecor & Cochran, 1980). This analysis was performed using Minitab release 10.1 (© 1994, Minitab incorporated). The association between pathogens (as detected by PCR analysis) was tested using Fisher`s exact test (Everitt, 1986) for which a correction according to Rom (1990) was used.

1.3 RESULTS

1.3.1 Visual disease assessment

This experiment was part of a larger field trial set up by P. Jenkinson to investigate the relationship between disease severity and yield loss, a field trial in which the sample size harvested per sub-plot increased with proximity to the inoculation zone. As a result, the number of harvested samples available per sub-plot for the present study varied accordingly. Visual disease assessment was performed at GS 80 on a range of single ear samples from each of the 15 sub-plots within the field plot (Table 1.1 & Fig. 1.1a and b). According to these results, the mean visual disease score attributed to the wheat ears taken from within the *F. culmorum*-inoculated sub-plot (sub-plot 8, samples 56-68) was 96 % of spikelets infected with *F. culmorum,* the majority (77 %) of ears from this sub-plot being given scores of 100 % spikelets infected. In the sub-plots outside the inoculated area (sub-plots 1-7, samples 1-55 and sub-plots 9-15, samples 69-123), although disease score varied from 0 to 100 %, the majority of ears showed disease symptoms. In the sub-plot adjacent to the inoculated sub-plot (sub-plot 9, samples 69-81), 12 of the 13 ears scored had disease ratings of 50 % or greater (mean = 70 %) (Fig. 1.1). In the other adjacent sub-plot (sub-plot 7, samples 43-55), disease severity was much reduced and no wheat ear had greater than 35 % spikelets infected (mean = 20 %). The disease severities for sub-plots 1-6 were generally low, mean disease scores being 2, 5, 21, 10, 12 and 12 %, respectively. Only one sample from this region (sample 8, sub-plot 3) had a relatively high disease severity (60 %). The disease severities for the corresponding sub-plots

on the other side of the inoculated sub-plot (sub-plots 10-15, samples 82-123) were generally higher, mean disease scores being 18, 31, 17, 19, 50 and 28 %, respectively. Compared with sub-plots 1-6, sub-plots 10-15 showed greater variation in disease severity from sample to sample, disease scores fluctuating between 0 and 100 %. Several samples from these sub-plots had disease severities greater than 50 % (i.e. samples 90, 96, 99, 103, 116, 119, 120 and 122 from sub-plots 10, 11, 11, 11, 13, 14, 14 and 15, respectively). Therefore, according to the visual disease assessment results, *F. culmorum* was detected in each sub-plot within the field trial. With the exception of the inoculated sub-plot (sub-plot 8), an adjacent sub-plot (sub-plot 9) and sub-plot 14, the mean disease severities of sub-plots were low to moderate, with no significant evidence of the development of a disease severity gradient from the inoculated sub-plot (Fig. 1.1b).

The visual disease results were used to classify each single ear sample as infected or not infected with *F. culmorum* (scores of 1 and 0 respectively) so as to enable comparison with the PCR results on the basis of the percentage of infected samples per sub-plot. The percentage of infected samples within each sub-plot are shown in Table 1.1, as are the number of samples taken from each sub-plot. Based on these results, all of the samples in the inoculated sub-plot appeared to be infected with *F. culmorum* and, even in the non-inoculated sub-plots, the frequency of infected samples was moderate to high (Table 1.1 & Fig. 1.2a). For example, 75 % of samples examined from sub-plot 14 exhibited head blight symptoms, presumed to be caused by *F. culmorum*.

Table 1.1 Field trial sampling plan and incidence of FEB symptoms.

Sub-plot (2 x 2 m)	No. ears sampled	Sample codes	Percentage samples infected[a]
1	3	1-3	33
2	4	4-7	75
3	6	8-13	83
4	7	14-20	57
5	9	21-29	78
6	13	30-42	77
7	13	43-55	85
8[b]	13	56-68	100
9	13	69-81	100
10	13	82-94	85
11	9	95-103	78
12	7	104-110	71
13	6	111-116	67
14	4	117-120	75
15	3	121-123	67

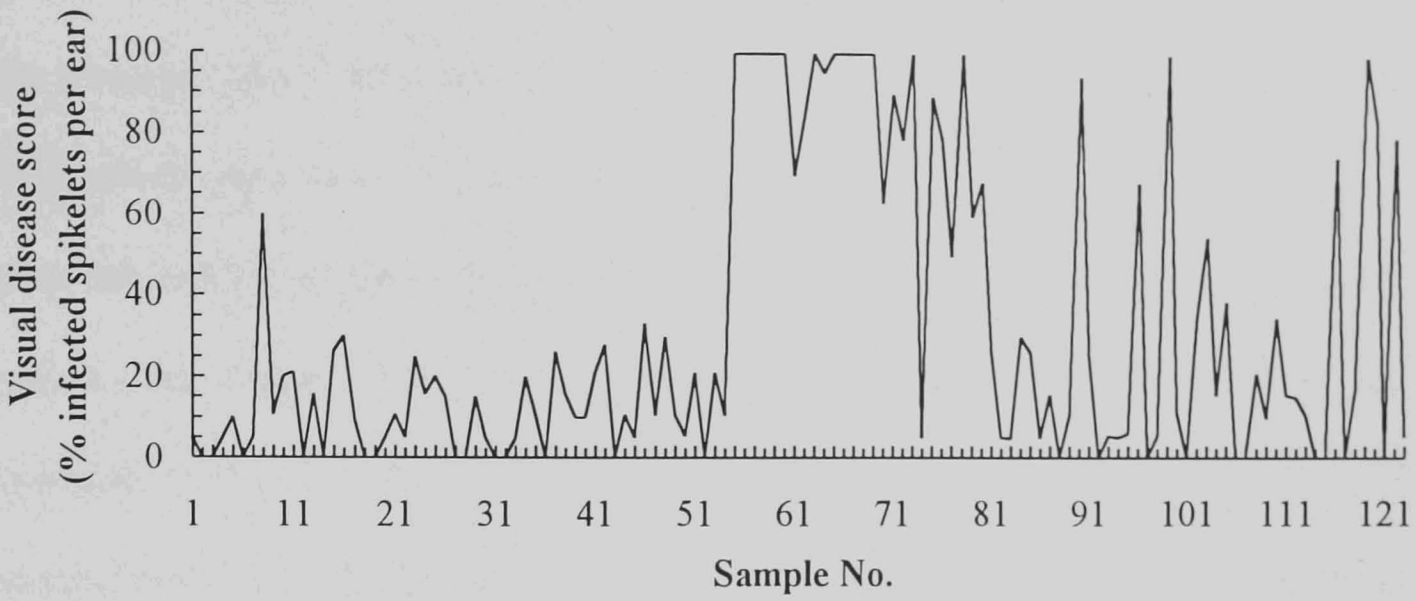
[a]Based on visual disease assessment results.

[b]Sub-plot inoculated with *Fusarium culmorum* (strain Fu 42) at GS 65 (2.1×10^7 conidia m^{-2}).

Table 1.2 Code and origin of fungal species

Species	Code	Origin (all isolated from wheat)
Fusarium culmorum	Fu 42	U.K.
F. graminearum	F 705	France
F. poae	F 62	Poland
F. avenaceum	F 720	Germany
Microdochium nivale var. *majus*	Mn 18	U.K.
M. nivale var. *nivale*	M 58	U.K.

a



b

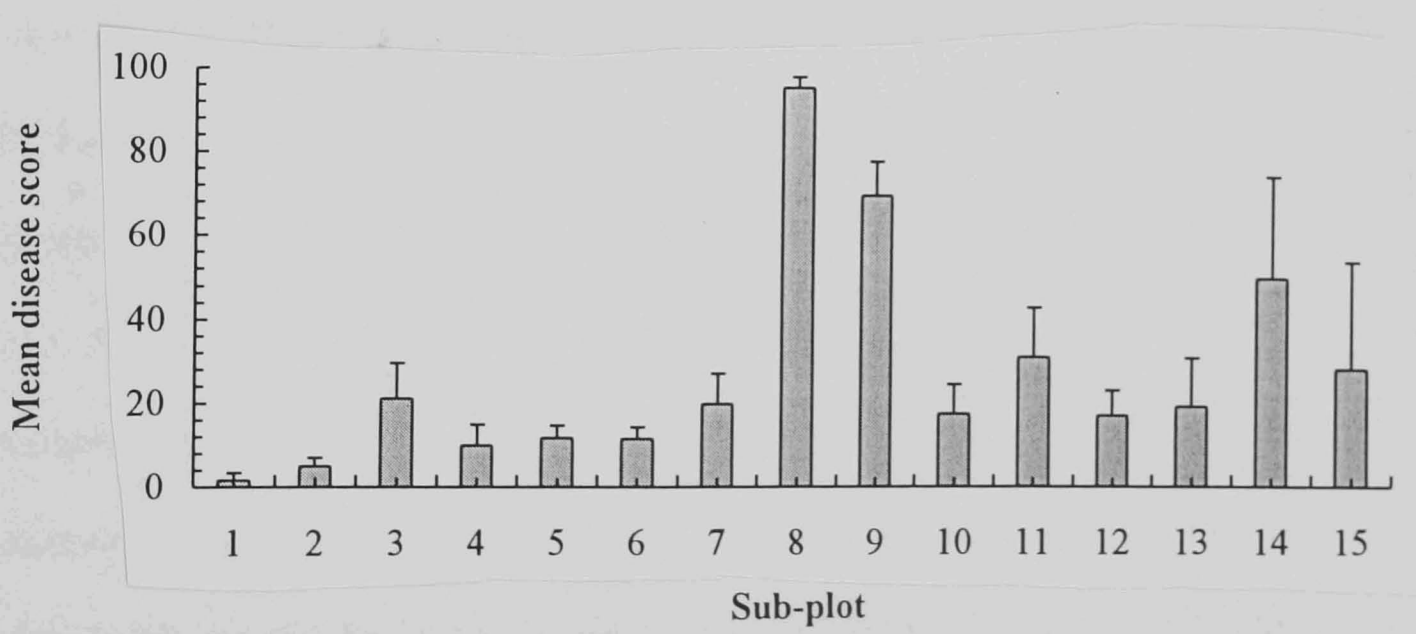


Fig. 1.1 Visual disease assessment results of ear blight of wheat in a field trial in which the central sub-plot (sub-plot 8, samples 56-68) was inoculated with conidia of *Fusarium culmorum* (2.1 x 10^7 conidia m^{-2}) at GS 65. Results expressed as (a), disease score for each sample and (b), mean disease score per sub-plot. Bars in graph b indicate the standard errors of the means (S.E.M.).

1.3.2 *F. culmorum* and *F. graminearum* PCR analysis

Fusarium graminearum-specific PCR analysis (primers Fg11F/R) did not detect this pathogen in any of the samples from the field plot, whether from grain, glume or rachis tissue. Therefore, it was concluded that any PCR amplification that occurred when using the *F. culmorum/F. graminearum*-specific primers (FcF/R) was due to the presence of *F. culmorum* DNA. As with visual disease assessment results, the PCR results were used to classify the ears within each sub-plot as diseased or non-diseased, and the results obtained for grain samples are shown in Fig. 1.2a. Similar results were obtained for glume and rachis samples (results not shown). Within the inoculated sub-plot (sub-plot 8) there was a high incidence of *F. culmorum* (100 % samples infected). PCR detected *F. culmorum* in the grain, glume and rachis of every sample from this sub-plot. Similarly, PCR analysis showed a high incidence of *F. culmorum* in one of the sub-plots adjacent to the inoculated sub-plot (sub-plot 9). PCR analysis detected *F. culmorum* in the grain, glume and rachis of 11 of the 13 samples from this sub-plot, with the exception of sample 75 for which the pathogen was detected in the glume and rachis, but not in the grain tissue. The other two samples taken from sub-plot 9 had lower disease scores (samples 74 and 81, disease scores of 5 and 26 %, respectively) and PCR analysis did not detect *F. culmorum* in sample 74, but did detect the pathogen in the glume and rachis of sample 81.

According to the PCR analysis, the incidence of *F. culmorum* outside sub-plots 8 and 9 was relatively low, although most of the ears outside the inoculated sub-plot that had disease scores of 50 % or greater were found to contain *F. culmorum* by PCR analysis (samples 8, 55, 69-73, 75-80, 90, 96, 99, 103, 116, 119 and 122

from sub-plots 3, 7, 9, 9, 10, 11, 11, 11, 13, 14 and 15 with disease scores of 60, 100, 100, 63, 90, 79, 100, 90, 50, 100, 60, 68, 95, 68, 100, 55, 75, 100 and 80 %, respectively). The results of *F. culmorum/F. graminearum*-specific PCR analysis of 8 of these 'high score' samples are shown in Fig. 1.3a. Of the 20 samples from outside the inoculated sub-plot attributed high disease scores, 11 were among 13 of the samples from sub-plot 9 which was adjacent to the inoculated sub-plot (samples 69-73 and 75-80).

In the other sub-plot adjacent to the inoculated sub-plot (sub-plot 7), PCR analysis only detected *F. culmorum* in 8 % of samples, but this 8 % corresponded to the only sample from this sub-plot attributed a high disease score (100 %). PCR analysis did not detect the pathogen in the other 92 % of samples from sub-plot 7 which were attributed disease scores of between 0 and 33 %.

Although FEB symptoms were observed in 30 of the 42 samples from sub-plots 1-6, PCR analysis detected *F. culmorum* in only a single sample (sample 8, sub-plot 3) where it was present in grain (Fig. 1.3a), glume and rachis tissue. Similarly, 32 of the 42 of the samples from sub-plots 10-15 had FEB symptoms while PCR analysis only detected *F. culmorum* in 13 of the samples from this region. PCR analysis detected *F. culmorum* in samples 82, 84, 85, 89-91, 93, 96, 99, 107, 119, 122 and 123 from sub-plots 10-15. Disease scores for samples 82, 89, 93 and 123 were relatively low (5, 11, 5 and 5 %, respectively) and the pathogen was only detected in one component part of each sample, i.e. glume (82 and 93) or rachis (89 and 123). Samples 84, 85 and 91 had moderate disease scores (30, 26 and 25 %, respectively) and PCR analysis detected the pathogen in the grain and glume of

sample 91, in the grain and rachis of sample 84 and in the rachis of sample 85. *F. culmorum* was detected in samples 90, 96, 99, 119 and 122 which had severe FEB symptoms (disease scores of 95, 68, 100, 100 and 80 %, respectively). PCR analysis detected the pathogen in the grain, glume and rachis components of samples 99 and 119, in the grain and rachis components of sample 90, the grain and glume components of sample 122 and only in the rachis component of sample 96. Sample 107 had exhibited no FEB symptoms, but PCR analysis detected *F. culmorum* in the grain of this sample. When the *F. culmorum* PCR results were correlated with visual disease assessment (Table 1.3), the highest correlation coefficient was obtained for the PCR results from glume samples ($r = 0.822$), as opposed to grain ($r = 0.759$) samples, rachis sample ($r = 0.782$) or the ear (combined grain, glume and rachis) results ($r = 0.675$).

1.3.3 *M. nivale* PCR analysis

The PCR results obtained for the *M. nivale* var. *nivale* and var. *majus* were analysed in the same way as those obtained for *F. culmorum* PCR analysis (Fig. 1.2b, c and d). When compared to the surrounding sub-plots, the frequency of *M. nivale* var. *nivale* and var. *majus* detection was relatively low in samples from the inoculated sub-plot (sub-plot 8) which had a high incidence of *F. culmorum* infection. This is particularly evident in rachis samples (Fig. 1.2d). *M. nivale* var. *majus* was not detected in any of the glume or rachis components, and only detected in 8 % of grain components of samples from sub-plot 8. Similarly, *M. nivale* var. *nivale* was not detected in any of the glume components and only detected in 15 % of the rachis and 8 % of the grain components

of samples from sub-plot 8. Sub-plot 9 also had a high incidence of *F. culmorum* infection and *M. nivale* var. *nivale* was not detected in any samples from sub-plot 9 (Fig. 1.2b, c and d). In sub-plot 9, var. *majus* was not detected in the glume components of any samples, while it was detected in the grain, rachis or both components of 25 % of the samples from this sub-plot. PCR analysis revealed that there was a particularly high incidence of these pathogens in the 13 samples from sub-plot 7, with 69 % of samples infected with *M. nivale* var. *nivale* and 92 % of samples infected with var. *majus*. The pathogen was predominantly detected in the rachis components of these samples (Fig. 1.2d).

PCR analysis detected *M. nivale* var. *nivale* in 33, 25, 0, 0, 56, and 62 % of samples from sub-plots 1-6, respectively, while var. *majus* was detected in 66, 25, 50, 71, 33 and 77 % of samples from sub-plots 1-6, respectively. Again, both pathogens were detected predominantly in the rachis components, often of the same sample. PCR analysis had not detected *F. culmorum* in any of these samples, many of which had low to moderate disease scores (section 1.3.2). *M. nivale* var. *nivale* was detected in 8, 11, 29, 0, 25 and 0 % of samples from sub-plots 10-15, respectively, while var. *majus* was detected in 23, 66, 86, 50, 50 and 66 % of samples from sub-plots 10-15, respectively. *M. nivale* var. *nivale* was found predominantly in the rachis component of these infected samples, often in conjunction with var. *majus*, while *M. nivale* var. *majus* was often detected in both the glume and rachis components. Fourteen of the 21 *M. nivale* var. *majus*- infected samples from sub-plots 10-15 were also infected with *F. culmorum*, and both pathogens were detected in the rachis component of 13 of the co-infected samples. *F. culmorum* had not been detected in any of the five *M. nivale* var. *nivale*-infected samples from sub-plots 10-15 (section 1.3.2). Most *M. nivale* var. *nivale* and/or var.

majus-infected plants from sub-plots 10-15 had been given low to moderate disease scores, although in a few cases no FEB symptoms had been detected (samples 97, 106 and 107 from sub-plots 11, 12 and 12, respectively). *M. nivale* was also detected in some samples from sub-plots 10-15 for which disease scores were relatively high, with var. *majus* in samples 96, 99, 103, 116, 120 (sub-plots 11, 11, 11, 13 and 14, respectively) and var. *nivale* in sample 120 (sub-plot 14). *M. nivale* var. *majus* and var. *nivale* PCR analysis for some of these samples is shown in Fig. 1.3b. *M. nivale* var. *majus* was detected in the rachis of samples 96 and 99 which were also infected with *F. culmorum* (Fig. 1.3a), and which had disease scores of 68 and 100 %, respectively. *M. nivale* var. *majus* was detected in the grain, glume and rachis component of samples 116 & 120, and in the rachis component of sample 103, while var. *nivale* was detected in the glume component of sample 120. *F. culmorum* was not detected in these samples which had disease scores of 75, 84 and 55 %, respectively. PCR analysis showed that var. *majus* was the predominant *M. nivale* sub-species within the field plot, with 64 % of *M. nivale* infected ears due to var. *majus* and 36 % due to var. *nivale*. Since both *M. nivale* var. *majus* and var. *nivale* occurred on symptomatic and asymptomatic wheat ears, neither pathogen served to increase the correlation coefficient between visual disease assessment and PCR-based assays (Table 1.3). In tests of the association between pathogens (Everitt, 1986; Rom, 1990), the only significant association found was between *F. culmorum* and *M. nivale* ($p < 0.05$) and it was found that there was an excess of samples where only one of the pathogens was present, i.e. *F. culmorum* or *M. nivale* var. *majus* / var. *nivale* (Appendix 1.1).

1.3.4 *F. poae* and *F. avenaceum* PCR analysis

PCR analysis was also employed to determine if any of the samples were infected with *F. poae*. Again, the results were analysed as described previously, and are depicted in Fig. 1.2b, c & d. In sub-plots 8 and 9 which had a high incidence of *F. culmorum* infection, the highest incidence of *F. poae* occurred in the glume component of samples, being detected in 23 and 15 % of glumes from sub-plots 8 and 9, respectively. *F. poae* was detected in many glume samples throughout the field plot. For example, *F. poae* was detected in 54 % of the glume samples from sub-plot 7, while it was not detected in any of the corresponding grain or rachis samples. These infected samples had disease scores of between 0 and 33 % and PCR analysis did not detect *F. culmorum* in any of these samples. *M. nivale* var. *nivale* and var. *majus* were detected in many of the *F. poae*-infected samples from sub-plot 7, although these pathogens were predominantly found in the rachis rather than the glume components of the samples.

PCR analysis detected *F. poae* in 66, 100, 50, 58, 56 and 38 % of samples from sub-plots 1-6, respectively. As is evident from Fig. 1.2, the incidence of *F. poae* in grain and glume components of samples from sub-plots 1-6 was relatively high, and the pathogen was detected in either or both of these components of infected samples. The pathogen was not detected in most of the rachis components of samples from sub-plots 1-6. For many of the *F. poae*-infected glume and grain samples, the corresponding rachis components were infected with *M. nivale* var. *nivale* and/or var. *majus*. In sub-plots 10-15, *F. poae* was detected in 23, 66, 71, 17, 0 and 33 % of samples, respectively. Fig. 1.2 shows that the pathogen was predominantly present

in the glume component and was rarely detected in the rachis component of samples from sub-plots 10-15. The majority of infected samples had low to moderate disease scores (5-35 %), although a few showed no FEB symptoms (samples 97, 106 and 107). Also *F. poae* was detected in samples which had relatively high disease scores (samples 96, 99, 103 and 116) and the PCR analysis of these samples is illustrated in Fig. 1.3c. *F. poae* was found in conjunction with *F. culmorum* (Fig. 1.3a) and *M. nivale* (Fig. 1.3b) in samples 96 and 99 (disease scores of 68 and 100 %, respectively). *F. poae*, *F. culmorum* and *M. nivale* were detected in the rachis component of sample 96. Both *F. poae* and *F. culmorum* were detected in the grain, glume and rachis tissues of sample 99, while *M. nivale* was detected in the rachis of this sample. *F. poae* was detected in conjunction with *M. nivale* in samples 103 and 116 (disease scores of 55 and 75 %, respectively), samples in which *F. culmorum* was not detected. In sample 103, *F. poae* was detected in the grain and glume components and *M. nivale* was detected in the rachis. Both *F. poae* and *M. nivale* were detected in the grain, glume and rachis of sample 116. Like *M. nivale* var. *majus* and var. *nivale,* when the results from *F. poae* PCR were combined with those from *F. culmorum* PCR and/or *M. nivale* PCR and correlated with the visual disease assessment results, the various correlation coefficients obtained were lower than that obtained for visual disease assessment and *F. culmorum* PCR results (Table 1.3).

PCR revealed a low incidence of *F. avenaceum* within the field plot and it was detected in only 18 of the 123 samples (results not shown). These infected plants generally had low to moderate disease scores (5-33 %), although FEB symptoms were not detected in two of the *F. avenaceum*-infected samples (samples 6 and 43). Also,

F. avenaceum was detected (along with *M. nivale* var. *majus* and *F. poae*) in the grain component of sample 116 which had a disease score of 75 % (Fig. 1.3). In contrast to *M. nivale* var. *majus, M. nivale* var. *nivale* and the *F. poae* PCR, when the *F. avenaceum* PCR results were combined with the *F. culmorum* PCR results and correlated with visual disease assessment (r = 0.843), a closer relationship was observed than between visual disease assessment and *F. culmorum* PCR results (r = 0.822) (Table 1.3).

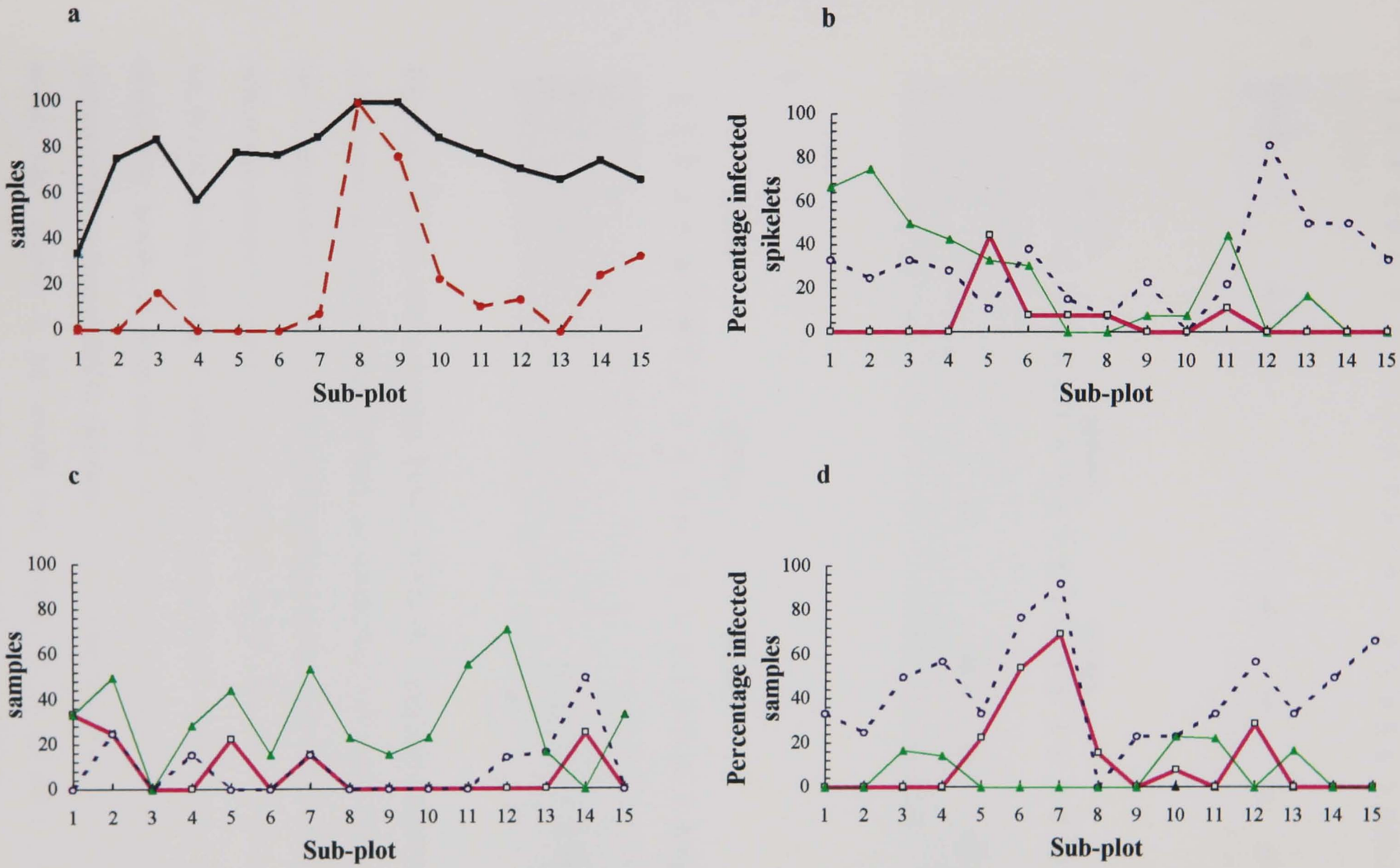


Fig. 1.2 Comparison of the frequency of occurrence of visual disease symptoms (■——■) with the frequency of PCR detection of *Fusarium culmorum* (●— —●) in grain tissue of ear samples taken from sub-plots of a field trial (a), and comparison of the frequency of PCR detection of *M. nivale* var. *majus* (○- - - -○) and var. *nivale* (□——□) and *F. poae* (▲——▲) in the corresponding grain (b), glume (c) and rachis (d) components of these ear samples.

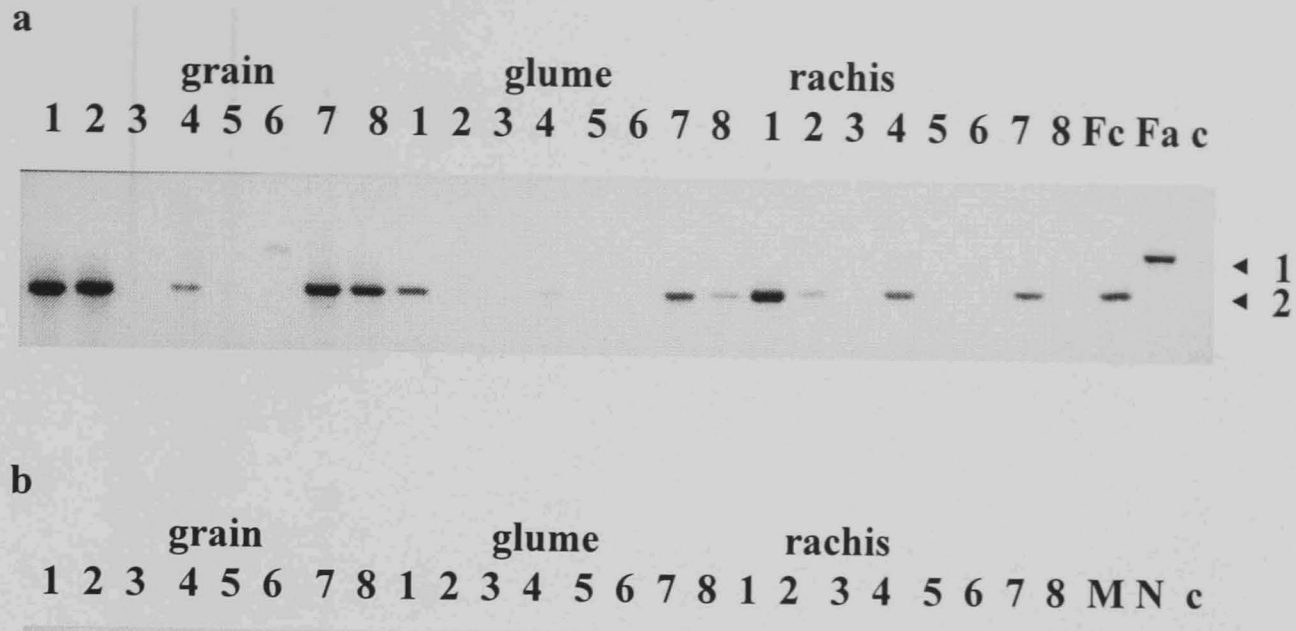


Fig. 1.3 Detection of the specific PCR products for *Fusarium culmorum* and *F. avenaceum* (**a**), *Microdochium nivale* var. *nivale* and var. *majus* (**b**) and *F. poae* and *F. graminearum* (**c**) in the corresponding grain, glume and rachis tissue of wheat ear samples. Lanes: 1--8, samples 8, 90, 96, 99, 103, 116, 119 and 122; Fa, Fc, M, N, Fp and Fg, *F. culmorum, F. avenaceum, M. nivale* var. *majus, M. nivale* var. *nivale*, *F. poae* and *F. graminearum* genomic DNA (Table 1); c, control without fungal DNA. Arrows: 1, *F. avenaceum*; 2, *F. culmorum*; 3, *M. nivale* var. majus; 4, M. *nivale* var. *nivale; 5, F. graminearum*; 6, *F. poae*-specific DNA products (920, 700, 750, 310, 400 and 220 bp, respectively).

Table 1.3 Correlation between visual disease assessment results and various PCR-based diagnostic assay results obtained for wheat ears

and their component parts (grain, glume and rachis tissue).

PCR assays	Pearsons Correlation coefficient (r)[a]			
	Grain	Glume	Rachis	ear
Fusarium culmorum	0.759	0.822	0.782	0.675
F. culmorum/F. poae	0.191	0.584	0.779	0.314
F. culmorum/Microdochium nivale var. *majus*	0.244	0.775	0.657	0.365
F. culmorum/M. nivale var. *nivale*	0.783	0.695	0.821	0.669
F. culmorum/F. avenaceum	0.647	0.843	0.371	0.352
F. culmorum/F. poae/M. nivale var. *majus*	-0.298	0.600	0.639	-0.058
F. culmorum/F. poae/M. nivale var. *nivale*	0.226	0.616	0.820	0.387
F. culmorum/F. poae/F. avenaceum	0.161	0.581	0.355	-0.031
F. culmorum/M. nivale var. *majus/M. nivale* var. *nivale*	0.297	0.661	0.667	0.094
F. culmorum/M. nivale var. *majus/F. avenaceum*	0.364	0.675	0.175	0.009
F. culmorum/M. nivale var. *nivale/F. avenaceum*	0.724	0.588	0.466	0.447
F. culmorum/F. poae/M. nivale var. *majus/M. nivale* var. *nivale*	-0.304	0.600	0.650	-0.042
F. culmorum/F. poae/M. nivale var. *majus/F. avenaceum*	-0.291	0.598	0.154	-0.056
F. culmorum/F. poae/M. nivale var. *nivale/F. avenaceum*	0.202	0.605	0.464	-0.017
F. culmorum/M. nivale var. *majus/M. nivale* var. *nivale/F. avenaceum*	0.182	0.665	0.175	0.028
F. culmorum/F. poae/M. nivale var. *majus/M. nivale* var. *nivale/F. avenaceum*	-0.296	0.590	0.154	-0.042

[a]Based on arcsine-transformed sub-plot frequency data.

1.4 DISCUSSION

The use of PCR analysis to identify the fungal species present in wheat ears which exhibited FEB symptoms within the field plot has highlighted some potential problems associated with visual assessment of a disease complex. Only within the central *F. culmorum*-inoculated sub-plot and an adjacent sub-plot (sub-plot 9) was a correlation found between visual disease assessment and the presence of *F. culmorum* as detected by PCR analysis. PCR analysis revealed the presence of *F. culmorum* in all 13 ears sampled in the inoculated sub-plot. Similarly, all of the ears exhibiting symptoms in sub-plot 9 were found to contain *F. culmorum* as determined by PCR analysis. However, PCR analysis failed to detect *F. culmorum* in some of the samples taken from the remaining sub-plots which were given disease scores assumed to be related to the presence of *F. culmorum* originating from the inoculated sub-plot. *F. culmorum* was detected in most of the ears (both within and outside the inoculated sub-plot) exhibiting the highest disease scores (above 50 %). However, since PCR analysis did not indicate that there was a gradient in the field plot in terms of the frequency of detection of the pathogen, it cannot be assumed that the *F. culmorum* inoculum for all of these samples originated from the inoculated sub-plot. In a recent review of FEB, Parry *et al.* (1995a) suggested that *F. culmorum,* along with *F. graminearum* was consistently the most pathogenic of the *Fusarium* species infecting cereal ears. In the present study, it could be inferred that perhaps *F. culmorum* was more pathogenic than the other *Fusarium* species and *Microdochium* sub-species detected within the field plot, under the prevailing environmental conditions.

PCR analysis was also used to detect other *Fusarium* species within the field

plot and the presence of some of these pathogens may account for the disease observed in many areas of the field trial, particularly where low to moderate disease scores were recorded (sub-plots 1-6 and 10-15). The presence of other *Fusarium* species within the field plot is understandable since conditions necessary for *Fusarium* ear blight were optimized within the field trial by the use of misting to generate warm humid conditions, during and following the period of anthesis. The source of inoculum is unclear, although possible sources include crop debris (Cook, 1981a; Sutton, 1982), alternative hosts such as grass and broad-leaved weeds (Gordon, 1959; Jenkinson & Parry, 1994b) or indeed *Fusarium* foot rot or seedling blight of cereals (Parry *et al.*, 1994).

The separation of ear samples into their component parts (i.e. grain, glume and rachis) for the purpose of PCR analysis permitted tissue localisation of the various *Fusarium* species and *M. nivale* sub-species. This highlighted the fact that *F. poae* was predominantly present in glume material, but often not detected in the corresponding grain tissue and was rarely detected in rachis tissue. In a survey of *Fusarium* ear diseases of winter wheat in Great Britain during 1989-1990, Polley & Turner (1995) reported that *F. poae* was the *Fusarium* species most commonly isolated from glumes affected by ear blight symptoms and that this pathogen was associated with distinct glume spot lesions.

The presence of *F. poae* in the glume, but not in the corresponding grain or rachis of some wheat ears suggests that initial infection by this pathogen was via the glumes. Although Tu (1930) achieved successful infection of ears by *F. graminearum* when anthers were absent, convincing evidence by other workers suggests that initial

infection of wheat ears by *F. graminearum* occurs predominantly via anthers and not via glumes (McKay & Loughnane, 1945; Pugh *et al.*, 1933; Strange & Smith, 1971). Strange & Smith (1971) showed that the emasculation of wheat ears significantly reduced the incidence of florets infected with *F. graminearum* and later these workers demonstrated that two compounds isolated from anther extracts, choline chloride and betaine hydrochloride, significantly promoted hyphal extension, but not spore germination of *F. avenaceum, F. culmorum* and *F. graminearum* (Strange & Smith, 1978). Thus it is suggested that either *F. poae* infection did not occur at anthesis or that the mode of infection and colonisation by *F. poae* differs from that of other pathogens associated with ear blight.

Outside the inoculated and adjacent sub-plots, *F. poae, F. avenaceum* and *M. nivale* were detected in some ears which did not contain *F. culmorum* and which had particularly high disease scores. Indeed, one interesting observation was the occurrence of *F. culmorum, F. avenaceum* and *M. nivale* on the grain and rachis components, but not the corresponding glume components, of several high disease score samples taken from outside the inoculated sub-plot. Although these samples only accounted for a small proportion of the total amount taken, these results implied that the bleaching of ears which resulted in high visual disease scores for these samples, was not as a result of glume infection, but rather was the indirect result of pathogen infection, possibly following infection of the rachis and the impediment of nutrient translocation to the glume.

PCR analysis revealed that *M. nivale* sub-species were more frequently detected in rachis than in grain or glume tissue. Also, PCR analysis indicated that *M.*

nivale var. *majus* was more abundant on ears than var. *nivale*. This is in accordance with the results of Parry *et al.* (1995b) who, using a PCR-restriction fragment length polymorphism (RFLP) technique, found that, of 91 *Microdochium* isolates obtained from grain taken from 7 sites throughout the U.K., 93 % were var. *majus* and 7 % var. *nivale*.

PCR analysis detected *F. avenaceum* in comparatively few samples and *F. graminearum* was not detected within the field plot. Tests using *F. avenaceum* as inoculum have resulted in severe ear blight in the U. K. (Parry *et al.*, 1995a), although infection by this species generally represents a small proportion of the isolates obtained from FEB affected crops in the U.K. and other cool maritime regions of Northwest Europe. There are few reports of *F. graminearum* on wheat crops in the UK (Moore, 1948) and this species is generally important in hotter regions of the world (Parry *et al.*, 1995a).

The fact that pathogens were detected in asymptomatic samples may be due to several factors including sensitivity of PCR-based assays and variability of disease symptoms (i.e. distinctiveness from *F. culmorum* disease symptoms). Among the pathogens, the only significant association was that found between *F. culmorum* and *M. nivale* var. *majus*/var. *nivale*. The significant probability of independence found between *F. culmorum* and *M. nivale* var. *majus*/var. *nivale* may suggest some sort of antagonistic interaction between these pathogens.

This work has demonstrated how PCR analysis may be used to gain insight into and overcome some of the problems associated with the diagnosis and understanding of FEB of wheat.

2. EVALUATION OF THE EFFICACY OF FUNGICIDES AGAINST *FUSARIUM* EAR BLIGHT OF WHEAT

2.1 INTRODUCTION

In the past, fungicidal control of *Fusarium* ear blight (FEB) of wheat has received relatively little attention and there are few reports of successful fungicidal control of FEB in the field. This is probably due to the difficulties associated with successful and consistent fungicidal control of the disease. These include the restricted timing of application necessary, the complex of causal organisms involved in the disease (Parry *et al.*, 1994), the development of fungicide resistance (Locke *et al.*, 1987; Pettitt *et al.*, 1993), discrepancies between *in vitro* activity and field performance of fungicides (Parry *et al.*, 1994) and the effect of fungicides on mycotoxin concentrations (Martin & Johnston, 1982; Moss & Frank, 1985; Boyacioglu *et al.*, 1992; Gareis & Ceynowa, 1994).

Conventionally, fungicide evaluation in the field has employed techniques such as visual disease assessment (percentage of ears diseased), the number of grains per ear and thousand grain weight. Hutcheon & Jordan (1992) used these parameters to measure the efficacy of fungicides against FEB of wheat, under glasshouse conditions. Techniques based on the quantification of chemical constituents common to fungal pathogens have also been used to evaluate the efficacy of fungicides, such as the method developed by Ride & Drysdale (1972), which is based on the degradation of chitin. However, these techniques cannot distinguish between the complex of causal organisms involved in FEB, which may be important under field conditions. For example, some

Fusarium species such as *Fusarium culmorum, F. poae, F. graminearum* and *F. sporotrichoides* have the potential to produce trichcothecene mycotoxins, whereas there is controversy as to whether species such as *F. avenaceum* or *Microdochium nivale* sub-species produce trichothecene mycotoxins (Thrane, 1989). Therefore, it would seem important to understand the efficacy of fungicides against the individual species which can cause FEB and to additionally understand the effect of fungicides on mycotoxin production.

Polymerase chain reaction (PCR)-based assays have been developed which enable identification, differentiation and quantification of the common causal agents of FEB of wheat. Qualitative PCR assays have been developed for the detection of *F. culmorum*(Schilling *et al.*, 1996; Nicholson *et al.*, unpublished), *F. avenaceum* (Nicholson & Parry, 1996; Schilling *et al.*, 1996), *F. poae* (Parry & Nicholson, 1996), *F. graminearum* (Ouellet & Seifert, 1993; Schilling *et al.*, 1996), *M. nivale* var. *nivale* and *M. nivale* var. *majus* (Nicholson & Parry, 1996; Nicholson *et al.*, 1996) in infected plant material. Those PCR assays developed in the Cereals Research Department of the John Innes Centre (U.K.) have also been adapted to enable quantification of the pathogens (Nicholson *et al.*, unpublished). Quantitative PCR is based on competition between an unknown amount of target fungal DNA and a constant amount of a competitor template for the same primers and other reagents in the reaction mix. The resulting PCR product ratio (ratio between the amount of target fungal DNA and the amount of competitor template) can be converted to nanograms of fungal DNA using a standard graph. One potential application of quantitative PCR may be as a screening tool to assess the differential effects of fungicides on *Fusarium* species. Use of quantitative PCR-based assays would enable quantitative determination of the effects of

fungicides on either individual *Fusarium* species or the effects on individual species present as a complex of causal organisms. Because of the specificity of quantitative PCR, it has the potential to play a major role in the understanding of the epidemiology and control of plant disease complexes.

Similar quantitative PCR systems have been developed for the analysis of *Verticillium* species pathogenic on potato (Hu *et al.*, 1993; Moukhamedov *et al.*, 1994) and for the analysis of *Leptosphaeria maculans* during blackleg development in oilseed rape (Mahuku *et al.*, 1995). Quantitative PCR has been shown to corroborate previous cytological studies of colonisation patterns in infected alfalfa systems which are resistant or susceptible to *V. albo-atrum* (Hu *et al.*, 1993).

As stated earlier, there are surprisingly few reports of successful fungicidal control of FEB of wheat. In the past methyl benzimidazole carbamate (MBC) fungicides were widely used to control early-season diseases of wheat. This group of fungicides includes benomyl, carbendazim and thiophanate-methyl, and were the first systemic fungicides with a broad antifungal spectrum, possessing both eradicant as well as protective properties (Davidse, 1986). These fungicides bind to β-tubulin, affecting the function of cellular micro tubules, causing inhibition of mycelial growth and distortion of germ tubes (Davidse, 1986). Resistance to benzimidazole (an MBC fungicide) in *M. nivale* isolates is now widespread in the UK (Locke *et al.*, 1987; Polley *et al.*, 1991; Parry, 1990; Pettitt *et al.*, 1993). However, Parry *et al.* (1994) found resistance to benomyl to be rare in populations of *F. avenaceum* and *F. culmorum*.

There are two main groups of sterol biosynthesis-inhibiting fungicides (SBI's) used in agriculture; the first group is the demethylase inhibiting fungicides (DMI's), and the second is the morpholine and 3-phenylpropylamine fungicides. These differ with

respect to the enzymes they inhibit in the post-squalene segment of the fungal sterol biosynthetic pathway (Mercer, 1991). DMI fungicides inhibit sterol 14α-demethylation, whereas morpholines and 3-phenylpropylamines inhibit sterol Δ^{14} reductase (SR) and Δ^{8}-Δ^{7} isomerase (Δ^{8}-Δ^{7} SI).

Tebuconazole and prochloraz, both DMI's, have been reported as effective chemicals against *Fusarium* species in glasshouse trials (Hutcheon & Jordan, 1992) and in the field (Fehrmann & Ahrens, 1984; Suty *et al.*, 1996). Of a range of fungicides, they found that tebuconazole and prochloraz gave the most effective control against *F. avenaceum, F. culmorum, F. graminearum* and *M. nivale,* with up to 91% reductions in ear disease. To date, there have been no reports of *Fusarium* species resistant to DMI fungicides.

The morpholines (fenpropimorph and tridemorph) and the 3-phenylpropylamines (fenpropidin) constitute the second group of SBI's. In *in vitro* studies, Debieu *et al.* (1992) found that fenpropimorph inhibited mycelial growth of various *Fusarium* species and *M. nivale* to varying degrees. According to these workers, in the presence of fenpropimorph, sensitive strains, with the exception of *M. nivale,* accumulate mainly $\Delta^{8,14}$ sterols and tolerant strains accumulate either both $\Delta^{8,14}$ and Δ^{8} sterols or only Δ^{8} sterols.

New fungicides with different modes of action are continuously being developed and may prove useful in controlling FEB of wheat. Pyrimethanil is a new anilino-pyrimidine fungicide which is effective against all strains of *Botrytis* (Neumann *et al.*, 1992). It was proposed that the target activity of this fungicide on *Botrytis* species is on the secretion of cell wall degrading enzymes by the fungus which are important in initial establishment of the pathogen in host tissue (Milling & Richardson, 1995). The novel

mode of action of pyrimethanil contrasts with the known activity of other commercially available fungicides.

Further work on the rate and timing of fungicide application is required to optimize their efficacy against FEB of wheat. In a field trial set up to evaluate the effect of fungicides on *F. graminearum* ear blight, Boyacioglu *et al.* (1992) found that maximum decrease in deoxynivalenol (DON) occurred if thiabendazole was applied two days pre-inoculation. However, the best disease control was observed with triadimefon and propiconazole applied 2 days post-inoculation. Application of these two fungicides prior to inoculation had little effect on disease.

In the future, biological control agents and/or their fungitoxic metabolites may provide an additional component, or indeed, an alternative to the current FEB control strategies. Several fungal antagonists have shown potential for use as biological control agents of *Fusarium* species (De Cal *et al.*, 1995; Knudsen *et al.*, 1995; Schisler *et al.*, 1995; Carver *et al.*, 1996). Knudsen *et al.* (1995) set up a study to verify the biological control effects of various candidates antagonistic towards *F. culmorum* and *Bipolaris sorokiniana* and found that the most successful antagonists against seedborne *F. culmorum* and *B. sorokiniana* were isolates of *Chaetonium* sp., *Idriella bolleyi* and *Gliocladium roseum*. In a field experiment, *G. roseum* controlled *F. culmorum* seedling blight in terms of increasing plant establishment, tiller number and thousand grain weight, compared to untreated plots (Knudsen *et al.*, 1995). *In vitro* studies carried out by Etheridge (1997) showed that substances secreted by *G. roseum* during culture in potato dextrose broth significantly inhibited the radial growth of various *Fusarium* species and *M. nivale* sup-species, when incorporated into potato dextrose agar. *Fusarium* growth inhibition was greatest when media of either pH 8.5 or 5.5 were used

for the production of culture filtrate, indicating that *G. roseum* may secrete more than one inhibitory substance.

The present work was carried out to evaluate the efficacy of fungicides prochloraz, tebuconazole and pyrimethanil and the efficacy of *G. roseum* culture filtrate against FEB of wheat, under glasshouse conditions. The fungicides and culture filtrate were applied 2 days post inoculation, as preliminary experiments had indicated that this was the optimum time for fungicide treatment under glasshouse conditions (J. Liggitt, unpublished). These experiments were also used to determine the effect of using different disease assessment techniques, i.e. visual disease assessment and quantitative PCR analysis, to evaluate fungicide efficacy. Yield analysis (1000 grain weight and the number of grains/ear) was also performed and the relationships between yield, quantitative PCR and visual disease assessment results were investigated.

2.2 MATERIALS & METHODS

2.2.1 Origin and maintenance of fungal isolates

Isolates of *Fusarium* species and *Gliocladium roseum* used in this study were obtained from the John Innes Centre facultative pathogen culture collection (Table 2.1). Media and conditions used for maintenance and subculturing of isolates and for DNA preparation were as described earlier (Chapter 1, section 1.2.2). *G. roseum* culture filtrate was prepared as follows: Erlenmeyer flasks containing 100 ml of potato dextrose broth (PDB) were inoculated with a 1 ml conidial suspension containing 10^6 conidia ml^{-1} (inoculum was prepared as described in Chapter 1, section 1.2.2). Cultures were grown at 30 ^{0}C, 150 rpm. After 3 weeks, culture filtrate was harvested by filtration through sterile Whatman No. 1 filter paper followed by sterile filtration using 0.2 µm Nalgene sterile filtration units (Nalge, UK).

2.2.2 Glasshouse trials

Three glasshouse trials were carried out (1994/5, 1995/6 and 1996/7) to evaluate the effectiveness of fungicides against *Fusarium* ear blight of wheat using quantitative PCR analysis, and to compare the results with those obtained using conventional visual disease assessment. Seeds of winter wheat cultivar Avalon were germinated in Petri dishes containing damp filter paper for 4 days at 5 °C. Germlings were transferred to Jiffy pots containing John Innes compost No.1 and were vernalised at 10 ^{0}C for eight weeks. They were then transferred to 15 cm pots containing a 2:1 mixture of John Innes compost No.1 and sand, one plant per pot, and pots were placed in a well ventilated

Table 2.1 Code and origin of fungal isolates

Code	Origin (all isolated from wheat)	Species
Fu 42[a]	U.K.	*Fusarium culmorum*
Fu 60[a]	U.K.	*F. culmorum*
Fu 72[a]	U.K.	*F. culmorum*
Fu 15[a]	U.K.	*F. culmorum*
Fu 3[c]	U.K.	*F. culmorum*
Fu 53[b]	U.K.	*F. poae*
F. 18[b]	U.K.	*F. poae*
CSL 8[b]	U.K.	*F. poae*
4/3084/2[b]	U.K.	*F. poae*
F. 731[c]	U.K.	*F. poae*
Gr P1	U.K.	*Gliocladium roseum*

[a]*F. culmorum* isolates used for 1995/6 and 1996/7 glasshouse trial inoculation.

[b]*F. poae* isolates used for 1995/6 and 1996/7 glasshouse trial inoculation.

[c]Isolates used for generation of standard curves for quantitative PCR analysis.

glasshouse on an irrigated sand bench.

The first glasshouse trial (1994/5) was set up to evaluate the efficacy of the fungicides prochloraz and pyrimethanil against *F. culmorum* ear blight of wheat, using *F. culmorum* (strain Fu 42) as inoculum. At mid-anthesis, wheat ears were inoculated with conidia of *F. culmorum*. Conidia were produced by growing the isolate on SNA (Nirenberg, 1976) under near-U.V. light for 18-24 days. Conidia were harvested using a 0.2% Tween 20 solution (Sigma, UK) and the concentration was adjusted to 10^6 conidia ml^{-1}. Three ears per wheat plant were inoculated with a total of 5 ml each of the conidial suspension. Uninoculated controls were treated in a similar manner, using a 0.2% (v/v) Tween 20 solution instead of conidial suspensions. Fungicides (prochloraz and pyrimethanil) were applied to the appropriate plants 2 days post-inoculation using a pressurised hand sprayer with a flat fan nozzle. Information regarding prochloraz and pyrimethanil, including rates of application, active ingredient content, formulation type and manufacturers' address are detailed in Table 2.2. Water volumes of 250 l ha^{-1} and pressures of 200-300 kPa were used. Treatments involved in this experiment are described in Table 2.3. Following inoculation, all plants were covered with polyethylene bags for 1 week to maintain high humidity.

The second glasshouse trial (1995/6) was set up to evaluate the efficacy of the fungicides prochloraz and tebuconazole and the efficacy of *G. roseum* culture filtrate against *F. culmorum* and *F. poae* ear blight of wheat, using a range of *F. culmorum* and *F. poae* isolates (Table 2.1). Conidial suspensions were prepared and inoculated as described above for *F. culmorum* and, since a mixed strain inoculum was used in the second glasshouse trial, the full inoculum density was divided equally between the strains involved. The treatments involved in this experiment are outlined in Table 2.3.

Fungicides (prochloraz and tebuconazole) were applied as above and details regarding the fungicides prochloraz and tebuconazole are given in Table 2.2. *G. roseum* culture filtrate was applied at a rate of 4 ml of culture filtrate per treated wheat ear. Water volumes and pressures were as described above and plants were covered with polyethylene bags for 1 week to maintain high humidity. A third glasshouse trial was conducted in 1996/7 to confirm the effects of the fungicides prochloraz and tebuconazole against *F. culmorum* and *F. poae* ear blight of wheat (Table 2.3) and this experiment was performed as described above for the 1995/6 glasshouse trial.

2.2.3 Visual disease assessment and yield determination

Visual disease assessment was carried out at GS 70, 75 and 80 on three treated ears from each plant. Disease assessment was based on the percentage of infected spikelets per ear. Ears were harvested at GS 90. The three ears from each plant were bulked together and the 1000 grain weight and the number of grains per ear calculated.

2.2.4 DNA extraction

Fungal DNA was extracted as described previously (Chapter 1, section 1.2.3) and DNA was diluted to 10 ng μl^{-1} TE buffer (10 mM Tris-HCl, 1 mM EDTA) for use in amplification reactions. At harvest, all the component parts of the three treated wheat ears from each plant were bulked together in 25 ml tubes for DNA extraction, which was as described earlier for grain samples (Chapter 1, section 1.2.3). DNA pellets were dissolved in TE buffer (400 µg dry weight equivalent μl^{-1}).

Table 2.2 Fungicide treatments.

Fungicide	Formulation type	Active ingredient (a.i.) content (g l^{-1})	Application rates (g a.i. ha^{-1})[a]	Manufacturer
Prochloraz	Sportak©	450	450	AgrEvo Ltd., Saffron Walden, Essex, U K.
Pyrimethanil	Technical grade	400	400	AgrEvo Ltd., Saffron Walden, Essex, UK.
Tebuconazole	Folicur©	250	400	Bayer UK. Ltd., Bury St. Edmunds, UK.

[a]Under glasshouse conditions, these application rates were achieved by using a pressurised hand sprayer with a flat fan nozzle, water volumes of 250 l ha^{-1} and pressures of 200-300KPa.

Table 2.3 Treatments used in 1994/5, 1995/6 and 1996/7 glasshouse trials.

Treatment number	1994/5		1995/6		1996/7	
	Inoculum	Fungicide	Inoculum	Fungicide	Inoculum	Fungicide
1	None	None	None	None	None	None
2	None	Pyrimethanil	None	Prochloraz	None	Prochloraz
3	None	Prochloraz	None	Tebuconazole	None	Tebuconazole
4	*Fusarium culmorum*	None	None	*Gliocladium roseum* culture filtrate	*F. culmorum*	None
5	*F. culmorum*	Pyrimethanil	*F. culmorum*	None	*F. culmorum*	Prochloraz
6	*F. culmorum*	Prochloraz	*F. culmorum*	Prochloraz	*F. culmorum*	Tebuconazole
7	-	-	*F. culmorum*	Tebuconazole	*F. poae*	None
8	-	-	*F. culmorum*	*Gliocladium roseum* culture filtrate	*F. poae*	Prochloraz
9	-	-	*F. poae*	None	*F. poae*	Tebuconazole
10	-	-	*F. poae*	Prochloraz	-	-
11	-	-	*F. poae*	Tebuconazole	-	-
12	-	-	*F. poae*	*Gliocladium roseum* culture filtrate	-	-

2.2.5 Qualitative PCR amplification and agarose gel electrophoresis

Qualitative PCR analysis was used to determine if DNA extracts from glasshouse experiments contained *F. culmorum* or *F. poae* genomic DNA, and qualitative PCR signals were also used as an indication of the dilution of DNA extracts to include in quantitative PCR analysis. PCR reactions contained 800 µg dry weight equivalent of DNA extracts (2 µl of DNA extract solutions). Control reactions included a negative control sample in which no DNA was added and a positive control sample containing 1-10 ng of the appropriate genomic fungal DNA template. All the other PCR reaction components, except primers, were as described earlier (Chapter 1, section 1.2.4). Two different primer pairs, FcF/R (Nicholson *et al.*, unpublished) (Chapter 1, section 1.2.4) and C51F/R: (5'-ATGGTGAACTCGTCGTGGC-3'/5'-CCCTTCTTACGCCAATCTC G-3') (Nicholson *et al.*, unpublished), were used for *F. culmorum* PCR analysis. The reason for this is that, in the first glasshouse experiment (1994/5), only primers specific for *F. culmorum/F. graminearum* (FcF/R) DNA were available. In this experiment, these primers were used in conjunction with *F. graminearum*-specific PCR primers (11F/R) (Chapter 1, section 1.2.4) to determine if plants were infected with *F. culmorum*. During 1995, the *F. culmorum*-specific C51F/R primers were designed and these primers were used for *F. culmorum*-specific PCR analysis of DNA extracts from the 1995/6 and 1996/7 glasshouse experiments. The primers P82F/R (Parry & Nicholson, 1996) (Chapter 1, section 1.2.4) were used for *F. poae*-specific PCR analysis.

Amplification was performed in a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin Elmer, USA). The PCR programme used to amplify fungal DNA from plant samples using primers FcF/R and 11F/R was as described in Chapter 1 (section 1.2.4). The PCR amplification programme used for primers C51F/R was as follows: 5 cycles

of 95 ^{0}C for 30 s, 66 ^{0}C for 20 s and 72 ^{0}C for 45 s, 5 cycles of 95 ^{0}C for 30 s, 64 ^{0}C for 20 s and 72 ^{0}C for 45 s and 30 cycles of 95 ^{0}C for 30 s, 62 ^{0}C for 20 s and 72 ^{0}C for 45 s 95 ^{0}C, using the fastest possible transitions between temperatures. A final extension step of 72 ^{0}C for 5 min was included in the programme followed by cooling to 4 ^{0}C until recovery of the samples. Aliquots (10-15 µl) of amplification products were electrophoresed through agarose gel as described earlier (Chapter 1, section 1.2.4).

2.2.6 Quantitative PCR amplification and agarose gel electrophoresis

2.2.6.1 Production of competitor templates

In order to quantify *F. culmorum* and *F. poae* DNA, it was necessary to produce competitor templates. A competitive DNA fragment specific for *F. culmorum* was produced by Lees (1995), based on the method of Forster (1994). Isolated *F. culmorum* genomic DNA (2 µl) was amplified using *F. culmorum/F. graminearum* competitive primers (Cu17F/R: 5'-TCGATATACCGTGCGATTTCC-3'/5'-TACAGACACCGTCAG GGGG-3') designed by Lees (1995). For all PCR amplifications, the other reaction components were as described earlier (Chapter 1, section 1.2.4). The 350 bp product was diluted (1 in 1000) and 2 µl was amplified using the same 5' primer (Cu17F) and an internal 3'-linker primer (Cu17CR: 5'-CGTCAGGGGGAGATACTGACACAGCGC-3'). The 300 bp product was diluted (1 in 100) and 2 µl of this dilution was amplified using the original 5'- primer (Cu17F) and 3'- primer (Cu17R). The PCR programme used for amplifications consisted of 30 cycles of 95 ^{0}C for 30 s, 60 ^{0}C for 20 s and 72 ^{0}C for 45 s using the fastest possible transitions between temperatures. A final extension step of

72 ^{0}C for 5 min was included in the programme followed by cooling to 4 ^{0}C until recovery of the samples. Aliquots of the resulting 300 bp competitor template were electrophoresed through a 2% (w/v) low melting point agarose gel (Fluka), prepared using TAE buffer (40 mM Tris base, 1 mM EDTA, 20 mM acetic acid). The 300 bp band was excised from the gel, incubated at 75 °C with 100 µl sterile d.H_2O, mixed, diluted using TE buffer (see section 2.2.4) and the DNA concentration was determined using a spectrophotometer. Quantification of *F. culmorum* for all three years was achieved using this competitor and Cu17F/R primers.

The *F. poae*-specific competitive DNA fragment was produced by A. Turner (John Innes Centre, UK) as follows. Hybrid primers were used to attach *F. poae*-specific primer sites (P82F/R) to a DNA fragment from the gene encoding the 23 Kda extrinsic polypeptide of the oxygen-evolving complex from pea (*Pisum savitum* cv. Feltham First) photosystem II (Wales *et al.*, 1989), by means of two PCR reactions. The first PCR reaction was carried out using linker primers consisting of pea DNA-specific sites, with the first 10 bp (5'-3') analogous to the last 10 bp (5'-3') of the P82F/R primers. The remainder of the P82F/R primer sequences were attached in a second PCR reaction, using P82F/R primers. Other PCR reaction components were as described earlier (Chapter 1, section 1.2.4), and the PCR programme was as described above for construction of the *F. culmorum* competitive template, except that an annealing temperature of 55 ^{0}C was used. Aliquots of the resulting 500 bp competitive template were electrophoresed through a 2% (w/v) low melting point agarose gel (Fluka), prepared using TAE buffer. The 500 bp band was excised from the gel, and ligated in the pGEM-T vector (Promega) as decribed in Sambrook *et al.* (1989). The plasmid was transformed into *Escherichia coli* DH5α (Biorad) according to the manufacturers'

recommendations. Positive clones were identified using blue/white selection on X-gal IPTG media, as described by Sambrook *et al.* (1989). Bulk preparations of plasmid were isolated and purified using the Qiagen plasmid purification kit (Qiagen), according to the manufacturers' recommendations. The vector was diluted in TE buffer (see section 2.2.4) and the DNA concentration was determined using a spectrophotometer.

2.2.6.2 Standard curves for *F. culmorum* and *F. poae* DNA

Competitive PCR relied on the construction of a standard graph from which the concentration of fungal DNA (ng) could be deduced. The *F. culmorum* standard graph was prepared as described by Hu *et al.* (1993) using the reaction conditions described above. Reactions (50µl) contained differing amounts of *F. culmorum* (strain Fu 3) DNA and 10 µl of a 0.12 ng μl^{-1} (1.2 ng) dilution of the competitor template. This resulted in two bands per reaction over the range 0.183-11 ng of *F. culmorum* DNA. Using similar conditions, the *F. poae* (strain F 731) standard curve was constructed by D. Simpson (John Innes Centre, UK). Reactions contained 10 µl of a 0.25 x 10^{-4} ng μl^{-1} dilution (0.25 pg) of the competitive template and two bands per reaction were obtained over the range 0.008-10 ng of *F. poae* DNA. Amplification products were separated by electrophoresis through a 2% (w/v) agarose gel (Gibco BRL) prepared as described earlier (Chapter 1, section 1.2.4). Gels were analysed using the Gel Doc Molecular Analyst programme (Biorad Laboratories Ltd., UK). Densitometric analysis permitted the PCR product ratios to be calculated (ratio between the densitometric units obtained for the amount of fungal product amplified and the amount of competitor product amplified) for the standard curves. The standard curves related the log PCR product

ratios to log ng fungal DNA and were based on the average results obtained from 3 replicates of each reaction.

2.2.6.3 Quantitative *F. culmorum* and *F. poae*-specific PCR analysis

Samples which were found to contain either *F. culmorum* or *F. poae* by conventional PCR were then subjected to quantitative PCR analysis. Quantitative PCR reactions contained 10 µl of either a 0.12 ng μl^{-1} dilution (1.2 ng) of the *F. culmorum* competitor template, or 10 µl of a 0.25 x 10^{-4} ng μl^{-1} dilution (0.25 pg) of the *F. poae* competitor template, and 10 µl of an appropriate dilution of DNA from the plant sample. Each quantitative PCR assay included four control reactions: one reaction contained 10 µl of the relevant competitor template dilution and 10 µl of the appropriate fungal genomic DNA template. The second control contained 10 µl of the competitor template dilution alone, and the third contained the appropriate fungal genomic DNA template alone. The fourth control contained water rather than DNA, i.e. negative PCR control. The other quantitative PCR reaction components (except primers) were as described above for PCR analysis (section 2.2.5), The primer sets used were specific for either *F. culmorum/F. graminearum* DNA and *F.culmorum* competitor template DNA (Cu17F/R) or *F. poae* DNA and *F. poae* competitor template (P82F/R) and the programme used to amplify DNA was as described for PCR analysis using primers C51F/R (section 2.2.5). The resulting PCR product ratio (ratio of fungal product to competitor template product) was converted to ng fungal DNA mg^{-1} plant material using the standard curve for *F. culmorum* or *F. poae* DNA. Each quantitative PCR result was based on three replicates of each reaction.

2.2.7 Statistical analysis

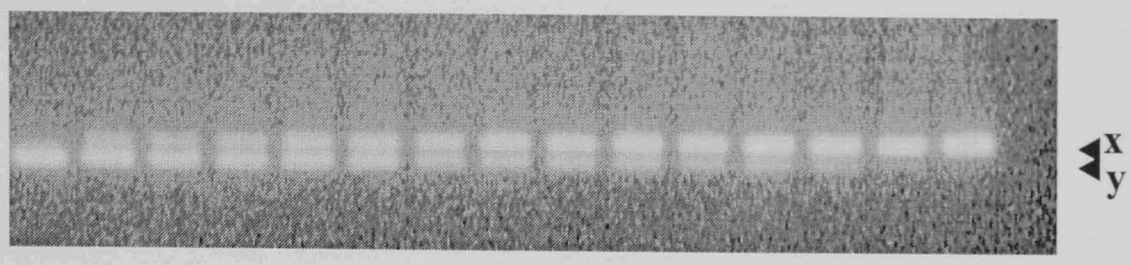
The square roots of quantitative PCR data and yield data (both number of grains per ear and 1000 grain weight) were used for statistical analysis in order to approximate normal distribution of the data (Snedecor & Cochran, 1980). Such transformations were unnecessary for the visual disease assessment data. General linear model analysis was used to assess the variability among replicate PCR reactions. Statistical analysis of treatment differences based on visual disease assessment, quantitative PCR and yield data consisted of one way analysis of variance (ANOVA) incorporating Tukeys` pairwise comparison test (at a 5 % level of significance) (Snedecor & Cochran, 1980). Pearson`s product moment correlation was used to investigate the linearity of the relationship between visual disease assessment and quantitative PCR analysis (Snedecor & Cochran, 1980). All analysis was performed using Minitab release 10.1© (1994, Minitab incorporated).

2.3 RESULTS

2.3.1 Standard curves

A standard curve for *F. culmorum* (Fig. 2.1a and b) was generated which enabled conversion of the PCR product ratios (ratios between the amount of fungal product and the amount of competitor product) obtained in quantitative PCR to ng fungal DNA. Fig. 2.1a shows the PCR products obtained when a range of *F. culmorum* DNA concentrations (0.183-11 ng) were amplified in the presence of a constant amount of competitor template. Following amplification of fungal DNA or competitor template alone, only a single product was observed in agarose gels. The product from the *F. culmorum* DNA was 350 bp, while that of the competitor template was smaller by 50 bp (Fig. 2.1a, lanes 1 and 15, respectively). In reactions which contained both *F. culmorum* DNA (0.183-11 ng) and competitor template, two products were detected in agarose gels (Fig. 2.1a, lanes 2 to 14). PCR product ratios obtained were plotted against the amount of fungal DNA (Fig. 2.1b) and the equations of the curve ($y = 1.35x + 0.43$, $R^2 = 0.96$) enabled conversion of the PCR product ratios to ng *F. culmorum* DNA. A similar standard curve, functional over the range 0.008-10 ng fungal DNA, was generated for *F. poae* by D. Simpson (John Innes Centre, UK). In the case of *F. poae*, the product of the fungal DNA was 220 bp, while that of the competitor template was larger by 280 bp. The equation of the *F. poae* standard curve was $y = 0.6559x + 0.1344$ ($R^2 = 0.99$).

a



b

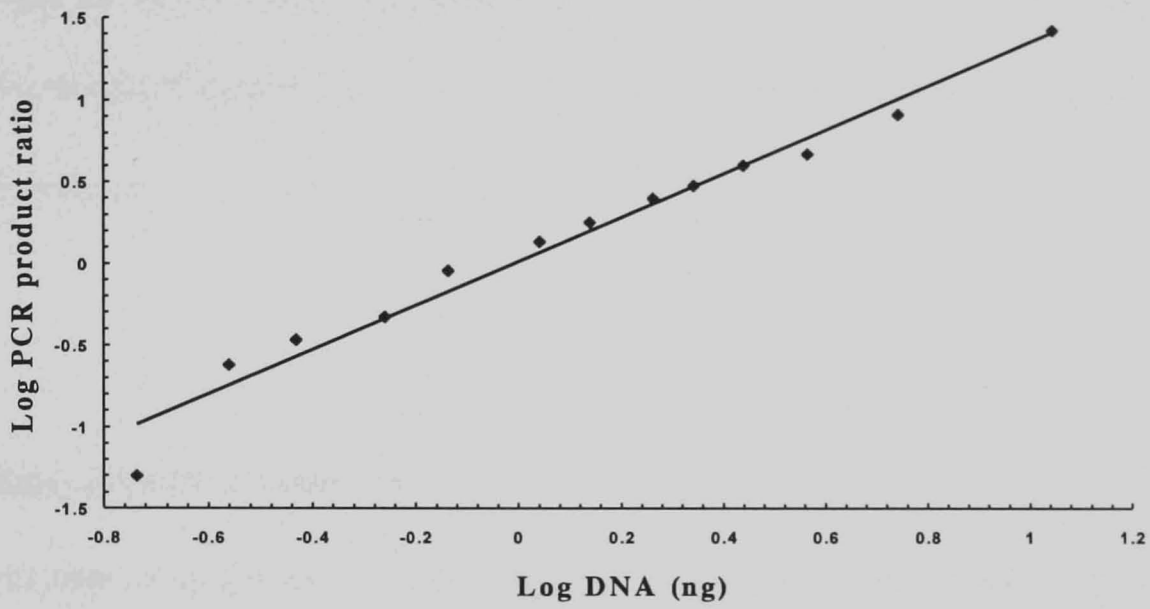


Fig. 2.1 Relationship between the amount of *Fusarium culmorum* (strain Fu 3) DNA and the resulting PCR product ratio. PCR products were a, visualised by UV trans-illumination following gel electrophoresis and b, PCR product ratios were determined by densitometry and related to the amount of *F. culmorum* DNA. Lanes: 1-14, reactions contained constant amount of competitor template (1.2 ng) plus varying amounts of *F. culmorum* genomic DNA (lanes 1-14 contained 0.000, 0.183, 0.275, 0.370, 0.550, 0.730, 1.100, 1.375, 1.830, 2.200, 2.750, 3.667, 5.50 and 11.0 ng, respectively); 15, *F. culmorum* genomic DNA (11 ng); c, control reaction without fungal DNA or competitor template. PCR product ratios on graph b represent the average of three replicates of the PCR reactions illustrated in lanes 2-14 of graph a. Arrows: x, *F. culmorum*-specific DNA product (350 bp); y, competitor template-specific DNA product (300 bp).

2.3.2 Effect of fungicides on *Fusarium* ear blight of wheat

Glasshouse trials were set up to investigate the effect of fungicides and the effect of *G. roseum* culture filtrate on *F. culmorum* and *F. poae* ear blight of wheat using quantitative PCR, visual disease assessment and yield analysis. In the first trial (1994/5), the efficacy of the fungicides prochloraz and pyrimethanil against *F. culmorum* ear blight were evaluated, and the second trial (1995/6) was set up to evaluate the efficacy of prochloraz, tebuconazole and *G. roseum* culture filtrate against *F. culmorum* and *F. poae* ear blight of wheat. The third glasshouse trial (1996/7) was set up to confirm the effects of the fungicides prochloraz and tebuconazole against ear blight of wheat caused by the two species.

2.3.2.1 Visual disease assessment

The *F. culmorum* visual disease assessment results obtained for the 1994/5, 1995/6 and 1996/7 trials are illustrated graphically in Figs. 2.2, 2.3 and 2.4, respectively. Treatments are detailed in Table 2.3. In all three years, there was a general increase in both the *F. culmorum* and *F. poae* disease scores of all inoculated treatments from GS 70 to 75, and from GS 75 to 80. *F. culmorum* disease severity was higher in both 1994/5 and 1996/7 than in 1995/6, mean GS 80 disease scores for inoculated untreated plants being 99, 100 & 87 %, respectively. The *F. poae* GS 80 disease scores were also higher in 1996/7 than 1995/6, mean disease scores for *F. poae*-inoculated untreated plants being 77 & 44 %, respectively. As is evident from Figs. 2.3c and 2.4c, in both 1995/6 and 1996/7, *F. culmorum*-inoculation resulted in higher disease severity than inoculation with *F. poae*. For example, in 1995/6 the mean disease scores at GS 80 for inoculated untreated plants

were *F. culmorum,* 86.7 % and *F. poae,* 44.4%.

The 1994/5 results showed that, at each disease scoring, the prochloraz-treated samples had significantly less disease than both the pyrimethanil-treated samples and inoculated samples untreated with fungicides (Fig. 2.2). Statistical analysis of visual disease assessment results (GS 70, 75 & 80) (Appendix 2.1) showed that, in 1994/5, pyrimethanil had no significant effect on *F. culmorum* disease. According to the final disease scores (GS 80), treatment with prochloraz had resulted in a 22 % reduction in disease severity compared to inoculated untreated plants. Analysis of both the 1995/6 and 1996/7 results (Appendix 2.1.2) showed that both the prochloraz and tebuconazole treatments significantly decreased the *F. culmorum* disease scores (GS 70, 75 and 80), compared to the *F. culmorum*-inoculated untreated plants (Fig. 2.3 & 2.4). In the 1995/6 experiment, prochloraz treatment resulted in a 51% reduction in mean disease score (GS 80) and tebuconazole treatment in a 49% reduction. The 1996/7 results showed that at GS 80, prochloraz and tebuconazole treatments had resulted in a 26 % and 27 % reduction in mean disease score, respectively. In both experiments, there was no significant difference between the efficacy of the fungicides prochloraz and tebuconazole against *F. culmorum* ear blight of wheat.

Visual disease assessment results for *F. poae* for the 1995/6 and 1996/7 trials are depicted in Figs. 2.3 & 2.4 and treatments are explained in Table 2.3. In the 1995/6 experiment, treatment with *G. roseum* culture filtrate did not decrease *F. culmorum* disease severity at GS 70, 75 or 80. Instead, it resulted in a significant increase in disease compared to inoculated untreated plants. Statistical analysis (Appendix 2.1.2 & 2.1.3) showed that in both years, prochloraz and tebuconazole treatments resulted in significant decreases in *F. poae* disease scores (GS 70, 75 & 80) when compared to *F. poae*-

inoculated plants untreated with fungicides. Based on the final disease scores (GS 80), both the 1995/6 and 1996/7 results suggested that there was no significant difference between the efficacy of the two fungicides against *F. poae*. In 1995/6, prochloraz and tebuconazole treatments resulted in a 41 % and 51 % reduction in mean disease score, respectively, and in 1996/7 the respective reduction in mean disease score due to these treatments were 82 % and a 73 %, when compared to inoculated untreated plants. In the 1995/6 trial, treatment with *G. roseum* culture filtrate did not significantly affect *F. poae* disease scores at GS 70, 75 or 80, compared to inoculated untreated plants.

2.3.2.2 PCR analysis

PCR analysis detected *F. culmorum* in all inoculated samples, both fungicide treated and untreated. However, no *F. culmorum* was detected in the uninoculated samples. These results agreed with those obtained using visual disease assessment.

Quantitative PCR was employed to assess the effect of the fungicides on fungal DNA as a measure of fungal biomass. The quantitative PCR results obtained for *F. culmorum* in 1994/5, 1995/6 & 1996/7 are shown in Figs. 2.5, 2.6 and 2.7, respectively. An example of *F. culmorum* quantitative PCR analysis of DNA from untreated plants which were inoculated with *F. culmorum* is shown in Fig 2.8a. Statistical analysis of the quantitative PCR results (Appendix 2.2) showed that, in the 1994/5 glasshouse trial, pyrimethanil had no significant effect on *F. culmorum* ear blight, compared to inoculated untreated plants (Fig. 2.5). Also, in the 1995/6 experiment, treatment of *F. culmorum*-inoculated samples with *G. roseum* culture filtrate did not result in a decrease, but in a significant increase in DNA content, compared with inoculated untreated plants.

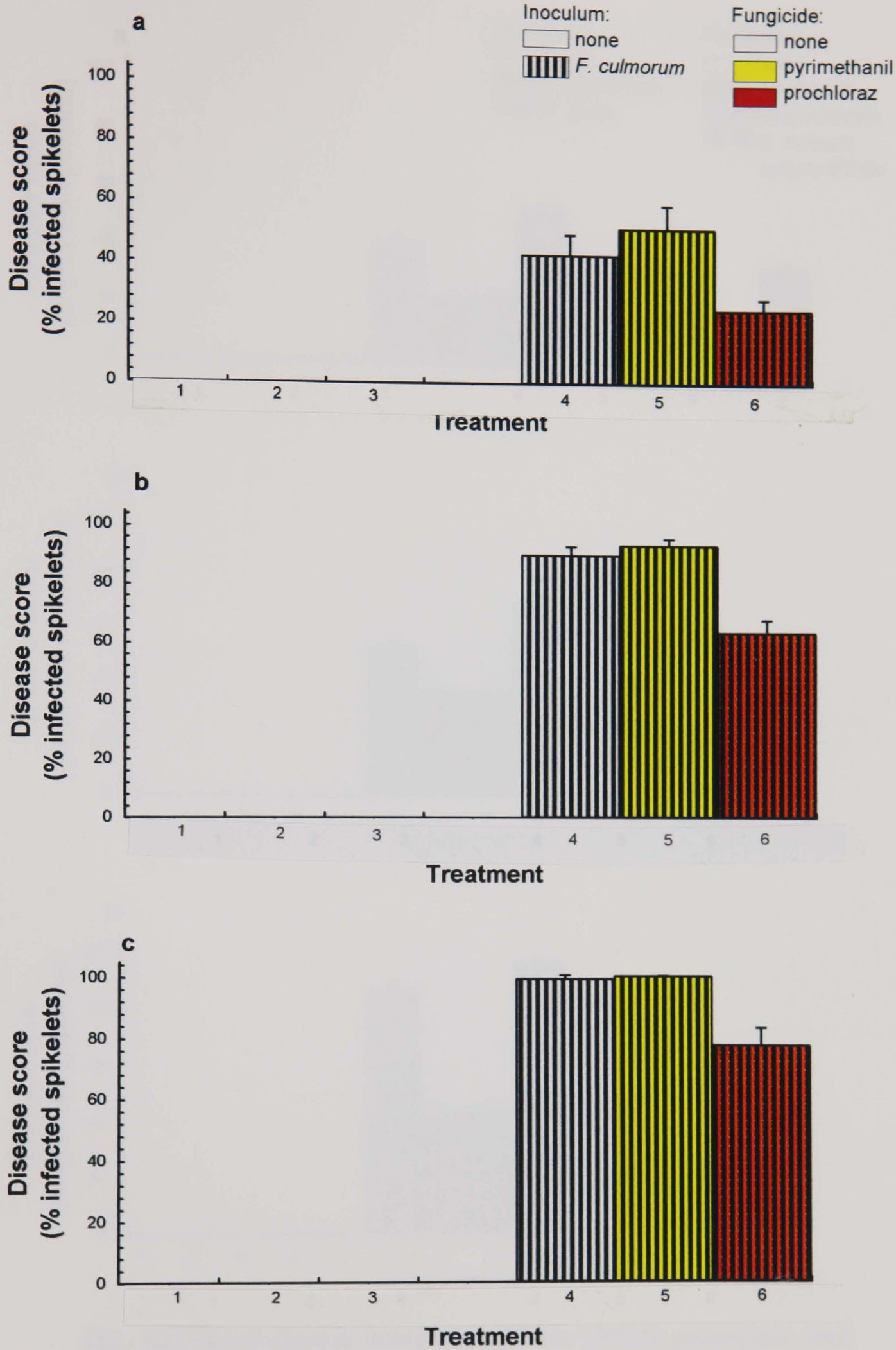


Fig. 2.2 Visual disease assessments of the 1994/5 glasshouse trial to evaluate the efficacy of the fungicides prochloraz and pyrimethanil against *Fusarium culmorum* ear blight of wheat (cv. Avalon). Disease scored at: a, GS 70; b, GS 75; c, GS 80. Bars indicate standard error of the means (S.E.M.). For statistical analysis see Appendix 2.1.1.

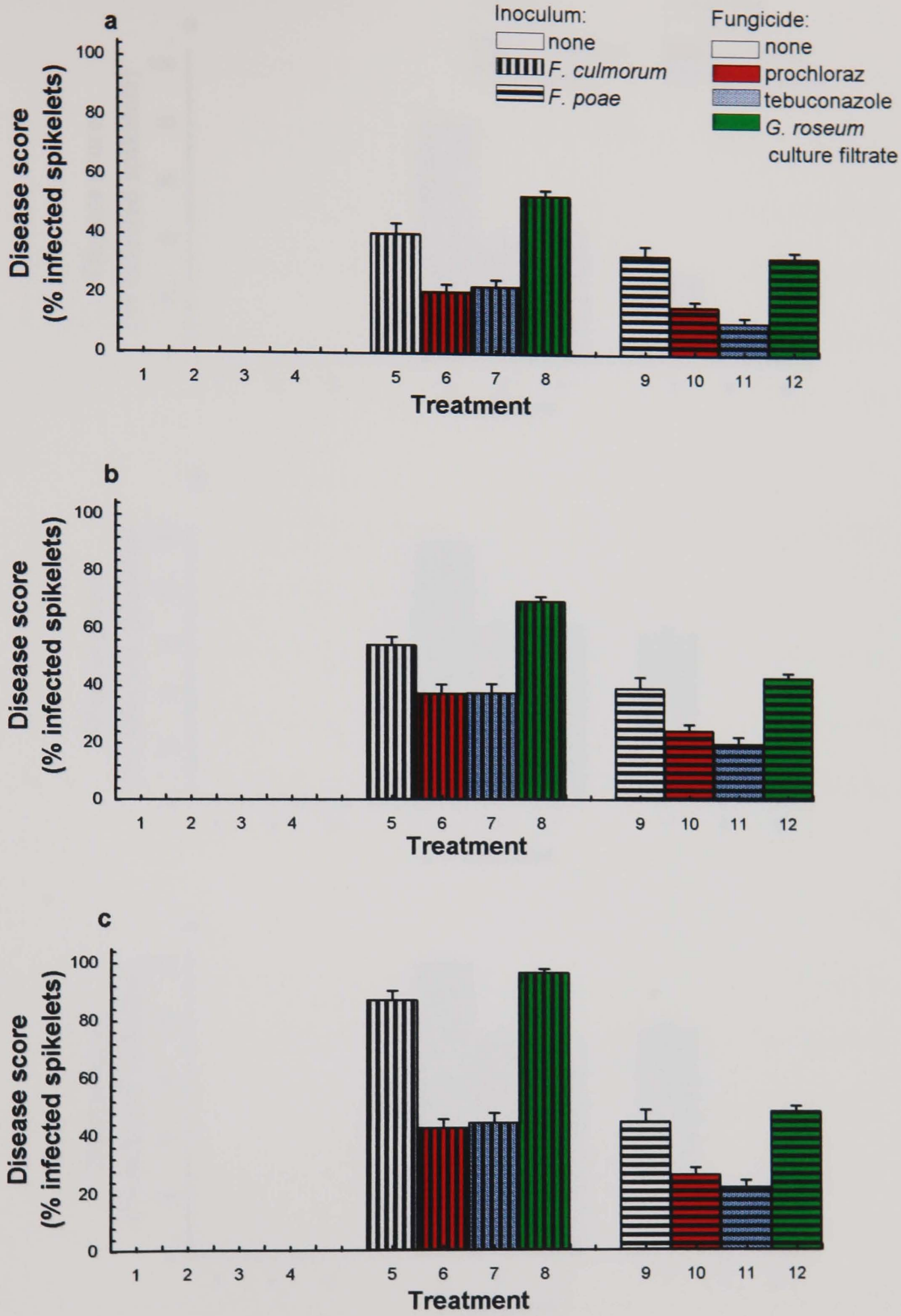


Fig. 2.3 Visual disease assessment of the 1995/6 glasshouse trial to evaluate the efficacy of the fungicides prochloraz and tebuconazole and the efficacy of *Gliocladium roseum* culture filtrate against *Fusarium culmorum* and *F. poae* ear blight of wheat (cv. Avalon). Disease scored at: a, GS 70; b, GS 75; c, GS 80. Bars indicate standard error of the means (S.E.M.). For statistical analysis see Appendix 2.1.2.

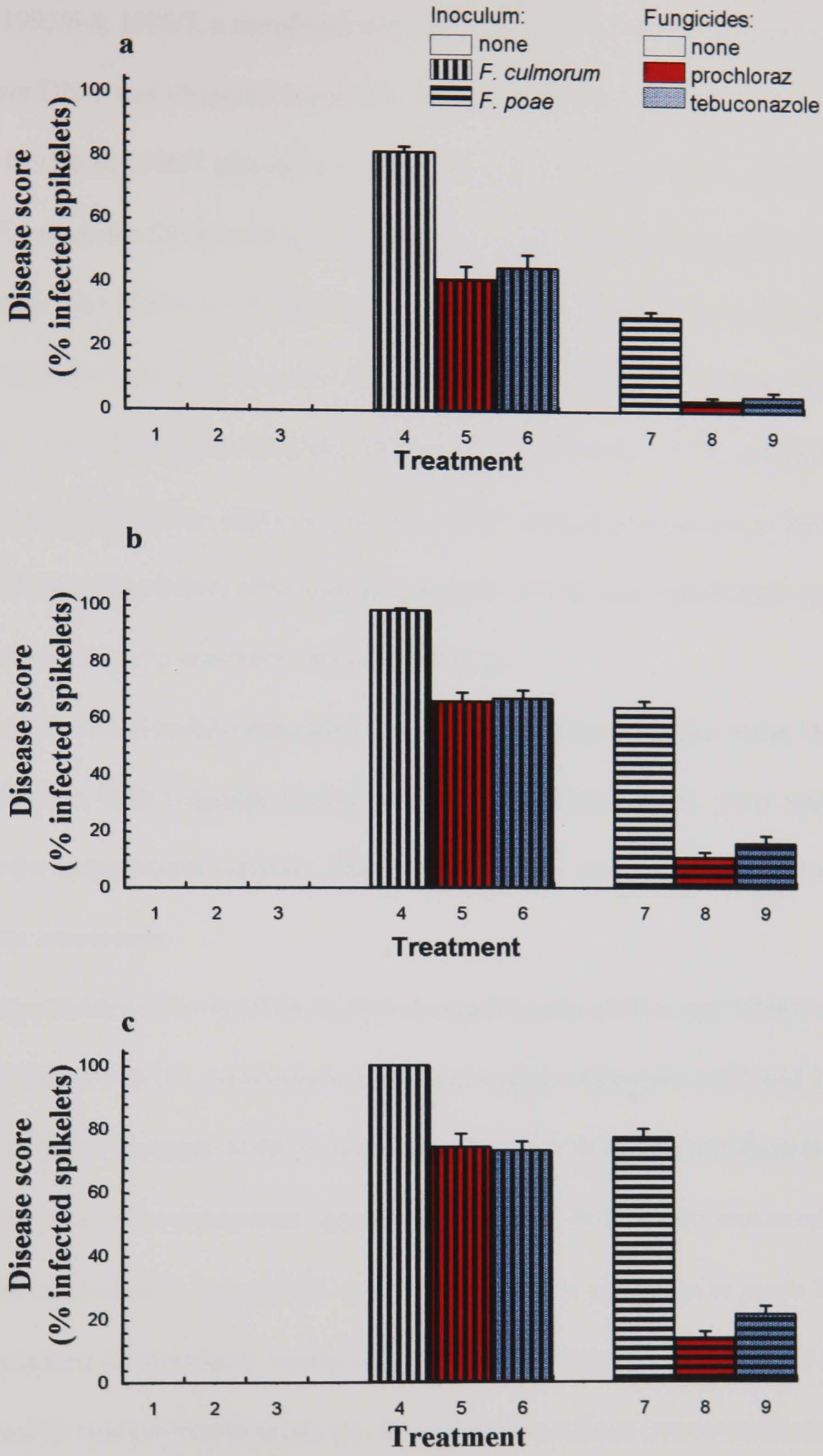


Fig. 2.4 Visual disease assessment of the 1996/7 glasshouse trial to evaluate the efficacy of the fungicides prochloraz and tebuconazole against *Fusarium culmorum* and *F. poae* ear blight of wheat (cv. Avalon). Disease scored at: a, GS 70; b, GS 75; c, GS 80. Bars indicates standard error of the means (S.E.M.). For statistical analysis see Appendix 2.1.3.

In 1994/5, 1995/6 & 1996/7, a significant decrease of 26, 67 & 49 % in the amount of *F. culmorum* DNA was observed in prochloraz treated samples (Figs. 2.5, 2.6 & 2.7) and in the 1995/6 & 1996/7 glasshouse trial, tebuconazole treatment also significantly decreased *F. culmorum* DNA content, when compared with inoculated plants untreated with fungicides. The 1995/6 results showed no significant difference between prochloraz and tebuconazole treatments in terms of *F. culmorum* DNA content, both treatments resulting in a 67% decrease in the mean amount of *F. culmorum* DNA, compared to inoculated untreated plants. However, in the 1996/7 trial, the reduction in mean *F. culmorum* DNA content due to tebuconazole treatment (69 %) was significantly greater than the reduction due to prochloraz treatment (49 %).

Qualitative PCR analysis detected *F. poae* in all inoculated samples in the 1995/6 and 1996/7 trials, both fungicide treated and untreated. However, *F. poae* was not detected in the uninoculated samples. These results agreed with those obtained using visual disease assessment.

The quantitative *F. poae* PCR results obtained for the 1995/6 and 1996/7 trials are shown in Figs. 2.6 & 2.7, respectively (statistical analysis Appendix 2.2.2 and 2.2.3, respectively), and an example of the *F. poae* quantitative PCR results are illustrated in Fig. 2.8b. In the 1995/6 experiment, quantitative *F. poae* PCR results indicated that tebuconazole decreased *F. poae* DNA content most (84 % reduction in mean DNA content, compared to inoculated untreated plants), and, while DNA content of both prochloraz and *G. roseum*-treated plants was lower than inoculated untreated plants (54 and 51 % reduction, respectively), the differences were not statistically significant. Also, the difference between the prochloraz and tebuconazole treatments and between the prochloraz and *G. roseum* culture filtrate treatments were not statistically significant,

although there was a significant difference between the tebuconazole and *G. roseum* culture filtrate treatments. In the 1996/7 experiment, both prochloraz and tebuconazole treatments significantly reduced *F. poae* DNA content, compared to inoculated untreated plants. In this experiment the reduction due to prochloraz treatment (66 %) was significantly greater than the reduction due to tebuconazole treatment (43 %).

The correlation between visual disease assessment and quantitative PCR analysis was investigated, as measured by Pearson`s product moment correlation coefficient. In both 1995/6 and 1996/7, the correlation between visual disease assessment and quantitative PCR was closer for *F. culmorum* than for *F. poae* ear blight of wheat (Table 2.4). The highest correlation coefficient was obtained for *F. culmorum* 1995/6 GS 80 results (r = 0.88). When compared to *F. poae*, the closeness of the linear relationship between *F. culmorum* quantitative PCR and visual disease assessment appeared more dependent on the growth stage at which disease symptoms were assessed. This was particularly evident in 1994/5, when the correlation coefficients varied from 0.37 at GS 70 to -0.17 at GS 75 and to 0.61 at GS 80 (Table 2.4).

2.3.2.3 Yield assessment

Yield was assessed by measurement of the number of grains/ear and 1000 grain weight (Tables 2.5, 2.6 and 2.7 for 1994/5, 1995/6 & 1996/7 results, respectively). Statistical analysis (Appendix 2.3) showed that in the 1994/5 & 1996/7 trial, inoculation with *F. culmorum* did not significantly affect the number of grains/ear, when compared to uninoculated untreated plants. However, analysis of the 1995/6 glasshouse trial showed that in this experiment *F. culmorum* inoculation significantly decreased the number of

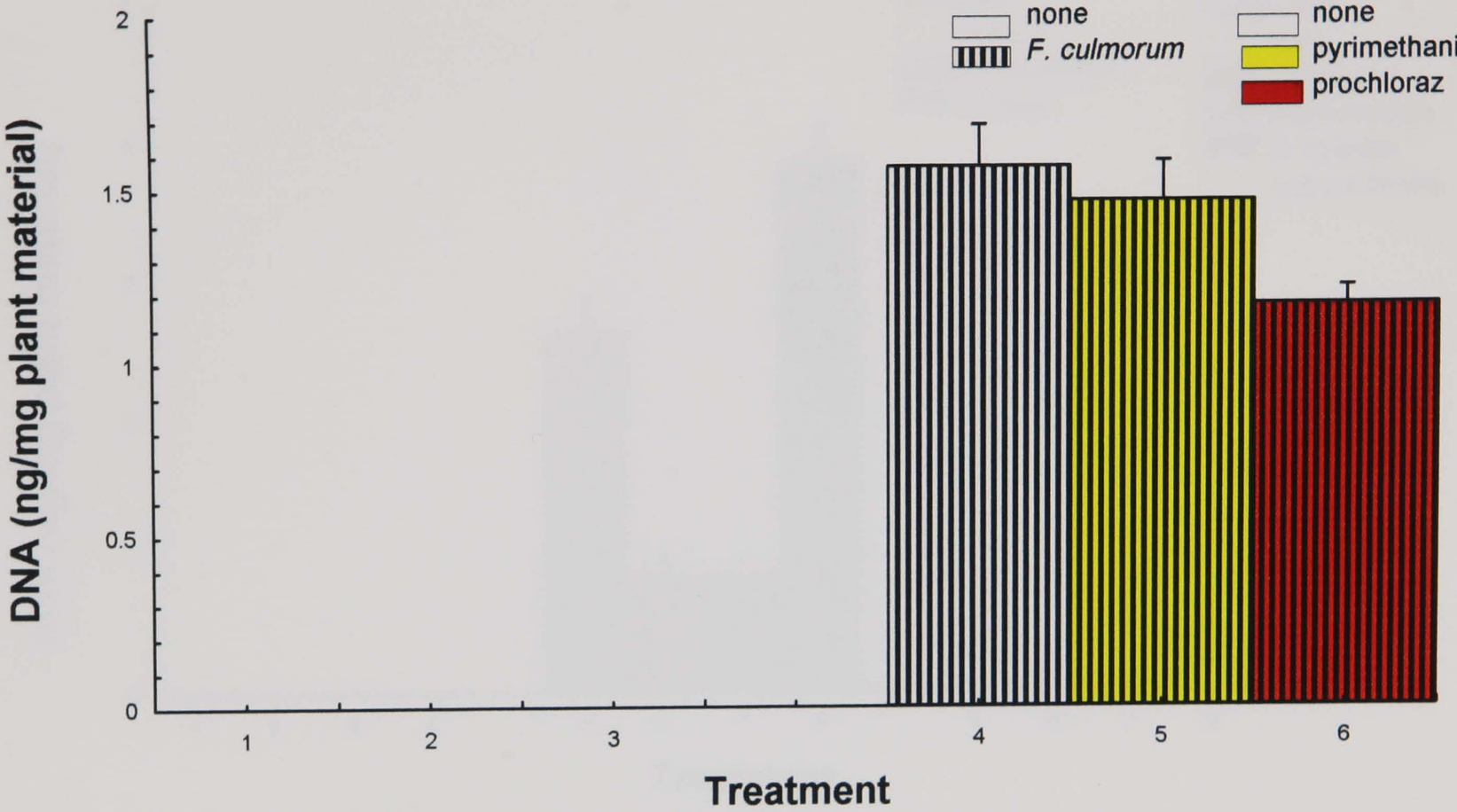


Fig. 2.5 Quantitative PCR analysis of the 1994/5 glasshouse trial to evaluate the efficacy of the fungicides prochloraz and pyrimethanil against *Fusarium culmorum* ear blight of wheat (cv. Avalon). Bars indicate standard error of the means (S.E.M.). For statistical analysis see Appendix 2.2.1.

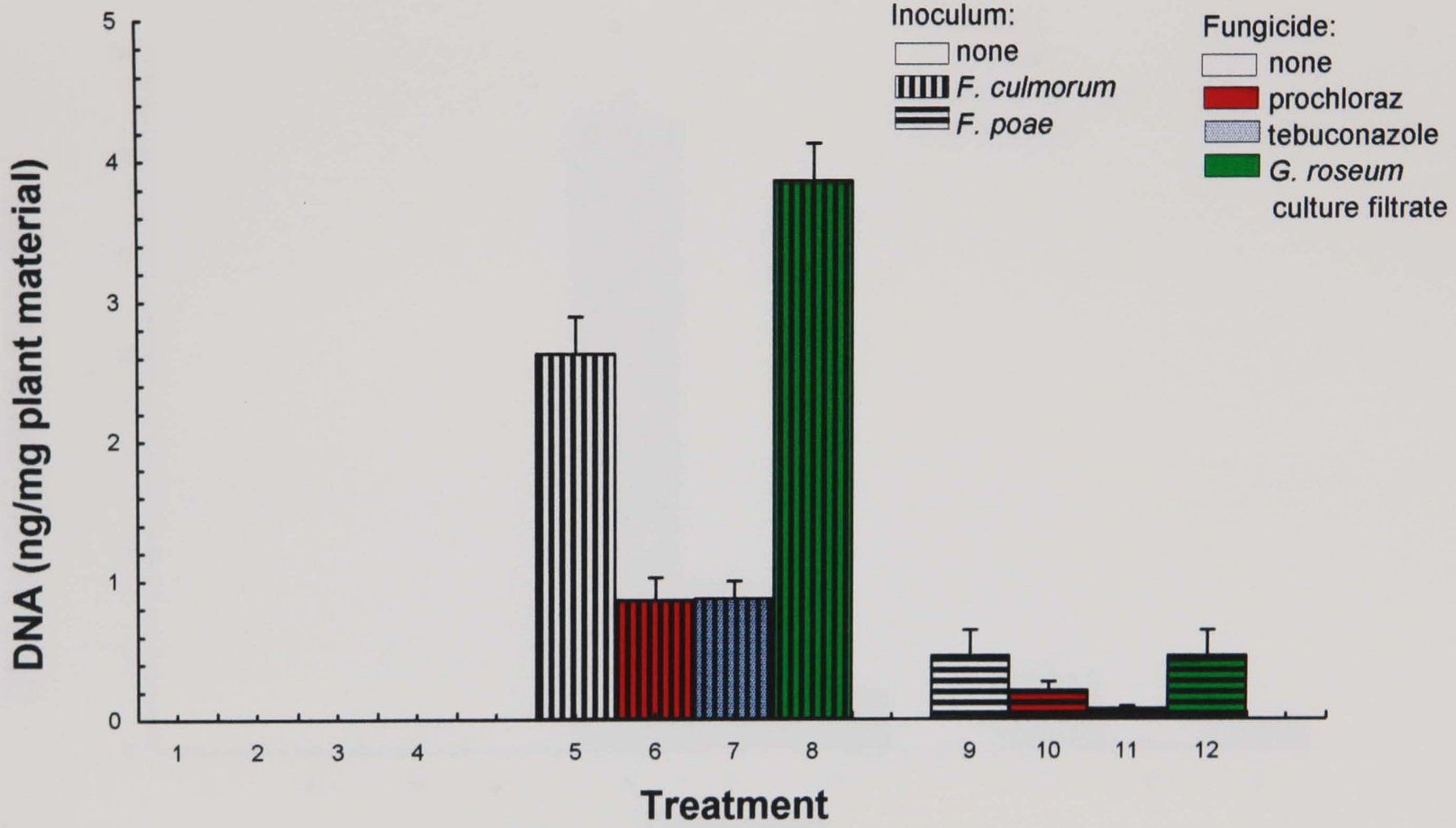


Fig. 2.6 Quantitative PCR analysis of the 1995/6 glasshouse trial to evaluate the efficacy of the fungicides prochloraz and tebuconazole and the efficacy of *Gliocladium roseum* culture filtrate against *Fusarium culmorum* and *F. poae* ear blight of wheat (cv. Avalon). Bars indicate standard error of the means (S.E.M.). For statistical analysis see Appendix 2.2.2.

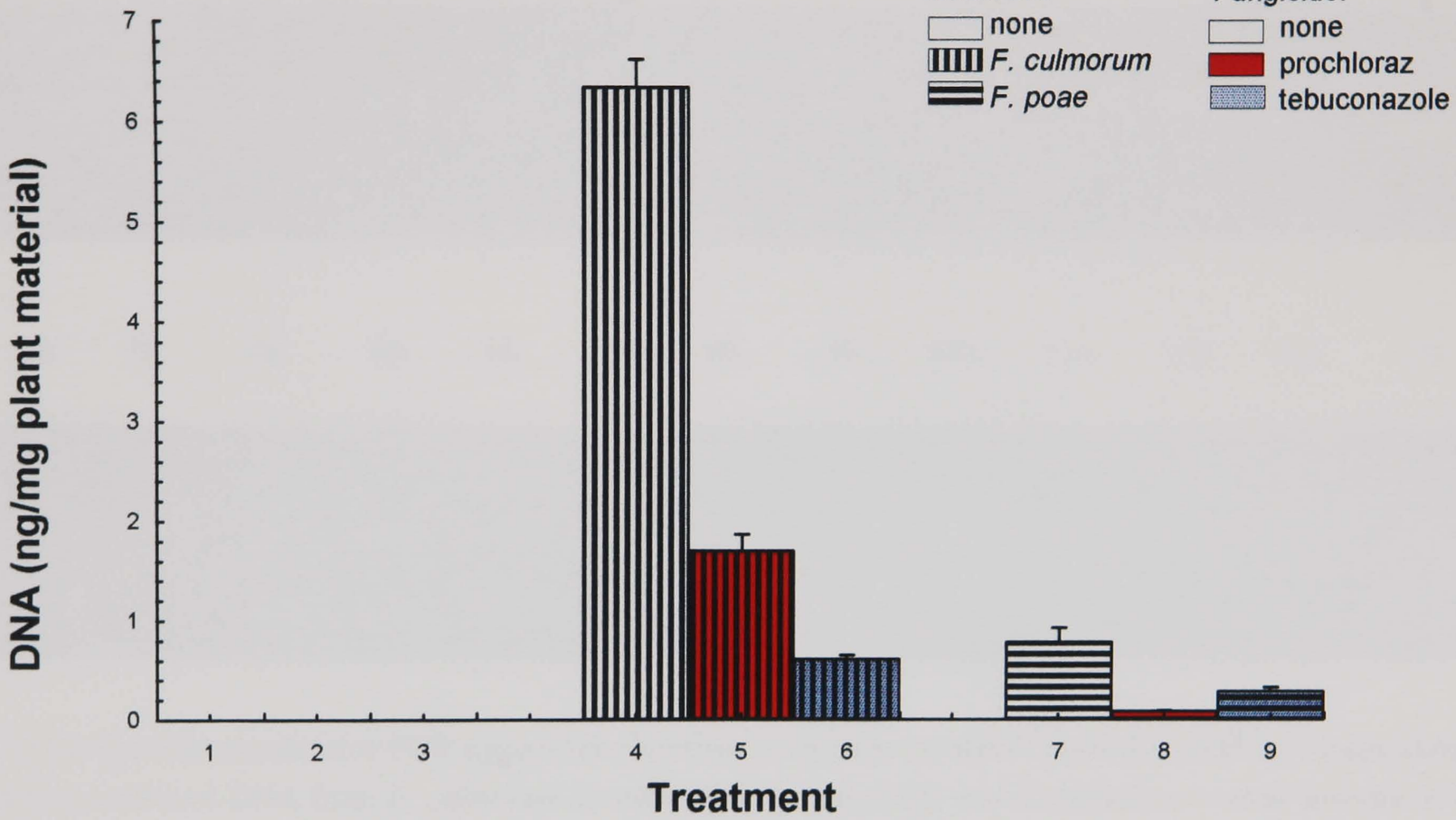


Fig. 2.7 Quantitative PCR analysis of the 1996/7 glasshouse trial to evaluate the efficacy of the fungicides prochloraz and tebuconazole against *Fusarium culmorum* and *F. poae* ear blight of wheat (cv. Avalon). Bars indicate standard error of the means (S.E.M.). For statistical analysis see Appendix 2.2.3.

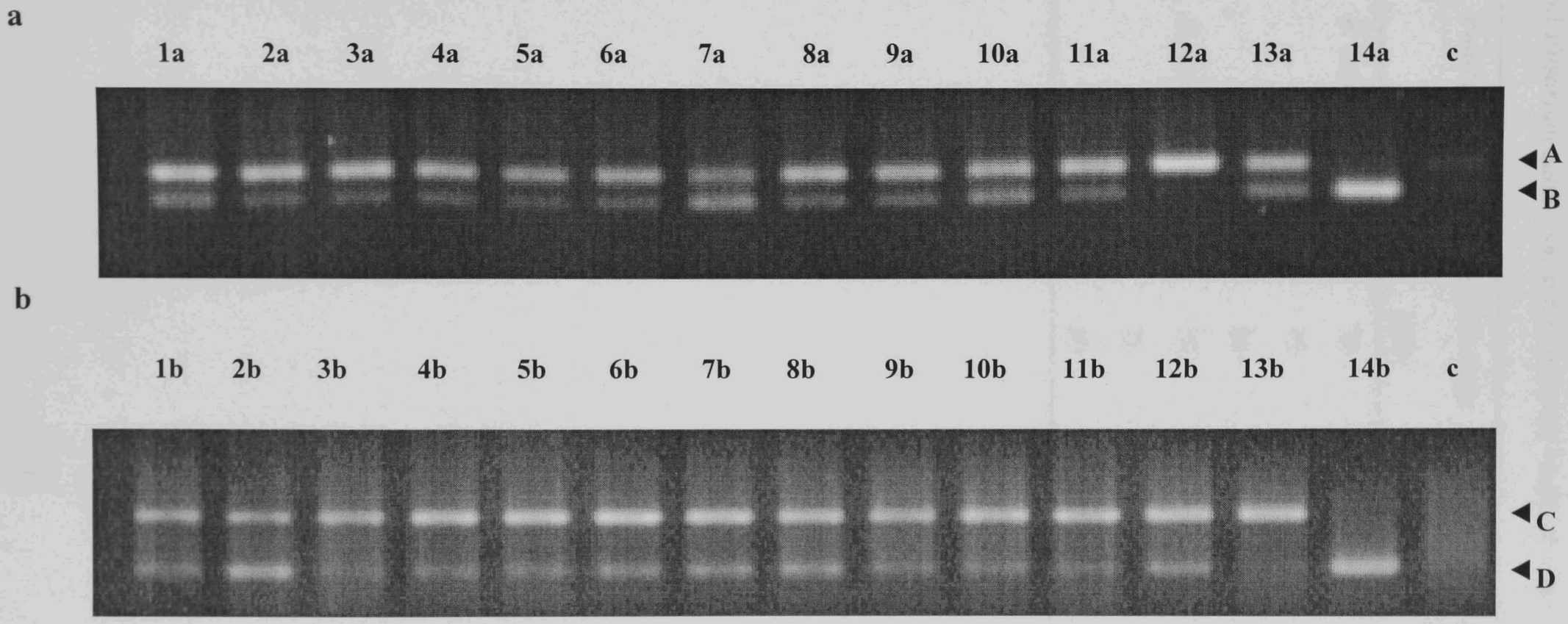


Fig. 2.8 Detection of the specific quantitative PCR signals for *Fusarium culmorum* (a) and *F. poae* (b) in DNA extracts from infected wheat ears. Lanes: 1-11a, reactions contained DNA from *F. culmorum*-inoculated untreated plants and contained a constant amount of *F. culmorum*-specific competitor template; 12a *F. culmorum* genomic DNA; 13a, *F. culmorum* genomic DNA plus competitor template; 14a, *F. culmorum* competitor template; 1-11b, reactions contained DNA from *F. poae*-inoculated untreated plants and contained a constant amount of *F. poae*-specific competitor template; 12b, *F. poae* genomic DNA plus competitor template; 13b, *F. poae*-specific competitor template DNA; 14b, *F. poae* genomic DNA; c, controls without fungal DNA or competitor template. Arrows; A, *F. culmorum*-specific DNA product (350 bp); B, *F. culmorum* competitor template-specific DNA product (300 bp); C, *F. poae* competitor template-specific DNA product (500 bp); D, *F. poae*-specific DNA product (220 bp).

Table 2.4 Correlation between visual disease assessment and quantitative PCR analysis of *Fusarium culmorum* and *F. poae* ear blight of wheat.

Pathogen	Growth stage	Pearson correlation coefficient (*r*)		
	(GS)	1994/5	1995/6	1996/7
F. culmorum	70	0.37	0.74	0.69
	75	-0.17	0.76	0.80
	80	0.61	0.88	0.69
F. poae	70	-	0.51	0.60
	75	-	0.55	0.64
	80	-	0.55	0.65

grains/ear by 17 %, compared to uninoculated untreated plants (Table 2.6). In all three experiments (1994/5, 1995/6 and 1996/7) application of the various fungicides or *G. roseum* culture filtrate to uninoculated plants (Tables 2.5, 2.6 & 2.7) had no significant effect on the number of grains/ear, when compared to uninoculated untreated control plants. Also, in the 1994/5 experiment, treatment of *F. culmorum*-inoculated samples with either prochloraz or pyrimethanil had no significant effect on the number of grains/ear, when compared to inoculated untreated plants (Table 2.5), and, in 1996/7, treatment of *F. culmorum*-inoculated samples with either prochloraz or tebuconazole did not result in any significant differences in the number of grains/ear (Table 2.7). However, in the 1995/6 experiment, the number of grains/ear obtained for *F. culmorum*-inoculated and prochloraz, tebuconazole and *G. roseum* culture filtrate-treated plants was greater than the number obtained from inoculated untreated plants (30, 14 & 39 % increase in number of grains/ear, respectively), although only the prochloraz and *G. roseum* culture filtrate results were statistically significant at the 5 % level (Table 2.6).

In all three experiments (1994/5-1996/7), inoculation with *F. culmorum* resulted in a significant reduction in 1000 grain weight, when compared to uninoculated plants (Tables 2.5, 2.6 & 2.7, respectively) (Appendix 2.4). Application of pyrimethanil (1994/5) or *G. roseum* culture filtrate (1995/6) to uninoculated plants did not significantly affect the 1000 grain weight (Tables 2.5 & 2.6) and results from all three trials showed that application of prochloraz to uninoculated plants did not significantly affect the 1000 grain weight, compared to untreated uninoculated plants (Tables 2.4, 2.5 & 2.6). In 1995/6 tebuconazole treatment of uninoculated plants resulted in a 25 % reduction in 1000 grain weight, compared to uninoculated untreated control plants. But in 1996/7, tebuconazole had no significant effect on 1000 grain weight.

The results from all three experiments (1994/5, 1995/6 and 1996/7) showed that treatment of *F. culmorum* - inoculated samples with prochloraz significantly increased the 1000 grain weight, compared to the untreated inoculated plants. Tebuconazole also significantly increased the 1000 grain weight (1995/6 & 1996/7) and there was no significant difference between the effect of prochloraz and tebuconazole on 1000 grain weight. The 1994/5 experiment showed that treatment of *F. culmorum*-inoculated samples with pyrimethanil had no significant effect on 1000 grain weight and, in 1995/6, a significant reduction (26 %) rather than an increase was observed in 1000 grain weight obtained for the *G. roseum* culture filtrate treatment compared to inoculated untreated plants.

Although the results of the 1995/6 glasshouse trial showed that *F. poae* inoculation resulted in a 15 % decrease in the number of grains/ear compared to uninoculated control plants, this decrease was not statistically significant at the 5 % level, and in the 1996/7 experiment, inoculation with *F. poae* did not reduce the number of grains/ear. (Tables 2.6 & 2.7, Appendix 2.3.2 & 2.3.3). Also, while in the 1995/6 experiment treatment of *F. poae*-inoculated samples with prochloraz, tebuconazole or *G. roseum* culture filtrate resulted in significant increases in the number of grains/ear compared to inoculated untreated plants (26, 27 & 20 % increases, respectively), in the 1996/7 experiment application of the fungicides prochloraz and tebuconazole to *F. poae*-inoculated plants had no significant effect on the number of grains/ear (Tables 2.6 & 2.7, Appendix 2.3.2 & 2.3.3).

In the 1995/6 and 1996/7 glasshouse trials, although inoculation with *F. poae* resulted in a reduction in 1000 grain weight (13 % and 19 % reduction, respectively), compared to uninoculated plants, the reductions were not significant at the 5 % level.

In the 1995/6 experiment, treatment of *F. poae*-inoculated plants with prochloraz, tebuconazole or *G. roseum* culture filtrate did not significantly increase the 1000 grain weight compared to inoculated untreated plants (Table 2.6 & Appendix 2.4.2) and similar results were again obtained for prochloraz and tebuconazole in the 1996/7 experiment (Table 2.7, Appendix 2.4.3).

Significant relationships were found to exist between 1000 grain weight and disease score (GS 80) and between 1000 grain weight and the *F. culmorum* DNA content in all three experiments (Figs. 2.9, 2.10 & 2.11, respectively). A "linear" trend ($y = ax + b$) was observed between disease score and 1000 grain weight, whereas a "power" trend ($y = ax^b$) was observed between between DNA content and 1000 grain weight. When the 1000 grain weight was plotted against either the disease score (GS 80) or DNA content, the 1000 grain weight decreased as disease score or DNA content increased (R^2= 0.49 & 0.48 in 1995, R^2 = 0.72 & 0.69 in 1996 & R^2 = 0.71 & 0.83 in 1996/7, respectively). The closest relationship was observed in 1996/7 between DNA content and 1000 grain weight (R^2 = 0.83). In all three experiments, the relationship between visual disease score and 1000 grain weight was seen to improve from GS 70 [R^2 = 0.43 (1994/5), 0.50 (1995/6) & 0.59 (1996/7)] to GS 80 [R^2 = 0.49 (1994/5), 0.72 (1995/6) & 0.71 (1996/7)]. However, in the 1994/5 trial, no significant relationship was observed between disease score and 1000 grain weight based on GS 75 visual disease assessment results (R^2 = 0.02), and while in the 1995/6 trial the relationship between disease score and 1000 grain weight remained virtually unchanged from GS 70 to GS 75 (R^2 = 0.50 and 0.49, respectively), in the 1996/7 trial the relationship improved from GS 70 to GS 75 and remained virtually unchanged from GS 75 to GS 80 (R^2 = 0.59, 0.70 & 0.71, respectively). No significant relationship existed between 1000 grain

weight and number of grains/ear, between disease score and number of grains/ear or between DNA content and number of grains/ear (results not shown). When the 1000 grain weight or the number of grains/ear obtained for *F. poae*-infected samples was plotted against either the corresponding disease score (GS 70, 75 or 80) or the corresponding amount of *F. poae* DNA, no significant relationship was observed (results not shown).

Table 2.5 The effect of *Fusarium culmorum* inoculation and fungicide treatments on number of grains/ear and thousand grain weight of winter wheat (cv. Avalon) in the 1994/5 glasshouse trial.

Treatment number	Inoculum	Fungicide application	[a]No. grain/ear	[a]1000 grain weight (g)
1	None	None	59.2 ± 2.9	31.6 ± 2.3
2	None	Pyrimethanil	57.5 ± 1.9	27.8 ± 2.1
3	None	Prochloraz	60.7 ± 2.3	29.7 ± 2.2
4	*F. culmorum*	None	58.1 ± 2.5	11.0 ± 1.2
5	*F. culmorum*	Pyrimethanil	62.8 ± 2.6	10.6 ± 1.6
6	*F. culmorum*	Prochloraz	63.7 ± 3.0	15.8 ± 1.1

[a]Mean value for each ± treatment standard error of the mean (S.E.M.). For statistical analysis see Appendix 2.3.1 & 2.4.1.

Table 2.6 The effect of *Fusarium culmorum* and *F. poae* inoculation and fungicide treatments on the number of grains/ear and thousand grain weight of winter wheat (cv. Avalon) in the 1995/6 glasshouse trial.

Treatment number	Inoculum	Fungicide application	[a]No. grain/ear	[a]1000 grain weight (g)
1	None	None	63.5 ± 2.0	47.4 ± 1.1
2	None	Prochloraz	65.0 ± 1.8	43.2 ± 2.1
3	None	Tebuconazole	60.1 ± 2.4	36.1 ± 2.2
4	None	*G. roseum* culture filtrate	64.0 ± 3.3	43.8 ± 2.8
5	*F. culmorum*	None	52.8 ± 2.5	18.5 ± 1.3
6	*F. culmorum*	Prochloraz	68.6 ± 3.6	34.8 ± 1.6
7	*F. culmorum*	Tebuconazole	60.3 ± 2.7	31.3 ± 1.8
8	*F. culmorum*	*G. roseum* culture filtrate	73.2 ± 2.0	13.6 ± 0.7
9	*F. poae*	None	54.8 ± 3.9	41.9 ± 1.6
10	*F. poae*	Prochloraz	69.0 ± 2.3	42.5 ± 3.2
11	*F. poae*	Tebuconazole	69.5 ± 1.7	41.4 ± 2.8
12	*F. poae*	*G. roseum* culture filtrate	65.7 ± 3.3	45.7 ± 2.6

[a]Mean value for each ± treatment standard error of the mean (S.E.M.). For statistical analysis see Appendix 2.3.2 & 2.4.2.

Table 2.7 The effect of *Fusarium culmorum* and *F. poae* inoculation and fungicide treatments on the number of grains/ear and thousand grain weight of winter wheat (cv. Avalon) in the 1996/7 glasshouse trial.

Treatment number	Inoculum	Fungicide application	[a]No. grain/ear	[a]1000 grain weight (g)
1	None	None	64.4 ± 2.2	42.5 ± 2.9
2	None	Prochloraz	70.5 ± 2.8	35.5 ± 4.3
3	None	Tebuconazole	63.7 ± 3.1	44.8 ± 3.1
4	*F. culmorum*	None	61.0 ± 1.8	3.9 ± 0.1
5	*F. culmorum*	Prochloraz	57.0 ± 1.7	17.1 ± 2.1
6	*F. culmorum*	Tebuconazole	48.9 ± 7.3	24.9 ± 1.8
7	*F. poae*	None	68.8 ± 3.4	34.5 ± 2.5
8	*F. poae*	Prochloraz	63.7 ± 1.6	38.7 ± 2.2
9	*F. poae*	tebuconazole	67.4 ± 2.0	33.7 ± 3.7

[a]Mean value for each ± treatment standard error of the mean (S.E.M.). For statistical analysis see Appendix 2.3.3 & 2.4.3.

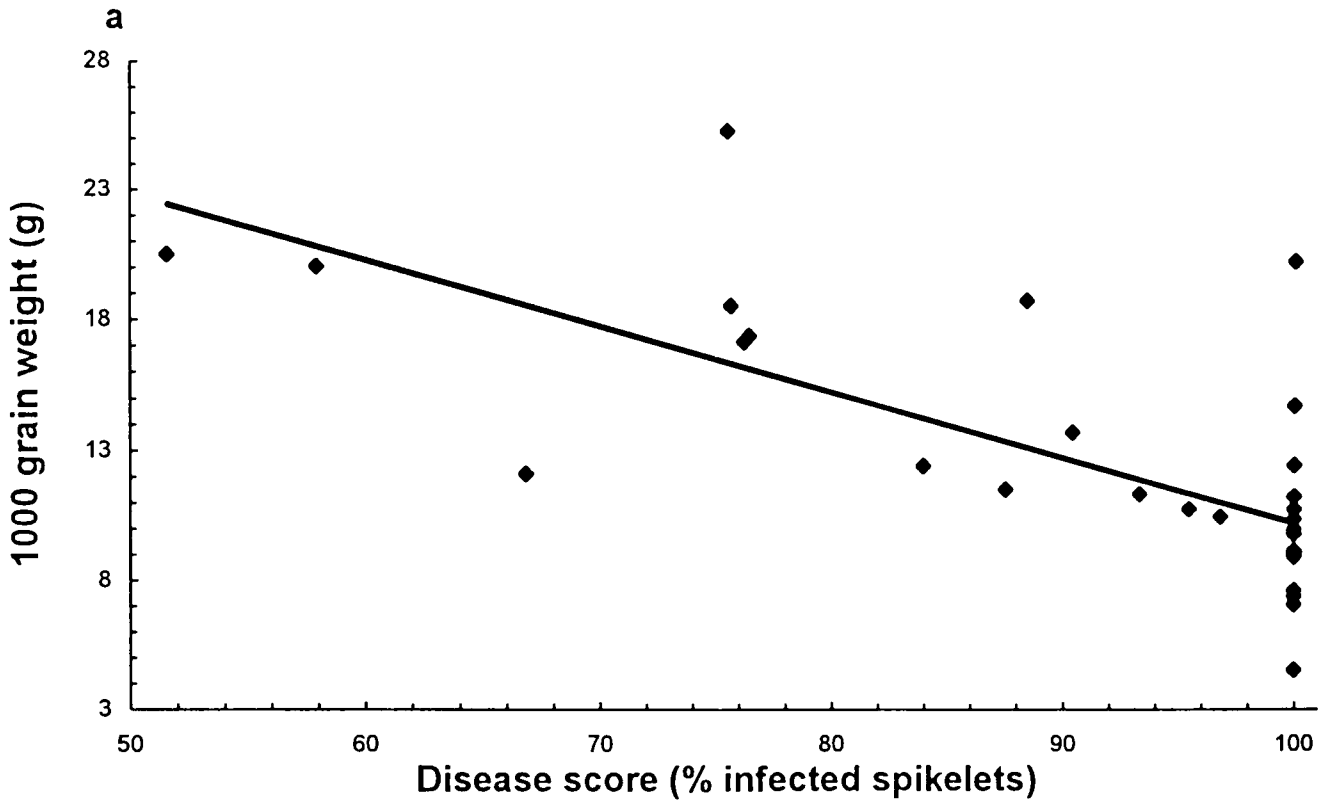
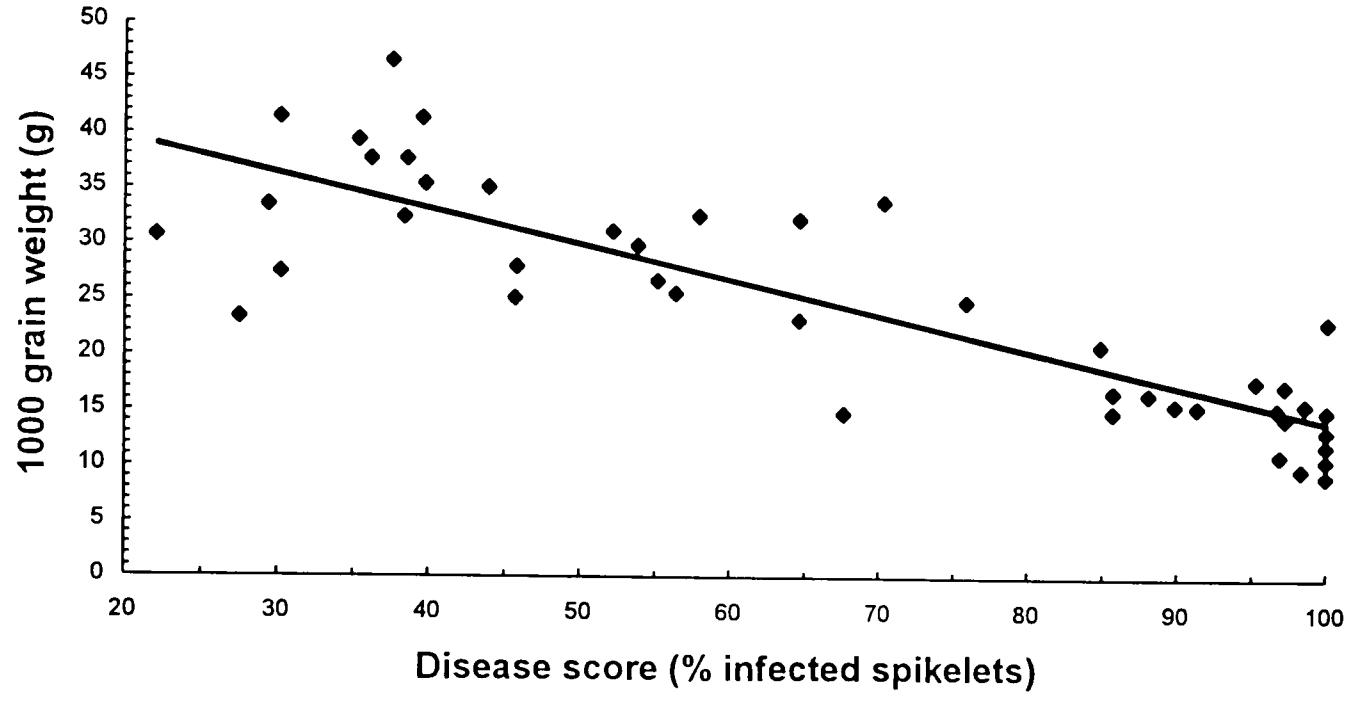


Fig. 2.9 Relationship between *Fusarium culmorum* disease and yield (1000 grain weight) for infected plant samples from 1994/5 glasshouse trial. Disease based on: a, visual disease assessment at GS 80 ($R^2 = 0.49$, $y = -0.2509x + 35.408$); b, quantitative PCR analysis ($R^2 = 0.48$, $y = 15.74x^{-0.98}$).

a



b

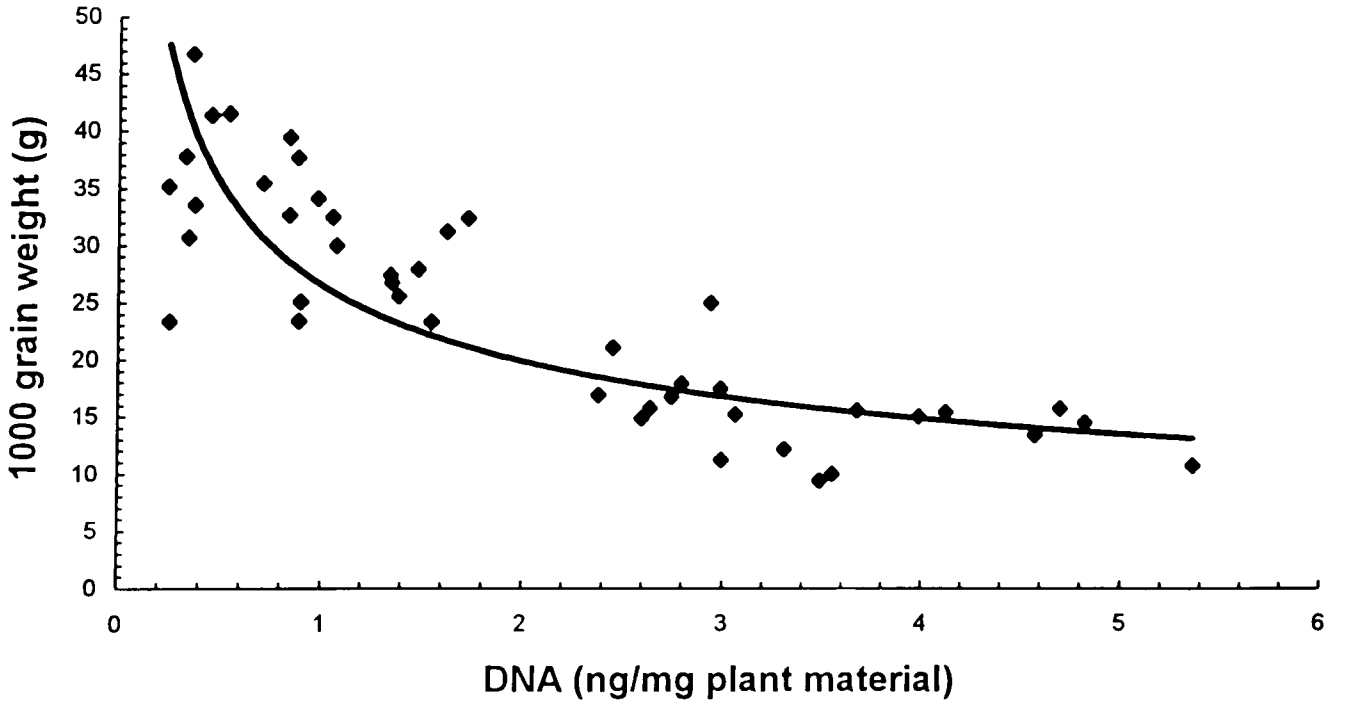


Fig. 2.10 Relationship between *Fusarium culmorum* disease and yield (1000 grain weight) for infected plant samples from 1995/6 glasshouse trial. Disease based on: a, visual disease assessment at GS 80 ($R^2 = 0.72$, $y = -0.32 + 45.88$); b, quantitative PCR analysis ($R^2 = 0.69$, $y = 26.65x^{-0.42}$).

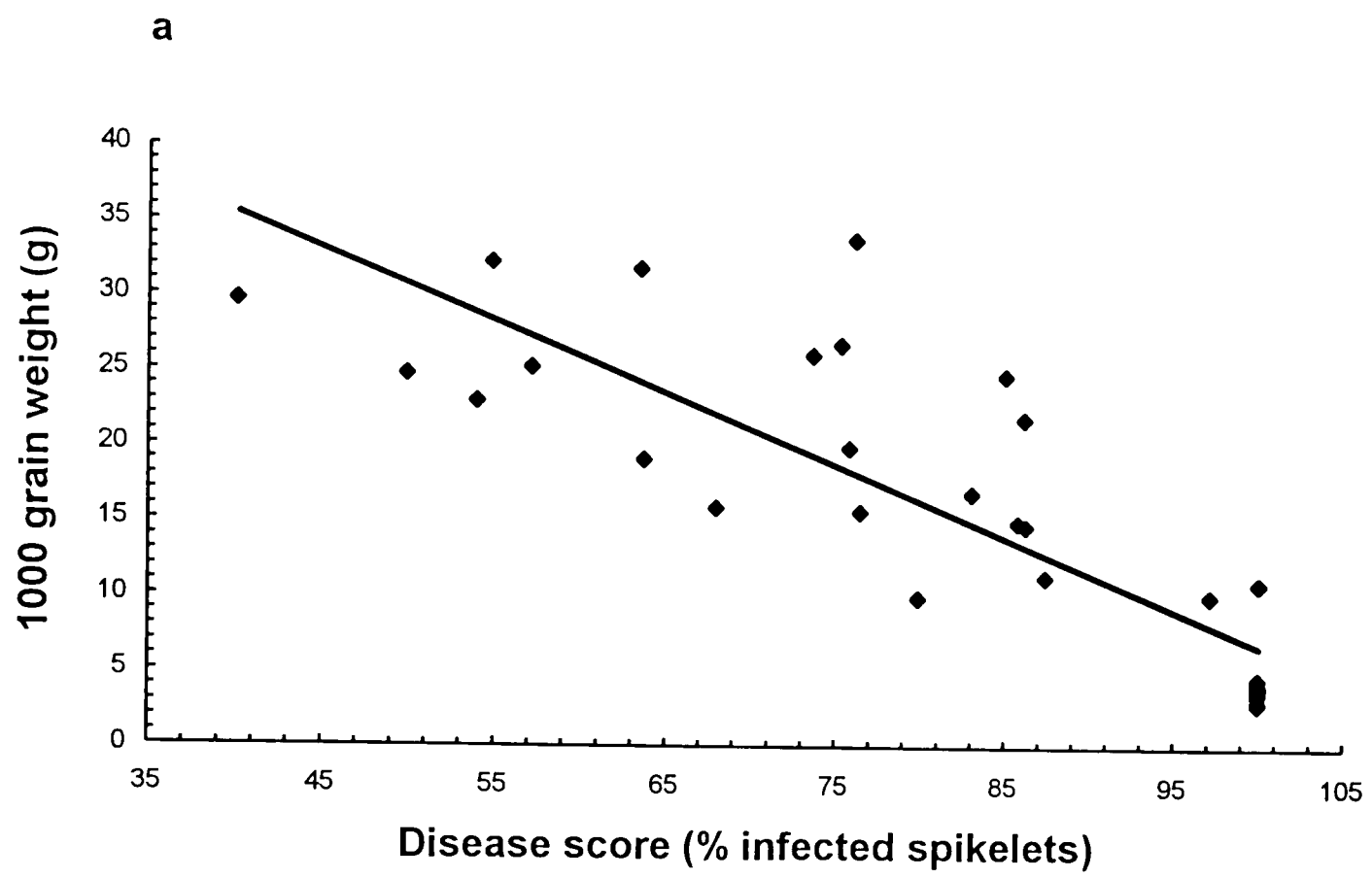


Fig. 2.11 Relationship between *Fusarium culmorum* disease and yield (1000 grain weight) for infected plant samples from 1996/7 glasshouse trial. Disease based on: a, visual disease assessment at GS 80 ($R^2 = 0.71$, $y = -0.48 + 54.76$); b, quantitative PCR analysis ($R^2 = 0.83$, $y = 17.22x^{-0.71}$).

2.4 DISCUSSION

Many *Fusarium* species have been implicated in FEB of wheat but *F. culmorum* and *F. poae* are among the *Fusarium* species that tend to predominate in the cooler maritime regions of Northwest Europe (Parry *et al.*, 1995a). Both *F. poae* and *F. culmorum* have the potential to produce mycotoxins and the recent report of the mycotoxin 3,15-diacetyl deoxynivalenol (3,15-diacetyl DON) as a natural contaminant in Canadian (Manitoba) barley (Usleber *et al.*, 1996) highlights the necessity for a greater contribution towards the understanding of the efficacy of both chemical and biological control agents against *Fusarium* species and of their effect on mycotoxin production.

Glasshouse experiments were carried out over three years to examine the efficacy of various fungicidal treatments against *Fusarium* ear blight of wheat caused by *F. culmorum* and *F. poae*. Both conventional visual disease assessment and quantitative PCR analysis were used to evaluate the results from the glasshouse experiments. Many of the visual disease symptoms detected for the two pathogens were similar to those described previously (Parry *et al.*, 1995a). The *F. culmorum* ear blight symptoms observed included premature bleaching of spikelets and in severe cases, mycelial growth was visible, emerging from spikelets (see General Introduction, Fig. I.1a). As time progressed, this premature bleaching of spikelets was difficult to distinguish from bleaching due to senescence of plant tissue. In severe cases, grains were characteristically smaller than normal, shrivelled and pale pink in colour. Such grains were described as 'tombstone' kernels by Abramson *et al.* (1987) who observed similar symptoms for FEB of wheat in Canada. In the glasshouse experiments, *F. poae* ear blight symptoms were distinctly different from those caused by *F. culmorum* and were similar to those described by Polley *et al.* (1991) for *F. poae* ear blight, lesions having

a bleached centre and dark brown margins visible on the glumes (see General Introduction, Fig I.1b). However, Parry *et al.* (1995a) have observed these *F. poae*-like symptoms as the initial FEB symptoms following *F. culmorum* inoculation.

This is the first time that quantitative PCR analysis has been used as a screening tool to evaluate the efficacy of chemical compounds against *Fusarium* ear blight of wheat. PCR-based assays have been developed for the quantification of several *Fusarium* species. These enable detection, differentiation and quantification of *F. culmorum, F. poae, F. graminearum, F. avenaceum, M. nivale* var. *majus* and *M. nivale* var. *nivale* in infected plant material (Nicholson *et al.*, 1996; Parry & Nicholson, 1996; Nicholson *et al.*, unpublished). The author notes that, even if DNA content is presumed to directly reflect fungal biomass, the fate of DNA from "dead" fungal cells in plant tissue and the effect of fungicides on such DNA has not been investigated.

Both *F. culmorum* and *F. poae* DNA were quantified using internal control templates in competitive PCR-based assays (Lees, 1995; Nicholson *et al.*, unpublished). Standard curves enabled conversion of the ratio between competitor template and target DNA sequences to fungal DNA quantities. *F. culmorum* quantitative PCR employed a homologous competitor template (Lees, 1995), while a heterologous template was employed for *F. poae* quantitative PCR (Nicholson *et al.*, unpublished). Hu *et al.* (1993) prepared both heterologous and homologous internal control templates for the quantification of *V. albo-atrum* and *V. dahliae* and found that in subsequent assays, calculations with homologous templates were complicated by additional products of *in vitro* recombination. This problem did not occur when using the *F. culmorum*-specific homologous competitor template. However, use of a heterologous template, prepared as described for *F. poae*, would exclude the possibility of such heteroduplex formation.

One potential disadvantage of heterologous competitors is that they may not be amplified with the same efficiency as the target sequences (Pannetier *et al.*, 1993). However, this did not appear to be the case for the *F. poae* heterologous template, and other workers have found that heterologous control templates used for quantification appeared to have the same amplification kinetics as the target sequences (Siebert & Larrick, 1993; Telenti *et al.*, 1992; Förster, 1994; Liu *et al.*, 1994; Mahuku *et al.*, 1995).

Several fungicides have been reported to reduce FEB of wheat (Martin & Johnston, 1982; Fehrmann & Ahrens, 1984; Boyacioglu *et al.*, 1992; Hutcheon & Jordan, 1992; Suty *et al.*, 1996), but the extensive post-harvest analysis work in these fungicide efficacy experiments meant that glasshouse trials had to be limited in terms of the number of chemicals tested for efficacy against FEB of wheat. The fungicide prochloraz was chosen as one of the fungicides for these efficacy studies, because, other reports have shown that this fungicide is among the most effective chemical agents for controlling FEB of wheat (Fehrmann & Ahrens, 1984; Hutcheon & Jordan, 1992). In the three glasshouse experiments (1994/5-1996/7), both quantitative PCR and visual disease assessment showed a significant decrease in disease in *F culmorum* - inoculated samples treated two days later with prochloraz, when compared with inoculated untreated samples. In the 1994/5 experiment, both visual disease assessment and quantitative PCR analysis suggested similar decreases in disease severity due to prochloraz treatment (22 & 24 %, respectively). However, in both the 1995/6 and 1996/7 the reduction in disease severity according to quantitative PCR analysis (66 & 49 %, respectively) was much greater than the reduction according to visual disease assessment (50 & 26 %, respectively). In any case, the *F. culmorum* prochloraz results confirm those of Hutcheon & Jordan (1992), who found that, in inoculated glasshouse

trials, this fungicide greatly reduced the percentage of wheat ears infected with either *F. culmorum, F. avenaceum, F. graminearum* and *M. nivale* (82, 87, 82 and 89 % reductions, respectively). In a field trial inoculated with *F. culmorum*, prochloraz treatment was found to reduce yield loss due to *F. culmorum* (Fehrmann & Ahrens, 1984), however visual disease symptoms were not assessed. *In vitro,* Polley *et al.* (1991) found that prochloraz was particularly effective against *F. culmorum, M. nivale* and *F. avenaceum* isolates tested. Prochloraz has also shown activity against eyespot and sharp eyespot, pathogens that are often found in conjunction with *Fusarium* species at the stem base (Bateman, 1990).

The fungicide tebuconazole is known to effectively reduce FEB of wheat, both under glasshouse conditions (Hutcheon & Jordan, 1992) and in the field (Suty *et al.*, 1996). This fungicide was included in 1995/6 and 1996/7 glasshouse experiments in order to relate the efficacy of prochloraz against FEB of wheat to that of tebuconazole. In 1995/6, tebuconazole was as effective as prochloraz in reducing *F. culmorum* ear blight of wheat, according to both quantitative PCR and visual disease assessment. Likewise, in the 1996/7 experiment, visual disease assessment results suggested that there was no significant difference in the efficacy of the two fungicides against this pathogen. In contrast, the 1996/7 quantitative PCR analysis suggested that tebuconazole was significantly more effective against *F. culmorum* than prochloraz (49 % and 69 % reduction in DNA content, compared with 22 % and 26 % reduction in disease symptoms due to tebuconazole and prochloraz treatments, respectively). Thus, the 1996/7 PCR results agreed with the results obtained by Hutcheon & Jordan (1992), who found that, under glasshouse conditions, tebuconazole was significantly better than prochloraz in controlling *F. culmorum* ear blight of wheat, as measured by percentage

of infected ears. However, they found no significant difference in the efficacy of prochloraz and tebuconazole against *F. avenaceum, F. graminearum* and *M. nivale* ear blight of wheat, these two fungicides being the most effective of a range of fungicides tested against FEB of wheat. In the field, Suty *et al.* (1996) showed that, while tebuconazole treatment reduced *F. culmorum* ear blight of wheat, the timing of application affected the degree of control achieved. Tebuconazole treatment reduced the percentage of infected spikelets by an average of 60 % (range: 40 to 95 %) for treatments applied within four days pre- or post-inoculation. The efficacy of tebuconazole was reduced (range: 30 to 50 %) for treatments applied five to ten days pre- or post-inoculation, and depending on the difference between inoculation and actual infection date, treatments carried out one to five days post-inoculation were slightly more effective than treatments applied one to five days pre-inoculation.

Given the significance of *F. poae* as a causal agent of FEB of wheat in cooler maritime climates such as the UK (Polley *et al.*, 1991), it is surprising that fungicidal control of this pathogen has not received any direct attention. In these glasshouse experiments, according to the visual disease assessment results, there was no significant difference between the efficacy of the fungicides prochloraz and tebuconazole against *F. poae* ear blight of wheat. The two treatments resulted in significant decreases in disease symptoms in both the 1995/6 trial (41 & 51 %, respectively) and 1996/7 trial (82 & 73 %, respectively). In 1995/6, the quantitative *F. poae* PCR results indicated that the decrease observed in DNA content due to prochloraz treatment (55 %) was not significant, while tebuconazole significantly decreased DNA content (81 %). The reduction due to prochloraz was not significant due to variable DNA content of samples. However, in 1996/7 the decrease in DNA content due to prochloraz (67 %) was

significantly greater than the decrease due to tebuconazole (38 %). This variation in efficacy from year to year is probably due to the variability of the *F. poae* ear blight severity, particularly in 1995/6, when disease levels were less than those of 1996/7 and, within individual treatments, *F. poae* disease severity appeared more variable than *F. culmorum* disease severity.

Overall, although the visual disease assessment and quantitative PCR analysis showed similar disease profiles, differences between treatments that were non-significant according to visual disease assessment were, in some cases, significant by quantitative PCR analysis. This suggested greater resolution of the PCR system. There is only one other report that details the use of both quantitative PCR analysis and visual disease assessment of cereal diseases (Nicholson *et al.*, 1997). Nicholson *et al.* (1997) used both techniques to quantify *Tapesia yallundae* (anamorph: *Pseudocercosporella herpotrichoides*) (WCS-type eyespot) and *Tapesia acuformis* (R-type eyespot) seedling diseases of wheat. Although, based on their results, the authors suggested that the DNA content of wheat seedlings with similar disease score might differ greatly, depending on the species of *Tapesia* involved, they also emphasised that only a single isolate of each species and relatively few samples were used for this test. Other disease assessment techniques, such as ergosterol analysis (Miedaner & Perkowski, 1996), the GUS reporter gene system (De la Peña & Murray, 1994) and enzyme linked immunosorbent assays (Höxter *et al.*, 1991) have been compared with traditional methods of visual disease assessment of cereal pathogens. In a two-year study (1992 & 1993) of the relationships between *F. culmorum* ear blight resistance, fungal colonisation and mycotoxin contents in inoculated winter rye field trials, Miedaner &Perkowski (1996) found that while the mean head blight rating (1-9) was similar in both 1992 (4.5) and

1993 (4.6), the mean ergosterol content (mg/kg fresh weight of grain) in 1992 (84.5) was much greater than in 1993 (65.9). The disadvantage of using ergosterol analysis to estimate disease is that, unlike PCR analysis, the GUS reporter gene system and certain ELISA-based systems, this technique cannot discriminate between plant pathogens. Use of a GUS (β-glucuronidase reporter gene) assay to quantify wheat stem base disease caused by a GUS transformant of *Tapesia yallundae* was found to resolve resistant and susceptible wheat varieties more clearly than either visual disease assessment or ELISA-based analysis (De la Peña & Murray, 1994).

In terms of the correlation between visual disease symptoms and quantitative PCR analysis, the closest was between the 1995/6 *F. culmorum* GS 80 visual disease score and DNA content of wheat ears. Indeed, in two of the three years, the closest correlation for this pathogen was obtained using GS 80 results. The correlation between *F. poae* visual disease score and DNA content appeared to be independent of the growth stage at which disease was assessed. Accurate inter-annual correlation (across years) between visual disease assessment and quantitative PCR analysis requires either simultaneous quantitative PCR analysis of 1994-7 samples, or preparation of individual standard curves for each new competitor template dilution prepared. Because the quantitative PCR analysis was performed each year within weeks of harvest using competitor template solutions prepared each year from stock, and because analysis was based on the original 1994/5 *F. culmorum* and 1995/6 *F. poae* standard curves, inter-annual correlations were deemed invalid.

Nicholson *et al.* (1997) used regression analysis to evaluate the relationship between *T. yallundae* and *T. acuformis* visual disease assessment and quantitative PCR analysis and found that the relationship was better for the former ($R^2 = 0.61$) rather than

the latter pathogen (R^2 = 0.38). In a two-year study of the relationships between *F. culmorum* ear blight resistance, fungal colonisation and mycotoxin contents in winter rye, Miedaner &Perkowski (1996) found no correlation between ergosterol content and visual disease assessment in 1992 (r = 0.17), but in 1993, the correlation (r = 0.68) was similar to some of those observed in the present work between visual disease assessment and quantitative PCR analysis. Höxter *et al.* (1991) found a close correlation between ELISA-based analysis and visual disease assessment of *M. nivale* foot rot of rye (r = 0.9). De la Peña & Murray (1994) found that for quantification of wheat stem base disease caused by a GUS transformant of *Tapesia yallundae*, the correlation coefficients between visual disease ratings and GUS activity were numerically larger (r = 0.59-0.76) than between visual disease ratings and ELISA (r = 0.45-0.66).

The effect of *Fusarium* inoculation and fungicide treatment on yield, under glasshouse conditions, was also assessed, (based on 1000 grain weight and the number of grains per ear), although yield estimates are usually field based. The 1000 grain weights were, in general, much greater in 1995/6 than in 1994/5 or 1996/7, suggesting that environmental factors may greatly influence the yield. Inoculation with *F. culmorum* resulted in a significant decrease in thousand grain weight. Other workers have observed yield losses due to *F. culmorum* inoculation (Häni, 1981; Snijders & Perkowski 1990; Saur, 1991). After inoculating wheat ears in Switzerland with *F. culmorum* and *M. nivale*, Häni (1981) observed grain yield reductions of 60% and 15%, respectively and according to Snijders & Perkowski (1990), *F. culmorum* reduced thousand kernel weight by 2.8-22.4% in 10 winter wheat genotypes. Similarly, Saur (1991) reported a yield reduction of 6.4-39.2% when over 500 wheat genotypes were inoculated with *F. culmorum*.

Tebuconazole differed from prochloraz in that, in 1995/6, application of this fungicide resulted in a 25 % reduction in 1000 grain weight compared with untreated control. However, no such effect was observed in 1996/7 and one possible explanation may be that, in 1995/6 the glasshouse environmental conditions were such that this fungicide had a phytotoxic effect on the plants, and thus affected yield. Both prochloraz and tebuconazole treatments significantly increased the 1000 grain weight of *F. culmorum*-inoculated samples. These results agreed with those of other workers, who found that these fungicides significantly reduced yield loss due to *F. culmorum* inoculation (Fehrmann & Ahrens, 1984; Hutcheon & Jordan, 1992) In the glasshouse, Hutcheon & Jordan (1992) reported a significant yield response (37 % increase in thousand grain weight) in plants inoculated with *F. culmorum* post ear emergence (GS 69) and treated with tebuconazole 3 days post-inoculation. In the field, Suty *et al.* (1996) showed that after natural *Fusarium* infection, treatment with products containing tebuconazole resulted in a 21 % increase in yield and a 73 % decrease in the concentration of the trichothecene mycotoxin, deoxynivalenol (DON). In the field, yield loss due to *F. culmorum* inoculation was reduced by prochloraz treatment, treatment with a preventative spray, a curative spray and a combined spray resulting in yield increases of 6, 16 and 19 %, respectively (Fehrmann & Ahrens, 1984).

Because of the significant effect of *F. culmorum* inoculation on thousand grain weight, the relationships between 1000 grain weight and *F. culmorum* disease [as measured by disease score (GS 80) or amount of *F. culmorum* DNA/ mg plant material] were investigated, and were found to be significant, with yield decreasing as disease levels increased. The most significant relationship was that observed between the 1996/7 1000 grain weights and DNA contents ($R^2 = 0.83$, $y = 17.22x^{-0.71}$, when x = DNA

content and y = 1000 grain weight). Due to several factors, including the variability of yield from year to year, the variability of DNA quantification from year to year as discussed earlier and the phytotoxic effect of tebuconazole in 1995/6, use of inter-annual results to investigate the relationship between disease and yield was deemed invalid. Other workers have reported significant relationships between 1000 grain weight and *F. culmorum* visual disease scores, under field conditions (Snijders, 1990b; Parry, 1991). Using the same wheat cultivar (Avalon), Parry (1991) reported a significant relationship between FEB ear blight of wheat and 1000 grain weight in three field trials inoculated with *F. culmorum* and *F. avenaceum*. In 1983, the regression of yield reduction (y) on *Fusarium* head blight (x) was $y = 1.17x$ ($R^2 = 0.64$), in 1988 it was $y = 2.12x$ ($R^2 = 0.23$) and in 1990 the relationship was $y = 0.8x$ ($R^2 = 0.23$). Snijders (1990b) reported a significant relationship between FEB and yield of wheat in field plots inoculated with *F. culmorum*. The regression of yield reduction (y) on *Fusarium* head blight (x) was estimated as $y = 6\sqrt{x}$ ($R^2 = 0.49$) for 32 genotypes in 1987 and as $y = 7.2\sqrt{x}$ ($R^2 = 0.63$) for 54 genotypes in 1989.

Inoculation with *F. poae* did not significantly affect the thousand grain weight and neither prochloraz nor tebuconazole significantly increased the thousand grain weight when applied to *F. poae*-inoculated plants. Therefore, it is not surprising that no significant relationship was observed between *F. poae* disease and yield. The fact that *F. poae* disease did not significantly affect yield suggests that either, infection by this pathogen does not significantly affect grain weight, or disease severity was not sufficiently high enough in these experiments to see a yield loss effect. However, the former is more likely, as comparable *F. culmorum* disease scores and DNA contents resulted in significant yield losses. These results, together with the results from the field

trial discussed in Chapter 1, where this pathogen was predominantly found in the glume, rather than grain or rachis component of wheat ears, support the theory that the mode of infection and colonisation of this pathogen may differ from that of *F. culmorum.* Also, in hindsight, the results of the fungicide experiment may have been more meaningful, particularly for *F. poae*, if PCR analysis had been based solely on the grain component rather than whole wheat ears.

For the most part, neither inoculation with *F. culmorum* or *F. poae* nor treatment of inoculated plants with fungicides had an effect on the number of grains/ear. Overall, the number of grains/ear did not seem to reflect disease levels and one possible explanation for the 1995/6 *F. culmorum* results is that the number of grains/ear for the *F. culmorum*-inoculated untreated plants was unusually low in this year. Other workers, such as Hutcheon & Jordan (1992), used the number of grains/ear as a criterion for differentiating fungicide efficacy against FEB of wheat under glasshouse conditions. However, in the field, Wong *et al.* (1992) observed that while treatment of *F. culmorum*-inoculated ears with the fungicide propiconazole had no effect on the grain number, it resulted in increased thousand grain weight. One of the possible explanations as to why no effect was seen in the present experiments is that ears were not mechanically thrashed, rather grains were separated by hand. Thus, even small diseased grains that often adhered to the chaff were removed and accounted for. It is the authors observation that such grains are often unaccounted for when a mechanical thrasher is used to separate grains from the chaff.

The anilino-pyrimidine fungicide pyrimethanil has been proposed to target the secretion of cell wall degrading enzymes in *Botrytis* species (Milling & Richardson, 1995). Because of this novel mode of action, and because preliminary *in vitro* experiments indicated that this fungicide had some activity against *Fusarium* species (J. Liggitt, pers. comm.), pyrimethanil was included in the first glasshouse experiment (1994/5). Results indicated that this fungicide did not affect *F. culmorum* disease as measured by visual disease assessment or quantitative PCR analysis and did not affect yield (as measured by 1000 grain weight and number of grains per ear).

In the 1995/6 experiment, *G. roseum* culture filtrate was included because preliminary *in vitro* experiments indicated that fungitoxic compounds present in the culture filtrate inhibited the growth of *F. culmorum* and *F. poae,* among other *Fusarium* species (Etheridge, 1997). However, in the glasshouse, the culture filtrate did not cause a reduction in *F. culmorum* or *F. poae* disease severity, as determined by either quantitative PCR or visual disease assessment and did not result in increased yield (as measured by 1000 grain weight and number of grains per ear). There may be many reasons why the culture filtrate was inactive against the pathogens, but one possible explanation is that the culture filtrate served to inhibit the growth of other naturally occurring saprophytes and thus allowed *F. culmorum* to flourish without any competition. Another possible explanation is that, since the active ingredient and hence its concentration was unknown, the culture filtrate could have had a phytotoxic effect on the plant tissue, predisposing it to attack by the pathogen, although any such effect was not reflected in yield (1000 grain weight).

Overall, the general outcome of the fungicide trials was relatively constant from year to year, considering the complexity of the many variable environmental factors

involved, even under glasshouse conditions. Quantitative PCR analysis enabled determination of the level of disease within the ear, not just on the outside of the spikelets. A comparison with visual disease assessment results showed that results were, for the most, broadly similar. However, results from quantitative PCR analysis revealed differences in fungal DNA content of ears that were not obvious from visual disease assessment. PCR results indicated that both tebuconazole and prochloraz treatments caused a reduction of colonization of tissues by both *F. culmorum* and *F. poae* that was not reflected to the same degree in the visual disease symptoms. In future experiments, use of grain samples, rather than whole wheat ears used in the present work, may show even greater differences between visual disease assessment and quantitative PCR analysis, particularly for *F. poae* ear blight experiments. Quantitative PCR may prove particularly useful for analysing the efficacy of fungicides against natural inoculum under field conditions, as it would enable detection, differentiation and quantification of the individual *Fusarium* species present in the field.

3. TRANSFORMATION OF *FUSARIUM CULMORUM* WITH THE β-D-GLUCURONIDASE (GUS) REPORTER GENE: A SYSTEM FOR STUDYING THE HOST/PATHOGEN RELATIONSHIPS AND DISEASE CONTROL

3.1 INTRODUCTION

The complex of cereal diseases caused by *Fusarium* species include seedling blight, brown foot rot and ear blight (Parry *et al.*, 1994). *Fusarium culmorum* can cause all three diseases and is among the most abundant and aggressive pathogens of small grain cereals (Nelson *et al.*, 1981). Significant yield losses have been observed in grain contaminated with this pathogen (Snijders & Perkowski, 1990, Parry, 1991; Saur, 1991) and the ability of *F. culmorum* to produce mycotoxins in grain poses a serious threat to both human and animal health (Pomeranz *et al.*, 1990). It is therefore surprising, given the potential of *F. culmorum* as a pathogen of wheat, that research into the host-pathogen relationship (and control of *Fusarium* cereal diseases) has been so limited.

Elucidation of the mode of infection and colonization of the pathogen through detailed physiological, biochemical and/or molecular studies on the host-pathogen relationship may provide some insight into the factors involved in the pathogenicity of *F. culmorum*. In the long term, such studies may provide new targets for control of the pathogen. Ideally, such elucidation requires a technique that would not only allow the quantification of fungal biomass, but also enable visualisation of the pathogen *in planta*. Diagnostic techniques such as qualitative and quantitative polymerase chain reaction (PCR) assays have been developed which enable the specific detection of *F. culmorum*

in infected plant tissue (Ouellet & Seifert, 1993; Lees, 1995; Nicholson & Parry, 1996; Nicholson *et al.*, 1996; Parry & Nicholson *et al.*, 1996; Schilling *et al.*, 1996; Nicholson *et al.*, unpublished). Therefore these techniques provide a useful tool for studying the infection and colonization of wheat by *F. culmorum* and allow quantitative evaluation of the *in vivo* effect of host resistance or fungicides on the pathogen. However, while such species-specific PCR-based assays allow differentiation and quantification of the pathogen during infection and colonization of the host, or following fungicide application, they do not discriminate between strains of the pathogen and do not enable visualisation of the hyphae within the host. While microscopic studies should enable visualisation of the infection and colonization of the host by a pathogen, such studies are not straightforward. In studies of natural infection and colonisation, it is necessary to differentiate not only between the host and the pathogen, but also between the pathogen of interest and other possible pathogenic organisms that may be present within the host tissue.

The introduction of a genetic marker into the fungal genome via genetic transformation would provide a tool not only for the quantification of the genetically transformed strain within infected plant tissue, but such an isolate would also allow microscopic visualisation of the transformed isolate within the infected host. One such genetic marker is the *Escherichia coli* β-D-glucuronidase (GUS) reporter gene (known as *gusA* or *uidA*) (Jefferson, 1987a,b, 1989). The GUS reporter gene system was originally developed for use in the nematode *Caenorhabditis elegans* (Jefferson *et al.*, 1986, 1987a,b), but has since been used extensively in studies of bacteria, animals and plants (Jefferson, 1989), in yeast studies (Pobjecky *et al.*, 1990) and in studies on filamentous fungi (Roberts *et al.*, 1989; Ashby & Johnstone, 1993; Couteaudier *et al.*,

1993; Mönke & Schäfer, 1993; Liljeroth *et al.*, 1993; Green & Jensen, 1995). The GUS enzyme is relatively stable and can be assayed using various methods (Jefferson, 1987a,b; Gould & Smith, 1989; Gallagher, 1992).

Several plant pathogenic fungi have been successfully transformed with the β-D-glucuronidase (GUS) reporter gene, without adverse effects on the pathogenicity of the fungi. GUS-transformed pathogens include *Fulvia fulva* (synamorph *Cladosporium fulvum*) (Roberts *et al.*, 1989; Oliver *et al.*, 1993; Wubben *et al.*, 1994), *Bipolaris sorokiniana* (Liljeroth *et al.*, 1993), *Fusarium oxysporum* (Couteaudier *et al.*, 1993), *Cochliobolus heterostrophus* (Mönke & Schäfer, 1993), *Pyrenopeziza brassicae* (Ashby & Johnstone, 1993) and *Leptosphaeria maculans* (Oliver *et al.*, 1993). The use of fungal transformants expressing GUS activity to detect infection and measure hyphal biomass in infected plant tissue using only standard laboratory equipment was first described by Oliver *et al.*(1993). Strains of the tomato pathogen *C. fulvum* and the brassica pathogen *L. maculans* that constitutively expressed the GUS enzyme were used to detect, histochemically, the presence of fungal hyphae in host tissue. Also, GUS activity has been used to quantify biomass in the cotyledons of near-isogenic lines of tomato differing in resistance to *C. fulvum*. Oliver *et al.* (1993) showed that GUS activity was significantly lower in incompatible interactions as compared with compatible interactions. Ashby & Johnstone (1993) transformed the heterothallic light leaf spot brassica pathogen, *P. brassicae*, with the GUS reporter gene and used this system to monitor the behaviour of the transformed isolate in crosses with non-transformed isolates of opposing mating type. This system enabled them to mark and differentiate otherwise indistinguishable compatible mating-type fungal spores and mycelia.

A member of the genus *Fusarium*, *F. oxysporum*, was transformed with the GUS

reporter gene and this reporter system allowed rapid quantification of *F. oxysporum* in infected flax (*Linum usitatissimum*) roots (Couteaudier *et al.*, 1993). Couteaudier *et al.* (1993) showed that, in the presence of the β-glucurionidase substrate X-glucuronide, hyphae of the transformed strain were clearly visible on root tissues and could be easily observed after different contact times between pathogenic strains and flax roots. These workers also demonstrated how the GUS gene fusion system provided a useful tool for the study of competition between pathogenic and non-pathogenic strains of the fungus on flax roots.

Genetic transformation makes it possible to introduce a marker such as the GUS reporter gene into a fungal genome. A variety of transformation systems have been developed for filamentous fungi, although most protocols involve the production of fungal protoplasts followed by the introduction of plasmid DNA in the presence of polyethylene glycol and divalent cations (Fincham, 1989). This transformation system has already been successfully used for transformation of *F. culmorum* with the *E. coli* hygromycin phoshotransferase (*hph*) gene (Curragh *et al.*, 1992).

The efficiency of transformation of filamentous fungi seems dependent on many factors. These include the organism and indeed the specific strain used in transformation experiments (Mohr & Esser, 1990), the ability to obtain high protoplast yields, the selectable marker used and the regulatory signals used to direct the expression of foreign genes (Fincham, 1989). Because of these, and other variable factors, drawing comparisons between the transformation efficiencies achieved for different fungi is often difficult.

Polyethylene glycol-mediated fungal transformation requires the production of high yields of fungal protoplasts. Production of protoplasts from filamentous fungi has

been shown to be dependent on several factors, including pH, osmotic stabilizer and mycelial concentration (Peberdy *et al.*, 1976; Lynch *et al.*, 1985; Fincham, 1989; Curragh *et al.*, 1992). Curragh *et al.* (1992) investigated the effect of both by varying the *F. culmorum* culture conditions and varying the lytic mix conditions for protoplast production. They found that younger, actively growing cultures gave higher protoplast yields than older cultures and they also found that protoplast production was optimal at a pH of 6.4 and a temperature of 30^0C. Younger actively growing mycelia were also found to give higher protoplast yields for the pathogens *F. oxysporum* and *F. tricintum* (Lynch *et al.*, 1985; Sallen *et al.*, 1988) and Peberdy *et al.* (1976) found that, for a range of fungi, the optimum pH for protoplast production was between 6.0-6.5. Various osmotic stabilizers have been used in the production and regeneration of fungal protoplasts including the inorganic stabilizers NH_4Cl and KCl (Peberdy *et al.*, 1976; Lynch *et al.*, 1985), $MgSO_4$ (Lynch *et al.*, 1985) and KCl and $CaCl_2$ (Sallen *et al.*, 1988) and organic stabilizers such as sorbitol and sucrose (Curragh *et al.*, 1992). Curragh *et al.* (1992) found that for the production of protoplasts of *F. culmorum*, higher yields and more rapid regeneration were obtained with the organic stabilizers sucrose and sorbitol than with the inorganic stabilizer NH_4Cl.

The choice of selectable marker and the regulatory signals used to direct the expression of foreign genes also influences the efficiency of transformation (Fincham, 1989). The *E. coli* hygromycin phosphotransferase (*hph*) gene has been successfully used as a heterologous selectable marker in many fungal transformation experiments (Roberts *et al.*, 1989; Bunkers, 1991; Curragh *et al.*, 1992; Hohn & Desjardins, 1992; Mönke & Schäfer, 1993; Oliver *et al.*, 1993; Walz & Kück, 1995; Weltring, 1995). Other homologous and heterologous selectable markers used in fungal transformation

experiments include the β-tubulin genes from *Neurospora crassa*, *A. niger* and *Septoria nodorum* (Orbach *et al.*, 1986; Bernier *et al.*, 1989; Cooley *et al.*, 1990, 1991), the *A. nidulans* acetamidase enzyme structural gene (*amdS*) (Tilburn *et al.*, 1983; Kelly & Hynes, 1985; Turgeon *et al.*, 1985) and the *A. nidulans* nitrate reductase structural gene (*niaD*) (Couteaudier *et al.*, 1993). Garcia-Pedrajas & Roncero (1996) reported the development of a highly efficient transformation system for *F. oxysporum* f.sp. *lycopersici*, based on the complementation of a nitrate reductase mutant with the homologous *nit1* gene and on the presence of autonomously replicating sequences (*ARS*) and telomeric sequences in the vector. Cooley *et al.* (1991) developed a homologous transformation system for the wheat-pathogenic fungus *S. nodorum* based on a benomyl-(MBC-) resistant allele of the β-tubulin gene. However, this homologous selectable marker did not show any improvement on the transformation frequency obtained using the heterologous hygromycin resistance encoded by pAN7-1 (Cooley *et al.*, 1988, 1991).

Another important factor in transformation is the choice of regulatory sequences used to control the expression of selectable markers and/or other genes of interest such as the *gusA* gene. The use of the GUS reporter system as a tracking device and genes such as *hph* as a selectable marker requires that they be constitutively expressed. For the transformation of *F. culmorum*, Curragh *et al.* (1992) used two plasmid vectors (pAN7-1 & pHRC3), both of which contained the *E. coli* hygromycin B phosphotransferase gene (*hph)* flanked by the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and *trp*C terminator sequences (Punt *et al.*, 1987; Kistler & Benny, 1988). These plasmids have been used to transform other filamentous fungi and yeast (Punt *et al.*, 1987; Cooley *et al.*, 1988; Kistler & Benny,

1988; Oliver *et al.*, 1993) and the *gpd* promoter and *trp*C terminator sequences have been widely used in other vectors for the introduction of foreign genes into filamentous fungi (Roberts *et al.*, 1989; Cooley *et al.*, 1990, 1991; Couteaudier *et al.*, 1993; Liljeroth *et al.*, 1993; Salch & Beremand, 1993; Thrane *et al.*, 1995). Other homologous and heterologous regulatory sequences which have been used successfully in fungal transformation experiments include a 1-kb promoter region from the *Erysiphe graminis* f.sp. *hordei* β-tubulin gene (Christiansen *et al.*, 1995), the *Cochliobolus heterostrophus* promotors *GPD1* (glyceraldehyde-3-phosphate dehydrogenase) and *P1* (promoter 1) (Mönke & Schäfer, 1993; Christiansen *et al.*, 1995) and the *Podospora anserina* and *Claviceps purpurea gpd1* promoters (Ridder & Osiewacz, 1992; Jungehulsing *et al.*, 1994). The use of homologous promoters fused to the gene of interest has been shown to improve transformation efficiency for the fungi *P. anserina* and *C. purpurea* (Ridder & Osiewacz, 1992; Jungehulsing *et al.*, 1994). Using a "promoter-probe library" strategy, Weltring (1995) isolated two genomic DNA fragments from the phytopathogenic fungus *Gibberella pulicaris* (anamorph: *F. sambucinum*) which could drive the expression of the *hph* gene.

Several filamentous fungi have been co-transformed with two plasmids, one carrying the selectable marker, the other carrying the gene of interest (Kelly & Hynes, 1985; Punt *et al.*, 1987; Cooley *et al.*, 1990; Ashby & Johnstone, 1993; Thrane *et al.*, 1995). The use of such a transformation system means that incorporating the desired gene sequences into the vector carrying the selectable marker or vice-versa is not necessary. Ashby & Johnstone (1993) co-transformed *P. brassicae* with plasmid pAN7-1 carrying the hygromycin resistance gene and plasmid pNOM102 carrying the *gusA* gene and found that, on average, approximately 80 % of transformants were co-

transformants. Cooley *et al.* (1990) also obtained high co-transformation frequencies of *S. nodorum* with three different unselected DNA species and plasmid pAN7-1 carrying the *hph* gene. High co-transformation frequencies have also been observed in other fungal species (Kelly & Hynes, 1985; Punt *et al.*, 1987; Roberts *et al.*, 1989; Bunkers, 1991).

The first objective of this study was to produce stable *F. culmorum* GUS transformants. The influence of different regulatory sequences on transformation efficiency was studied by transformation with two plasmids which contained the *gusA* gene and the *hph gene* under the control of different promotors. Also, the effect of co-transformation of the plasmids with cosmid pHPC1 (Desjardins *et al.*, 1992) which contained the *hph* gene under the control of a *Fusarium* promotor, was investigated.

The second objective of this study was to determine the usefulness of the GUS gene fusions system for the detection and quantification of *F. culmorum* in infected plant material, and the feasibility of using such a system to evaluate the efficacy of fungicides against *F. culmorum.*

3.2 MATERIALS & METHODS

3.2.1 Strains and Media

Fusarium culmorum strain Fu 5 was obtained from the John Innes Centre facultative pathogen collection and the media and conditions used for maintenance, growth, DNA preparation and production of conidia of the wild type isolate and transformants were as described previously (Chapter 1, section 1.2.1 & Chapter 2, section 2.2.2). For protoplast formation, the fungus was grown on complex yeast medium (CYM) broth which contained: glucose, 20 g l^{-1}; yeast extract, 2 g l^{-1}; bacto peptone, 2 g l^{-1}; $MgSO_4.7H_2O$, 1.02 g l^{-1}; KH_2PO_4, 0.46 g l^{-1}; K_2HPO_4, 1.31 g l^{-1} (Curragh *et al.*, 1992). Protoplasts were regenerated in a non-selective regeneration broth (SYC) which contained: yeast extract, 1.0 g l^{-1}; caesin-enzyme hydrolysate, 1.0 g l^{-1}; sucrose, 342.3 g l^{-1} (Hohn & Desjardins, 1992). For selection, protoplasts were transferred to molten (50 ^{0}C) SYC agar (SYC broth plus 16.0 g l^{-1} bacto agar) (Hohn & Desjardins, 1992) containing hygromycin B (50 µg ml^{-1}) (Boehringer Mannheim GmbH, Germany). Potato dextrose agar (PBA) (Difco, UK) was used for subculturing of transformants and to determine the hygromycin B stability of transformants (70 µg hygromycin B ml^{-1} PDA). The *Escherichia coli* (strain DH5α) was used for propagation of plasmids (Biorad Laboratories Ltd., UK).

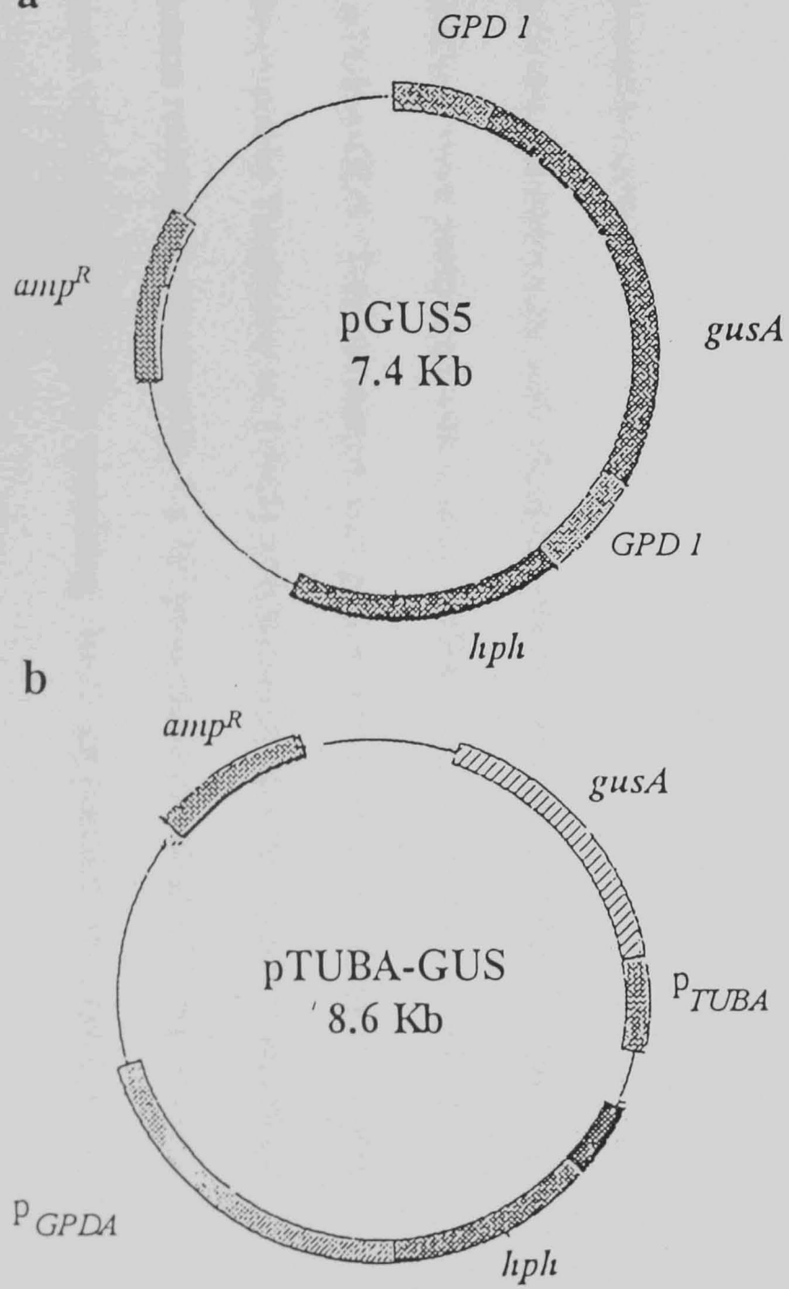
3.2.2 Plasmids

Plasmid pGUS 5 (Mönke & Schäfer, 1993) (Fig. 3.1a) was kindly provided by W. Schäfer, Institut für Genbiologische Forschung Berlin GmbH, Berlin, Germany and

plasmid pTUBA GUS (N. Cooley, pers. comm.) (Fig. 3.1b) by N. Cooley, AgrEvo UK Ltd., Essex, UK. Plasmids pGUS5 and pTUBA-GUS both harboured the *E. coli* GUS gene (*gusA*) and the *E. coli* hygromycin phosphotransferase gene (*hph*). In plasmid pGUS 5, both genes were under the control of the glyceraldehyde-3-phosphate dehydrogenase (*GPD 1*) promoter from *Cochliobolus heterostrophus* (anamorph: *Helminthosporium maydis: Bipolaris maydis*), with a transcriptional fusion between *gusA* as well as *hph* and the *GPD 1* promoter. In plasmid pTUBA-GUS, the *hph* gene was under the control of *GPD* expression sequences from *Aspergillus nidulans* and *gusA* was under the control of a β-tubulin (*tubA)* promoter sequence from *Septoria nodorum* (telomorph: *Leptosphaeria maculans*). Cosmid pGPC1 was donated by B. Brückner, Universitat Munster, Munster, Germany. This cosmid harboured the *hph* gene flanked by a promoter sequence from *Gibberella pulicaris* (anamorph: *F. sambucinum*) and a transcription termination sequence of the *A. nidulans trpC* gene (Desjardins *et al.*, 1992, Weltring, 1995) (Fig. 3.1c).

3.2.3 Protoplast isolation

Protoplasts were prepared using a method similar to the techniques described by Curragh *et al.* (1992) and Salch and Beremand (1993). Four mycelial plugs (approx 8 mm diameter) from 7 day old *F. culmorum* PDA (Difco, UK) plate cultures were used to inoculate 100 ml flasks of CYM broth. Cultures were incubated at 20 ^{0}C on a Rotatest R100 bench top orbital shaker (Luckham Ltd., UK), speed 3, for 2 days. Mycelia were transferred to 250 ml tubes, harvested by centrifugation (1500 x g for 5 min) and resuspended in the minimum of culture medium. Mycelia were transferred to



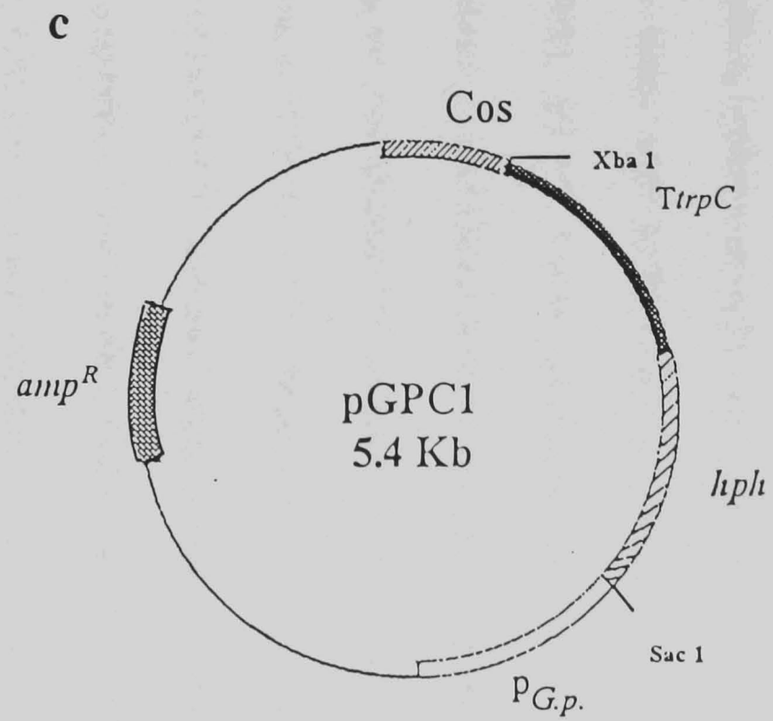


Fig. 3.1 Vectors used for transformation of *Fusarium culmorum*. Vectors: a, pGUS5; b, pTUBA-GUS; c, pGPC1. Promoters: *GPD1*, *Cochliobolus heterostrophus* glucose phosphate dehydrogenase promoter; ^{P}*GPDA*, *Aspergillus nidulans GPD* promoter; ^{P}*G.p.*, *Gibberella pulicaris* (*F. sambucinum*) promoter sequence; ^{P}*TUBA*, *Septoria nodorum* β-tubulin promoter sequence. Genes: *gusA*, *Escherichia coli* β-D-glucuronidase gene; *hph*, *E. coli* hygromycin phosphotransferase resistance gene; *amp*^{R}. *E. coli* β-lactamase ampicillin resistance gene. After: a, Mönke & Schäfer (1993); b, N. Cooley (pers. comm.); c, B. Bruckner (pers. comm.).

15 ml tubes and homogenised for approx 1 min using an electric drill (AEG, Germany) and a 'custom made' drill bit (John Innes Centre, UK). After passing through two layers of cheese cloth, the supernatant resulting from 2 x 100 ml CYM cultures was used to inoculate 250 ml of CYM broth which was incubated as above for 18 h. After incubation, hyphae were collected by centrifugation at 1500 x g for 12 min and washed three times with buffer/stabiliser [10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 6.3, 1.0 M sorbitol] as described by Curragh *et al.* (1992). Washed mycelium was added to the lytic mix which consisted of 10 ml buffer/stabiliser, 0.1 g lysing enzyme (Sigma, UK) and 0.15 g driselase (Sigma, UK) and incubated without shaking at 30 ^{0}C for 3.5 h. The protoplasts were collected by centrifugation at 1000 x g for 12 min at 4 ^{0}C, and then washed twice in buffer/stabiliser and once in STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, 10 mM $CaCl_2$) (Salch and Beremand, 1993), each wash being followed by a centrifugation, as above. Protoplasts were resuspended in STC buffer and protoplast concentration was determined using a haemocytometer (Weber Scientific International Ltd., UK). Protoplasts were used immediately for transformation.

3.2.4 Transformation

Protoplasts were transformed with plasmids pGUS5 and pTUBA-GUS. Also, co-transformations were performed with cosmid pGPC1 and either plasmid pGUS5 or plasmid pTUBA-GUS. Transformation was performed using a modification of the method developed by Turgeon *et al.* (1985) with two replicates per transformation/co-transformation reaction. Approximately 5 x 10^7 protoplasts in 100 µl STC buffer were gently mixed with 50 µl STC buffer containing 30-32 µg plasmid DNA (pGUS5 or

pTUBA-GUS DNA). Co-transformation reactions received a 1:1 molar ratio of cosmid pGPC1 DNA and either plasmid pGUS5 or plasmid pTUBA-GUS DNA. The control protoplast suspension received 50 µl STC. The suspensions were placed on ice and after 20 min polyethylene glycol 4000 (60 % w/v in 10 mM Tris-HCl pH 7.5 and 10 mM $CaCl_2$) was added to each suspension in three aliquots of 200, 200 and 800 µl, mixing gently between additions. After incubation for 40 min on ice, protoplasts were pelleted at 1000 x g for 12 min. Protoplasts were resuspended in 100 µl STC buffer, mixed with 1 ml of SYC broth and allowed to regenerate for 18 h at 10 ^{0}C. Following regeneration, each suspension was added to 20 ml molten (50 ^{0}C) regeneration agar (SYC) containing 50 µg ml^{-1} hygromycin B, and poured into two Petri dishes (10 ml each). Hygromycin B-resistant colonies emerged after 6-8 days incubation at 15 ^{0}C. These colonies were sub-cultured onto fresh PDA plates containing hygromycin B (70 µg ml^{-1}). Colonies were finally transferred to a 10 % (v/v) glycerol solution for storage at - 70 ^{0}C.

3.2.5 DNA extraction

Plasmid DNA was isolated and purified through anion-exchange columns (Qiagen Ltd., UK), according to the suppliers recommendations. Both *F. culmorum* genomic DNA and plant DNA were extracted as described previously (Chapter 1, section 1.2.3), except that a phenol/chloroform /iso-amyl alcohol (25:24:1) extraction step (1:1) was included prior to the chloroform extraction step. DNA pellets were resuspended in TE buffer (10 mM Tris-HCL, 1 mM EDTA).

3.2.6 PCR analysis of transformants

Following two sub-cultures on PDA containing hygromycin B (70 ug ml^{-1}), DNA was extracted from tolerant cultures (as described in section 3.2.5) and screened for the presence of plasmid DNA using PCR analysis. PCR amplification conditions were as described earlier (Chapter 1, section 1.2.4). Primers specific for the *E. coli* β-glucuronidase gene (*gusA*) were designed using the Primer (version 0.5) program (© 1991, Whitehead Institute for Biomedical Research, USA) and synthesised using an Expedite™ Nucleic Acid Synthesis System (Millipore, UK). The *gusA*-specific forward and reverse primers (GuF/R: 5′-AACCCCAACCCGTGAAATC-3′/5′-AATAACATAC GGCGTGACATCG-3′) were based on the *E. coli gusA* gene sequence (Jefferson *et al.*, 1986). A fragment of 359 bp was expected for GuF/R genomic DNA PCR amplification. Primers specific for the *E. coli* hygromycin B phosphotransferase gene (*hph*) (Gritz & Davies, 1983) (ArF/mm104: 5′-CTGTCGAGAAGTTTCTGATCG-3′/5′-TTTCCACTA TCGGCGAGT AC-3′) were obtained from P. Smith (ArF) and P. Christou (mm104), John Innes Centre, Norwich, UK. A fragment of 1170 bp was expected for ArF/mm104 genomic DNA PCR amplification. Each PCR assay included the appropriate positive plasmid control reaction (pGUS 5 or pTUBA-GUS) and a negative control reaction to which no DNA was added. The programme was based on 'touchdown' PCR (Don *et al.*, 1991), as follows: 95 ^{0}C for 30 s, annealing for 20 s, 72 ^{0}C for 45 s, annealing temperature being 66, 64 and 62 ^{0}C for 5, 5 and 15 cycles, respectively. A final extension step of 5 min was included, followed by cooling to 10 ^{0}C until recovery of samples. Aliquots (10-15 µl) of amplification products were electrophoresed through agarose gel (1.5 % w/v) (Sigma, UK) as described earlier (Chapter 1, section 1.2.4).

3.2.7 Southern analysis of *F. culmorum* transformants

Southern hybridisation analysis was used to identify the integration pattern of the transforming DNA in the genomic DNA extracts found to contain plasmid DNA by PCR analysis. Total genomic DNA from transformants (1 µg) was digested with various restriction enzymes according to manufacturers' recommendations (Northumbria Biologicals Ltd., UK). DNA from transformants arising from protoplasts treated with either pGUS5 or pGUS5 and pGPC1 were digested with *Xba* I. Both pGUS5 and pGPC1 have one *Xba* I site. DNA from transformants arising from protoplasts treated with either pTUBA GUS or pTUBA-GUS and pGPC1 were digested with *Sac* I. Both pTUBA-GUS and pGPC1 have one *Sac* I site. These enzymes do not restrict the *gusA* gene. DNA fragments were separated by agarose gel electrophoresis (0.8 % w/v agarose) as described in Chapter 1 (section 1.2.4) and transferred by diffusion to a nylon membrane (Hybond N+, Amshersham International, UK) by alkaline Southern blotting (Reed & Mann, 1985). Membranes were probed with the *gusA*-specific PCR product (see section 3.2.6). PCR amplification products were labelled with ^{32}P-dCTP (Amshersham International, UK) by the oligolabelling method of Feinberg & Vogelstein (1984). Membranes were washed twice for 15 min with 2 x saline sodium citrate (SSC) buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) containing 0.1 % sodium dodecyl sulphate (SDS), washed a further two times with 0.2 x SSC (30 mM NaCl, 3 mM sodium citrate, pH 7.0) containing SDS (0.1 % w/v), and then exposed to Kodak XAR-5 film (Kodak, UK) at -70 ^{0}C between two intensifying screens for 2-4 days.

3.2.8 Stability of transformants

Transformants were subjected to a preliminary qualitative GUS assay. This involved the assay of transformants (2 replicate assays per transformant) on SYC plates by addition of 30 µl MUG substrate [20 mM 5-bromo-4-chloro-3-chloro β-D-glucuronide (MUG) (Sigma B-0522), 50 mM sodium-phosphate buffer, pH 7.0]. After overnight incubation at 30 ^{0}C, 30 µl of stop buffer (0.3 M Na_2CO_3) was added and plates were examined under UV light for the presence of the MUG reaction product as described by Gould & Smith (1989). Blue fluorescence under UV light indicated the presence of GUS activity within cultures, no blue fluorescence indicating the absence of GUS activity. Isolates found to express GUS were then successively sub-cultured five times on non-selective PDA over a period of five weeks. Growth of transformants on PDA containing hygromycin B (70 µg ml^{-1}) was then compared with growth on non-selective PDA to determine hygromycin B tolerance. Conidia of HygB-tolerant isolates were produced as described earlier (Chapter 2, section 2.2.2) and these were used to prepare eight single spore cultures of each isolate. These single spore cultures were then tested for GUS stability in a microtitre plate assay similar to that described by Thrane *et al.* (1995). For this test, isolates were subcultured on PDA (3 replicate plates per isolate) and fresh mycelium scrapes taken from 3 d old cultures were incubated in 200 µl of MUG substrate (as above). Plates were incubated in the dark for 1 h at room temperature and examined under UV light. Transformants/isolates were deemed 'GUS-stable'if all cultures, i.e. 3 replicates x eight single spore cultures per isolate, expressed GUS activity, i.e. fluoresced blue under UV light (Gould & Smith, 1989).

3.2.9 Quantitative GUS assay

Quantitative assay of GUS activity was performed on selected, stable transformants (arising from pGUS5 transformation, pTUBA-GUS transformation and pGUS5/pGPC1 co-transformation) and for wild type Fu 5. Conidial suspensions (10^6 conidia ml^{-1} H_20) of transformants and wild-type were produced as described earlier (Chapter 2, section 2.2.2). 1 ml of conidial suspensions were added to 50 ml of potato dextrose broth (PDB) (Difco, UK) and incubated at room temperature on a Rotatest R100 bench top rotary shaker (Luckham Ltd., UK), at speed 3, for 3 days. Mycelium was harvested by filtration through Mira cloth, washed with sterile distilled water and squeezed dry. The resulting mycelia were weighed and transferred to a 5 ml tissue homogeniser (BDH, UK) containing 0.75 ml extraction buffer (50 mM $NaPO_4$ pH 7.0, 10 mM dithriothreitol, 1 mM EDTA, 0.1 % sodium lauryl sarcosine, 0.1 % Triton X-100) (Jefferson, 1987) per g fresh weight of mycelium. Tubes were spun at 10,000 x g for 15 min and the supernatant was removed to a fresh tube and centrifuged again, as above. The resulting supernatant/ extracts were stored at -70 ^{0}C. GUS was assayed quantitatively by spectrophotometry using a microtitre plate assay, based on an adaptation of the method of Jefferson (1987a). Extract (20 µl) was added to 180 µl of pre-warmed assay buffer (1 mM MUG in GUS extraction buffer). Reactions were incubated at 37 ^{0}C and, at timed intervals (0, 15, 30 & 60 min), 20 µl aliquots were removed and added to 180 µl stop buffer (0.2 M Na_2CO_3) in black fluoroplates held at room temperature. 4-methylumbelliferone (MU) concentrations were determined using a Titertek Fluoroscan II (ICN Biomedicals Ltd., UK), excitation at 365 nm, emission at 455 nm. Protein concentration was estimated using the Bio-rad assay (Bio-rad Laboratories Ltd., UK) and one unit of GUS activity

was defined as the amount of enzyme producing one nanomole of MU min^{-1} mg of $protein^{-1}$ at 37 ^{0}C. Results were based on three replicate tests per transformant.

3.2.10 Pathogenicity of *F. culmorum* transformants

Pathogenicity of transformants was determined using a procedure similar to that described by Bunkers (1991). Seeds of winter wheat (cv. Avalon) were germinated as described previously (Chapter 2, section 2.2.2) and germlings were transferred to small pots (5 cm diameter) containing John Innes compost No. 2 and incubated at 15 ^{0}C. Stem bases of 10-day-old wheat seedlings were inoculated with 1 ml of spore suspensions (10^6 conidia ml^{-1}, prepared as described in Chapter 2, section 2.2.2) of the transformants (19 seedlings per treatment). Plants were then covered with polyethylene bags for 3 days to maintain high humidity. Ten days after inoculation, 10 seedlings per treatment were harvested and GUS expression was measured qualitatively by incubating stem base sections (2.5 cm) in GUS extraction buffer containing 1 mM MUG overnight at 37 ^{0}C. Fluorescence was visualised under UV light. After 5 weeks, stem base sections (2.5 cm) of the remaining plants were rated for disease.

Disease was assessed both visually and using quantitative *F. culmorum*-specific PCR analysis. Visual disease symptoms were scored according to lesion length and colour (Table 3.1) (Lees, 1995). DNA was extracted from wheat stem base sections as described above (section 3.2.5) and *F. culmorum* quantitative PCR analysis was performed using new *F. culmorum*-specific primers (C51F/R) (Chapter 2, section 2.2.5) and a new competitor template, constructed as described previously for the *F. poae* competitor template (Chapter 2, section 2.2.6.1), was obtained from A. Turner (John

Innes Centre, UK). Reactions contained 10 µl plant DNA extract (4 ng dry weight equivalent ul^{-1} H_2O) and 10 µl of competitor template DNA (10^{-3} ng DNA μl^{-1} H_2O) and each PCR assay included both a fungal control reaction (10 ng DNA), a competitor template control reaction (10^{-2} ng DNA) and a negative control (no DNA). Amplification reaction conditions, the amplification programme, electrophoresis and densitometic analysis was as described previously for *F. poae* competitive PCR (Chapter 2, section 2.2.6.3). *F. culmorum* DNA content of samples was extrapolated from a standard curve constructed by D. Simpson (John Innes Centre, UK), which related the PCR product ratio (ratio between the amount of target PCR product and the amount of competitor template) to the amount of *F. culmorum* DNA. Quantitative PCR analysis results were based on two replicate reactions per DNA extract.

3.2.11 Disease detection and histochemical localization of fungal hyphae

GUS transformant G514 was selected to test the feasibility of using the GUS system to detect disease and histochemically localize fungal hyphae within infected plant tissue. Transformant G514 was derived from wild type isolate Fu 5 and transformed with plasmid pGUS 5 containing the *E. coli* GUS gene (*gusA*) and the hygromycin B resistance gene (*hph*). Wheat seedlings (cv. Avalon) (3 seedlings per pot and six pots per treatment) were cultivated and inoculated with either G514 conidia, Fu 5 conidia or water as described above for the pathogenicity test (conidia produced as described in Chapter 2, section 2.2.2). Plants were then covered with polyethylene bags for 3 days to maintain high humidity. After 2 weeks, plants were harvested and stem base sections (2.5 cm) were infiltrated at reduced pressure with x-glucuronide (0.5 mg ml^{-1}) in 50 mM

Table 3.1 Stem base disease scoring system, based on the colouration and length of disease lesions.

Disease category[a]	Colouration
0	Green/lesion absent
1	Honey-brown lesions
2	Brown lesions
3	Dark brown lesions
4	Black lesions

[a] Disease score = A x lesion length (cm), where

A = the disease category.

After: Lees (1995).

phosphate buffer, pH 7.0, 1 mM KFeCN and 0.05 % (v/v) triton X-100 and incubated overnight at 37 ^{0}C. The stem base sections were then partially cleared with 70 % ethanol (v/v) and 1 mm long stem base sections were mounted on microscope slides in 50 % (v/v) glycerol in phosphate-buffered saline. The sections were examined using a Nikon Microphot-SA microscope (Nikon Europe B.V., The Netherlands) and photographed using Ektachrome Panther 100 colour reversal film (Kodak, UK).

3.2.12 Evaluation of fungicide efficacy

The feasibility of using the GUS reporter gene system as a screening tool to evaluate fungicide efficacy was assessed using GUS transformant G514. Wheat seedlings (cv. Avalon) (3 seedlings per pot and six pots per treatment) were cultivated and inoculated as described above (see section 3.2.11). Plants were then covered with polyethylene bags to maintain high humidity. The fungicides prochloraz and tebuconazole were applied to the appropriate plants at approximately half rate (200 g a.i. ha^{-1}) 7 d post-inoculation. Information regarding active ingredient content, formulation type and manufacturers' addresses of the fungicide and fungicide application are as detailed previously (Chapter 2, Table 2.2 & section 2.2.2). After 2 weeks, plants were harvested. Stem base sections (2.5 cm) of the plants within each pot were bulked together and homogenised in a 1 ml aliquot of MUG assay buffer and GUS was extracted and activity quantified as described previously (section 3.2.9).

3.2.13 Statistical analysis

The pathogenicity test was performed twice and fungicide efficacy experiment once. Statistical analysis was carried out using analysis of variance (ANOVA) and Tukey`s Pairwise Comparison test (Snedecor & Cochran, 1980), performed using Minitab release 10.1 (©1991, Minitab incorporated).

3.3 RESULTS

3.3.1 Transformation

Prior to transformation, the wild-type *F. culmorum* isolate Fu 5 was tested for its endogenous hygromycin B tolerance using SYC agar supplemented with hygromycin B. Growth of wild-type *F. culmorum* on SYC was completely inhibited when supplemented with 70 μg ml^{-1} hygromycin B (results not shown). *F. culmorum* was transformed with both plasmids pGUS 5 and pTUBA-GUS, and co-transformed with cosmid pGPC1 and either plasmid pGUS5 or plasmid pTUBA-GUS. Many of the regenerated colonies failed to grow after being transferred from the original to fresh SYC plates containing hygromycin. These were presumed to be abortive transformants similar to those reported for other fungi (Salch & Beremand, 1993) and were not considered as true transformants. Table 3.2 shows the number of regenerated protoplasts, the number of abortive and non-abortive transformants and the transformation frequencies obtained with the different transforming vectors. Two thirds of the pGUS5-transformed regenerated colonies were abortive and the frequency of transformation to hygromycin B tolerance was approximately 0.5 transformants per μg of pGUS5 DNA. Most (93 %) of pTUBA-GUS-transformed regenerated colonies were abortive and the frequency of transformation to hygromycin B tolerance was lower than that observed for plasmid pGUS5 (approximately 0.1 transformants per μg of pTUBA-GUS DNA). Many of the pGUS5/pGPC1 and pTUBA-GUS/pGPC1 co-transformants were also abortive (65 & 63 %, respectively). The frequency of hygromycin B tolerance arising from co-transformation reactions with either pGPC1 and pGUS5 or pGPC1 and pTUBA-GUS was approximately 0.5 transformants per μg of pGUS5 or pTUBA-GUS DNA.

Therefore, co-transformation with either pGPC1 and pGUS5 or pTUBA-GUS and pGPC1 did not enhance the efficiency of transformation to hygromycin B resistance, compared to transformation with pGUS5 alone. Thirty four of the 'faster growing' hygromycin B tolerant cultures were then selected for further study (Table 3.3).

3.3.2 PCR analysis of transformants

Following a further subculture on hygromycin B selective medium, DNA was extracted from the 34 selected transformants. A PCR screen was performed using primers specific to the *E. coli* GUS gene (*gusA*) and primers specific to the *E. coli* hygromycin phosphotransferase gene (*hph*) to detect plasmid DNA in genomic DNA extracts of hygromycin B tolerant isolates. Fig. 3.2 shows some of the *gusA*- and *hph*-specific PCR results obtained from some of the hygromycin B tolerant isolates. Both GUS-specific and hygromycin B-specific PCR analysis yielded positive results for all of the hygromycin B tolerant isolates, thus indicating the presence of plasmid DNA in genomic DNA extracts. Also, the *gusA* PCR results indicated the presence of pGUS5 or pTUBA-GUS plasmid DNA in the isolates which had been co-transformed with either of these plasmids along with pGPC1. However, since both the plasmid DNA (pGUS5 or pTUBA-GUS) and the co-transformed cosmid pGPC1 DNA contained the *hph* gene, *hph* PCR analysis did not determine whether cosmid pGPC1 DNA was present in the genomic DNA extracts from co-transformants. No further studies were undertaken to determine if pGPC1 DNA had integrated into genomic DNA, and therefore it was not determined if the co-transformed isolates were true co-transformants.

Table 3.2 *Fusarium culmorum* (strain Fu 42) transformation efficiency.

Transforming vector(s)	No. of regenerated colonies[a]	No. of abortive transformants[b]	No. of non-abortive transformants[c]	Transformation efficiency (No. transformants ug^{-1} plasmid DNA)
none	0	-	-	-
pGUS5	90	60	30	0.5
pTUBA-GUS	90	84	6	0.1
pGUS5/pGPC1	85	55	30	0.5[d]
pTUBA-GUS/pGPC1	80	50	30	0.5[d]

[a]Colonies which grew on original selective medium (SYC agar containing 50 μg ml^{-1} hygromycin B). Results based on two replicate transformation reactions per transforming vector(s)

[b]Colonies which failed to grow when transferred to fresh SYC + hygromycin B (50 μg ml^{-1} SYC) agar.

[c]Colonies which grew when transferred to fresh SYC + hygromycin B (50 μg ml^{-1} SYC) agar.

[d]Co-transformation efficiency expressed as No. of transformants μg^{-1} pGUS5 or pTUBA-GUS DNA.

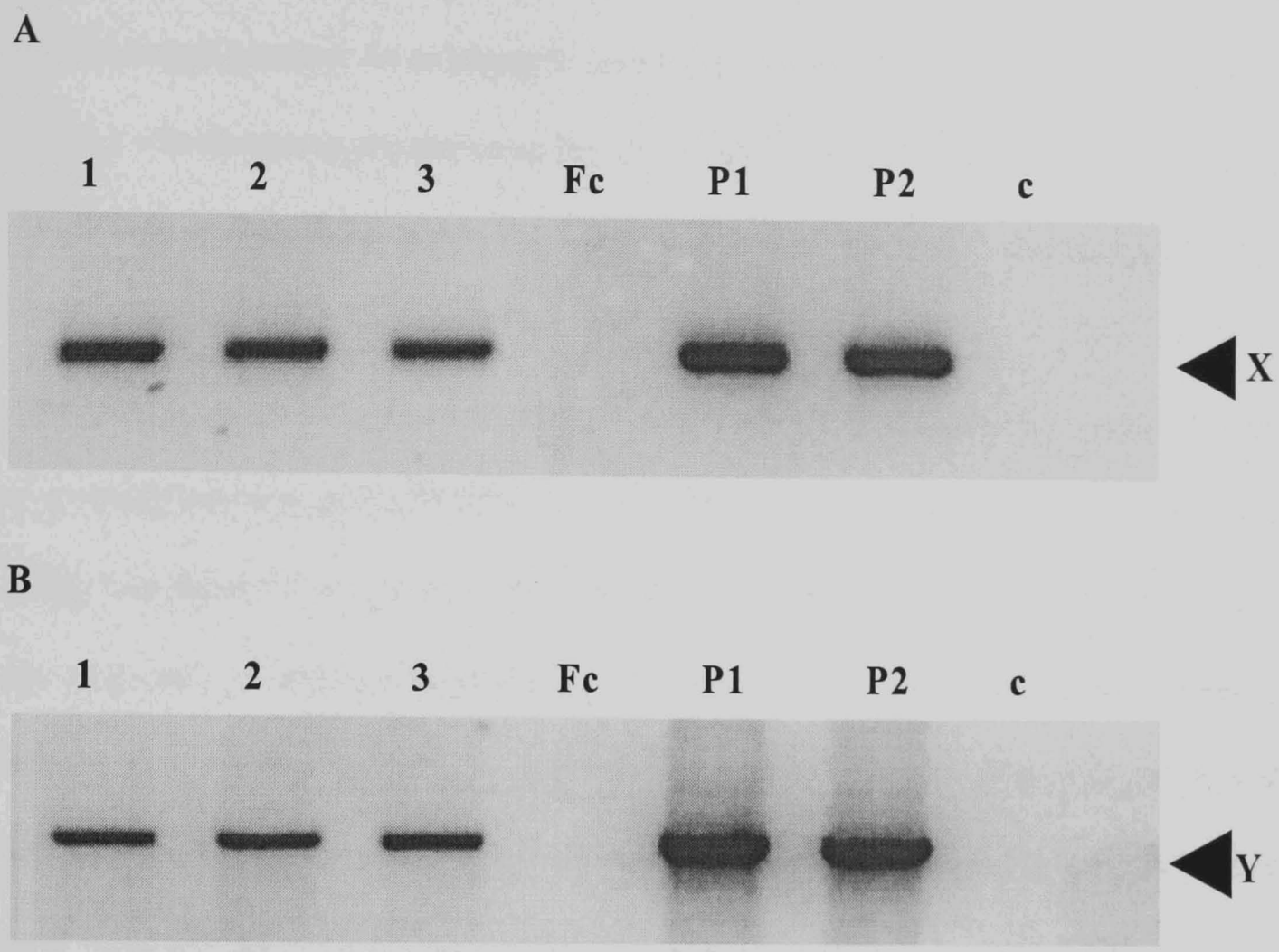


Fig. 3.2 PCR amplification using (A), *Escherichia coli* β-glucuronidase (GUS) gene- (*gusA*) specific primers and (B), *E. coli* hygromycin phosphotransferase gene- (*hph*) specific primers to detect the presence of transforming vector DNA in hygromycin B tolerant transformants. Lanes: 1 & 2, transformants G506 & G508 genomic DNA (transforming vector pGUS5); 3, transformant T516 genomic DNA (transforming vector pTUBA-GUS); Fc, wild type (strain Fu 5) genomic DNA; P1, plasmid pGUS5; P2 DNA, plasmid pTUBA-GUS DNA; c, control (no DNA). Arrows: X, *gusA*-specific PCR band (351 bp); Y, *hph*-specific PCR band (1170 bp).

3.3.3 Southern blot analysis of transformants

Southern blot analysis was used to determine if plasmids carrying the *gusA* gene had integrated into the fungal genome and to determine the pattern of integration of plasmids into the fungal genome. As an example, some of the results obtained for a number of the pGUS5 transformants are shown in Fig. 3.3. This analysis was performed on genomic DNA isolated from wild-type *F. culmorum* isolate Fu 5 and from isolates arising from transformation with either pGUS 5 (14 transformants) or pTUBA-GUS (3 transformants), or co-transformation with either pGUS5 and pGPC1 (8 transformants) or co-transformation with pTUBA-GUS and pGPC1 (9 transformants). As demonstrated in Fig 3.3a (lanes G506-G509), when the undigested DNA from the transformants and the wild-type was hybridized with the a *gusA* gene fragment, hybridization only occurred with high molecular weight DNA, indicating integration of vector DNA into the fungal genome. Similar results were obtained for the other transformants (Table 3.3). The *gusA* gene fragment did not hybridise to the wild-type DNA (Fig. 3.3a, lane Fu 5).

To determine the pattern of integration of pGUS5 or pTUBA-GUS within the transformants, DNA was digested with enzymes which had one site (*Xba* I for pGUS5 and pGPC1, *Sac* I for pTUBA-GUS and pGPC1) within the transforming vector and probed with the GUS gene fragment. Several patterns of integration were observed, examples of which are shown in Fig. 3.3b. In many cases two or three moderately intense hybridization bands were detected, one of which corresponded in size to the linearized transforming vector (pGUS5 or pTUBA-GUS) and the associated bands occurred at larger and smaller sizes (Fig. 3.3c, lanes G506-G508). These results indicated single or multiple tandem copy integrations of the plasmid at the same site, resulting in one or two flanking fragments, depending on the recombination event within

the plasmid (Table 3.3). Smaller intense bands were observed for some transformants (less than 2 Kb), possibly indicating integration within the GUS gene. Seven of the thirty four transformants showed integration of only a single plasmid copy (Fig. 3.3, lane G509). Five of these seven transformants resulted from pGUS5 transformation and two from pTUBA-GUS transformation (Table 3.3). None of these five pGUS5 or two pTUBA-GUS transformants showed hybridization bands of equal size, indicating different sites of integration (results not shown).

3.3.4 Stability of transformants

All transformants were initially subjected to a qualitative MUG assay and all tested positive. Then, after five successive transfers on non-selective medium, hygromycin B tolerance was assessed and compared to growth under non-selective conditions (Table 3.3 & Appendix 3.1). For the fourteen isolates arising from transformation with plasmid pGUS5, growth on selective medium relative to growth on non-selective medium ranged between 16.4 to 81.1 %. Of the three pTUBA-GUS transformants, T515 failed to grow on selective medium and T516 and T517 showed 79.6 % and 39.2 % relative growth, respectively. One of the nine transformants which originated from co-transformation with plasmid pTUBA-GUS and cosmid pGPC1 failed to grow on selective medium, and the other eight such transformants showed between 61.8 and 79.2 % growth on selective medium, relative to non-selective medium. For the eight transformants arising from co-transformation with plasmid pGUS5 and cosmid pGPC1, growth on selective medium was between 9.2 and 33.6 % of the growth on non-selective medium. Twenty of the hygromycin B tolerant isolates were then selected (nine originating from pGUS5

transformation, one from pTUBA-GUS transformation, seven from pGUS5 and pGPC1 co-transformation and three from pTUBA-GUS and pGPC1 co-transformation), and the mitotic stability of these transformants was determined using a qualitative microtitre plate assay for GUS activity. Transformants were considered stable if all eight single spore cultures tested (3 replicates assays/culture) showed GUS expression. Of these twenty transformants, fourteen showed stable inheritance of GUS activity (Table 3.3). All nine of the transformants originating from transformation with plasmid pGUS5 were stable, as was the single transformant originating from transformation with pTUBA-GUS. Four of the seven transformants originating from co-transformation with plasmid pGUS5 and cosmid pGPC1 were stable. None of the three isolates tested which originated from pTUBA-GUS and pGPC1 co-transformation showed stable inheritance of GUS activity.

3.3.5 Quantification of GUS activity

GUS activity was quantified for transformants G501, G506, G508 and G514 (from pGUS5 transformation), T516 (from pTUBA-GUS transformation), GGP531 and GGP532 (from pGUS5/pGPC1 co-transformation) and for wild type Fu 5 (Table 3.4). Background fluorescence detected in the wild type isolate (Fu 5) was extremely low compared with enzyme activity detected in transformants ($\leq$ 0.05 nmol MU min^{-1} mg $protein^{-1}$). Highest activity was detected in transformant G514 (418 nmol MU min^{-1} mg $protein^{-1}$). In contrast, the level of activity detected for transformant GGP531 was extremely low (0.4 nmol MU min^{-1} mg $protein^{-1}$).

Table 3.3 Stability of transformants

Transformant/isolate[a]	Copy number[b]	Hygromycin B tolerance[c] (% relative growth)	GUS stability[d]
G501	> 1	81.1	s
G502	1	24.6	nd.
G503	1	44.7	s
G504	1	20.8	nd.
G505	> 1	16.4	s
G506	> 1	67.0	s
G507	> 1	27.3	nd.
G508	> 1	76.7	s
G509	1	40.3	s
G510	1	40.0	nd.
G511	> 1	42.5	s
G512	> 1	54.9	nd.
G513	> 1	75.4	s
G514	> 1	33.9	s
T515	> 1	00.0	nd.
T516	> 1	79.6	s
T517	1	39.2	nd.
TGP518	> 1	61.6	nd.
TGP519	> 1	74.4	u
TGP520	> 1	79.8	u
TGP521	> 1	00.0	nd.
TGP522	> 1	50.5	u
TGP523	> 1	70.0	nd.
TGP524	> 1	71.1	nd.
TGP525	> 1	72.2	nd.
TGP526	> 1	107.9	nd.
GGP527	1	30.1	u
GGP528	> 1	21.3	nd.
GGP529	> 1	29.2	u
GGP530	> 1	33.6	s
GGP531	> 1	33.6	s
GGP532	> 1	29.7	s
GGP533	> 1	12.4	s
GGP534	> 1	09.2	u
Fu 5 (wild type)	0	0	-

[a]Transforming vector: G, pGUS5; T, pTUBA-GUS; TGP, pTUBA-GUS and pGPC1; GGP, pGUS5 and pGPC1.

[b]Number of genome-integrated plasmid/cosmid copies.

[c]Relative growth of isolates on PDA containing hygromycin B (75 $\mu g\ ml^{-1}$) as compared to growth on PDA, after 5 successive sub-cultures over 5 weeks on non-selective media (PDA). Standard error of the mean (SEM) ≤ 7.4 %. For statistical analysis see Appendix 3.1.

[d]GUS expression: s, stable; u, unstable; nd., no data, -, not applicable.

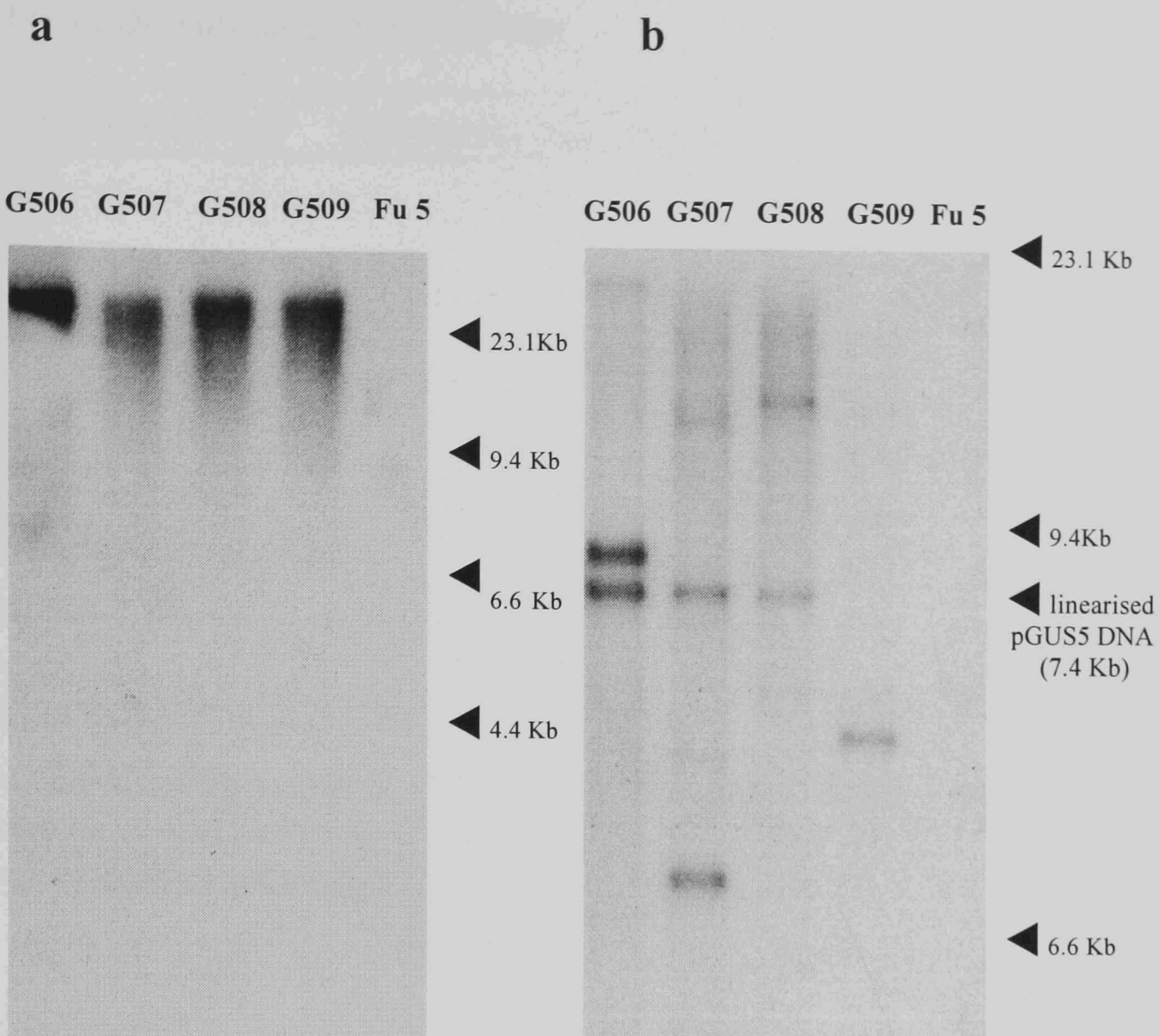


Fig. 3.3 Southern blot analysis of DNA of hygromycin B resistant colonies of *F. culmorum* transformed with plasmid pGUS5. Undigested genomic DNA (a) and genomic DNA digested with Xba I was electrophoresed on a 0.8 % agarose gel, transferred to a nylon membrane, and probed with a ^{32}P-labelled 0.3 Kb region of the *gusA* gene (PCR product, see section 3.2.6). Lanes: transformants G506, G507, G508, G509 DNA (transforming vector pGUS5); Fu 5, wild type control DNA. Arrows indicate molecular markers (kilobases) based on Hind III-digested Lambda DNA and Xba I-linearised pGUS5 DNA (7.4 Kb).

Table 3.4 Quantification of GUS activity.

Transformant/isolate[a]	GUS activity (nmol MU min^{-1} mg $protein^{-1}$)[b] ± S.E.M.[c]
G501	40 ± 4
G506	42 ± 7
G508	228 ± 16
G514	418 ± 39
T516	13 ± 3
GGP531	0.4 ± 0.4
GGP532	156 ± 13
Fu 5	≤ 0.05

[a]See Table 3.3 for transforming vectors.

[b]Mean GUS (β-D-glucuronidase) activity based on three replicates and expressed as nmol MU min^{-1} mg $protein^{-1}$.

[c]S.E.M.: standard error of the mean.

3.3.6 Pathogenicity of transformants

The pathogenicity of six transformants, G501, G506, G508, G514 (from pGUS5 transformation), T516 (from pTUBA-GUS transformation) and GGP531 (from pGUS5 and pGPC1 co-transformation) was determined using both visual disease assessment (Table 3.1 & 3.5) and quantitative *F. culmorum* PCR analysis (Table 3.6) of two replicate glasshouse trials. According to visual disease assessment, although disease scores were significantly higher in the first of the two glasshouse trials ($p < 0.01$, $F = 1154.92$), statistical analysis (based on analysis of variance and Tukey`s pairwise comparison) of the results from both trials indicated no significant difference between the pathogenicity of the wild type isolate (Fu 5) and transformants ($p > 0.1$, $F = 0.69$ & 0.81 for trial I and II, respectively) (Appendix 3.2.1). In contrast to the visual disease assessment results, quantitative PCR results indicated that disease severity (DNA content) in glasshouse trials I and II were not significantly different ($p > 0.1$, $F = 0.00$) and therefore results for the two tests were combined for statistical analysis. Quantitative PCR analysis agreed with the visual disease assessment results in that the statistical analysis indicated that there was no significant difference between the pathogenicity of the wild type isolate (Fu 5) and transformants ($p > 0.1$, $F = 0.48$ for both test combined) (Appendix 3.2.2). All transformants tested expressed GUS during growth in plant tissue, while no activity was detected in uninfected or wild type infected tissue (Fig. 3.4).

Table 3.5 Use of visual disease assessment to evaluate the pathogenicity of transformants

Transformant/isolate[a]	Visual disease score[b] ± S.E.M.[c]	
	Glasshouse trial I	Glasshouse trial II
G501	7.7 ± 0.3	2.4 ± 0.1
G506	7.2 ± 0.2	2.7 ± 0.3
G508	7.5 ± 0.3	2.5 ± 0.1
G514	7.2 ± 0.3	3.1 ± 0.4
G516	7.0 ± 0.3	2.5 ± 0.2
G531	7.4 ± 0.3	2.8 ± 0.3
Fu 5	7.3 ± 0.3	2.6 ± 0.2
Control	0.0 ± 0.0	0.0 ± 0.0

[a]See Table 3.2 for transforming vectors.

[b]Mean disease scores based on nine replicates. See Table 3.1 for stem base disease assessment key.

[c]S.E.M.: standard error of the mean. For statistical analysis see Appendix 3.2.1

Table 3.6 Use of quantitative PCR analysis to evaluate the pathogenicity of transformants.

Transformant/isolate[a]	DNA content (ng/mg plant material)[b] ± S.E.M.[c]
G501	4.0 ± 1.5
G506	5.5 ± 2.2
G508	4.7 ± 2.5
G514	3.5 ± 1.0
G516	2.6 ± 0.7
G531	6.8 ± 3.3
Fu 5	0 ± 0
Control	

[a]See Table 3.2 for transforming vectors.

[b]Based on two glasshouse trials, nine replicates per trial.

[c]S.E.M.: standard error of the mean. For statistical analysis see Appendix 3.2.2.

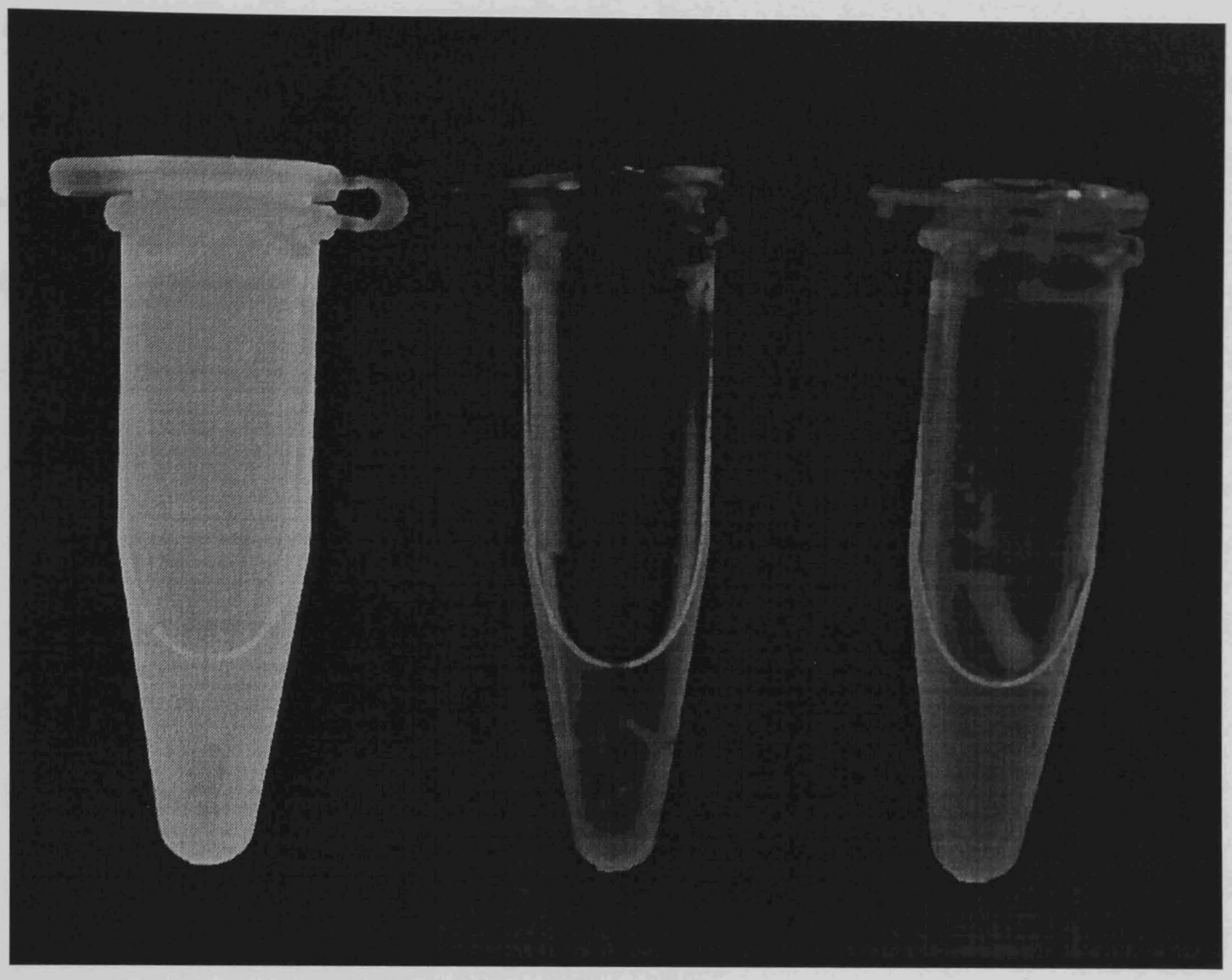


Fig. 3.4 Detection of β-D-glucuronidase (GUS) activity in wheat (cv. Avalon) stem base sections infected with *Fusarium culmorum.* Inoculum: a: GUS transformant G506 (transformed with plasmid pGUS5); b: wild type (strain Fu 5); c: control (uninoculated). GUS activity detected as methylumbelliferone (MU) fluorescense under UV light.

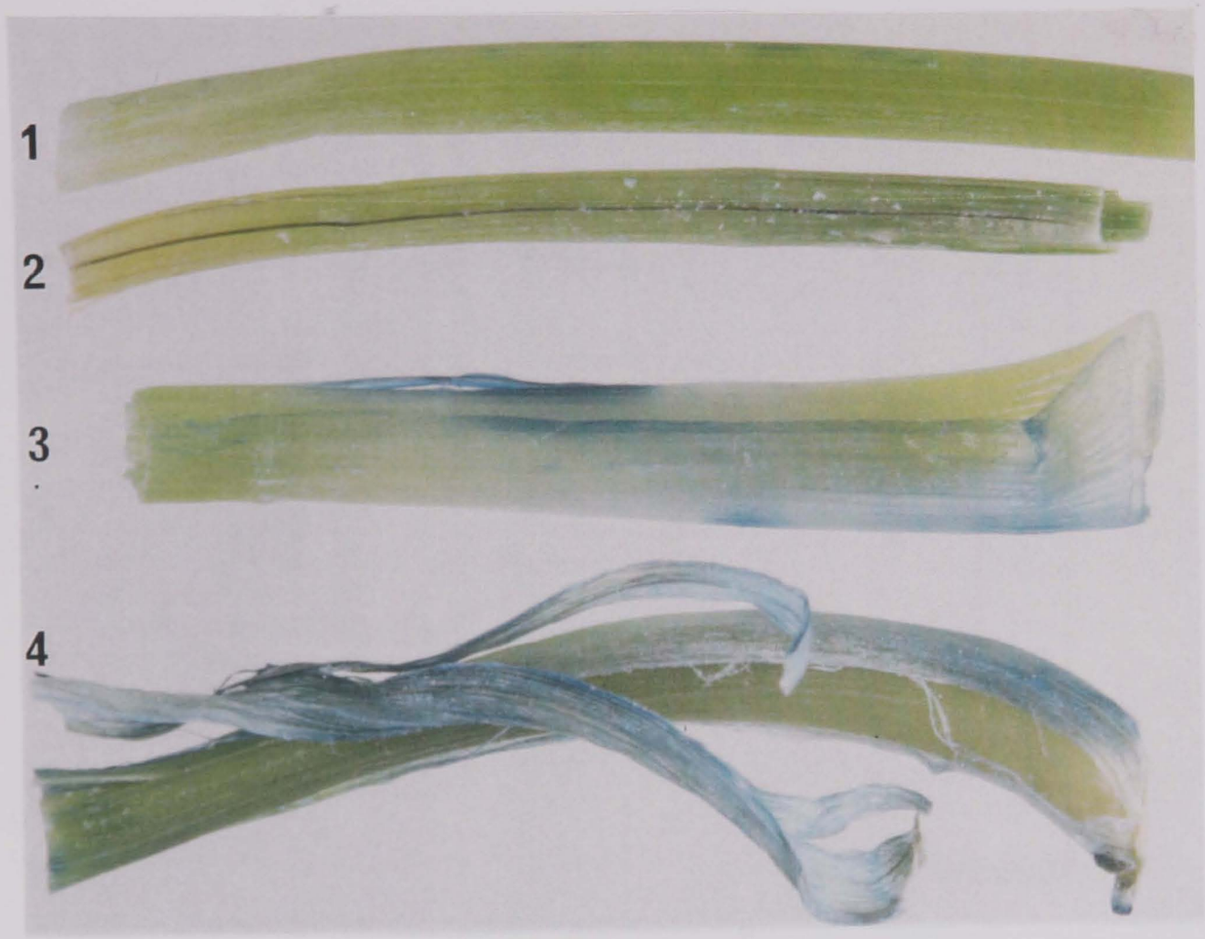
3.3.7 Disease detection and histochemical localization of fungal hyphae

The feasibility of using a GUS transformant (G514, transforming vector pGUS5) to detect disease and to localize, histologically, fungal hyphae in infected wheat (cv. Avalon) seedlings under non-sterile conditions was assessed (Fig. 3.5). Following histochemical staining, a blue colouration was clearly visible , to the naked eye, within diseased tissue on the surface of stem base section infected with GUS transformant G514, but generally not detected on uninoculated and wild-type-inoculated stem base sections (Fig. 3.5a), except for occasional blue dots detected on all tissues. Microscopically, the blue-stained hyphae and spores of the transformed pathogen were visible within both necrotic tissue and often within surrounding asymptomatic stem base tissue (Fig. 3.5b). No such colouration was observed in plant tissue inoculated with wild-type Fu 5. Microscopic examination revealed that the occasional discrete blue dots observed on tissue were due to bacterial contamination and any GUS stained bacterial cells were easily distinguishable from fungal growth.

3.3.8 Evaluation of fungicide efficacy

The object of this experiment was to investigate the feasibility of using a GUS transformant as a screening tool to evaluate the efficacy of fungicides against *F. culmorum* foot rot of wheat under non-sterile conditions. The effect of fungicides on *F. culmorum* stem base disease was assessed by measurement of GUS activity of protein extracts (Fig. 3.6). GUS activity levels were very low for control uninoculated plants and for plants inoculated with wild-type *F. culmorum* (Fu 5), compared to plants inoculated with transformant G514. Statistical analysis of the results (Appendix 3.1) showed that

there were significant differences between the treatments ($F = 15.45$, $p < 0.05$, Appendix 3.3.1) and the use of Tukey`s Pairwise Comparison test (Appendix 3.3.2) showed that, at the 5 % level, there was a significant difference between prochloraz-treated G514-inoculated samples and the G514-inoculated untreated samples as measured by the mean GUS activity (mean GUS activity of 1.07 and 1.95 nmol MU min^{-1} mg $protein^{-1}$) . Although the mean GUS activity of tebuconazole-treated inoculated samples was lower than that of untreated plants (1.48 nmol MU min^{-1} mg $protein^{-1}$), at the 5 % level, it was not significantly different from from untreated samples. However, the GUS activity of tebuconazole-treated inoculated samples was also not significantly different (at the 5 % level) from the GUS activity of prochloraz-treated inoculated samples.



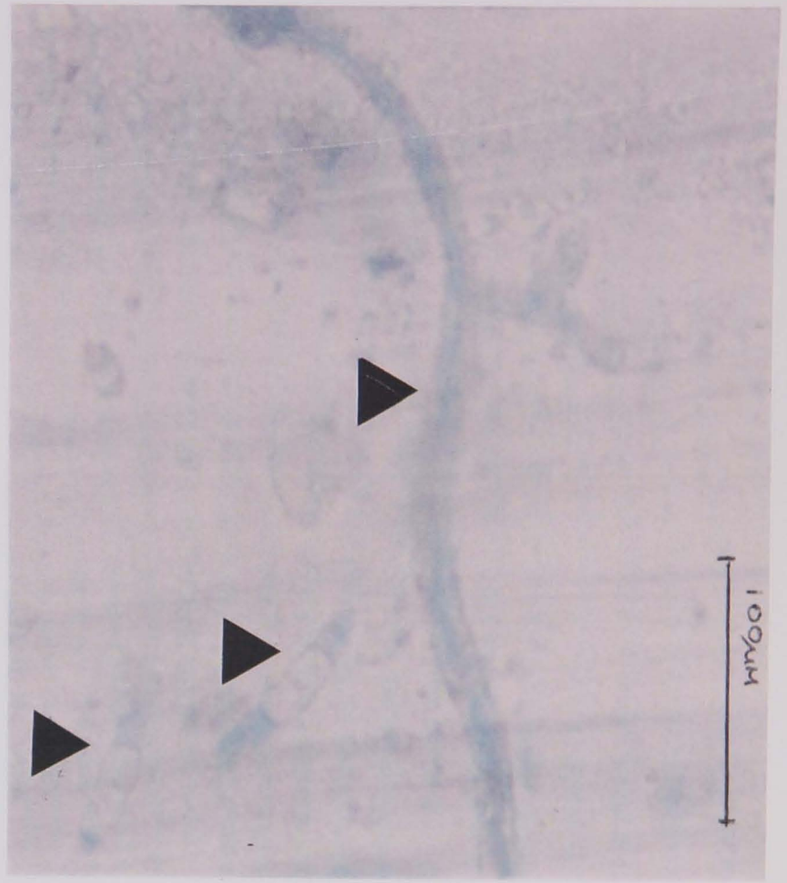


Fig. 3.5 Macroscopic detection of β-D-glucuronidase (GUS) activity in wheat (cv. Avalon) stem base sections infected with *Fusarium culmorum* GUS transformant G514 (transforming vector pGUS5)(a) and microscopic localisation of GUS activity (blue precipitate) in a wheat stem base section infected with transformant G514 (b). Stem base sections in (a) were derived from wheat seedlings inoculated with: 1, water (negative control); 2, wild type *F. culmorum*; 3 & 4, GUS transformant G514. Arrows in (b) indicate localisation of the GUS activity (blue precipitate) within spores and mycelia of transformant G514.

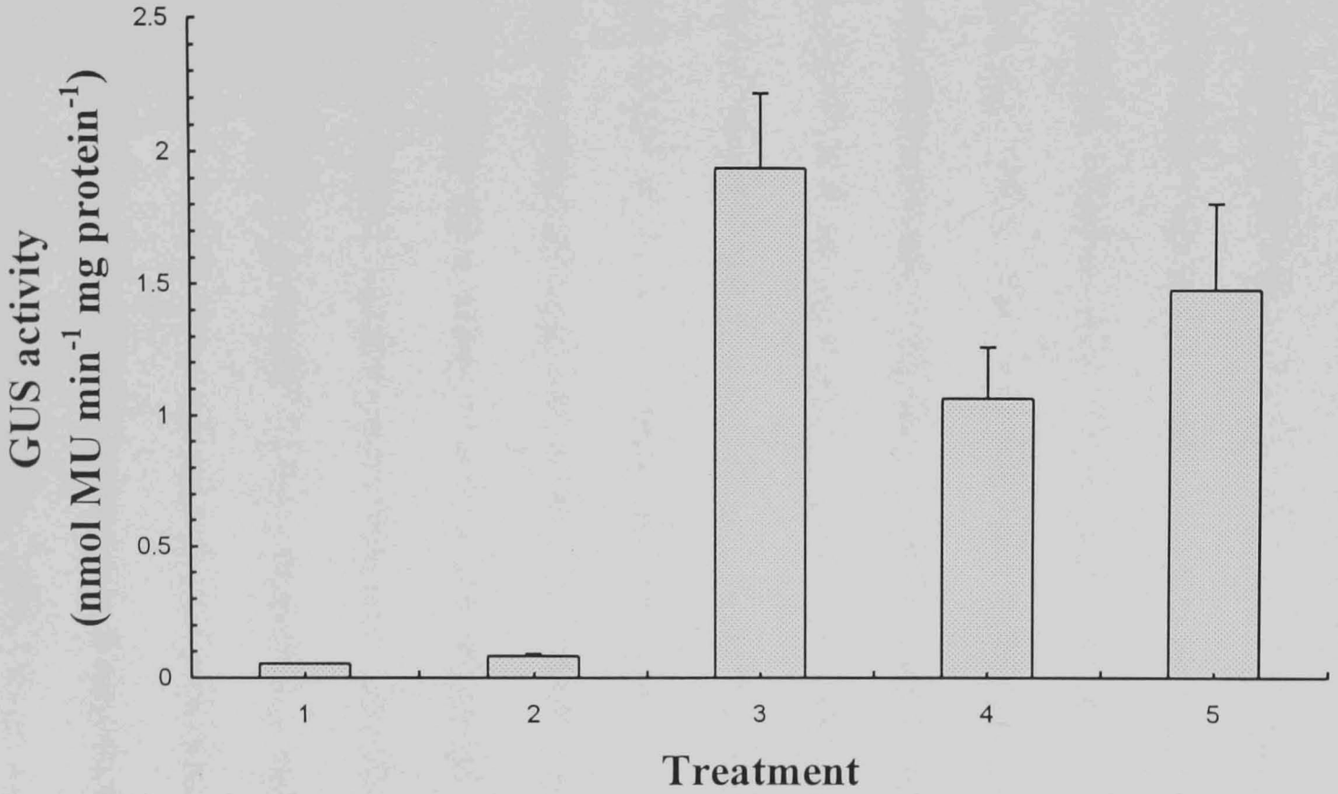


Fig 3.6 Effect of fungicide treatment of *Fusarium culmorum* stem base disease as measured by GUS activity of protein extracts. Treatments: 1, uninoculated untreated control plants; 2, wild type (Fu 5)-inoculated plants; 3, GUS transformant (G514)-inoculated plants; 4, G514-inoculated and prochloraz-treated plants; G514-inoculated and tebuconazole-treated plants. Bars represent the standard error of the mean. For statistical analysis see Appendix 3.3.

3.4 DISCUSSION

F. culmorum was successfully transformed with the *E. coli* β-glucuronidase gene (*gusA*) (GUS) reporter gene, using the *E. coli* hygromycin phosphotransferase gene (*hph*) as a selectable marker. Two plasmids, pGUS5 (Mönke & Schäfer, 1993) and pTUBA-GUS (N. Cooley, pers. comm.), both carrying the *gusA* and *hph* genes, were used to transform *F. culmorum* with the GUS reporter gene. Plasmids pGUS5 and pTUBA-GUS were also co-transformed with cosmid pGPC1 that contained the *hph* gene under the control of a *Gibberella pulicaris* (*F. sambucinum*) promoter fragment (Desjardins *et al.*, 1992). The highest transformation efficiency (to hygromycin B tolerance) was 0.5 transformants μg^{-1} DNA. This was achieved using plasmid pGUS5, while the transformation efficiency achieved using pTUBA-GUS was 0.1 transformants μg^{-1} DNA. In plasmid pGUS5, both the *gusA* and *hph* genes were under the control of the *GPD1* promotor from *C. heterostrophus,* with a transcriptional fusion between *gusA* as well as *hph* and the *GPD1* promoter. *F. culmorum* has previously been transformed to hygromycin B resistance using plasmids pHRC 3 and pAN7-1, both of which contained the *hph* gene flanked by the *A. nidulans GPD1* promoter and *trpC* terminator sequences. However, they achieved low transformation efficiencies with both plasmids (0.1 and 0.04 transformants μg^{-1} pHRC 3 and pAN7-1 DNA, respectively), lower than those achieved with pGUS5. Other workers have reported similar problems of low frequency of transformation of filamentous fungi to hygromycin B tolerance (Cooley *et al.*, 1988; Kistler & Benny, 1988; Dickman & Partridge, 1989; Oliver *et al.*, 1993).

The transformation efficiency to hygromycin B resistance achieved by co-transformation of pGUS5 with pGPC1 (0.5 transformants μg^{-1} pGUS5 DNA) was no greater than that achieved with pGUS5 alone. Co-transformation of pTUBA-GUS with

pGPC1 resulted in 0.5 transformants μg^{-1} pTUBA-GUS DNA, a five-fold increase in the efficiency of transformation to hygromycin B resistance compared with transformation with pTUBA-GUS. But, again, transformation efficiency was no greater than that achieved using pGUS5 alone (0.5 transformants μg^{-1} DNA). Therefore, since pGPC1 did not contain the *gusA* gene and since plasmid pGUS5 seemed most suitable for this and future *F. culmorum* transformation experiments, no studies were undertaken to find out if pGPC1 had integrated into the fungal genome. As a result it was not determined if transformants arising from co-transformation experiments were true co-transformants.

A preliminary PCR screen confirmed the presence of both *hph* and *gusA* gene sequences in genomic DNA isolated from transformants. This screen allowed discrimination between true transformants and any spontaneous mutations to hygromycin B tolerance. Therefore, although this screen did not show whether the vectors had been integrated into the genomic DNA, it provided a simple method for screening a large number of isolates to confirm that vector DNA had been transformed into fungal cells. PCR-based assays have been used to detect gene-disruption events in *Aspergillus* transformants (Aufauvre-Brown *et al.,* 1993; Hohn *et al.,* 1993). According to Aufauvre-Brown *et al.* (1993), use of a PCR-based assay meant that identifying gene disruption events among the more common ectopic integrations was possible approximately four hours after sporulating transformants appeared on selective media.

Southern blot analysis enabled confirmation that vector DNA was integrated into the genomic DNA of the transformants tested. Several patterns of integration were observed in transformants, and most transformants appeared to contain more than one copy of the pGUS5 or pTUBA-GUS vectors. Following successive sub-culturing of transformants in the absence of hygromycin B selective pressure, their stability was

assessed, firstly in terms of hygromycin B tolerance, and secondly in terms of mitotic stability of GUS expression. Only two transformants failed under hygromycin B selective pressure. However, according to Sivan *et al.* (1992) only a low percentage of the nuclei within a mycelium need to be transformed with the *hph* gene to allow growth under hygromycin B selective pressure. Therefore, the mitotic stability of transformants was also assessed, in terms of the stability of GUS expression. Of the transformants tested for the mitotic stability of GUS expression, all the pGUS5 transformants showed stable expression of the enzyme, as did the one pTUBA-GUS transformant tested. However, all three of the pTUBA-GUS/pGPC1 co-transformants and three of the seven pGUS5/pGPC1 co-transformants tested showed unstable expression of the enzyme. As a result, none of the pTUBA-GUS/pGPC1 co-transformants were studied further.

Various levels of GUS activity were observed among a selection of transformants. The levels of GUS activity were comparable to those observed for some *F. oxysporum* transformants (Couteaudier *et al.*, 1993) and higher than those observed for transformants of *C. fulvum* (Roberts *et al.*, 1989), *L. maculans* (Oliver *et al.*, 1993) and *P. brassicae* (Ashby & Johnstone, 1993). However, activity levels were generally lower than those observed for *A. nidulans* transformants (Roberts *et al.*, 1989), and *P. herpotrichoides* transformants (Bunkers, 1991). Of the transformants tested, highest GUS activity was observed for a pGUS5 transformant (G514). Differences in expression may reflect promoter strength (Mönke & Schäfer, 1993), plasmid copy number (Bunkers, 1991) and/or the integration positions of plasmids (Couteaudier *et al.*, 1993). In transient GUS expression studies, Mönke & Schäfer (1993) found that at peak activity, expression due to the *GPD1* promoter was five fold stronger than expression due to another *C. heterostrophus* promoter fragment (*P1*). The same difference in

promoter strength was observed when the vectors were stably integrated into the genome. Bunkers *et al.* (1991) found that for *P. herpotrichoides* transformants, GUS activity levels were highest in transformants with a high *gusA* copy number, but differences in GUS activity levels between transformants may also be related to the site of integration of plasmid(s). Couteaudier *et al.* (1993) found that, in *F. oxysporum* transformants, the highest level of GUS activity occurred in a single plasmid copy transformant. On analysis of the structure of the rescued plasmid, their results indicated that *F. oxysporum* sequences located near the integrated plasmid promoted or enhanced GUS gene activity. As pointed out by Couteaudier *et al.* (1993), the rescue of such sequences could provide alternative plasmids for future *F. culmorum* transformation experiments.

Since the objective was to obtain GUS transformants that could be used to localize and quantify active fungal biomass in plant tissue, it was important that transformation did not affect the pathogenicity of the fungus. No significant differences were observed between the pathogenicity of transformants and the pathogenicity of the wild type isolate, as measured by visual disease symptoms or quantitative PCR. Use of a simple fluorescence assay showed that transformants could be easily detected in wheat stem base tissue at the early stages of disease development. Indeed, such an assay could form the basis of a semi-quantitative disease score index for GUS transformants, thus providing a very simple and rapid screening system for studying host resistance or fungicide efficacy.

Having obtained stable GUS transformants, similar in pathogenicity to the wild-type isolate, the next objective was to examine the potential of detecting and discriminating such an isolate in infected plant tissue under non-sterile conditions.

Following X-glucuronide staining of wheat stem base sections infected with a *F. culmorum* GUS transformant, macroscopic examination revealed blue staining of the necrotic regions and often of the surrounding asymptomatic tissue. Microscopic examination revealed blue stained hyphae and fungal spores. Any colouration due to bacterial contamination was easily distinguishable, particularly at the microscopic level. In contrast wild-type hyphae and spores did not stain blue and were often extremely difficult to differentiate from plant tissue, especially necrotic plant tissue. Therefore, such a GUS-tagged *F. culmorum* isolate could prove extremely useful in studies of fungal infection and colonization of wheat tissue. Oliver *et al.* (1993) showed that histochemical staining of tomato cotyledons infected with *C. fulvum* GUS transformants enabled both macroscopic and microscopic localization of fungal hyphae within plant tissue. Couteaudier *et al.* (1993) found that X-glucuronide-stained hyphae of a *F. oxysporum* transformant were clearly visible on flax root tissues and could be easily observed after different contact times between roots and pathogenic strains. Since a simple staining procedure allows the detection and differentiation of GUS-transformed isolates from their surroundings, it may be possible to combine this and other histochemical techniques to facilitate detailed microscopic studies of the interaction between *Fusarium* species. Also, it may be possible to use these techniques to study the *in planta* interaction between *F. culmorum* isolates differing in pathogenicity.

Use of a *F. culmorum* GUS transformant as a screening tool to evaluate the efficacy of fungicides against *Fusarium* foot rot of wheat showed that, even under non-sterile conditions, the GUS reporter gene system provides a useful tool for such experiments. GUS activity was also detected in uninoculated and wild-type-inoculated wheat stem bases, however, the activity levels were much lower than those detected in

stem base sections infected with the GUS transformant. The most probable source of this background activity was bacterial contamination and/or perhaps autofluorescent of plant tissue. Application of the fungicides prochloraz and tebuconazole to GUS transformant-inoculated plants resulted in decreased GUS activity, although only the decrease in activity due to prochloraz treatment was statistically significant at the five percent level. As seen in Chapter 2, both these fungicides significantly decreased the *F. culmorum* visual disease scores and DNA content of wheat ears inoculated with untransformed *F. culmorum* isolates. Oliver *et al.* (1993) used GUS transformants of *C. fulvum* as a screening tool to differentiate compatible and incompatible interactions between the pathogen and a near isogenic tomato line (cv. Moneymaker) carrying resistance genes (*Cf-3, Cf-5, Cf-9*) or no resistance genes (*Cf-0*).

GUS-transformed plant pathogens could prove extremely useful as screening tools. *F. culmorum* GUS transformants, such as those developed here, could play an important role, not only for evaluating fungicide efficacy, but also for evaluating future biological control agents and in studies of host disease resistance.

4. DEVELOPMENT OF *TRI5*-SPECIFIC PCR AND RT-PCR-BASED ASSAYS: TOOLS FOR THE DETECTION OF TOXIGENIC *FUSARIUM* AND FOR INVESTIGATING THE EFFECT OF FUNGICIDES ON TRICHOTHECENE PRODUCTION BY *FUSARIUM* SPECIES

4.1 INTRODUCTION

Several *Fusarium* species are known to produce trichothecene mycotoxins, including small grain cereal pathogens such as *F. culmorum*, *F. graminearum* (teleomorph: *G. zeae)*, *F. poae*, *F. crookwellense, F. sporotrichoides, F. sambucinum* (teleomorph: *G. pulicaris*) (Desjardins & Hohn, 1997). These mycotoxins have been shown to be naturally occurring in cereal grains (Côté *et al.*, 1984; Tanaka *et al.*, 1988; Lacey, 1990; Ryu *et al.*, 1996) and such contamination of cereals may result in serious economic losses (Moss, 1991) and has been linked to mycotoxicosis in both humans and animals (Joffe, 1978; Ueno, 1980; Marasas *et al.*, 1984). Clinically significant concentrations of mycotoxins have been detected in cereals grains and animal feed (Gareis *et al.*, 1989; Perkowski *et al.*, 1990a,b,c). In Poland, Perkowski *et al.* (1990a,b,c) detected significant concentrations of the trichothecene deoxynivalenol (DON) (up to 40 mg/kg) in wheat kernels, and in a European survey conducted during 1979-1988, DON was detected at concentrations of up to 43.8 mg/kg in maize, and up to 20 mg/kg in wheat (Gareis *et al.*, 1989).

The trichothecenes comprise a large group of closely related sesquiterpene epoxides which inhibit eukaryotic protein synthesis (Fig 4.1) (Desjardins *et al.*, 1993). Details of the trichothecene biosynthetic pathway have been established by experimentation on various *Fusarium* species in several laboratories in the US, Canada and in the UK (Desjardins *et al.*, 1993). Biosynthesis of trichothecenes proceeds from trichodiene (Hohn & Van Middlesworth, 1986; Zamir *et al.,* 1989) through a sequence of complex oxygenations, isomerizations, cyclizations and esterifications leading to the production of more complex trichothecenes including, diacetoxyscirpenol (DAS), T-2 toxin and 3-acetyl deoxynivalenol (3-acetyl DON) (Desjardins *et al.*, 1993). In total, the structure of more than eighty trichothecene biosynthetic pathway intermediates and end-products have been reported (Betina, 1989). Experiments with *F. culmorum, F. sporotrichoides* and *F. sambucinum* (Desjardins *et al.*, 1993) showed that these pathogens share most of the initial scheme of oxygenations and cyclizations in trichothecene biosynthesis and that the branch point between *F. culmorum* and *F. sporotrichoides* appeared to occur after didecalonectrin (Fig. 4.1). *F. culmorum, F. graminearum, F. crookwellense* and *F. sambucinum* produce mainly DON and DAS, while *F. poae* and *F. sporotrichoides* produce mainly T-2 toxin and DAS (Marasas *et al.*, 1984; Lauren *et al.*, 1987).

Isolates of a particular species may also produce different spectrums of trichothecene mycotoxins (Miller *et al.,* 1983a; Ichinoe *et al.*, 1983). In Japan, Ichinoe *et al.* (1983) showed that trichothecene production could not be detected in a small proportion of their *G. zeae* isolates collected during 1981. Most of their *G. zeae* isolates did produce trichothecenes and could be divided into two chemotaxonomic groups:

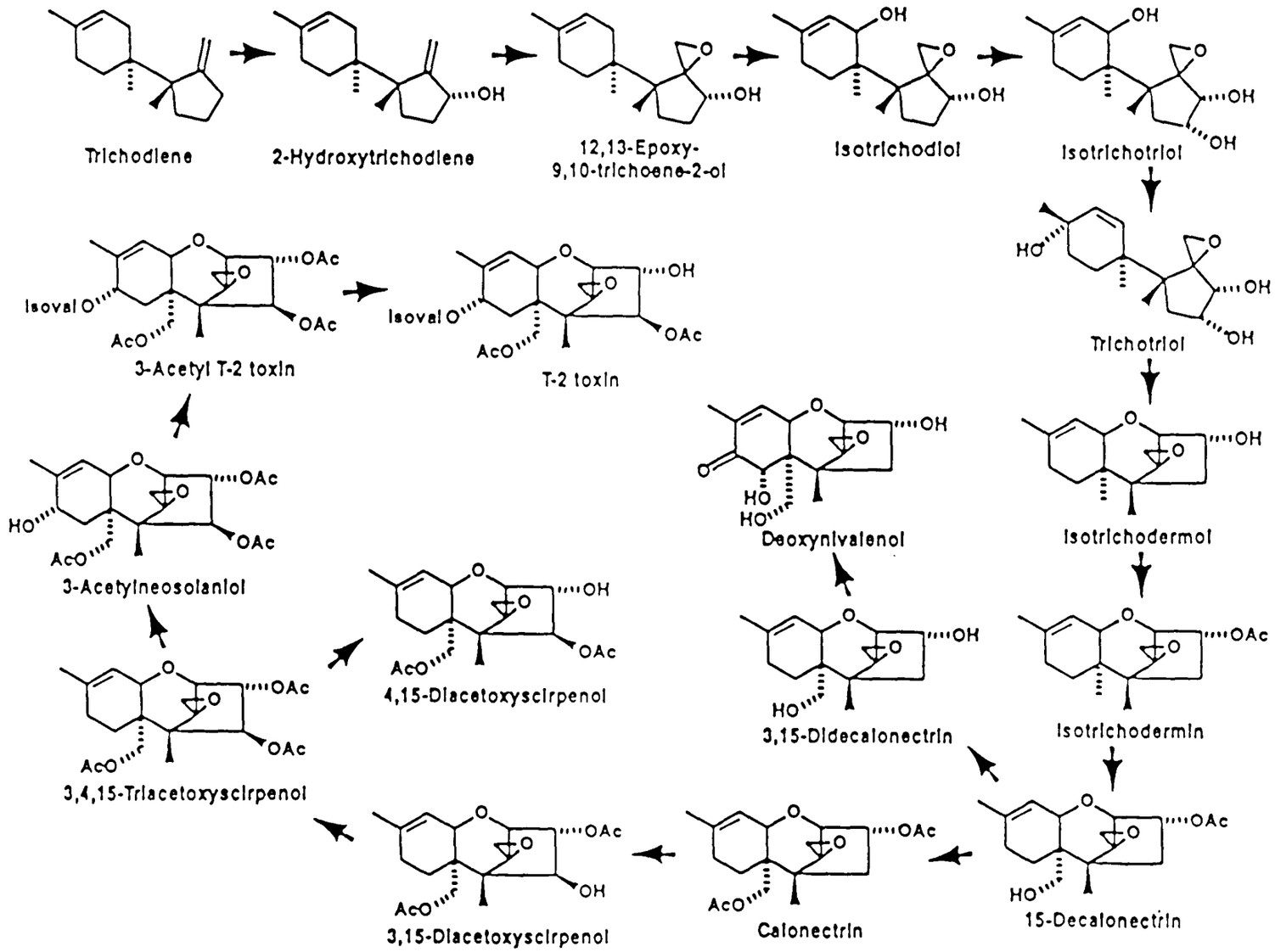


Fig. 4.1 Trichothecene biosynthetic pathway in *Fusarium* species.
After Desjardins *et al.* (1993).

nivalenol and fusarenone-X producers and DON and 3-acetyl DON producers. No cross production of these two types of trichothecenes was observed in isolates. Miller *et al.* (1983a) showed that there was a widely varying potential for DON accumulation and toxin profile between *F. graminearum* isolates.

Environmental factors may also influence the toxin profile of a particular trichothecene-producing species or isolate (Miller *et al.*, 1983a; Ramakrishna *et al.*, 1996). Miller *et al.* (1983a) found that aeration, i.e. oxygen and/or carbon dioxide and nutrients influenced toxin production by *F. graminearum*. Ramakrishna *et al.* (1996) studied the effect of water activity (a_w), temperature and competition on the ability of an isolate of *F. sporotrichoides* to produce the trichothecene T-2 toxin. They found that T-2 toxin production occurred at 20 ^{0}C, but not at 30 ^{0}C, and at 0.97 or 0.95 a_w, but not at 0.90 a_w. They also found that, under most environmental conditions tested, T-2 toxin production was significantly greater in the presence of either of the filamentous fungi *Aspergillus flavus* or *Penicillium chrysogenum*, and slightly decreased in the presence of the yeast *Hyphopivhia burtonii*, throughout a three week period.

There is controversy regarding the effect of fungicide treatments on trichothecene production by *Fusarium* species. The fungicide tebuconazole has been regarded as being among the most effective chemicals for controlling FEB of wheat (Hutcheon & Jordan, 1992; Suty *et al.*, 1996), and has been associated with the control of naturally occurring FEB of wheat accompanied by a simultaneous decrease in the concentrations of the trichothecene DON in infected grain (Suty *et al.*, 1996). However, Gareis & Ceynowa (1994) found that although a combination of the fungicides tebuconazole and triadimenol reduced *F. culmorum* ear blight of wheat in the field, this

treatment resulted in a 16-fold increase in the nivalenol content of grains. Martin & Johnston (1982) showed that although the sterol biosynthesis inhibiting (SBI) fungicide propiconazole reduced *Fusarium* disease in the field, the concentration of the mycotoxin DON was not significantly reduced in treated plots. However, in ear blight field trials, Boyacioglu *et al.* (1992) found that the two SBI fungicides propiconazole and triadimefon decreased *F. graminearum* ear blight disease by 39-61 % and also decreased DON concentrations by 34-79 %, while the benzimidazole fungicide thiabendazole had no effect on disease but decreased DON concentrations by 83 %. *In vitro,* Moss & Frank (1985) found that the fungicide tridemorph (6-8 ppm) inhibited the production of T-2 toxin by *F. sporotrichoides* and enhanced fungal growth slightly. However, at higher concentrations (30-50 ppm) the fungicide inhibited fungal growth by approximately 50 %, but stimulated T-2 toxin production.

One of the problems with studying the effect of fungicides on trichothecene production by *Fusarium* species may stem from the fact that there are up to eighty intermediates of the trichothecene biosynthetic pathway which are known (Betina, 1989) and many of the methods used to analyse toxin production are based on the detection of a limited number of specific compounds. Various chemical methods such as preparative thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography/mass spectroscopy (GC-MS) have been used to detect specific trichothecenes in *Fusarium*-infected cereal grains (Miller *et al.*, 1983a & b, 1985; Visconti *et al.*, 1990; Ryu *et al.*, 1996; Schaafsma *et al.*, 1993). Immunological methods have also been developed for the detection of trichothecene mycotoxins, such as the dipstick enzyme immunoassay developed by De Saeger & Van

Peteghem, (1996) for the detection of *Fusarium* T-2 toxin in cereals. All these methods, both chemical and immunological, are usually used to detect a small proportion of the products of the trichothecene biosynthetic pathway. However, it is possible that fungicide treatment could cause 'shunting' between different branches of the pathway, resulting in the accumulation of different pathway products to those normally produced by a particular pathogen in the absence of fungicide treatment. Such 'shunting' within the trichothecene biochemical pathway may explain why treatment with the fungicide tebuconazole has been associated with decreases in DON concentrations of infected grains (Suty *et al.*, 1996), but has also been associated with increases in nivalenol concentrations of infected grains (Gareis & Ceynowa, 1994). If treatment with fungicides can cause such 'shunting', it would require chemical or immunological analysis of a much wider variety of the products of the trichothecene biosynthetic pathway to reveal the true effect of fungicides on trichothecene production.

One way of overcoming problems associated with the multitude of potential trichothecene mycotoxins which may be produced by a potential trichothecene producer is to follow the flow through the trichothecene biosynthetic pathway using gene expression studies. Northern blot analysis (Farrell, 1993) has been widely used for studying gene expression. This technique involves the electrophoresis of RNA extracts from samples of interest, transfer of nucleic acid to nylon membranes which are then incubated with labelled (usually radioactively) DNA or RNA probes of the gene of interest. Gene expression is detected (by autoradiography) through hybridization of probes to RNA templates within the membrane-bound extracts (Farrell, 1993). Therefore in order to obtain the appropriate probes for such analysis, some knowledge

of the genetics and biochemical pathway of interest is essential.

To date, ten genes involved in trichothecene biosynthesis in *F. sporotrichoides* have been localised to a 25 kb region of chromosomal DNA and the characterisation of this trichothecene gene cluster is ongoing (Desjardins *et al.,* 1993). The single copy gene, *Tri5,* codes for the enzyme trichodiene synthase (Desjardins *et al.*, 1993) which catalyses the first step in the trichothecene biosynthetic pathway, which involves the isomerisation-cyclization of farnesyl pyrophosphate (FPP), resulting in the formation of the alicyclic hydrocarbon trichodiene (Hohn & Beremand, 1989). The sequence of the *Tri5* gene has been deduced for *F. culmorum* (Smith, 1997), *F. graminearum* (Proctor *et al.*, 1995a), *F. poae* (L. Hornock, pers. comm.), *F. sporotrichoides* (Hohn & Beremand, 1989) and *F. sambucinum* (Hohn & Desjardins, 1992) (Fig. 4.2). Proctor *et al.* (1995a) disrupted the *Tri5* gene of *G. zeae* (*F. graminearum*) via fungal transformation and experimentation with these transformants in wheat pathogenicity tests suggested that trichothecene production contributes to the virulence of *G. zeae* on some cereal hosts. Three of the other genes involved in trichothecene biosynthesis, *Tri3, Tri4* and *Tri6*, have been studied in detail in *F. sporotrichoides*, but not in other *Fusarium* species (Hohn *et al.*, 1995; Proctor *et al.*, 1995b; McCormick *et al.*, 1996). *Tri3* encodes a transacetylase that converts 15-decalonectrin to calonectrin (McCormick *et al.*, 1996) and *Tri4* encodes a cytochrome P-450 monooxygenase that converts trichodiene to 2-hydroxytrichodiene (Hohn *et al.*, 1995) (Fig. 4.1). Hohn *et al.* (1993) showed that *Tri3, Tri4* and *Tri5* genes are clustered within a 9-kb region of the *F. sporotrichoides* genome. *Tri6* encodes an unusual zinc finger protein which is required for pathway gene expression (Proctor *et al.*, 1995b).

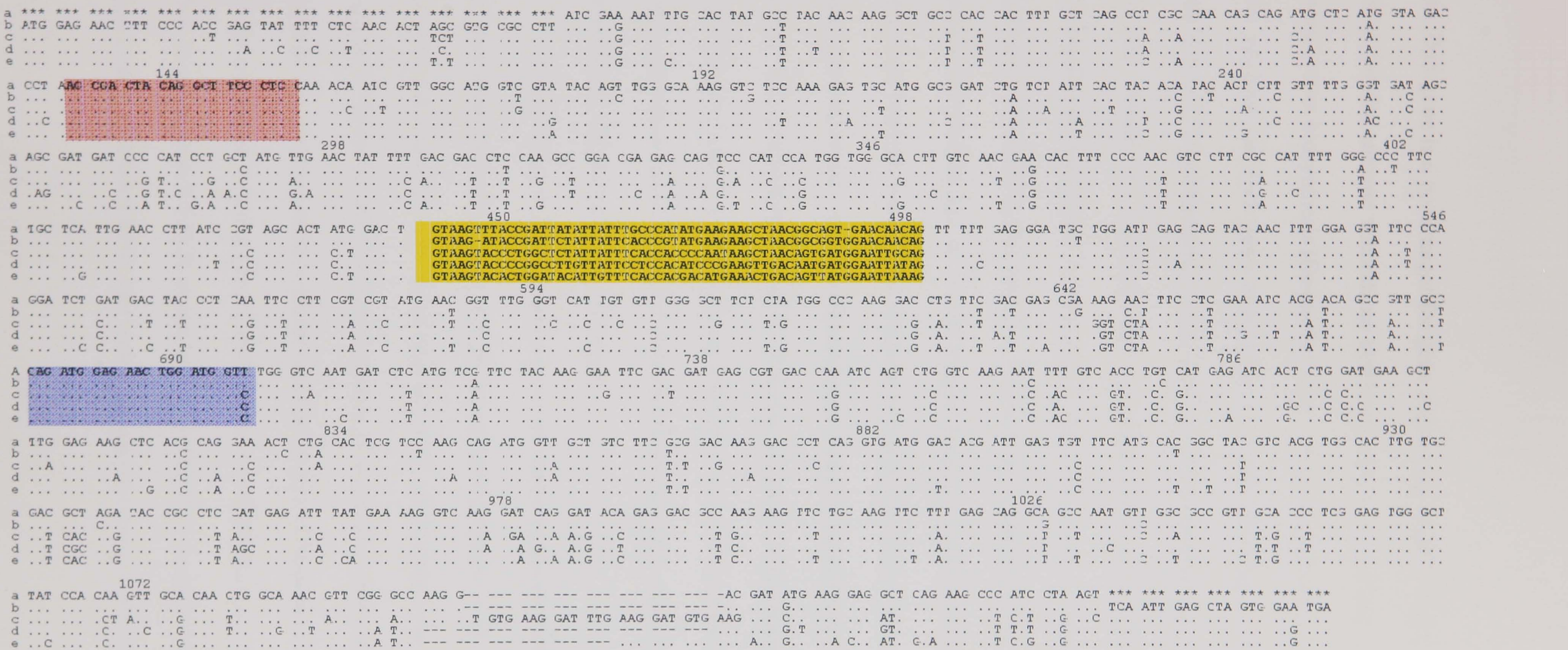


Fig. 4.2 Comparison of the nucleotide sequence for the trichodiene synthase (*Tri5*) gene from from various *Fusarium* species. Species: a, *F. culmorum* (Smith, 1997); b, *Gibberella zeae* (*F. graminearum*) (Proctor *et al.*, 1995a); c, *G. pulicaris* (*F. sambucinum*) (Hohn & Desjardins, 1992); d, *F. sporotrichoides* (Hohn & Beremand, 1989); e, *F. poae* (L. Hornock, pers. comm.). Codes: * nucleotides not yet deduced for *F.culmorum*, - nucleotides deleted in a given species; . identical nucleotide to the one stated for *F.culmorum* except where this has not yet been deduced (in which case comparison is made with *F.graminearum*). Red and blue boxes indicate the sequences of the *Tri5* forward and reverse primers (Tr5F/R) and the yellow box denotes the intron sequence. After: Smith (1997).

The *Tri5* gene is a good target for gene expression studies, since, as pointed out, it codes for the enzyme trichodiene synthase, which is involved in the first step of trichothecene biosynthesis (Desjardins *et al.*, 1993). Hohn *et al.* (1993) used northern blot analysis of *Tri5* gene expression to study trichothecene production in *F. sambucinum*. "Housekeeping" genes which are expressed at approximately constant 'per-cell' levels, i.e. constitutively expressed, such as β-tubulin, β-actin and glyceraldehyde phosphate dehydrogenase have been used as endogenous internal standards for northern blot analysis. Hohn *et al.* (1993) did not standardize their samples, but workers such as van der Vlught-Bergmans *et al.* (1997) used the β-tubulin gene as an internal standard for northern blot analysis of *Botrytis cinerea* catalase A (*catA*) gene expression studies. A limited experiment performed by L. Boyd (John Innes Centre, UK, unpublished) and C. Placinta (Scottish Agricultural College, UK, unpublished) using *F. sporotrichoides* total RNA extracts harvested at different time points post-inoculation indicated that the β-tubulin gene may be approximately constitutively expressed by this pathogen.

An alternative technique known as reverse transcription PCR (RT-PCR) analysis has been widely used in human and animal research (Murphy *et al.*, 1990; Horikoshi *et al.*, 1992; Reiner *et al.*, 1993; Kan-Mitchell *et al.*, 1993; Klebe *et al.*, 1996). RT-PCR analysis is based on the reverse transcription of mRNA using oligo dT, random hexamers or a primer specific to the downstream region of the gene of interest, followed by PCR amplification using gene-specific primers (Gilliland *et al.*, 1990; Rashtchian, 1994). Because these RT-PCR assays have been shown to be 1000-10,000 times more sensitive than traditional RNA blot techniques (Byrne *et al.*, 1988; Wang *et al.*, 1989;

Morcharla *et al.*, 1990), it may be possible to develop a *Tri5*-specific RT-PCR-based assay for more sensitive detection of *Tri5* gene expression. To date, the use of RT-PCR-based assays in plant pathology has been limited (Stewart *et al.*, 1992; Jones & Dunkle, 1995; Lamar *et al.*, 1995; Bogan *et al.*, 1996a,b). Jones & Dunkle (1995) developed an RT-PCR-based assay for the analysis of a *Cochliobolus carbonum* race 1 multi-functional peptide synthetase gene (*HTS1*), an enzyme which is involved in the synthesis of a host-selective toxin.

"Housekeeping" genes have also been used in RT-PCR-based assays as internal controls to account for RNA degradation and variation in reverse transcription efficiency (Jones & Dunkle, 1995, Bogan *et al.*, 1996a,b). Ideally the gene-specific primers for both the target template and internal control 'housekeeping' gene should be chosen so that they flank at least one intron within the genes. Amplified transcript is then readily distinguishable from amplified genomic DNA in subsequent RT-PCR assays. Fortunately, the *Tri5* genes which have been sequenced all contain a 59-61 bp intron, depending on *Fusarium* species of origin (Smith, 1997) (Fig. 4.2). Also, although the sequences of the β-tubulin genes from many of the *Fusarium* species of interest are not known, the sequence of the *G. pulicaris* (*F. sambucinum*) gene is known to contain several introns (Genbank accession number U27303).

Non-competitive RT-PCR-based assays have been used for quantification and semi-quantification of gene transcripts (Kellogg *et al.*, 1990; Horikoshi *et al.*, 1992; Kinoshita *et al.*, 1992; Kan-Mitchell *et al.*, 1993). These RT-PCR-based systems were semi-quantitative in that they estimated relative rather than absolute quantities of gene expression. The relative gene expression was determined by comparing the ratio of PCR

products generated by simultaneous or separate amplification of the target cDNA segment and "housekeeping" cDNA. Horikoshi *et al.* (1992) developed such a semi-quantitative system for analysing thymidylate synthase, dihydrofolate reductase and DT-diaphorase gene expression relative to β-microglobulin and β-actin gene expression in RNA extracts from human tumour specimens. Kan-Mitchell *et al.* (1993) developed a semi-quantitative system to study the expression of a calcium-modulated protein (S100β) relative to β-actin gene expression in RNA extracts from choroidal and skin melanomas.

Quantitative RT-PCR-based assays have been developed for analysis of manganese peroxidase, cellobiohydrolase and lignin peroxidase gene expression in the white rot fungus *Phanerochaete chrysosporium* (Stewart *et al.*, 1992; Lamar *et al.*, 1995; Bogan *et al.*, 1996a,b). These assays relied on the presence of introns within the genomic DNA of the target gene sequences. This involved the addition of competitor templates: DNA fragments produced by PCR amplification of genomic DNA (which contained introns) using the gene-specific primers used for cDNA amplification. A series of dilutions of competitor template (genomic DNA) of known concentration were added to reactions containing a constant amount of cDNA, and introns within the competitive templates enabled size fractionation of the target cDNA product and genomic DNA product by agarose gel electrophoresis. Riedy *et al.* (1995) developed a technique for preparing an RNA competitive reference standard (RNA-CRS) template, synthesised using non-plasmid-based PCR techniques. The advantage of such a system over a DNA competitive template is that the RNA-CRS template and target RNA template simultaneously undergo both reverse transcription and PCR analysis.

Therefore it reduces the variation due to reverse transcription efficiency. The disadvantages of this system include the fact that the RNA-CRS template is more difficult to prepare and more labile than a DNA competitive template.

Comparison of the *Tri5* gene sequences of *F. culmorum, F. graminearum, F. poae, F. sporotrichoides* and *F. sambucinum* has shown that there is a high degree of homology between the genes from the different species (Smith, 1997). Therefore, it should be possible to design primers to conserved regions of the *Tri5* gene which, in theory, could not only be used for reverse transcription polymerase chain reaction analysis (RT-PCR) of trichothecene expression, but could also be used in *Tri5*-specific PCR analysis for the identification of potential trichothecene-producing *Fusarium* species. Several PCR-based assays have been developed for the identification of *Fusarium* species, including several potential trichothecene-producing *Fusarium* species; *F. culmorum* (Schilling *et al.*, 1996, Nicholson *et al.*, unpublished), *F. graminearum* (Ouellet & Seifert, 1993; Schilling *et al.*, 1996; Nicholson *et al.*, unpublished), *F. poae* (Parry & Nicholson, 1996) and *F. crookwellense* (Turner *et al.*, unpublished). The species-specific primers for these PCR-based assays were derived from random amplified polymorphic DNA (RAPD) fragments which were cloned and sequenced. In general, species-specific primers for diagnostic PCR analysis of plant-pathogenic fungi have been based upon variable portions of the internal transcribed spacer regions of rDNA (Poupard *et al.*, 1993; Moukhamedov *et al.*, 1994). Although the *Fusarium* PCR assays enable identification and differentiation of trichothecene-producing *Fusarium* species, PCR primers designed to conserved regions of the *Tri5* gene could provide a simple PCR-based assay for the detection of potential

trichothecene-producing *Fusarium* species in plant tissue samples. This assay could be particularly useful in cases where the presence of potential trichothecene-producing *Fusarium* species in infected cereals is the important factor, rather than the individual species involved in the disease. Use of such an assay as a preliminary screen for the detection of potential trichothecene-producing *Fusarium* species in cereal crops could reduce the time and cost involved in subsequent chemical detection (Miller *et al.*, 1983a & b, 1985; Visconti *et al.*, 1990; Ryu *et al.*, 1996; Schaafsma *et al.*, 1993) or immunological detection (De Saeger & Van Peteghem, 1996) of trichothecene mycotoxins.

The objective of this work was to develop a semi-quantitative *Tri5*-specific RT-PCR-based assay, in which the level of *Tri5* gene expression in total RNA extracts was expressed relative to that of the endogenous internal control "housekeeping gene", β-tubulin. The potential of this RT-PCR-based assay for studying the effect of fungicides on trichothecene biosynthesis, as measured by *Tri5* gene expression, was investigated. During the course of this work, a *Tri5*-specific PCR-based assay was developed for the detection of potential trichothecene-producing *Fusarium* species in infected cereals.

4.2 MATERIALS & METHODS

4.2.1 Origin and maintenance of fungal isolates

Isolates of *Fusarium* species used in this study were obtained from the John Innes Centre facultative pathogen culture collection (Table 4.1). Media used for maintenance, subculturing of isolates and for DNA preparation were as described earlier (Chapter 1, section 1.2.2). The media used for fungicide-amended cultures contained: glucose, 10.0 g l^{-1}; yeast extract, 1.0 g l^{-1}; peptone, 1.0 g l^{-1} (GYEP) (Glass & Donaldson, 1995). *F. culmorum* (strain Fu 42) was used as inoculum for shake flask liquid cultures used in the fungicide experiment and the conidial suspension (1 x 10^6 conidia ml^{-1}) was prepared as described earlier in Chapter 2, (section 2.2.2).

4.2.2 Infected plant material

Wheat plants (cv. Avalon) were cultivated, inoculated with mixed isolates of *F. culmorum* or *F. poae* (Table 4.1) and harvested as described for the 1996/7 fungicide glasshouse experiment in Chapter 2 (section 2.2.2). *F. graminearum* (strain Z 3639) infection of wheat plants (cv. Avalon) was carried out by P. Smith, using the same cultivation, inoculation and harvesting conditions. At harvest, wheat ears from the *F. graminearum* experiment were divided into grain, glume and rachis components.

Table 4.1 Code and origin of fungal isolates

Isolate	Code	Origin	*Tri5* amplification[c]
Fusarium culmorum[b]	Fu 3	UK	+ +
F. culmorum[a]	Fu 5	UK	+ +
F. culmorum[ab]	Fu 15	UK	+ +
F. culmorum[ab]	Fu 42	UK	+ +
F. culmorum[b]	Fu 60	UK	+ +
F. culmorum[a]	F 77	France	+ +
F. culmorum[a]	F 200	France	+ +
F. culmorum[a]	F 400	France	+ +
F. graminearum[a]	F 86	France	+ +
F. graminearum[a]	F 500	France	+ +
F. graminearum[a]	F 508	France	+ +
F. graminearum[a]	F 604	France	+ +
F. graminearum[a]	F 700	France	+ +
F. graminearum[a]	F 740	France	+ +
F. graminearum[ab]	Z 3639	USA	+ +
F. poae[ab]	Fu 53	UK	+ +
F. poae[b]	F 18	UK	+ +
F. poae[a]	F 62	Poland	+ +
F. poae[ab]	CSL 8	UK	+ +
F. poae[ab]	4/3084	UK	+ +
F. poae[a]	4/3155	UK	+ +
F. poae[a]	4/4343	UK	+ +
F. crookwellense[a]	C 108	Poland	+ +
F. crookwellense[a]	C 233	Poland	+ +
F. crookwellense[a]	C 581	Poland	+ +
F. crookwellense[a]	C 582	Poland	+ +
F. crookwellense[a]	C 804	Poland	+ +
F. crookwellense[a]	C 1160	Poland	+ +
F. sambucinum[a]	F 64	France	+ +
F. sambucinum[a]	F 108	Poland	+ +
F. sambucinum[a]	F 111	Poland	+ +
F. sambucinum[a]	F 133	France	+ +
F. sambucinum[a]	F 153	Germany	+ +
F. sporotrichoides[a]	F 95	Poland	+ +
F. sporotrichoides[a]	F 627	France	+ +
F. avenaceum[a]	Fu 17	UK	+
F. avenaceum[a]	76-1	Canada	-
F. avenaceum[a]	78-1	Canada	-
F. avenaceum[a]	96UKR2	UK	-
F. avenaceum[a]	FARS 41.1	France	-
F. avenaceum[a]	239	UK	-
F. tricintum[a]	F 308	France	-
F. tricintum[a]	F 402	France	-
F. moniliforme[a]	A 3733	USA	-
F. moniliforme[a]	B 3853	USA	-
F. moniliforme[a]	C 53	USA	-
F. moniliforme[a]	C 93	USA	-
F. moniliforme[a]	C 95	USA	-
F. moniliforme[a]	D 3736	USA	-

[a]Isolates used to test specificity of *Tri5* PCR primers (Tr5F/R).

[b]Isolates used for inoculation of wheat ears in glasshouse trials.

cDetermined by *Tri5*-specific PCR analysis: + + strong PCR amplification signal detected; + weak PCR amplification signal detected; - no PCR-amplified signal detected.

4.2.3 Fungicide experiment

Erlenmeyer flasks containing 100 ml of sterile GYEP broth (section 4.2.1) were aseptically inoculated with 0.5 ml of a *F. culmorum* (strain Fu 42) conidial suspension, to a final concentration of approximately 5 x 10^4 conidia per ml of media. Flasks were incubated at 25 ^{0}C, 150 rpm. After 24 h, the fungicides prochloraz and tebuconazole (see Chapter 2, Table 2.2) were added to the appropriate flasks, in a volume of 0.5 ml. Two concentrations (2 and 8 µg a.i. ml^{-1} media) of both fungicides were examined. Water (0.5 ml) was added to control cultures. Two cultures per treatment were harvested 24, 36, 48 & 96 h post-inoculation (0, 12 , 24 & 72 h after addition of fungicides). Mycelium was freeze-dried, transferred to 10 ml flat-ended tubes, and dry weight recorded. Dried mycelium was then stored at - 70 ^{0}C prior to RNA extraction.

4.2.4 DNA and RNA extraction

DNA was extracted from fungal cultures and from grains of *F. graminearum*-inoculated wheat ears as described previously (Chapter 1, section 1.2.3) and DNA was extracted from the combined grain, glume and rachis of *F. culmorum*- and *F. poae*-inoculated wheat ears using the procedure outlined in Chapter 2 (section 2.2.4). Total RNA was extracted using a modification of the method described by Logemann *et al.* (1987). Mycelium was taken from -70 ^{0}C storage and immediately ground to a fine powder using a mill (milling time approximately 1 min), as described previously for DNA extraction (Chapter 1, section 1.3.3). Approximately 1 ml extraction buffer [8 M guanidine hydrochloride, 20 mM MES (4-morpholineethan-sulfonic acid), 20 mM

EDTA and 50 mM β-mercaptoethanol, pH 7.0] (Logemann *et al.*, 1987) was added per 50-100 mg dry weight of mycelium and this mixture was milled for an additional 30 s. The extract was transferred to 15 ml tubes and an equal volume of phenol/chloroform (5:1) (Sigma, UK) was added to the tubes, which were vortexed for approximately 1 min and centrifuged at 2600 xg for 20 min. The aqueous phase was removed to a fresh tube and re-extracted with an equal volume of phenol/chloroform (1:1) using the vortexing and centrifugation conditions described above. Following this, the aqueous phase was re-extracted with an equal volume of chloroform using the vortexing and centrifugation conditions as described above. The aqueous phase was then collected into fresh 10 or 30 ml Nalgene polypropylene tubes (Nalge, UK) and mixed with precooled 0.7 volume of ethanol and 0.2 volume of 1 M acetic acid. Tubes were incubated overnight at -20 ^{0}C to precipitate the RNA, which was then pelleted by centrifugation at 10,000 xg for 10 min. The pellet was washed twice with sterile 3 M sodium acetate, pH 5.2 at room temperature and salt was removed by washing with 70 % (v/v) ethanol followed by centrifugation as described above. The RNA pellets were dissolved in 400 µl of sterile diethylpyrocarbonate (DEPC)-treated water by heating to 55 ^{0}C for 5 min. RNA was quantified using an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech, UK) (optical density of 260 nm). An aliquot (10-20 µg) of each RNA preparation was removed to a fresh 1.5 ml tube for DNase I digestion and the remainder of the RNA was mixed with 0.1 volume sodium acetate and 2.5 volume ethanol and stored at -70 ^{0}C.

DNase 1 treatments were performed in a total volume of 50 µl and contained 10-20 µg total RNA extracts, 3.5 units of RNase-free DNase I (Gibco, UK), 50 units of

ribonuclease inhibitor (Pharmacia Biotech, UK), 0.1 mM dithiothreitol (DTT), 40 mM Tris-HCl, pH 8.3 and 6 mM $MgCl_2$. Reactions were incubated for 30 min at 37 ^{0}C using a thermal cycler (Perkin Elmer, USA). They were then diluted to 200 µl and terminated by the addition of sodium dodecyl sulphate (SDS) and EDTA to final concentrations of 0.2 % and 10 mM, respectively (Sambrook *et al.*, 1989). Solutions were then extracted with phenol/chloroform (1:1), followed by chloroform extraction (1:1), RNA was precipitated and finally washed with 70 % (v/v) ethanol, all as described above. RNA was resuspended in 30 µl of DEPC-treated water, quantified as above, and stored at -20 ^{0}C.

4.2.5 *Tri5* PCR analysis

Primers for the trichodiene synthetase (*Tri5*) gene (Tr5F/R: 5'-AGCGACTACAGGCTT CCCTC-3'/5'-AAACCATCCAGTTCTCCATCTG-3') were derived from DNA sequences known to be conserved among transcribed regions of the gene from *F. culmorum* (Smith, 1997), *F. graminearum* (Proctor *et al.*, 1995a), *F. poae* (L. Hornock, pers. comm.), *F. sporotrichoides* (Hohn & Beremand, 1989) and *F. sambucinum* (Hohn & Desjardins, 1992) (Fig. 2.1). Primers were designed and synthesised as described previously (Chapter 3, section 3.3.6). According to analysis of *Tri5* gene sequences (Smith, 1997), a fragment of approximately 544 bp was expected for Tr5F/R genomic DNA PCR amplification (544-546 bp, length variation being due to the presence of introns within the target sequences which are 59-61 bp in length, depending on *Fusarium* species). These primers (Tr5F/R) were tested against a range of *Fusarium*

species (Table 2.1), including *F. culmorum, F. graminearum. F. poae, F. sambucinum, F. sporotrichoides, F. avenaceum, F. tricintum* and *F. moniliforme*. The *Tri5* primers were also tested against DNA extracts from wheat ears/grains inoculated with *F. culmorum, F. graminearum* or *F. poae.* Reactions contained 10 μg of fungal DNA or DNA from 0.8 mg dry weight equivalent of plant DNA. Negative control reactions contained no DNA. PCR reactions contained 10 pmol each of the *Tri5*-specific forward and reverse primers (Tr5F/R) and the other reaction components and the amplification conditions were as described earlier (Chapter 1, section 1.2.4). Amplification was performed in a Perkin-Elmer Cetus DNA thermal cycler (Perkin Elmer, USA). The programme used for *Tri5*-specific PCR amplification consisted of 30 cycles of 95 ^{0}C for 30 s, 62 ^{0}C for 20 s and 72 ^{0}C for 45 s, using the fastest possible transition between temperatures.

Reactions for multiplex species-specific PCR analysis to detect *F. culmorum, F. graminearum* and *F. poae* in infected plant tissue contained 0.8 mg dry weight equivalent of plant DNA, *F. culmorum, F. graminearum* and *F. poae*-specific PCR primers. Primers used for *F. culmorum* (C51F/R), *F. poae* (P82F/R) and *F. graminearum* (11F/R) species-specific multiplex PCR analysis have been described previously (Chapter 2, section 2.2.5). The other reaction components, the amplification conditions and the PCR programme used for multiplex PCR were as described in Chapter 2 (section 2.2.5).

Aliquots (10 μl) of all resulting PCR products were electrophoresed through agarose gel (1.5 % w/v) and visualised as described previously (Chapter 1, section 1.2.4).

4.2.6 Development of *Tri5*-specific RT-PCR assay

RNA extracts from *F. culmorum* GYEP liquid shake cultures harvested 24, 36, 48 and 96 h post-inoculation (control cultures from fungicide experiment) were used to optimise the RT-PCR efficiency. RNA extracts from two flasks per time point were used for this analysis, with two RT-PCR replicates per extract. The effect of using different primers (oligo dT, random hexamers or *Tri5*-specific Tr5R) for reverse transcription of total RNA extracts from *F. culmorum* liquid cultures on subsequent *Tri5*-specific PCR amplification of cDNA aliquots was investigated.'Two-step' RT-PCR assays were used for this analysis, i.e. reverse transcription (cDNA synthesis) and PCR reactions were performed in separate tubes.

Reverse transcription reactions were performed in a Perkin-Elmer Cetus thermal cycler (Perkin Elmer, USA). An initial mixture (12 µl) containing 1 µg of total RNA and either 0.5 µg of oligo dT (Gibco, UK), 0.5 µg of random hexamers (Gibco, UK) or 34 pmol of downstream *Tri5* primer (Tr5R) in DEPC-treated water was overlaid with mineral oil (Sigma, UK) and incubated for 10 min at 70 ^{0}C. Negative control reactions contained no RNA. These tubes were then chilled on ice for 1 min, adjusted to a volume of 20 µl which contained 100 units of Superscript reverse transcriptase (Gibco, UK), 20 units of ribonuclease inhibitor (Pharmacia Biotech, UK), 10 mM DTT, 500 µM each of dATP, dCTP, dGTP and dTTP, 10 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 50 mM KCl and 100 µg ml^{-1} gelatine, and incubated for 30 min at 42 ^{0}C. Reactions were terminated by incubating at 99 ^{0}C for 5 min, diluted to 100 µl with sterile distilled H_20 and stored at -20 ^{0}C.

PCR analysis was performed using 10 µl of cDNA solution in a total volume of

50 µl. Genomic DNA control reactions contained 10 ng of *F. culmorum* (strain Fu 42) DNA. The other PCR reaction components were as described above for *Tri5*-specific PCR analysis (section 4.2.5). RT-PCR amplification programmes were preceded by a 3 min incubation at 95 ^{0}C and the PCR programme used, gel electrophoresis conditions and PCR product visualisation used for *Tri5*-specific RT-PCR amplification, were as described above for *Tri5*-specific PCR (section 4.2.5). Because primer pair Tr5F/R flanks a 59 bp intron within the *Tri5* gene of *F. culmorum* (Smith, 1997), the expected product size for RT-PCR analysis of total RNA was smaller (485 bp) than that expected for any contaminating genomic DNA or genomic DNA controls included in PCR amplification assays (544 bp).

The potential of performing RT-PCR analysis in a single tube, as opposed to separate reverse transcription and PCR reactions, was also investigated. For single tube RT-PCR analysis, an initial mixture (12 µl) containing 100 ng of total RNA, 34 pmol of downstream *Tri5* primer (Tr5R) in DEPC-treated water was overlaid with mineral oil (Sigma, UK) and incubated for 10 min at 70 ^{0}C. Negative control reactions contained no RNA. These tubes were then chilled on ice for 1 min, adjusted to a volume of 20 µl which contained 25 pmol each of *Tri5*-specific forward and reverse primers (Tr5F/R), 100 units of Superscript reverse transcriptase (Gibco, UK), 20 units of ribonuclease inhibitor (Pharmacia Biotech, UK), 1 unit of Amplitaq Gold Taq DNA polymerase (Perkin Elmer, USA), 1 mM DTT, 500 µM each of dATP, dCTP, dGTP and dTTP, 10 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 50 mM KCl and 100 µg ml^{-1} gelatine. Reactions were overlaid with mineral oil and the reverse transcription conditions and reaction termination were as described above for 'two-step' RT-PCR analysis. Reverse

transcription was immediately followed by PCR amplification which was as described above for 'two-step' RT-PCR analysis, except that reactions were preceded by a 30 min incubation at 95 ^{0}C which was required to activate the Amplitaq Gold Taq DNA polymerase enzyme. Gel electrophoresis conditions and PCR product visualisation were as described above for *Tri5*-specific PCR (section 4.2.5).

4.2.7 Development of semi-quantitative *Tri5*-specific RT-PCR assay

RNA extracts from *F. culmorum* GYEP liquid shake cultures harvested 24, 36, 42 and 96 h post-inoculation (control cultures from fungicide experiment) were used to develop the semi-quantitative RT-PCR-based assay for *Tri5* gene expression. RNA extracts from two flasks per time point were used for this analysis, with two RT-PCR replicates per RNA extract. Semi-quantitative RT-PCR analysis was based on co-amplification of *Tri5* cDNA and cDNA of an endogenous control 'housekeeping' gene, β-tubulin.

Tri5 gene expression was quantified by determining the ratio of *Tri5* RT-PCR amplification product to that of β-tubulin using 'two-step' RT-PCR analysis. Three β-tubulin primers were used for the development of this assay: the forward and reverse primers Bt-2a/b have been described previously (Glass & Donaldson, 1995), and the downstream primer (B531R:5'-GACTGACCGAAAACGAAGTTG-3') was derived from a transcribed region conserved between the *G. pulicaris* (*F. sambucinum*) β-tubulin gene (*tub2*) (Genbank accession number U27303) and the β-tubulin gene from a *Neurospora crassa* benomyl resistant mutant (Orbach *et al.*, 1986). Primers were designed and synthesised as described previously (Chapter 3, section 3.2.6). Because

primer pair Bt-2a/b flanks an intron (42 bp) within the *G. pulicaris* (*F. sambucinum*) gene (Genbank accession number U27303), the expected product size for RT-PCR analysis of total RNA was smaller (approximately 298 bp) than that expected for any contaminating genomic DNA or genomic DNA controls included in PCR amplification assays (approximately 340 bp). Similarly, the expected product sizes for Bt-2a/B531R RT-PCR analysis were 244 bp for total RNA and 286 bp for any contaminating genomic DNA or genomic DNA controls included in PCR amplification assays.

Reverse transcription was performed as described above (section 4.2.6), using the *Tri5*-specific Tr5R primer and one of two β-tubulin downstream primers: Bt-2b or B531R. PCR amplification was performed using 10 pmol each of primers specific for *Tri5* gene (Tr5F/R) and 10 pmol each of primers specific for the β-tubulin gene: either primer pair Bt-2a/b or primer pair Bt-2a/B531R. PCR reaction components, amplification conditions and the PCR programme used was as described above for 'two-step' *Tri5*-specific RT-PCR (section 4.2.6), except that an annealing temperature of 60 ^{0}C was used. Aliquots of the RT-PCR products were separated by agarose gel electrophoresis (2 % w/v) and visualised as described previously (Chapter 1, section 1.2.4).

4.2.8 Semi-quantitative RT-PCR analysis of the effect of fungicides on *Tri5* gene expression

To increase the accuracy of semi-quantitative RT-PCR, the linear amplification range for *Tri5* and β-tubulin co-amplification was determined for RNA extracts from the

fungicide experiment, i.e. the target cDNA dilutions (from reverse transcription) which gave amplification within the exponential phase of PCR, under particular cycling and reaction conditions. This ensured that semi-quantitative RT-PCR analysis was not performed on samples which had entered the plateau phase of PCR amplification. Subsequently, all samples from the fungicide experiment were subjected to semi-quantitative RT-PCR analysis using cDNA dilutions within the linear amplification range.

4.2.8.1 Determination of the linear range of PCR *Tri5* and β-tubulin co-amplification and semi-quantification of *Tri5* gene expression

Addition of serial dilutions (5, 10 15 and 20 µl) of the cDNA resulting from reverse transcription of RNA extracts from control samples and fungicide amended cultures harvested 24, 36, 48 and 96 h post-inoculation (1 sample per time point, 4 samples in total) was used to determine the linear range for PCR co-amplification of *Tri5* and β-tubulin. RT-PCR analysis was performed exactly as described previously (section 4.2.7) using the downstream TR5R (*Tri5*-specific) and B531R (β-tubulin-specific) primers for reverse transcription and primers Tr5F/R and Bt-2a/B531R for PCR co-amplification reactions. Following agarose gel electrophoresis, the *Tri5* and β-tubulin PCR products were quantified by densitometry, which was performed as described earlier in Chapter 2 (section 2.2.6.2). For this experiment, RT-PCR reactions were performed at least three times and the linear range for *Tri5* and β-tubulin PCR co-amplification was determined by plotting the mean band densities (densitometric units) against the volume of reverse

transcription product (cDNA) added to the corresponding PCR reactions.

4.2.8.2 Effect of time and fungicides on relative *Tri5* gene expression

Semi-quantitative RT-PCR analysis was then used to determine the effect of time and the effect of the different concentrations of the fungicides prochloraz and tebuconazole on relative *Tri5* gene expression in total RNA extracts from *F. culmorum* GYEP liquid shake cultures harvested 24, 36, 48 and 96 h post-inoculation (fungicides added 24 h post-inoculation). Two samples per treatment per time point were used for this analysis, with two replicate RT-PCR reactions per sample. Based on linear PCR amplification range of both *Tri5* and β-tubulin, 10 µl of reverse transcription products were used in *Tri5* and β-tubulin RT-PCR co-amplification assays, which were performed as described previously (section 4.2.8.1 & section 4.2.7).

Following agarose gel electrophoresis, the *Tri5* and β-tubulin PCR products were quantified by densitometry, which was performed as described earlier in Chapter 2 (section 2.2.6.2). The correction described by Menzo *et al.* (1992) was used in order to account for the reduced incorporation of ethidium bromide incorporation by β-tubulin relative to *Tri5,* which was due to the smaller size of the β-tubulin cDNA (244 bp) as compared with *Tri5* cDNA (485 bp). β-tubulin band density (DA) was multiplied by *Tri5* cDNA bp length/β-tubulin cDNA bp length, i.e. 544 bp/244 bp = 1.53, therefore DA x 1.53 = corrected β-tubulin band density. The expression of *Tri5* was related to that of the housekeeping gene, β-tubulin, based on the technique described by Kan-Mitchell *et al.* (1993). The ratio of the *Tri5* RT-PCR product to that of β-tubulin (i.e.

Tri5 band density/corrected β-tubulin band density) was used as an index of the relative expression of *Tri5* among fungicide cultures.

4.2.9 Statistical analysis

The significance of the effect of time and fungicide treatments on the *Tri5*/β-tubulin ratios was investigated using two-sided Wilcoxon rank-sum test (Snedecor & Cochran, 1980), performed using Minitab release 10.1 (©1994, Minitab incorporated).

4.3 RESULTS

4.3.1 *Tri5*-specific PCR analysis

Primers Tr5F/R were used to amplify DNA from isolates of *Fusarium* species known to produce trichothecene mycotoxins (Desjardins *et al.*, 1993) (Table 4.1). These included isolates each of *F. culmorum, F. graminearum, F. poae, F. sambucinum, F. sporotrichoides* and *F. crookwellense* (Fig. 4.3). A single fragment of expected size (544 bp) was amplified from DNA from all of the isolates of these species tested. This primer pair was also tested against *F. moniliforme* isolates, a species for which there are no reports of trichothecene production. No fragment was amplified from DNA of the selected isolates of this species. In addition, these primers were tested against isolates of *F. avenaceum,* a pathogen for which there are only a few reports of trichothecene production (Marasas *et al.*, 1984; Thrane *et al.*, 1989). No fragment was amplified from DNA of five of the six *F. avenaceum* isolates tested, but a 544 bp fragment was amplified from the DNA extract of the *F. avenaceum* isolate Fu 17. However, the PCR amplification product detected for this isolate was much weaker than those detected for DNA extracts from the *F. culmorum, F. graminearum, F. poae, F. sambucinum, F. sporotrichoides* and *F. crookwellense* isolates (Table 4.1). Tr5F/R primers were also tested against two isolates of *F. tricinctum* and no fragment was amplified from the DNA of these isolates.

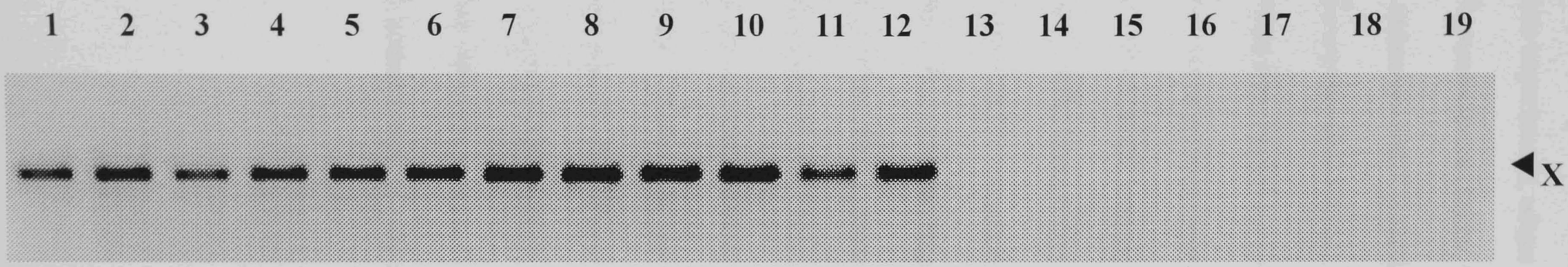


Fig. 4.3 Detection of the *Tri5*-specific PCR signal in genomic DNA extracts from various *Fusarium* species. Lanes: 1-2, *F. culmorum* strains Fu 15 & Fu 42; 3-4, *F. graminearum* strains F 508 & F 604; 5-6, *F. poae* strains Fu 53 & F 62; 7-8, *F. crookwellense* strains C 233 & C 581; 9-10, *F. sporotrichoides* strains F 95 & F 627; 11-12, *F. sambucinum* strains F 64 & F 108; 13-14, *F. avenaceum* strains 76-1 & 96UKR2; 15-16, *F. tricintum* strains F 308 & F 402; 17-18, *F. moniliforme* strains A 3733 & B 3853; 19, negative control (no DNA added). Arrow (x) indicates the *Tri5*-specific PCR product (544 bp).

4.3.2 Detection of specific PCR products in infected wheat ears

In glasshouse experiments, wheat ears were inoculated with *Fusarium culmorum, F. poae* and *F. graminearum* (Smith, 1997) (3 isolates of each). DNA was extracted from ears for *F. culmorum* and *F. poae*-inoculated samples and from the grain component of *F. graminearum*-inoculated samples and multiplex species-specific PCR analysis was used to confirm that samples contained DNA from the appropriate pathogen (Fig. 4.4a). No fragment was amplified from DNA extracts of uninoculated control plants (Fig. 4.4a; lanes 1-3). A single fragment of 550, 400 and 220 bp was amplified from *F. culmorum* (Fig. 4.4a; lanes 4-6), *F. graminearum* (Fig. 4.4a; lanes 7-9) and *F. poae* (Fig. 4.4a; lanes 10-12)-inoculated samples, respectively. The size of the fragments corresponded to the size of the fragments expected from *F. culmorum, F. graminearum* and *F. poae* genomic DNA extracts (550, 400 and 220 bp, respectively) (Fig. 4.4a; lane x). Plant DNA extracts were then analysed by *Tri5*-specific PCR analysis (Fig. 4.4b). Again, no fragment was amplified from DNA extracts of uninoculated control plants (Fig. 4.4b; lanes 1-3). A single 544 bp fragment was amplified from each DNA extract from *F. culmorum, F. graminearum* and *F. poae*-inoculated plant samples (Fig. 4.4b; lanes 4-6, 7-9 and 10-12, respectively). The size of the fragments corresponded to the fragment size obtained previously (section 4.3.1) for genomic DNA extracts from potential trichothecene-producing fungi (Fig. 4.3).

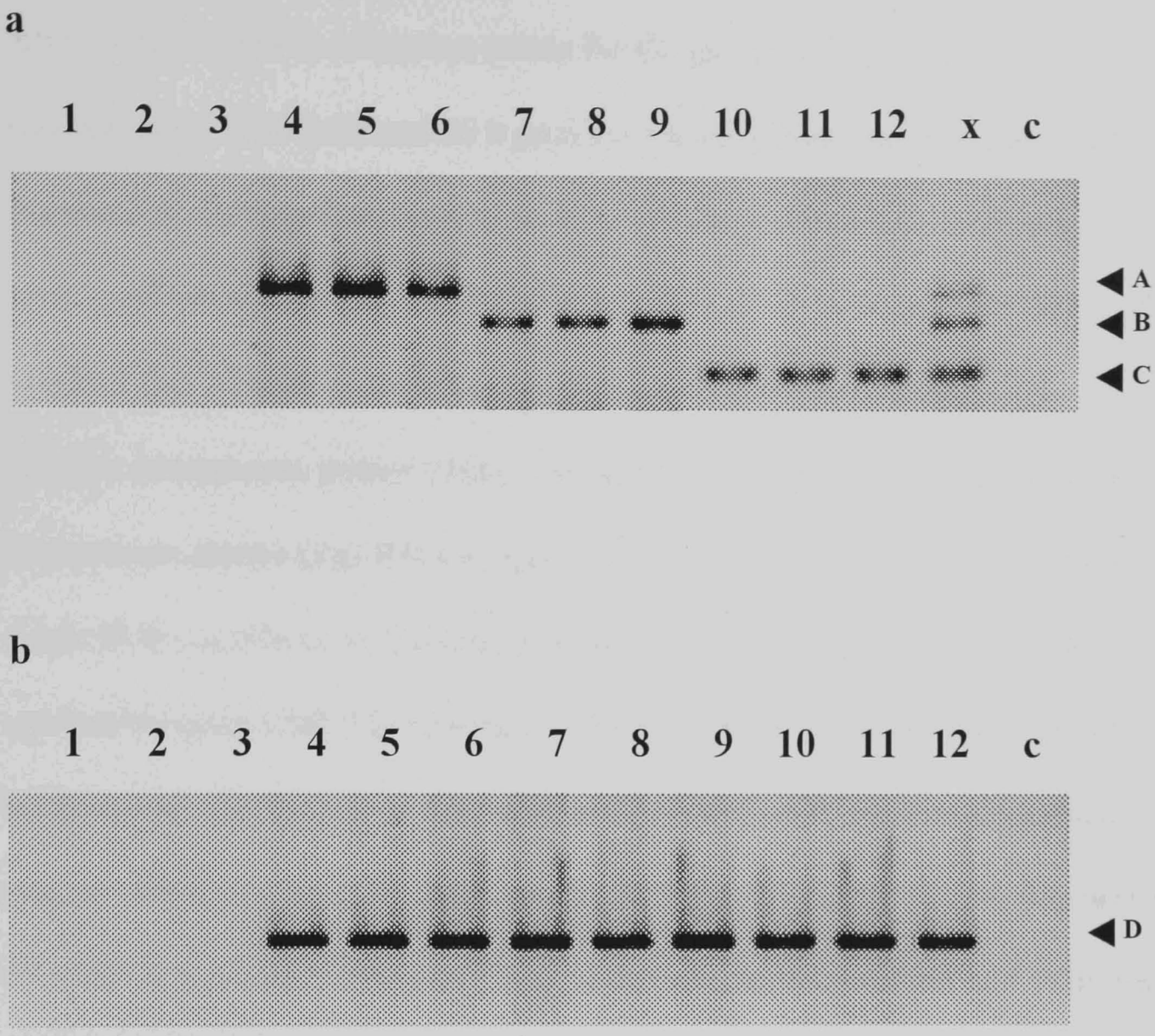


Fig. 4.4 Detection by species-specific PCR (a) and *Tri5*-specific PCR (b) of *Fusarium culmorum, F. graminearum* and *F. poae* in DNA extracts of wheat (cv. Avalon) ears/grains. Lanes: 1-3, uninoculated wheat ears; 4-6, *F. culmorum*-inoculated ears; 7-9, *F. graminearum*-inoculated ears; 10-12, *F. poae*-inoculated ears; x, *F. culmorum, F. graminearum* and *F. poae* genomic DNA extracts; c, negative PCR control reaction (no DNA added). Arrows: A, *F. culmorum*-specific PCR product (550 bp); B, *F. graminearum*-specific PCR product (400 bp); C, *F. poae*-specific PCR product (220 bp); D. *Tri5*-specific PCR product (544 bp).

4.3.3 Development of an RT-PCR based assay to detect *Tri5* gene expression

RNA extracts from *F. culmorum* (strain Fu 42) grown in GYEP liquid shake cultures and harvested 24, 36, 48 and 96 h post-inoculation (control cultures from fungicide experiment) were used to develop an RT-PCR based assay for the detection of *Tri5* gene expression. Fig 4.5a illustrates the 'two-step' *Tri5*-specific RT-PCR results obtained (i.e. separate reverse transcription and PCR reactions) when either the *Tri5*-specific downstream primer (Tr5R), oligo d(T) or random hexamers was used for reverse transcription of an RNA extract of a culture harvested 36 h post-inoculation. A single PCR amplification fragment of expected size (485 bp) was detected for cDNA synthesised using Tr5R (Fig 4.5a, lane 1). This fragment was of expected size (485 bp), being smaller than the size of the fragment detected from *F. culmorum* genomic DNA extracts included in RT-PCR-based assays (544 bp) (Fig 4.5a, lane x). No fragment was detected in negative controls included in the reverse transcription and PCR assays (results not shown). PCR analysis showed that much higher concentrations of *Tri5* cDNA were obtained when the *Tri5*-specific downstream primer (Tr5R) was used for reverse transcription as opposed to when oligo d(T) was used for cDNA synthesis (Fig 4.5a, lanes 1 & 2, respectively). Also, for some samples, substantial amounts of non-target PCR products were obtained for cDNA synthesised using random hexamers (Fig. 4.5a, lane 3). Similar RT-PCR results were obtained for the other RNA extracts (from cultures harvested 24,36, 48 and 96 h post-inoculation) used for development of the *Tri5*-specific RT-PCR-based assay (results not shown). When reverse transcription and PCR amplification were performed in a single tube, multiple amplification fragments (≥ 4 fragments) were observed following agarose gel electrophoresis, with non-target

fragments being ≥ 550 bp in length (results not shown). Therefore, for all subsequent RT-PCR analysis, the *Tri5*-specific downstream primer TR5R was used for 'two-step' RT-PCR analysis where reverse transcription and PCR amplification were performed separately.

4.3.4 Development of a semi-quantitative RT-PCR assay for *Tri5* gene expression

RNA extracts from *F. culmorum* (strain Fu 42) grown in GYEP liquid shake cultures and harvested 24, 36, 48 and 96 h post-inoculation (control cultures from fungicide experiment) were used to develop a semi-quantitative RT-PCR based assay for the analysis of *Tri5* gene expression. The semi-quantitative system was based on the RT-PCR co-amplification of *Tri5* and the endogenous control 'housekeeping' gene, β-tubulin. Initially the potential of using the published primers Bt-2a/b (Glass & Donaldson, 1995) for the detection of β-tubulin gene expression by RT-PCR analysis was investigated. However, incorporation of the downstream primer (Bt-2b) in *Tri5* reverse transcription reactions and subsequent PCR amplification using *Tri5* (TR5F/R) and β-tubulin-specific primers (Bt-2a/b) generated multiple PCR products, one of expected size for *Tri5* cDNA amplification (485 bp), one of expected size for β-tubulin cDNA amplification (approx 250-260 bp), and one or more unexpected amplification products (< 150 bp) (results not shown). Therefore a new downstream primer was designed for β-tubulin amplification (B531R). When this downstream primer was included in *Tri5* reverse transcription reactions, subsequent PCR co-amplification using *Tri5* (TR5F/R) and the β-tubulin-specific primers Bt-2a/B531R resulted in two amplification of products of the expected sizes. Fig. 4.5b shows the results obtained for

one of the two RNA extracts from each harvest time point. Similar results were obtained for the other set of replicate cultures (results not shown). The *Tri5*-specific RT-PCR fragment corresponded to the fragment size detected by independent *Tri5*-specific RT-PCR analysis (485 bp), while the β-tubulin-specific RT-PCR fragment was approximately 244 bp in size (Fig 4.5b, lanes 4-7). The β-tubulin-specific and *Tri5*-specific PCR fragments detected in the *F. culmorum* genomic DNA extracts included in PCR assays were approximately 286 bp and 544 bp, respectively (Fig. 4.5b, lane x).

4.3.5 Linear *Tri5* and β-tubulin RT-PCR co-amplification range

RNA extracts from several of the control samples and fungicide-amended samples harvested 24, 36, 48 and 96 h post-inoculation (four samples in total) were used to determine the dilution of cDNA required such that, for the fungicide experiment, *Tri5* and β-tubulin RT-PCR co-amplification was within the exponential phase of PCR. When serial dilutions of cDNA (5, 10, 15 & 20 µl of reverse transcription reactions) were added to *Tri5* and β-tubulin PCR co-amplification reactions, two fragments were detected in each of the RNA extracts. The results obtained from the cDNA resulting from a *F. culmorum* liquid culture unamended with fungicide and harvested 48 h post-inoculation are illustrated in Fig. 4.6a. In order to determine the linear range for both *Tri5* and β-tubulin cDNA amplification in co-amplification reactions, the mean densitometric units (band densities) (DA) (based on two samples with 3 replicate RT-PCR reactions per sample) obtained for both the *Tri5* and β-tubulin-specific RT-PCR products were plotted against the volume of cDNA added to the corresponding PCR

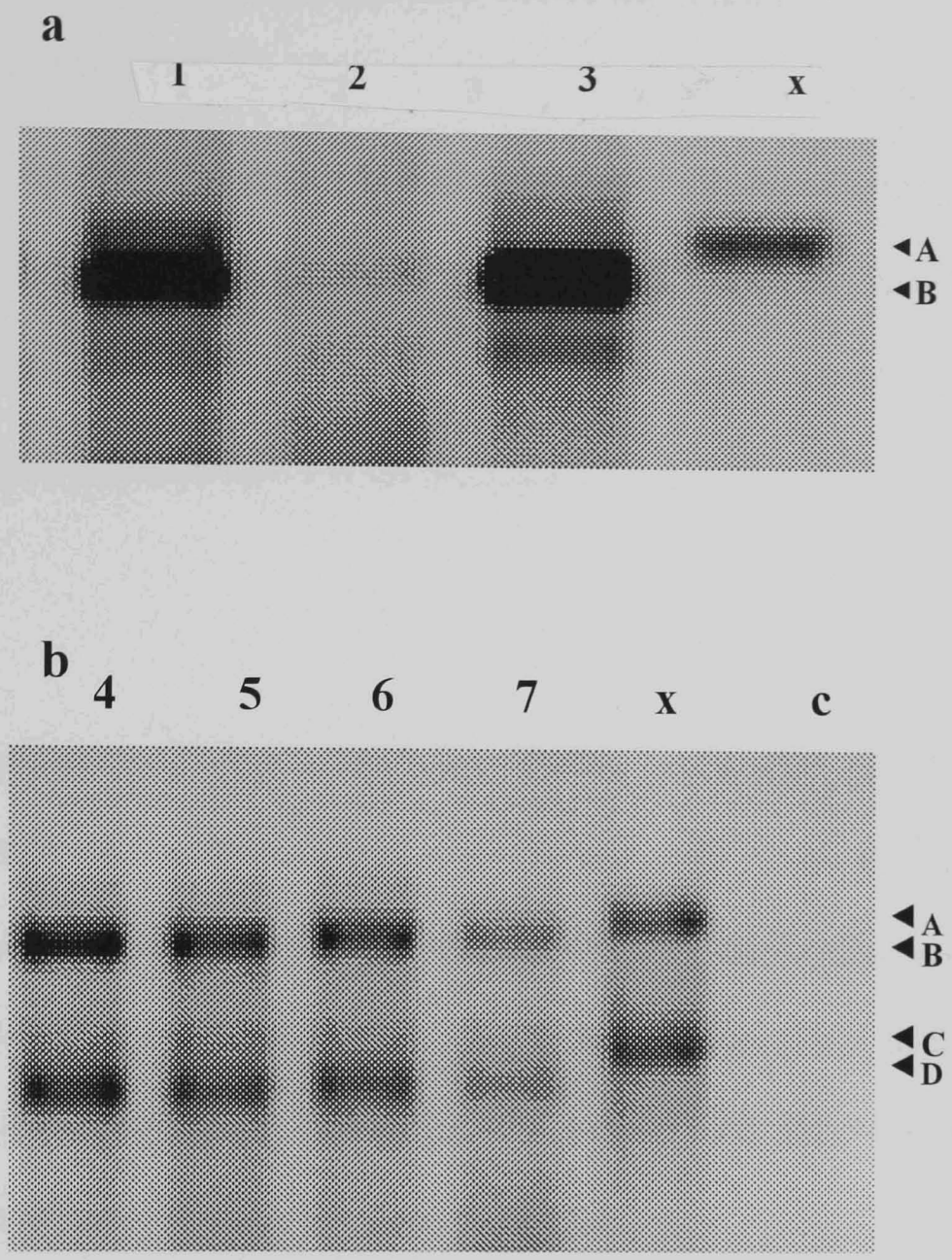


Fig. 4.5 Development of *Tri5*-specific and *Tri5*-/β-tubulin-specific RT-PCR-based assays using *Fusarium culmorum* (strain Fu 42) total RNA extracts from cultures grown in GYEP medium for various lengths of time. a: Optimisation of cDNA synthesis for *Tri5*-specific PCR analysis, using an RNA extracted from a culture harvested 36 h post-inoculation. Primers: 1, *Tri5*-specific dowmstream primer (Tr5R); 2, Oligo d(T); 3, random hexamers; x, *F. culmorum* genomic DNA positive PCR control. b: *Tri5*-/β-tubulin-specific PCR co-amplification of cDNA synthesised by *Tri5*/β-tubulin-specific reverse transcription. Lanes: 4-7, RNA extracts, respectively harvested 24, 36, 48 & 96 h post-inoculation, respectively; x, *F. culmorum* genomic DNA; c, RT-PCR negative control (no RNA or DNA added). Arrows: A, *Tri5*-specific genomic DNA RT-PCR product (544 bp); B *Tri5*-specific total RNA RT-PCR product (485 bp); C, β-tubulin-specific genomic DNA RT-PCR product (286 bp); D, β-tubulin-specific total RNA RT-PCR product (244 bp).

reactions (Fig. 4.6b). Amplification of *Tri5* cDNA was linear over the range tested, as was β-tubulin cDNA amplification. Similar results were obtained for the other three RNA extracts from the experiment used to determine the linear co-amplification range and therefore, a cDNA volume within this range, i.e. 10 µl of the diluted reverse transcription product (corresponding to 100 ng of input total RNA), was used for all subsequent semi-quantitative RT-PCR analyses.

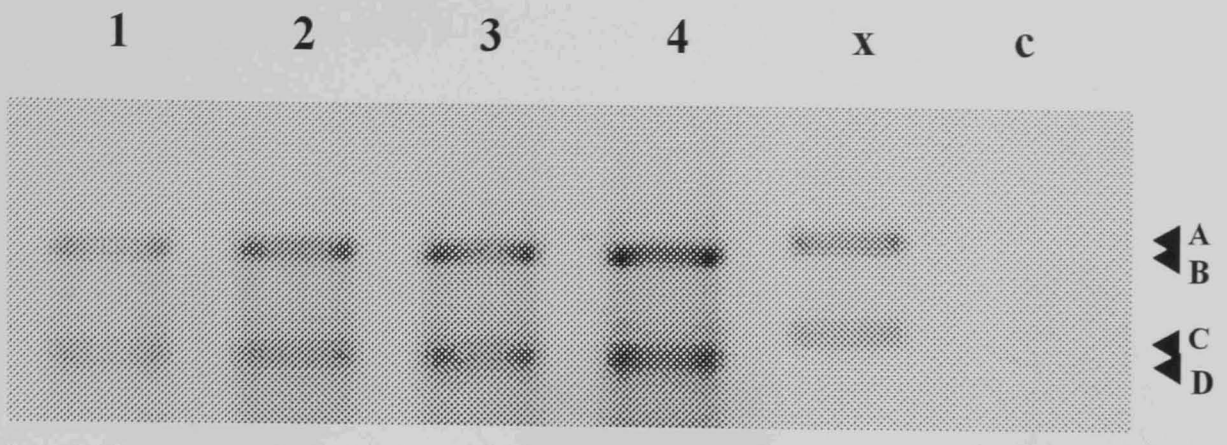
4.3.6 Effect of time and fungicides on the relative *Tri5* gene expression

The effect of time on *F. culmorum Tri5* gene expression and the effect of the fungicides prochloraz and tebuconazole on *Tri5* gene expression in liquid cultures harvested 24, 36, 48 and 96 h post-inoculation (fungicides added to appropriate flasks 24 h post-inoculation) was estimated by comparing the ratio of *Tri5* RT-PCR amplification product to that of the housekeeping gene, β-tubulin.

For control cultures (no fungicide added), the largest relative *Tri5* mRNA level in cultures was observed 48 h post-inoculation (mean ratio = 0.98), but these *Tri5*/β-tubulin ratios were not significantly different from those obtained at 24, 36 and 96 h (mean ratios of 0.82, 0.73 & 0.59, respectively) (Fig 4.7, Appendix 4.).The effect of fungicides on the ratio of *Tri5*/β-tubulin gene expression was investigated using RNA extracts from liquid cultures amended with either prochloraz or tebuconazole at two different concentrations (2 or 8 µg ml^{-1} media) 24 h post-inoculation and harvested 36, 48 and 96 h post-inoculation (Fig 4.7 & Appendix 4.). Thirty six hours post-inoculation (12 h after addition of fungicides) RNA extracts from both prochloraz and tebuconazole-amended cultures had significantly higher *Tri5*, β-tubulin RT-PCR

product ratios, compared to control cultures (Fig. 4.7). The higher prochloraz and tebuconazole concentration (8 μg ml^{-1} media) resulted in higher *Tri5*/β-tubulin RT-PCR product ratios (mean ratios = 1.15 & 1.11, respectively) than 2 μg fungicides ml^{-1} media (mean ratios = 0.95 & 0.88, respectively). However, these differences were not statistically significant at the 5 % level, and also, there was no significant difference between the two fungicides in terms of their effect on *Tri5*/β-tubulin RT-PCR product ratios. For cultures harvested at 48 and 96 h post-inoculation, although the ratios observed for both prochloraz and tebuconazole-amended cultures were lower than those observed for control cultures, differences in ratios were not significant at the 5 % level (Fig. 4.7).

a



b

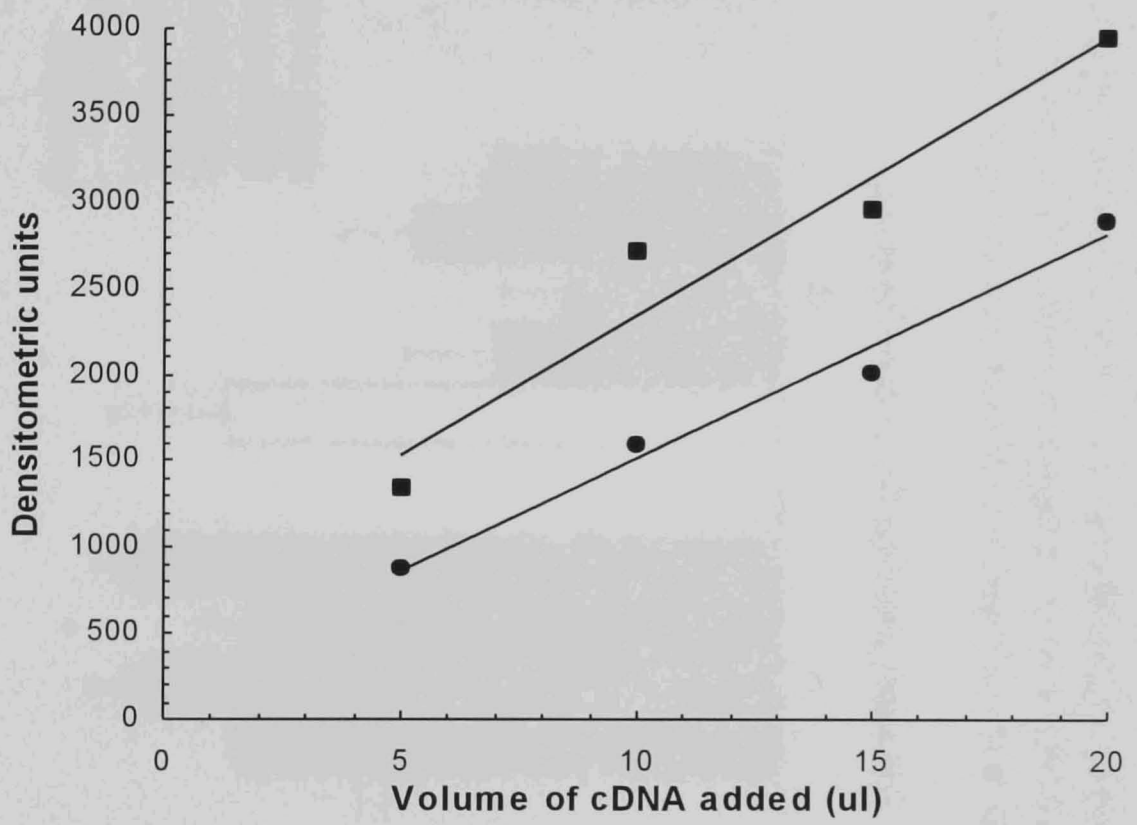


Fig. 4.6 Determination of the linear range for *Tri5* and β-tubulin PCR co-amplification for cDNA from *Fusarium culmorum* GYEP liquid culture harvested 48 h post-inoculation. PCR products were a, visualised by UV trans-illumination following gel electrophoresis and b, densitometric units (band densities) were determined by densitometric analysis and related to the volume of cDNA added. Lanes: 1-4, 5, 10, 15 and 20 µl of cDNA (corresponding to 50, 100, 150 and 200 ng of input total RNA); x, *F. culmorum* (strain Fu 42 genomic DNA); c, control reaction without cDNA or genomic DNA added. Densitometric units of both *Tri5* (■) and β-tubulin (●) PCR product densities on Fig. 4.6b represent the average of three replicates RT-PCR reactions, one of the relicates being illustrated in Fig. 4.6a. Arrows: A, *Tri5*-specific genomic DNA fragment (544 bp); B, *Tri5*-specific cDNA fragment; C, β-tubulin-specific genomic DNA fragment (286 bp); D, β-tubulin-specific cDNA fragment (244 bp).

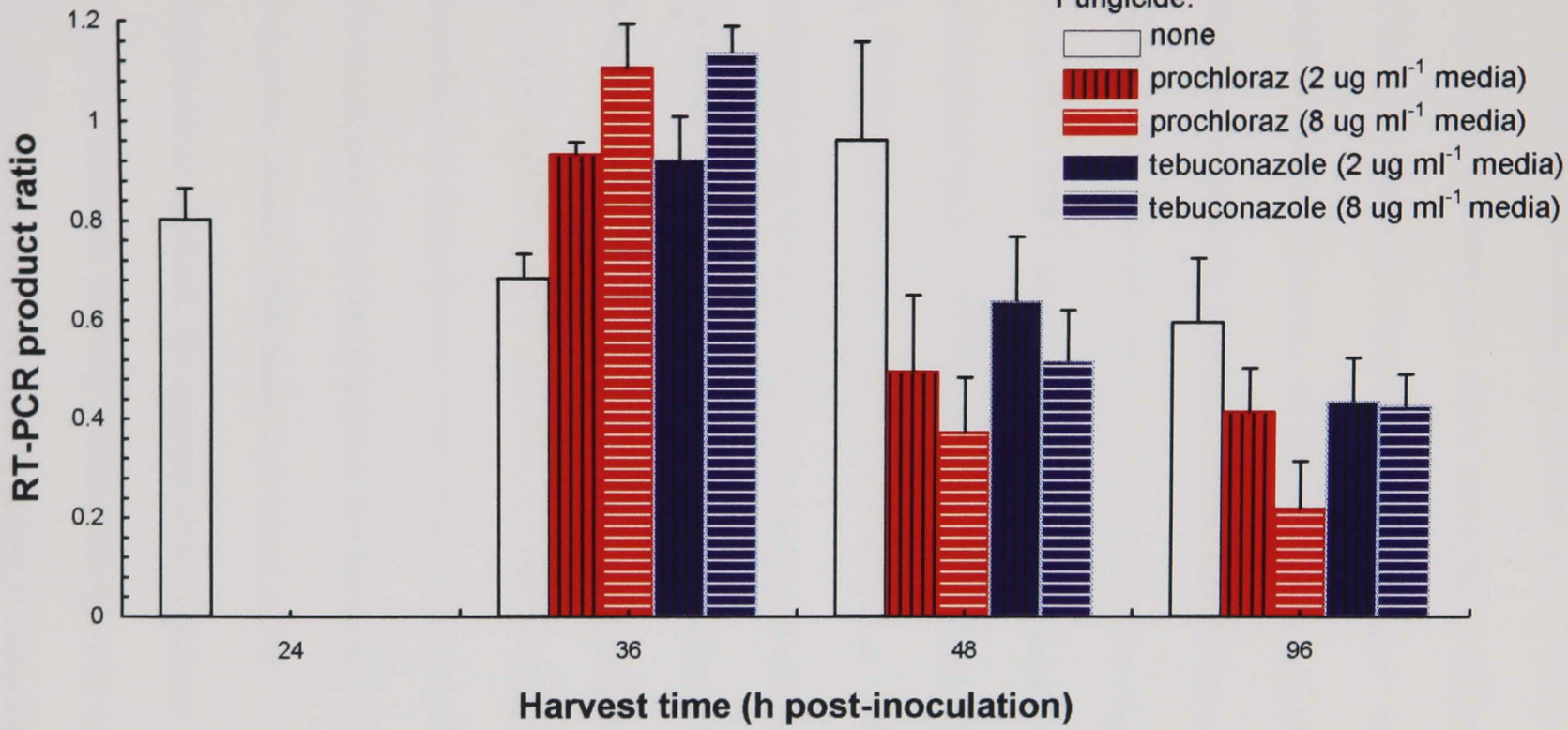


Fig. 4.7 Effect of time, fungicides and fungicide concentration on RT-PCR product ratio (ratio of *Tri5* - specific amplified product to β-tubulin-specific amplified product) of *Fusarium culmorum* liquid cultures. GYEP liquid cultures were amended with fungicides 24 h post-inoculation and harvested 24, 36, 48 and 96 h post-inoculation. Bars indicate standard error of the means. For statistical analysis see Appendix 4.

4.4 DISCUSSION

Several *Fusarium* species which are important pathogens of small grain cereals, including *F. culmorum, F. graminearum* and *F. poae,* have been shown to produce trichothecene mycotoxins in cereals (Desjardins *et al.*, 1993). Chapter two has shown how, under glasshouse conditions, sterol biosynthesis inhibiting fungicides such as prochloraz and tebuconazole offered moderate control of both *F. culmorum* and *F. poae* ear blight of wheat. However, the effect of such fungicides on the biosynthesis of trichothecene mycotoxins by *Fusarium* species has not been well studied. Given the potential dangers associated with mycotoxin contamination of grain (Joffe, 1978; Ueno, 1980; Marasas *et al.,* 1984; Moss, 1991) and the controversy regarding the effect of other chemical control agents on trichothecene production by *Fusarium* species (Martin & Johnston, 1982; Moss & Frank, 1985; Boyacioglu *et al.,* 1992; Gareis & Ceynowa, 1994), it is clear that the effect of fungicides such as prochloraz and tebuconazole on trichothecene production by *Fusarium* species warrants further investigation.

Although the main objective of this work was to develop an RT-PCR-based assay which could be used to study trichothecene expression, in particular to study the effect of fungicides on trichothecene expression, in the course of this work a *Tri5*-specific PCR-based assay was developed for the detection of potential trichothecene-producing *Fusarium* species. This assay was based on the *Tri5* gene which codes for the enzyme trichodiene synthase, the enzyme which catalyses the first unique step of the trichothecene biosynthetic pathway. Primers designed to conserved regions of the *Tri5* genes from several *Fusarium* species, including *F. culmorum, F. graminearum, F. poae, F. sporotrichoides* and *F. sambucinum* were used to develop the *Tri5*-specific

PCR-based assay which amplified a single 544 bp fragment from *F. culmorum, F. graminearum, F. poae, F. crookwellense, F. sporotrichoides* and *F. sambucinum* genomic DNA extracts. These pathogens are well documented as being trichothecene producers (Thrane, 1989; Desjardins *et al.,* 1993). *Tri5*-specific PCR analysis also detected a *Tri5* gene fragment in one of the six *F. avenaceum* isolates tested (strain Fu 17). However, this PCR signal was weaker than those detected for known trichothecene producers. Therefore, it is probable that the *Tri5*-specific PCR signal observed in the present work was due to PCR contamination. There are only a few reports of trichothecene production by *F. avenaceum* (Marasas *et al.,* 1984; Möller & Åkerstrand; 1986). The identity of the producer strains used by Marasas *et al.* (1984) has not been verified and Thrane (1989) could not detect trichothecene production by the strains used by Möller & Åkerstrand (1986). Similarly, the production of trichothecenes by *F. tricinctum* is questionable (Thrane, 1989) and in this work, the *Tri5* gene fragment was not detected in DNA extracts from two isolates of this species. Several authors have reported trichothecene production by strains of *F. tricinctum* (Kuang *et al.,* 1984; Engelhardt *et al.,* 1986; Möller & Åkerstrand, 1986). However, according to Thrane (1989), the taxonomic systems used by authors such as Kuang *et al.* (1984) and Engelhardt *et al.* (1986) are unknown and the production of trichothecenes by the *F. tricinctum* strains used by Möller & Åkerstrand (1986) has not been confirmed. There are no reports of trichothecene production by *F. moniliforme* and PCR analysis did not detect the *Tri5* gene fragment in the six *F. moniliforme* isolates tested. Another *Tri5*-specific PCR assay using primers designed to conserved regions of the *Tri5* genes of *F. sporotrichoides, F. poae, G. pulicaris* (*F. sambucinum*) and *G. zeae* (*F.*

graminearum) has recently been developed by Niessen & Vogel (1997). These workers showed that their *Tri5*-specific PCR primers amplified a 650 bp *Tri5* gene fragment from 18 different *Fusarium* species, including *F. graminearum, F. culmorum, F. poae, F. sambucinum* and *F. sporotrichoides* isolates.

The present PCR-based assay also detected the *Tri5* target sequence in *F. culmorum-, F. graminearum-* and *F. poae*-infected wheat ears. The *Tri5*-specific PCR-based assay developed by Neissen & Vogel (1997) has also been used to detect trichothecene-producing *Fusarium* species in contaminated wheat malt samples. They showed that a positive PCR signal was produced using a DNA sample extracted from wheat which had been inoculated with *F. cerealis* and single wheat malt grains, heavily contaminated with *F. graminearum* or *F. culmorum,* also tested positive in their assay.

Although the *Tri5* gene has been shown to be highly conserved over a range of *Fusarium* species (Smith, 1997), Neissen & Vogel (1997) sequenced the PCR fragments amplified by their *Tri5*-specific primers and showed that species- and sub-species-specific differences could be identified within the *Tri5* gene sequences and these differences allowed the development of PCR primers specific to sub-groups of trichothecene producers. Other PCR-based assays have been developed for the detection of specific *Fusarium* species, including assays specific for *F. culmorum* (Schilling *et al.,* 1996; Nicholson *et al.*, unpublished), *F. graminearum* (Ouellet & Seifert, 1993; Schilling *et al.,* 1996; Nicholson *et al.,* unpublished), *F. poae* (Parry & Nicholson, 1996) and *F. crookwellense* (Turner *et al.* unpublished). Unlike these assays, the present *Tri5*-specific PCR-based assay cannot differentiate between *Fusarium* species, but could provide a screening tool for preliminary detection of trichothecene producers in

plant tissue.

Having ensured that the Tr5F/R primers were specific for the *Tri5* gene, they were then used for the development of an RT-PCR-based assay for the detection of *Tri5* gene expression and for the analysis of the effect of fungicides on *Tri5* gene expression. Messenger RNA amplification has all the attributes of DNA amplification, but has the advantage that it simultaneously provides additional information regarding physiological processes, i.e. *Tri5* gene expression, within the substrate and can be used to monitor low level expression or where the amount of tissue is limited. The use of the *Tri5*-specific downstream primer Tr5R, rather than oligo dT or random hexamers for *Tri5* mRNA reverse transcription and the performance of 'two-step' RT-PCR assays, i.e. separate reverse transcription and PCR amplification rather than combined single-tube RT-PCR, appeared optimal for *Tri5*-specific RT-PCR amplification. A single 485 bp fragment was amplified following *Tri5*-specific RT-PCR based analysis of RNA extracts from *F. culmorum* liquid shake cultures. The *Tri5*-specific PCR primers were designed to flank the intron (59-61 bp intron, depending on the origin of the gene, i.e. *Fusarium* species) present in the *Tri5* gene. This allowed size differentiation of amplified genomic DNA and cDNA.

Tri5 mRNA was detected in all cultures from the fungicide experiment, irrespective of post-inoculation harvest time (i.e. 24, 36, 48 and 96 h post-inoculation), irrespective of whether or not they were amended with fungicides and irrespective of fungicide concentration (2 or 8 $\mu g\ ml^{-1}$ media). These results were as expected, since it has been demonstrated that this culture medium (GYEP) stimulated trichothecene production in liquid culture by various *Fusarium* species (Miller *et al.*, 1983a; Hohn &

Beremand, 1989; Hohn *et al.*, 1993). Hohn *et al.* (1993) used northern blot analysis to show that when *G. pulicaris* (*F. sambucinum*) was grown in GYEP culture broth, *Tri5* gene expression increased 47-fold between 18 and 42 h post-inoculation. It would have been preferable to use culture media which did not stimulate trichothecene production in liquid culture in order to emphasise any *Tri5* gene expression that might have been due to fungicide treatments. However, limited time meant that it was not possible to investigate the effect of other media on *Tri5* gene expression and therefore, GYEP medium was used.

The incorporation of PCR primers specific for the housekeeping gene β-tubulin in the RT-PCR-based assays acted as a control to ensure that the absence of a *Tri5*-specific RT-PCR product following RT-PCR would have been a reflection of the absence of *Tri5* expression rather than failure of either the reverse transcription or PCR reactions. Although β-tubulin-specific primers (Bt-2a/b) were available for amplification of this gene from genomic DNA extracts of *Fusarium* species (Glass & Donaldson, 1997), problems of cross-reactivity were encountered when these primers were used in combination with the *Tri5*-specific primers (Tr5F/R) for RT-PCR analysis. Therefore, a new reverse β-tubulin primer (B531R) was designed and when the *Tri5*-specific primers were used in combination with Bt2a/B531R β-tubulin primers, problems of cross reactivity were not encountered. The *Tri5*- and β-tubulin-specific RT-PCR co-amplification assays yielded two fragments, one fragment corresponding to *Tri5* and one corresponding to β-tubulin. The β-tubulin primers were similar to the *Tri5* primers in that they flanked an intron within the gene, allowing size differentiation of amplified genomic DNA and cDNA. In a study of virulence gene expression by

Cochliobolus carbonum during conidial germination, Jones & Dunkle (1995) detected two products when RNA was amplified in β-tubulin-specific RT-PCR-based assays. They presumed that this was due to processing of the transcripts and splicing out of introns between the primers. In the present study, a single β-tubulin product was detected for all RNA extracts amplified in *Tri5*/β-tubulin-specific RT-PCR-based assays.

The incorporation of PCR primers specific for the "housekeeping" gene β-tubulin in the RT-PCR-based assays also enabled quantification of *Tri5* gene expression relative to that of β-tubulin (i.e. semi-quantitative RT-PCR analysis). The empirical values obtained by obtaining the ratio of *Tri5* RT-PCR product to that of β-tubulin co-amplified product could be used to compare the levels of gene expression in different samples. The underlying assumption in this experiment was that β-tubulin gene expression was constitutive. Results from a limited northern blot analysis experiment performed by L. Boyd and C. Placinta indicated that the β-tubulin gene may be constitutively expressed in *F. sporotrichoides*. However, this requires further verification by comparison of β-tubulin gene expression with some quantitative measure of fungal biomass, such as dry weight, ergosterol content or DNA content.

This semi-quantitative RT-PCR-based assay was used to evaluate the effect of the fungicides prochloraz and tebuconazole on *Tri5* gene expression over time in *F. culmorum* liquid cultures. Kan-Mitchell *et al.* (1990), Murphy *et al.* (1990) and Horikoshi *et al.* (1992) performed separate amplification reactions for the gene of interest and the internal reference standard used. Kan Mitchell *et al.* (1993) compared the expression of a calcium-modulated protein (S100β) in choroidal and skin

melanomas to the expression of the β-actin "housekeeping" gene in order to estimate the relative S100β gene expression. Horikoshi *et al.* (1992) used separate PCR amplification of β_2-microglobulin and β-actin cDNA as internal reference standard "housekeeping" genes to measure the relative gene expressions of thymidylate synthase, dihydrofolate reductase and DT-diaphorase genes from human tumour specimens. According to Murphy *et al.* (1990), simultaneous amplification of cDNA from "housekeeping" genes and target genes results in lower levels of PCR products due to competition, which varies from sample to sample. The disadvantage of independent (separate tube) amplification of the internal reference standard and control genes is the variability which may exist between individual reaction tubes.

Co-amplification systems have also been used to estimate the expression of target sequences of interest relative to the expression of internal control "housekeeping" genes (Hurwitz *et al.*, 1985; Kinoshita *et al.*, 1992). The relative gene expression of a retroviral mRNA sequence was estimated by co-amplifying the cDNA of interest with endogenous β-actin cDNA (Kinoshita *et al.*, 1992). Hurwitz *et al.* (1985) used co-amplification of a structurally unrelated endogenous gene as internal standard for estimating the relative amounts of target DNA in different samples. For this reason, co-amplification of *Tri5*- and β-tubulin were used for semi-quantitative *Tri5* RT-PCR analysis. In this work, the levels of *Tri5* gene expression detected were relatively high compared to β-tubulin expression. However, since internal control genes such as β-tubulin are often expressed at higher levels than most messages, in cases of low *Tri5* expression it may be necessary to use independent *Tri5* and β-tubulin amplification systems.

Although it has not been discussed in this work, an exogenous competitor template has also been produced for quantitative *Tri5* RT-PCR analysis. This competitor was constructed by the method described for the *F. poae*-specific competitive template in Chapter 2 (section 2.3.6.1) using an unrelated DNA fragment from the gene encoding a 23 Kda extrinsic polypeptide of the oxygen-evolving complex from pea (*Pisum savitum* cv. Feltham First) photosystem II (Wales *et al.*, 1989). By this method of quantification, the competitor template is added to the PCR reaction and both the competitor fragment and target sequence compete for the same primers within the reaction. This method of quantitative RT-PCR has previously been used for quantitative analysis of gene expression in the plant pathogenic fungus *Phanerochaete chrysosporium* (Stewart *et al.*, 1992; Lamar *et al.*, 1995; Bogan *et al.*, 1996a,b). One of the advantages of this method over the semi-quantitative RT-PCR method described above is that absolute rather than empirical values are obtained for *Tri5* expression. One of the disadvantages of this method is that, unlike the semi-quantitative RT-PCR method, this system does not take into account the accuracy of the RNA quantification step (by spectrophotometry) or the efficiency of the reverse transcription step. In any case, both techniques are now available and can be compared in future *Tri5*-specific RT-PCR experiments.

With the semi-quantitative system, it is important that ratios are based on PCR products from the linear exponential range of amplification and are not influenced by the 'plateau' effect which occurs in PCR reactions when reagents become limiting, i.e. following extensive PCR cycling or in the presence of excess template. The linear range of amplification can be determined either by serially diluting the amount of cDNA in

the PCR or by terminating the PCR at sequential cycles (Murphy *et al.*, 1990; Kan-Mitchell *et al.*, 1993). In this work, serial dilutions of cDNA were used to determine the linear range of amplification and it was found that incorporation of 10 µl of 100 µl diluted reverse transcription reactions, the equivalent of 100 µg of input total RNA, was optimal for both *Tri5* and β-tubulin. The optimal quantity of input RNA should, ideally, be determined for every sample in each individual experiment, although in the present work the optima were only based on a limited number of samples.

The potential use of this semi-quantitative *Tri-5*-specific RT-PCR-based assay as a tool to monitor *Tri5* gene expression over time and as a method for studying the effect of fungicides on trichothecene expression in liquid culture was investigated. *F. culmorum* was the pathogen of choice for this study because of its importance in cooler maritime regions of the world, such as the UK (Parry *et al.*, 1995a). For control *F. culmorum* liquid cultures, the relative *Tri5* gene expression was found to vary over time, being greatest 48 h post-inoculation, although differences in expression between time points were not statistically significant. Using northern blot analysis of gene expression, Hohn *et al.* (1993) showed that the level of *Tri5* mRNA in *G. pulicaris* increased 47-fold between 18 and 42 h post-inoculation, although these workers did not use any internal reference "housekeeping" genes to normalise (standardise) their results. Enzyme assays showed that trichodiene synthase activity increased approximately 10-fold during the same period. At the genetic level, research conducted by Hohn *et al.* (1993) indicated that transcriptional controls play an important role in the regulation of *Tri5* gene expression and that other genes involved in trichothecene biosynthesis in *F. sambucinum* may be linked to *Tri5*. Using chemical methods of analysis, Miller *et al.*

(1983a) showed that major environmental factors which stimulate trichothecene production in *F. graminearum* liquid cultures include oxygen levels, depletion of carbohydrate in the medium, pH and possibly a low concentration of an organic nitrogen source (Miller *et al.*, 1993a).

The present work indicated that, at least *in vitro,* fungicides can significantly affect the production of trichothecene mycotoxins by *F. culmorum*. The fungicides prochloraz and tebuconazole were chosen as test compounds since work discussed in Chapter 2 showed that both these fungicides reduced FEB of wheat caused by *F. culmorum*. Initially, both fungicides caused significant increases in the relative *Tri5* expression (36 h post-inoculation), but later (48 & 96 h post-inoculation), the situation was reversed, both fungicides decreasing *Tri5* expression, although not significantly. Results also indicated that there may be a relationship between fungicide concentration and relative *Tri5* expression, higher levels being detected at the higher fungicide concentrations, although again, differences between concentrations were not statistically significant. It is recognised that in the present work, only a limited number of replicate samples were used and the experiment was not repeated, therefore no firm conclusions can be drawn with regard to the effect of these particular fungicides on *Tri5* gene expression.

Overall, this work has produced a semi-quantitative RT-PCR-based assay for analysis of *Tri5* gene expression and a preliminary experiment has indicated that fungicide treatments may significantly affect the expression of *Tri5* and hence the production of trichothecene mycotoxins by *F. culmorum*. In the future, this assay should allow further, more detailed *in vitro* studies on the effect of fungicides on trichothecene

expression. Also, further investigations will determine if this assay can be used to detect *Tri5* gene expression in infected plant material, and will therefore determine if this assay can be used to evaluate the *in planta* effect of fungicides on *Tri5* gene expression by *Fusarium* species. In the present work, the *Tri5*-specific RT-PCR-based assay was developed and used for the detection and semi-quantification of *Tri5* gene expression in RNA extracts from *F. culmorum* liquid cultures. But, because the primers used for *Tri5* RT-PCR analysis were designed to conserved regions of the *Tri5* genes from *F. culmorum, F. graminearum, F. poae, F. sporotrichoides* and *F. sambucinum,* in theory, it should be possible to use this RT-PCR assay to study *Tri5* gene expression by any these pathogens. This work has also provided a *Tri5*-specific PCR assay for the detection of potential trichothecene-producing *Fusarium* species in infected wheat tissue. Such an assay could be used to pre-screen grain samples for the presence of potential trichothecene-producing *Fusarium* species prior to using classical chemical analysis for the detection of trichothecene mycotoxins.

CONCLUSIONS

Fusarium ear blight (FEB) of wheat can be caused by a range of different *Fusarium* species, but most reports of disease have been associated with *F. culmorum*, *F. graminearum*, *F. poae*, *F. avenaceum* and/or *Microdochium nivale* (Parry *et al.*, 1995a). These pathogens can occur either independently, or as shown in Chapter 1, as a complex of causal organisms, which greatly complicates the study of FEB of wheat.

Comparison of visual disease assessment and species-specific PCR analysis for the detection of *F. culmorum* ear blight of wheat in a field trial in which the central sub-plot was inoculated with *F. culmorum* demonstrated that visual disease assessment could not distinguish between the inoculated pathogen and the complex of other FEB causal organisms which were naturally present in the field trial. These results indicated that PCR-based assays rather than visual methods should be used in FEB related studies where natural inoculum may be present. In Chapter 1, separation of wheat ears into their component parts, i.e. grain, glume and rachis tissue for PCR analysis permitted tissue localisation of the pathogens. This showed that *F. poae* was predominantly present in the glume, rather than grain or rachis components, which agreed with the findings of Polley *et al.* (1991). Also, *M. nivale* was predominantly present in rachis tissue and *M. nivale* var. *majus* was more abundant than var. *nivale*.

The present work provides the first report of the use of quantitative PCR analysis to evaluate the efficacy of fungicides against FEB of wheat (Chapter 2). Under glasshouse conditions, *F. culmorum* ear blight disease symptoms were distinct from those observed for *F. poae*. *F. culmorum* ear blight was associated with premature bleaching of spikelets, while *F. poae* disease was associated with the formation of brown glume spots surrounded by a dark brown margin. Overall, results obtained by

visual disease assessment and quantitative PCR analysis of the glasshouse fungicide trials of *F. culmorum* and *F. poae* ear blight of wheat were broadly similar. However, the reductions observed in DNA contents of wheat ears due to the fungicides prochloraz and tebuconazole were consistently greater than the reductions in visual disease scores. In general, visual disease assessment did not resolve fungicide efficacy to the same degree as seen by PCR analysis. This was particularly evident with ear blight due to *F. poae*, where fungicide efficacy of tebuconazole and prochloraz was much greater as measured by quantitative PCR analysis rather than by visual disease assessment.

Significant relationships were observed between *F. culmorum* disease and yield in the fungicide experiments (Chapter 2), irrespective of whether disease was measured by visual assessment or quantitative PCR analysis, although the best relationship was observed between DNA content and yield in the 1996/7 experiment. Interestingly, *F. poae* did not, in general, significantly affect yield, even where comparable disease levels caused by *F. culmorum* significantly reduced yield. It is suggested that *F. culmorum* and *F. poae* may differ in their mode of infection and/or colonisation of wheat ears. This is supported by Chapter 1, where *F. poae* was predominantly found in the glume, rather than grain or rachis tissue, whereas *F. culmorum* was generally found in all component parts of the ear. Although Parry *et al.* (1995a) suggested that *F. culmorum* and *F. graminearum* were consistently the most pathogenic *Fusarium* species infecting cereal ears, no detailed studies have yet been undertaken to investigate the pathogenicity, mode of infection or possible tissue specialisation among the common causal organisms of FEB of wheat.

Quantitative PCR analysis of fungicide efficacy (Chapter 2) showed that the sterol biosynthesis inhibiting fungicides (SBI's) prochloraz and tebuconazole effectively controlled *F. culmorum* and *F. poae* ear blight of wheat under glasshouse

conditions, and that (overall), both fungicide treatments were equally effective, resulting in moderate to good disease control. Neither fungicide totally eradicated *F. culmorum* or *F. poae* disease, but the disease pressure (i.e. inoculum loads used for the glasshouse experiments) were high, probably much greater than any naturally occurring inoculum. However, it is questionable whether moderate to good control of the disease is acceptable, since there is controversy regarding the effect of fungicides on the production of mycotoxins by *Fusarium* species (Martin & Johnston, 1982; Moss & Frank, 1985; Boyacioglu *et al.*, 1992; Suty *et al.*, 1996; Gareis & Ceynowa, 1994). Therefore, more detailed studies are required on the effect of existing fungicides on the production of mycotoxins by *Fusarium* species, and any new compounds developed to control FEB of wheat should combine eradicant properties with inhibition of mycotoxin production.

Within this work (Chapter 3), *F. culmorum* has been transformed with the β-glucuronidase (*gusA*) (GUS) reporter gene. It has been shown that GUS-transformed isolates can be used to evaluate the efficacy of fungicides against *F. culmorum* foot rot of wheat. Traditional stem base disease assessment for stem base diseases are highly subjective. The use of GUS transformed isolates may permit more objective studies of fungicide efficacy and host resistance against this pathogen.

Problems inherent in studying the effect of fungicides on the production of trichothecene mycotoxins by *Fusarium* ear blight pathogens, such as the multitude of potential pathway intermediates and end-products (Betina, 1989), led to the development of the semi-quantitative RT-PCR-based assay discussed in Chapter 4, which was specific for the *Tri5* gene which codes for trichodiene synthase; the enzyme that catalyses the first step of the trichothecene biosynthetic pathway. The potential use of this technique as a tool for evaluating the effect of fungicides on *Tri5* gene

expression, and hence trichothecene production by *F. culmorum* was demonstrated. Preliminary results indicated that, in *F. culmorum* liquid cultures, the fungicides prochloraz and tebuconazole can significantly affect the expression of trichothecene mycotoxins. This tool could also be used for studying the effect of environmental factors on trichothecene production by *Fusarium* species, including, temperature, moisture, aeration, fungal competition and nutrients such as starch, oils etc. present in grain. It could also be used to study the effect of the *Fusarium* hyphal growth stimulators choline chloride and betaine hydrochloride, which are present in anthers (Strange & Smith, 1978), on *Tri5* gene expression. The sensitivity of this technique over traditional RNA blot techniques (Byrne *et al.*, 1988; Wang *et al.*, 1989; Mocharla *et al.*, 1990) may permit *in planta* studies of *Tri5* gene expression. Similar systems could also be developed to study the effects of fungicides and other environmental factors on the expression of additional genes within the trichothecene biosynthetic pathway which have been cloned and sequenced, such as *Tri3* (McCormick *et al.*, 1996), *Tri4* (Hohn *et al.*, 1995) and *Tri6* (Proctor *et al.*, 1995b). RT-PCR assays could be developed for the study of other biosynthetic pathways of the pathogens, once the sequence of the genes coding for the enzyme(s) involved in the initial steps of the biosynthetic pathway are known.

In conclusion, the work described here and the techniques developed to study FEB of wheat will allow more in-depth investigations into the different aspects of FEB of wheat, including fungal pathogenicity, competition between pathogens, mycotoxin production, host resistance and fungicide application. In the long term, such in-depth studies should provide a better understanding of the FEB disease complex, and this deeper understanding of the disease may ultimately point towards new approaches to disease control.

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APPENDICES

APPENDIX 1. TESTS OF ASSOCIATION BETWEEN PATHOGENS (SEE CHAPTER 1, SECTION 1.3.3)

Association between pathogens (*Fusarium culmorum, F. poae, F. avenaceum* and *Microdochium nivale)* was tested using Fisher's exact test (Everitt, 1986) and the probabilities of two species being independent are shown below. A correction to these values was needed because there were several tests (Rom, 1990). On this basis, the only significant association was that between *F. culmorum* and *M. nivale* ($0.05 > P > 0.01$), with a comparison of the observed and expected values showing that there was an excess of samples where only one of these two pathogens was present.

Table 1.1 Fisher's exact test (probability of two species being independent).

	Fungal species			
Fungal species	*Fusariumculmorum*	*F. poae*	*F. avenaceum*	*Microdochium nivale*
F. culmorum	X	0.182	0.012	1.94×10^{-3}
F. poae		X	0.207	0.152
F. avenaceum			X	0.192
M. nivale				X

APPENDIX 2. ANALYSIS OF THE EFFECT OF FUNGICIDES ON *FUSARIUM* EAR BLIGHT OF WHEAT (SEE CHAPTER 2)

APPENDIX 2.1 ONE WAY ANALYSIS OF VISUAL DISEASE ASSESSMENT RESULTS (SEE CHAPTER 2, SECTIONS 2.4.2.1 & 2.4.3.1)

Appendix 2.1.1 1994/5 results

GS 70

Analysis of Variance on GS 70

Source	DF	SS	MS	F	p
TREATMENT	2	12476	6238	14.31	0.000
Error	96	41848	436		
Total	98	54324			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	------+---------+---------+---------+
1*	33	42.62	22.93	(-----*-----)
2*	33	51.76	25.24	(-----*-----)
3*	33	24.73	12.04	(-----*-----)

------+---------+---------+---------+

Pooled StDev = 20.88 24 36 48 60

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.0192 Critical value = 3.37

Intervals for (column level mean) - (row level mean)

	1*	2*
2*	-21.39 3.11	
3*	5.64 30.14	-39.28 -14.78

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & pyrimethanil-treated; 3, *F. culmorum*-inoculated & prochloraz-treated

GS 75

Analysis of Variance on GS 75

Source	DF	SS	MS	F	p
TREATMENT	2	17818	8909	40.32	0.000
Error	96	21210	221		
Total	98	39029			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	-+---------+---------+---------+-----
1*	33	91.02	14.26	(---*---)
2*	33	93.64	9.23	(---*---)
3*	33	63.96	19.34	(---*----)

-+---------+---------+---------+-----

Pooled StDev = 14.86 60 72 84 96

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.0192 Critical value = 3.37

Intervals for (column level mean) - (row level mean)

	1*	2 *
2*	-11.35 6.09	
3*	18.33 35.77	-38.40 -20.96

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & pyrimethanil-treated; 3, *F. culmorum*-inoculated & prochloraz-treated.

GS 80

Analysis of Variance on GS 80

Source	DF	SS	MS	F	p
TREATMENT	2	10834	5417	42.30	0.000
Error	96	12295	128		
Total	98	23130			

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
1*	33	99.11	3.81	(---*---)
2*	33	99.86	0.78	(---*---)
3*	33	77.30	19.21	(---*---)
Pooled StDev =		11.32		80 90 100

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.0192 Critical value = 3.37

Intervals for (column level mean) - (row level mean)

	1*	2*
2*	-7.40 5.88	
3*	15.16 28.44	15.92 29.20

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & pyrimethanil-treated; 3, *F. culmorum*-inoculated & prochloraz-treated.

Appendix 2.1.2 1995/6 results

GS 70

Analysis of Variance on GS70

Source	DF	SS	MS	F	p
TREATMENT	7	44728	6390	29.75	0.000
Error	256	54992	215		
Total	263	99719			

Individual 95% CIs For Mean Based on Pooled StDev.

Level	N	Mean	StDev	-------+---------+---------+---------
1*	33	40.38	19.66	(--*--)
2*	33	20.92	14.93	(--*--)
3*	33	22.70	13.78	(--*--)
4*	33	53.48	12.04	(---*--)
5*	33	33.70	19.61	(--*---)
6*	33	16.43	10.68	(--*--)
7*	33	11.02	11.63	(--*---)
8*	33	33.30	11.86	(--*---)
Pooled StDev =		14.66		-------+---------+---------+--------- 15 30 45

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00269 Critical value = 4.29

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*	6*	7*
2*	8.51 30.40						
3*	6.74 28.63	-12.72 9.17					
4*	-24.04 -2.15	-43.50 -21.61	-41.73 -19.84				
5*	-4.27 17.62	-23.73 -1.84	-21.95 -0.06	8.83 30.72			
6*	13.00 34.89	-6.46 15.43	-4.68 17.21	26.10 47.99	6.33 28.22		
7*	18.41 40.30	-1.05 20.84	0.73 22.62	31.51 53.40	11.73 33.63	-5.54 16.35	
8*	-3.86 18.03	-23.32 -1.43	-21.55 0.34	9.23 31.13	-10.54 11.35	-27.81 -5.92	-33.22 -11.33

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & prochloraz-treated; 3, *F. culmorum*-inoculated & tebuconazole-treated; 4, *F. culmorum*-inoculated & culture filtrate-treated; 5, *F. poae*-inoculated; 6, *F. poae*-inoculated & prochloraz-treated; 7, *F. poae*-inoculated & culture filtrate-treated.

GS 75

Analysis of Variance on GS75

Source	DF	SS	MS	F	p
TREATMENT	7	58619	8374	31.67	0.000
Error	256	67693	264		
Total	263	126312.			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	---+---------+---------+---------+---
1*	33	54.34	16.72	(--*--)
2*	33	37.39	19.02	(--*-)
3*	33	37.49	19.51	(--*--)
4*	33	69.94	9.82	(--*--)
5*	33	39.33	22.52	(--*-)
6*	33	24.35	13.58	(--*--)
7*	33	43.06	9.91	(--*-)
8*	33	19.74	14.40	(--*--)

Pooled StDev = 16.26 ---+---------+---------+---------+---
20 40 60 80

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00269 Critical value = 4.29

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*	6*	7*
2*	4.81 29.10						
3*	4.70 28.99	-12.25 12.04					
4*	-27.74 -3.45	-44.69 -20.41	-44.59 -20.30				
5*	2.87 27.16	-14.09 10.20	-13.98 10.31	18.46 42.75			
6*	17.85 42.14	0.90 25.18	1.00 25.29	33.45 57.73	2.84 27.13		
7*	-0.86 23.42	-17.82 6.47	-17.71 6.57	14.73 39.02	-15.88 8.41	-30.86 -6.57	
8*	22.46 46.75	5.50 29.79	5.61 29.90	38.05 62.34	7.45 31.73	-7.54 16.75	11.18 35.47

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & prochloraz-treated; 3, *F. culmorum*-inoculated & tebuconazole-treated; 4, *F. culmorum*-inoculated & culture filtrate-treated; 5, *F. poae*-inoculated; 6, *F. poae*-inoculated & prochloraz-treated; 7, *F. poae*-inoculated & culture filtrate-treated.

GS 80

Analysis of Variance on GS 80

Source	DF	SS	MS	F	p
TREATMENT	7	164910	23559	86.81	0.000
Error	256	69477	271		
Total	263	234388			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	----+---------+---------+---------+--
1*	33	86.73	18.42	(--*-)
2*	33	42.26	17.78	(-*-)
3*	33	44.13	18.96	(--*-)
4*	33	96.32	7.21	(--*-)
5*	33	44.39	24.36	(--*-)
6*	33	25.98	13.91	(-*--)
7*	33	21.59	14.49	(--*-)
8*	33	48.07	10.37	(-*-)

Pooled StDev = 16.47 ----+---------+---------+---------+--
25 50 75 100

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00269 Critical value = 4.29

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*	6*	7*
2*	32.16						
	56.77						
3*	30.30	-14.17					
	54.90	10.44					
4*	-21.90	-66.36	-64.50				
	2.71	-41.75	-39.89				
5*	30.04	-14.42	-12.56	39.63			
	54.65	10.18	12.05	64.24			
6*	48.45	3.98	5.85	58.04	6.10		
	73.05	28.59	30.45	82.65	30.71		
7*	52.84	8.37	10.24	62.43	10.49	-7.91	
	77.44	32.98	34.84	87.04	35.10	16.69	
8*	26.36	-18.11	-16.24	35.95	-15.99	-34.39	-38.78
	50.96	6.50	8.36	60.56	8.62	-9.79	-14.18

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & prochloraz-treated; 3, *F. culmorum*-inoculated & tebuconazole-treated; 4, *F. culmorum*-inoculated & culture filtrate-treated; 5, *F. poae*-inoculated; 6, *F. poae*-inoculated & prochloraz-treated; 7, *F. poae*-inoculated & culture filtrate-treated.

Appendix 2.1.3 1996/7 results

GS 70

Analysis of Variance on Gs 70

Source	DF	SS	MS	F	p
TREATMENT	5	138818	27764	115.96	0.000
Error	192	45970	239		
Total	197	184788			

Individual 95% CIs For Mean
Based on Pooled StDev

Level	N	Mean	StDev	-+---------+---------+---------+-----
1*	33	81.27	9.43	(--*-)
2*	33	41.29	24.51	(--*-)
3*	33	45.15	23.15	(-*-)
4*	33	29.86	10.22	(-*-)
5*	33	3.50	6.36	(-*--)
6*	33	4.88	8.14	(-*-)

Pooled StDev = 15.47 -+---------+---------+---------+----- 0 25 50 75

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00444 Critical value = 4.07

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*
2*	29.02				
	50.95				
3*	25.16	-14.83			
	47.08	7.10			
4*	40.45	0.46	4.33		
	62.37	22.39	26.25		
5*	66.81	26.82	30.69	15.40	
	88.73	48.75	52.61	37.32	
6*	65.43	25.44	29.31	14.02	-12.34
	87.35	47.37	51.23	35.94	9.58

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & prochloraz-treated; 3, *F. culmorum*-inoculated & tebuconazole-treated; 4, *F. poae*-inoculated; 5, *F. poae*-inoculated & prochloraz-treated; 6, *F. poae*-inoculated & tebuconazole-treated.

GS 75

Analysis of Variance on GS 75

Source	DF	SS	MS	F	p
TREATMENT	5	188421	37684	193.70	0.000
Error	192	37354	195		
Total	197	225774			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	--------+---------+---------+--------
1*	33	98.41	3.80	(-*)
2*	33	66.21	18.69	(-*-)
3*	33	67.28	17.52	(*-)
4*	33	64.31	12.60	(*-)
5*	33	10.87	10.89	(-*)
6*	33	15.97	14.81	(*-)

Pooled StDev = 13.95 30 60 90

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00444 Critical value = 4.07

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*
2*	22.32 42.08				
3*	21.25 41.01	-10.95 8.81			
4*	24.21 43.98	-7.99 11.78	-6.92 12.85		
5*	77.65 97.42	45.45 65.22	46.52 66.29	43.56 63.32	
6*	72.56 92.32	40.35 60.12	41.43 61.19	38.46 58.22	-14.98 4.79

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & prochloraz-treated; 3, *F. culmorum*-inoculated & tebuconazole-treated; 4, *F. poae*-inoculated; 5, *F. poae*-inoculated & prochloraz-treated; 6, *F. poae*-inoculated & tebuconazole-treated.

GS 80

Analysis of Variance on GS80

Source	DF	SS	MS	F	p
TREATMENT	5	195249	39050	167.43	0.000
Error	192	44781	233		
Total	197	240030			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	--------+---------+---------+--------
1*	33	100.00	0.00	(*-)
2*	33	74.08	23.57	(-*)
3*	33	73.00	16.50	(*-)
4*	33	77.05	14.89	(-*)
5*	33	13.61	11.73	(-*)
6*	33	21.18	14.57	(-*-)

Pooled StDev = 15.27 30 60 90

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00444 Critical value = 4.07

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*
2*	15.10 36.74				
3*	16.18 37.82	-9.74 11.90			
4*	12.13 33.77	-13.79 7.85	-14.87 6.77		
5*	75.57 97.21	49.65 71.29	48.57 70.21	52.62 74.26	
6*	68.00 89.64	42.08 63.72	41.00 62.64	45.05 66.69	-18.39 3.25

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & prochloraz-treated; 3, *F. culmorum*-inoculated & tebuconazole-treated; 4, *F. poae*-inoculated; 5, *F. poae*-inoculated & prochloraz-treated; 6, *F. poae*-inoculated & tebuconazole-treated.

APPENDIX 2.2 ANALYSIS OF QUANTITATIVE PCR RESULTS FOLLOWING SQUARE ROOT TRANSFORMATION (SEE CHAPTER 2, SECTIONS 2.4.2.2 & 2.4.3.2)

Appendix 2.2.1 1994/5 results

General Linear Model

Factor	Levels	Values
PCR REPLICATE	3	1 2 3
TREATMENT	3	1 2 3

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F	P
PCR REPLICATE	2	0.01343	0.01343	0.00671	0.35	0.709
TREATMENT	2	0.47034	0.47034	0.23517	12.10	0.000
Error	94	1.82702	1.82702	0.01944		
Total	98	2.31078				

One Way Analysis of Variance

Source	DF	SS	MS	F	p
TREATMENT	2	0.4703	0.2352	12.27	0.000
Error	96	1.8404	0.01921		
Total	98	2.3108			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	---+---------+---------+---------+---
1*	33	1.2447	0.1556	(------*------)
2*	33	1.2031	0.1655	(------*------)
3*	33	1.0822	0.0769	(------*-----)

Pooled StDev = 0.1385 ---+---------+---------+---------+--- 1.050 1.120 1.190 1.260

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.0192 Critical value = 3.37

Intervals for (column level mean) - (row level mean)

	1*	2*
2*	-0.0396 0.1228	
3*	0.0813 0.2437	-0.202 -0.0397

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & pyrimethanil-treated; 3, *F. culmorum*-inoculated & prochloraz-treated

Appendix 2.2.2 1995/6 results

General Linear Model

Factor	Levels	Values
PCR REPLICATE	3	1 2 3
TREATMENT	8	1 2 3 4 5 6 7 8

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F	P
PCR REPLICATE	2	0.1887	0.1887	0.0944	1.36	0.258
TREATMENT	7	85.4268	85.4268	12.2038	176.02	0.000
Error	254	17.6101	17.6101	0.0693		
Total	263	103.2256				

One-Way Analysis of Variance

Source	DF	SS	MS	F	p
TREATMENT	7	85.4268	12.2038	175.53	0.000
Error	256	17.7989	0.0695		
Total	263	103.2256			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	--------+---------+---------+--------
1*	33	1.6003	0.2855	(-*)
2*	33	0.8844	0.2928	(-*)
3*	33	0.9030	0.2434	(*-)
4*	33	1.9435	0.3232	(*-)
5*	33	0.4392	0.1877	(*-)
6*	33	0.2474	0.1103	(*-)
7*	33	0.3827	0.2513	(*-)
8*	33	0.5682	0.3387	(*-)

Pooled StDev = 0.2637 --------+---------+---------+-------- 0.60 1.20 1.80

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00269 Critical value = 4.29

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*	6*	7*
2*	0.5191						
	0.9129						
3*	0.5004	-0.2156					
	0.8942	0.1783					
4*	-0.5401	-1.2560	-1.2374				
	-0.1462	-0.8622	-0.8435				
5*	0.9642	0.2482	0.2669	1.3073			
	1.3580	0.6420	0.6607	1.7012			
6*	1.1560	0.4400	0.4587	1.4991	-0.0051		
	1.5498	0.8339	0.8525	1.8930	0.3887		
7*	1.0207	0.3047	0.3234	1.3638	-0.1404	-0.3322	
	1.4145	0.6985	0.7172	1.7577	0.2534	0.0616	
8*	0.8352	0.1192	0.1379	1.1784	-0.3259	-0.5177	-0.3824
	1.2290	0.5131	0.5317	1.5722	0.0679	-0.1239	0.0114

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & prochloraz-treated; 3, *F. culmorum*-inoculated & tebuconazole-treated; 4, *F. culmorum*-inoculated & culture filtrate-treated; 5, *F. poae*-inoculated; 6, *F. poae*-inoculated & prochloraz-treated; 7, *F. poae*-inoculated & culture filtrate-treated.

Appendix 2.2.3 1996/7 results

General Linear Model

Factor	Levels	Values
PCR REPLICATE	3	1 2 3
TREATMENT	6	1 2 3 4 5 6

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F	P
PCR REPLICATE	2	0.025	0.025	0.012	0.14	0.865
TREATMENT	5	106.294	106.294	21.259	249.21	0.000
Error	190	16.208	16.208	0.085		
Total	197	122.527				

One-Way Analysis of Variance

Source	DF	SS	MS	F	p
TREATMENT	5	106.2942	21.2588	251.45	0.000
Error	192	16.2326	0.0845		
Total	197	122.5267			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	--------+---------+---------+--------
1*	33	2.4966	0.3508	(-*)
2*	33	1.2620	0.3411	(*)
3*	33	0.7693	0.1438	(*)
4*	33	0.7788	0.4271	(*-)
5*	33	0.2618	0.1153	(-*)
6*	33	0.4843	0.2269	(-*)
				--------+---------+---------+--------

Pooled StDev = 0.2908 0.70 1.40 2.10

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00444 Critical value = 4.07

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*
2*	1.0287				
	1.4407				
3*	1.5213	0.2867			
	1.9333	0.6987			
4*	1.5118	0.2772	-0.2155		
	1.9239	0.6892	0.1965		
5*	2.0288	0.7941	0.3015	0.3110	
	2.4408	1.2061	0.7135	0.7230	
6*	1.8063	0.5716	0.0789	0.0884	-0.4285
	2.2183	0.9836	0.4910	0.5004	-0.0165

*Treatments: 1, *F. culmorum*-inoculated; 2, *F. culmorum*-inoculated & prochloraz-treated; 3, *F. culmorum*-inoculated & tebuconazole-treated; 4, *F. poae*-inoculated; 5, *F. poae*-inoculated & prochloraz-treated; 6, *F. poae*-inoculated & tebuconazole-treated.

APPENDIX 2.3 ANALYSIS OF NUMBER OF GRAIN PER EAR FOLLOWING SQUARE ROOT TRANSFORMATION OF DATA (SEE CHAPTER 2, SECTIONS 2.4.2.3 & 2.4.3.3)

Appendix 2.3.1 1994/5 results

One-Way Analysis of Variance

Source	DF	SS	MS	F	p
TREATMENT	5	1.426	0.285	0.97	0.442
Error	60	17.591	0.293		
Total	65	19.017			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	---------+---------+---------+-------
1*	11	7.6721	0.5944	(----------*----------)
2*	11	7.7762	0.5003	(----------*----------)
3*	11	7.5728	0.4306	(----------*----------)
4*	11	7.6015	0.5524	(----------*----------)
5*	11	7.9615	0.6136	(----------*----------)
6*	11	7.9073	0.5370	(----------*----------)

Pooled StDev = 0.5415 ---------+---------+---------+-------
7.50 7.80 8.10

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00463 Critical value = 4.16

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*
2*	-0.7833 0.5750				
3*	-0.5798 0.7785	-0.4757 0.8826			
4*	-0.6086 0.7497	-0.5045 0.8538	-0.7079 0.6504		
5*	-0.9686 0.3897	-0.8644 0.4939	-1.0679 0.2904	-1.0391 0.3192	
6*	-0.9144 0.4439	-0.8103 0.5480	-1.0137 0.3446	-0.9849 0.3734	-0.6250 0.7333

*Treatments: 1, control (uninoculated & untreated); 2, prochloraz-treated; 3, pyrimethanil-treated; 4, *F. culmorum*-inoculated; 5, *F. culmorum*-inoculated & prochloraz-treated; 6, *F. culmorum*-inoculated & pyrimethanil-treated.

Appendix 2.3.2 1995/6 results

One-Way Analysis of Variance

Source	DF	SS	MS	F	p
TREATMENT	11	18.222	1.657	5.03	0.000
Error	117	38.513	0.329		
Total	128	56.735			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	-----+---------+---------+---------+-
1*	11	7.9625	0.3980	(-----*----)
2*	11	8.0540	0.3815	(----*-----)
3*	11	7.7364	0.5120	(-----*-----)
4*	9	7.9667	0.6885	(------*-----)
5*	11	7.2452	0.5980	(-----*----)
6*	11	8.2499	0.7306	(----*-----)
7*	11	7.7390	0.5649	(-----*-----)
8*	11	8.5363	0.3802	(----*-----)
9*	11	7.3419	0.8977	(----*-----)
10*	11	8.2843	0.4556	(-----*-----)
11*	10	8.3249	0.3267	(-----*-----)
12*	11	8.0768	0.6645	(-----*----)

-----+---------+---------+---------+-
Pooled StDev = 0.5737 7.20 7.80 8.40 9.00

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00114 Critical value = 4.72

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*	6*	7*
2*	-0.9080 0.7250						
3*	-0.5904 1.0426	-0.4989 1.1341					
4*	-0.8648 0.8565	-0.7733 0.9481	-1.0909 0.6305				
5*	-0.0992 1.5338	-0.0077 1.6253	-0.3253 1.3077	-0.1392 1.5821			
6*	-1.1039 0.5291	-1.0123 0.6207	-1.3299 0.3031	-1.1439 0.5775	-1.8211 -0.1881		
7*	-0.5930 1.0400	-0.5014 1.1316	-0.8190 0.8140	-0.6330 1.0883	-1.3103 0.3227	-0.3056 1.3274	
8*	-1.3903 0.2427	-1.2988 0.3342	-1.6164 0.0166	-1.4303 0.2910	-2.1076 -0.4746	-1.1030 0.5300	-1.6138 0.0192
9*	-0.1959 1.4371	-0.1044 1.5286	-0.4220 1.2110	-0.2359 1.4854	-0.9132 0.7198	0.0914 1.7244	-0.4194 1.2136
10*	-1.1383 0.4947	-1.0467 0.5863	-1.3643 0.2687	-1.1783 0.5430	-1.8556 -0.2226	-0.8509 0.7821	-1.3618 0.2712
11*	-1.1991 0.4743	-1.1075 0.5658	-1.4251 0.2482	-1.2381 0.5216	-1.9163 -0.2430	-0.9117 0.7616	-1.4226 0.2507
12*	-0.9308 0.7022	-0.8393 0.7937	-1.1569 0.4761	-0.9708 0.7505	-1.6481 -0.0151	-0.6435 0.9895	-1.1543 0.4787

	8*	9*	10*	11*
9*	0.3779 2.0109			
10*	-0.5645 1.0685	-1.7589 -0.1259		
11*	-0.6253 1.0481	-1.8196 -0.1463	-0.8773 0.7961	
12*	-0.3570 1.2760	-1.5514 0.0816	-0.6090 1.0240	-0.5886 1.0847

*Treatments: 1, control (uninoculated & untreated); 2, prochloraz-treated; 3, tebuconazole-treated; 4, culture filtrate-treated; 5, *F. culmorum*-inoculated; 6, *F. culmorum*-inoculated & prochloraz-treated; 7, *F. culmorum*-inoculated & tebuconazole-treated; 8, *F. culmorum*-inoculated & culture filtrate-treated; 9, *F. poae*-inoculated; 10, *F. poae*-inoculated & prochloraz-treated; 11, *F. poae*-inoculated & tebuconazole-treated; 12, *F. poae*-inoculated & culture filtrate-treated.

Appendix 2.3.3 1996/7 results

One Way Analysis of Variance

Source	DF	SS	MS	F	p
TREATMENT	8	6.633	0.829	4.34	0.000
Error	90	17.174	0.191		
Total	98	23.807			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	---------+---------+---------+--------
1*	11	8.0112	0.4511	(-----*------)
2*	11	8.3806	0.5244	(------*-----)
3*	11	8.0862	0.4182	(-----*------)
4*	11	7.7995	0.3883	(------*------)
5*	11	7.5397	0.3663	(-----*------)
6*	11	7.7043	0.2046	(------*-----)
7*	11	8.2664	0.6835	(------*-----)
8*	11	7.9726	0.3388	(-----*------)
9*	11	8.1989	0.3940	(------*------)
				---------+---------+---------+-------

Pooled StDev = 0.4368 7.60 8.00 8.40

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00203 Critical value = 4.50

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*	6*	7*
2*	-0.9622 0.2232						
3*	-0.6677 0.5177	-0.2983 0.8871					
4*	-0.3811 0.8043	-0.0116 1.1738	-0.3060 0.8794				
5*	-0.1213 1.0641	0.2482 1.4336	-0.0463 1.1391	-0.3329 0.8525			
6*	-0.2858 0.8996	0.0836 1.2690	-0.2108 0.9746	-0.4974 0.6880	-0.7572 0.4282		
7*	-0.8479 0.3375	-0.4784 0.7070	-0.7729 0.4125	-1.0595 0.1259	-1.3193 -0.1339	-1.1548 0.0306	
8*	-0.5541 0.6313	-0.1847 1.0007	-0.4791 0.7063	-0.7658 0.4196	-1.0256 0.1598	-0.8610 0.3244	-0.2989 0.8865
9*	-0.7805 0.4049	-0.4110 0.7744	-0.7054 0.4800	-0.9921 0.1933	-1.2519 -0.0665	-1.0874 0.0980	-0.5253 0.6601

	8*
9*	-0.81902 0.36648

*Treatment: 1, control (uninoculated & untreated); 2, prochloraz-treated; 3, pyrimethanil-treated; 4, *F. culmorum*-inoculated; 5, *F. culmorum*-inoculated & prochloraz-treated; 6, *F. culmorum*-inoculated & tebuconazole-treated; 7, *F. poae*-inoculated; 8, *F. poae*-inoculated & prochloraz-treated; 9, *F. poae*-inoculated & tebuconazole-treated.

APPENDIX 2.4 ANALYSIS OF 1000 GRAIN WEIGHT FOLLOWING SQUARE ROOT TRANSFORMATION OF DATA (SEE CHAPTER 2, SECTION 2.4.3.3)

Appendix 2.4.1 1994/5 results

One-Way Analysis of Variance

Source	DF	SS	MS	F	p
TREATMENT	5	66.349	13.270	34.05	0.000
Error	60	23.386	0.390		
Total	65	89.735			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	--+---------+---------+---------+----
1*	11	5.5822	0.6728	(---*---)
2*	11	5.4095	0.6570	(---*---)
3*	11	5.2329	0.6594	(--*---)
4*	11	3.2726	0.5884	(---*--)
5*	11	3.9511	0.4668	(---*--)
6*	11	3.1924	0.6748	(---*---)

Pooled StDev = 0.6243 --+---------+---------+---------+----
3.0 4.0 5.0 6.0

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00463 Critical value = 4.16

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*
2*	-0.6104 0.9558				
3*	-0.4337 1.1324	-0.6064 0.9597			
4*	1.5266 3.0927	1.3539 2.9200	1.1772 2.7434		

	1*	2*	3*	4*	5*
5*	0.8481	0.6754	0.4987	-1.4616	
	2.4142	2.2415	2.0649	0.1046	
6*	1.6067	1.4341	1.2574	-0.7029	-0.0244
	3.1729	3.0002	2.8235	0.8632	1.5417

*Treatments: 1, control (uninoculated & untreated); 2, prochloraz-treated; 3, pyrimethanil-treated; 4, *F. culmorum*-inoculated; 5, *F. culmorum*-inoculated & prochloraz-treated; 6, *F. culmorum*-inoculated & pyrimethanil-treated.

Appendix 2.4.2 1995/6 results

One-Way Analysis of Variance

Source	DF	SS	MS	F	p
TREATMENT	11	121.595	11.054	35.13	0.000
Error	120	37.758	0.315		
Total	131	159.353			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	---+---------+---------+---------+---
1*	11	6.8820	0.2773	(-*--)
2*	11	6.5528	0.5232	(--*-)
3*	11	5.9372	0.6338	(-*--)
4*	11	6.5504	0.6969	(--*-)
5*	11	4.2671	0.5341	(--*-)
6*	11	5.9021	0.4642	(--*--)
7*	11	5.5719	0.5343	(-*--)
8*	11	3.6804	0.3218	(--*-)
9*	11	6.4367	0.4269	(--*-)
10*	11	6.4771	0.7863	(--*--)
11*	11	6.4046	0.6691	(-*--)
12*	11	6.7321	0.6323	(--*--)

Pooled StDev = 0.5609 ---+---------+---------+---------+--- 3.6 4.8 6.0 7.2

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.0011 Critical value = 4.71

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*	6*	7*
2*	-0.4675						
	1.1257						
3*	0.1481	-0.1810					
	1.7413	1.4122					
4*	-0.4650	-0.7941	-1.4097				
	1.1282	0.7991	0.1835				
5*	1.8183	1.4892	0.8736	1.4867			
	3.4115	3.0823	2.4667	3.0799			
6*	0.1832	-0.1459	-0.7615	-0.1483	-2.4316		
	1.7764	1.4473	0.8317	1.4448	-0.8384		
7*	0.5134	0.1843	-0.4313	0.1818	-2.1014	-0.4664	
	2.1066	1.7775	1.1619	1.7750	-0.5083	1.1268	
8*	2.4050	2.0758	1.4602	2.0734	-0.2099	1.4251	1.0949
	3.9981	3.6690	3.0534	3.6666	1.3833	3.0183	2.6881
9*	-0.3514	-0.6805	-1.2961	-0.6830	-2.9662	-1.3312	-1.6614
	1.2418	0.9127	0.2971	0.9102	-1.3731	0.2620	-0.0682
10*	-0.3917	-0.7209	-1.3365	-0.7233	-3.0066	-1.3716	-1.7018
	1.2015	0.8723	0.2567	0.8699	-1.4134	0.2216	-0.1086
11*	-0.3192	-0.6483	-1.2639	-0.6508	-2.9341	-1.2990	-1.6292
	1.2740	0.9449	0.3293	0.9424	-1.3409	0.2942	-0.0360
12*	-0.6467	-0.9759	-1.5915	-0.9783	-3.2616	-1.6266	-1.9568
	0.9464	0.6173	0.0017	0.6148	-1.6684	-0.0334	-0.3636

	8*	9*	10*	11*
9*	-3.5529			
	-1.9597			
10*	-3.5933	-0.8370		
	-2.0001	0.7562		
11*	-3.5207	-0.7644	-0.7241	
	-1.9276	0.8288	0.8691	
12*	-3.8483	-1.0920	-1.0516	-1.1241
	-2.2551	0.5012	0.5416	0.4690

*Treatments: 1, control (uninoculated & untreated); 2, prochloraz-treated; 3, tebuconazole-treated; 4, culture filtrate-treated; 5, *F. culmorum*-inoculated; 6, *F. culmorum*-inoculated & prochloraz-treated; 7, *F. culmorum*-inoculated & tebuconazole-treated; 8, *F. culmorum*-inoculated & culture filtrate-treated; 9, *F. poae*-inoculated; 10, *F. poae*-inoculated & prochloraz-treated; 11, *F. poae*-inoculated & tebuconazole-treated; 12, *F. poae*-inoculated & culture filtrate-treated.

Appendix 2.4.3 1996/7 results

One-Way Analysis of Variance

Source	DF	SS	MS	F	p
TREATMENT	8	202.751	25.344	63.00	0.000
Error	90	36.204	0.402		
Total	98	238.955			

Individual 95% CIs For Mean Based on Pooled StDev

Level	N	Mean	StDev	----------+---------+---------+------
1*	11	6.4822	0.7172	(-*--)
2*	11	6.2855	0.6755	(--*-)
3*	11	6.6545	0.7401	(-*--)
4*	11	1.9782	0.1266	(-*--)
5*	11	4.0618	0.8005	(-*--)
6*	11	4.9600	0.6196	(-*--)
7*	11	5.8309	0.7190	(--*-)
8*	11	6.1973	0.5674	(-*--)
9*	11	6.0245	0.4716	(-*--)

Pooled StDev = 0.6342 3.0 4.5 6.0

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00203 Critical value = 4.50

Intervals for (column level mean) - (row level mean)

	1*	2*	3*	4*	5*	6*	7*
2*	-0.6638						
	1.0573						
3*	-1.0329	-1.2296					
	0.6882	0.4915					
4*	3.6435	3.4467	3.8158				
	5.3646	5.1678	5.5369				
5*	1.5598	1.3631	1.7322	-2.9442			
	3.2809	3.0842	3.4533	-1.2231			
6*	0.6616	0.4649	0.8340	-3.8424	-1.7587		
	2.3827	2.1860	2.5551	-2.1213	-0.0376		
7*	-0.2093	-0.4060	-0.0369	-4.7133	-2.6296	-1.7315	
	1.5118	1.3151	1.6842	-2.9922	-0.9085	-0.0104	
8*	-0.5756	-0.7724	-0.4033	-5.0796	-2.9960	-2.0978	-1.2269
	1.1455	0.9487	1.3178	-3.3585	-1.2749	-0.3767	0.4942
9*	-0.4029	-0.5996	-0.2305	-4.9069	-2.8233	-1.9251	-1.0542
	1.3182	1.1215	1.4905	-3.1858	-1.1022	-0.2040	0.6669

	8*
9*	-0.6878
	1.0333

*Treatment: 1, control (uninoculated & untreated); 2, prochloraz-treated; 3, tebuconazole-treated; 4, *F. culmorum*-inoculated; 5, *F. culmorum*-inoculated & prochloraz-treated; 6, *F. culmorum*-inoculated & tebuconazole-treated; 7, *F. poae*-inoculated; 8, *F. poae*-inoculated & prochloraz-treated; 9, *F. poae*-inoculated & tebuconazole-treated.

APPENDIX 3 (SEE CHAPTER 3)

Appendix 3.1 Stability of transformants (see Chapter 3, section 3.3.4)

Table A3.1 Growth of transformants/isolates on PDA & PDA containing hygromycin B.

Transformant/isolate[a]	Mean radial growth (mm) +/- S.E.M[b].	
	PDA	PDA + hygromycin B (70 μg ml^{-1})
G501	23.8 +/- 1.0	19.3 +/- 0.2
G502	34.2 +/- 1.3	08.4 +/- 1.2
G503	48.5 +/- 1.0	21.7 +/- 1.3
G504	43.2 +/- 1.5	09.0 +/- 1.0
G505	39.8 +/- 0.4	06.5 +/- 1.0
G506	36.4 +/- 1.3	24.4 +/- 2.1
G507	36.1 +/- 0.8	09.8 +/- 0.3
G508	31.8 +/- 1.0	24.4 +/- 1.0
G509	40.9 +/- 2.4	16.5 +/- 0.9
G510	43.3 +/- 2.3	17.3 +/- 0.5
G511	29.0 +/- 1.9	12.3 +/- 1.2
G512	34.9 +/- 1.3	19.2 +/- 1.2
G513	23.4 +/- 0.2	17.7 +/- 0.2
G514	26.8 +/- 1.3	09.1 +/- 0.5
T515	51.2 +/- 0.6	00.0 +/- 0.0
T516	46.5 +/- 1.0	37.0 +/- 0.9
T517	35.8 +/- 1.3	14.0 +/- 0.7
TGP518	48.2 +/- 1.1	29.7 +/- 0.7
TGP519	52.7 +/- 1.9	47.0 +/- 2.1
TGP520	47.9 +/- 1.4	38.3 +/- 0.9
TGP521	40.8 +/- 1.2	00.0 +/- 0.0
TGP522	33.7 +/- 2.1	17.0 +/- 1.2
TGP523	47.3 +/- 0.6	41.8 +/- 1.4
TGP524	37.8 +/- 1.2	26.8 +/- 0.9
TGP525	49.5 +/- 1.0	35.8 +/- 1.8
TGP526	47.3 +/- 1.1	43.2 +/- 0.5
GGP527	37.0 +/- 2.7	11.2 +/- 0.4
GGP528	33.7 +/- 1.3	07.2 +/- 0.3
GGP529	22.6 +/- 1.0	06.6 +/- 0.7
GGP530	44.8 +/- 0.5	15.1 +/- 1.0
GGP531	44.8 +/- 0.5	15.1 +/- 1.0
GGP532	32.6 +/- 1.0	09.7 +/- 0.6
GGP533	25.5 +/- 0.8	04.8 +/- 0.3
GGP534	43.4 +/- 1.9	04.0 +/- 0.0
Fu 5 (wild-type)	31.5 +/- 1.0	00.0 +/- 0.0

[a]For information regarding transformants see Chapter 3, Table 3.3.

[b]S.E.M.: standard error of the mean, based on 6 replicates per treatment.

Appendix 3.2 **Analysis of the Pathogenicity of transformants**

(see chapter 3, section 3.3.6)

3.2.1 Analysis of visual disease assessment results.

3.2.1.1 Comparison of pathogenicity tests I & II using one way ANOVA of visual disease assessment results.

Source	DF	SS	MS	F	p
Pathogenicity test	1	683.669	683.669	1154.92	0.000
Error	124	73.403	0.592		
Total	125	757.072			

Level	N	Mean	StDev
Test I	63	7.3175	0.8027
Test II	63	2.6587	0.7345

Pooled StDev = 0.7694

Individual 95% CIs For Mean
Based on Pooled StDev

```
----+---------+---------+---------+--
                                  (*)
(-*)
----+---------+---------+---------+--
   3.0       4.5       6.0       7.5
```

3.2.1.2 Comparison of pathogenicity of transformants/isolates in pathogenicity tests I & II using one way ANOVA of visual disease assessment results.

Pathogenicity test I
One Way Analysis of Variance

Source	DF	SS	MS	F	p
Isolate	6	2.766	0.461	0.69	0.655
Error	56	37.184	0.664		
Total	62	39.951			

Isolate	N	Mean	StDev
Fu 5	9	7.3444	0.7282
G501	9	7.6556	0.5659
G506	9	7.2333	0.8559
G508	9	7.5000	0.8660
G514	9	7.1778	0.8393
G516	9	6.9556	0.9139
GGP531	9	7.3556	0.8805

Pooled StDev = 0.8149

Individual 95% CIs For Mean
Based on Pooled StDev

```
-+---------+---------+---------+---
      (---------*---------)
           (---------*---------)
      (---------*---------)
           (---------*---------)
      (---------*---------)
 (---------*---------)
      (---------*---------)
--+---------+---------+---------+----
  6.50      7.00      7.50      8.00
```

*See Chapter 3 (Table 3.3) for transforming vectors.

Tukey's pairwise comparisons
Family error rate = 0.0500 Individual error rate = 0.00345
Critical value = 4.32

Intervals for (column level mean) - (row level mean)

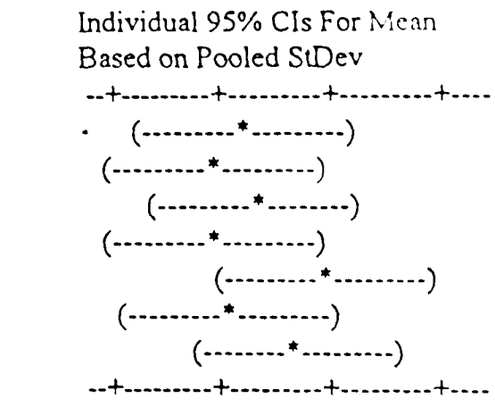
	Fu 5	G501	G506	G508	G514	G516
G501	-1.4845 0.8623					
G506	-1.0623 1.2845	-0.7512 1.5956				
G508	-1.3290 1.0179	-1.0179 1.3290	-1.4401 0.9067			
G514	-1.0067 1.3401	-0.6956 1.6512	-1.1179 1.2290	-0.8512 1.4956		
G516	-0.7845 1.5623	-0.4734 1.8734	-0.8956 1.4512	-0.6290 1.7179	-0.9512 1.3956	
GGP531	-1.1845 1.1623	-0.8734 1.4734	-1.2956 1.0512	-1.0290 1.3179	-1.3512 0.9956	-1.5734 0.7734

Pathogenicity test II
One Way Analysis of Variance

Source	DF	SS	MS	F	p
Transformant/isolate	6	2.659	0.443	0.81	0.570
Error	56	30.793	0.550		
Total	62	33.453			

Individual 95% CIs For Mean
Based on Pooled StDev

Level	N	Mean	StDev
Fu 5	9	2.6000	0.5292
G501	9	2.4444	0.3575
G506	9	2.6778	0.7775
G508	9	2.4667	0.4472
G514	9	3.0667	1.1800
T516	9	2.5333	0.4796
GGP531	9	2.8222	1.0072



Pooled StDev = 0.7415
*See Chapter 3 (Table 3.3) for transforming vectors.

Tukey's pairwise comparisons
Family error rate = 0.0500 Individual error rate = 0.00345
Critical value = 4.32

Intervals for (column level mean) - (row level mean)

	Fu 5	G501	G506	G508	G514	G516
G501	-0.9123 1.2234					
G506	-1.1456 0.9900	-1.3012 0.8345				
G508	-0.9345 1.2012	-1.0900 1.0456	-0.8567 1.2789			
G514	-1.5345 0.6012	-1.6900 0.4456	-1.4567 0.6789	-1.6678 0.4678		

	Fu 5	G501	G506	G508	G514	G516
G516	-1.0012	-1.1567	-0.9234	-1.1345	-0.5345	
	1.1345	0.9789	1.2123	1.0012	1.6012	
GGP531	-1.2900	-1.4456	-1.2123	-1.4234	-0.8234	-1.3567
	0.8456	0.6900	0.9234	0.7123	1.3123	0.7789

Pathogenicity tests I & II combined

Source	DF	SS	MS	F	p
Transformant/isolate	6	1.67	0.28	0.04	1.000
Error	119	755.40	6.35		
Total	125	757.07			

Individual 95% CIs For Mean
Based on Pooled StDev

Level	N	Mean	StDev	------+---------+---------+---------+
Fu 5	18	4.972	2.518	(----------*----------)
G501	18	5.050	2.720	(----------*----------)
G506	18	4.956	2.474	(----------*----------)
G508	18	4.983	2.675	(----------*----------)
G514	18	5.122	2.337	(----------*----------)
T516	18	4.744	2.383	(----------*----------)
GGP531	18	5.089	2.506	(----------*----------)
				------+---------+---------+---------+
Pooled StDev =	2.520			4.00 4.80 5.60 6.40

*See Chapter 3 (Table 3.3) for transforming vectors.

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00329

Critical value = 4.24

Intervals for (column level mean) - (row level mean)

	Fu 5	G501	G506	G508	G514	G516
G501	2.440					
G506	-2.501	-2.423				
	2.535	2.612				
G508	-2.529	-2.451	-2.546			
	2.507	2.585	2.490			
G514	-2.668	-2.590	-2.685	-2.657		
	2.368	2.446	2.351	2.379		
G516	-2.290	-2.212	-2.307	-2.279	-2.140	
	2.746	2.823	2.729	2.757	2.896	
GGP531	-2.635	-2.557	-2.651	-2.623	-2.485	-2.862
	2.401	2.479	2.385	2.412	2.551	2.173

3.2.2 Analysis of the quantitative PCR results.

3.2.2.1 Comparison of pathogenicity test I & II using one way ANOVA of quantitative PCR results.

Source	DF	SS	MS	F	p
Pathogenicity test	1	0.1	0.1	0.00	0.970
Error	124	8957.3	72.2		
Total	125	8957.4			

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
Test I	63	4.678	11.173	(----------*----------)
Test II	63	4.622	4.431	(----------*----------)
Pooled StDev =		8.499		3.6 4.8 6.0

3.2.2.2 Comparison of transformants/isolates using one way ANOVA and Tukeys Pairwise Comparison of quantitative PCR results.

Source	DF	SS	MS	F	p
Transformant/isolate	6	213.7	35.6	0.48	0.819
Error	119	8743.7	73.5		
Total	125	8957.4			

Level*	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
Fu 5(wild type)	18	5.352	7.654	(----------*----------)
G501	18	3.967	6.478	(----------*----------)
G506	18	5.549	9.534	(----------*----------)
G508	18	4.735	10.395	(----------*----------)
G514	18	3.486	4.09	(----------*----------)
T516	18	2.641	2.820	(----------*----------)
GGP531	18	6.819	13.789	(----------*----------)
Pooled StDev =		8.572		0.0 3.5 7.0 10.5

*See Chapter 3 (Table 3.3) for transforming vectors.

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00329
Critical value = 4.24

Intervals for (column level mean) - (row level mean)

	Fu 5	G501	G506	G508	G514	G516
G501	-7.181 9.952					
G506	-8.764 8.369	-10.149 6.984				
G508	-7.949 9.184	-9.334 7.799	-7.752 9.381			
G514	-6.701 10.432	-8.086 9.047	-6.504 10.629	-7.318 9.815		
G516	-5.855 11.278	-7.240 9.893	-5.658 11.475	-6.472 10.661	-7.721 9.412	
GGP531	-10.033 7.100	-11.418 5.715	-9.836 7.297	-10.650 6.483	-11.899 5.234	-12.745 4.388

Appendix 3.3 Analysis of the effect of fungicides on *F. culmorum* stem base disease as measured by GUS activity of protein extracts (see Chapter 3, section 3.3.8).

One-Way Analysis of Variance

Source	DF	SS	MS	F	p
Treatment	4	17.071	4.268	15.98	0.000
Error	25	6.675	0.267		
Total	29	23.746			

Treatment	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
1	6	0.0536	0.0052	(-----*----)
2	6	0.0830	0.0256	(----*----)
3	6	1.9464	0.6809	(----*-----)
4	6	1.0701	0.4847	(----*-----)
5	6	1.4839	0.7974	(-----*----)

Pooled StDev = 0.5167

0.00 0.80 1.60 2.40

*See Chapter 3, Fig. 3.6 for explanation of treatments

Tukey's pairwise comparisons

Family error rate = 0.0500 Individual error rate = 0.00706
Critical value = 4.15

Intervals for (column level mean) - (row level mean)

	1	2	3	4
2	-0.9048 0.8461			
3	-2.7683 -1.0174	-2.7389 -0.9880		
4	-1.8919 -0.1411	-1.8626 -0.1117	0.0009 1.7518	
5	-2.3058 -0.5549	-2.2764 -0.5255	-0.4130 1.3379	-1.2893 0.4616

APPENDIX 4. ANALYSIS OF EFFECT OF FUNGICIDES ON THE *TRI5*/β-TUBULIN RATIOS OF RNA EXTRACTS FROM *FUSARIUM CULMORUM* LIQUID CULTURES AND FUNGICIDE-SPIKED CULTURES USING THE TWO-SIDED WILCOXON RANK-SUM TEST (AT THE 5 % LEVEL OF SIGNIFICANCE) (SEE CHAPTER 4, SECTION 4.3.6).

Table A4.1 Mean and standard deviation of *Tri5*/β-tubulin ratios obtained from RT-PCR products of RNA extracts from *Fusarium culmorum* liquid cultures spiked with fungicides (harvested at 24, 36, 48 and 96 h post-inoculation).

Treatment (ug fungicide[a] per ml of media)	24 h			36 h			48 h			96 h		
	Number of observations[b]	Mean	Standard deviation	Number of observations[b]	Mean	Standard deviation	Number of observations[b]	Mean	Standard deviation	Number of observations[b]	Mean	Standard deviation
water (control)	4	0.81	0.13	4	0.69	0.10	4	0.96	0.40	4	0.60	0.26
Prochloraz (2)	-	-	-	4	0.93	0.05	4	0.50	0.31	4	0.42	0.18
Prochloraz (8)	-	-	-	4	1.11	0.18	4	0.37	0.22	3[c]	0.22	0.17
Tebuconazole (2)	-	-	-	4	0.93	0.17	4	0.64	0.26	4	0.44	0.18
Tebuconazole (8)	-	-	-	4	1.14	0.10	4	0.52	0.21	4	0.43	0.13

[a]Cultures spiked with fungicides 24 h post-inoculation

[b]Two replicate flasks per treatment and two replicate RT-PCR reactions per flask.

[c]Values missing due to loss of sample.

Table A4.2 Two-sided Wilcoxon rank-sum test (p values given) analysis of the effect of time on the relative *Tri5*/β-tubulin RT-PCR product ratios of RNA extracts from unspiked *Fusarium culmorum* liquid cultures harvested at 24, 36, 48 and 96 h after post-inoculation (fungicides added 24 h post-inoculation).

Time (h)	Time (h)			
	24	36	48	96
24	-	-	-	-
36	0.19	-	-	-
48	0.67	0.31	-	-
96	0.31	1.00	0.19	-

Table A4.3 Two-sided Wilcoxon rank-sum test (p values given) analysis of the effect of fungicides on the relative *Tri5*/β-tubulin RT-PCR product ratios of RNA extracts from *Fusarium culmorum* liquid cultures harvested at 36, 48 and 96 h post-inoculation (fungicides added 24 h post-inoculation).

Treatment[a]	36 h					48 h					96 h				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	0.030	-	-	-	-	0.11	-	-	-	-	0.31	-	-	-	-
3	0.030	0.312	-	-	-	0.11	0.31	-	-	-	0.11	0.60	-	-	-
4	0.030	0.470	0.31	-	-	0.31	0.67	0.11	-	-	0.31	0.67	0.22	-	-
5	0.030	0.030	1.00	0.19	-	0.11	1.00	0.31	0.89	-	0.47	1.00	0.22	1.00	-

[a]Treatments: 1, control unspiked cultures; 2, prochloraz-spiked (2 μg ml^{-1}); 3, prochloraz (8 μg ml^{-1}); 4, tebuconazole (2 μg ml^{-1}); 5. tebuconazole (8 μg ml^{-1}).

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THE USE OF SPECIES-SPECIFIC PCR-BASED ASSAYS TO ANALYZE *FUSARIUM* EAR BLIGHT OF WHEAT

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The use of species-specific PCR-based assays to analyze *Fusarium* ear blight of wheat

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ABSTRACT

Polymerase chain reaction (PCR) assays for the detection of various *Fusarium* species and *Microdochium nivale* sub-species were compared with conventional visual disease assessment using a field plot of wheat in which the central sub-plot was inoculated with *F. culmorum*. Visual disease assessment was performed on a range of samples taken from each of 15 sub-plots at growth stage 80. At harvest, each sample was divided into its component parts, i.e. grain, glume and rachis, and species-specific PCR analysis was used to detect the presence of *F. culmorum, F. poae, F. avenaceum, F. graminearum, M. nivale* var. *majus* and *M. nivale* var. *nivale*. Within the inoculated sub-plot there was good correlation between visual disease assessment and PCR analysis, both techniques indicating a high incidence of *F. culmorum* in this region. According to the visual disease assessment results, there was also a relatively high incidence of *F. culmorum* in most other regions of the field plot. However, according to PCR analysis the incidence of *F. culmorum* in many of the other sub-plots was relatively low and *F. poae, M. nivale* var. *majus* and *M. nivale* var. *nivale* and *F. avenaceum* were detected within the grain, glume and rachis tissues of many of the ear samples from these sub-plots. *F. poae* was predominantly found in the glume component of ears and *M. nivale* var. *majus* and *M. nivale* var. *nivale* were predominantly found in the rachis component of ears. *M. nivale* PCR results revealed that 64 % of infected samples were due to var. *majus*, and 36 % due to var. *nivale*. The use

of PCR analysis has highlighted some difficulties which may arise when using visual disease assessment when studying disease complexes.

INTRODUCTION

Fusarium ear blight (FEB) of wheat has been linked to at least 17 causal organisms, with most records of disease being associated with 5 species: *Fusarium culmorum, F. avenaceum* (*Gibberella avenacea*), *F. graminearum* (*G. zeae*, formerly known as *G. saubinetii*), *F. poae* and *Microdochium nivale* (*Monographella nivalis,* formerly known as *F. nivale*) (Parry *et al.,* 1995a). *F. culmorum, F. poae* and *M. nivale* are regarded as important causal agents of FEB in the cooler maritime regions of Northwest Europe, whereas *F. graminearum* assumes a greater importance in hotter regions of the world such as parts of the USA, Canada, Australia and Central Europe. *F. poae* was the most frequently isolated species in a UK survey of affected ears carried out in 1989 and 1990 (Polley *et al.,* 1991).

This complex of causal organisms greatly complicates the study of FEB of wheat and conventional disease assessment techniques, such as visual disease assessment and culture methods, require a degree of taxonomic expertise to distinguish them at the species level. Visual disease assessment is based on the recognition of the classical symptoms of FEB, such as premature bleaching of spikelets (Wiese, 1987). However, there has been controversy regarding the

symptoms of FEB produced by some of the causal agents (Rapilly *et al.*, 1973; Cassini, 1981; Inglis & Cook, 1981; Parry *et al.*, 1995a). This had led to increasing interest in obtaining more sensitive methods for the identification of *Fusarium* species in infected plant material, including techniques involving molecular diagnosis. Several such diagnostic assays have been developed for the identification and detection of the major fungi involved in FEB of wheat (Höxter *et al.*, 1991; Koopman *et al.*, 1994; Ouellet & Siefert, 1993; Parry & Nicholson, 1996; Nicholson *et al*, 1996). Most of these are based on the polymerase chain reaction (PCR) and include diagnostic assays specific for *F. graminearum* (Ouellet & Siefert, 1993; Schilling *et al.*, 1996), *F. poae* (Parry & Nicholson, 1996) and *F. culmorum* and *F. avenaceum* (Schilling *et al.*, 1996). Two varieties are recognised within *M. nivale*, var. *majus* and var. *nivale* (Lees *et al.*, 1995) and species specific assays have been developed for each of these (Nicholson & Parry, 1996; Nicholson *et al.*, 1996). Using these assays, it is possible to identify and distinguish between the individual pathogens within the disease complex.

In the present work, samples obtained from a field trial inoculated with *F. culmorum* were analysed by PCR and the results related to visual disease assessment.

MATERIALS & METHODS

Field trial

A field experiment was conducted during the 1993/94 season at Harper Adams Agricultural College, Shropshire. The winter wheat cultivar Avalon was used due to its susceptibility to *Fusarium* ear blight pathogens (authors observation) and the field plot was 30 x 2 m, sub-divided into 15 sub-plots, each 2 x 2 m. Wheat was planted in early October and was kept free from weeds and foliar diseases by application of the appropriate agrochemicals, according to manufacturers' recommendations. At mid-anthesis (GS 65) the central sub-plot (sub-plot 8) was inoculated with 167 ml of a conidial suspension of *F. culmorum* (strain Fu 42) at a rate of 2.1×10^7 conidia m^{-2} using a pressurised hand sprayer. Following inoculation, the field trial was mist-irrigated until harvest. Visual disease assessment (based on the percentage infected spikelets/ear) was performed at GS 80 on a range of samples randomly chosen from each sub-plot, numbers of samples varied according to sub-plot (Table 1). FEB symptoms scored were premature bleaching of ears, formation of small pink grains and, in severe cases, mycelial growth. At GS 90, those wheat ears examined at GS 80 were harvested, separated into grain, glume and rachis tissue, DNA was extracted and PCR analysis was used to confirm visual diagnosis.

Origin and maintenance of fungal isolates

DNA from isolates of *Fusarium* species and *M. nivale* sub-species obtained from the John Innes Centre facultative pathogen culture collection (Table 2) were used as positive controls for PCR analysis. The isolates were maintained on potato dextrose agar (PDA) (Difco) containing Penicillin G (50 μg ml^{-1}) and streptomycin sulphate (100 μg ml^{-1}). For DNA preparations, mycelium from 7-day-old colonies grown on PDA were used to inoculate aseptically 50 ml of potato dextrose broth (PDB), using a sterile scalpel. PDB cultures were incubated on an orbital shaker at 20-22 ^{0}C, 110 rpm, for 7 days. *Fusarium culmorum* was grown at 15 ^{0}C on 1 % agar (w/v) containing milled wheat straw for the production of conidia. After 7 days conidia were washed from plates with sterile distilled water and adjusted to 5 x 10^{5} conidia ml^{-1} for inoculation of the field trial.

DNA extraction

Wheat ears were harvested, separated into glume, grain and rachis tissue, freeze-dried and the dry weight recorded. Rachis material was ground to a fine powder which was incubated at 65 ^{0}C for 2 h in 7 ml CTAB buffer (sorbitol 2.3 g, n-lauryl sarcosine 1.0 g, hexadecyl trimethyl-ammonium bromide 0.8 g, sodium chloride 4.7 g, polyvinylpolypyrolidone 1.0 g, water to 100 ml) together with 15 μl proteinase K (10 mg ml^{-1}) and 10 μl RNAase (10 mg ml^{-1}). Following incubation, an equal volume of chloroform/isoamyl alcohol (24:1) was added to

the tubes, mixed, and centrifuged at 3000 rpm for 15 min. The aqueous phase was removed to a fresh tube and two volumes of ethanol (100 %) were added followed by centrifugation as above to precipitate the DNA. The pellet was washed in a 70 % solution of cold ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA).

The method used for DNA extraction from glume and grain samples was similar except that 20 ml of CTAB buffer together with 50 μl of proteinase K and 30 μl of RNAase were used. Due to the presence of substances which inhibited the PCR reaction, glume samples were also subjected to a phenol/chloroform (1:1) extraction step prior to chloroform/isoamyl alcohol extraction, using the same procedure. DNA from all tissues was diluted in TE for use in PCR amplification reactions (1 mg dry weight equivalent 2.5 μl^{-1}). DNA was extracted from fungal isolates using a method similar to that described by Nicholson & Parry (1996). Mycelium was harvested onto Whatman No.1 filter paper and DNA was extracted as described for rachis material. Fungal DNA was diluted to 10 ng μl^{-1} in TE for use in PCR reactions.

PCR amplification and agarose gel electrophoresis

Amplification conditions were similar to those described by Nicholson & Parry (1996). Reactions were performed in volumes of 50 μl and contained DNA from 0.8 mg dry weight of plant material or 10 ng of fungal DNA. The reaction buffer

consisted of 100 μM each of dATP, dCTP, dGTP and dTTP, 100 nM each of forward and reverse primer for PCR reactions, and 0.8 units of Taq polymerase (Boehringer Mannheim Ltd., Germany) in 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 μg ml^{-1} gelatine and 0.05 % (w/v) each of Tween 20 and Nonidet P-40. Primers used were those for *F. culmorum/F. graminearum* (Fc F/R: CAAAAGCTTCCCGAGTGTGTC/GGCGAAGGTTCAAGGATGAC) (Lees, 1995), *F. graminearum* (Fg11F/R: CTCCGGATATGTTGCGTCAA/GGTAGGTA TCCGACATGGCAA) (Nicholson *et al.*, manuscript in preparation), *F. poae* (Fp82F/R: CAAGCAAACAGGCTCTTCACC/TGTTCCACCTCAGTGACAGGT T) (Parry & Nicholson, 1996), *F. avenaceum* (AF/R: CAAGCATTGTCGCCACT CTC/GTTTGGCTCTACCGGGACTG) (Lees, 1995), *M. nivale* var. *majus* (Mnm2F/R: TGCAACGTGCCAGAAGCT/AATCGGCGCTGTCTACTAAAAGC) (Nicholson & Parry, 1996) and *M. nivale* var. *nivale* (Y13NF/R: ACCAGCCGAT TTGTGGTTATG/GGTCACGAGGCAGAGTTCG) (Nicholson *et al.*, 1996). Reaction mixtures were overlaid with mineral oil prior to PCR.

Amplification was performed in a Perkin-Elmer Cetus 480 DNA thermal cycler. The programme used to amplify fungal DNA from infected plant samples varied depending on the specific primers used. When using *F. culmorum/F. graminearum* or *F. avenaceum*-specific primers the cycler was programmed for 40 cycles of 30 s at 95 ^{0}C, 30 s at 60 ^{0}C and 40 s at 72 ^{0}C. Programmes used with *M. nivale* var. *nivale*, *M. nivale* var. *majus*, *F. graminearum* and *F. poae* primers were similar except that annealing temperatures of 61, 61, 62 and 62 ^{0}C respectively, were used. Aliquots (15 μl) of amplification products were

electrophoresed through agarose gels (1.5 % w/v), prepared using TAE buffer (40 mM Tris base, 1 mM EDTA, 20 mM acetic acid) and containing 0.05 mg ethidium bromide per 100 ml TAE buffer.

Statistical analysis

Statistical analysis consisted of determination of the correlation coefficients between visual disease assessment and PCR-based assays using the Pearson Product Moment Correlation of arcsine-transformed sub-plot frequency data. This analysis was performed using Minitab release 10.1© (1994, Minitab incorporated). The association between pathogens was tested using Fisher's exact test (Everitt, 1986) for which a correction according to Rom (1990) was used.

RESULTS

Visual disease assessment

This experiment was part of a larger field trial set up to relate disease severity to yield loss, a field trial in which the sample size harvested per sub-plot increased with proximity to the inoculation zone. As a result, the number of harvested

samples available per sub-plot for the present study varied accordingly. Visual disease assessment was performed at GS 80 on a range of single ear samples from each of the 15 sub-plots within the field plot (Table 1 & Fig. 1a and b). According to these results, the mean visual disease score attributed to the wheat ears taken from within the *F. culmorum*-inoculated sub-plot (sub-plot 8, samples 56--68) was 96 % of spikelets infected with *F. culmorum,* the majority (77 %) of ears from this sub-plot being given scores of 100 % spikelets infected. In the other sub-plots (sub-plots 1--7, samples 1--55 and sub-plots 9--15, samples 69--123), although disease score varied from 0 to 100 %, the majority of ears showed disease symptoms. In the sub-plot adjacent to the inoculated sub-plot (sub-plot 9, samples 69--81), 12 of the 13 ears scored had disease ratings of 50 % or greater (mean = 70 %) (Fig. 1). In the other adjacent sub-plot (sub-plot 7, samples 43--55), disease severity was much reduced and no wheat ear had greater than 35 % spikelets infected (mean = 20 %). The disease severities for sub-plots 1--6 were low to moderate, mean disease scores being 2, 5, 21, 10, 12 and 12 %, respectively. Only one sample from this region (sample 8, sub-plot 3) had a relatively high disease severity (60 %). The disease severities for the corresponding sub-plots on the other side of the inoculated sub-plot (sub-plots 10--15, samples 82--123) were generally higher, mean disease scores being 18, 31, 17, 19, 50 and 28 %, respectively. Compared with sub-plots 1--6, sub-plots 10--15 showed greater variation in disease severity from sample to sample, disease scores fluctuating between 0 and 100 %. Several samples from these sub-plots had disease severities greater than 50 % (i.e. samples 90, 96, 99, 103, 116, 119, 120

and 122 from sub-plots 10, 11, 11, 11, 13, 14, 14 and 15, respectively). Therefore, according to the visual disease assessment results, *F. culmorum* was detected in each sub-plot within the field trial. With the exception of the inoculated sub-plot (sub-plot 8), an adjacent sub-plot (sub-plot 9) and sub-plot 14, the mean disease severities of sub-plots were low to moderate, with no significant evidence of the development of a disease severity gradient from the inoculated sub-plot (Fig. 1b).

The visual disease results were used to classify each single ear sample as infected or not infected with *F. culmorum* (scores of 1 and 0 respectively) so as to enable comparison with the PCR results on the basis of the percentage of infected samples per sub-plot. The combined results for the samples within each sub-plot are shown in Table 1, as are the number of samples taken from each sub-plot. Based on these results, all of the samples in the inoculated sub-plot appeared to be infected with *F. culmorum* and, even in the non-inoculated sub-plots, the frequency of infected samples was moderate to high (Table 1 & Fig. 2a). For example, 75 % of samples examined from sub-plot 14 exhibited head blight symptoms, presumed to be caused by *F. culmorum*.

F. culmorum and *F. graminearum* PCR analysis

Fusarium graminearum-specific PCR analysis (primers Fg11F/R) did not detect this pathogen in any of the samples from the field plot, whether from grain,

glume or rachis tissue. Therefore, it was concluded that any PCR amplification that occurred when using the *F. culmorum/F. graminearum*-specific primers (FcF/R) was due to the presence of *F. culmorum* DNA. As with visual disease assessment results, the PCR results were used to classify the ears within each sub-plot as diseased or non-diseased, and the results obtained for grain samples are shown in Fig. 2a. Similar results were obtained for glume and rachis samples (results not shown). Within the inoculated sub-plot (sub-plot 8) there was a high incidence of *F. culmorum* (100 % samples infected). PCR detected *F. culmorum* in the grain, glume and rachis of every sample from this sub-plot. According to the PCR analysis, the incidence of *F. culmorum* outside sub-plots 8 and 9 was relatively low, although most of the ears outside the inoculated sub-plot which had disease scores of 50 % or greater were found to contain *F. culmorum* by PCR analysis (samples 8, 55, 69--73, 75--80, 90, 96, 99, 103, 116, 119 and 122 from sub-plots 3, 7, 9, 9, 10, 11, 11, 11, 13, 14 and 15 with disease scores of 60, 100, 100, 63, 90, 79, 100, 90, 50, 100, 60, 68, 95, 68, 100, 55, 75, 100 and 80 %, respectively). The results of *F. culmorum/F. graminearum*-specific PCR analysis of some of these 'high score' samples are shown in Fig. 3a. Of these 20 samples from outside the inoculated sub-plot attributed high disease scores, 11 were among the 13 of the samples taken from sub-plot 9 adjacent to the inoculated sub-plot. PCR analysis detected *F. culmorum* in the grain, glume and rachis of all of these 11 samples, with the exception of sample 75 for which the pathogen was detected in the glume and rachis, but not in the grain tissue. The other two samples taken from sub-plot 9 had lower disease scores (samples 74 and 81, disease scores of

5 and 26 %, respectively) and PCR analysis did not detect *F. culmorum* in sample 74, but detected the pathogen in the glume and rachis of sample 81. In the other sub-plot adjacent to the inoculated sub-plot (sub-plot 7), PCR analysis only detected *F. culmorum* in 8 % of samples, but this 8 % corresponded to the only sample from this sub-plot attributed a high disease score (100 %). PCR analysis did not detect the pathogen in the other 92 % of samples from sub-plot 7 which were attributed disease scores of between 0 and 33 %.

Although FEB symptoms were observed in 30 of the 42 samples from sub-plots 1--6, PCR analysis detected *F. culmorum* in only a single sample (sample 8, sub-plot 3) where it was present in grain (Fig. 3a), glume and rachis tissue. Similarly, 32 of the 42 of the samples from sub-plots 10--15 had FEB symptoms while PCR analysis only detected *F. culmorum* in 13 of the samples from this region. *F. culmorum* PCR analysis detected *F. culmorum* in samples 82, 84, 85, 89--91, 93, 96, 99, 107, 119, 122 and 123 from sub-plots 10--15. Disease scores for samples 82, 89, 93 and 123 were relatively low (5, 11, 5 and 5 %, respectively) and the pathogen was only detected in one component part of each sample, i.e. glume (82 and 93) or rachis (89 and 123). Samples 84, 85 and 91 had moderate disease scores (30, 26 and 25 %, respectively) and PCR analysis detected the pathogen in the grain and glume of sample 91, in the grain and rachis of sample 84 and in the rachis of sample 85. *F. culmorum* was detected in samples 90, 96, 99, 119 and 122 which had severe FEB symptoms (disease scores of 95, 68, 100, 100 and 80 %, respectively). PCR analysis detected the pathogen in the grain, glume and rachis components of samples 99 and 119, in the grain

and rachis components of sample 90, the grain and glume components of sample 122 and only in the rachis component of sample 96. Sample 107 had exhibited no FEB symptoms, but PCR analysis detected *F. culmorum* in the grain of this sample. When the *F. culmorum* PCR results were correlated with visual disease assessment (Table 3), the highest correlation coefficient was obtained for the PCR results from glume samples ($r = 0.822$), as opposed to grain ($r = 0.759$) samples, rachis sample ($r = 0.782$) or the combined grain, glume and rachis results ($r = 0.675$).

M. nivale var. _nivale_ and var. _majus_ PCR analysis

The PCR results obtained for the *M. nivale* var. *nivale* and var. *majus* were analyzed in the same way as those obtained for *F. culmorum* PCR analysis (Fig. 2b, c and d). When compared to the surrounding sub-plots, the frequency of *M. nivale* var. *nivale* and var. *majus* detection was relatively low in samples from the inoculated sub-plot (sub-plot 8) which had a high incidence of *F. culmorum* infection. This is particularly evident in rachis samples (Fig. 2d). *M. nivale* var. *majus* was detected in none of the glume components, and only 8 % of grain and 15 % of rachis components of samples from sub-plot 8. Similarly, *M. nivale* var. *nivale* was not detected in any of the glume or rachis components and only 8 % of grain components of samples from sub-plot 8. Sub-plot 9 also had a high incidence of *F. culmorum* infection and *M. nivale* var. *nivale* was not detected in

any samples from sub-plot 9 (Fig. 2b, c and d). In sub-plot 9, var. *majus* was not detected in the glume components of any samples, while it was detected in the grain, rachis or both components of 25 % of the samples from this sub-plot. PCR analysis revealed that there was a particularly high incidence of these pathogens in the 13 samples from sub-plot 7, with 69 % of samples infected with *M. nivale* var. *nivale* and 92 % of samples infected with var. *majus*. The pathogen was predominantly detected in the rachis components of these samples.

PCR analysis detected *M. nivale* var. *nivale* in 33, 25, 0, 0, 56, and 62 % of samples from sub-plots 1--6, respectively, while var. *majus* was detected in 66, 25, 50, 71, 33 and 77 % of samples from sub-plots 1--6, respectively. Again, both pathogens were detected predominantly in the rachis components, often of the same sample, and as shown earlier, PCR analysis did not detect *F. culmorum* in any of these samples, many of which had low to moderate disease scores. *M. nivale* var. *nivale* was detected in 8, 11, 29, 0, 25 and 0 % of samples from sub-plots 10--15, respectively, while var. *majus* was detected in 23, 66, 86, 50, 50 and 66 % of samples from sub-plots 10--15, respectively. *M. nivale* var. *nivale* was found predominantly in the rachis component of these infected samples, often in conjunction with var. *majus*. *M. nivale* var. *majus* was often detected in the corresponding glume and rachis components of samples from sub-plots 10---15. Of the total of 21 *M. nivale* var. *majus*- infected samples from sub-plots 10--15, 63 % of these samples were also infected with *F. culmorum,* and both pathogens were detected in the rachis component of 13 of the 14 co-infected samples. None of the five *M. nivale* var. *nivale*-infected samples from sub-plots 10--15 were

infected with *F. culmorum*. Most *M. nivale* var. *nivale* and/or var. *majus*-infected plants from sub-plots 10--15 had been given low to moderate disease scores, although in a few cases no FEB symptoms had been detected (samples 97, 106 and 107 from sub-plots 11, 12 and 12, respectively). *M. nivale* was also detected in some samples from sub-plots 10--15 for which disease scores were relatively high, with var. *majus* in samples 96, 99, 103, 116, 120 (sub-plots 11, 11, 11, 13 and 14, respectively) and var. *nivale* in sample 120 (sub-plot 14). *M. nivale* var. *majus* and var. *nivale* PCR analysis for some of these samples is shown in Fig. 3b. *M. nivale* var. *majus* was detected in the rachis of samples 96 and 99 which were also infected with *F. culmorum* (Fig. 3a), and which had disease scores of 68 and 100 %, respectively. *M. nivale* var. *majus* was detected in the grain, glume and rachis component of sample 116 & 120, and in the rachis component of sample 103, while var. *nivale* was detected in the glume component of sample 120. *F. culmorum* was not detected in these samples which had disease scores of 75, 84 and 55 %, respectively. PCR analysis showed that var. *majus* was the predominant *M. nivale* sub-species within the field plot, with 64 % of *M. nivale* infected ears due to var. *majus* and 36 % due to var. *nivale*. Since both *M. nivale* var. *majus* and var. *nivale* occurred on symptomatic and asymptomatic wheat ears, neither pathogen served to increase the correlation coefficient between visual disease assessment and PCR-based assays (Table 3). In tests of the association between pathogens (Everitt, 1986; Rom, 1990), the only significant association found was between *F. culmorum* and *M. nivale* ($p < 0.01$) and it was found that there were an excess of samples where only one of the pathogens was present, i.e.

F. culmorum or *M. nivale* var. *majus*/var. *nivale*.

F. poae and *F. avenaceum* PCR analysis

PCR analysis was also employed to determine if any of the samples were infected with *F. poae*. Again, the results were analyzed as described previously, and are depicted in Fig. 2. In sub-plots 8 and 9 which had a high incidence of *F. culmorum* infection, the highest incidence of *F. poae* occurred in the glume component of samples, being detected in 23 and 15 % of glumes from sub-plots 8 and 9, respectively. *F. poae* was detected in many glume samples throughout the field plot. For example, *F. poae* was detected in 54 % of the glume samples from sub-plot 7, while it was not detected in any of the corresponding grain or rachis samples. These infected samples had disease scores of between 0 and 33 % and PCR analysis did not detect *F. culmorum* in any of these samples. *M. nivale* var. *nivale* and var. *majus* were detected in many of the *F. poae*-infected samples from sub-plot 7, although these pathogens were predominantly found in the rachis rather than the glume components of the samples.

PCR analysis detected *F. poae* in 66, 100, 50, 58, 56 and 38 % of samples from sub-plots 1--6, respectively. As is evident from Fig. 2, the incidence of *F. poae* in grain and glume components of samples from sub-plots 1--6 was relatively high, and the pathogen was detected in either or both of these components of a sample. The pathogen was generally not detected in the rachis components of

samples from sub-plots 1--6. For many of the *F. poae*-infected glume and grain samples, the corresponding rachis components were infected with *M. nivale* var. *nivale* and/or var. *majus*. In sub-plots 10--15, *F. poae* was detected in 23, 66, 71, 17, 0 and 33 % of samples, respectively. Fig. 2 shows that the pathogen was predominantly present in the glume component and was rarely detected in the rachis component of samples from sub-plots 10--15. The majority of infected samples had low to moderate disease scores (5--35 %), although a few showed no FEB symptoms (samples 97, 106 and 107). Also *F. poae* was detected in samples which had relatively high disease scores (samples 96, 99, 103 and 116) and the PCR analysis of these samples is illustrated in Fig. 3c. *F. poae* was found in conjunction with *F. culmorum* (Fig. 3a) and *M. nivale* (Fig. 3b) in samples 96 and 99 (disease scores of 68 and 100 %, respectively). *F. poae*, *F. culmorum* and *M. nivale* were detected in the rachis component of sample 96. Both *F. poae* and *F. culmorum* were detected in the grain, glume and rachis tissues of sample 99, while *M. nivale* was detected in the rachis of this sample. *F. poae* was detected in conjunction with *M. nivale* in samples 103 and 116 (disease scores of 55 and 75 %, respectively), samples in which *F. culmorum* was not detected. In sample 103, *F. poae* was detected in the grain and glume components and *M. nivale* was detected in the rachis. Both *F. poae* and *M. nivale* were detected in the grain, glume and rachis of sample 116. Like *M. nivale* var. *majus* and var. *nivale*, when the *F. poae* PCR results were combined with *F. culmorum* PCR results and/or *M. nivale* PCR results and correlated with the visual disease assessment results, the various correlation coefficients obtained were lower than that obtained for visual

disease assessment and *F. culmorum* PCR results (Table 3).

PCR revealed a low incidence of *F. avenaceum* within the field plot and it was detected in only 18 of the 123 samples (results not shown). These infected plants generally had low to moderate disease scores (5--33 %), although FEB symptoms were not detected in two of the *F. avenaceum*-infected samples (samples 6 and 43). Also, *F. avenaceum* was detected (along with *M. nivale* var. *majus* and *F. poae*) in the grain of sample 116 which had a disease score of 75 % (Fig. 3). In contrast to the *M. nivale* var. *majus, M. nivale* var. *nivale* and the *F. poae* PCR results, the *F. avenaceum* PCR results when combined with the *F. culmorum* PCR results and correlated with visual disease assessment ($r = 0.843$), a more linear relationship was observed than between visual disease assessment and *F. culmorum* PCR results ($r = 0.822$) (Table 3).

DISCUSSION

The use of species-specific PCR analysis to identify the fungal species present in wheat ears which exhibited FEB symptoms within the field plot has highlighted many potential problems associated with visual assessment of a disease complex. Only within the central *F. culmorum*-inoculated sub-plot and an adjacent sub-plot (sub-plot 9) was a high correlation found between visual disease assessment and the presence of *F. culmorum* as detected by PCR analysis. Both visual disease

assessment and PCR analysis revealed the presence of *F. culmorum* in all 13 ears sampled in the inoculated sub-plot and in all of the ears exhibiting symptoms in the adjacent sub-plot. However, PCR analysis failed to detect *F. culmorum* in some of the samples taken from the remaining sub-plots which were given disease scores assumed to be related to the presence of *F. culmorum* originating from the inoculated sub-plot. *F. culmorum* was detected in the most of the ears (both within and outside the inoculated sub-plot) exhibiting the highest disease scores (above 50 %). However, since PCR analysis did not indicate that there was a gradient in the field plot in terms of the frequency of detection of the pathogen, it cannot be assumed that the *F. culmorum* inoculum for all of these samples originated from the inoculated sub-plot. It could be inferred that perhaps *F. culmorum* was more pathogenic than the other *Fusarium* species and *Microdochium* sub-species detected within the field plot, under the prevailing environmental conditions. In a recent review of FEB, Parry *et al.* (1995a) suggested that *F. culmorum* along with *F. graminearum* were consistently the most pathogenic of the *Fusarium* species infecting cereal ears.

PCR analysis indicated that the presence of other *Fusarium* species and *M. nivale* sub-species within the field plot may account for the disease observed in many areas of the field trial, particularly where low to moderate disease scores were recorded in sub-plots 1--6 and 10--15 of the field trial. It is likely that the high level of the pathogenic isolate of *F. culmorum* applied to the inoculated region limited the establishment of other species within this area of the field plot.

The separation of ear samples into their component parts (i.e. grain, glume

and rachis) for the purpose of PCR analysis permitted tissue localisation of the various *Fusarium* species and *M. nivale* sub-species. This highlighted the fact that *F. poae* was predominantly present in glume material, but often not detected in the corresponding grain tissue and was rarely detected in rachis tissue. This suggests that initial infection by this pathogen was via the glumes. In a survey of *Fusarium* ear diseases of winter wheat in Great Britain during 1989--1990, Polley & Turner (1995) reported that *F. poae* was the *Fusarium* species most commonly isolated from glumes affected by ear blight symptoms and that this pathogen was associated with distinct glume spot lesions. Thus it may be that the mode of infection and colonisation by *F. poae* differs from that of other pathogens associated with *Fusarium* ear blight.

Outside the inoculated and adjacent sub-plots, *F. poae, F. avenaceum* and *M. nivale* were detected in ears which did not contain *F. culmorum* and which had moderate to high disease scores. Indeed, one interesting observation that emerged from this experiment was the occurrence of *F. culmorum, F. avenaceum* and *M. nivale* on the grain and rachis components, but not the corresponding glume components, of several high disease score samples taken from outside the inoculated sub-plot. Although these only accounted for a small proportion of samples, these results implied that the bleaching of ears which resulted in high visual disease scores for these samples, was not as a result of glume infection, but rather was the indirect result of pathogen infection, possibly following the impediment of nutrient translocation to the glume.

PCR analysis also confirmed that *M. nivale* var. *majus* was more abundant

in ears than var. *nivale*. Using a PCR-restriction fragment length polymorphism (RFLP) technique, Parry *et al.* (1995b) found that, of 91 *Microdochium* isolates obtained from grain taken from seven sites throughout the U.K., 93 % were var. *majus* and 7 % var. *nivale*. In the present study it appeared that *M. nivale* var. *majus* was more frequently detected in rachis than in grain or glume tissue.

PCR analysis detected *F. avenaceum* in comparatively few samples and *F. graminearum* was not detected within the field plot. Tests using *F. avenaceum* as inoculum have resulted in severe ear blight in the U.K. (Parry *et al.*, 1995a), although this species generally represents a small proportion of the isolates obtained from FEB affected crops in the U.K. and other cool maritime regions of Northwest Europe. There are few reports of *F. graminearum* on wheat crops in the U.K. (Moore, 1948) and this species is generally important in hotter regions of the world (Parry *et al.*, 1995a).

The fact that pathogens were detected in asymptomatic samples may be due to several factors including sensitivity of PCR-based assays and variability of disease symptoms (i.e. distinctiveness from *F. culmorum* disease symptoms). Among the pathogens, the only significant association was that found between *F. culmorum* and *M. nivale* var. *majus*/var. *nivale*. The significant probability of independence found between *F. culmorum* and *M. nivale* var. *majus*/var. *nivale* may suggest some sort of antagonistic interaction between these pathogens.

This work has demonstrated how PCR analysis may be used to gain insight into and overcome some of the problems associated with the diagnosis and understanding of FEB of wheat.

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Table 1 Field trial sampling plan

Sub-plot (2 x 2 m)	No. ears sampled	Sample codes	Percentage samples infected[a]
1	3	1--3	33
2	4	4--7	75
3	6	8--13	83
4	7	14--20	57
5	9	21--29	78
6	13	30--42	77
7	13	43--55	85
8[b]	13	56--68	100
9	13	69--81	100
10	13	82--94	85
11	9	95--103	78
12	7	104--110	71
13	6	111--116	67
14	4	117--120	75
15	3	121--123	67

[a]Based on visual disease assessment results.
[b]Sub-plot inoculated with *F. culmorum* (Table 2) at GS 65 (2.1×10^7 conidia m^{-2}).

Table 2 Code and origin of fungal species

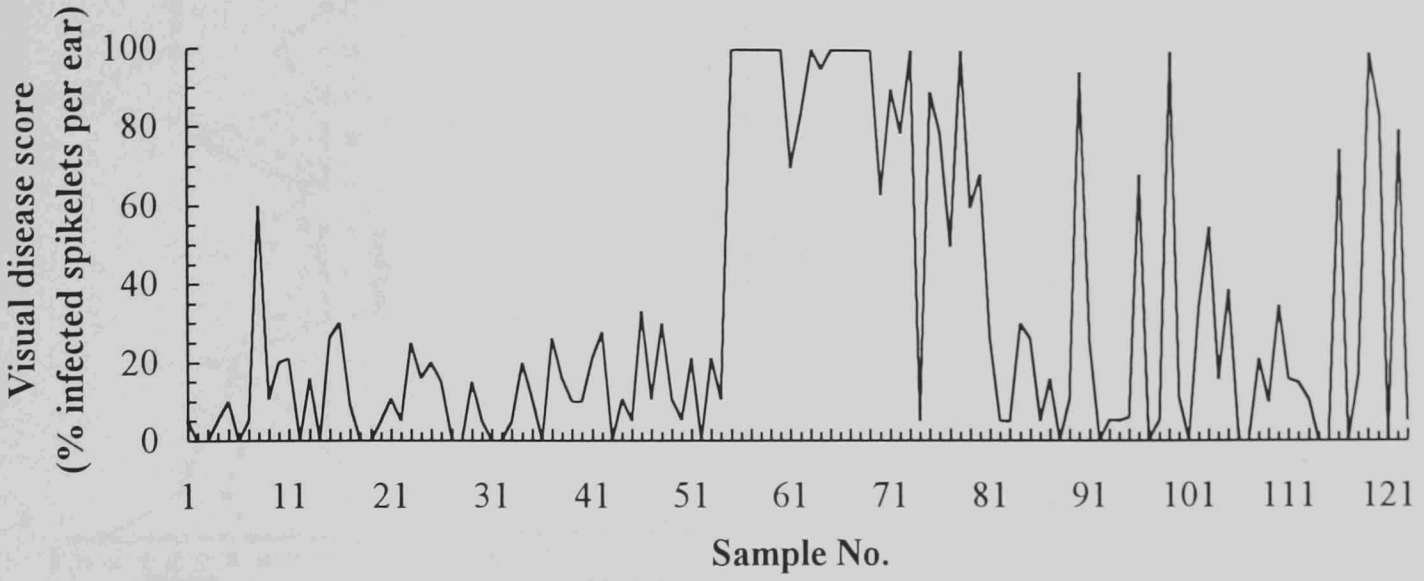
Species	Code	Origin (all isolated from wheat)
Fusarium culmorum	Fu 42	U.K.
F. graminearum	F 705	France
F. poae	F 62	Poland
F. avenaceum	F 720	Germany
Microdochium nivale var. *majus*	Mn 18	U.K.
M. nivale var. *nivale*	M 58	U.K.

Table 3 Correlation between visual disease assessment results and various PCR-based diagnostic assay results obtained for wheat ears and their component parts (grain, glume and rachis tissue).

PCR assays	Pearsons Correlation coefficient (r)[a]			
	Grain	Glume	Rachis	ear
F. culmorum	0.759	0.822	0.782	0.675
F. culmorum/*F. poae*	0.191	0.584	0.779	0.314
F. culmorum/*M. nivale* var. *majus*	0.244	0.775	0.657	0.365
F. culmorum/*M. nivale* var. *nivale*	0.783	0.695	0.821	0.669
F. culmorum/*F. avenaceum*	0.647	0.843	0.371	0.352
F. culmorum/*F. poae*/*M. nivale* var. *majus*	-0.298	0.600	0.639	-0.058
F. culmorum/*F. poae*/*M. nivale* var. *nivale*	0.226	0.616	0.820	0.387
F. culmorum/*F. poae*/*F. avenaceum*	0.161	0.581	0.355	-0.031
F. culmorum/*M. nivale* var. *majus*/*M. nivale* var. *nivale*	0.297	0.661	0.667	0.094
F. culmorum/*M. nivale* var. *majus*/*F. avenaceum*	0.364	0.675	0.175	0.009
F. culmorum/*M. nivale* var. *nivale*/*F. avenaceum*	0.724	0.588	0.466	0.447
F. culmorum/*F. poae*/*M. nivale* var. *majus*/*M. nivale* var. *nivale*	-0.304	0.600	0.650	-0.042
F. culmorum/*F. poae*/*M. nivale* var. *majus*/*F. avenaceum*	-0.291	0.598	0.154	-0.056
F. culmorum/*F. poae*/*M. nivale* var. *nivale*/*F. avenaceum*	0.202	0.605	0.464	-0.017
F. culmorum/*M. nivale* var. *majus*/*M. nivale* var. *nivale*/*F. avenaceum*	0.182	0.665	0.175	0.028
F. culmorum/*F. poae*/*M. nivale* var. *majus*/*M. nivale* var. *nivale*/*F. avenaceum*	-0.296	0.590	0.154	-0.042

[a]Based on arcsine-transformed sub-plot frequency data.

a



b

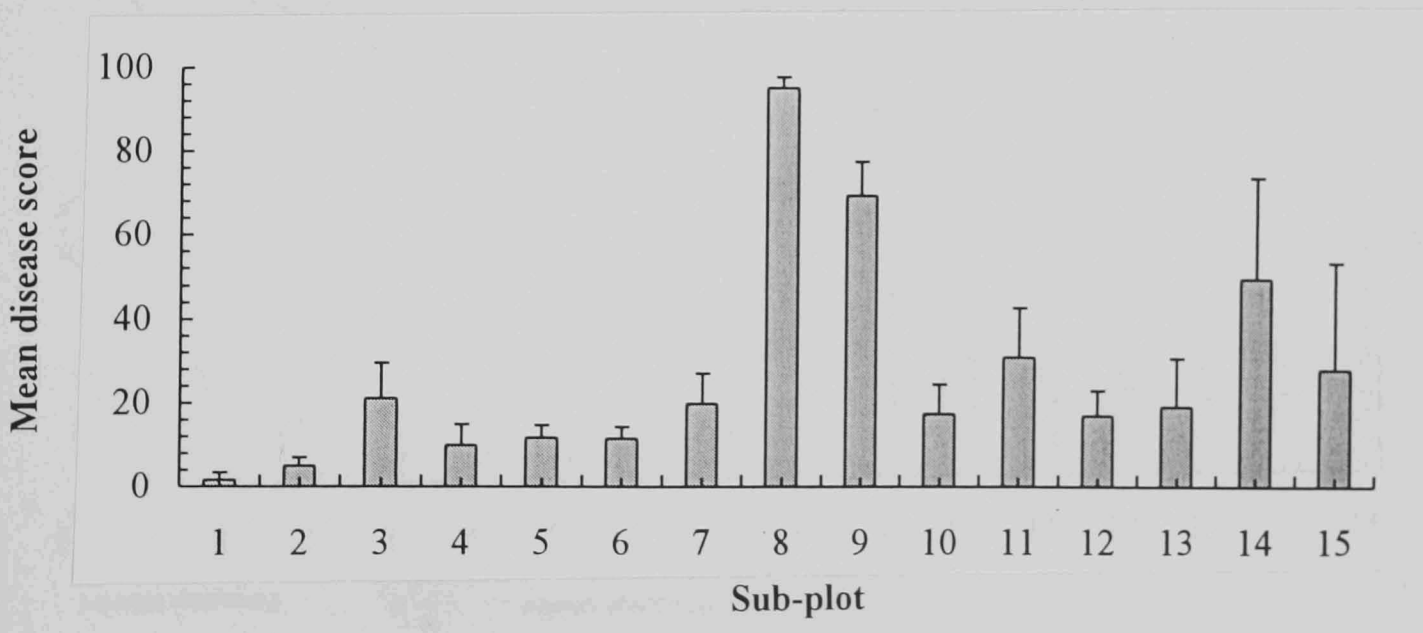


Fig. 1 Visual disease assessment results of ear blight of wheat in a field trial in which the central sub-plot (sub-plot 8, samples 56-68) was inoculated with conidia of *Fusarium culmorum* (2.1×10^7 conidia m^{-2}) at GS 65. Results expressed as (a), disease score for each sample and (b), mean disease score per sub-plot. Bars in graph b indicate the standard errors of the means (S.E.M.).

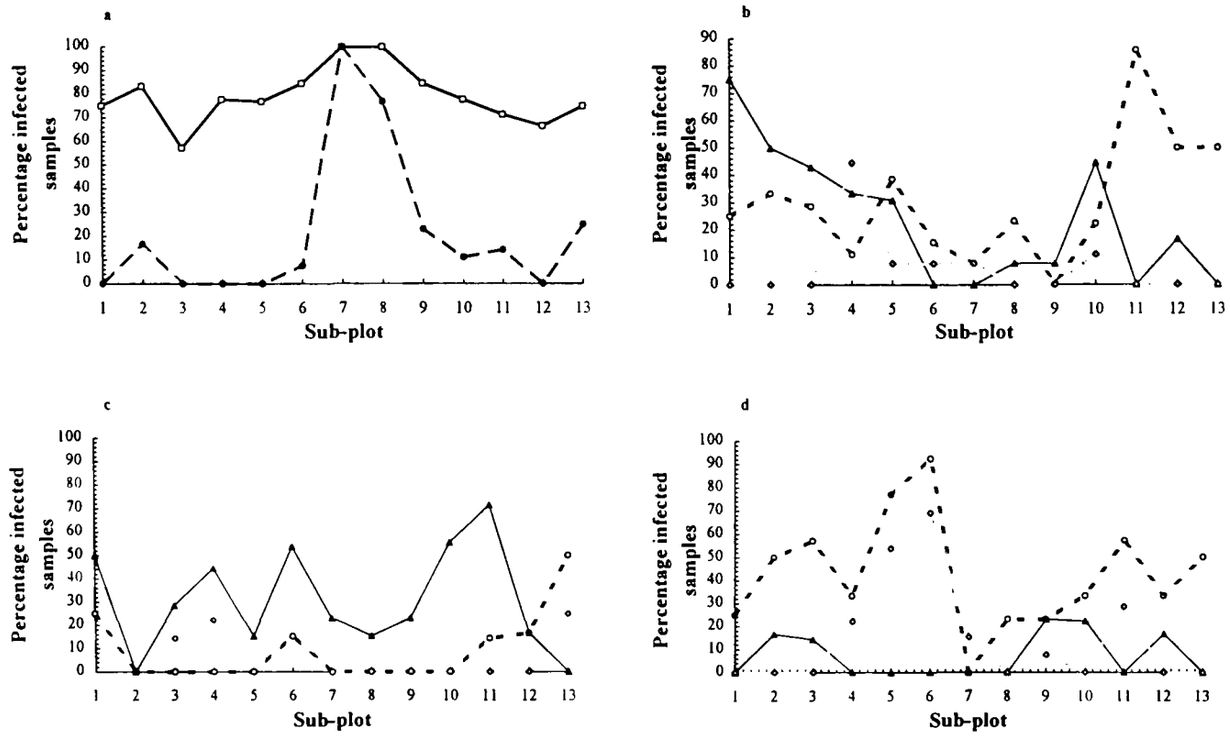


Fig. 2 Comparison of the frequency of occurrence of visual disease assessment (□▬▬□) with the frequency of PCR detection of *Fusarium culmorum*(●▬ ▬●) in grain samples of wheat ears taken from a field trial (a), and comparison of the frequency of PCR detection of *M. nivale* var. *majus* (○▬ ▬ ▬○) and var. *nivale* (◇┈┈┈◇) and *F. poae* (▲▬▬▬▲) in the corresponding grain (b), glume (c) and rachis (d) components of these ear samples.

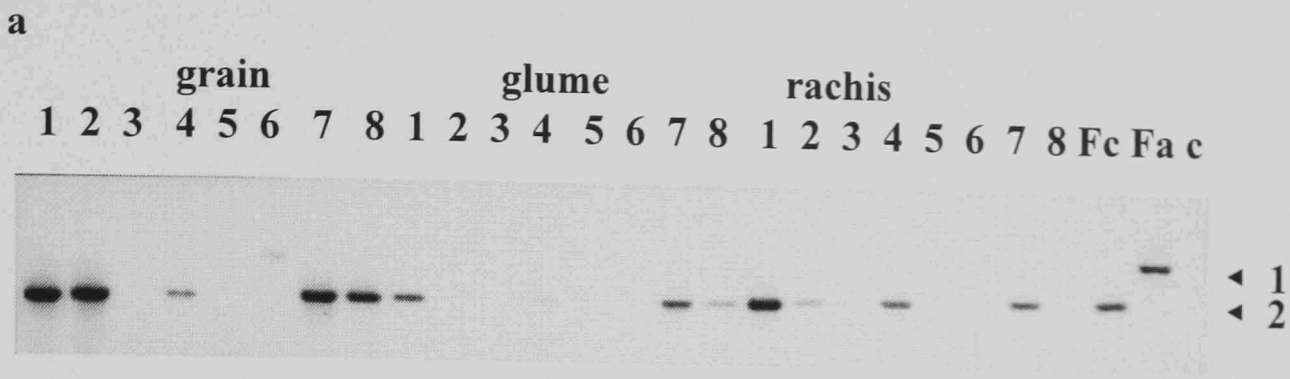


Fig. 3 Detection of the specific PCR products for *Fusarium culmorum* and *F. avenaceum* (**a**), *Microdochium nivale* var. *nivale* and var. *majus* (**b**) and *F. poae* and *F. graminearum* (**c**) in the corresponding grain, glume and rachis tissue of wheat ear samples. Lanes: 1--8, samples 8, 90, 96, 99, 103, 116, 119 and 122; Fa, Fc, M, N, Fp and Fg, *F. culmorum, F. avenaceum, M. nivale* var. *majus, M. nivale* var. *nivale*, *F. poae* and *F. graminearum* genomic DNA (Table 1); c, control without fungal DNA. Arrows: 1, *F. avenaceum*; 2, *F. culmorum*; 3, *M. nivale* var. majus; 4, M. *nivale* var. *nivale; 5, F. graminearum*; 6, *F. poae*-specific DNA products (920, 700, 750, 310, 400 and 220 bp, respectively).