European Journal of Plant Pathology **109:** 691–703, 2003.

Molecular tools to study epidemiology and toxicology of fusarium head blight of cereals

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Key words: Microdochium, trichothecene, PCR, chemotype

Abstract

Fusarium head blight (FHB) of cereals is a disease complex. *Fusarium graminearum* is the major pathogen worldwide, while *F. culmorum*, *F. avenaceum* and *F. poae* are also associated with this disease. In addition to the true *Fusarium* species, *Microdochium nivale* may also cause head blight and is particularly prevalent where cooler, wetter conditions prevail. Other species such as *F. sporotrichioides, F. equiseti* and even *F. verticillioides* may also be of significance in particular situations. FHB is of particular concern because of the ability of the *Fusarium* species to produce mycotoxins in the grain that are harmful to human and animal consumers. The predominant mycotoxins within cereals are the trichothecenes, chiefly deoxynivalenol, nivalenol and their acetylated derivatives, along with T-2, HT-2, diacetoxyscirpenol and neosolaniol. This paper reviews the use of molecular techniques to identify the individual causal agents and to quantify their relative amounts within plant tissue. Diagnostic and quantitative polymerase chain reaction assays have been developed to detect and quantify individual fungal species within the disease complex and, where relevant, to differentiate between chemotypes within a single species. Assays to determine the type of toxin produced, or monitor the regulation of toxin production also provide valuable tools for understanding this disease. These techniques are being used to dissect the disease complex into its component parts in order to study interactions between the pathogens and their host and between the pathogens themselves as well as to determine the influence of environmental factors on the disease and the toxins produced by these fungi.

Introduction

Fusarium head blight (FHB) of wheat and other small-grain cereals constitutes a disease complex in which several fungal species may cause largely indistinguishable symptoms (Parry et al., 1995). Within the complex are fungi that produce mycotoxins (*Fusarium* species) and others that do not (*Microdochium nivale* varieties). To further complicate the situation, the different *Fusarium* species, and even different isolates, produce different toxins. Although a large number of *Fusarium* species have been isolated from blighted wheat, relatively few are considered to be of overall significance (Parry et al., 1995). *Fusarium graminearum* is the major pathogen worldwide, while *F. culmorum* tends to predominate in cooler, maritime regions where *F. cerealis* (syn. *F. crookwellense*) (Nirenberg, 1990) may also be found. *F. avenaceum* and *F. poae* are also frequently associated with FHB, with *F. poae* causing symptoms termed 'glume spot' (Kemp et al., 1996). In addition to the actual *Fusarium* species, *M. nivale* (formerly *F. nivale*) may also cause head blight and is particularly prevalent where cooler, wetter conditions prevail. Although these *Fusarium* species are of the greatest overall significance, it should be emphasised that other species such as *F. sporotrichioides, F. equiseti* and even *F. verticillioides* may be of significance in particular situations (Sugiura et al., 1993; Miller 1994; Tekauz et al., 2000). *F. verticillioides* is generally considered to be a pathogen of maize but has also been isolated from wheat (Chelkowski et al., 1995).

In addition to causing disease which leads to reduced yield and quality of grain, FHB is of particular concern because of the ability of the majority of the causal organisms to produce mycotoxins in the grain that are harmful to human and animal consumers (Joffe, 1986). Among these are trichothecenes, zearalenone, moniliformin, enniatins, beauvericin, and fumonisins. The predominant mycotoxins produced within cereals are the trichothecenes, a group of sesquiterpenoid

secondary metabolites. Four basic classes have been designated within the trichothecenes with types A and B being of most relevance to FHB. These two differ in the presence of a carbonyl group at position C-8 of type B trichothecenes. Type A trichothecenes such as T-2, HT-2, diacetoxyscirpenol (DAS) and neosolaniol (NEO) are significantly more toxic than type B trichothecenes such as deoxynivalenol (DON), acetylated derivatives including 3-acetyl (3-ADON) and 15-acetyl (15-ADON) and nivalenol (NIV) (Madhyastha et al., 1994; European Commission, 2002).

Fusarium graminearum and *F. culmorum* produce type B trichothecenes including DON, 3-ADON, 15-ADON and NIV. DON and NIV chemotypes (chemotypes I and II, respectively) are recognised within both species although the majority of isolates of *F. graminearum* and, possibly also *F. culmorum*, produce DON. With these species being the chief cause of FHB it is not surprising that DON and NIV are the most common trichothecenes detected in cereal grain worldwide (Placinta et al., 1999). It is unclear how or whether these compounds differ with respect to their phytotoxicity but chemotype and aggressiveness may be linked (see below). NIV is believed to be more toxic to humans and animals than DON or its acetylated derivatives, and hence is of importance with respect to food safety (Perkowski et al., 1997). NIV is particularly significant, as the monoclonal ELISA-based detection kits currently commercially available do not react to this compound.

Fusarium cerealis, F. poae and *F. equiseti* have also been reported to produce NIV (Sugiura et al., 1993; Liu et al., 1998; Langseth et al., 1999). In addition, *F. poae* and *F. equiseti* also produce type A trichothecenes including DAS (Langseth et al., 1999) and hence can produce both type A and type B trichothecenes. *F. sporotrichioides* produces type A trichothecenes including T-2 and HT-2 (Langseth et al., 1999). *F. avenaceum* is not known to produce trichothecenes but does produce other mycotoxins including moniliformin, enniatins and beauvericin (Golinski et al., 1996; Herrmann et al., 1996a; Logrieco et al., 1998). *F. verticillioides* also is not known to produce trichothecene mycotoxins but produces fumonisins (B1, B2 and B3) as well as beauvericin and moniliformin. Two groups, termed var. *nivale* and var. *majus* have been identified within *M. nivale* (Woolenweber and Reinking, 1935; Lees et al., 1995) but, in contrast to the *Fusarium* species, neither variety is known to produce mycotoxins (Logrieco et al., 1991).

Several parameters are used to quantify disease levels in epidemiological studies and in studies of host resistance and fungal pathogenicity. Visual symptoms of necrosis/bleaching of the glume tissues are assessed on a severity scale or as a percentage of infected spikelets either at a single time point or over several dates to produce an area under the disease progress curve (AUDPC). Harvested grain can be visually assessed for discolouration and shrivelling (tombstone kernels) and for yield loss (percentage yield or 1000 grain weight relative to controls). These disease parameters may then be related to the amount of mycotoxin present in order to establish the relationship between disease and toxin accumulation. Even when crops are inoculated with a single species or isolate the relationship between disease parameters and toxin content may not be closely related (Mesterhazy et al., 1999). Miedaner et al. (2001a) reported that no relationship could be observed between disease severity and DON content across environments with similar disease severities. Such results indicate the important influence of the environment although differences in the status of the inoculum may also be important. Under natural conditions, where pathogen populations are involved in producing disease, the relationship between disease and toxin content will inevitably be yet more complicated.

Dissection of the disease complex: The need for new tools

The presence of toxin-producing and non-toxin-producing species within the disease complex, along with isolates of differing chemotype greatly complicates attempts to understand the factors that influence disease development and toxin accumulation. The relative contribution of each of the species in causing disease in a particular situation will depend upon a range of variables, as will the amount of toxin produced by that species. Only by identifying and understanding the nature of the interactions between the environment and the causal organisms will it be possible to understand the consequences with respect to mycotoxin accumulation in grain and, ultimately, the subsequent threat posed to human and animal consumers.

Attempts to evaluate the relative role of each fungal species in a particular context by means of isolation into axenic culture may be confounded, because in the absence of selective media, the relative amounts of

each pathogen may not be accurately determined. For example, *F. culmorum* tends to out-compete *M. nivale* on most growth media and this may result in an underestimation of the prevalence of the latter in mixed infections (Pettitt et al., 1993). Whatever the method, the isolation of pathogens from plant tissues can only reveal what may be grown out of the plant rather than what is within the plant. It would be most desirable to be able to determine the identity, amount and, if possible, the location of each pathogen within the host tissues.

A number of molecular techniques are being utilised in order to understand the nature and diversity of the pathogens responsible for FHB. Molecular techniques are also being used to study the interactions between the pathogens and their hosts and between the pathogens themselves along with the influence of environmental factors such as fungicides on these interactions. The majority of techniques are based upon analysis of DNA or RNA although measurement of ergosterol using high pressure liquid chromatography (HPLC) is also used as a generic estimator of fungal biomass in many studies involving inoculation of plants with characterised isolates of known species.

An important aspect of these studies is the influence of the environment (in the broadest sense) on the production and accumulation of mycotoxins within grain and other plant tissues. With respect to the mycotoxins, most attention is paid to the trichothecenes but the potential of species to produce many different related compounds complicates attempts to study the factors affecting overall regulation of toxin biosynthesis. Assays to permit study of the regulation of trichothecene biosynthesis would aid investigations into the effect of host and environment on the toxigenic fungi involved in FHB. It is likely, however, that detailed chemical analysis will continue to be required to determine the amount of each trichothecene product within harvested grain.

Detection and identification of the causal agents by polymerase chain reaction (PCR)

The PCR offers a sensitive and potentially specific means to detect, identify and quantify the species present within plant tissues. A number of assays have been produced to permit detection of many of the major pathogens associated with FHB. The PCR assays are available for *F. graminearum* (Schilling et al., 1996; Niessen and Vogel, 1998, Nicholson et al., 1998), *F. culmorum* (Schilling et al., 1996; Nicholson et al., 1998), *M. nivale* var. *majus* and var. *nivale* (Nicholson et al., 1996), *F. poae* (Parry and Nicholson, 1996), *F. cerealis* (syn. *F. crookwellense*) (Yoder and Christianson, 1998), *F. avenaceum* (Schilling et al., 1996; Turner et al., 1998) and *F. verticillioides* (Möller et al., 1999). The development and use of these assays is complicated by the difficulty of correctly identifying isolates in axenic culture and our still limited understanding of phylogeny within the *Fusarium* genus. For example, several assays for *F. avenaceum* have subsequently been shown to cross react with *F. tricinctum* (Turner et al., 1998). Conventional taxonomy places these two species in separate sections (*Roseum* and *Sporotrichiella*, respectively). Molecular studies, however, have demonstrated that the *Sporotrichiella* section is a phenetically diverse group and that *F. tricinctum* and *F. avenaceum* are indeed closely related (Bateman et al., 1996; Turner et al., 1998). This demonstrates the importance of testing the specificity of assays against all species with the potential of being co-isolated and not just those believed to be closely related on the basis of our current understanding.

FHB pathogen diversity and populations

Molecular phylogeny is revealing the presence of cryptic variation that may have significance with respect to the epidemiology of FHB. Although *F. graminearum* has been isolated in all major agricultural regions of the world little was known, until recently, about the population structure of *F. graminearum* from different geographical areas and whether these differences were related to pathogenicity or any other trait. Genetic variation within *F. graminearum* has been detected by random amplified polymorphic DNA (RAPD) analysis. Although isolates from different continents have been examined (Schilling et al., 1994; Carter et al., 2002), most analyses have compared isolates from closer geographical sites, e.g. within Europe (Schilling et al., 1997), Canada (Dusabenyagasani et al., 1999), Nepal (Carter et al., 2000) and North Carolina, USA (Walker et al., 2001). While isolates from two regions (Ontario and Quebec) in Canada were considered to form a single population pool (Dusabenyagasani et al., 1999), two distinct groups were identified among Nepalese isolates (Carter et al., 2000). The latter study also characterised isolates using a sequence characterised amplified region (SCAR). This revealed

that all isolates of one group (termed B) possessed a single SCAR polymorphism while those of the second group (group A) had one of five different SCAR polymorphisms (Carter et al., 2000).

RAPDs have also been used to assess variability within *F. culmorum* (Miedaner et al., 2001b). These workers reported a relatively high level of genotypic variability within a population of *F. culmorum* from a field in Novgorod, Russia. The level of variability was even greater than that within a population of *F. graminearum* from a field in Sersheim, Germany. The reasons for the high level of variability within a fungus with no known teleomorph are unclear but these findings may reflect the consequences of a wide range of selective pressures upon fungi, such as *F. culmorum,* which may have both saprophytic and parasitic phases within their life-cycle.

Isolates of *F. graminearum* collected from wheat, maize and rice in Nepal separated into two groups (termed A and B) on the basis of RAPD analysis (Carter et al., 2000). The distinction might be related to host preference, with isolates of group B being less frequently isolated from wheat or rice than maize. Similarly, Satyaprasad and Bateman (1997) observed two groups within *F. avenaceum* on the basis of PCR-RFLP of the internal transcribed spacer (ITS) region of ribosomal DNA, with one being more frequently isolated from white lupin than the other. It was concluded that sub-groups related to host preference may exist within *F. avenaceum* (Satyaprasad and Bateman, 1997).

Isolates of *F. graminearum* from different geographic origins vary in their pathogenicity towards different hosts. The pathogenicity of Chinese and USA isolates has been tested on wheat, and in a comparison of six isolates, one Chinese isolate caused significantly greater disease symptoms than did any of four isolates from the USA (Bai and Shaner, 1996). In a study of pathogenicity towards rye, the genetic variation among isolates from three continents, America, Australia and Europe, was greater than that obtained for strains from populations of *F. graminearum* from individual sites in Europe (Miedaner and Schilling, 1996). The individual field populations, however, contained 60% of the total variation observed, indicating that a high degree of variation in pathogenicity was present at each site. Isolates of *F. graminearum* across Europe and the USA differ from those in the two groups (A and B) found in Nepal and form a distinct group (Carter et al., 2002). This group (group C) was found to be more aggressive towards wheat and maize seedlings than group A or B although the pathogenicity of individual isolates within each group also varied.

DNA sequence analysis has been used to differentiate *F. graminearum* into at least seven biogeographically structured lineages (O'Donnell et al., 2000). The relationship between the RAPD groups (Carter et al., 2000; 2002) and those of the sequence-derived lineages (O'Donnell et al., 2000) has yet to be fully determined but groups A, B and C appear to be largely congruent with lineages 6, 2 and 7, respectively. Evidence obtained to date indicates that these groupings are of biological significance with respect to host preference and aggressiveness (Carter et al., 2002) and hence it is important that epidemiological studies define the causal agent down to the level of group/lineage in order to place the results into context.

Serendipitously, the region amplified by the *F. graminearum* PCR assay developed by Nicholson et al. (1998), is polymorphic and amplicon size and/or sequence is generally associated with a RAPD group. This permits an immediate tentative designation of *F. graminearum* isolate/causal agents within plant tissues to different RAPD groups (Carter et al., 2000; 2002). The use of single strand conformational polymorphism (SSCP) enables differentiation between amplicons that differ only in sequence (Qu Bo, unpublished results), thus increasing the resolving power of this assay. These assays can be used to establish the distribution of FHB causal agents, down to the level of group/lineage, across regions and within crops and even within individual plants. At present the groups/lineages appear to be related to geographic regions, but such distinctions may disappear as a result of global trade in grain and plant products aiding distribution of these pathogens.

Diagnostic PCR assays for FHB pathogens

The annual Department for Environment, Food and Rural Affairs (DEFRA)-funded survey of winter wheat diseases revealed that the incidence of FHB was significantly greater in 1998 than in previous years (J.A. Turner and P. Jennings, Central Science Laboratory, personal communication). Harvested grain from severely affected sites was analysed for trichothecene mycotoxins and for the major FHB pathogens using competitive PCR. The predominant pathogen was *M. nivale* var. *majus*, which was present in 94% of samples. The preponderance of non-mycotoxin producing species in the UK epidemic of 1998, may

largely have accounted for the relatively low levels of mycotoxins present in the majority of samples. The detection of *F. graminearum* in over 40% of samples was of particular significance as *F. graminearum* had previously been found only very rarely in the UK. The cause of the apparent increase in this species is not known but it may be related to altered agronomic practices, such as the increased area of forage maize in the UK, or to environmental factors. DON and NIV were the major toxins detected in grain and there was a strong correlation (0.68) between the levels of these two toxins and the amount of *F. culmorum* and *F. graminearum* detected, indicating that these two species were probably largely responsible for the presence of the type B trichothecenes detected.

HT-2 toxin was detected in 38% of the samples but the causal agent could not be determined because, at that time, no PCR assays were available for species known to produce these toxins. Recently, Wilson et al. (unpublished) have developed assays for *F. sporotrichioides* and *F. langsethiae*, both producers of T-2 and HT-2. When comparisons were made between the incidence of *F. langsethiae* and *F. sporotrichioides*, and the incidence of toxin in the UK samples from 1998 (HGCA, 1999), it was found that up to 90% of type A trichothecene (T-2 and HT-2) production could be accounted for by the presence of these two species within the samples. Moreover, it was found that the incidence of *F. langsethiae* and *F. sporotrichioides* were independent of each other but the factors that determine the distribution of the two species are not known.

PCR has also been used to detect individual species in single field plot experiments and to determine their tissue localisation and relationship to visual disease symptoms (Doohan et al., 1998). Whereas *F. culmorum* was detected in grain, glume and rachis tissues, *M. nivale* varieties were found mainly in rachis tissue. *F. poae* was detected predominantly in glume tissues and was rarely found in the rachis indicating that this pathogen may be less able to colonise wheat heads than *F. culmorum.* Polley and Turner (1995) reported that *F. poae* was most commonly isolated from glumes, a finding in agreement with that of Doohan et al. (1998). PCR also detected *Fusarium* species in tissues from heads lacking disease symptoms. This is of significance because other workers have reported finding trichothecene mycotoxins in apparently healthy wheat heads (A. Mesterhazy, personal communication).

Quantification of FHB causal agents

Ergosterol has been used in a number of studies to estimate the degree of fungal colonisation of grain (Miedaner and Perkowski, 1996; Gang et al., 1998; Miedaner et al., 2000). Such studies are possible where known isolates are used to inoculate plants and where the influence of secondary infection is minimal. Host resistance was found to be the major factor affecting fungal colonisation and disease development on winter rye by *F. culmorum*. Mycotoxin content, however, was most influenced by environmental factors interacting with the host genotype (Miedaner and Perkowski, 1996). These results support those of others who reported the strong influence of environmental factors on the relationship between disease severity and DON content (Mesterhazy et al., 1999). In a separate study, Miedaner et al. (2001) reported that, while disease levels were similar in wheat, triticale and rye cultivars, the level of DON was generally higher in wheat than in rye indicating a potential role of the host species in determining the accumulation of DON. The ability to degrade DON has been proposed as a potential resistance mechanism (Miller et al., 1985) and naturally occurring plant compounds have been shown to inhibit trichothecene toxin biosynthesis by *F. sporotrichioides in vitro* (Desjardins et al., 1988). While the ratio of DON to disease differed among the cultivars, the ratio of DON to ergosterol was similar in wheat and rye indicating that similar levels of visual disease may not reflect similar levels of colonisation and, hence DON content, in different species.

Ergosterol measurement has also been used to assess aggressiveness among FHB pathogens. When isolates of *F. culmorum* and *F. graminearum* of differing aggressiveness were examined, increased levels of disease correlated with increased colonisation, as measured by ergosterol content, and with increased DON content of grain (Miedaner et al., 2000; Gang et al., 1998). Thus the greater the amount of fungus present in the grain, the higher the level of DON. The ratio of DON to ergosterol, however, did not correlate with disease in either study indicating that the differences in aggressiveness are probably not due to differences in the rate of production of DON. The use of ergosterol to estimate fungal colonisation is severely limited as the assay measures the total amount of all fungi present rather than the amount of a specific species.

Although conventional PCR is an extremely sensitive and potentially specific technique, due to the nature of the assay it is generally unsuitable for quantification.

Minor differences in amplification efficiencies, such as might occur where two samples differ in the level of inhibitory impurities present, would result in large differences in amplicon yield at the end of the reaction. Competitive PCR and, more recently, 'real-time' PCR assays have been developed to address this problem with respect to the study of FHB. These assays can be used to detect and quantify the individual fungal species in plant tissues even where they are present as part of mixed infections. Competitive PCR assays to detect and quantify the amount of each of the target species, based upon the amount of DNA of that species present, have been developed for *F. graminearum* and *F. culmorum* (Nicholson et al., 1998), *M. nivale* var. *majus* and var. *nivale* (Nicholson et al., 1996), *F. poae* (Parry and Nicholson, 1996) and *F. avenaceum* (Turner et al., 1998). Recently, Simpson et al. (unpublished) developed a 'real-time' PCR assay for *F. culmorum*. These assays provide the means to dissect the FHB complex into its component parts and thus determine the prevalence and role of each component in disease development and under various conditions.

Competitive PCR has been used in a number of studies of FHB, principally to study the effect of fungicides on disease and mycotoxin accumulation. Doohan et al. (1999) found that competitive PCR provided greater resolution than visual disease assessment for determination of fungicide efficacy and highlighted the difficulty of relating symptoms on glume tissues to colonisation of grain, even where only a single pathogen is present.

Attempts to determine fungicide efficacy against disease complexes such as FHB are complicated because of the potential for fungal components controlled by the treatment to be replaced by others less susceptible to the fungicide. It is conceivable for non-toxin producing species to be replaced by toxin producers. Control of FHB by fungicides has often proved to be erratic and it is possible that environmental factors and replacement of one pathogen by others may have contributed to this. Simpson et al. (2001) determined the levels of FHB pathogens in naturally infected and inoculated field experiments. The results demonstrated that fungicide application influenced the pathogen population through selective suppression of particular species by individual fungicides. Application of tebuconazole suppressed *F. culmorum* whereas azoxystrobin suppressed *M. nivale*. Suppression of either species tended to result in an increase in the amount of the other, indicating that an antagonistic/competitive interaction exists between these two species. The finding from other studies that *F. culmorum* and *M. nivale* were found together less often in single wheat heads than expected by chance supports the view that a competitive/antagonistic interaction exists between these species (Doohan et al., 1998).

The interaction between *Fusarium* species and *M. nivale* has important consequences for mycotoxin accumulation. Grain from plots treated with azoxystrobin had more DON than untreated plots or those treated with tebuconazole. The increase in DON may largely be due to increased colonisation by *Fusarium* species where the azoxystrobin fungicide has suppressed the competing *M. nivale* (Edwards et al., 2002; Nicholson et al., unpublished). However, in some instances the increase in DON is not associated with an increase in the amount of fungus detected in the grain suggesting that the fungicide may have a stimulatory effect on the production of toxin (Simpson et al., 2001; Nicholson et al., unpublished).

Quantitative PCR enables the detection and quantification of individual species within plant tissues or other matrices. Thus this technique is ideal for studying the interactions between FHB species. Simpson et al. (2000) co-inoculated seedlings of wheat, rye and oats with *M. nivale* vars. *majus* and *nivale*. At 15 °C var. *majus* colonised wheat and oat stems more than var. *nivale.* In contrast, on rye var. *nivale* had a significant competitive advantage over var. *majus*. Further studies have shown that the effect of the host species on competition between the two varieties also occurs at 10 and 20 °C (Simpson et al., unpublished).

More recently, competitive PCR has been used to investigate interactions between *F. culmorum* and *M. nivale* vars. *majus* and *nivale* on seedling cereal plants (Simpson et al., unpublished). When wheat seedlings were inoculated with both *F. culmorum* and *M. nivale* var. *majus*, colonisation by both species was reduced relative to when they were inoculated alone (Figure 1). Such studies confirm that these head blight pathogens are antagonistic to one another. When *F. culmorum* and *M. nivale* var. *nivale* were inoculated together, *F. culmorum* appeared to be unaffected whereas colonisation by *M. nivale* var. *nivale* was significantly reduced (Figure 1) which would suggest that *F. culmorum* interacts differently with the two varieties of *M. nivale*. At present, it is not known whether competition between *Fusarium* species is influenced by the host species. Such information would yield important information regarding the relative abilities of particular

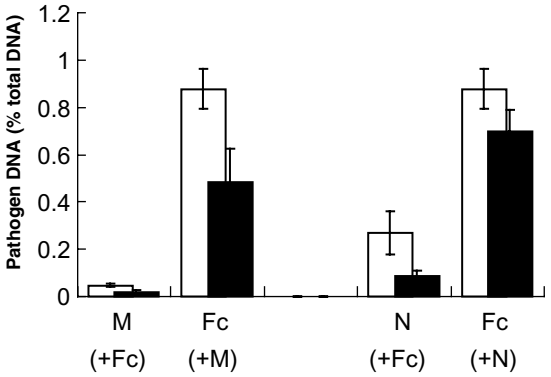

Figure 1. Amount of pathogen DNA detected (mean and one standard error) of *M. nivale* var. *majus* (M), *M. nivale* var. *nivale* (N) and *F. culmorum* (Fc) inoculated on wheat seedlings grown at 20 °C for 18 days. Open columns represent the DNA of each pathogen when inoculated alone. Hatched columns represent the amount present following mixed inoculation of *F. culmorum* and either variety of *M. nivale*. Error bars are one standard error.

fungal species to develop on particular cereal hosts and thus highlight the potential for particular mycotoxins to accumulate in grain of infected plants.

As well as interactions between the FHB pathogens themselves, it should be borne in mind that saprophytic elements of the microflora may also affect growth of pathogenic species (Liggitt et al., 1997) and also influence accumulation of mycotoxins (Ramakriskna et al., 1996). The use of similar assays designed for relevant species such as *Alternaria alternata*, and *Cladosporium herbarum* is highly desirable to increase our ability to identify all the players present in a particular niche and to partition effects accordingly. Competitive or 'real time' PCR assays such as those described above provide a means to detect and quantify each pathogen species and thus determine how they interact with one another and with other components of the microflora. These tools can also reveal how the interactions between pathogens and the host are influenced by environmental factors, including fungicide application.

The work described above highlights the role of host resistance and host species in influencing the development of FHB disease and hence mycotoxin accumulation. It is important to bear in mind that many of the FHB pathogens may also have a significant saprophytic element to their life-cycle, either through survival on colonised crop debris or as resting structures such as chlamydospores (Cook and Bruel, 1968; Nyvall, 1970). The ability to survive and multiply to become a significant source of inoculum for FHB may differ between species (Sitton and Cook, 1981) and contribute to determine the particular species that predominate in a given situation. There have been few attempts to examine this element of FHB epidemiology with molecular tools, but they should provide an insight into this component of a pathogen's life-cycle.

Detection and quantification by metabolite (toxin) profile

While determination of the relative proportions of individual FHB species is important, attention in many studies focuses on the amount of toxin accumulating in grain. In some instances, it is of more interest to determine whether toxigenic species are present than to identify exactly what species are present. The PCR-based tools have been developed to address diagnosis of pathogens by their toxigenic potential. For example, the first dedicated step in the trichothecene biosynthetic pathway is the isomerisation and cyclisation of farnesyl pyrophosphate to trichodiene by the enzyme trichodiene synthase (encoded by the gene *tri5*) (Proctor et al., 1995). The *tri5* gene is common to all known trichothecene-producing *Fusarium* species. PCR assays based upon sequences of this gene conserved among *Fusarium* species have been developed to enable the detection of the presence of fungi with the potential to produce trichothecenes. Doohan et al. (1999) and Edwards et al. (2001) developed competitive PCR assays based upon *tri5* to quantify the amount of potential trichothecene-producing *Fusarium* species present in plant tissues. Schnerr et al. (2001) have recently produced a 'real time' PCR assay for this gene. Similarly, a PCR assay has been developed to the *esyn1* gene that encodes enniatin synthetase to permit detection of *Fusarium* species with the potential to produce enniatins (Doohan et al., unpublished; Simpson et al., unpublished).

The importance of trichothecenes to the producing fungus and their role in causing disease has been much debated (Adams and Hart, 1989; Atanassov et al., 1994). Trichothecene-deficient mutants of *F. graminearum* have been produced through molecular gene disruption of *tri5* (Proctor et al., 1995) and a number of studies have provided information on the role of trichothecenes in pathogenicity of *Fusarium* species towards wheat and other species (Proctor et al., 1995; Desjardins et al., 1992; 1996) These studies have demonstrated that trichothecenes are not essential for

pathogenicity but may act as virulence/aggressiveness factors that result in toxin producers causing more disease than non-toxin producers (see below). Using competitive PCR strains of *F. graminearum* capable of producing trichothecenes have been shown to colonise wheat grain to a greater extent than non-producing strains (Nicholson et al., 1998; Smith unpublished). Similar studies of the infection of maize indicate that trichothecene-producing isolates of *F. graminearum* also colonise maize kernels to a greater extent than non-producing strains (Harris et al., 1999). The effect of trichothecenes on the aggressiveness of the pathogen appears, however, to be dependent upon the host species. Disruption of the *tri5* gene of *F. sambucinum* led to reduced virulence on parsnip root slices but not upon slices of potato tuber (Desjardins et al., 1992). Similarly, while disruption of the enniatin synthetase gene (*esyn1*) of *F. avenaceum* led to reduced levels of virulence against slices of potato tuber (Herrman et al., 1996b), it has not been possible to detect any effect on virulence against wheat and rye seedlings (Thomsett et al., unpublished). Trichothecenes and other toxins produced by *Fusarium* species may therefore aid infection and colonisation (aggressiveness) on some hosts while they have no such apparent role on others.

It is recognised that environmental factors play an important role in infection and development of FHB and mycotoxin accumulation (Miller, 1994; Schaafsma et al., 2001). The weather conditions during the latter stages of development are most important because wheat is most susceptible to infection at anthesis (Miller, 1994). The amount of mycotoxin accumulating also depends upon the resistance to infection of the host (Schaafsma et al., 2001), although significant genotype × environment interactions have also been observed in many studies (Miedaner et al., 2001). Trichothecene accumulation has been assessed in grain and chaff components of cultivars that differ in their susceptibility to FHB caused by *F. culmorum*. Ears of a susceptible wheat cultivar and a moderately resistant cultivar were spray-inoculated with conidia of *F. culmorum* at mid-anthesis (GS 65) (Zadoks et al., 1974). Inoculated ears were harvested at GS 90 and separated into chaff and grain components. Fungal colonisation was assessed using competitive PCR and levels of DON were determined using a commercial antibody test kit (Ridascreen – DON fast kit). Similar levels of fungal colonisation and toxin content were observed in the chaff of both cultivars (Gosman, 2001). As anticipated, the grain of the susceptible cultivar contained significantly greater amounts of DNA of *F. culmorum* than that of the resistant cultivar. The grain of the susceptible variety also contained higher levels of DON than the resistant variety. However, the ratio of DON to fungal DNA was significantly greater in the resistant than in the susceptible cultivar. A similar situation was observed among doubled haploid lines produced from a cross between a susceptible wheat cultivar (Riband) and a resistant cultivar (Arina) (Figure 2). Lines with higher resistance to FHB had much lower amounts of fungal DNA in the grain and reduced levels of DON, with the consequence that the ratio of DON to fungal DNA increased with greater resistance. Such findings suggests that, in the more hostile environment of the resistant cultivar, the fungus may produce more toxin than in the relatively benign environment of the susceptible host. Further studies are required to determine what temporal and/or spatial differences in the production of trichothecenes occur during infection of cultivars differing in their resistance to FHB. It is conceivable that toxin biosynthesis is enhanced when the fungus is experiencing stresses such as nutrient deprivation or exposure to antifungal substances produced by the resistant host cultivar. The form and level of stress will probably also vary between tissues.

Regulation of trichothecene biosynthesis

Because of the potential number of trichothecenes produced by a particular fungal species, or even isolate,

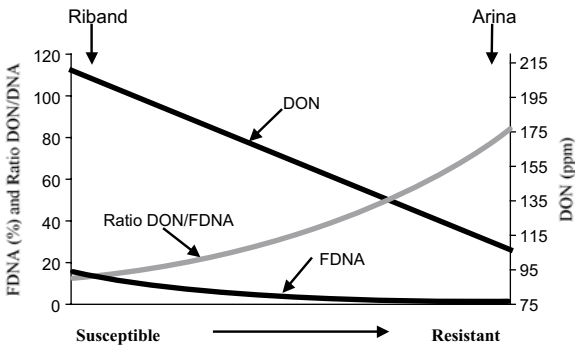

Figure 2. Trend for fungal colonisation, measured as fungal DNA (FDNA; fungal DNA as % of total DNA per sample) content determined using competitive PCR, DON (ppm) content and ratio of DON/FDNA in grain of a doubled haploid population (60 lines) derived from a cross between a susceptible (Riband) and a resistant (Arina) cultivar of wheat. Lines are ordered according to increasing resistance.

techniques to permit study of the overall regulation of the trichothecene biosynthetic pathway might be particularly useful for the identification of factors that prevent or enhance toxin production. The isomerisation and cyclisation of farnesyl pyrophosphate to trichodiene by the product of *tri5* occurs before the branching off to form the various trichothecenes and can act as a marker of overall activity in the pathway. Previous studies have shown that transcriptional control plays an important part in regulation of *tri5* expression, making this a suitable target for the study of factors affecting regulation of the biosynthesis of trichothecene mycotoxins (Hohn et al., 1993). A reverse transcription (RT)-PCR assay has been developed to enable study of *tri5* gene expression in trichothecene-producing strains of *Fusarium* species (Doohan et al., 1999). As noted above, stress may play a role in the regulation of trichothecene biosynthesis. As well as host-derived factors, environmental factors, including fungicides, may affect toxin production. There is growing evidence that sub-lethal concentrations of certain fungicides may increase mycotoxin production by *Fusarium* species (D'Mello et al., 1998). The possibility that this may occur under field conditions is supported by a limited number of reports in which the mycotoxin content of grain has increased following fungicide treatment (Simpson et al., 2001). *In vitro* studies using the *tri5* RT-PCR assay demonstrated that Folicur and Sportak fungicides induced a transient increase in expression of *tri5* of *F. culmorum* before levels fell to below those in the control cultures (Doohan et al., 1999).

Very little is known about the temporal and spatial regulation of trichothecene biosynthesis during infection and colonisation of wheat heads. Preliminary studies of the expression of *tri5* in grain and chaff of heads of a susceptible cultivar have been undertaken (Doohan et al., 1999). Expression of *tri5* was significantly greater in chaff (glume tissues) than in grain at GS 70 and GS 80. While expression levels in the chaff remained similar at GS 70 and GS 80, *tri5* expression was not detected in grain at this time (Doohan et al., 1999). These results indicate that, at least until GS 80, expression of *tri5* declines in grain while remaining high in glume tissues. It is of interest to determine which factors underlie the differential regulation of expression in the different tissues. Differential tissue-related patterns of *tri5* expression have since been observed in other wheat cultivars that differ in their susceptibility to FHB (Draeger et al., unpublished).

From the toxicological perspective, focusing upon *tri5* as a generic marker for trichothecenes is problematic since the toxicity of type A and type B trichothecenes differs markedly (Madhyastha et al., 1994; European Commission, 2002). Sequence analysis of 28S ribosomal DNA revealed that trichothecene producing *Fusarium* species can be resolved into two monophyletic groups (Mulè et al., 1997). The phylogenetic relatedness of species producing type A or type B trichothecenes has been utilised to develop PCR assays to detect species on the basis of trichothecene type (Simpson et al., unpublished). Such assays may provide sufficient resolution for many epidemiological studies.

Identification of chemotype and relevance to FHB

Even within a species, the potential for different isolates to produce different trichothecene toxins is significant when attempting to relate environmental factors to disease development and toxin accumulation. Although DON is the predominant toxin produced by most isolates of *F. graminearum* found across Europe (group C or lineage 7) some isolates produce NIV. The aggressiveness of DON-producers towards rye was found to be slightly greater than that for NIV-producers (Miedaner et al., 2000). To date, all isolates of *F. graminearum* group B/lineage 2 produce NIV, while both chemotypes are present within group A/lineage 6 isolates (Carter et al., 2002). While isolates of both DON and NIV chemotypes of group A were similarly aggressive towards wheat seedlings, NIV chemotypes were more aggressive towards maize than DON chemotypes (Carter et al., 2002). Thus, when evaluating the effect of particular factors upon disease development the chemotype of the isolate may be of significance. Both chemotypes have been observed within *F. culmorum* and both types appear common in Europe (Bakan et al., 2001). The aggressiveness of DON chemotypes of *F. culmorum* is generally greater than that of NIV types against both wheat and rye (Gang et al., 1998; Muthomi et al., 2000). Only very recently has the genetic basis for the difference in chemotype been determined (Lee et al., 2001; 2002). Two genes (*tri7* and *tri13*) appear non-functional among isolates of the DON chemotype, making them unable to produce NIV. Assays for both genes have been developed to allow the detection and differentiation of NIV and DON chemotypes among type B

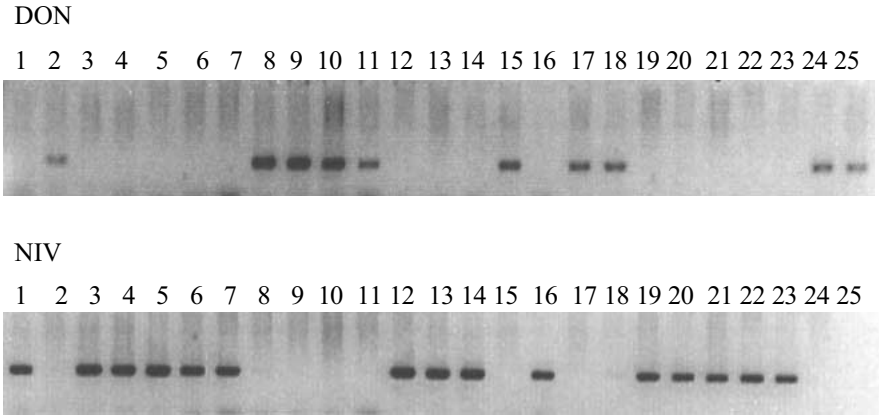

Figure 3. PCR assays of 25 isolates of *F. graminearum* with primers designed to specifically amplify from non-functional DON and functional NIV alleles of *tri7* in order to determine anticipated trichothecene production: DON or NIV.

trichothecene producing species (Chandler et al., unpublished) (Figure 3). These primers will permit detailed study of interactions between, and the competitive advantage of, NIV and DON chemotypes on different cereal hosts and against different saprophytic and pathogenic species associated with FHB.

Summary

The study and control of FHB is complicated because of the involvement of several fungal species and several mycotoxins in the disease. A number of molecular tools have been developed to permit dissection of this complex and enable the identification of the factors important in determining where the disease becomes established and which species might predominate in a particular situation. Diagnostic PCR assays have contributed to the detection of species new to the UK, such as *F. graminearum*, which was formerly rare in this country. Competitive and 'real time' PCR assays permit the relative amount of each species to be determined, even where they are present as part of a mixture. These assays are revealing how the different species interact with each other and with the cereal host. As the number of assays and ease of use increases, our ability to relate the presence and amount of particular causal agents to the amount and type of mycotoxin present in grain will also improve. This will contribute towards our understanding of the disease complex to reveal how host and environmental factors such as fungicide application affect the balance between species as well as the consequences for mycotoxin accumulation. Molecular tools such as the *tri5* RT-PCR assay provide the ability to study temporal and spatial regulation of the trichothecene biosynthetic pathway and to understand the effect of environmental factors and other components of the microflora of the wheat plant on mycotoxin production. Where isolates of the same species differ in chemotype, molecular assays related to the relevant genes provide a means to include the aspect of chemotype into efforts to understand the complex interactions between the FHB pathogens, the host, other microbes and the environment. These assays, along with those developed elsewhere, are useful tools with which to increase our understanding of the factors that influence FHB and, ultimately, our ability to control this disease and eliminate the risk of mycotoxin contamination of grain and foodstuffs.

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