THE APPLICATION OF HACCP TECHNIQUES TO THE MANAGEMENT OF *FUSARIUM* TOXINS IN CEREALS

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This document provides additional information from the literature searches used in the hazard/risk analyses described in the main body of the report.

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A PROJECT CONTEXT

1.0 CEREALS PRODUCTION IN THE EUROPEAN UNION

1.1 Climate

The European Union (EU) occupies most of the central and western part of the European land-mass, together with the principal off-shore islands (British Isles). Broadly speaking, continental Europe can be divided into five broad climatic zones (287).

Northwest:- Including the United Kingdom, Denmark, Benelux Western Germany, Western Austria and most of France. These areas are generally characterised by relatively mild summers and cool winters.

Scandinavia: - Including Norway, Sweden and Finland. In the extreme south, this area experiences mild summers with cold winters, in the north - a cold to mild summer with an arctic winter

Northeast: - Including Eastern Germany and Central and Eastern Austria, Poland, Hungary. These areas are often charactrerised by relatively warm summers and cold winters, often with heavy snow cover.

Southeast: - Including Southern Balkan states and Greece and most of Italy. These countries generally experience hot dry summers in the south with mild winters, but snow in mountainous areas.

Southwest: - Principally the Iberian Peninsula. Generally this area has hot and relatively dry summers with mild cool winters.

This is a very broad description and growing seasons, even within a particular climatic zone can vary considerably. Although areas with growing periods in excess of 240 days are generally found in Southern Europe, some areas in North-western Europe, e.g. Brittany, France and the Southwest of the UK and Ireland also benefit from equally long periods.

1.2 Grain Production and Consumption in the European Union

This project is principally concerned with the cereals: barley, wheat and maize. The EU currently produces approximately 16.5%, 38.0% and 6.0% of the world's wheat, barley and maize respectively (1999 data, reference 22). Broadly speaking there is a shift in the types of crops grown within the EU with barley and wheat in the north and wheat and maize in the south.

During the period 1990 to 1999 there was an increase in European cereals production from approximately 158 million tonnes to a peak of 192 million tonnes in 1998 and 182 million tonnes in 1999 (2). During this period, wheat production within the European Union rose from 84.7 million tonnes in 1990 to a peak of 103.8 million tonnes in 1998 and to 96.7 million tonnes in 1999. In the

same period there was a fall in barley production (50.4 to 48.6 million tonnes) and a rise in the maize crop from 22.6 to 36.6 million tonnes in 1999. These figures can be misleading and do not reflect the situation in individual Member States. For example in Italy, while wheat production has remained relatively static, at approximately 8 million tonnes per year, maize production has increased from 5.9 million tonnes in 1990 to 9.7 million tonnes in 1999. Most cereals grown within the EU are destined for the human food chain either directly or as a component of animal feed. In the case of wheat approximately equal proportions are used in food (38%) and animal feed (39%) production. In terms of coarse grains (primarily maize and barley) the distribution is more biased to animal feed (60%) with approximately 5% being used directly for human foods.

2.0 AGRICULTURAL AND PUBLIC HEALTH SIGNIFICANCE OF *FUSARIUM* SPECIES

2.1 Plant Diseases Associated With *Fusarium* spp.

2.1.1 Introduction

Members of the genus *Fusarium* are important plant pathogens, particularly of cereals. These diseases are of considerable commercial importance both with regard to yield and technological quality (reviewed by Bai and Shaner, 32).

2.1.2 Wheat & Barley

Fusarium spp. are associated with three principle types of disease in small grain (e.g. wheat and barley) crops (reviewed by Miedaner, 255).

Seedling Blight

This is generally caused by *F. culmorum* and/or *F. graminearum.* It occurs in humid climates, usually a consequence of seed borne infection. The disease leads to both a reduction in the number of viable plants and the promotion of secondary attack by pests. A second type of seedling blight caused by *F. pseudograminearum* (*F graminearum* type I, reference 13) results from soil borne infection and only occurs in dry soils (63). Cultivars resistant to *Fusarium* spp. induced seedling blight may also be resistant to ear blight, the key fusarial disease involved in mycotoxin contamination (249).

Stem Base Diseases

Members of the *Fusarium* genus are associated with three types of this disease:

Brown Foot Rot This is caused by *F. culmorum* and *F. graminearum* in areas of high soil moisture and humidity (97). The disease is usually the consequence of a more complex attack including fungi from other genera (e.g. *Microdochium nivale*). This disease begins with an attack

from an above ground inoculum. It is associated with reduced yields due to impaired capacity of the stem to transfer nutrients to the ear, as well as an increased risk of lodging (255), itself a risk-factor in *Fusarium* spp. mycotoxin contamination of small grain cereals (211).

Crown Rot Mediated by *F. pseudograminearum* under dry weather and soil conditions. In contrast to brown foot rot, this is associated with a below ground inoculum entering the plants around the emerging roots and crowns (97) and remains latent, until the plant is put under extreme water stress (98). The disease leads to premature ripening of the crop with reductions in kernel number and/or weight). Although not probably such a risk factor in the humid climatic areas, found through central and northern parts of the EU, at a global level, crown rot is probably the most destructive of the *Fusarium* spp. linked diseases (255).

Common Foot Rot Often caused by a complex attack involving *F. culmorum, F. graminearum* and *Bipolaris sorokiniana* and seen in North America (395).

Fusarium Ear Blight

Early studies by Tu (375) proposed that *Fusarium* ear blight was associated with infection of the wheat shortly before, during or just after anthesis and was favoured by humid conditions. In terms of mycotoxin contamination this is considered to be the key causative disease (80). Epidemics of *Fusarium* ear blight were reported as long ago as 1884 in England and 1890 in USA (80). In the twentieth century, epidemics of *Fusarium* ear blight diseases first came to light in Japan in 1962 (400) and in North America in the beginning of the 1980s (343). The significance of early root infection to subsequent *Fusarium* ear blight and mycotoxin contamination is uncertain. However, no link appears to be mediated by a systemic route. Studies with winter wheat, where splash contamination of the plant was prevented (95), revealed that, when grown in *Fusarium* spp. contaminated compost; fungi were recovered in the stem tissue above soil level, even in apparently symptomless plants. However, no fungi were recovered from the ears.

2.1.3 Maize

In maize, *Fusarium spp.* mediate three types of ear disease closely related to the risk of mycotoxin contamination (reviewed in 52):

Gibberella Ear Rot (also called pink ear rot) Associated with *Gibberella zeae* (the sexual stage of *F. graminearum*) and *F. culmorum*; these fungi can contaminate maize with deoxynivalenol, zearalenone and T-2 toxin. Climate appears to be determining factor on the causative organism, with *F. graminearum* being associated with outbreaks of the disease in Canada and northern Europe, while *F. culmorum* tends to be the dominant organism in north central and Eastern Europe.

Fusarium Ear Rot Mediated mainly by *F. monoliforme, F. subglutans* and *F. proliferatum.* These organisms are associated with the production of moniliformin and the fumonisins. The environmental conditions which favour this disease are dry weather and insect damage.

Red Ear Rot (red fusariosis) Less common than the other two diseases and is associated with the organisms *F. equiseti, F. sporotrichoides, F. crookwellense,* and *F. avenaceum.* These fungi are associated with the production of the A-type trichothecenes, including T-2 toxin.

2.1.4 *Fusarium* Diseases and Mycotoxin Contamination of Cereals

Plant diseases associated with *Fusarium* spp. also have implications regarding public health. As alluded to above, different members of this genus can also produce mycotoxins known to be harmful to both man and livestock (15). It has been estimated that worldwide, 25% of food crops are contaminated with mycotoxins, with those from *Fusarium* spp. making a significant contribution (79). Examples of some of the principal mycotoxins produced by *Fusarium* spp. are detailed in Table 1. Chelkowski (80) has listed over 20 species of *Fusarium* associated with grain. To this list he added *Microdochium nivale.* Although not a member of the genus *Fusarium,* it is a significant pathogen that can induce ear blight but does not appear to produce any mycotoxins. *F. culmorum* is frequently found in the cooler maritime regions of northern Europe, while *F. graminearum* predominates on a global scale (292).

It is important to note that *a priori,* all strains of the same species do not necessarily produce one or more of the mycotoxins listed. Logrieco *et al.* (225) observed that all of the *F. proliferatum* strains, isolated from Italian maize exhibiting preharvest maize rot were capable of producing fumonisin B_1. However, Chelkowski *et al.* (84) found that four out of thirteen isolates of *F. culmorum* isolated from wheat produced deoxynivalenol, while twelve produced zearalenone. Work by L'-vova *et al* (204) with *F. graminearum* strains isolated from scabby wheat, found that all of the strains studied were capable of producing deoxynivalenol and zearalenone, while 36% and 17% were capable of producing acetyl-deoxynivalenol and zearalenol respectively. Visconti and Doko (385) examined strains of *Fusarium* spp. isolated from a variety of crops and mixed feed from France, Italy, Poland and Spain. They found that all of the strains of *F. moniliforme* were capable of producing fumonisin B_1 in varying amounts while little or no toxin production was found in cultures of *F. subglutinans.*

Table 1 *Fusarium* species and the principal toxins they produce (after 15)

Species	Principal Toxins Produced
F. acuminatum	T-2 toxin, HT-2 toxin
F. avenaceum	Moniliformin
F. cerealis (crookwellense)	Nivalenol, Fusarenon X, Zearalenone
F. culmorum	Deoxynivalenol, Zearalenone
F. equiseti	Diacetoxyscipernol, Zearalenone, Fusarochromanone
F. graminearum	Deoxynivalenol, Zearalenone, Acetyldeoxynivalenol
F. oxysporum	Moniliformin
F. poae	Diacetoxyscipernol, Monoaceteoxyscirpenol, Nivalenol, Fusarenon X.
F. proliferatum	Fumonisins, Moniliformin
F. sacchari (subglutans)	Moniliformin
F. sambucinum	Diacetoxyscipernol
F. semitectum (incarnatum)	Zearalenone
F. sporotrichioides	T-2 toxin, HT-2 toxin
F. torulosum	Wortmanin
F. tricinctum	Fusarochromanone
F. verticillioides	Fumonisins

2.2 Public Health Implications

The toxicological status of *Fusarium* spp. mycotoxins has recently been reviewed by the EU Scientific Committee on Food (335-340). Of direct relevance to all three crops (wheat, barley and maize) considered in this study are the trichothecenes (e.g. T-2 toxin, HT-2 toxin, deoxynivalenol and nivalenol) and zeralenone. Another group of *Fusarium* spp. mycotoxins of significance to man, the fumonisins, are associated with maize. Mycotoxins produced by the genus *Fusarium* are chemically diverse and elicit a diverse range of toxic effects. A number of these compounds have been shown to be involved in human toxicoses. The trichothecenes T-2 toxin and HT-2 toxin have been associated with outbreaks of alimentary toxic aleukia in the Soviet Union in the period 1941-47 (discussed in 336). Toxicoses with other mycotoxins have also been recorded. Deoxynivalenol and other trichothecenes have been implicated in food poisoning incidents in China, India and Japan (reviewed in 81). Zearalenone may have been a contributor to precocious pubertal changes in young children in Puerto Rico between 1978 and 1981 (discussed in 15) and more recently South East Hungary (362). Epidemiological evidence has correlated exposure to fumonisins (90, 401) and other *Fusarium* spp. mycotoxins (229) to the occurrence of oesophageal cancer, in certain parts of China,

where the incidence of the disease is abnormally high. In light of this and other evidence, fumonisins have been evaluated as being type 2b carcinogens - possibly carcinogenic to humans (174).

While no major human toxicoses attributable to *Fusarium* spp. mycotoxins have been reported within the EU, levels of contamination in both commodities and finished products in certain Member States has prompted cause for concern. One such example relates to the elevated levels of deoxynivalenol associated with the 1998 wheat harvest in some parts of the Western European mainland (163). As a consequence, some Member States have introduced monitoring and intervention programmes (354). Although levels of contamination subsequently decreased in harvests from following years, occasional product recalls from the public as a result of levels exceeding national limits for deoxynivalenol have occurred (e.g. 21).

In response to this problem, a number of papers have been produced to suggest strategies to control mycotoxins at the level of Good Agricultural Practice (20, 21, 24). It has also been proposed that subsequent developments should involve application of the HACCP approach to the control of mycotoxins (16, 21). Such an approach has already been used with regard to the question of ochratoxin A contamination in the UK cereal crop (11), and the concept has also been proposed and discussed in outline for *Fusarium* spp. mycotoxins (330).

3.0 FACTORS AFFECTING FUNGAL GROWTH AND MYCOTOXIN DEVELOPMENT

3.1 Laboratory Studies

Both fungal growth and mycotoxin production are governed by two environmental variables, temperature and water activity. Comerio *et al.* (96) looked at deoxynivalenol production in wheat maintained at different water activities (0.90 - 0.98) stored at 25°C over a 10 weeks period. The highest levels of mycotoxin production were found in wheat stored at a water activity of 0.98 and decreased with progressive reductions in water activity. No deoxynivalenol was found in samples stored at water activity of 0.90. Studies (282) using different substrates (oat, wheat or straw) and *F. sporotrichioides Sherb.* have shown mycotoxin production (T-2 toxin) to occur at water activities as low as 0.84-0.88.

Marin *et al.* (235, 238) have demonstrated that growth and mycotoxin production by *Fusarium* spp. (e.g. *F. proliferatum or F. moniliforme*) and fumonisin B_1 and B_2 production requires relatively high water activities (0.93 - 0.97). Further studies by this group (232, 234) have characterised fumonisin B_1 production over different water activities and temperature profiles. They have used these data to generate predictive models for the production of this mycotoxin by *F. moniliforme* and *F. prolifeatum.* Similar observations with regard to the requirement for high water activity levels have been made by Cahagnier *et al.* (65). They observed that reducing the water activity from 1.0 to 0.9, led to 20-fold reduction in fungal growth and 300-fold reduction in fumonisin production. Subsequent work by this group (247) looked at two isolates of *F. proliferatum* from maize grown

in France, which differed in their ability to produce fumonisin B_1. For both strains, the optimum temperature for mycotoxin production was 15°C, however, toxin production by the two strains differed at low temperature (10°C), with one strain producing almost the same amount as at 15°C, while the other, almost 1,000-fold less than the optimum.

The kinetics of fumonisin B_1 production in maize by *F. moniliforme* have been described by Alberts *et al.* (10). They observed that mycotoxin production began 2 weeks into the active growth phase of the mould and continued to increase during the stationary phase (achieved at 4 to 6 weeks). Mycotoxin levels reached a maximum at 13 weeks and then decreased. Other studies (39) looking at a range of *F. moniliforme* isolates found that optimum mycotoxin production took place at temperatures between 20° and 25°C, with no mycotoxin production occurring at a lower range of 5-10°C, nor at a higher range of 30-35°C.

Laboratory studies have also shown that the composition of the mycoflora can affect both *Fusarium* spp. growth and mycotoxin productions. Marin *et al.* (236) investigated the effect of water activity and temperature on the colonization of maize grain by isolates of *F. moniliforme* and *F. proliferatum* with different spoilage fungi (*Aspergillus* spp. and *Penicillium* spp.). At low water activities (0.93 or 0.95) mutual inhibition on contact or overgrowth by the spoilage fungi was observed, however, this was not accompanied by a decrease in fumonisin production. At higher water activities (0.98), interaction with *Aspergillus* spp. led to stimulation of fumonisin production by both *Fusarium* species, while interaction with *Penicillium implicatum,* led to a reduction in mycotoxin production by *F. moniliforme*. This same group (381) also investigated the effects of competition between a fumonisin producing isolate of either *F. moniliforme* or *F. proliferatum* and an isolate of *F. graminearum* that produced zearalenone. Under the conditions studied (water activities, 0.98, 0.95, 0.93 and temperatures, 15 or 25°C), populations of the fumonisin B_1 producing strains were reduced in the presence of a zearalenone producing strain of *F. graminearum.* In addition, while zearalenone production was not affected under any of the conditions studied, the presence of *F. graminearum* inhibited fumonisin B_1 production by *F. proliferatum.* In the case of the *F. moniliforme* strain, mycotoxin production was inhibited at 15°C but enhanced at 25°C.

Studies have also been performed looking at T-2 toxin produced by *F. sporotrichioides*, using barley infected with *Aspergillus flavus, Penicillium verrucosum or Hyphopichia burtonii* as substrates (315). While the presence of these fungi did not inhibit germination of the *Fusarium,* subsequent colonisation was inhibited at all of the conditions studied. T-2 toxin production only took place at 20°C, at water activity values of 0.97 or 0.95 and mostly during the first 7 days. Toxin production was significantly greater when *F. sporotrichioides* was grown in the presence of *P. verrucosum* and *A. flavus* and only slightly lower in the presence of *H. burtonii,* despite an apparent lack of *F. sporotrichioides* growth.

Zearalenone production in a number of different *Fusarium* species (*F. graminearum, F. culmorum* and *F. oxysporum*) has been studied *in vitro* (187, 260). Optimum production was found at a water activity of 0.97 and various temperatures at or below 30°C. Little or no mycotoxin production was seen when cultures were incubated between 12 -14°C or at 37°C. Similar studies (269) looking at

zearalenone production by *F. graminearum* indicated that the optimum water activity for mycotoxin production was at least 0.97, while no toxin production was observed at a water activity of 0.90.

3.2 Field Studies

3.2.1 Wheat & Barley

The general process by which *Fusarium* spp. infection takes place has been reviewed by Smith *et al.*, (353) and further characterised in more recent studies (140, 297).

In general, *Fusarium* ear-blight epidemics appear to be linked with multiple inoculation events with coincident wet periods (143). Surface wetness for 48-60 hrs is also required for substantial infection. In broad outline, *Fusarium* spp. are harboured within plant debris in the soil (396) where ascophores and macroconidia form the resistant stage for survival. The ascophores are dispersed mainly at night, requiring a relative humidity of 95-100% and ambient temperatures in the range of 11°-23°C. Typically, peak ascosphore release occurs two to four days after major rainfall. High rainfall during anthesis (flower opening) and kernel development has been associated with both elevated incidences of *Fusarium* spp. linked diseases and mycotoxin (e.g. deoxynivalenol) production (5). Laboratory experiments (182) involving simulations of rain showers suggest that maximum splash heights for the transmission of *F. culmorum* and *F. avenaceum* were 60 and 45cm in wheat at maximum horizontal distances of >100 and 90 cm respectively from the source. For the disease to progress to epidemic proportions, it is usually necessary for relative humidities to remain high sometime after anthesis. This enables fungus on the ears to sporulate and infect neighbouring plants as well as late flowering ones (52).

3.2.2 Maize

As in the case of wheat and barley, optimum weather conditions for the release of fungal spores must coincide with the time that the maize crop is at its most receptive. Optimum temperatures for infection are between 25° and 32°C (170). Epidemics of fusarial diseases are usually completed within one reproductive cycle, since the corn ears are only receptive for about 10 to 20 days after silking. Spore dispersal is favoured by rain, which promote splashing and/or are wind driven. These climatic conditions plus persistent wetness of the ear (>48-60 hours) promote infection (358). In the case of *F. graminearum*, maize appears to be susceptible to airborne infection for between 7 and 10 days after silking has commenced. The inoculum can be conidia or ascospores, chlamidospores and hyphal fragments. These originate from the previous crop debris left on or near the soil surface (195).

With regard to *F. moniliforme* infections, the situation is more complicated. Early work (376) demonstrated that diseases associated with fungus were affected by moisture during silk emergence and were favoured by wet weather later in the season. Experimental studies with *F. moniliforme* (197) observed that its infection of maize kernels began approximately 2 weeks after mid silk and

then increased progressively. Subsequent studies (162) have indicated that the browning and senescence of silks appeared to be important in initiating infection. *F. moniliforme* is something of an enigma, since it can sometimes exist systemically within the maize plant without showing any disease symptoms (142), leading to the suggestion that not all strains of this species are pathogenic (45, 46).

B GRAIN PRODUCTION (SEED TO PRIMARY PROCESSOR)

Author's note: stage numbers referred to in the text, cross reference to those quoted in the flow diagram (Figure 3) used for the hazard/risk analyses described in the main body of the report

1.0 FARM - FIELD (STAGES 1 -4)

1.1 Field Preparation (Stage 1)

1.1.1 Geography

Fusarium spp. are usually a normal constituent of the soil mycoflora. However, the numbers (both in absolute and relative terms) together with the genotypes and phenotypes present in the soil differ with location. Studies in North America, performed over a number of growth seasons, have demonstrated geography not only to influence the nature of the species and frequency of occurrence of *Fusarium* organisms (93, 227), but also the amounts and types of mycotoxin produced (383, 390). Matters are made more complicated in that the proportion of the crop contaminated with *Fusarium* spp. toxins varies from year to year (341, 373, 374). A similar phenomenon has been observed in other parts of the world e.g. South Africa (199, 379) and Brazil (144). Parallel studies have also been performed in Europe, including, Bavaria (218), United Kingdom (309), Russia (206, 403), Czech Republic (179), the Netherlands (281), Norway (208) and Poland (153, 222) - all with similar results. The situation is further complicated, in that, at least one study (257) has shown that considerable amount (about 60%) of the genetic variation associated with *Fusarium* spp. virulence takes place within the individual field population.

Bottalico (60) has reviewed the production of *Fusarium* produced mycotoxins in European cereals. While the most frequently encountered mycotoxins were deoxynivalenol and zearalenone, regional variations between warmer and cooler climates and between central Europe and the maritime regions were also noted. For example the major organism producing deoxynivalenol and zearalenone in Southern Europe was *F. graminearum*, while *F. culmorum* predominated in the north of the continent. Subsequently a survey of the Italian mycoflora was published (36). In all, 16 species of *Fusarium* were found. While *F. oxysporum, F. equiseti, F. solani* and *F. compactum* were the most frequently occurring species found, *F. oxysporum* was predominantly found in Northern and Central Italy, while *F. equiseti* was mainly found in central and southern Italy. Paradoxically a French study (35) appeared to show that when looking at one particular fungal species (in this case *F. culmorum*), although the species could be divided into two chemotypes, based on the types of mycotoxins they produced, there was no geographical determinant in their distribution.

The consequences of differences in geographical distribution can have a direct influence as to whether or not they provide a mycotoxin hazard. For instance studies on the *Fusarium* spp. mycoflora found in Sicilian cereals (101) demonstrated that while mycotoxigenic *Fusarium* spp. were present, the predominant species isolated (*F. moniliforme*) did not produce any of the

mycotoxins studied (including deoxynivalenol and zearalenone). Defining a precise geographical area, in order to focus resources, can be difficult given the geographical variations in both Fusarium infection and mycotoxin production. These phenomena can operate at quite a low level. For example studies in Bavaria (218) have observed regional differences with highest levels of deoxynivalenol contamination being found in Middle and Lower Franconia. In contrast Gilbert *et al.* (148) found no geographic trend with regard to the UK barley crop.

The effects of geographical factors can change with time. For example de Nijs *et al.* (109) compared the wheat harvest in north and south Netherlands in 1991 with that of 1993. Although total fungal loading in the two crops was similar, the number of *Fusarium* spp. contaminated samples changed. In 1991, 34% of samples (all from the north of the country) were contaminated with *Fusarium* spp., while in 1993, 83% (from both north and south) were found to be contaminated. Analysis of the 1993 crop revealed 3% of cereal samples to contain deoxynivalenol and zearalenone at significant levels.

A key component of the contribution made to the role played by geography is climate. Climatic conditions play a role not only in the genotypes of *Fusarium* spp. present in the soil, but also in the infective process and subsequent events. Given this significance, the subject of climate is dealt in its entirety in section 1.3.1 below. In addition to climate another geographical factor that needs to be considered is the geology of the location and its effects on plant health. Thus the mineral content of the soil may also be a contributory factor to the ease with which *Fusarium* spp. can infect cereals. It has been demonstrated that zinc deficiency in the soil contributes an increased risk of *Fusarium* spp. infection. This risk can be reduced by addition of zinc based soil fertilisers or the use of crop varieties more efficient in their uptake of zinc (155, 355)

1.1.2 Ground Preparation

Certain types of ground preparation and crop rotation appear to affect the incidence of *Fusarium* spp. disease (166). As discussed previously (Part A, sections 3.2.1, 3.2.2), crop residues from the previous harvest have the potential of acting as sources on inoculum. The amount of *Fusarium* spp. spores being produced in the spring being inversely proportional to the degree of decomposition of the plant debris present (44). *Fusarium* spp. linked wheat ear blight is also favored by increased amounts of surface residue from previous crops. Plant debris can therefore act as a long-term reservoir of infection by *Fusarium* spp., this ability being dependent on the original plant source and its location (143, 149, 157, 316).

Debris management is therefore an element of the system that has to be controlled. It has been known for some time, that stubble management techniques contribute not only to the efficiency of *Fusarium* spp. infection but also to its route (99, 357). In particular, the importance of stubble as the source of the field inoculum has been demonstrated. American studies have shown that maize stalks still left standing after harvest in October were heavily contaminated with toxigenic *Fusarium* spp. (267). Studies (99) with plant debris inoculated with spores of *Fusarium moniliforme, F. proliferatum,* or *F. subglutinans,* have demonstrated that the fungi can persist for considerable

periods of time, in excess of two growing seasons. In the long term (630 days), crop rotation and burial of the debris in the soil led to the lowest survival rates for the fungi. Overall fungal survival decreased more slowly in the surface residues than in the buried ones.

Maize residues in particular, take longer to decompose than those from other crops and provide a suitable environment for *F. graminearum* to survive over the winter (44). The advent of late varieties and their cultivation in cooler climes appears to be a significant confounding factor in the incidence of fusarial infections. As in North America (94) there is an increasing preponderance of *F. graminearum* over *F. culmorum* in Northern Europe (94, 319), accompanied by an increased incidence of *Fusarium* ear blight (246). This has been associated, at least in part, with the inclusion of maize within the crop rotation and the adoption of no tillage systems (245, 319). Plant debris management also includes weed control. There is some evidence that certain plants, whilst not exhibiting any symptoms themselves, can act as hosts for pathogenic *Fusarium* spp. (139, 181, 239).

Introduction of no-tillage systems to combat soil erosion (246) appear to exacerbate the situation with regard to the amount of plant residue remaining on the surface. Moldboard ploughing has been shown to lower incidences of *Fusarium* ear blight and deoxynivalenol contamination (116). Stubble management is therefore important and extends beyond tillage practices. For example, studies in Austria (216) have shown that the practice of stubble burning may actually favour zearalenone formation in the subsequent year's crop. Later studies by others (352) have shown that stubble burning can promote *Fusarium* ear blight.

In countries with the potential of a long growing season and where winter varieties of wheat are sown (e.g. Italy), a common practice is to plough the soil some time after harvest and allow it to remain fallow under the autumn sun before sowing the following year's crop. This practice ('solarization') has been shown to reduce *Fusarium* spp. loading in the soil and reduce the incidence of Fusariosis and mycotoxin production in maize. Ahmad *et al* (8), studying the maize crop, reported that exposing ploughed irrigated soil to sunlight for seven weeks led to substantial reductions in the incidence of: *F. moniliforme, Macrophomina phaseolina and F. graminearum.* The degree to which solarization was effective was determined in part by the variety of maize grown.

Ground fertiliser treatments can also contribute to the incidence of the *Fusarium* ear blight and by inference mycotoxin contamination. The incidence of disease was less where nitrogen was added in an organic (e.g. pea vines or manure) rather than inorganic form (352). In contrast Bateman and Coskun (40) observed that propagule numbers of *F. culmorum* were highest during July in fields treated with manure. This information is consistent with other observations in northern Italy. Here it has been observed that in the organic wheat market, a switch from traditional urea to manure as a field nitrogen source has led to increased incidences of Fusariosis in the crop (58). The efficacy of urea in the management of *Fusarium* spp. diseases was also noted by Teich *et al.* (368) who observed that wheat fertilized with urea exhibited less ear blight than crops treated with ammonium nitrate.

1.1.3 Crop Rotation

Crop rotation also plays a role in either increasing or reducing the risk of *Fusarium* spp. infection. From section 1.1.2, it is evident that certain types of plant debris are better substrates for *Fusarium* spp. Reduction of infection is associated with those rotational systems which break the infective cycle (223). Rotations involving legumes (116, 196, 308, 352), *Brassicas* (198, 203), or leaving the field fallow (207), have all been shown to lead to reduced disease incidence. In the case of *Brassicas*, it has been suggested that the suppressive effects on *Fusarium* spp. may be mediated by the isothiocyanate compounds produced by such plants (198). There is evidence to suggest that such rotations may also (as a consequence of reducing the incidence of fusarial diseases?) lead to reduced mycotoxin production (116, 203)

There are also crop-rotation systems that favour increased infection. Teich and Hamilton (367) working in Ontario, Canada observed that both the incidence of ear blight and production of deoxynivalenol were higher in wheat which was rotated with maize as opposed to soybeans, barley, and mixed grains where no such increase was observed. Other factors considered (e.g. fertiliser regime, disease, cultivar) did not appear to contribute an effect. A subsequent Canadian study (92), this time in Manitoba, also identified a wheat/maize rotation as favouring the incidence of head blight and mycotoxin contamination. Similar observations have been reported elsewhere in North America (116). Others (33) have demonstrated that crop rotation becomes increasingly important when no-tillage systems are used. One particular mechanism by which this occurs, is the observation that experimentally at least, certain crops for example wheat, actually favour a change in the soil *Fusarium* spp. population (128).

Studies in Europe such as those by Plaskowska (308) have confirmed the results of the studies in North America. In Plaskowska's studies, field bean was a beneficial fore-crop with regard to risk of *Fusarium* spp. infection, while spring barley was the worst. Similar results were obtained in Switzerland with winter wheat (203). In the Swiss studies, the highest incidence of *Fusarium* infection was obtained in crops grown in fields where the preceding crops had been either maize or wheat and no tillage had been used. Ploughing or digging the field with a cultivator led to reductions in deoxynivalenol concentrations of 79% and 47% respectively. In fields where no tillage was used, introduction of rape as the prior rotational crop led to a reduction of 90% in deoxynivalenol concentrations.

1.2 Sow Seed (Stage 2)

1.2.1 Crop density

As discussed in Part A section 3.2.1, a key factor contributing to epidemic spread of the *Fusarium* fungi is the spread of spores from plants already infected by the disease (93), particularly under humid conditions (160). It has therefore been recommended that seeding rates should be no higher than that for optimal yield (160).

1.2.2 Species and Variety.

Both the species and variety of the crop sown are contributory factors in determining which particular fungal organism will be responsible for any pathogenesis (66, 67, 241, 256, 380, 397). Laboratory studies in Canada, working with spring varieties of cereals (100), showed significant differences between cultivars. This was more marked in wheat than in barley. It was also demonstrated that there was a negative correlation between plant height and seed infection. Moreover, hard wheat cultivars were shown to be more resistant than the soft wheat varieties, as too, cultivars of malting barley compared with feed varieties. Intra-species variation has also been shown in the field. Work such as that by Celletti *et al.* (77) has demonstrated that in the same geographical area (Prince Edward Island, Canada), infection of winter wheat ears was often effected by *F. avenaceum* and *F. sambucinum* as opposed to *Bipolaris sorokiniana* and *F. graminearum* in barley. They also observed that soil populations *F. sambucinum* were higher in fields where wheat as opposed to barley was grown.

Generally speaking, while trichothecene mycotoxins have been found in all three cereals considered in this study, contamination by fumonisin mycotoxins of field grown commodities appeared to be restricted to maize. Studies in Spain (71) however, have demonstrated that these mycotoxins can be found in other commodities (e.g. wheat and barley) albeit at low levels. This observation may be in part be explained by laboratory studies. In one study (385), it was observed that *F. moniliforme* isolates from maize produced higher quantities of the toxin than those isolated from wheat and barley, which in turn produced more than those isolated from sorghum. Other studies (235) using *F. moniliforme* stains isolated from maize revealed that while these strains grew well in culture on wheat, barley and maize, it was only possible to demonstrate mycotoxin production, when the strains were grown on maize.

Wheat

At an agronomic level, cultivar has long been shown to play an important role. For example in a study published by Clear and Patrick (93), the lowest frequency of *Fusarium* spp. in wheat was found in the Canadian Western Red Spring class of wheats, and the highest in the Canadian Western Amber Durum class. The additional sensitivity of some varieties of Durum wheat has also been seen in Europe. Cvirn *et al.*, (102), based in Austria, noted that durum wheat (*Triticum durum*) was more susceptible to deoxynivalenol contamination than standard wheat (*Triticum aestivi*).

Studies in Germany (43, 218, 218) and Switzerland (185) have provided some evidence to correlate cultivar with mycotoxin production. In the Swiss study (185), it was observed in field trials with *F. culmorum* and *F. graminearum* that deoxynivalenol concentrations tended to be higher in late as opposed to early varieties of wheat. In addition, studies with varieties used in Argentina (226) suggest that one of the routes that cultivar affects susceptibility to *Fusarium* spp. disease may be in respect of the way a particular cultivar responds to particular environmental phenomena. Pan-European ring trials with winter wheat varieties performed by Ruckenbauer *et al* (322) in the UK, the Netherlands, Austria and Germany and using experimental inoculations with *Fusarium* spp. (e.g.

F. culmorum) have shown considerable cultivar variation in terms of disease susceptibility and the amount of mycotoxin (deoxynivalenol) produced. Interestingly, while geographic differences in the incidence of disease and levels of deoxynivalenol were seen, the over overall ranking of the varieties was consistent over the four locations.

Given the commercial importance of *Fusarium* spp. linked diseases, much attention has been paid to the breeding of resistant wheat strains both at fundamental genetic (185, 254) and traditional breeding (110, 185) levels. Techniques have been developed to help plant breeders to screen early on in the breeding process for *Fusarium* spp. resistant varieties (114, 180, 248, 250, 251, 258, 365) and can include screening for increased resistance to deoxynivalenol (399). The relative success of breeding programmes has been limited due to difficulties in understanding the genetics of resistance (389). However, there has been some success in determining the location of some of the genes involved in resistance to *Fusarium* spp. infection in spring (350) and winter (175) wheats. Additionally there has been progress in gaining some insight into the genetics of resistance at a molecular level (29, 268). The use of these and other conventional breeding programmes have led to some progress not only towards developing *Fusarium* spp. resistant crops (9, 31, 154) but also in identifying resistant wheat varieties which do not appear to promote deoxynivalenol accumulation (30). Modern developments in molecular biology have also been used to produce transgenic crops containing genes from other cereal species, e.g. rice (86, 268) which confer increased resistance to *Fusarium* spp. linked infection.

It has been observed that in some cases *Fusarium* spp. mycotoxins are also toxic to some plants (1, 3, 27). Earlier, Chelkowski and Manka, (83) had noted that in the case of zearalenone contamination effected by *F. culmorum,* the more pathogenic the fungi, the more toxin produced. Studies looking at the molecular mechanisms of resistance, such as those by Miller *et al* (263) and Savard (327) have demonstrated that resistance to the pathogenic effects of experimental infection with some *Fusarium* spp. can sometimes be linked with mechanisms that either prevent synthesis and/or promote degradation of deoxynivalenol. Recent work (312) has shown that genetic manipulation, leading to the disruption of the trichothecene biosynthetic pathway can lead to reduced virulence in *G. zeae.* Additional mechanisms of resistance have also been identified with respect to deoxynivalenol, and include cultivars that produce an altered peptidyl transferase, resistant to the toxic effects of deoxynivalenol (262, 327).

Other defence mechanisms against *Fusarium* spp. include the possession of antifungal properties (70, 119). However, it is clear that mycotoxin (deoxynivalenol) toxicity is not the only route by which *Fusarium* spp. exerts its pathogenic effects on plants. Experimental work by Procter *et al.* (311) and Arsenuik (25) suggests that generally the system regulating deoxynivalenol accumulation is different to that eliciting the disease symptoms.

Barley

Experimental studies by Chelkowski's group (6, 85), investigating the susceptibility of barley double haploids to *Fusarium* headblight using both double and six-rowed varieties, and Evans *et al.* (136)

in commercial varieties, have demonstrated that variety itself can be a contributory factor in the incidence of mycotoxin (deoxynivalenol) contamination. One confounding factor in developing resistant strains is that performance is strongly influenced by the environmental conditions in the year the crop is harvested (136).

Breeding programmes to improve resistance against *Fusarium* linked diseases with barley have also proved difficult (107). Methods for screening new varieties more resistant to *Fusarium* spp. infection have been described (364) and quantitative trait loci (QTL) associated with disease resistance and resistance to deoxynivalenol accumulation have been identified (107, 230, 404). One possible factor influencing the development of *Fusarium* spp. infection is the amount of flavonoid synthesis in the testa. Skadhauge *et al.* (351) observed that mutations in the flavonoid biosynthetic pathway had the potential to either enhance or diminish the ability of the fungus to successfully infect the kernel. In the latter case this appeared to be related to the ability of a particular mutant to accumulate small amounts of dihydroquercetin. The authors however presented no data on the consequences that this had on mycotoxin production.

Maize

Cultivar plays an important role in the susceptibility of maize to *Fusarium* spp. linked diseases. Late developing varieties, whose moisture content reduces to below 30% relatively slowly, are the most susceptible to *F. graminearum* infection (231). These varieties may give higher yields or permit their cultivation in more northern latitudes; however, the increased risk of adverse summer or autumn conditions may also encourage higher incidences of fungal infection and consequent elevated levels of mycotoxin contamination (52). *F. graminearum* infection is also thought to be favoured in hybrids which produce upright ears and tight husks (132). Hybrids with a greater risk of kernel splitting also appear to an increased risk of disease (286). Variety may also dictate in part the ability of *Fusarium* spp. to survive in store after harvest. Orsi *et al* (289) observed that post-harvest production of fumonisins was lower in some hybrids compared to others.

Strategies for developing resistant varieties have recently been discussed by Duvik (124) and more specifically regarding mycotoxin production by Miller (261). Breeding resistant varieties of maize has been a principal means of reducing *Fusarium* spp. based infection (106, 108, 146, 161, 162, 176, 202, 252). Methods for screening resistant hybrids have been described (293) and QTL's for resistance to infection have been mapped (298). In some cases, the mechanism of resistance is through the plant producing antifungal agents which can either be relatively small (approximately 4kDalton) peptides (127) or specific enzymes (e.g. chitinase) (173). Another route is to breed hybrids with increased resistance to husk splitting. This, together with intra-ear thirps infestation has been identified as a factor which contributes to the incidence of *Fusarium* induced disease in maize (391).

A second approach has been to develop maize varieties more resistant to the mycotoxins, in particular fumonisins. Doko *et al.* (117), looked at the natural fumonisin contamination of maize grown in a number of European and African countries. They observed that there was a trend towards

higher contamination in maize genotypes with higher FAO maturity class or dent type endosperm. Breeding varieties, which are not only disease resistant, but also give reduced risk of substantial mycotoxin contamination, has proved more difficult. For example in one study (294) only one out of fourteen hybrids studied exhibited all of the desired characteristics. More recently, possible strategies for breeding maize with reduced risk of fumonisin contamination have also been described (125). These involve the use of molecular biology and genetic manipulation (GM). Among such approaches proposed is the cloning of genes expressing fumonisin degrading enzymes from other sources into maize (126).

Physical damage to the developing maize crop has also been shown to promote fungal infection. Early studies (360) have shown that transmission of *F. graminearum* in maize could be increased as a consequence of physical damage, mediated by either birds or insects. They further demonstrated a positive correlation between the amount of bird damage and the level of zearalenone contamination. Gilbertson *et al.* (150) observed higher levels of fungal infection in crops infested with the western corn rootworm beetle (*Diabrotica virgifera*) than those which were not. They also demonstrated that the beetle was a vector for *F. moniliforme* and *F. subglutinans* infection. Other studies (221) have shown a positive correlation between the incidence of European core borer damage and *F. moniliforme* disease, together with fumonisin contamination. Development of genetically modified (GM) maize varieties which are more resistant to insect damage (e.g. those producing the *Bacillus thuringiensis* Cry IA(b) gene product) have also been observed, in some cases, not only to be more resistant to *Fusarium* spp. infection (120, 275) but also, on occasions to exhibit less fumonisin contamination (120, 274). In one case (34), it was reported that the fungal biomass recovered from transgenic maize crops was 4-18x lower than in conventional crops. This was accompanied by an order of magnitude reduction in the levels of fumonisin B_1 contamination.

1.2.3 Seed Treatment.

The use of bacterial control agents as seed dressings has been shown to have potential benefit in preventing *Fusarium* spp. infections. Pre-treatment of seeds with various microbiological preparations has been shown to significantly reduce disease caused by *Fusarium* spp. Examples include *Erwinia herbicola* against *F. culmorum* (194) and *E. herbicola* together with *Pseudomonas syringiae* against *F. nivale* (7) - all in wheat. Other examples include: *Enterobacter cloacae* (167) against *F. monoliforme* in maize; *Gliocladium roseum* against *F. culmorum* in wheat and barley (200), *Streptomyces griseoviridis* in barley and spring wheat, naturally infected with *Fusarium* spp. (363), *Clonostachys rosea* against *F. culmorum* (186) and attenuated *Fusarium* spp. strains against *F. culmorum* in wheat (304).

Attempts have also been made to develop chemical treatments. Early studies with maize (217) showed that fungicide (furadan, captan and benlate) pre-treatment of seed failed to have any effect in the control of ear rot. However, pre-treatment of seed with certain chemical pesticides in wheat and barley, e.g. maneb, carbathiin and triadimenol (76) or thiabendazole (188), can afford some protection against *F. graminearum* infection. Interactions between treatments and subsequent *Fusarium* spp. infection may be more indirect. For example, failure to dress seed to prevent 'Bunt

Smut' caused by *Tilletia caries* was observed to lead to an increased incidence of this disease, followed by increased infection by *Fusarium* spp. (388). However, the relevance of this to mycotoxin production in the grain is uncertain. As discussed in Part A section 3.2.1, the mechanism for infection relating to mycotoxin production appears to be by a splash mechanism, rather than direct infection through the seed. There may, however, be an indirect benefit in that reduced root infection contributes to the general vigour of the plant, rendering it more resistant to *Fusarium* ear blight (see also Part A section 2.1 and section 1.3 below).

1.3 Crop Development & Maturity (Stages 3 & 4)

1.3.1 Climate

Climate plays a key role in the incidence of mycotoxin contamination, through its effects in three areas:

- Composition of natural mycoflora and its cyclical development through the seasons (Mycoflora);
- Capacity of the mould to successfully infect the crop (Infection);
- Ability of the infective inoculum to grow and eventually produce mycotoxins (Mycotoxin Production).

As will be discussed below, climate has been identified as a factor that contributes to determining the make up of the soil mycoflora and hence the composition of the *Fusarium* spp. based soil population. Climate makes a significant contribution to the occurrence of mycotoxins in grain. Lew (220) has observed that in central Europe, where grain (with the exception of maize) is usually harvested at moisture contents less than 16%, field mycotoxins, in particular those produced by *Fusarium* spp. are of greater relevance than storage mycotoxins (e.g. ochratoxin A).

Mycoflora

The geographical variation in the incidence of *Fusarium* spp. has already been broadly discussed in Part A, section 2.1 and section 1.1.1 of this section. What follows, addresses in more detail the role of climatic factors themselves.

Studies in Australia (64) looking at *F. crookwellense* demonstrated the significance of climatic zone by showing that the fungus was generally more abundant in temperate areas experiencing high rainfall or in irrigated areas. Another factor to be considered is the season of the year. It has been shown both in the UK (40) and Egypt (271) that the *Fusarium* spp. population varies with the time of year. In the UK it was observed that the greatest preponderance was in July, as opposed to February or April (40). Atypical climatic conditions can also contribute to sudden changes in the mycoflora. For example Perkowski *et al.* (301) observed that following a long snowy winter, *F. avenaceum* was replaced by *M. nivale* as the dominant species in head-blight infected crops of

triticale. Similarly, work in China (138) has shown marked differences in contamination with deoxynivalenol and zearalenone between the 1998 and 1999 harvests. These differences were attributable to a combination of unusual winter weather which permitted larger numbers of *Fusarium* spp. to survive, followed by heavy rains during anthesis.

As already discussed above, climate may also be a factor contributing to the diversity and numbers of species occurring. Experimental work (325) has demonstrated that that the propagule density and infectivity of certain *Fusarium* spp. is a function of temperature. High soil temperatures (25-30°C) favoured *F. solani* and *F. compactum*, lower soil temperatures (13-18°C) favoured *F. torulosum.* Temperature did not appear to affect the viability and infectivity of *F. equiseti*. Work by another group (59), compared the pathogenicity of *F. graminearum* and *F. crookwellense* in wheat. They showed that although the route of infection was the same for both species, *F. graminearum* was more pathogenic at higher temperatures (22-24.6°C) than *F. crookwellense*, with the later exhibiting greater pathogenicity at lower temperatures (13.8°C).

Infection

The mechanism by which infection takes place and the general climatic conditions necessary for successful establishment of the mould has been discussed previously (see Part A, section 3.2.1). There is evidence to indicate that wheat seedlings when placed under drought-induced stress are more prone to infection and the development of both root (47) and ear diseases (69). Managing the risk of drought is therefore important. Early studies in the USA (356) reported that ear blight was most common in irrigated as opposed to dry-land fields. However, Mihuta-Grimm and Forster (259) reported on an outbreak of *Fusarium* spp. induced ear-blight in wheat and barley following prolonged rainy and cloudy weather in normally semi-arid areas of Idaho. This epidemic was associated with farms where sprinkler as opposed to rill irrigation was used. Since high moisture at anthesis plays a key role in infection (see Part A, section 3.2.1), it has been recommended (52) that irrigation should not coincide with heading and anthesis. However, it should be noted that climatic extremes of any nature can have a deleterious effect and promote mycotoxin accumulation. An example of this being the elevated occurrence of deoxynivalenol in US cereal-based foods produced in 1989, following a major drought while the crop was being cultivated (4).

There is thus a clear linkage between climate, *Fusarium* spp. infection and subsequent mycotoxin production. While computer models have been developed using climatic variables as predictors of *Fusarium* spp. disease for particular areas (270), the utility of weather reports for particular growing areas as an aid to predict whether in-field mycotoxin contamination has occurred is at best an imprecise science (171).

Mycotoxin Production

The role of climate in the development of *Fusarium*-linked mycotoxins is complicated by the interdependence of three factors: climate; causative organism and crop. In the case of small grain cereals (e.g. wheat and barley), contamination with the trichothecenes such as deoxynivalenol

occurs following the growth of (principally) *F. graminearum* and *F. culmorum* during prolonged cool, wet growing and harvest seasons (306). Langseth *et al.* (209) looking at the incidence of deoxynivalenol in oats crops harvested over the five year period in different parts of Norway, determined that while rainfall was a significant risk factor with regards to deoxynivalenol contamination, moisture content at harvest was not. A more detailed analysis (208) determined a positive statistical correlation between rainfall in July (when anthesis takes place) and levels of subsequent deoxynivalenol contamination. Early studies looking at zearalenone contamination of maize by *F. graminearum* in Canada (359), showed that the incidence of zearalenone contaminated maize significantly correlated with rainfall during August, probably because rainfall promoted epidemic development of the infecting mycotoxigenic fungi.

Wheat & Barley

By the time the crop has reached maturity, the moisture content of the kernel is usually sufficiently low not to favour further mycotoxin production. Studies such as those of Prom *et al.* (314) with barley, have shown that *Fusarium* activity (both in terms of disease and mycotoxin (deoxynivalenol) production) takes place during the early stages of grain development. However, exceptions have been noted. Workers in Russia (205) have observed reactivation of *F. graminearum* due to precipitation during harvest accompanied by production of deoxynivalenol. Researchers in Norway (212) have demonstrated that, where kernel moisture is high, mycotoxin production can continue post-harvest until the grain is adequately dried. Experimental field studies by the same group (211) with barley and oats, have shown that the deoxynivalenol contamination of grain at harvest was influenced in descending order by: lodging, cultivar, experimental field, year and time of harvest.

Over-wintering of grain, in areas where weather (or other circumstances) does not permit an autumn harvest, also has a contributory role. Over-wintering was the cause of an outbreak of alimentary toxic aleukia of epidemic proportions in Western Siberia during the second World War. It was caused principally by the toxic metabolites of *F. sporotrichioides* and *F. poae*, following a mild winter with heavy snow, accompanied by a subsequent spring characterised by repeated freezing and thawing (378). More recently Langseth *et al* (210) observed that concentrations of deoxynivalenol in grain were quantitatively lower and that *F. avenaceum* was more common in over-wintered crops as opposed to crops harvested in autumn. Paradoxically, preparations from over-wintered crops were more cytotoxic in *in vitro* tests. There was a positive correlation between the incidence of *F. avenaceum* and cytotoxicity.

Maize

As with the small grain crops, climate can directly influence the composition of the mycoflora present on the maize kernel and its consequences for mycotoxin production. Reid *et al.* (318) working in Canada and looking at maize harvests over a 4 year period, concluded that temperature and rainfall during July and August were key factors in the incidence and levels of deoxynivalenol contamination. Subsequent work (317) looked at the interaction between *F. graminearum* and *F. moniliforme* in artificially inoculated maize. They found that fumonisin B_1 production by *F.*

moniliforme did not differ if using a single or mixed inoculation. They also observed that silk temperatures favoured the growth of *F. moniliforme*. This was attributed to the observation that whereas *F. graminearum* was able to grow well between 26 to 28°C, *F. moniliforme* grew well over a wider range of temperatures. Fumonisin production also appears to be related to the degree of stress. Production of fumonisins by *F. moniliforme* and *F. proliferatum* in maize appears to be promoted by stress induced by heat or drought (346).

As the maize kernels develop, their moisture content decreases from about 80% to between 15 and 30%. Mould growth is favoured by moisture contents in excess of 30%; however, it can occur to a limited extent at moisture contents as low as 20% in ears on the cob (52). Studies in Argentine maize (91), looking at the ripening cob from 45 days after flowering, found that fumonisin production only took place over 60 days after flowering. Laboratory studies in the USA (392) suggest that the growth phase of the kernel may also play role, by virtue of the amount and types of nutrient available. Evidence for this was the observation of a transition in the *Fusarium* spp. population with time, with the non-toxin producing *F. subglutans* being replaced by toxigenic *F. moniliforme* and *F. proliferatum* strains. Other studies in Canada (402) looking at experimental infection of maize ears with *F. graminearum*, revealed that while deoxynivalenol concentrations rose to a peak after about 6 weeks, zearalenone was only recorded near harvest time. A subsequent study (276) suggested that production of this mycotoxin takes place late in the growth phase of the mould, which is typical for secondary metabolites.

1.3.2. Crop Treatments

Various factors contribute to the risk of *Fusarium* spp. infection and subsequent mycotoxin production. One such example is the plant's disease status. For example Koch and Huth (201) demonstrated that infection with barley yellow dwarf virus could increase the sensitivity of wheat to successful *F. culmorum* infection. In order to help maintain a healthy crop therefore, one or a combination of five groups of compounds (fertilisers, growth modifiers, fungicides, herbicides and insecticides) might have be administered to it during its growth and development. Administration of these compounds can influence not only the plant's development but also the ability of the mould to develop and produce mycotoxins.

Fungicides

Evidence that pesticides, in particular fungicides, have an effect on *Fusarium* spp. infection and mycotoxin production is confusing. One group working with malting barley has even reported that pesticide treatment regimes had no significant effect on the composition of the mycoflora present on the kernel (12). Even when optimally applied, fungicides appear to be only 60-70% effective in controlling *Fusarium* ear blight (183).

Despite the confusion in the literature, fungicides probably have the most direct effect. Choice of fungicide is important not only in terms of the compound's action against *Fusarium* spp. (discussed below), but also against other fungal species with which the *Fusarium* strains must compete to

establish themselves within the local environment. For example, Arseniuk *et al.* (26) noted that foliar fungicides developed for *Stagnospora nodorum* infections in wheat were not effective against *Fusarium* spp. and could even enhance infestation by this group of fungi. In terms of *Fusarium* ear blight, timing of application (137, 172) is also important for successful control (discussed further below).

A number of fungicides, for example tebuconazole, have been shown to be efficient in the management of *Fusarium* spp. mediated plant diseases (137, 387). While tebuconazole has been shown to be effective against members of the *Fusarium* genus, it is ineffective against *M. nivale* (184). In contrast use of strobilurin based fungicides controls *M. nivale* but not *Fusarium* spp. (349). With regard to mycotoxin production the situation is more complex. It has been noted that application of some fungicides (including tebuconazole) can actually stimulate increased toxin production for example nivalenol production by *F. culmorum* in wheat (145). Studies with winter wheat (62, 87) incubated with *F. graminearum* have demonstrated that while fungicides such as triadimefon not only suppress pathogen growth but also deoxynivalenol production, application of tebuconazole led to increased contamination with the mycotoxin 3-acetyldeoxynivalenol. The situation regarding tebuconazole is made even more complicated in the light of the work by a number of other workers (168, 189) who have reported tebuconazole to be an effective inhibitor of both *F. culmorum* pathogenesis and deoxynivalenol contamination in wheat.

Other field studies (349) found that while tebuconazole was efficient against a number of *Fusarium* spp, treatment with azoxystrobulin appeared to promote deoxynivalenol production in wheat, despite no increase in the amount of *F. culmorum* present in the crop. This may in part have been due to a selective action against the non-toxic mould *Microdochium nivale* which also induces symptoms similar to *Fusarium* ear blight. Work with barley (189) identified the fungicide Fludioxonil as having the potential to not only reduce disease but also the severity of deoxynivalenol contamination.

In common with other biological contaminants, strains of *Fusarium* spp. arise which are insensitive to fungicides. This may have an impact on the risk of mycotoxin production. For example D'Mello *et al* (105) identified a difenoconazole insensitive strain of *F. culmorum*, which produced 3-acetyldeoxynivalenol on exposure to this fungicide. Subsequent work (104) demonstrated that the fungicide induced biosynthetic enzymes involved in the production of the mycotoxin. In the light of this work, a recent review (103) questioned the overall efficacy of fungicide use as a means of controlling mycotoxin contamination.

The key elements in the use of fungicides to manage mycotoxin contamination revolve around both the timing in relation to anthesis and the rate of application. As discussed previously (Part A section 3.2.1) the window for successful infection is relatively short. Experimental studies (168, 243) have shown that the closer application of fungicides after infection the more effective the treatment. The optimum time for application being approximately two days after infection (243). Others (264) have shown that the most effective control was effected when the fungicide was applied at mid-anthesis. As well as when the fungicide is applied there is also the question of how much. Studies (23, 244)

have shown that application below recommended rates can promote mycotoxin (deoxynivalenol) production. Guidelines (23) for the use of foliar fungicides in the management of mycotoxins have been published and include the following recommendations:

- Immediate preparation of fungicide spray if the weather is wet at anthesis;
- The use of mixtures of fungicides to ensure a broad spectrum effect to include mycotoxigenic species;
- Application in accordance with manufacturers' specifications and not at reduced doses;
- Spray as soon as possible after infection (or not at all).

Herbicides and Fertilizers

A second factor to consider is the chemical management of the crop itself, in particular the use of other pesticides and fertilisers. Ruppel *et al.* (323) examined the effect of various herbicides (cyanazine, desmedipham, dicamba, EPTC, ethofumesate, pendimethalin, phenmedipham, trifluralin and 2,4-D amine) on the soil mycoflora of a barley-maize-pinto bean-sugar-beet cropping system. They found that neither the type nor quantity of herbicide used affected populations of a number of fungal generas, including *Fusarium*. With regard to fertilisers, although studies in North America (367) have failed to show a relationship between nitrogen treatments and mycotoxin contamination, European studies (130) have. In the latter case, delayed initial and/or supplemental nitrogen applications predisposed plants to infection by fungi causing ear blight. Other studies (211) have shown that lodging was a key risk factor in mycotoxin contamination. Application of growth modulators to reduce risk of lodging might therefore contribute to reduced risk of contamination.

Insecticides

Insecticide treatments may be of relevance to both wheat and barley. Reference has already been made to the contributory effect made by insect and bird damage to *Fusarium* spp. infection of maize (Part A, section 2.1; Part B, section 1.2.2). Diehl and Fehrmann (115) observed that the damage accompanying aphid attack during anthesis led to increased severity of attack by *Fusarium* spp. Dowd *et al* have shown both experimentally (121) and using aerial spraying (122) that treatment of maize with the insecticide malathion not only effects insect control but also indirectly brings about a reduction in contamination by mycotoxigenic fungi. Insecticides can, however, directly inhibit mycotoxin production. Beresford and Ayres (49) have demonstrated that the insecticide naled could inhibit zearalenone production by *F. graminearum* in culture. Subsequently, it has been shown that fonophos, carbaryl and maneb all inhibited zearalenone production by *F. graminearum* on maize both *in vitro* and also in field trials, if the insecticide was applied after silking (123)

Organic (Eco) Farming

Products grown under organic farming conditions are experiencing increased consumer demand. Work (131) characterising the mycoflora of fields either in transition for use in producing organic products or already used for organic crops have shown differences between these and more intensive

systems. Fields in transition to organic status were observed to have a compositionally far more diverse *Fusarium* spp. flora. Early studies performed in Germany (240) looking at rye and wheat grown under conventional and alternative/organic conditions found that on average, deoxynivalenol and zearalenone concentrations were higher in organically grown wheat and rye compared with the conventionally grown crops. Subsequent work (28) analysed the microbiological and mycotoxicological (deoxynivalenol and ochratoxin A) quality of organic winter wheat, grown over three seasons. At harvest or during storage, no ochratoxin A was found, although deoxynivalenol was, albeit at levels below current guideline limits. In contrast, more recent work from Germany (329) suggests that levels of deoxynivalenol contamination in conventionally grown wheat were greater than those found in organic crops.

2.0 HARVEST TO FARM- OR THIRD-PARTY FINISHED GRAIN STORES (STEPS 5 TO 10; W8 &W9; T10 TO T13)

This section is concerned with the flow of grain from harvest until it is stored ready for sale. Storage can either be on farm or effected by a third party.

2.1 Harvest (Stage 5)

As discussed above (Part A section 3.1; Part B, section 1.3.2), grain moisture at harvest is usually too low to enable *Fusarium* spp. to either grow or produce mycotoxins. However, under certain circumstances the potential remains for post-harvest mycotoxin synthesis, for example the production of zearalenone produced in harvested wheat, which had not been combined (204) and deoxynivalenol production following heavy rains at harvest time (205). Given that the productivity of any chemical reaction is a function of time, temperature and moisture, it is logical to expect that, in the case of wet-harvested grain, the faster at-risk grain is transferred to the dryer, the lower the risk of contamination. This topic is discussed further under 2.3 (Storage).

One of the problems identified with storage of mycotoxins (e.g. the aflatoxins and ochratoxin A) is that one is unable to correlate the level of mycotoxin contamination with either the amount of fungal biomass present nor observed defects in the crop. Whether the same applies for *Fusarium* spp. toxins is the matter of some debate. Some studies have shown strong correlations between wheat ear blight damage and the presence of mycotoxin (300, 377, 384). In contrast, Liu *et al.* (224) found significant correlations between the degree of experimental infection with deoxynivalenol contamination, but not between field ear blight assessments and mycotoxin contamination. A more recent study (25) has shown that a better statistical correlation exists between the number of visually 'scabby' kernels and the level of deoxynivalenol contamination than with either grain density or thousand grain weight measurements. The authors sounded a note of caution, in that it is still possible to detect significant amounts of mycotoxin contamination in grain that apparently meets its harvest potential. Others (129) have failed to observe any correlation between visual assessments of *Fusarium* ear blight disease and levels of tricothecene contamination in winter wheat.

In the case of barley, less information is available. Abramson *et al.* (5), investigating an outbreak of *Fusarium* ear blight in barley grown in Manitoba during 1993 & 1994 observed poor correlation between head blight damage and trichothecene contamination. They also stated that, in the case of deoxynivalenol, an enzyme based immunoassay may be an appropriate method for screening suspect batches of harvested grain. This recommendation did not, however, extend to 3- and 15-acetyldeoxynivalenol. Similar observations showing poor correlations between grain attributes and mycotoxin contamination in barley were made by Jones and Mirocha (190) working in Minnesota. In contrast, Schwarz *et al.* (332) looking at *Fusarium* spp. infected barley harvested in the midwest of the USA, observed that there was a correlation between deoxynivalenol contamination and grain weight (but no other quality marker). Similarly studies in Europe by Perkowski and co-workers, working with experimentally infected barley yielded mixed results. Field studies with spring barley (302) experimentally infected with *F. culmorum* demonstrated a relationship between a reduction in yield and toxin production with one particular strain. Other work by this group (303) using *F. graminearum* failed to show any correlation in experimentally infected (*F. culmorum* or *F. graminearum*) barley. However it was subsequently reported (299) that between 77 and 94% of the contamination by deoxynivalenol produced by these fungi were associated with small grains (< 2.5mm diameter).

With regard to maize, distribution of contamination may not only vary from plant to plant but also within the cob. Studies of an epidemic of *F. graminearum* infection of maize in Maryland, USA (393) demonstrated that is possible for apparently non-infected kernels, showing no evidence of deoxynivalenol contamination, to co-exist in the same cob with obviously infected ones, which were contaminated with the mycotoxin. Desjardins *et al.* (111), studying fumonisin production in maize ears experimentally infected with *F. moniliforme,* observed that while both symptomatic and symptom free kernels were colonised by the fungus, the highest levels of toxin contamination were observed in symptomatic kernels.

2.2 On Site (Farm or Third Party) Transport (Stages, 6, 8, W9 & T12)

Vehicles and trailers used for the transportation of grain need to be of an appropriate hygienic standard. Cleaning regimes and accepted practices have been set out in documents issued by third-party accreditation schemes at either a trade level as in the UK (19) or a legislative one, as in the case of the Netherlands (313). While strictly not part of the remit of this project, it is worth noting that recent work (38) has shown that grain contamination with *P. verrucosum* appears to be attributable to contamination in the combine, trailers and store. The source of the inoculum being residues which had not been removed during cleaning operations.

As well as ensuring the hygienic status of the combines and trailers, a key factor is ensuring that wet crop is transferred to the dryer as quickly as possible. Workers in Brazil (288) have shown that even short delays in drying can lead to 10-fold increases in harvest levels of fumonisin contamination.

2.3 On Farm/3rd Party Storage (Buffer & Finished Grain Farm Storage, Stages, 7, 9, T10, & T13)

Once the grain is harvested and goes into storage, it is known that the predominant grain mycoflora changes with a shift from the 'field' mycoflora such as *Fusarium* spp. to 'storage' mycoflora such as *Penicillium* spp. (147). A number of groups have reported on the absence of *Fusarium* spp. mycotoxin production in stored grain. Etchevers *et al.* (134), studying malting barley observed that field fungi (e.g. *Fusarium* spp.) were not viable for more than a few (fifteen) weeks when stored at low (14%) moisture contents. Beattie *et al* (42), again working with malting barley, have reported no changes in deoxynivalenol concentrations in earblight-infected malting barley. Experiments by Lund *et al.*, (228) observed *Fusarium* spp. growth in stored grain was only promoted at grain moisture contents greater than 26%. In the case of wheat and barley, as the grain ripens, it gradually becomes dryer and eventually, by the time of harvest, the grain water activity is usually too low to sustain *Fusarium* spp. activity. Earlier work, in naturally infected wheat (344) actually observed a decline in the concentration of deoxynivalenol as the grain ripened.

The factors that need to be addressed at this stage of the product flow are:

- *Physiological,* those which directly affect fungal metabolism (time, temperature, moisture and use of modified storage conditions);
- *Quality*, those which can lead to an increase in mycotoxin loading either as a consequence of further promoting fungal metabolism or through cross contamination between wholesome and contaminated grain.
- *Application*, of scientific and technological information to current commercial practices to provide maximum assurance against mycotoxin production.

2.3.1 Physiological Factors (temperature, moisture and modified storage conditions)

As will be discussed further under 2.4 (Grain Drying) a key challenge is to reduce and maintain the grain moisture, and hence water activity, to a level that does not does permit fungal metabolism (see also Part A, section 3.1). Thus while post-harvest grain moisture contents have been considered critical for the, 'storage' mycotoxins (e.g. ochratoxin A and aflatoxin B_1), less emphasis appears to have been placed on the moisture conditions required for the production of 'field' mycotoxins such as those produced by *Fusarium* spp. This applies particularly where moisture content is not so well managed, for example grain produced for feeding to livestock on farm. Early studies (156) using field-inoculated barley with *F. culmorum* demonstrated that zeralenone contamination did not arise until after prolonged storage at 34% moisture. It has been suggested that livestock intoxication by zeralenone contaminated maize may also reflect improper storage conditions rather than pre-harvest events (394). Toxin contamination in maize appears to be more common in wet maize cobs stored in cribs over winter (394).

Early experimental studies (348) investigating the growth of *Fusarium* spp. and production of zeralenone in stored grain (maize, wheat, barley or oats), reported that all grains were susceptible to invasion by *Fusarium* spp. at moisture contents above 18%, even when stored at 7 °C. The risk of

zeralenone contamination in grain held at between 15 and 18% moisture increased as a function of temperature. Maximum zeralenone yields (500-1000 µg/kg) were obtained in cultures stored at 12 and 18 °C even though mycelial growth had been reduced. Under conditions where fungal growth was optimised, zeralenone yields were considerably reduced (approximately 100 µg/kg). More recently, studies in Brazil have shown that *Fusarium* spp. can persist in parcels of maize, dried to average moisture contents of 11 - 14% over a 12 month storage time. However, no additional fumonisin production was observed while the crop was stored. Subsequently others have reported similar findings in other cereals. Both Birzele *et al.* (53) and Hormdork (169) have reported increased levels of deoxynivalenol with storage in German wheat samples. In the case of Birzele's studies this was accompanied by a progressive reduction in the *Fusarium* spp. population. Studies in Russia (14) have demonstrated deoxynivalenol production in wheat stored at 20°C after 2-3 days when the moisture content was 20-25% and 7-10 days when moisture contents were in the range 16.5-18%.

The problem is particularly acute when the grain is harvested at high moisture contents that permit continued mycotoxin production (e.g. in the case of wheat and barley in the Nordic countries, 212). Post-harvest activities which lead to delays in achieving relatively low moisture contents can also promote *Fusarium* spp. mycotoxin production. L'voya *et al.*, (204) reported zearalenone production in wheat infected with *F. graminearum* either left in swathes or on the threshing floor. In the case of maize, Ono *et al* (288) have reported that even short delays in the drying of freshly harvested wet maize can lead to 10-fold increases in fumonisin contamination. Other studies (289) have reported high numbers of *Fusarium* spp. surviving in maize after 5 months in storage. This is further compounded by the fact that grain moisture distribution within a bulk is heterogeneous (89). Farmers and other grain handlers often rely on average moisture contents and ignore the authors' observation (89) that it is the highest moisture content within the parcel which determines mould growth.

Where it is necessary to store grain with relatively high moisture contents for prolonged periods (this applies mainly to cereals intended for animal feed) other strategies need to be applied. This can involve either modifying the environment to suppress fungal activity or the addition of agents which inhibit fungal metabolism. In terms of environment modification, the most commonly used approach is to ensile the crop. Use of silage techniques makes the environment anaerobic and prevents the aerobic *Fusarium* spp. from producing mycotoxins such as zearalenone (it does not however lead to the degradation of the mycotoxin). However, where anaerobic conditions are compromised, for example due to air leaks, fungal activity can resume (347) and mycotoxin production can occur as in the case of zearalenone production within ensiled maize (133)

The use of chemical preservatives for stored grain began in the late 1960's with the use of propionic acid (326). Generally speaking, it is either applied in a pure form or in a mixture with other organic preservatives (e.g. acetic acid, isobutyric acid or formaldehyde). Typically the acid is sprayed onto the grain in an auger and the grain transferred to storage bins. Common rates of application range from 0.3-0.6% for grain at a moisture content of 18% to 0.8-1.2% for grain at 30% moisture. Treatment with organic acids reduces seed viability and can give rise to taint. Consequently it is usually only applied to grain intended for on-farm use (326). Attention has been paid to the effect

such treatments can have on the survival of *Fusarium* species and on mycotoxin production. Early studies (347) looking at barley, wheat and oats showed inhibition of toxigenic strains of *Fusarium* at 2 000 ppm propionic acid. Other organic acids (formic, acetic and butyric) were effective, albeit at much higher concentrations. Storage conditions are a critical factor contributing the efficacy of preservatives. Mueller and Thaler (272) observed that when stored in bins; treated maize would remain free of fungal growth for up to one year. In contrast, in grain stored in piles, mould development was seen after six months of storage. More recent laboratory studies by Marin *et al.* (233, 237) observed that propionic acid could inhibit fumonisin B_1 production by some species of *Fusarium* but not by others. Use of the preservative did not appear to enhance fumonisin production. Paster *et al.* (295, 296) have demonstrated that that the efficacy of propionate treatment can be further enhanced through synergistic effects with other treatments such as irradiation, co-addition of nisin and storage under high carbon dioxide partial pressures.

The potential of other compounds has also been investigated. Studies with sorbic acid (41) showed that T-2 toxin production by *F. acuminatum* increased when the mould was grown on maize meal containing between 0.025-0.05% sorbic acid. Thompson (369) has reported that a number of food grade phenolic preservatives (e.g. benzoic acid) are effective inhibitors of *Fusarium* spp. growth.

2.3.2 Quality Factors

Management practices for grain coming into store need to address two key factors:

- Segregation of at-risk material (either showing signs of *Fusarium* infection or at a critical moisture content) from wholesome grain, suitable for immediate storage. If necessary these should include procedures for the removal of grain showing evidence of *Fusarium* infection;
- Maintenance and monitoring of the storage environment to ensure that mycotoxin production does not occur.

Segregation requires assessments to be made at the point of entry to the store. Two parameters need to be considered; grain moisture and the amount of *Fusarium* ear blight damage in the batch of grain. The latter is probably the most commercially challenging and has yet to be fully addressed.

In the case of freshly harvested grain, a decision will need to be made as to whether the crop needs to be dried (see, 2.4 below) or whether it can be transferred directly to the finished grain store. Since the beginning of the century, control of grain moisture in storage has been identified as a critical factor in spoilage prevention (89). Storage practices can lead to temperature differentials and moisture migration due to convection. This leads to areas of high moisture content, as too can blending of grain with different moisture contents to obtain satisfactory average moisture content (89). Temperature/moisture interactions can also have more subtle effects. Grain metabolism of carbohydrates leads to the generation of moisture and heat. Below 15% moisture and 15°C, little activity is seen. Above 16% moisture, enzyme activity increases, even at low temperatures (discussed in 310). Localised areas of high moisture can also be generated by insect activity, or by poor blending. This inevitably leads to increased grain water-activity, with the concomitant risk of

mould growth in those areas (88), together with the potential for mycotoxin production. The key parameter that therefore needs to be measured within any lot of grain is the highest moisture content within it (89), rather than the moisture content of a composite sample (average). Reliance on average moisture content measurements has on occasion led to fungal spoilage and commercial loss (88).

2.3.3 Application of lessons from scientific observations to commercial practices.

The relationship between grain moisture and water-activity is complex. At a fundamental level it is not only influenced by other physical parameters such as temperature, but also cereal variety (307). A key factor is that hysterisis is observed, such that for any particular grain water-activity value, the desorption moisture value is higher than that found for absorption (164). This difference in values can be marked. However, at high water-activity values the difference becomes smaller, particularly at water-activities above 0.90. A second and, probably more significant problem, is the methodology used to determine moisture contents. Given the commercial importance of grain moisture, although commercial moisture determinations can be made using different methods, these are normally validated against a commonly accepted reference method. This is usually defined at a national level (e.g. 17)) and based on an international standard (e.g. ISO 712 : 1998).

A failure to validate either individual methods (e.g. at a research laboratory level) with that set out in a recognised standard and/or to regularly calibrate moisture measuring equipment against standards (e.g. as in ISO 7700-1 : 1984) will obviously lead to inaccuracies. There is, therefore, not only a potential that data derived from laboratory studies might not be immediately capable of being translated into advice for industry, but also that industrial measurements with moisture meters may not reflect the true situation. In one report (88) of a commercially significant loss due to fungal damage, moisture contents measured with a meter were at least one percentage point lower than true.

2.4 Drying - On Farm (Stage W8) - or by Third Parties (Stage T11)

As discussed earlier, members of the genus *Fusarium* are considered to be field as opposed to storage mycotoxins. However, as already detailed, under certain circumstances, *Fusarium* spp. do have the potential to produce mycotoxins post-harvest. Reference has already been made to the additional risk conferred by delaying drying of maize with respect to fumonisin contamination (288). Under these circumstances, it has been demonstrated that incorrect drying practices can also contribute to elevated levels of *Fusarium* spp. toxins (212). Studies addressing the question primarily of mycotoxins in small grain crops, such as those by Jonsson (191) have been performed with respect to ochratoxin A. These have demonstrated that the both geographical location and the method of grain drying are of significance, both on the incidence of contamination by storage fungi and also mycotoxins, particularly ochratoxin A. It should be noted that even with the use of drying with heated air, average grain moistures of greater than 14% were reported after 5 months storage. In the case of grain dried with near ambient air or near ambient air plus added heat, average grain moistures in the range 15 to 20% were found. Values at the top end of this scale might be high

enough to permit *Fusarium* spp. mycotoxin production as well as ochratoxin A by *P. verrucosum.* Studies such as those by Mueller and Thaler (273) with maize support this observation. Drying with ambient air therefore can be disadvantageous in cooler temperate climates. Attempts have been reported to improve the process by including an agent, which inhibits fungal activity such as treatment with ammonia (283).

3.0 FINISHED GRAIN STORE TO PRIMARY PROCESSOR (STAGES 10, F11, M11 C11 & T14)

Events following dispatch of the grain from the farm to the eventual user are considered under a single heading. With the exception of the standard pre-requisites (e.g. moisture,) the major risk factor is essentially the entry of contaminated grain onto the market. By this time, grain for sale has a moisture content too low to sustain *Fusarium spp.* activity. Both in the UK and elsewhere within the EU, the supply of grain is usually governed by industry-wide codes. For example in the UK flour industry, codes of practice cover not only the transport of grain to flour mills (371) but also grain quality (18). These specifications are enforced by weighbridge checks and are increasingly being managed through industry-based 3rd party quality assurance schemes for both farmers and transport/intermediaries.

C WHEAT PROCESSING (BREAD): PRIMARY PROCESSOR - RETAILER

Author's note: stage numbers referred to in the text, cross reference to those quoted in the flow diagram (Figures 6&7) used for the hazard/risk analyses described in the main body of the report

1.0 FLOUR-MILL GRAIN RECEPTION TO BAKERY FLOUR SILO (STAGES F11 TO F25)

1.1 Background

Toxicoses have been associated with bakery products in areas of the world where mould contamination cannot be so rigorously controlled, (e.g. 50). Elsewhere, both in Member States (e.g. the Netherlands, 163) and elsewhere (e.g. Argentina, 291) survey data suggests that bakery products can make a make a significant contribution to the population's deoxynivalenol body burden and may have public health implications. In addition, numerous workers have shown that flour produced from *Fusarium* spp. damaged wheat is often inferior, leading to poor baking and product quality (112, 113, 253). The degree of impairment is, in part, a function of the wheat variety (112, 113).

1.2 Flour Milling (Stages F11 - F25)

Aspects relating to storage have been addressed in Part B, section 2.3; this section relates to the effect that various components of the milling process can have on mycotoxin contamination.

1.2.1 Screen Room (Stage F13)

The key purpose of the screen room is to effect the separation of foreign matter and inferior grain (grain fragments, small grains and large grains). The process uses several pieces of equipment used in sequence. Their actions are usually based on sieving or centrifugal principles.

Work by Abbas *et al* (2) has demonstrated that unless contaminated grains are actually physically segregated, the cleaning operations performed in the screen room will not be effective in removing mycotoxins from the flour. Physical separation and segregation methods based on the observation that severely infected kernels are concentrated in the least dense fractions of the wheat parcel have been described, for example, the use of gravity tables (370). Other methods of density separation, based on liquid systems have shown that deoxynivalenol and zearalenone concentrations in the finished flour can be reduced by as much as 96% and 55% respectively (78). Additional approaches that have been proposed to reduce levels of mycotoxin (deoxynivalenol and zearalenone) contamination include selective removal of the hulls (e.g. in barley) and bran layers or selective sieving of kibbled grains or washing in sodium carbonate solutions (372). At a commercial level, while pre-kibbling grains or use of liquid-based removal systems would not probably be feasible, the use of gravity tables is, and is practiced in certain parts of the world. A key point in the use of such systems, is the need to continuously monitor performance. Recent work (321) has described

the use of image analysis systems connected to neural networks to monitor the outflow from such equipment.

1.2.2 Flour Production (Stages F14 - F20)

Flour milling is essentially a process of reduction and (in the case of most flours, except wholemeal) fractionation. In terms of fungal distribution within the wheat kernel, Chelkowski *et al.* (82) observed that grains naturally infected with *F. culmorum* exhibited more damage than those infected with, *F. graminearum, F. avenaceceum,* and *M. nivale.* Mycelial growth was most extensive between the pericarp and aleurone layers and alongside the scutellum. Invasion by the mycelia of both the embryo and endosperm was also observed. Given this distribution, removal of the outer bran layers (scouring) might be considered to be effective in reducing mycotoxin loading. Such studies that have been performed (78) have resulted in reductions of between 20 and 40% in the mycotoxin content of the finished flour.

Other studies have been performed looking at the distribution of mycotoxins in various mill fractions. Abbas *et al.* (2) working in North America with *F. graminearum* contaminated wheat, observed something similar, in that while deoxynivalenol was distributed throughout the grain, it tended to accumulate in the mill fractions derived from the outer layers of the grain (e.g. bran). A similar finding was made by Lee *et al.*, (215). However, they also found differences between *Fusarium* mycotoxins. Thus while deoxynivalenol and nivalenol appeared to permeate the endosperm to one degree or another, no contamination of the endosperm by zearalenone was found, as evidenced by its absence in white flours produced from contaminated wheat. Within the context of the milling process, removal of the outer bran layers of the grain can therefore result in some reduction in the amount of mycotoxin present. However, there is still residual (60-80%) contamination. Findings with bread-making wheats (*Triticum aestivi*) grown and produced in Europe have also been reported (219). In this case, up to 50% reductions in deoxynivalenol concentrations were reported, depending on the extraction rate used. These effects may be species dependent since, Nowicki *et al.* (284) working with deoxynivalenol contaminated durum wheat (*T. durum*); found that although milling effected a slight partition of the toxin, losses were not significant.

2.0 BAKERY FLOUR SILO TO RETAILER (STAGES F24 TO F34)

2.1 Dough Preparation and Bread Baking (Stages F25-F32)

The key factors, in terms of *Fusarium* spp. mycotoxin production, which have to be addressed, are the role of any minor ingredients (e.g. improving agents), yeast fermentation and the thermal environment during baking.

2.1.1 Role of Minor Ingredients

Studies by Boyacioglu *et al.* (61) demonstrated that the permitted process aid, ascorbic acid, had no effect on deoxynivalenol levels in bread baked from contaminated wheat. However, inclusion of either sodium bisulphite or L-cysteine (which are routinely used in the production of certain biscuit types) did effect a significant reduction in deoxynivalenol levels in the bread model used. Bench experiments such as those of Pineda & Bullerman (305) have demonstrated that some *Fusarium* spp. mycotoxins (e.g. moniliformin) are degraded under alkaline conditions. Alkaline production processes, such as those for maize tortillas may therefore contribute to a reduction in contamination

2.1.2 Yeast Fermentation (Prove)

Studies by Boeswald *et al.* (57) using pure culture systems and investigating a range of yeasts of industrial significance, observed that while strains *Saccharomyces cerevisiae* were observed to metabolise zearalenone by reducing it to both alpha- and beta- zearalenol, no metabolism of deoxynivalenol was observed. However, under industrial conditions, yeast fermentation has been observed to lead to reductions in deoxynivalenol concentrations originating from natural contamination of the original grain (277). This observation is at variance with those of Savard (327) who suggested the possibility that fatty-acid and glycoside conjugates of deoxynivalenol can be deconjugated by industrial yeasts to release the active toxin.

2.1.3 Baking

Fusarium spp. mycotoxins appear to exhibit a degree of heat resistance and are not significantly degraded during the baking process (342, 366).

D BARLEY PROCESSING (BEER) PRIMARY PROCESSOR - RETAILER

Author's note: stage numbers referred to in the text, cross reference to those quoted in the flow diagram (Figures 8&9) used for the hazard/risk analyses described in the main body of the report

1.0 MALTSTER GRAIN RECEPTION TO BREWERY MALT SILO (STAGES M11 TO M24)

1.1 Background

As in the case of wheat and bread, *Fusarium* spp. damage to barley is of commercial significance irrespective of any public health implications. *Fusarium* damage has been implicated both with impaired malting and brewing performance. In the case of malt, this has been particularly in respect of poor germination (152, 285) and other malting characteristics (328, 334). While in brewing, *Fusarium* spp mycotoxins have been observed to have an inhibitory effect on yeast fermentation (54, 55), other metabolites have been associated with the 'gushing' phenomenon (278, 331), a quality defect particularly associated with bottled beer.

1.2 Barley Malting (Stages M11-M24)

1.2.1 Grain Receipt and Grain Drying

Given the stringent requirements of the malting industry, barley is transferred direct from the farm to the maltster. In Northern Europe, grain moisture contents will invariably be high enough to support toxigenic storage fungal growth (e.g. *Penicillium verrucosum)* and sometimes that of *Fusarium* spp. Drying is therefore undertaken on a priority basis with batches with high moisture contents being dried first (37). Correct processing of barley at this stage can lead to substantial reductions in the numbers of contaminating field fungi (159). The importance of the drying process and of reducing the water-activity of the grain have been discussed in Part B, sections 2.1-2.4).

1.2.2 Steeping & Germination

Steeping involves raising the average moisture content of the barley from 11-12% commonly associated with long-term storage to between 43 and 46%. The latter moisture contents being sufficiently high enough to promote further *Fusarium* spp. outgrowth. Flanagan *et al.* (141) reported that the number of *Fusarium* spp. infected kernels could rise from 15 to 90% during the steeping process. Schwarz *et al.*, (333) studied *Fusarium* spp. growth and mycotoxin production during steeping and germination. While steeping was accompanied by a reduction in deoxynivalenol concentrations, germination was often associated with fungal outgrowth and mycotoxin production. However, considerable variability was observed (18-114% of original contamination), suggesting differences in fungal viability or in the nature of infection.

2.0 BREWERY MALT SILO TO RETAILER (STAGES M24 TO M34)

2.1 Wort Preparation and Beer Brewing (Stages M25-M32)

The key factors, in terms of *Fusarium* spp. mycotoxin production, which have to be addressed, are the leeching of mycotoxins into the wort, thermal treatment of the wort and yeast fermentation.

2.1.1 Wort Production

The principle problem associated with wort production is the extraction of mycotoxins from the malt into the wort. The 'extractability' of the mycoxins varies with the chemical structure. Hernandez *et al* (165) determined that while deoxynivalenol was readily extractable, zearalenone was not. This work confirmed previous studies (333) where between 80 and 93% of contaminating deoxynivalenol present in the original malt was found in beer brewed from it. In contrast, when malt contaminated with zearalenone was studied, little leaching into beer was observed, with most of the mycotoxin being found in the spent-grains.

2.1.2 Wort Boiling & Cooling

There is little published information on the effect of wort boiling and cooling on the stability or otherwise of *Fusarium* spp. mycotoxins. However, certainly in the case of deoxynivalenol (333), it is unlikely that any significant thermal destruction takes place.

2.1.3 Fermentation

This term is used to describe the fermentation process and subsequent steps leading to the finished packaged product. Reference has already been made to the degradation of zearalenone by *Saccharomyces cerevisiae* in the context of bread production (56) similar results have been found in the context of brewing (242). Deoxynivalenol, however, does not appear to be metabolised by brewing yeast (56).

E PRIMARY PROCESSOR - RETAILER, BREAKFAST CEREALS (CORN FLAKES), WITH A NOTE ON WET MILLING

Author's note: stage numbers referred to in the text, cross reference to those quoted in the flow diagram (Figure 10-12) used for the hazard/risk analyses described in the main body of the report

1.0 MILL GRAIN RECEPTION TO FACTORY STORES (STAGES B11 TO B26)

1.1 Background

Maize-based products are of interest from three points:

- The diversity of the processes which the kernel can undergo prior to becoming a finished consumer product;
- For reasons discussed elsewhere (Part A, section 2.1), maize appears to be unique as the botanical source of the fumonisin group of mycotoxins;
- Outbreaks of food poisoning attributable to fumonisin B_1 have been reported (51).

Fumonisin contamination appears to vary internationally and risk of exposure may reflect different national dietary practices. Surveillance work in the UK has shown that in over a nine month period (1998-1999), some 48% of imported maize samples had concentrations of total fumonisins in excess of 1000 µg/kg total fumonisins and 42% contained zearalenone at levels in excess of 100 µg/kg (266). Subsequent work expanding on this study (345) showed that, particularly in the case of fumonisin contamination, there were clear geographical differences between shipments, with higher levels of contamination being seen in Argentine compared with European maize. Of further interest was the correlation between the latitude of European port from where the maize was shipped and the level of fumonisin contamination. In this case, the further south the port, the greater the mean concentration of fumonisins measured.

In terms of maize-based foods intended for human consumption, a previous UK study (265) had found that, while low levels of total fumonisins were found in breakfast cereals, higher levels (>1000 µg/kg) could occasionally be found in other foods (e.g. polenta). Studies in the USA (73, 158) have found levels of fumonisin B_1 at levels which could give cause for concern. Similar results have been reported from Italy (118), the Netherlands (279, 280) and the Czech Republic (290). In the latter case, the highest concentrations of fumonisins were found in products based on maize meal (e.g. polenta) and the lowest in heavily processed food such as cornflakes, a finding supported by other work in Italy (386) and Spain (382).

1.2 Dry Milling

Studies (361) on maize imported from the USA into South Africa have shown that introduction of a pre-cleanup step, which removed fines, could lead to between a 29 and 69% reduction in the fumonisin load. Similar results have been reported for fumonisins in the UK (266) but not zearalenone. Studies on the distribution of *Fusarium* spp. and mycotoxins in maize and their subsequent distribution during the dry milling process have been described (192). Overall, *Fusarium* counts and fumonisin concentrations tended to increase as grit size decreased and high counts and levels of fumonisins were found in the germ, bran and fines fractions.

2.0 FACTORY INGREDIENTS STORE TO RETAILER (STAGES B26 TO B37)

Generally speaking *Fusarium* mycotoxins are heat stable (214); however, as discussed above, survey data has shown that levels of mycotoxin contamination in this type of product tends to be low.

Thermal processing can reduce the level of fumonisin contamination. Early studies (213) demonstrated that the use of alkali (ammonia solution) or oxidizing agents (hydrogen peroxide) followed by heating and drying could effect reductions in fumonisin contamination. Experiments in model systems (177) have shown fumonisin B_1 to be hydrolysed under acid (pH 4.0) or alkali conditions (pH 10.0). Hydrolysis was to a degree temperature dependent with 18-90% lost at 60 minutes at 150°C, depending on pH). However, use of higher temperatures (>175°C) led to over 90% reduction, irrespective of pH.In contrast, when these studies were repeated in real food systems, where such temperatures might be expected (baking and frying) no such reductions were seen (178). Studies (74) investigating wet-heat systems, such as canning showed only slight reductions (< 15%) in contamination, while those based on baking showed reductions of between 0 and 48% in levels of contamination.

There is some evidence that HTST (High Temperature Short Time) processes may be effective in reducing levels of mycotoxin contamination. Using model systems, extrusion cooking has also been shown to reduce fumonisin contamination (72). The degree of reduction was dependent on the configuration of the screws and the amount of water present. Similar results were obtained for zearalenone contaminated maize (324). This type of reduction in the presence of sodium metabisulphite has also shown to be effective (>95% reduction) with regard to deoxynivalenol contaminated maize (75). However, others (398) found no such effect for deoxynivalenol. Experiments, looking at systems analogous to those found in the industry (193), determined that extrusion cooking could lead to a reduction in fumonisin contamination. Reduction was found to be a function of temperature and holding time (the greater the heat supplied, the greater the inactivation). Under conditions which generated a commercially acceptable product, reductions between 46 and 76% were obtained. With regard to cornflake manufacture, based on an extrusion step; de Girolamo *et al* (151) have shown that the whole method of manufacture contributed to substantially reducing the level of fumonisin contamination.

3.0 WET MILLING (STAGES B11, B38 TO B48)

Within the context of wet milling, the process is of concern in that the process may lead to a concentration of mycotoxins within a particular fraction. For example, studies with zearalenone or fumonisin contaminated maize (48) have shown that, while it is possible to obtain toxin free starch, there is a positive concentration effect within the gluten fraction. The process of steeping itself has been shown to have an extractive effect on fumonisin B_1 and B_2 contaminated grain (68). Similar results have been found with T-2 toxin (320).

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Food Contaminants (Mycotoxins, Nitrate & Process Contaminants)

Programme Review

Project Number(s):	CO4022 CO4034
Project Title:	Investigation of levels of Fusarium mycotoxins in UK wheat production Investigation of Fusarium mycotoxins in UK barley and oat production
Research programme requirements	Many foods contain naturally occurring chemicals, which may have deleterious effects on human health. The Food Standards Agency commissioned a survey of wheat for a range of mycotoxins to assess overall safety risks, to explore ways of minimising such risks and to aid advice to consumers on specific foods. *(Requirement reference: RRD5/C04/ B. Survey of wheat, barley and oats for a range of mycotoxins).*
Technical Aims and Objectives	CO4022 1. Determine the range of trichothecene and zearalenone contamination of grain within organic and conventional wheat production. Determine the effect of T3 fungicide applications within conventional wheat production on trichothecene and zeearalenone contamination of grain. Determine how other agronomic factors affect trichothecene and zearalenone contamination of grain. These will include variety, cultivation methods, crop rotation and region. 2. Determine the relationships between the amount of trichothecenes within grain, the amount of trichothecene-producing *Fusarium* within grain and a visual assessment of grain quality. CO4034 3. Determine the range of trichothecene, zearalenone and moniliformin contamination of grain within organic and conventional barley and oat production over a four year period (2002-2005). Determine how agronomic factors affect trichothecene, zearalenone and moniliformin contamination of grain. These will include fungicide usage, variety, cultivation methods, crop rotation and region. 1. Determine the relationships between the amount of fusarium mycotoxins within grain and visual assessments of grain quality.
Objectives achieved	The projects are ongoing.
Observations	

Publications

Edwards SG (2003) Fusarium mycotoxins in UK wheat. *Aspects of Applied Biology* **68**, 35-42.

Presentation at the 7th European Fusarium Seminar, Poznan, Poland, 4-7th September 2002

Presentation at the COST 835 (WG-3) meeting, HRI East Malling, 13-14th September 2002

Presentation at the Campden & Chorleywood Food Research Association Cereals, Milling and Baking Panel Meeting, Chipping Campden, 7th October 2002

Presentation at the Campden & Chorleywood Food Research Association conference "Managing mycotoxins", Chipping Campden, 29th October 2002

Poster at the BCPC Brighton Conference, Pests and Diseases in 18th-21stNovember, 2002.

Presentation at a European BASF Fusarium mycotoxin workshop, Frankfurt, Germany, 12th December 2002.

Presentation at the AAB conference "Mycotoxins in food production systems," Bath, 25-27th June 2003.

Presentation at the International Life Sciences Institute conference "Trichothecenes with a special focus on DON" Dublin, Ireland, 10-12 September 2003.

Presentation at the HGCA breakfast meeting "Mycotoxins in Grains" Taunton, UK, 5th May 2004.

Food Contaminants (Mycotoxins, Nitrate & Process Contaminants)

Programme Review

Project Number(s):	C03026
Project Title:	Use of DNA microarray technology to detect genes involved in the production of mycotoxins
Research programme requirements	Current methods of mycotoxin analysis are concerned with the presence or absence of the compound. There are currently few methods, which can reliably predict the potential of contaminating fungi to produce mycotoxins subsequently. Test-systems based on genetic markers for particular genes involved in mycotoxin biosynthesis may provide a route forward.
Technical Aims and Objectives	1. Demonstrate, in the short term, that DNA microarray technology can provide a short-cut to identification of the genes that are switched on in ochratoxin A-producing strains of Penicillium verrucosum 2. Monitor, in the longer term, the control of these genes in toxigenic, non-toxigenic and facultative strains of P. verrucosum 3. Identify a method that could be used for identification of the genes involved in the production of mycotoxins by other fungi
Objectives achieved	1. cDNA library constructed and probes produced. 2. Study demonstrated the utility of DNA microarray technology for identifying genes that are differentially expressed in toxigenic and non-toxigenic strains.
Observations	Production of the final report was delayed due to a number of factors, including staff illness. The report (including a cDNA library) and a concept note for further research were received from the contractor in February 2003. The concept note was further discussed internally.
Publications	Reported in the FSA News.