Food safety of cereals: A chain-wide approach to reduce Fusarium Mycotoxins

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Contents

Preface

The document 'Food safety of cereals: A chain wide approach to reduce Fusarium mycotoxins' is the final deliverable of EU FAIR-CT98-4094, i.e. the Concerted Action 'Quality Control Measures in the Production and Processing Chain to Reduce Fusarium Mycotoxin Contamination of Food and Feed Grains' with the acronym 'Mycotochain'. The Concerted Action started in January 1999 and involved three well attended general meetings of all participants from eight European countries: Austria, Denmark, France, Germany, Italy, Sweden, the Netherlands and the United Kingdom. Partners belong to fundamental research organisations, such as universities and governmental institutes, commercial breeding companies, the trade, a millers association, a milling company and food and feed safety organisations. The number of partners has steadily grown throughout the course of the project. The meetings led to extensive exchange of information between chain partners and young scientists took part in exchange and mobility programs as part of this Concerted Action.

The Concerted Action has divided its activities in the following task groups:

- Reduction of Mycotoxin Contamination during Crop Production
- Reduction of Mycotoxin Contamination during Storage and Processing of Grain
- Improved Methodology to Measure Mycotoxin Contamination
- Integrated Chain Wide Approach

For each task group a chairman was appointed.

This document presents an overview regarding Fusarium research and mycotoxin contamination of cereals, mainly wheat, but also maize, the effect of processing and possibilities of decontamination of cereals and the state of the art of methodologies to measure mycotoxins in the chain. The document is the result of collaboration between European partners, which represent all parts of the production chain of cereals and cereal based products and can be regarded as an integrated chain wide approach. The task group chairmen have played a major role in assembling the present document. They brought up information about their specific activities. Apart from the task group activities, information is presented about risk analysis, HACCP, legislation and EUREPGAP, followed by a final chapter with conclusions and recommendations for further research to be financed by the Sixth Framework Programme of the European Union. During the course of the project, we have felt an increasing awareness of the mycotoxin problem amongst the participants and growing sense of urgency to invest in solutions for this threat to our food and feed chain. Therefore several participants have contributed to an Expression of Interest regarding mycotoxin control. We trust that this document may be useful as means to re-affirm the links between the partners in the cereal chains, so that the ultimate goal: mycotoxin-free cereals and cereal products, may become a reality to the benefit of European consumers.

June 2002, A.P.M. (Ton) den Nijs & Olga E. Scholten
(chairman and secretary of Mycotochain)

Note: also visit our internet site, created especially for the project
http://www.mycotochain.org

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Summary

Fusarium head blight (FHB) in wheat and barley and Fusarium ear rot in maize is caused by several *Fusarium* species. Infection with *Fusarium* fungi firstly decreases the yield of the crop due to the production of shrunken kernels. More importantly, however, the disease reduces the quality of the seed since several of these fungi produce mycotoxins. Examples of mycotoxins produced by *Fusarium* in cereals are deoxynivalenol, nivalenol, fumonisins and zearalenone. From a food safety point of view, consumption of mycotoxin-infected cereals is dangerous as it threatens the health of men and animals. Currently, the EU is working on legislation and set maximum tolerated levels of mycotoxin concentrations in flour and cereal products.

This working document 'Food safety of cereals: A chain wide approach to reduce Fusarium mycotoxins' is the final deliverable of EU FAIR-CT98-4094, i.e. the Concerted Action 'Quality Control Measures in the Production and Processing Chain to Reduce Fusarium Mycotoxin Contamination of Food and Feed Grains' with the acronym 'Mycotochain'. The Concerted Action has brought together over 20 European actors in the cereals production chain during the period January 1999 through June 2002, to discuss the possibilities to reduce Fusarium mycotoxin contamination of food and feed grains. Partners originate from eight European countries: Austria, Denmark, France, Germany, Italy, Sweden, the Netherlands and the United Kingdom and belong to fundamental research organisations, such as universities and governmental institutes, commercial breeding companies, the trade, a millers association, a milling company and food and feed safety organisations. This document describes the activities of the Concerted Action, which was structured in four task groups.

An Introduction to the Concerted Action is presented in chapter 1. An out-line is given of the objectives as well as of the research tasks. The main objective of this Concerted Action is the exchange of knowledge between partners. To stimulate this exchange of knowledge three general meetings were organised in which partners were invited to present their research results, to share information regarding the Fusarium-mycotoxin problem and to discuss relevant topics in the mycotoxin field. Exchange of information between partners resulted in the writing of the chapters 2, 3 and 4 of this document.

Chapter 2 deals with various aspects of crop production. The first part of this chapter informs about breeding for resistance in wheat. A description is given of the Fusarium species that are involved in the infection process and the types of mycotoxins that are being produced. The problem of mycotoxin contamination is a problem that starts at the beginning of the production chain where susceptible wheat varieties are used. The use of resistant varieties is important to reduce mycotoxin contamination. Since, however, no

high-yielding resistant varieties exist, breeding for resistance is necessary. Breeding for resistance is important to reduce mycotoxin contamination. Resistance inherits mostly dominantly, but is controlled by a number of genes. Molecular markers seem interesting to accelerate the breeding process. Based on experiments, it is expected that resistance to *Fusarium* is quite durable. The same chapter also informs about the results of a ring test carried out by three partners involved in breeding. In this test, 17 varieties were screened for resistance to FHB on 5 locations in Europe of which three were artificially infected and the other two naturally. In general, disease estimates for varieties ranked similarly at different sites. Although some varieties were only slightly infected, the results clearly showed that none of them was completely resistant. The correlation between disease incidence and DON content for these samples was estimated as 0.80. Fungicides may be used to control the disease. So far, however, the effect of fungicides has been inconsistent. Product choice and timing of application as well as rate of application are important factors to keep in mind to achieve optimal control of FHB.

In chapter 3 an overview is presented of problems occurring after harvesting the grain: during storage, processing and decontamination. The humidity and the temperature are important factors that influence fungal growth. During storage also damaging of seeds may result in higher contamination, especially in maize. Food processing that may involve physical and/or chemical decontamination could be considered as a strategy to destroy mycotoxins. The ideal decontamination procedure should be easy to use, inexpensive and should not lead to the formation of compounds that are still toxic or can alter the nutritional and palatability properties of the grain or grain products. Strategies for intentional detoxification or decontamination of commodities containing mycotoxins can be classified as chemical, microbiological or physical and are explained in detail. It is concluded that more research is needed to further optimise decontamination procedures.

The state of the art regarding the analysis of relevant Fusarium mycotoxins in cereals is described in chapter 4. There is a need for good and standardised analytical methods to make surveys and control of these toxins possible, but in most cases they are still lacking. The methods for many of the toxins are complicated and often lead to high variations both within and between laboratories. Activities supported by the European Commission are going on to improve the analytical methods. Results from such studies and others are presented. For trichothecenes (e.g. deoxynivalenol, nivalenol) HPLC has been used as well as capillary gas chromatographic methods with EC or MS-detection, which are preferred for their higher sensitivity and selectivity. Also methods for rapid screening, such as ELISA and colorimetric bioassays are discussed. For zearalenone, although TLC, GC and GC-MS methods are available, HPLC is mostly used. For fumonisins, a validated HPLC method has been developed that meets CEN criteria. The chapter ends with recommendations for further optimisation of LC-MS(-MS), near infra red transmittance and biosensors.

In the meantime in Europe and the USA, project partners were aware of or played a role in many other activities concerning the problem of mycotoxin contamination in cereals, such as e.g. HACCP, good agricultural practice, legislation, the Codex Alimentarius. These activities have been compiled in chapter 5. To apply HACCP to establish minimum mycotoxin contamination, the following prerequisites should be taken into account: good hygiene practice, good agricultural practice, good storage practice, good manufacturing practice, management/stake holder commitment as well as training. Recommendations to improve good agricultural practice during the whole cereal production chain are presented. Under legislation EU opinions on Fusarium mycotoxins is listed together with their internet addresses.

The final chapter 6 is the result of a discussion among the co-ordinator, the task group chairmen and the secretary. It identifies the needs for further concerted research and breeding and gives recommendations for the chain-wide approach. This evolves into the expression of interest regarding the mycotoxin control problem in which several of the participants of the concerted action participate.
The group has not drawn up a protocol for use throughout the chain, since organisations such as EUREPGAP and the Codex Alimentarius, have already studies underway for establishing such protocols. The data in this report may be used to underpin such protocols as needed.

1. The Concerted Action Mycotochain: an introduction

1.1 Objectives

Fusarium fungi are an important problem in the cereal food and feed chain because of their ability to produce mycotoxins in the grain, which cause serious illness and immuno-repression in humans and animals, as well as yield loss per se. This mycotoxin problem is the result of events at the start of the chain, due to fungal infestation during the growing of the cereal crop, while the negative effects are found at the end of the chain, in food and feed products. The control of this problem therefore calls for collaboration throughout the chain of production and processing.

The long term objective of this Concerted Action was to establish a working relationship between European partners of the production chain of cereal based food and feed products to be able to prevent mycotoxin contamination due to *Fusarium* fungi (see Appendix I for an overview of the partners). The concerted action started in January 1999 with a general meeting and ran through 2001. It was extended with an extra half year in order to produce the final document with the Taskgroup chairmen and ended July 1, 2002.

Within the timeframe of this Concerted Action we achieved

- Exchange of knowledge on problems caused by infection due to *Fusarium* fungi throughout the production chain of cereals
- Identification of possibilities for effective collaboration to minimise mycotoxin contamination of cereal based products
- Identification of suitable quality control opportunities for mycotoxin content throughout production and manufacturing of food and feed products
- Establishment of opportunities for future collaboration
- Identification of gaps in knowledge which require further research

1.2 Research Tasks

Within this Concerted Action partners were brought together that represent all parts of the production chain of cereals and cereal based products. Initially the project was divided into five Task groups, which concentrated on various parts of the production chain:

- Task 1. Reduction of mycotoxin contamination during Crop Production
- Task 2. Reduction of mycotoxin contamination during Transportation and Storage
- Task 3. Reduction of mycotoxin contamination during Processing of grain
- Task 4. Improved methodology to Measure trichothecene and fumonisin
- Task 5. Integrated Chain Wide Approach

During the project phase, it became clear that Task 2 overlapped with other Tasks. Therefore, it was concluded to leave out Task 2 and consider the tasks like investigation of cereal samples at the farms for Task 1, the storage for Task 3 and the methodology needed to investigate samples during transportation and storage for Task 4. Within these specific task groups the various partners exchanged results of their own activities first, and subsequently interacted with other task groups to achieve improved quality throughout the production chain.

The project has resulted in three well-attended general meetings where scientific and practically oriented partners discussed freely and enthusiastically about possible solutions for the problems encountered. Exchange of information among partners resulted in the writing of the chapters 2, 3 and 4 of this document. In the meantime in Europe and the USA, project partners were aware of or played a role in many other activities concerning the problem of mycotoxin contamination in cereals, such as e.g. HACCP, good agricultural practice, legislation, the Codex Alimentarius, which have been compiled in chapter 4 (see also Appendix II for an overview of running European research). The final chapter 5 is the result of a discussion among the Task group chairmen. It identifies the needs for further concerted research and breeding and gives recommendations for the chain-wide approach. This evolves into the expression of interest regarding the mycotoxin control problem in which several of the participants of the concerted action participate. The group has not drawn up a protocol for use throughout the chain, since organisations such as EUREPGAP and the Codex Alimentarius, have already studies underway for establishing such protocols. The data in this report may be used to underpin such protocols as needed.

2. Crop production

2.1 Introduction

Minimizing mycotoxin contamination starts with clean starting material, i.e. seed. The primary production is at the start of the chain and sits at the basis of any program to reduce the risk of mycotoxins further down the chain and to the end user, albeit human or animal. Breeding for resistance to Fusarium head blight (FHB) in wheat and Fusarium ear rot in maize is the potentially most rewarding strategy, but awkwardly difficult and time-consuming. In this chapter various aspects of breeding for resistance are considered in relation to sustainability and mycotoxin accumulation. Fungicides have a long history in controlling FHB but with various degrees of success. Efficacy in relation to especially time of application is discussed. Resistance levels may vary between varieties depending on their location throughout Europe. To critically assess this supposed variation, a ring test with a set of common European wheat varieties was performed as part of the concerted action project. In this test attention is focussed on the relationship between (partial) resistance and mycotoxin accumulation.

2.2 Breeding for resistance to *Fusarium* spp. in wheat

T. Miedaner[1]

Fusarium head blight (FHB) is caused by several *Fusarium* species. *Fusarium* species are economically important pathogens in most agricultural crops. They occur on all vegetative and reproductive organs of plants causing wilts, rots or blights. Moreover, they have been isolated from soils of every continent except Antarctica (Windels, 1992). In small-grain cereals, about 20 *Fusarium* species have been regularly associated with disease symptoms (Duben & Fehrmann, 1979a; Gerlach & Nirenberg, 1982). *F. culmorum* (W.G. Smith) Sacc., *F. graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schw.) Petch], and *F. avenaceum* (Corda ex Fries) Sacc. [teleomorph: *Gibberella avenacea* Cook] were most frequently isolated (Cook, 1968; Duben & Fehrmann, 1979a; Mesterházy, 1995a). This introduction will focus on *F. culmorum* and *F. graminearum* because *F. avenaceum* isolates were generally found to be less aggressive in small-grain cereals (Colhoun *et al.,* 1968; Mesterházy, 1978; Duben & Fehrmann, 1979b; Diehl, 1984; Stack & McMullen, 1985; Wilcoxson *et al.,* 1988).

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1 This version is specially updated by the author for this document and has been published before as part of the paper 'Breeding wheat and rye for resistance to Fusarium diseases' in Plant-Breeding 116 (1997) 201-220.

F. culmorum and *F. graminearum* are 'generalists', infecting all cereal species including wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.), and a large number of non-gramineous hosts (Gerlach & Nirenberg, 1982). *F. culmorum* was isolated frequently in the cooler maritime regions of Northern Europe (Parry *et al.,* 1995), whereas *F. graminearum* is the predominating species on a global basis. The kind of disease they cause is predominantly a function of inoculum, plant growth stage, and environment (Cook, 1981a). Two ecologically and genetically different subpopulations of *F. graminearum* were described by Purss (1971) and Francis & Burgess (1977) and originally designated as Groups I and II. Recently, Group 1 isolates have been classified as the new species *Fusarium pseudograminearum* as judged by conidial morphology and molecular markers (Aoki & O'Donnell, 1999).

Seedling blight caused by *F. culmorum* and *F. graminearum* is mainly seed-borne in the humid climates and leads to a reduced number of plants and secondary attack by pests (e.g. *Oscinella frit*) due to thinned stands and delayed plant development. Seedling blight caused by *F. pseudograminearum* results, in contrast, from soil-borne infection and occurs in dry soils only (Burgess *et al.,* 1981).

At the stem base of small-grain cereals three epidemiological distinct Fusarium-incited diseases may occur: (1) brown foot rot caused by *F. culmorum* and *F. graminearum* in areas with high soil moisture and humidity (Cook, 1981a), (2) crown rot caused by *F. pseudograminearum* under dry weather and soil conditions, especially in the North-western U.S.A. (Cook, 1968), Eastern Australia (Burgess *et al.,* 1981), South Africa (Marasas *et al.,* 1988), and (3) common root rot caused by a complex attack of *F. culmorum*, *F. graminearum* and *Bipolaris sorokiniana* in the Great Plains of the U.S.A. and the Prairie Provinces of Canada (Windels & Holen, 1989). The attack of brown foot rot starts from aboveground inoculum (Cook, 1981a). The fungus penetrates the successive layers of the leaf sheaths during the growth period and finally reaches the stem. The lowest internodes, but not the crowns or roots, show necrotic lesions and may develop a soft rot that cannot be seen before flowering (Fehrmann, 1988). Brown foot rot is often the result of a complex attack of *F. culmorum*, *F. avenaceum*, *Pseudocercosporella herpotrichoides* and *Microdochium nivale* (Duben & Fehrmann, 1979a; Miedaner *et al.,* 1993b). Brown foot rot causes yield losses due to a reduced capacity of the stem for the movement of water and nutrients and an increased risk of lodging. Additionally, lodged wheat and rye crops impair baking and feeding quality.

Crown rot is caused by belowground inoculum entering the plants around emerging roots and crowns (Cook, 1981a). The infection remains latent unless the plant is subjected to heavy water stress reaching plant water potentials between -1.5 and -2.5 MPa (Cook, 1981b). The characteristic symptoms are scattered, bleached, and dead plants amongst unaffected plants on fields exposed to water stress. Crown rot causes premature ripening and thus a reduction of kernel number and/or kernel weight (Burgess *et al.,* 1981). In the more humid climate of Central Europe the combination of heavy water stress and crown

rot is unlikely to occur in wheat (Jenkins *et al.,* 1988), but in dry wheat growing areas crown rot is the most destructive stem disease (Cook, 1981a; Burgess *et al.,* 1981; Wildermuth & McNamara, 1994). *Fusarium* species are usually not primary pathogens of healthy upper leaf blades but they frequently enter lesions caused by powdery mildew (Mathis *et al.,* 1986) and aphids, or through mechanical wounds (Diehl, 1984).

Head blight is caused by ascospore or macroconidia infecting in periods with high humidity (>92-94% relative humidity, Cook, 1981a) and temperatures above 15°C (Parry *et al.,* 1995). Infections may occur at any time from head emergence to maturity, but disease severity is highest when inoculum is present in the flowering period of both wheat and rye (Anderson, 1948; Diehl, 1984; Mielke, 1988; Gang, 1996).

Symptoms and disease development were extensively reviewed (Cook, 1981a; Teich, 1989; Sutton, 1982; Parry *et al.,* 1995). Head blight epidemics may result in severe yield loss by destruction of the embryo and/or reduction of kernel weight, poor milling and baking quality of wheat (Meyer *et al.,* 1986; Pomeranz *et al.,* 1990), or reduced germination rate and seedling vigour in the following crop (Manka, 1989). Devastating epidemics occurring since the early 1990s in Midwestern USA resulted in yield losses up to 30%, a severe reduction in grain quality and high mycotoxin contents (McMullen *et al.,* 1997; Windels, 2000). *F. culmorum* and F.*graminearum* are both capable of producing trichothecene type A toxins (HT-2 toxin, T-2 toxin), type B toxins (mainly deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, fusarenone-X, calonectrin), and zearalenone in epidemics in wheat, barley, triticale, and rye (Marasas *et al.,* 1984; Chelkowski, 1989; Perkowski *et al.,* 1995). Several mycotoxins may occur simultaneously in different composition and amounts. They are hazardous to animal and human health (Friend & Trenholm, 1988; Pomeranz *et al.;* 1990, Snijders, 1990a; D'Mello *et al.,* 1999).

Yield losses caused by the various Fusarium diseases were reported to range for natural infections from 7 to 17% for seedling blight (Greaney *et al.,* 1938; Duben, 1978), from 10 to 30% for foot rot (Duben, 1978; Meyer, 1985), from 0 to 17% for crown rot (Dodman & Wildermuth, 1987), and from 0 up to 30 - 70% for head blight (Martin & Johnston, 1982). With artificial inoculation much higher losses can be gained for the various diseases (e.g. Purss, 1966; Diehl, 1984; Miedaner & Walther, 1987; Chelkowski, 1989; Snijders, 1990b; Miedaner *et al.,* 1993a). Fungicide treatment and agricultural management practises are only reducing the damage but cannot prevent yield and quality losses (Mielke, 1988; Teich, 1989; Milus & Parsons, 1994). Thus, the development of cultivars with appropriate disease resistance is the most effective means of controlling Fusarium diseases.

The state of knowledge on *F. graminearum* and *F. culmorum* has been thoroughly reviewed with regard to symptomatology and epidemiology (Cook, 1981a; Burgess *et al.,* 1981; Sutton, 1982; Jenkins *et al.,* 1988; Teich, 1989; Miller, 1994; Parry *et al.,* 1995), breeding strategies (Miedaner, 1997; Bai & Shaner, 1994; Mesterhazy *et al.,* 1999), and toxicology (Marasas *et al.,* 1984; Chelkowski, 1989; Pomeranz *et al.,* 1990).

Mycotoxin production in wheat caused by *Fusarium* ssp.

F. graminearum produces a variety of mycotoxins, namely nonmacrocylic trichothecenes and the estrogenic zearalenone (ZEA). Among these, deoxynivalenol (DON) and its derivatives 3-acetyl DON and 15-acetyl DON, ZEA and, in some parts of the world, also nivalenol (NIV) are most often encountered in wheat (Tanaka *et al.,* 1988; Mirocha *et al.,* 1989; Scott, 1990; Placinta *et al.,* 1999). The co-occurrence of several of these mycotoxins in grain has often been reported (e.g. Müller & Schwadorf, 1993; Mirocha *et al.,* 1994) and the frequency of mycotoxin-producing isolates in natural populations seems to be high. Of 114 isolates of *F. graminearum* collected from soil or cereals on a world-wide basis, 95 and 89% were capable of producing DON and ZEA *in vitro*, respectively (Mirocha *et al.,* 1989).

In smaller percentages, however, other patterns can be found. Some isolates of *F. graminearum* produce NIV, but they were found only rarely in the U.S.A. (Miller *et al.,* 1991). They are more common in Japan (Ichinoe *et al.,* 1983; Miller *et al.,* 1991), but have also been detected in Hungary, Poland, and Italy (Miedaner *et al.,* 2000). And although DON and NIV are chemically related, DON producers do not produce NIV and *vice versa* (Ichinoe *et al.,* 1983; Miedaner *et al.,* 2000a).

ZEA can be found in small-grain cereals, the appearance of high amounts seems to be associated predominantly with corn (Yoshizawa, 1991). Out of 2403 samples of small-grain cereals analysed world-wide, about 20% were found to contain ZEA (Yoshizawa, 1991). *F. culmorum*, *F. graminearum* and *F. pseudograminearum* produce similar mycotoxins with minor differences in the relative amounts of each mycotoxin (Blaney & Dodman, 1988; Marasas *et al.,* 1984; Snijders, 1990a; Atanassov *et al.,* 1994). The ability of *F. culmorum* to produce NIV has only recently been detected (Atanassov *et al.,* 1994; Mirocha *et al.,* 1994). For both *F. graminearum* and *F. culmorum*, the mycotoxins produced and the amount of mycotoxin production highly depends on the isolate investigated (Marasas *et al.,* 1984; Miller *et al.,* 1991; Atanassov *et al.,* 1994; Miedaner *et al.,* 2000).

It has been suggested that DON may contribute to the pathogenicity of *F. graminearum* and *F. culmorum*. DON inhibits protein synthesis (Miller, 1989) and growth of wheat coleoptile tissue and seedlings (Bruins *et al.,* 1993). However, it appears that DON production may not be required for pathogenicity, i.e. the ability to cause disease, because *F. graminearum* isolates that were unable to produce DON and 3-acetyl DON *in vitro* were pathogenic on wheat, rye, and triticale seedlings (Manka *et al.,* 1985). Moreover, a simple qualitative reaction (DON/no DON) cannot explain the quantitative nature of aggressiveness within *F. graminearum* populations as reported earlier (Miedaner & Schilling, 1996; Mesterházy *et al.,* 1999). According to Vanderplank (1984), aggressiveness designates the quantity of disease induced by a pathogenic isolate on a susceptible host when the isolates do not

interact differentially with host cultivars. Thus, DON might have no relation to pathogenicity, but may contribute to aggressiveness, i.e. the extent of fungal colonisation within host tissue in the early stages of pathogenesis (Snijders & Krechting, 1992). This was recently demonstrated by the generation of a trichothecene-deficient isolate of *F. graminearum* that was less aggressive than the DON-producing wild type (Proctor *et al.,* 1995; Desjardins *et al.,* 1996). Interestingly, the trichothecene-deficient isolate was still able to produce symptoms, i.e. its pathogenicity was retained (Proctor *et al.,* 1995). The physiological factors leading to different aggressiveness levels among isolates are still unclear. Besides mycotoxins, cell-wall-degrading enzymes may also play a major role.

Susceptibility of wheat varieties to head blight – the problem in wheat products at the beginning of the production chain

Fusarium culmorum and *F. graminearum* are the main causal organisms of head blight in wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), and triticale (x *Triticosecale* Wittmack) in humid-temperate climates (Snijders, 1990a). Complete resistance appears to be rare but large quantitative variation for head blight resistance in winter wheat was found for both *F. culmorum* and *F. graminearum* in all varieties tested. Hanson *et al.* (1950) summarised the results of former U.S. evaluation trials across thousands of entries and reported that all genotypes became infected, i.e. no source of complete resistance was found. Moreover, most genotypes proved to be susceptible with only few exceptions. Similar conclusions were drawn from more recent tests (Walther, 1976; Miedaner & Walther, 1987; Mesterházy, 1987; Mielke, 1988; Tomasovic, 1989; Snijders, 1990b; Saur, 1991; Bai & Shaner, 1994; Mesterházy *et al.,* 1989). Durum wheat varieties were generally more susceptible than bread wheat but some resistance was recently reported(Stack *et al.,* 2001). Distinct resistance sources for F. *graminearum*-incited head blight in hexaploid wheat were reported from three origins: winter wheats from Eastern Europe, spring wheats from Japan and China (e.g. 'Nobeokabozu Komugi', 'Sumai 3', 'Ning' selections), and from Brazil (e.g. 'Frontana', 'Encruzilhada') (Schroeder & Christensen, 1963; Mesterházy, 1987). Snijders (1990b) confirmed resistance of some of these wheat materials for infections to *F. culmorum* and identified additional accessions from these gene pools.

Despite a high genotypic variance, genotype-by-environment interaction plays a major role in the wheat/Fusarium head blight pathosystem (Mesterházy, 1987, 1989, 1995a,b; Miedaner *et al.,* 2001b). Therefore, correlations of host resistance to *F. graminearum* between years may vary considerably. In experiments over six years, Mesterházy (1995a) reported correlation coefficients between each of two years ranging from 0.19 to 0.81 for head blight rating and from 0.32 to 0.67 for relative grain weight. Stability of resistance expression over environments greatly depended on the resistance level of the genotypes studied. Highly resistant materials showed less variation across environments than medium susceptible genotypes (Mesterházy1995a). These data indicate that tests across several

environments are necessary to rank genotypes properly for their resistance to Fusarium head blight.
In quantitative-genetic experiments, a preponderance of additive variance for resistance to *F. graminearum* and *F. culmorum* have been found (Gu, 1983; Bai *et al.,* 1993; Ban & Suenaga, 2000; Snijders, 1990c,d, respectively). In addition the mean head blight resistance of F_2 populations can be predicted by the resistance of the parental lines (Snijders, 1990d,e). The only exceptions were found for progenies from crosses where one awned genotype was involved (Snijders, 1990d). Within the non-additive components of genetic variation, dominance was found most often. However, dominance expressed as heterosis for resistance was significant in some F_1 crosses only (Hanson *et al.,* 1950; Tomasovic, 1989; Snijders, 1990c). Similarly, the occurrence of epistatic effects of the additive-by-additive component was reported for a minority of crosses (Snijders, 1990d; Bai *et al.,* 1993).

Nakagawa (1955) firstly published an estimate of the number of effective factors by generation mean analysis. He found three genes that controlled scab resistance. In more recent surveys, Bai & Xhiao (1989) and Bai *et al.* (1993) reported one to three genes responsible for resistance to *F. graminearum* head blight in Chinese materials. Snijders (1990d) found on the basis of 45 crosses that the number of effective factors varied from one to six for *F. culmorum* head blight. It should be noted that, from the theoretical point of view, all these estimates are only a rough indication of the true number of genes responsible for Fusarium head blight resistance. At least three assumptions cannot be fulfilled in the experiments: (1) equal gene action, (2) one parent supplies only positive, the other only negative alleles, and (3) equal degree of dominance (Wright, 1968). Moreover, in any one cross only a limited sample of genes contributes to segregation and, therefore, the real number of genes will most likely be underestimated (Geiger & Heun, 1989). High genotype-by-environment interaction will additionally affect the estimation of gene number when the experiments are not conducted in several environments.

Molecular marker analyses of some resistance sources revealed one to five quantitative-trait loci (QTL) for head blight resistance up to now (Kolb *et al.,* 2001). Bai *et al.* (1999) analyzed 133 RILs derived from a cross of `Ning7840' (resistant) x `Clark' (susceptible) with AFLP markers and identified one major QTL for scab resistance that explained almost 60% of the phenotypic variation for Type II resistance in that population. Later, this QTL was localized on chromosome 3BS. Waldron *et al.* (1999) identified several QTL for scab resistance by analyzing RFLP markers in 112 F5-derived RILs from the cross between `Sumai 3' (resistant) and `Stoa' (moderately susceptible). Two major QTL were located an 3BS of `Sumai 3' and 2AL of `Stoa', respectively. The most infonnative RFLP marker in the 3BS rcgion explained 15% of the plienotypic variation in that mapping population (Waldron *et al.,* 1999). Later, Anderson *et al.* (2001) reported several microsatellite markers linked to the same QTL for scab resistance an 3BS. Subsequently, other groups have confirmed the association of markers with a QTL on 3BS (Buerstmayr *et al.,* 2002; Chen *et al.,* 2000; Gupta *et al.,* 2000, 2001; Zhou *et al.,* 2000). Buerstmayr *et al.* (2002) reported three chromosome regions associated with FHB resistance in a double-

haploid mapping population with `Sumai 3' as resistant parent. Again, the most-prominent effect was detected an chromosome 3BS, explaining up to 60% of the phenotypic variation for type II resistance (Buerstmayr *et al.,* 2002). When analysing type I and II resistance together by spraying a conidia suspension onto the heads, two QTLs on 3B and 5A each were found to explain about 30% of phenotypic variance (Buerstmayr, pers. comm.).

Resistance to mycotoxin accumulation as a future breeding target in wheat breeding

Host genotypes suffering Fusarium head blight might accumulate several mycotoxins in their grains (see Mycotoxin Production earlier). Most commonly, DON and its metabolites were found on a worldwide basis (Snijders, 1990a). In naturally infected grain, mean DON concentrations of wheat samples collected arbitrarily ranged from 0.03 to 1.78 mg kg^{-1} with maximum values between 0.14 to 8.53 mg kg^{-1} (Snijders, 1990a). In a five-year analysis of wheat in Southwest Germany, Müller & Schwadorf (1993) found a mean DON content of 1.6 mg kg^{-1}, ranging from 0.004 to 20.5 mg kg^{-1}.

In artificial Fusarium inoculations much higher DON concentrations have been reported in wheat (e.g. Mesterházy & Bartok, 1993; Trissler, 1993). In a collaborative analysis of six wheat, six triticale and 12 rye genotypes, rye and triticale accumulated a comparable amount of DON, but wheat showed three times higher DON contents across two locations although disease severity in wheat was somewhat lower than in rye (Miedaner *et al.,* 2001b).

Because DON was detected also in healthy looking wheat (0.2-5.9 mg kg^{-1}) and rye (0.1-0.8 mg kg^{-1}) kernels (Perkowski *et al.,* 1990, 1995, respectively), and keeping in mind that mycotoxin analyses are expensive, an association between resistance traits and mycotoxin accumulation would greatly enhance progress in selection of less toxin-accumulating genotypes. Most studies found a low to medium correlation between resistance traits (such as head blight rating, relative grain weight or thousand-grain weight) and DON content in inoculation experiments.

However, disease incidence (% diseased heads per plot) and the seed infection rate seem to result in somewhat higher correlations to DON content than other factors. In most studies, the environment (location-year combination) strongly influenced the correlation. Environmentally stable conditions were only found in one winter wheat cross of largely differing parents (Miedaner, unpubl.) and in the study of Mesterházy & Bartok (1993).

A biometrical cause for only moderate correlation between resistance traits and DON accumulation might be the considerably higher genotype-environment and error variances of DON content (Miedaner *et al.,* 2001b) leading to a large bias in correlation estimates. Other causes might be rooted in the disease epidemiology. In years when disease is severe,

or in highly susceptible genotypes, kernel number per head will be low due to loss in threshing of severely shrivelled grains or their early abortion in the head. In such cases, mycotoxin content of grain samples may be underestimated. Additionally, when infections occur early, *F. culmorum* can invade the primary bundles in the head. The head sections above the infection site turn white due to water and nutrient depletion without being colonised ('wilting'). Thus, mycotoxin contamination is unlikely to occur in those parts of the head. Moreover, symptom expression or yield reduction and mycelial growth and mycotoxin production might be influenced, at least partially, by different environmental conditions. This hypothesis is supported by the moderate correlation between head blight rating and ergosterol content of infected heads (Trissler, 1993; Miedaner & Perkowski, 1996). Moreover, the correlation between a resistance trait and mycotoxin content depends on the fungal isolate used as reported for three and six individual *Fusarium* spp. isolates, respectively (Snijders & Perkowski, 1990; Atanassov *et al.,* 1994).

In general, highly resistant wheat genotypes such as Sumai 3, Wuhan 37E-OY-OFC, Wuhan 10B-OY-OFC from China, Nobeokabozu from Japan and materials developed from these sources showed very low DON contents even under favourable epidemic conditions (Mirocha *et al.,* 1994; Mesterházy, 1995b; Mesterházy *et al.,* 1999). However, the level of resistance in most breeders' materials is lower. In particular, moderate susceptible genotypes may sho high deviations from the mean regression of resistance traits on DON content (Teich *et al.,* 1987; Mesterházy *et al.,* 1994).

Durability of Fusarium resistance of wheat varieties

The success of resistant varieties in practise depends on the durability of the improved resistance. However, experimental results on this aspect are very limited for the wheat, rye/Fusarium pathosystems. Durability of resistance depends on pathogen variation, mechanisms and inheritance of resistance, and agricultural management practises (Parlevliet, 1993; Bowden & Leslie, 1994). However, the occurrence of specific adaptation of certain *F. culmorum* or *F. graminearum* isolates to a host cultivar seems to be unlikely because (1) a low degree of pathogenic specialisation was reported, (2) both *Fusarium* species are good saprophytes in soil habitats, (3) no consistent isolate-by-host genotype specificity was found in wheat and rye, and (4) host resistance was shown to be inherited quantitatively with no single genotype being completely resistant. Selection pressure on a *Fusarium* population, therefore, should be small. Even if a high level of quantitative resistance might cause a change in the composition of *Fusarium* populations in future, erosion of resistance should most likely be stepwise and slow making adequate resistance available for acceptable periods of time. These theoretical considerations are supported by experimental results of Mesterházy (1995a) who reported head blight resistance to be stable for one highly resistant genotype when tested across sixteen environments. However, the ultimate test of durability of resistance is to grow the resistant variety over a

longer period of time on a great acreage in areas where the disease occurs regularly (Johnson, 1993).

Conclusions from breeding for resistance to *Fusarium* spp. in wheat

Taking all experimental data together, resistance breeding to Fusarium diseases is not limited by the lack of genetic variability but by the limited selection response. Thus, mapping resistance gene complexes by DNA markers should provide a solution to this problem. Analysis of quantitative trait loci (QTL) are used by several groups to determine the number of genes involved, to assess gene action and interaction, to investigate the correlation between resistance and other agronomic traits, and finally to study the interactions with plant organs, plant growth stages and environments at the individual QTL level (see above). Molecular markers could be further valuable in transferring important QTLs from exotic germplasm to adapted breeding materials (marker-assisted backcrossing), or selecting within progenies of crosses between susceptible and resistant genotypes (marker-assisted selection). However, the precision of mapping QTLs for Fusarium resistances greatly depends on the heritability of resistance assessment, the number of effective factors, the distribution of QTLs across the genome (linkage) and the occurrence of non-genetic factors (Van Ooijen, 1992). In particular the high importance of host genotype-by-environment interaction and the association of host resistance with plant growth stage require a high experimental input (large number of environments, different inoculation treatments) for properly estimating the genotypic values needed for a precise QTL mapping of Fusarium resistances.

Doubled-haploid (DH) techniques might offer a further approach to enhance selection efficiency. DH lines derived from F1 crosses are completely homozygous. They allow selection for Fusarium resistance in multi-environmental tests with a maximal genetic variance between homogeneous entries and, therefore, a precise estimation of the genotypic value. Because this is not possible for selection in segregating generations, DH techniques would offer a perfect solution to select for quantitative resistant genotypes. A fast recurrent selection (RS) scheme could be realized that would be especially advantageous for inbreeding crops (Foroughi-Wehr & Wenzel, 1990). Genetically, a RS scheme based on the DH technique would be most advantageous when the resistance is mainly governed by recessive genes and these are not closely linked to undesired agronomic traits, because the probability of recombination between closely linked genes is lower in DH steps than by subsequent selfing (Becker, 1993). The inheritance studies showed a low importance of dominance for most of the pathosystems reported. A linkage to agronomically undesired traits is most probably occurring when the resistance genes are being introgressed from exotic germplasm. Then, the occurrence of undesired linkages should be either experimentally tested or the first cycle(s) of RS should be done by single-seed descent. A maximal selection gain would be achievable, if DH techniques could be

combined with efficient marker-assisted selection. A reliable selection for Fusarium resistances would then be possible directly with DNA from the regenerated single plants. Although the DH technique can be used successfully in wheat, this is not yet feasible for winter rye caused by the low regeneration rate in adapted breeding materials (Flehinghaus-Roux *et al.,* 1995).

Progress in improving Fusarium resistance may be gained in future by gene technology. Several procedures are under development, e.g. the transfer of defense-response genes, including those for anti-fungal proteins, and of genes for detoxification of DON and ZEA, the enhancement of the efficiency of transporter proteins within the plant cell for a rapid export of mycotoxins, and the production of an artificial avirulence by manipulating the host-pathogen recognition (for review see Dahleen *et al.,* 2001).

Another subject of interest for the breeder is whether reduced susceptibility of the host genotypes to Fusarium head blight will necessarily result in a correlated reduction of the mycotoxin content in the grains. This depends on the correlation between resistance traits and mycotoxin contents. Moreover, the number and relative importance of different mycotoxins should also be considered in future studies. Although DON is reported to be the most prevalent mycotoxin in *F. culmorum* and *F. graminearum* infections of small-grain cereals, seven out of 42 tested *F. culmorum* isolates were capable of producing high levels of nivalenol on a susceptible winter rye genotype (Gang, 1996). In addition, *F. graminearum* isolates can secrete high amounts of zearalenone (Marasas *et al.,* 1984). Considering the high importance of mycotoxin contamination for animal and human health, the interactions between *Fusarium* isolates, host genotypes, mycotoxin accumulation and environment should be analyzed in more detail.

The most serious lack of knowledge in resistance genetics of Fusarium diseases concerns the causes of host resistance and pathogen aggressiveness. For all Fusarium diseases in small-grain cereals, less susceptible host genotypes can be identified, but little is known about the molecular or physiological basis of the resistance mechanisms. The relative contribution of preinfectional mechanical barriers or postinfectional host defenses is also unknown. Similarly, the role of the mycotoxins in pathogenesis is still not clear. Does a highly aggressive isolate cause more disease because it produces more toxin, or does it produce more toxin because it causes more disease (Yoder, 1981)? This could only be answered if the kinetics of mycotoxin production during the very early processes of pathogenesis are monitored by highly sensitive assays. Additionally, the existence and possible role of other factors that may be responsible for aggressiveness, such as cell-wall degrading enzymes, hormones, or specific metabolites altering the host's resistance reaction, are not known. All these questions do not substantially impede selection efficiency for Fusarium resistance but their answer would greatly contribute to our understanding of the fascinating cereal-*Fusarium* interaction and may offer new approaches for resistance breeding.

2.3 Ring test with selected European winter wheat varieties

P. Ruckenbauer, T.W. Hollins, H.C. de Jong and O.E. Scholten

Genetic variation in resistance to the disease is well recognised in most parts of the world (Bai *et al.,* 2001; Miedaner *et al.,* 1999; Snijders, 1990b). Variety registration procedures in a number of countries assess genetic resistance to fusarium head blight and minimum standards for resistance are in place (Bundessortenamt, Beschreibende Sortenliste, 2000, - Hannover; NIAB, UK Recommended Lists of Cereals, 2000). For example such differences in resistance can be demonstrated clearly between 37 commercially grown varieties at the Monsanto Cambridge site in the trial year 2000 (Table 1).

Wheat varieties are most susceptible at the flowering stage, growth stage (GS) 61 – 69, (Tottman & Broad, 1987), so it is important when critically comparing varieties to inoculate at a consistent stage of growth. In this way lines with different maturity, often from different geographic regions, can be compared. In this experiment disease was then assessed at defined intervals after inoculation (350, 400 & 450 ^{o} centigrade days), which accounted for average temperature as well as time to minimise inaccuracies due to changing average temperatures during disease development.

Large differences in resistance among current European varieties were observed. In these very disease conducive conditions up to 60% infection was observed on some UK varieties (Charger) whereas the widely grown German variety Batis had 20% disease only. Several lines were even less diseased (Sumai#3, etc.) but most of these are low yielding and/or poorly adapted to northwest Europe. Their resistance is currently being incorporated in breeding programmes throughout the world.

Stability of resistance

Among the wheat breeders of the participants of Mycotochain (Monsanto at Cambridge, UK, IFA-Tulln at Tulln, Austria and Cebeco Seeds at Lelystad, The Netherlands) a ring test with currently grown winter wheat varieties within the EU was performed. These field trials were sown at locations in Cambridge, UK; Tulln, Austria; Lelystad, The Netherlands and Wallerfing, Germany, with variable treatments concerning irrigation and inoculation.

Within the ring test trials seventeen varieties from five different European countries were tested at four locations, some with artificial infection and some with natural infection. Disease estimates demonstrated that, in general, varieties ranked similarly at the different

sites (Table 2) although some varieties (e.g. Semper) showed some inconsistency across sites. Results of Wallerfing are not presented because infection levels were too low.

Table 1. Amount of Fusarium head blight on winter wheat varieties after artificial inoculation with *Fusarium culmorum* in Cambridge in 2000. Varieties inoculated individually at anthesis and assessed for disease by thermal time.

Variety	Date inoculated[1]	Assessed at: (°C days)			Average disease[3]
		350[2]	400	450	
95NYP1256	143	0.2	0.3	1.5	1
Sumai#3.2	141	0.2	0.3	1.7	1
Zhefeng	141	0.5	0.6	1.7	1
Ning 7840	148	1.8	1.8	3.2	2
Patton	148	0.7	2.5	6.4	3
Heyne	152	0.6	3.2	9.2	4
Jagger	143	0.3	5.7	9.9	5
Freedom	150	3.9	5.8	12.3	7
Cockpit	160	2.7	7.4	13.7	8
Piko	164	1.6	9.9	20.3	11
Soissons	152	3.4	8.0	21.9	11
Ludwig	160	3.9	12.0	20.0	12
Kraka	164	3.6	11.9	21.8	12
AC Winsloe	154	4.0	8.6	24.7	12
Greif	160	7.3	10.5	20.2	13
Frelon	154	6.0	9.2	23.1	13
Petrus	160	3.0	19.1	24.3	15
Achat	164	2.8	17.3	27.8	16
Astron	164	6.1	15.7	26.8	16
Batis	164	5.0	21.5	33.3	20
Isengrain	154	10.9	17.3	35.6	21
Huntsman	160	10.9	26.4	35.5	24
Tremie	152	12.7	22.7	40.9	25
Smart	164	7.3	27.9	47.1	27
Savannah	164	8.7	28.8	50.5	29
TP1689/-/-/18	164	13.1	31.5	45.5	30
Fruhgold	157	12.1	29.9	49.6	31
Semper	164	8.8	34.3	49.6	31
Ritmo	164	18.6	32.7	43.2	31
Rialto	160	15.5	36.6	58.0	37
Contra	160	15.6	38.1	58.8	37
Shango	164	19.7	39.6	61.2	40
Consort	164	15.3	44.7	69.5	43
Tower	164	13.1	46.7	70.0	43
Equinox	160	26.4	55.1	68.8	50
Hanseat	160	30.8	54.6	73.6	53
Charger	160	30.6	65.8	80.4	59
5% LSD		12.5 (within date)			7

Inoculated with a mixture of two F. culmorum isolates in 6 replicates and grown under irrigation

[1] *days after 1 January*

[2] *average of daily maximum and minimum temperature summed over days*

[3] *percentage of ear area with symptoms of FHB*

Table 2. Percentage of disease on heads of winter wheat at four locations in Europe carried out in 2000 under artificial infection in the UK and Austria and natural infection in Germany and The Netherlands.

Variety	Cambridge, UK	Tulln, Austria		Lelystad, Netherlands
	F.c[1].	F.c[1].	F.g.[2]	F.c[1].
Soissons	11	22	21	7
Ludwig	12	19	24	4
Petrus	15	14	15	1
Achat	16	26	32	4
Batis	20	18	15	3
Isengrain	21	26	26	6
Tremie	25	58	58	17
Ritmo	31	54	58	13
Semper	31	29	42	4
Contra	37	46	42	11
Shango	40	54	62	14
Consort	43	32	46	9
Tower	43	21	20	3
Hanseat	53	76	62	23
Charger	59	76	76	28
Equinox	50	62	46	11
Bercy	-	83	83	30
5%LSD	8.8	12.3	14.7	

[1] *Fusarium culmorum;* [2] *Fusarium graminearum*

In addition in Austria varieties were tested against six separate pathogens that have the potential to cause FHB (*F. culmorum, F. graminearum, F. avenaceum, F. poae, F. sporotrichoides, F. subglutinans*). Only two species caused substantial disease on the susceptible lines (see Table 2) and again variety ranking was similar. These results are in agreement with those reported by Van Eeuwijk *et al.* (1995) who similarly tested varieties covering a range of resistance to different pathogens in different regions of Europe.

Relationship between disease and mycotoxin content

Specific end use requires grain free from mycotoxins so it is important to the grower, and plant breeder, to know that decreased disease in the field leads to decreased mycotoxin content in the grain. Several studies have show this to be the case with either natural epidemics (Wosnitza, pers. comm.) or with artificial infection (Bai *et al.,* 2001; Miedaner *et al.,*, 1999), the latter often when disease levels, and mycotoxin content, are high. To confirm these observations, the same three partners of Mycotochain analysed the DON content of the 17 wheat samples of the ring trial by means of GC and HPLC methods (Table 3). In situations where M. nivale is involved, a high visual infestation in the field does not always mean that mycotoxins are present. It was noted that the variety Petrus, with the best field scores (Table 2), showed the lowest DON content across all trial sites whereas the samples of the most susceptible winter wheat variety of the ring test Hanseat had the highest DON contents, independently from sites and treatments.

Table 3. DON content of wheat samples in ppm and in % of the experimental mean determined in samples taken from 4 locations in 2000.

Cultivar	Tulln (A)		Cambridge (UK)		Lelystad (NL)		Wallerfing (D)		Mean over Locations	
	ppm	%	ppm	%	ppm	%	ppm	%	ppm	%
Petrus	2.51	14	13.02	48	2.32	18	0.20	21	4.51	31
Soissons	6.05	34	10.95	40	3.03	24	0.60	64	5.16	35
Tower	4.68	26	13.45	50	4.92	38	1.09	117	6.03	41
Batis	3.44	19	21.88	81	5.72	45	0.36	38	7.85	53
Achat	10.59	59	18.30	68	7.09	55	1.06	113	9.26	63
Isengrain	5.29	29	32.68	121	3.05	24	0.85	91	10.47	71
Ludwig	17.94	100	26.21	97	5.15	40	1.16	124	12.61	86
Semper	15.85	88	29.23	108	7.28	57	0.64	69	13.25	90
Contra	15.97	89	27.20	101	15.00	117	0.56	60	14.68	100
Bercy	33.42	186	21.54	80	13.09	102	0.77	82	17.20	117
Tremie	24.03	134	36.00	133	11.91	93	1.10	117	18.26	124
Equinox	16.05	89	36.00	133	23.54	184	0.97	104	19.14	130
Consort	31.91	178	32.55	120	16.70	130	1.72	184	20.72	141
Charger	27.42	153	36.00	133	18.62	145	0.97	104	20.75	141
Ritmo	27.07	151	32.67	121	25.89	202	1.02	109	21.66	148
Shango	26.79	149	36.00	133	24.39	190	2.08	222	22.31	152
Hanseat	36.00	201	36.00	133	30.06	235	0.74	79	25.70	175
Location-Mean	17.94	100	27.04	100	12.81	100	0.93	100	14.68	100

The correlation between DON and percentage of diseased spikelets after artificial inoculation in Tulln is 0.80 (Figure 1). This result clearly indicates that the levels of DON in resistant varieties in general are lower than in susceptible varieties. The Tulln samples were furthermore subjected to two DON analysis methods: GC (IFA-Tulln) and HPLC (PRI-Wageningen) in order to compare both methods (Figure 2). A high correlation of 0.87 was obtained between both analythical methods.

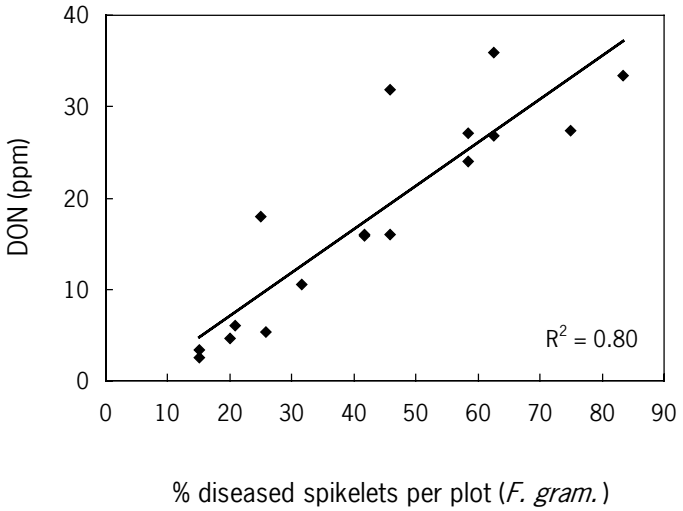


Figure 1. Correlation between DON (ppm) and percentage of diseased spikelets after artificial inoculation with *F. graminearum* (Tulln, 2000).

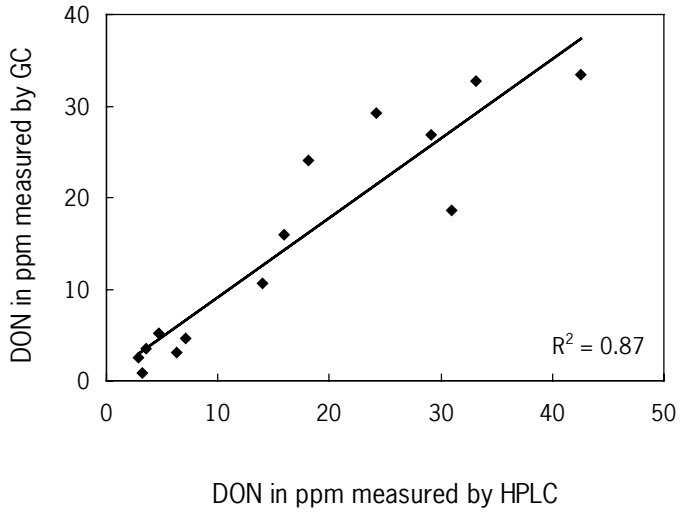


Figure 2. Correlation between DON (ppm) measured by HPLC and GC of diseased spikelets after artificial inoculation with *F. graminearum* (Tulln, 2000).

2.4 Control of the fungus through the use of fungicides

P. Jennings

Control of FHB by fungicides has been inconsistent. In practice fungicides are applied with the intention of controlling other head diseases as well as Fusarium and even when correctly timed for FHB may only be 60-70% effective. Agrochemical companies are addressing the problem of fungicide activity to FHB and improved active ingredients are continually being tested. There are several areas where important decisions have to be made in order to achieve optimal control of FHB.

Product choice

FHB is associated with a complex of species on the ear, a product which shows good activity against one FHB pathogen may not be active against another so correct product choice is of particular importance. Such differential control of FHB pathogens was shown in experimental field trials carried out by Jennings *et al.* (2000). Trials inoculated at mid-

anthesis with a mixed conidial suspension of FHB pathogens showed that tebuconazole (as Folicur) effectively controlled the toxigenic *Fusarium* species present on the ear, but showed little control of the non-toxigenic *M. nivale*. However, the application of a strobilurin fungicide, azoxystrobin (as Amistar), controlled *M. nivale* but not the *Fusarium* species present. Similar differential control by tebuconazole and azoxystrobin has also been reported in trials naturally infected by FHB pathogens (Simpson *et al.,* 2001). Differential control of fusaria and *M. nivale* also exists within the MBC group of fungicides, however this has arisen through the widespread development of resistance in populations of *M. nivale* (Locke *et al.,* 1987; Pettitt *et al.,* 1993).

Other products with good efficacy towards fusaria responsible for FHB, include metconazole [as Caramba (Jennings *et al.,* 2000)], prochloraz [as Sportak (Matthies & Buchenauer, 2000)], and epoxiconazole and carbendazim [as Opus and Derosal WDG respectively (Nicholson *et al.,* unpublished data from HGCA project No. 2067). Many of the strobilurin fungicides, such as azoxystrobin, trifloxystrobin and kresoxim methyl, show good efficacy towards *M. nivale*.

In most cases the reduction in disease symptoms which followed fungicide application also gave a reduction in deoxynivalenol (DON) contamination of grain compared to control plots. However, under certain circumstances it has been reported that application of some fungicides can lead to increases in DON levels in the field (Jennings *et al.,* 2000). Trials carried out in 1998 and 1999 showed that following the application of azoxystrobin DON levels in grain increased in 1998, but not in 1999. The difference in the two years was the FHB pathogen mixture found on the ear. In 1998, *M. nivale* and *F. culmorum* were both detected on the ear in control plots, whereas in 1999 *M. nivale* was not detected. This suggests that the combination of FHB pathogens found on the ear is important in determining whether increased DON production occurs following the application of azoxystrobin. Where both *M. nivale* and *F. culmorum* were present on the ear the removal of *M. nivale*, through use of azoxystrobin, reduced competition on *F. culmorum*, resulting in increased DON levels in grain. Where *M. nivale* was absent from the ear, the application of azoxystrobin did not alter competition between FHB pathogens and had therefore no effect DON levels. Similar increases in DON have also been seen from 'on-farm' data (Turner *et al.,* unpublished data). In 1998, a survey carried out on UK wheat grain indicated the predominant pathogen was *M. nivale*, with low levels of Fusaria also present (Turner *et al.,* 1999). Examination of the fungicides applied to each field analysed for toxin and FHB pathogen indicated that where azoxystrobin was applied to the ear the level of DON was higher than where there was no azoxystrobin application.

Timing of application

Arguably the timing of fungicide application is more important than product choice when trying to control FHB and mycotoxin contamination of grain; no matter how effective the fungicide if it is applied at the wrong time it will not control FHB. Mid-anthesis is the most susceptible time for infection of wheat by FHB pathogens (Sutton, 1982) and as such is the most appropriate time to apply a fungicide spray aimed at FHB. Work carried out by Homdork *et al.* (2000) and Matthies & Buchenauer (2000) have both highlighted just how narrow the window for fungicide application is for optimum control of FHB pathogens.

Matthies & Buchenauer (2000) investigated timing of fungicide application on disease development using trials artificially inoculated with *F. culmorum* at mid-anthesis. Fungicide treatments applied were tebuconazole or prochloraz at 8 days pre, 2 or 9 days post inoculation. The most effective treatment timing for both fungicides was 2 days post inoculation. The efficacy of the fungicide treatments decreased with increasing time interval between fungicide application and inoculation. A similar set of experiments carried out by Homdork *et al.* (2000) using *F. culmorum* inoculation with tebuconazole sprayed at either 3 days pre and/or 5 days post inoculation, again showed the closer the timing of the spray and inoculation, the better the efficacy of the fungicide.

Some reports of fungicide failure in the field can be directly attributed to incorrect timing of application. Milus & Parsons (1994) concluded after testing the efficacy of seven fungicides against *F. graminearum* that the prospects for chemical control of head blight were poor. Fungicides were applied to plots at the end of heading growth stage. To each fungicide treated plot *F. graminearum* was inoculated three times, at the beginning, mid and end of anthesis (this equated to 2, 5 and 7 days post fungicide application). The results showed no reduction in levels of either head blight or DON following fungicide treatment. However, as already highlighted, inoculum landing on the ear seven days post fungicide treatment would not be effectively controlled. A more appropriate growth stage for the fungicide application would have been mid-anthesis. At this growth stage the time interval between fungicide application and inoculum arrival at the ear would be at its optimum at between two and three days.

Application rate

To achieve the optimum efficacy against FHB pathogens a fungicide must be applied at the manufacturers recommended rate. Work carried out by Nicholson *et al.* (unpublished data from HGCA project No. 2067) showed that halving the rate of several fungicides led to significant reductions in control of FHB disease levels and mycotoxin production.

At present, the challenge of controlling FHB and mycotoxin contamination of wheat grain will only be met through an integrated approach to crop protection. The use of fungicides forms an essential part of this approach. However, the inappropriate use of fungicides through incorrect product choice, timing of application or rate of product can significantly reduce the efficacy of a fungicide application.

Conclusions

- Differential control of FHB pathogens exists between fungicides. It is important to determine which FHB pathogens are likely to be present on the ear in order to make an informed choice on the appropriate fungicide to use. In some instances it may be appropriate to apply a mixture of products.
- Timing of application is critical. For optimum control fungicides should be applied at mid-flowering.
- Fungicides should always be applied at the manufactures recommended rate. Any reduction in the rate applied will reduce the efficacy of the fungicide.

2.5 Risk factors in Fusarium head blight epidemics

E.T.M. Meekes and J. Köhl

Introduction

Fusarium head blight (FHB), also known as Fusarium ear blight or scab, can be linked with up to 17 causal agents in small grain cereals, although most records concern five species: *Fusarium culmorum*, *F. graminearum*, *F. avenaceum*, *F. poae* en *Microdochium nivale* (Parry *et al.*, 1995). FHB is considered to be part of a complex of cereal diseases, since many of the species responsible for FHB can also cause seedling blight and foot rot. However, the epidemiological relationship between the three diseases is not always clear.

Central to the disease cycle is the survival of these pathogens. The above-mentioned species, except for *F. poae*, are able to survive saprophytically on crop residues. The spores produced on this debris are considered to be the major source of FHB (Parry *et al.*, 1994).

Damaged caused by FHB can vary between regions, years and cropping systems. A combination of warm weather and rain before and during anthesis will enhance chances on FHB. In addition, several cultivation factors can influence the severity of a FHB epidemic like: 1) crop rotation, 2) soil preparation, 3) choice of wheat cultivar, 4) use of fungicides and/or plant grow promoters, 5) use of fertilizer, and 6) weed control (Bauer, 2000; Meier *et al.*, 2000), not necessarily in this order. All *Fusarium* species are able to produce one or more mycotoxins, but *M. nivale* is not (Chelkowski, 1998). The irregular pattern of FHB epidemics has led to an underestimation of the potential danger of a toxin contaminated wheat crop (Snijders, 1990a).

Crop rotation

In Europe as well as in North America, *F. graminearum* seems to replace other pathogens like *F. culmorum* and *F. avenaceum* as major causal agent of FHB (Clear & Patrick, 2000; Rintelen, 2000). This phenomenon is partly attributed to the inclusion of maize in the cropping system and an increase of short rotation intervals or abandonment of crop rotation al together (McMullen *et al.,* 1997; Rintelen, 2000).
Maize as previous crop of wheat provides a higher risk for FHB incidence and DON contamination of wheat than potato, sugarbeet, wheat, barley or soybean (Beck & Lepschy, 2000; Dill-Macky & Jones, 2000). The amount of decomposition of crop residues correlates negatively with the amount of *Fusarium* spores produced in spring especially when grain-maize is grown (Beck & Lepschy, 2000; Pereyra *et al.,* 1999). Maize is not only a

good host for *F. graminearum*, its residues also take longer to decompose than for instance wheat residues and therefore providing ample opportunity for *F. graminearum* to survive the winter saprophytically (Beck & Lepschy, 2000). In Northwestern Europe the increase of silage maize acreage coincided with the increase of FHB caused by *F. graminearum*. In Bavaria (Germany), for instance this increase was 2-3 fold from 1972 to 1988 (Eder, 2000), in the Netherlands this increase was even more extreme 50 times increase in area from in 1970 to 1999 (Anonymous, 1970; Anonymous, 1999). In the 1990's cultivation of grain-maize also increased, posing a higher risk, because it leaves more residues in the field than silage maize (Beck & Lepschy, 2000). In North America an additional explanation for further increasing of FHB is the high percentages of cultivated acres planted to susceptible host crops and short rotations (McMullen *et al.,* 1997).

Soil preparation

Crop residues play a role in the disease cycle, especially when decomposition is slow. Residues at the soil surface decompose considerable slower than buried residues, increasing the inoculum potential of fungi causing FHB (Pereyra *et al.,* 1999). The introduction of no-tillage practices – to reduce soil erosion – has led to an increasing amount of crop residue left at the soil surface, posing a higher risk for FHB. Moldboard plow treatments led to considerable lower crop residue cover, FHB incidence and DON content compared to chisel plow or no-tillage treatments (Dill-Macky & Jones, 2000). No tillage after a previous maize crop is the highest risk factor leading to high levels of DON contamination (Bauer, 2000; Dill-Macky & Jones, 2000).

Host range

Besides crop residues there are several other possible inoculum sources of which their role is not exactly known. Other grasses, like ryegrass, timothy and fescue are susceptible to the same causal agents of head blight (*e.g.* (Engels & Kramer, 1996; Holmes, 1983). In most cases the fungi are associated with seedling diseases and foot rot, but some report also ear infection (Cagas *et al.,* 1998). Grasses infected with *Fusarium* spp. also tested positive on toxin presence (Engels & Kramer, 1996). There is strong evidence that species causing seedling and foot diseases in grasses also cause the same diseases in wheat, but it is unknown what role they play in FHB.
The same fungal species causing head blight have been isolated from several dicotyl weeds, all of these isolates were able to infect wheat. Weed control did lead to lower Fusarium infection, although this effect was not quantified (Jenkinson & Parry, 1994a). *Fusarium* spp. potentially causing FHB can also be isolated from green manure plants like clover and lupin. However, the role of other host plants of *Fusarium* spp. and *M. nivale* in FHB epidemics is unknown.

Weather conditions

Crop rotation, soil preparation and presence of alternative hosts determine, among others, the inoculum potential at the beginning of the growing season and during anthesis. Severity of FHB is related to inoculum potential, but occurrence of FHB is highly dependent on weather conditions. If weather conditions for infection during anthesis are conducive the damage by FHB will be limited, despite high level of inoculum. Weather conditions influence different parts of the infection cycle, having the following influences on the complex of diseases caused by *Fusarium* spp. (Parry *et al.,* 1995):

- Warm dry soil conditions during the early part of the growing season promote the development of Fusarium foot rot and the production of inoculum on stem bases.
- Intense rainfall during the period of anthesis can effectively disperse *Fusarium* inoculum to ears when they are most susceptible to infection (Jenkinson & Parry, 1994b). But also wind dispersed ascospores of *Giberella zeae* (*F. graminearum*) can cause ear infections (Fernando *et al.,* 1997; Francl *et al.,* 1999), but rain is still needed to set these spores free.

- Prolonged periodes of warm humid conditions are conducive to infection of cereal ears by *Fusarium* spp.. Studies using *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *M. nivale*, have shown that, temperatures above 15 °C and wetness periods of at least 24 h are required for optimum infection of winter wheat ears (Parry *et al.*, 1995).

Conclusion

Preventative measures to reduce inoculum potential of FHB is a major option to reduce the risk of FHB epidemics. Survival of *Fusarium* spp. causing FHB should be limited by optimiaing crop rotation tillage and control of weeds as potential alternative hosts. Development of novel methods to enhance decomposition of cop residues of FHB infected crops may be helpful to achieve this goal.

3. Fusarium mycotoxins in cereals: storage, processing and decontamination

A. Visconti and A. De Girolamo

3.1 Introduction

Fungi of the genus *Fusarium* are common plant pathogens occurring world-wide in a variety of crops, although they are mainly associated with cereals. *Fusarium* species can produce over one hundred secondary metabolites, some of which can unfavourably affect human and animal health. The most important Fusarium mycotoxins, that can frequently occur at biologically significant concentrations in cereals, are fumonisins (mainly B_1 and B_2), zearalenone and trichothecenes (deoxynivalenol, nivalenol and T-2 toxin). These compounds can occur naturally in agricultural and food products, either individually or as specific clusters of two or more of them depending on the producing fungal species (or strain); they have been implicated (alone or in combination between themselves and/or with other mycotoxins) as the causative agents in a variety of animal diseases and have been associated to some human diseases. Corn is the crop most susceptible to contamination by all Fusarium mycotoxins (particularly important are fumonisins), while wheat and barley are subjected to contamination of deoxynivalenol, nivalenol and, at lesser extent, of zearalenone and T-2 toxin and related trichothecenes. Fungal infection and toxin production can occur in the field, during the storage period and post-harvest. Prevention through pre-harvest management is the main goal of agricultural and food industries for controlling mycotoxin contamination. When contamination occurs in the field and the product is to be used as human food or animal feed, the hazards associated with the toxin must be managed through harvest, storage, transportation and post-harvest. Consequently, an integrated management system, able to control every phase of production, is needed.

This chapter will review the main factors responsible for Fusarium mycotoxins contamination during grain storage and decontamination strategies, including food processing, used to reduce the risk associated with the consumption of mycotoxin contaminated food or feed.

3.2 Fungal and mycotoxin contamination in stored crops

During storage the cereal crop undergoes quality loss characterised by increased susceptibility to infection by fungi, insects and mites which directly or indirectly affect grain quality. Depending on the geographic origin and the storage conditions, fungi belonging to *Fusarium*, *Penicillium* and *Aspergillus* species, insects, yeast, and bacteria are the main responsible for spoilage of stored products. Nevertheless, fungal growth does not necessarily denote the presence of mycotoxins because not all fungal species and strains are toxigenic. Fungal invasion and mycotoxins contamination of agricultural products lead to losses in terms of quantity, market value and quality of food and feed production due to changes of colour, texture and taste (Mills, 1989; Brooker *et al.,* 1992), development of fungal doors (Abramson, 1991; Kaminski & Wasowicz, 1991) and reduction of seed germination (Sauer, 1988). Energy and nutritional value changes in term of losses of carbohydrates, proteins, amino acids and vitamins and increases of fatty acids may also occur (Ominski *et al.,* 1994). The species composition and the production of secondary metabolites in crop entering storage may vary depending on the presence of storage fungal infection and mycotoxins originated in pre-harvest and harvest.

Interactions of several factors, such as water activity, moisture content, rapidity of drying, temperature, time, composition of the substrate, mechanical damages to the seed, oxygen and carbon dioxide availability, fungal abundance, prevalence of toxigenic strains, spore load, microbial interactions and invertebrate are responsible for fungal growth in stored crops and the eventual development of mycotoxins (Abramson, 1998).

The water availability, that may be expressed as the moisture content (MC) or water activity (a_w), i.e. the ratio of vapour pressure of the product to that of pure water, influences fungal growth and stability of stored products (Pitt & Hocking, 1985). A tolerance to low a_w corresponds to the minimum a_w at which fungal spore germination and hyphal growth can occur. *Fusarium* spp. are usually described as field fungi but they occasionally develop in storage when a_w is higher than 0.90 (MC > 20-22%), with a minimum between 0.87 and 0.89 a_w and an optimum between 0.98 and 1.00 a_w. In order to obtain a good preservation of stored crops from fungal growing, the water content of grain must be removed by drying the crop until the necessary MC for safe crop storage reaches value ranging from 8% to 16.5% depending on the cereals (Bottalico, 1997; Lacey & Magan, 1991). However, too rapid heating may cause stress cracks in corn kernels increasing susceptibility to fungal invasion, while overheating may alter the relationship between water availability and water content (Lacey & Magan, 1991).

Fungal contamination is also influenced by the temperature and, as with water content, each fungal species has characteristic minimum, optimum and maximum temperature requirements for growth. Some fungal species may have minimum close to or below 0°C, whereas others have maximum up to 55°-60°C; the optimum value for the growing of

Fusarium spp. and production of mycotoxins on cereals is around 22°-27°C, except for T-2 toxin and zearalenone production that require lower temperatures, such as 2°-12°C and 12°-15°C, respectively (Bottalico, 1997).

Mycotoxigenic fungi are able to grow on several substrates and the nature and amount of mycotoxin production depend on physical and chemical characteristics of the substrates. These include several parameters such as available water, mechanical resistance to packing and thermal conductivity, fat and protein content, trace mineral, amino acid and fatty acid composition. Sometimes, the presence of other micro-organisms, such as bacteria or other filamentous fungi, may alter fungal growth and mycotoxin production. The growth rate of the fungus depends on the a_w, the temperature of the grain, the gaseous composition of the intergranular atmosphere and the biological properties of the competitive species (Ominski *et al.,* 1994; Lacey & Magan, 1991).

Mechanical damages from harvesting equipment together with insect, rodents or birds damage can break the outer seed coat and facilitate fungal infections. During respiration insects, together with other micro-organisms (e.g. bacteria or other fungi), can modify the environments releasing energy, which causes heating, and water, which causes moisture migration. Heating may occur in local 'hot spots' that, having a higher water content than the bulk, may represent suitable sites for fungal infection. Certain kinds of stored-grain insects develop larvae and pupae within the infested kernel and carry numerous spores of storage fungi or through their faecal material may provide substrates for colonisation. In addition, fungi may either attract or inhibit insects or mites and may also provide food for them.

3.3 Food processing and detoxification of Fusarium mycotoxins

Although the prevention of mycotoxin contamination in the field is the main goal of agricultural and food industries, once the crop becomes infected under field conditions, fungal growth will continue during post-harvest phases and storage. Therefore several strategies for detoxification or decontamination of commodities containing mycotoxins have been reported and may be classified as chemical, physical, microbiological. Food processing that may involve physical and/or chemical decontamination could be considered as a strategy to destroy or redistribute mycotoxins. The ideal decontamination procedure should be easy to use, inexpensive and should not lead to the formation of compounds that are still toxic or can alters the nutritional and palatability properties of the grain or grain products. Strategies for intentional detoxification or decontamination of commodities containing mycotoxins can be classified as chemical, microbiological or

physical. In addition to these methods, food processing, that may involve physical and/or chemical decontamination, could destroy or reduce mycotoxins.

Food processing

Processed foods are very complex systems because processing not only alters the food but also ads new ingredients and conditions, so many new interactions can occur. Fusarium mycotoxins are relatively stable under most food processing conditions and can be detected in most cereal based foods. These operations include wet and dry milling, fermentation, nixtamalization, and thermal processing such as baking, cooking, extrusion, roasting and malting. In general, factors that may influence the fate of a mycotoxin during food processing include the food matrix itself, the moisture content, whether the mycotoxin is introduced into the matrix for experimental study by natural contamination or by spiking, and its concentration. Wet and dry milling are procedures that distribute mycotoxins in the different fractions depending on the commodity and the type and level of contamination. In wet-milling of deoxynivalenol-contaminated corn much of the deoxynivalenol went into steep liquor, although measurable amounts remained in the starch (Scott, 1984). In wet-milling of zearalenone-contaminated corn it was shown that mycotoxin was mainly concentrated in the gluten (49% to 56%), followed by milling solubles (17% to 26%), while the starch fractions, corresponding to the moist products of the milling, were relatively free of zearalenone (Lopez-Garcìa & Park, 1998). Also for fumonisins-contaminated corn it was observed that the starch did not contain detectable fumonisin B_1 residues. Fumonisin B_1 remained in the fibber, gluten and germ fractions at 10-40% the level found in the starting corn (Bennett *et al.,* 1996). Dry-milling did not significantly reduce deoxynivalenol and zearalenone levels in grains, whereas it was effective on fumonisins; in particular Katta *et al.* (1997) in experimentally dry-milled corn samples found the highest concentration of fumonisins in the bran and fines, whereas germ, flaking grits and grits for extrusion processing contained little or no fumonisins. It is important to realise that in some cases there may be increases in mycotoxin levels in some processed products. For example, malt may contain more zearalenone and deoxynivalenol than the unmalted barley; bran obtained after polishing barley tends to contain higher concentrations of deoxynivalenol, nivalenol and zearalenone; levels of T-2 toxin increased in corn germ after wet milling of corn; yeast doughnuts were shown to have higher concentrations of deoxynivalenol than in the flour used (Visconti *et al.,* 2000).

The traditional method used to produce masa or tortillas flour, called nixtamalization, and consisting of boiling and soaking of corn in lime water ($Ca[OH]_2$) has been used to study the fate of fumonisin B_1. Nixtamalization considerably reduced fumonisin B_1 concentration in the finished products but produced hydrolysed fumonisin B_1 (HFB_1) which was still toxic (Murphy *et al.*, 1996). Treatments of fumonisin B_1-contaminated corn simulating modified nixtamalization (heat treatment with $NaHCO_3$ + H_2O_2 alone or with

$Ca(OH)_2$) gave 100% reduction of fumonisin B_1 and reduced brine shrimp toxicity by ca. 40% (Park *et al.,* 1996). A combination of heat and treatment with lime water in the process of making tortillas, reduced zearalenone by 59 to 100% and deoxynivalenol by 72 to 82% in two corn samples (Charmley & Prelusky, 1994).

The fermentation processes did not destroy fumonisin, and 85% of the toxin could be recovered in all products. Consumption of these products, still containing fumonisin, by pigs, horses or other animals sensitive to relatively low levels of this toxin, could be detrimental (Lopez-Garcìa & Park, 1998). Other investigations showed that there was no carryover of zearalenone to distilled ethanol during fermentation, however solids containing 2 to 2.5 times the level of zearalenone in the starting product, were recovered (Lopez-Garcìa & Park, 1998).

Fumonisins are considered to be fairly heat stable compounds. No loss of fumonisin B_1 was observed when *F. verticillioides* culture material was boiled in water for 30 min and dried at 60° for 24h (Alberts *et al.,* 1990) or during cooking of polenta for 20-30 min in boiling water (Pascale *et al.,* 1995). Moreover, several investigations, focused on the effect of thermal processing on the stability of fumonisins, showed that sometimes thermally processed corn products contained lower concentrations of fumonisins than unprocessed products depending on the time and the temperature of the processes. In particular, Jackson *et al.* (1997) found that baking corn muffins at 175°C and 200°C for 20 min result in 16.3% and 27.6% reduction of fumonisin B_1, respectively; no significant reduction in fumonisin B_1 level was found when spiked corn masa was fried at 140° to 170°C for 0 to 6 min, whereas frying chips for 15 min at 190°C resulted in 67% loss of fumonisin B_1. Pineiro *et al.* (1999) found that frying polenta or autoclaving corn meal produced reductions of fumonisin B_1 of 70-80% with no conversion to the hydrolysed form. Jackson *et al.* (1996a,b) found that the rate and extent of fumonisins decomposition in aqueous solutions increase with processing temperature; in particular from < 27% at ≤ 125° C to > 80% at ≥ 175° C, for 60 min, depending on buffer pH. Losses of fumonisin B_1 and fumonisin B_2 exceeding 70% were obtained in dry corn meal heated at 190° C for 60 min and complete loss at 220°C for 25 min (Scott & Lawrence, 1994). In another study it was observed that fumonisins in spiked and naturally contaminated corn meal were unstable under roasting conditions (218°C for 15 min) but were stable under canning (121°C up to 87 min) and baking conditions (204°-232° C for 20 min), probably because the canned and baked products reached lower internal temperatures than the roasted products (Castelo *et al.,* 1998).

Extrusion processing is one of the most versatile technologies available to the food industry and it is used in the production of breakfast cereals and snack foods. During extrusion cooking high temperature and pressure are reached causing gelatinization of corn starch. The stability of fumonisin B_1 and B_2 during processing of corn flakes was investigated by analysing the naturally contaminated raw material (corn flour), intermediate product (extruded, but not roasted corn flakes) and final product (roasted corn flakes). It

was observed that about 60-70% of the initial amount of fumonisins was lost during the entire cycle of corn flakes processing, with less than 30% losses occurring during the intermediate extrusion step (70°-170°C for 2-5 min) (De Girolamo *et al.,* 2001). However, corn flakes processing may vary considerably from plant to plant depending on the time and temperature of cooking, the kind and amount of additives (salts, iron, vitamins, sugars, etc.) and on the quality of the raw material (corn variety, with or without germ and bran layers, etc.). These different parameters, while being determinant for the quality of the final product, may also affect the degree of fumonisin reduction during processing. For studies on mycotoxin decontamination during food processing, it is essential to use analytical methods which are reliable for both the raw starting material and the processed product, such as the one adopted by the AOAC International for fumonisins in corn and corn flakes (De Girolamo *et al.,* 2001;Visconti *et al.,* 2001).

Heating zearalenone-contaminated corn at 150°C resulted in only 0% to 28% reduction of zearalenone, depending on the duration of the process, while a greater reduction (69%) was observed by heating wheat flour cake at 200°C for 60 min (Bennett *et al.*, 1980; Matsuura *et al.,* 1981). Similar or greater zearalenone reductions were achieved at lower temperature (120°C) with extrusion cooking (Ryu *et al.,* 1999).

Chemical decontamination

A wide variety of chemicals, including calcium hydroxide monomethylamine, sodium bisulfite, moist and dry ozone, chlorine gas, hydrogen peroxide, ascorbic acid, hydrochloric acid, sulfur dioxide, formaldehyde, ammonia and ammonium hydroxide, have been found to be effective (at different extents) against several Fusarium mycotoxins, including deoxynivalenol, zearalenone, T-2 toxin, diacetoxyscirpenol and fumonisin B_1 and B_2.

Calcium hydroxide monomethylamine has been used to decontaminate feeds containing T-2 toxin and diacetoxyscirpenol at 10 to 20 mg/kg; the success of the procedure is dependent on the moisture content of the feed and the processing temperature. In particular, about 50% mycotoxin reduction was observed when the treatment was performed at about 25°C and 10% moisture in 4 hours; when the moisture content was increased to 25% T-2 toxin level was reduced by 95 to 99% (Bauer *et al.,* 1987). Sodium bisulfite solutions are quite effective in reducing deoxynivalenol levels of contaminated corn and wheat; in particular up to 85% deoxynivalenol reduction was observed when corn contaminated with 4.4 mg/kg of deoxynivalenol was treated with aqueous sodium bisulfite (1.25%) at 80°C for 18 hours (Young *et al.,* 1987). A greatest reduction (up to 95% deoxynivalenol) was achieved when the corn contaminated with 540 mg/kg deoxynivalenol was autoclaved for 1 h at 121°C in the presence of 8.33% aqueous bisulfite (Young *et al.,* 1987). These treatments resulted in the formation of deoxynivalenol-sulfonate conjugate which is unstable due to its alkaline hydrolysis into deoxynivalenol

under certain baking and processing conditions (Young *et al.,* 1986). For this reason and because deoxynivalenol-sulfonate conjugate affects the rheological properties of flour, this treatment might not be suitable for direct application to human foods. Nevertheless, this treatment has been proposed for decontaminating deoxynivalenol-contaminated corn destined for use in pig feeds because deoxynivalenol-sulfonate conjugate was found nontoxic to pigs and reduced the short-term toxic effects of deoxynivalenol-contaminated corn on feed intake and body weight gain in pigs (Young *et al.,* 1987).

Moist ozone and dry ozone were able to reduce deoxynivalenol concentration in contaminated corn (at ca. 1mg/g) up to 90% and 70%, respectively (Young, 1986), whereas moist ozone had little effect on deoxynivalenol-contaminated wheat. Fumonisin B_1 was rapidly reduced by ozone gas without obtaining an effective detoxification since the resulting solutions were still positive in two bioassay systems (McKenzie *et al.,* 1997). The heating of fumonisin B_1 in an aqueous solution with reducing sugars such as D-fructose or D-glucose resulted in the formation of N-(carboxymethyl)-fumonisin B_1 that appeared less toxic than fumonisin B_1 when tested on cell tissue culture (Lu *et al.,* 1997; Howard *et al.,* 1998; Murphy *et al.,* 1995). A possible mechanism of fumonisin B_1 detoxification was suggested as the decreased inhibitory binding of fumonisin B_1 to ceramide synthase resulting from fumonisin B_1 reaction with reducing sugar (Lu *et al.,* 1997). Further studies are needed to compare the toxicity N-(carboxymethyl)-fumonisin B_1 and fumonisin B_1.

Ammoniation treatment combined with heat and pressure was able to reduce fumonisin level by 79% in corn contaminated with 86 mg/kg of fumonisin B_1 (Park *et al.,* 1992). Ammoniation of *Fusarium verticillioides* culture material as well as naturally contaminated corn, for 4 days at 50°C and atmospheric pressure, resulted in the reduction of fumonisin B_1 by 30-45%, although the toxicity of the culture material in rats was not altered by that treatment (Norred *et al.,* 1991). Deoxynivalenol content was reduced by 9% and 85% in contaminated corn (1 mg/g) exposed to 100% ammonia for 1h and 18h, respectively (Young, 1986). Ammonium hydroxide (3%) was able to reduce zearalenone by 64% in naturally contaminated corn (33.5 mg/kg) after 16 h of exposure (Chamley & Prelusky, 1994).

Formaldehyde, in vapour form or in aqueous solutions, was able to reduce zearalenone concentration in both naturally contaminated corn meal and spiked corn grits. A complete reduction of zearalenone in corn grits containing 3 or 5 mg/kg of zearalenone was observed after exposure to 3.7% of formaldehyde solution for 16h at 50°C (Bennett *et al.,* 1980).

Chlorine at a gas concentration of 30% was able to eliminate deoxynivalenol from contaminated corn at ca. 1 mg/g within 30 min (Young, 1986).

Physical decontamination

Physical methods used for removal or elimination of mycotoxins from contaminated commodities include density segregation and flotation, cleaning and washing, sieving, dehulling, hand picking and electronic sorting, screening, irradiation, milling, thermal degradation, solvent extraction and adsorption.

Trenholm *et al.* (1991) performed segregation experiments of coarsely ground barley, wheat and corn contaminated with deoxynivalenol and zearalenone into fractions of different particle sizes by sieving through a series of screens. Higher toxin concentrations were found in fractions containing smaller particles, and the removal of these fractions reduced the levels of deoxynivalenol and zearalenone from 73% to 83% and from 67% to 79%, respectively, in barley, wheat and corn. Due to relatively large losses of material during sieving, this technique would be advantageous only in case of extensive mycotoxin contamination of cereal crop.

Removing the hull portion from barley, grain and rye contaminated with 5 to 23 mg/kg of deoxynivalenol and 0.5 to 1.2 mg/kg of zearalenone resulted in a 40% to 100% reduction of deoxynivalenol and zearalenone, respectively, with a concomitant loss of 13% to 19% of the grain material (Trenholm *et al.,* 1991).

Simple washing procedures, using distilled water, resulted in 65% to 69% reductions of deoxynivalenol (16 to 24 mg/kg) and 2% to 61% of zearalenone (0.9 to 1.6 mg/kg) in barley and corn contaminated. Washing with sodium carbonate solution increased the removal of deoxynivalenol and zearalenone up to 74% and 87%, respectively. Washing might be a useful treatment to use prior to wet milling or ethanol fermentation, otherwise the cost of drying grains would be prohibitive (Trenholm *et al.,* 1992).

In some cases, mold-damaged and mycotoxin-contaminated kernels exhibit different physical properties with respect to undamaged kernels, therefore they may be separated by density segregation in certain liquids, or fractionation by specific gravity table. Density segregation and removal of kernels buoyant in water and saturated sodium chloride solution reduced deoxynivalenol and zearalenone in cereals up to 96% and 55%, respectively (Charmley & Prelusky, 1994).

Although significant amounts of deoxynivalenol can be removed by cleaning and polishing, the toxin remains in wheat flour at levels ranging from 60% to 80% of original toxin levels from the starting wheat (Charmley & Prelusky, 1994).

Corn screenings or broken corn kernels usually contain fumonisin levels about 10 fold higher than intact corn; therefore the separation of the screenings, based on size, has been suggested as a candidate method for decontamination. Nevertheless, since screenings

represent a significant proportion of the corn used for feed, further decontamination needs to be used (Murphy *et al.,* 1993).

While the above treatments remove or eliminate mycotoxins from contaminated commodities, irradiation (γ-irradiation, X-rays, ultraviolet light, visible light) has been used for inactivation or destruction of some mycotoxins. Gamma irradiation reduced T-2 toxin, zearalenone and deoxynivalenol levels of wheat, corn and soybeans, of 16, 25 and 33%, respectively, and deoxynivalenol and fumonisins in corn of 13% and 20%, respectively (Scott, 1998; Visconti, 2001). Detoxification of 70% to 90% of trichothecenes has been observed in Austria in contaminated corn by applying ultrasonication without altering its original taste and appearance (Lindner & Hasenhuti, 1996).

Within physical methods the utilisation of mycotoxin-binding adsorbents is the most widely used for protecting animals against the harmful effects of contaminated feed. Addition in the diet of nutritionally inert sorbents (hydrated sodium calcium aluminosilicates, zeolite, activated carbon, bentonite, clays and special polymers) reduces the absorption of mycotoxins from the gastrointestinal tract avoiding the toxic effects for livestock and their carryover into animal products (Ramos *et al.,* 1996). The efficiency of the adsorption depends on the chemical structure of both the adsorbent and the mycotoxin.

Activated carbon, a non soluble powder formed by pyrolysis of different kinds of organic materials, shows different adsorbing properties according to its origin. Surface area measurements for activated carbons may vary from 500 to 2000 m^2/g and up to 3500 m^2/g for superactive carbons. In aqueous solution it can efficiently adsorbs most of the mycotoxins but has less or no effects against mycotoxicosis. Activated carbon showed *in vitro* the capacity to adsorb fumonisin B_1 from aqueous solutions (200 mg/l) by 62%, but it was ineffective in reducing the toxic effects of fumonisins (increase of the sphinganine/sphingosine ratio in urine)in *in vivo* experiments performed on rats fed with fumonisin contaminated diets (Visconti *et al.,* 2000). Beneficial effects of activated carbon have been shown in rats intoxicated with T-2 toxin. The mechanism of this beneficial effect has been associated with the ability, shown *in vitro*, of the carbon to bind the mycotoxin, preventing its absorption and especially enterohepatic recirculation (Ramos *et al.,* 1996). Sodium bentonite has been used as a binding and lubricating agent in the production of pelletted foods (Ramos *et al.,* 1996). The addition of bentonite to a T-2-contaminated diet could have beneficial effects on the rats reducing the transit time of digestion through the gastrointestinal tract and promoting fecal losses of the toxin (Ramos *et al.,* 1996). Bentonite was ineffective against zearalenone and nivalenol in pigs.

Synthetic anion exchange zeolite was found to alleviate the adverse effects of zearalenone in rats by reducing the intestinal absorption and the enterohepatic circulation of this mycotoxin (Ramos *et al.,* 1996).

Cholestyramine has been used to adsorb zearalenone *in vitro* from gastric and intestinal simulated fluids. This resin, used extensively for decreasing total and LDL cholesterol, adsorbed almost 100% of the mycotoxin present in the medium when used at concentration over 1%. One gram of cholestyramine was able to adsorb over 1.76 to 2.00 mg of zearalenone (Ramos *et al.,* 1996). Experiments performed by incubating fumonisin B_1 (up to 200µg/ml) with 1mg/ml cholestyramine showed high affinity for fumonisin B_1, absorbing up to 85% of mycotoxin (Visconti *et al.,* 2000). The effectiveness of the cholestyramine against fumonisins was confirmed by *in vivo* experiments with rats, using the increase of sphinganine/sphingosine ratio in urine and kidney to display quantitatively the bioavailability of fumonisins. The addition of cholestyramine (20mg/g) to the fumonisins-contaminated diets (up to 20mg/kg fumonisin B_1+B_2) with toxigenic *F. verticillioides* culture material consistently reduced the effect of fumonisins, i.e. reduced significantly both urinary and renal sphinganine/sphingosine ratios (Solfrizzo *et al.,* 2001).
Divinylbenzene-styrene polymers (anion-exchange resins) exhibited beneficial effects when added to diet of T-2 intoxicated rats, minimising the reduction in feed consumption and the growth-depressing effect caused by T-2 toxin. The addition of the divinylbenzene-styrene to diets of rats supplemented with zearalenone resulted in a major decrease in urinary excretion of conjugated zearalenone and its metabolites (Ramos *et al.,* 1996).

Polyvinylpyrrolidone (0.2%) added to the diets of pigs contaminated with deoxynivalenol did not appear to alleviate the toxic effect of this toxin when fed to barrows and gilts over a period of 5 weeks (Ramos *et al.,* 1996).

Biological decontamination

An alternative approach to mycotoxins decontamination is the biological detoxification, intended as the enzymatic degradation or transformation of toxins leading to less toxic products. Duvick *et al.* (1994) isolated from moldy corn a black yeast fungus *Exophiala spinifera* and a Gram-negative bacterium that were able to grow on fumonisin B_1 as a sole carbon source. These micro-organisms hydrolysed fumonisin B_1 yielding free tricarballylic acid and aminopentol; hydrolysis was followed by oxidative deamination of the resulting aminopentol. Fumonisin esterase and deaminase enzymes were isolated from both *E. Spinifera* and the bacterium and expressed in transgenic corn plants showing a complete metabolization of fumonisin B_1 with release of CO_2 (Duvick *et al.,* 1998).

While lactic acid bacteria and yeast expressing mycotoxin-degrading enzymes may offer a natural way of providing these activities for fermentation processes, transgenic plants are being proposed as an economic approach to reduce fumonisins contamination of corn (Karlovsky, 1999). Corn hybrids genetically engineered with genes from the bacterium *Bacillus thuringiensis* (*Bt*), which express proteins toxic to the European corn borer (*Ostrinia nubilalis*), have shown to reduce fumonisins levels in field-harvested grain (Munkvold, 1999).

Deoxynivalenol was converted to 3-keto-deoxynivalenol during incubation with a soil bacterium isolated by enrichment culture and belonging to the *Agrobacterium-Rhizobium* group. This compound exhibited a reduced immunosuppressive toxicity compared to deoxynivalenol (Shima *et al.,* 1997).

Zearalenone degradation by a variety of micro-organisms including bacteria, yeast and fungi have been extensively reviewed by Karlovsky (1999), indicating the formation of various zearalenone metabolites that may still retain the original oestrogenic activity. The most effective detoxification was obtained by the mycoparasite *Gliocladium roseum* causing the cleavage of the zearalenone lactone ring followed by spontaneous decarboxylation, rendering the reaction irreversible. Fermentation by *Saccharomyces cerevisiae* of wort-containing zearalenone resulted in conversion of 69% of the toxin to beta-zearalenol, a metabolite with less activity than the parent compound (Scott *et al.,* 1992).

The only product of microbiological origin now on the market is Mycofix Plus, produced by Biomin in Austria, which is claimed to degrade mycotoxins in feeds by enzymatic activities. It is a feed additive that, in addition to a conventional sorbent, comprises a preparation declared to inactivates trichothecenes such as deoxynivalenol and T-2 toxin by enzymatic decomposition of the 12,13-epoxy ring, and zearalenone by enzymatic opening of the lactone ring (Karlovsky, 1999). The claimed lactonase activity of this product towards zearalenone could not be confirmed by Karlovsky (1999). Other microbial strains (yeast, fungi or bacteria) have been proposed for detoxification of deoxynivalenol, T-2 toxin and other trichothecenes, but their practical use has not been shown (Karlovsky, 1999).

3.4 Recommendations and future research

The use of an integrated management program based on the observance of EUREPGAP and HACCP principles contributes to prevent the risk of mycotoxin contamination of cereals at harvesting and post-harvest stages, including storage and processing.

Data gathered by food processors about the fate of Fusarium toxins during processing are seldom or only partially made available to the public. More work is needed to study the fate of mycotoxins during food and feed decontamination and food processing; in particular, reduction of toxicological risk associated with processed mycotoxins-contaminated commodities as well as prevention of recontamination during storage should be evaluated.

Contaminated feeds are frequently more toxic than the pure toxin in animals and humans, indicating possible interactions. It is recommended to perform further studies on

mycotoxins occurring concomitantly in foods, their possible interactions and how toxicological significance of such interactions could be assessed. Although certain treatments have been found to reduce concentrations of specific mycotoxins in cereal foods and feedstuffs, no single method has been developed that is equally effective against the wide variety of mycotoxins that may co-occur in different commodities. In addition, detoxification processes that may appear effective *in vitro* do not necessarily retain their efficacy when tested *in vivo*. The use of cell lines or artificial models to simulate animals is recommended as an important alternative to the use of living experimental animals.

4. Analysis of relevant Fusarium mycotoxins in cereals - the state of the art

W.A. van Osenbruggen and H. Pettersson

4.1 Introduction

Toxin producing *Fusarium* species and their toxins are commonly occurring on European cereals. The most common Fusarium toxins found in cereals are the trichothecenes(deoxynivalenol, nivalenol, HT-2 and T-2 toxins), zearalenone and the fumonisins. High concentrations of deoxynivalenol (DON) have also been found in North American and European wheat during recent years.

The risks for human and animal health by these fusarium toxins have been addressed by the international organisations WHO/FAO, the European Commission and national authorities in many countries. Toxic evaluation and risk assessments are going on or have been done for many of the toxins. The Nordic Working Group on Food Toxicology and Risk Assessment has presented a Risk Assessment of the most common fusarium toxins in cereals (Eriksen & Alexander, 1998). The European Commissions Scientific Committee on Food has given an opinion about DON, zearalenone, fumonisin B1, nivalenol and T-2/HT-2 toxin. JECFA (Joint FAO/WHO Expert Committee on Food Additives) has evaluated zearalenone, fumonisins and the trichothecenes (DON, T-2 toxin and HT-2 toxin) (JECFA, 2001). U.S. Food and Drug Administration have recently made an evaluation for fumonisins and published a final paper and recommendation on Internet http://www.cfsan.fda.gov/~dms/fumongui.html. Maximal limits for certain Fusarium toxins are already introduced in some countries or they are under preparation in the European Union and other countries. The procedure for DON in the EU seems to be quickened compared to earlier for other mycotoxin limits.

There is a need for good and standardised analytical methods to make surveys and control of these toxins, but in most cases they are still lacking. The methods for many of the toxins are complicated and often leading to high variations both within and between laboratories. Activities supported by the European Commission are going on to improve the analytical methods. Results from those studies and others concerning method development will be presented to give an overview of the analytical methods for Fusarium toxins.

Screening methods for the Fusarium toxins are also necessary for rapid detection of contaminated cereals. Some of the ELISA methods can be used for such purposes, but additional investigations are going on making use of techniques like NearInfraRed or

Biosensor-technology. Also results on cytotoxicity screening of trichothecenes with colorimetric bioassays will be presented.

4.2 Fusarium mycotoxins - analysis

Trichothecenes

The working group for Biotoxins of the European Committee for Standardisation (CEN) has expressed the need for a standardised method for DON. There is already an AOAC collaboratively studied gas chromatographic method for DON (Ware *et al.,* 1986). In that study there was, however, a very high variation (31-52%) between laboratories even for samples with relatively high concentration. In the BCR certification of reference materials for DON they obtained much lower variation (1-7%) by mainly using HPLC methods (Gilbert *et al.,* 1992b).

Other inter-comparison studies with different methods have also found high between laboratory variations for DON 19-60%, nivalenol 20-33%, HT-2 16-103% and T-2 19-131% (Gilbert, 1992a; Pettersson, 1995, 1998b; Schuhmacher *et al.,* 1997; Joseph & Krska, 1998). The variations were mostly lower when a common calibrant was used. The high variation even for DON is remarkable.

Capillary gas chromatographic methods using EC or MS-detection have been preferred for their higher sensitivity and selectivity. In the EU-project Intercomparision of Trichothecene Analysis (the Standard, Measurement and Testing, SMT Program) have those methods been studied for the analysis of DON, nivalenol, HT-2 and T-2 toxins during the last four years. The project has been co-ordinated by Hans Pettersson, Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden. The purity and the amount/concentration of commercial trichothecenes for calibration do vary and is one important cause for between laboratory variation. The project has revealed even more serious GC-method problems (Pettersson, 1998a,b; Pettersson & Langseth, 2002a). The GC-responses of the trichothecenes are higher in presence of matrix than as pure calibrants. This is mainly due to different adsorption of the derivatized trichothecenes in the injector and column in presence and absence of matrix and may lead to an overestimation of the trichothecenes in analysed samples by sometimes more than 100%. This matrix effect was more or less pronounced in all laboratories and with all methods. Regular changing of dirty injector liner and cutting/shorting the column will reduce but not completely eliminate the problem. Using cool on-column injector instead of splitless injector and injection of low sample amount

can also reduce the overestimation. Matrix assisted calibration curves can effectively compensate for the matrix effect. It is often used in pesticide analysis for the same reason, but is not (normally) allowed in certification exercises, which is one of the goals for the project. Matrices prepared by clean up of uncontaminated wheat, barley or oats give about the same compensation. To use an internal GC standard derivatized as the trichothecenes and relative response calculation can also compensate but unfortunately partly differently for type A and B trichothecenes. An internal GC standard is also needed to reduce the high variation in injection repeatability found with MS-detectors. Possibilities to improve clean up and by that way reduce the matrix effect have also been studied but without much success.

An inter-laboratory study after recommendations for method improvements and implementation showed still differences in slope between calibration curves with and without matrix (Pettersson & Langseth, 2002b). Matrix assisted calibration curves is thus needed in a GC-method suggested for standardisation. Regular use of reference material is also important for the method control of long time variation.

In the final Intercomparison of trichothecene analysis in contaminated wheat (Pettersson & Langseth, 2002b) the relative between-laboratory standard deviation (RSD_R) with both the matrix and non-matrix assisted calibration were relatively good (<17%) for all the analysed trichothecenes. They were similar and lower than for the determination of trichothecenes in solution and better compared with earlier collaborative studies. The Relative within-laboratory (RSD_r) or relative between sample standard deviation was on the other hand in most cases higher than the RSD_R. This means a high variation in the analyses, but the laboratory means are in relatively good agreement.

Immunoaffinity columns for clean up of DON are commercial available. The columns are mainly used in methods based on final HPLC separation and UV-determination of DON (e.g. Cahill *et al.,* 1999). It is sensitive to solvents, and DON is extracted with water-PEG and applied as such to the column followed by water washings. Although the recovery has been reported to be good, some laboratories have got only low values for the certified reference material or other naturally contaminated material. Also contamination of some column batches with a DON like compound has been observed. Also experiments are carried out with immunoaffinity column cleanup in combination with final GC-determination.

There is also a HPLC method for DON with Mycosep clean-up, which has been collaboratively studied (Trucksess *et al.,* 1998), but with only 3 laboratories and has therefore been recognised as an AOAC Peer-verified method. It had relatively good performance data, but a modification to use acetonitrile-water as mobile phase and a gradient to also separate and detect nivalenol and acetyl-DON may improve the method further in a new interlaboratory study.

A combination of charcoal and immunoaffinity clean-up has been found good in both UK and Germany. One method has collaboratively been studied by VDLUFA in Germany during several years with good results (Reuter, unpubl). The RSD_R has mostly been below 15% and the RSD_r below 9%. The cost and time with two clean-up columns will, however, be high.

The development of screening methods for trichothecenes has so far focused on the determination of DON as the most prevalent representative of this toxin group. The first methods developed for final separation and detection of mycotoxins, such as trichothecenes, in agricultural commodities were based on TLC. TLC is simple and economical and, with the introduction of high-performance TLC (HPTLC) and scanning instruments, separation efficiency and precision compete with those of other types of chromatography (eg. Trucksess *et al.,* 1984). Reagents such as aluminium chloride sulphuric acid or para-anisaldehyde are, however, necessary to visualise the non-fluorescent and only short wavelength (220 nm)-absorbing DON.

In addition to TLC, ELISA methods have been developed for rapid screening and quantification of trichothecenes in cereals. ELISA makes use of specific antibodies, derived by a series of complex procedures such as immunogen synthesis, immunisation of animals, isolation and characterisation of antibodies. ELISA methods are selective, sensitive and rapid. Because of the multi-functional properties of trichothecenes, however, production of useful antibodies against these compounds has proved difficult. The most sensitive ELISA methods have been developed for tri-acetylated DON; the LOD is 0.3-1 ng/g and the method entails acetylation of the toxin in the cereal extract before the DON assay and therefore results in the determination of the sum of DON and its acetylated deivatives! ELISA methods enabling direct determination of DON have also been developed, although they are less sensitive (LOD = 20-300 ng/g). Although accurate quantification of DON by immunological assays is often limited, because of remarkable cross reactivity with DON-related compounds, when chromatographic instruments are not available, ELISA methods are an interesting alternative for determination of DON. The AOAC and US Grain inspection has performance tested several kits for DON determination in cereals at specified levels (GIPSA, 2002). In Europe we may have other requirements, especially lower detection and the kits need to be tested also here.

Colorimetric bioassays for trichothecenes: Three new colorimetric bioassays (MTT, BrDU and LDH) were evaluated for detection of low concentrations of the most common trichothecenes (Widestrand *et al.,* 1999). The bioassays assessed DNA-synthesis (incorporation of 5-bromo-2´-deoxyuridine, BrDU), metabolic activity (cleavage of 3(4,5-dimethyltiazol-2-yl)-2,5-diphenylterazolium bromide; MTT) and cell membrane damage (release of lactatedehydrogenase; LDH), respectively. Different cells were also tested. The cells were in all cases incubated in 96-well microtiter plates followed by a colour reaction and measurement of absorbance on an ELISA-reader.

The BrdU-method together with Swiss mouse 3T3 fibroblasts was found to be most sensitive and reliable. The IC50 was 4.6 ng for T-2 toxin and 13, 263, 365 ng for HT-2, DON and nivalenol, respectively. A cell proliferation kit (Roche Diagnostics) to determine the amount of incorporated BrdU was used.

Co-extraction of normal but cytotoxic compounds in cereals has been a limitation in the use of celltoxicity testing for mycotoxins. Direct use of acetonitrile-water (84+16) extract (normally used in trichothecene analysis) from milled cereal samples shows also in this case a certain cytotoxic effect. A clean-up with MycoSep™ #225 multifunctional column has therefore been used in the screening method (Widestrand *et al.,* 2000). Relative sample amounts up to 400 mg/ml can be used for all cereals. The remaining purified extract can be evaporated and saved for eventual gas chromatographic determination of the individual trichothecenes.

The IC50 of the trichothecenes T-2, HT-2, DON and nivalenol in spiked wheat (400 mg/ml) corresponds to 9, 41, 1216 and 666 ppb respectively. The method is able to detect lower concentrations of T-2 and HT-2 toxins than most GC-methods. Trichothecenes often occur together in cereals and the response will be the sum of the effects and is expressed as T-2 toxin equivalents.

The method has up to now only been used to screen for cytotoxicity in cereals (wheat, barley and oats) with and without trichothecenes checked by gas chromatography. The cytotoxicity of the samples corresponds well with the occurrence of trichothecenes and the calculated T-2 toxin equivalents. Some oat samples without detected trichothecenes have, however, shown certain cytotoxicity.

BCR - Certified Reference Materials (CRM) have early been produced for DON in wheat and maize, which contain 0.67 and 0.43 mg/kg respectively (Gilbert *et al.,* 1992b). It is important to use such reference material for in laboratory validation of methods and for control of in-house produced reference material. It is especially important in trichothecene analysis to use reference material to control the long time variation in the methods. The short time variation or the repeatability can be good although there is a high variation from run to run.

Zearalenone

Although TLC, GC and GC-MS methods are available, HPLC is usually chosen for the determination of Zearalenone. Most of the HPLC methods have been developed for maize and other cereals. In the past there was for zearalenone an AOAC collaboratively studied method based on HPLC (Bennet *et al.,* 1985). The cleanup step was relatively laborious and the method variations both within (repeatability) and between (reproducibility) laboratories

were rather high in the collaborative study 25-33 respectively 13-41% on corn. Recent HPLC methods for Zearalenone have employed reversed-phase chromatography with direct fluorescence detection (275/450 nm). Fluorescence detection is also used in a recently described method based on immunoaffinity columns (IAC) and quantification by reaction with aluminium chloride hexahydrate (AlCl3.6H2O) then measurement with a fluorimeter. During recent years immunoaffinity columns for zearalenone have become available. Different HPLC methods using these immunoaffinity columns been developed (e.g. Schuhmacher *et al.,* 1998; Visconti & Pascale, 1998; Kruger *et al.,* 1999). They are relatively fast, sensitive and with low detection. It is, however, important to check the capacity of the columns and the recovery at also highest determination level. There is a risk for overload at high concentrations if the capacity, which can vary, is low.

In the EU - SMT project 'Preparation and Certification of Reference Material for the Determination of Zearalenone in Maize' most laboratories are using HPLC methods with immunoaffinity cleanup. The results from the method inter-comparisons with 29 laboratories were very good. The between laboratory variation was only 8% for a maize sample containing 129 μg zearalenone per kg. The mean of recovery rates was high (96%) with a CV of 10%.

Other inter-comparison studies using different methods have also been done (Schuhmacher *et al.,* 1997; Joseph & Krska, 1998). The between laboratory variations for zearalenone were 15-28%. The variation was as for the trichothecenes higher when the laboratories own calibrants were used. A zearalenone method based on immunoaffinity column cleanup and HPLC determination with fluorescence detection ought to be standardised. It will probably be done after the present EU project, which main goal is the production of maize CRM for zearalenone. Certification of both zearalenone calibrant and maize reference materials will probably soon be the result of the project.

An ELISA based method has been tested collaboratively and approved by the AOAC as a first-action screening method for zearalenone concentrations higher than 800 ug/kg. The method has been accepted by the AOAC for detection of zearalenone (> 800 μg/kg) in corn, wheat and pig feed (Bennet *et al.,* 1994). At this moment several ELISAs for detection of zearalenone are available commercially and can be considered as a major technique for the screening of zearalenone in cereals.

Fumonisins

A HPLC method for fumonisin B1, B2 and B3 in corn has been evaluated in a AOAC-IUPAC collaborative study with 9 laboratories (Sydenham *et al.,* 1996). The method is using ion exchange SPE-columns for cleanup. Repeatability and Reproducibility were good in the study (RSD_r 6-12% and RSD_R 14-19%). The recoveries of fumonisin B1 was

81-84% and fumonisin B2 76-82% but have been lower in other studies. Using long time shaking instead of short time blending for the extraction can enhance the extraction efficiency. The method however, can not be used for processed maize.

An HPLC method with improved extraction efficiency and immunoaffinity column clean-up has been validated by 23 laboratories in an EU - SMT-project (Visconti *et al.,* 2001) for fumonisin B1 and B2 in different maize products (maize flour, cornflakes, extruded maize, muffins and infant formula). The method is based on two consecutive extractions of the sample with ACN+MeOH+water (25+25+50, v+v+v), immunoaffinity column clean-up and HPLC analysis of FB1 and FB2 with fluorometric detection after derivatisation with ortho-phtaldialdehyde. Results of the collaborative study showed that the proposed method is quite satisfactory for its application to these materials and that also reproducibility data satisfy the CEN criteria.

Recoveries, repeatability (RSD_r) and reproducibility (RSD_R) for cornflakes were all within the criteria established by the CEN for the acceptability of the method. The method has been adopted by AOAC International as First Action Method and approved by the CEN.

There has been an EU-SMT-project, which have tried to produce CRM for fumonisins in maize (Visconti & Boenke, 1995). The project failed mainly due to low recoveries (<70%) for most of the methods used. There is, however, a need for a maize fumonisin CRM.

4.3 Recommendations and future research

LC-MS-(MS)

The availability of commercial, relatively easy-to-use LC-MS-(MS) instruments for routine analysis, which enable both quantification and identification, has led to an increasing use of this technique. The use of LC-MS-(MS), enabling simultaneous determination of several trichothecenes and zearalenone can be considered a major future trend in the analysis of these Fusarium mycotoxins in cereals.

NearInfraRed Transmittance (NIT)

In a Danish – Swedish project funded by the Nordic Industrial Fund we are investigating the possibilities to rapidly classify the hygienic quality of cereals delivered to mills. NIT-

instruments (Infratec™) from Foss-Tecator and their network, which is normally utilised for determination of protein and moisture, will be used for measurements and calibration trials of parameters like DON, ergosterol, ochratoxin, Fusarium, insects and mites.

A first calibration trial for DON has just been conducted (Pettersson & Åberg, 2002). Wheat samples (42) containing DON between 0 and 6100 μg/kg and variation in protein and fusarium infection have been used. Infratec 1241 was used with normal (850-1050 nm) and extended (570-1100 nm) wavelength area. The spectral scans (10/sample) were analysed in Win-Unscrambler. No samples deviated from the population. PLS model with cross validation was calculated on the whole material. Best results were obtained with extended wavelength and 10 principal components were used. A slope of 0.909 with a correlation of 0.97 was obtained. The standard error of prediction was 334 ppb. The results look promising for a future calibration for DON.

Highly DON-contaminated barley material (1-30 ppm) from USA was also obtained and used in calibration trials. DON was reference analysed in USA by both gas chromatography and ELISA. A PLS regression model based on 526 samples gave a correlation of 0.837 and a slope of 0.701 but the standard error of prediction became 3.6 ppm. A separate test set (n=150) used for validation of the calibration gave similar values. Calibration with artificial neural network and use of data from both USA, France and Austria improved the results further. The results on ergosterol were also promising in the trials, but not for insects. The other parameters have not yet been thoroughly investigated.

Biosensors

At TNO Nutrition and Food Research Institute, Zeist, The Netherlands, a biosensor for the simultaneous detection of four mycotoxins has been developed. The principle of the technique is as follows. The biosensor measurement is designed as an inhibition assay. A fixed amount of mycotoxin specific antibody is mixed with a sample containing an unknown amount of mycotoxin. The antibody and mycotoxin form a complex. The sample is then passed over a sensor surface to which mycotoxins have been immobilised. The amount of non-complexed antibody is determined as they bind to the immobilised mycotoxin on the sensor surface. The general measurement principle of the biosensor assay makes it possible to monitor the interaction between the antibody and mycotoxins on the sensor surface as long there is a change in mass. For the development a surface plasmon resonance (SPR) sensor device was used. Toxins were immobilised on the sensor surface of a dextrane-coated sensor chip. For the individual toxins, separate immobilisation procedures were applied. Sample extracts and reference solutions were mixed 1:1 with an antibody mixture and injected into the four mycotoxin flowcells for simultaneous detection of individual levels of four mycotoxins. Detection is possible at relevant concentration levels with sufficient reproducibility.

Recently a new coating based on self-assembling proteins has been developed. This protein coating is capable of withstanding many organic solvents including acetonitrile, methanol and ethanol. The deposition of these proteins on sensor surfaces is very easy and fast. This offers the possibility to transfer the complete analyses to any transduction system of choice. The combination of a multi-myctoxin biosensor assay with a very simple sample pre-treatment and an easy to use coating can make mycotoxin analysis fast and with low cost of ownership.

Rapid Test Kits

Commercial test kits for fusarium toxins in cereals and cereal products must be tested for suitability, sensitivity and reliability in the control procedure at different stages. New rapid and multi-toxin quantitative test methods should also be developed to improve and speed up the control of the most important fusarium toxins.

Standardised Quantitative Analytical Methods

Good, reliable and standardised analytical methods for zearalenone, DON and other trichothecenes, to be used in control of the mycotoxins, are still lacking. A HPLC method with immunoaffinity column clean-up and fluorescence detection ought to be collaboratively studied for possible standardisation. Both HPLC and GC-methods for DON and other trichothecenes needs to be tested and selected for interlaboratory studies and standardisation. The CEN, Workinggroup for Biotoxins has also pointed out the need for standardisation of methods for those toxins.

Certified Reference Materials and Calibrants

Calibrants with correct concentrations and CRM:s are vital for method validation, control and determination of the true mycotoxin content in the cereals. CRM:s for DON in maize and wheat as BCR reference material from Institute for Reference Materials and Measurements (IRRM, Geel) are at present the only certified material for Fusarium toxins. A Certified calibrant and maize CRM:s for zearalenone will probably soon also appear. Certified calibrants for fumonisins, DON and other trichothecenes need to be produced as well as CRM:s for fumonisins and trichothecenes in maize and wheat respectively.

5. Tools to improve food safety in the chain

5.1 Introduction

This chapter consists of various aspects of risk management in order to prevent Fusarium mycotoxins in the cereal chain. First there is information about risk analysis, followed by the introduction of a HACCP system, to be used to prevent the contamination of cereals with mycotoxins in the food and feed chain. The authors have tried to investigate which actions should be undertaken regarding Good Agricultural Practice to come to a Code of Practice to prevent chain wide mycotoxin problems. Finally, information is presented on the status of the legislation of Fusarium mycotoxins in the EU. World wide, mycotoxin problems are being discussed in the Codex Alimentarius.

5.2 Risk analysis

A. Visconti and A. De Girolamo

Mycotoxins can never be completely removed from the food supply, therefore there is a need to ensure these hazards are reduced to an acceptable level. It is the task of food producers to maintain the level of risk at the minimum that is practical and technologically feasible. The role of official bodies should be to use *risk analysis* to determine realistic and achievable risk levels for food-borne hazards and to base food safety policies on the practical application of the results of these analyses. *Risk analysis* consists of three parts: *risk assessment* (hazard identification, hazard characterisation, exposure assessment, risk characterisation), *risk management* and *risk communication*. Each of these major groups overlaps with the others.

Risk assessment is the scientific evaluation of known or potential adverse health effect resulting from human exposure to food-borne mycotoxins. It provides a qualitative and quantitative estimate of the severity and likelihood of harm resulting from exposure to these hazards.

Hazard identification is the first step of risk assessment and identifies the mycotoxins which may be present in food and may cause harm to human and animal health. For example fumonisins are carcinogenic, nephrotoxic and cause equine leukoencephalomalacia and porcine pulmonary oedema; zearalenone is estrogenic and

immunosuppressive; deoxynivalenol is immunosuppressive and causes anorexia and vomiting.

The end point of this step is the calculation of 'no-observed-effect-level' (NOEL, mg/kg of body weight per day, namely the greatest concentration or amount of mycotoxin that does not cause detectable adverse effects) or of 'lowest-observed-effect-level' (LOEL, mg/kg of body weight per day, namely lowest concentration or amount of mycotoxin that cause a detectable adverse effect). For example for fumonisins the NOEL for renal toxicity was 0.2 mg/kg of body weight per day; for deoxynivalenol the NOEL for body weight reduction in mice was 0.1 mg/kg of body weight per day.

Hazard characterisation is the qualitative and/or quantitative evaluation of the nature of the adverse effects associated with mycotoxins, which may be present in food. A dose-response assessment by combining exposure data with toxicity data should also be performed. The end point of this step is the estimation of a 'safe dose' such as a 'provisional-maximum-tolerable-daily-intake' (PMTDI, μg/kg of body weight per day) or equivalent. The PMTDI in humans is obtained by dividing the NOEL or LOEL extrapolated from animal experiments by a safety factor. For example the PMTDI as established by JECFA (2001) is 0.014, 0.06, 2 and 1 μg/kg body weight per day for ochratoxin A, T-2 and HT-2 toxin, fumonisins B_1 , B_2 and B_3 (alone or in combination) and deoxynivalenol, respectively. These PMTDIs contain safety factors of 1500 (in relation to nephrotoxicity and renal carcinogenicity in rats), 500 (in relation to a LOEL in rats), 100 and 100 for ochratoxin A, T-2 and HT-2 toxin, fumonisins B_1 - B_3 and deoxynivalenol, respectively. In this presentation the PTWI for OTA as established by JECFA has been recalculated to a PMTDI for comparison.

Exposure assessment is the qualitative and/or quantitative evaluation of the likely intake of mycotoxins via food. Various methods can be applied, such as direct analysis of duplicate diets or of foods or food groups as eaten, and/or calculation of the exposure of humans to these hazards by determining the levels of mycotoxin contamination in foods and combining these data with the consumption amounts of the specified foods. Intake calculations can be performed in a deterministic way, by using single figures as average or maximum levels in foods and combining these with average food consumption data for a national population of for a population group, e.g. children. When more advanced data are available about levels in foods and about food consumption patterns, probabilistic calculations can be made, which yield a probability pattern of intake levels for a population. In international studies, JECFA mostly uses average regional diets based on food balance sheets, e.g. the European diet and mean mycotoxin levels or proposed maximum levels. Intake results are mostly expressed as μg/kg or ng/kg of body weight. For example the mean total intake as calculated by the 2001 JECFA for the European diet and for the average consumer (assumed body weight of 60 kg) for ochratoxin A, aflatoxin M1, fumonisins , deoxynivalenol and T-2 + HT-2 toxin is 0,0064, 0,00011, 0.2, 1.4 and 0.0163 μg/kg of body weight per day, respectively.

Risk characterisation is the integration of hazard identification, hazard characterisation and exposure assessment into a qualitative and/or quantitative estimation of severity and occurrence of the adverse effects likely to occur in a given population, including attendant uncertainties. Risk characterisation can also be the establishment of levels of daily exposure at which the risk is insignificant over a lifetime (i.e. the exposure needs to be below the TDI or other measure of safe dose). For example, the mean exposure assessment figures can be compared with the PMTDIs mentioned earlier, but also calculated maximum intakes for specific population groups can be evaluated.

Risk management is defined as the process of weighing policy alternatives to accept, minimise or reduce assessed risks and to select and implement appropriate control options including the establishment and enforcement of maximum levels of mycotoxins in foods.

Risk communication is an interactive process of exchange of information and opinion concerning risk among risk assessors, risk managers and other interested parties.

5.3 HACCP principles as a tool in the prevention of Fusarium mycotoxins in the cereal chain

O.E. Scholten

HACCP stands for Hazard Analysis and Critical Control Points and can be defined as a system of food safety control based on the systematic identification and assessment of hazards in foods and the definition of means to control them (Park *et al.,* 1999). It is a preventive, rather than a reactive, tool that places the protection of the food supply from microbial, chemical and physical hazards into the hands of food management systems. Prevention through pre-harvest management is the best method for controlling mycotoxin contamination; however, should contamination occur, the hazards associated with the toxins must be managed through post-harvest procedures, if the product is to be used for food and feed purposes. In an ideal integrated management system, mycotoxin hazards would be minimised during production, harvesting, processing and distribution (Lopez-Garcia & Park, 1998; FAO, 1979, 2001).

The HACCP concept consists of the following seven principles, which will be explained in more detail regarding the problems encountered in the complexity of Fusarium mycotoxins in the cereal chain:

- Conduct a hazard analysis
- Determine the critical control points

- Establish critical limits
- Establish monitoring procedures
- Establish corrective actions
- Establish verification procedures
- Establish record-keeping and documentation systems

Within the Fusarium Mycotoxin Cluster of the European Research Program, one European Research Project in the framework deals with 'Hazard analysis and control of food contaminants in cereals', namely the project 'Prevention of Fusarium mycotoxins entering the human and animal food chain'. This research project aims at studying and unraveling all difficulties to set up a correct set of HACCP principles that can be verified, recorded and documented. Their work plan is divided into 5 major tasks:
Task 1: Development of critical control systems: Use of ecological and control data for developing a Hazard Analysis Critical Control Point (HACCP) system for identification, reduction and prevention of the risk of Fusarium mycotoxins entering the food chain.
Task 2: Pre-harvest Biocontrol: Development of biocompetitive strains for preharvest control and competitive exclusion of toxigenic fusaria, in cereal (wheat/barley/oats/maize) production.
Task 3: Post-harvest control: Novel natural control food-grade systems will be used for control of mycotoxigenic species and mycotoxins into food.
Task 4: Decontamination using microbial inoculants for prevention of entry into animal production systems: Bacteria and yeast will be used for the breakdown of mycotoxins in stored cereals.
Task 5. Decontamination using physico/chemical means. Adsorbent materials and biomarkers will be used to assess the exposure to Fusarium mycotoxins (i.e. sphinganine/sphingasine ratio for fumonisins) to quantify the effectiveness of treatments.
For information about the Mycotoxin cluster see http://WWW.mycotoxin-prevention.com

Principle 1. Conduct a hazard analysis

In this first step, investigation is needed of all hazards that occur during the stages in the cereal chain, which should be of such nature that their prevention, elimination, or reduction to an acceptable level is essential to the production of safe food. The cereal chain consists of the following stages: crop production (pre-harvest, harvest and post-harvest), transportation and storage and processing. In developed counties mycotoxin major problems are associated with animal health, because animal feeds are largely unregulated and animal mycotoxicoses frequently occur (Smith *et al.,* 1994). Contamination with mycotoxins occurs during the crop production phase, whereas an increase in mycotoxin contamination as well as carryover may take place during storage and processing. The most critical period for wheat to become contaminated by Fusarium

mycotoxins is during anthesis, when conidia of *Fusarium culmorum* and/or *F. graminearum* favourably infect ears. Environmental factors such as humidity or rain during the flowering period resulting in surface wetness for 48-60 hr and temperatures above 15 °C determine the success rate of the infection process. Maize is most susceptible after silk emergence, with rainfall of 70-80 mm during days 6-9. Furthermore the amount of primary inoculum, surviving on crop debris of e.g. maize is also an important factor. Insects play a role with maize, as was shown in studies with insect resistant maize plants, which appeared to be less sensitive for *Fusarium* fungi. The role of fungicides is not clear yet and needs further investigations. In developed countries, Fusarium mycotoxins are a typical pre-harvest problem. During and after harvest, no further increase in levels of mycotoxins is expected, due to the transportation and storage of cereals under optimal conditions which do not allow further fungal growth. In case storage conditions are not good, this may result in a further increase of fungal growth as well as in mycotoxin production.

Principle 2. Determine critical control points

Critical control points (CCPs) are points, steps or procedures at which control can be applied, essential to prevent or eliminate a food safety hazard, or reduce it to an acceptable level. CCPs can be identified by use of decision trees, in which questions are raised about the hazards. A first question could be 'do control measure(s) exist for the identified hazard? And if so, does this step eliminate or reduce the likely occurrence of a hazard to an acceptable level?

Critical control points are:

- The seed Are the seeds clean or do they contain Fusarium spp. on the surface?
  Have the seeds been treated with fungicides?
  Do the seeds originate from resistant cultivars?
- The soil Is crop debris present in the soil?
  When you grow wheat, did you avoid maize as the previous crop?
- Germination Do environmental conditions request for application of fungicides during growth and especially during anthesis?
  Or, in the case of maize, application of insecticides?
  Do mycotoxin-producing fungi occur in the field?
- The harvest Is moisture content of the seed low enough to harvest the seed?
  Does damaging of seed through harvesting machines not occur?
- Post-harvest Can the seed moisture easily be reduced by heating?
  Has physical cleaning been applied to get rid of small infected seeds with probably high mycotoxin content?
- The storage Are temperature and humidity low enough to prevent further increase of fungal growth and subsequent mycotoxin production?
  Are the storage facilities free from insects?

Principle 3. Establish critical limits

A critical limit is a maximum and/or minimum value to which a biological, chemical or physical parameter must be controlled at a CCP to prevent, eliminate or reduce to an acceptable level the occurrence of a food safety hazard. Critical limits are the boundaries of safety for preventive measures put in place at CCPs. A critical limit will usually be a reading or observation, a simple parameter, such as temperature, time, product property such as moisture content or water activity, or a chemical property such as available chlorine, salt concentrations or pH. Critical limits need to be exact and specific. According to Olsen mycotoxin levels should only be set as critical limits when easier/quicker/cheaper parameters are not adequate. In Table 4 an overview is presented of possible stages in the application of HACCP principles in cereals.

Table 4. Possible stages in the application of the HACCP principle to cereal commodities, food products and animal foodstuffs with respect to mycotoxins produced by *Fusarium culmorum* and/or *F. graminearum* (modified from Park *et al.*, 1999).

Stage	Hazard	Corrective action
Crop production		
Pre-harvest	Fungal infestation with subsequent mycotoxin formation	Utilise clean seeds Utilise resistant cultivars Perform good tillage and crop rotation Remove cereal crop debris Apply fungicides Apply insecticides (maize) Take care with irrigation (maize)
Harvest		Harvest at appropriate time
Post-harvest		Dry rapidly to below 15% moisture
Storage	Increase of mycotoxin	Store products dry and clean
Processing	Mycotoxin carryover or contamination	Physical separation of infected kernels Apply heating (fumonisins) Test all ingredients added Monitor processing/manufacturing Operation to maintain high-quality products Follow good manufacturing practices Decontamination

Principle 4. Establish a monitoring system

Monitoring is the continuous or scheduled measurement or observation at a CCP to access whether the step is under control, i.e. within the specified critical limits. Methods for monitoring should give rapid response and should warn that critical limits are being approached, e.g. ELISA, PCR.

Principle 5. Establish corrective actions

Specific corrective actions must be developed for each CCP in the HACCP system in order to deal with the deviations when they occur. The product produced whilst the CCP was out of order should be identified and re-processed, downgraded, or rejected. So when a mycotoxin critical control point moves out of control, the resulting contamination can often lead to rejection or down-grade of the batch.

Principle 6. Establish verification procedures

Independent inspection and audit of HACCP plan to verify that it is working.

Principle 7. Establish documentation system

HACCP procedures should be documented. Documentation and record keeping should be appropriate to the nature size of the operation.

To apply HACCP to establish minimum mycotoxin contamination, the following pre-requisites are needed to be taken into account:

- Good Hygienic Practice
- Good Agricultural Practice
- Good Storage Practice
- Good Manufacturing Practice
- Management/Stakeholder commitment
- Training

5.4 Good Agricultural Practice and EUREPGAP

A. De Girolamo, W.A. van Osenbruggen, P. Ruckenbauer, O.E. Scholten, A. Visconti and A.P.M. den Nijs

A prerequisite for the development of HACCP-based integrated mycotoxin management programs is the observance of Good Agricultural Practice (GAP) and Good Manufacturing Practice (GMP) (Park *et al.,* 1999). EUREP (Euro Retailer Produce Working Group) represents leading European food retailers and defines essential elements for the development of pest-practice for the global production of combinable crops (barley, beans, durum wheat, linseed, corn, oats, peas, rape seed/canola, rye, soybeans, sunflower, triticale, wheat and other combinable crops) and supports the principles and encourages the use of HACCP.

The Task groups recommend that the draft protocol of EUREPGAP Combinable Crops should be extended by paying attention to limiting or possibly excluding the occurrence of contamination in the cereal and maize chain for human and animal consumption.

The following operations are required or encouraged from EUREPGAP for pre harvest:

- A specific warning should be given to avoid rotations, which can induce Fusarium contamination e.g. maize followed by (durum) wheat.
- Only grow Fusarium resistant varieties of wheat and maize.
- In areas with high *Fusarium* incidence, the use of minimum tillage systems of should be reconsidered to minimise the danger of *Fusarium* carry-over.

The following operations are required or encouraged from EUREPGAP for post harvest, handling and storage:

- Buildings used for holding of grain must be weatherproof and all roof leaks, broken sheeting, guttering etc. must be repaired prior to storage of grain. They must have solid floors and suitable walls and doors. In the case of flat grain stores, hard outside loading areas must be maintained in a clean and well drained condition.
- Action must be taken to prevent bird, rodent and domestic animal entry to long term grain storage.
- Grain stored for more than a few days must have conditioning. Long term stored grain must have a moisture content and temperature suitable for storage. Over-drying and heat damage to the grain must be avoided.
- During longer term grain storage the temperature and condition of grain must be monitored and recorded weekly, and any rise in temperature must be investigated. Appropriate action must be taken to remedy water ingress, bird and rodent activity and hot spots.

- Growers must have easy access to moisture meter and temperature probe, if they store grain.
- Grain drying equipment must be regularly maintained in line with manufacturers instructions. All light bulbs, tubes, lamps, window or any other glass materials must be protected or constructed to avoid broken glass contaminating the grain. This applies to temporary holdings, long-term stores and all grain movement areas.
- Representative samples of each storage bin and/or silo and of each load leaving the farm must be taken and retained at time of filling. In addition a receipt for each load must be obtained.
- All handling and storage sites must have adequate and effective pest (including rodent) control measures.
- Samples should be taken regularly and these should be under the control of mycotoxin-analysis to ensure the level of mycotoxins below legal limits.
- In the HACCP procedure for the industry also mycotoxin detection should be carried out.

The following hygiene rules must be followed:
- All grain store walls, floors and horizontal surfaces of any storage, holding or reception facilities must be cleaned and where appropriate, washed and insecticide treated prior to use. Residues of previous crops must be cleaned from all areas including ventilated floors and beneath conveyers.
- Where livestock buildings are intended for use as grain storage or temporary holding facilities, they must be thoroughly cleaned and power washed at least 5 weeks prior to storage.
- Pre-harvest insect trapping in grain storage areas must be carried out to ensure cleaning operations have been successful.
- If any pre-harvest grain store pesticide are used, the product use, dose rate, date of application and operator must be recorded.
- All equipment used for the harvesting transportation handling, conveying and loading of grain must be thoroughly cleaned and the dates recorded.

5.5 Legal limits for Fusarium mycotoxins in the EU

G. Koornneef, D.G. Kloet and O.E. Scholten

At the European level no regulations have been determined yet for DON and ZEA. Nevertheless in the EU-regulation on contaminants (nr. 315/93), in article 2.1, there is a provision that foodstuffs with a content of a contaminant that is toxicological unacceptable, are not allowed to come on the market. This provision makes it possible for

the government of EU member states to act against foodstuffs that contains a contaminant in an amount that is considered to be dangerous to human health, even if the EU has not yet regulated this contaminant.

For example in Austria maximum tolerated levels of DON or ZEA in human food and animal feed have become valid since July 1, 1999. For humans, maximum tolerated levels of DON in wheat end products are 0.5 mg/kg and for ZEA 0.06 mg/kg. For pigs kept for meat as well as for breeding, maximum tolerated levels of DON are 0.5 mg/kg and for ZEA 0.05 mg/kg feed. For chickens kept for meat the maximum tolerated level of DON is 1.5 mg/kg feed, whereas for chickens kept for eggs and for breeding the level is reduced to 1.0 mg/kg feed. For cows kept for meat production the maximum tolerated level of DON is 1.0 mg/kg feed.

In Italy the Ministry of Health recommended the following maximum levels for ZEA, since June 10, 1999: 0.02 mg/kg in baby food and 0.1 mg/kg in cereals and cereal products.

In the Netherlands, wheat and DON were often in the news during the spring and summer of 1999, because of the detection of lots of winter wheat grown in 1997 and 1998 containing high levels of DON. As a result the Ministry of Health contacted the Dutch Board for Arable Crop Production and the Dutch Association of Flour Producers (NVM). Those contacts resulted in legal action limits for consumer products and for flour from the mills. The current action limit for cereal consumer products (as sold) is 0.5 mg/kg DON and the current action limit for flour from the mills (as sold to customers) is 0.75 mg/kg DON. There is no action limit for wheat, but in practice the Dutch millers buy wheat with a maximum of 0.75 mg/kg DON

In Germany a maximum tolerated level of 0.5 ppm DON in cereals and cereal products, 0,35 ppm in bread and bakeries with >30% cereals, and 0,1 ppm in food for infants are discussed by the government.

Also the EU-Commission considered DON as a problem and asked, as the first step in establishing legal limits, advice from the Scientific Committee for Food (SCF). The SCF preferred to consider all Fusarium toxins. Already on 2 December 1999 it expressed an opinion on DON, and opinions on other toxins were expressed later in 2000 and 2001 (see list below). The EU also gathers information on levels of Fusarium-toxins through a so-called SCOOP-Task in which 12 EU countries contribute. Germany is the co-ordinator of this task. Before December 31, 2002 this task should be completed. In 2000, the Commission became aware that the setting of maximum limits would take a long time. To help the member states and to prevent diverging maximum limits based on the EU-regulation on contaminants (nr. 315/93) article 2.1, the Commission has adopted the Dutch action limits for DON in cereal consumer products and in flour. On October 19, 2000 the Standing Committee for Foodstuffs agreed with the Recommendation of the

Commission in which these limits are contained. However, till now (31-10-02) this recommendation has not been published. It is expected that discussions about proposals for EU limits for trichothecenes and zearalenone will start soon after completion of the SCOOP-task.

Concerning Fumonisins an official tolerance value (Fumonisin B1+ B2) for dry maize products (1000 ug/kg) has been issued by the Swiss (Swiss Federal Office of Public health, 1997), while the French Council of Public Hygiene has recommended a maximum level of 3000 ug/kg for cereals.

List of opinions on Fusarium mycotoxins by the European Union:

- European Commission, Scientific Committee on Food, Opinion on Fusarium toxins, part 1: Deoxynivalenol (DON). Recommendation for a temporary tolerable daily intake (tTDI) for DON. Date 02-12-1999, see http://europa.eu.int/comm/food/fs/sc/scf/out44_en.pdf
- European Commission, Scientific Committee on Food, Opinion on Fusarium toxins, part 2: Zearalenone (ZEA). Recommendation for a temporary tolerable daily intake (tTDI) for ZEA. Date 22-06-2000, see http://europa.eu.int/comm/food/fs/sc/scf/out65_en.pdf
- European Commission, Scientific Committee on Food, Opinion on Fusarium toxins, part 3: Fumonisin B1 (FB1). Recommendation for a temporary tolerable daily intake (tTDI) for FB1. Date 17-10-2000, see http://europa.eu.int/comm/food/fs/sc/scf/out73_en.pdf
- European Commission, Scientific Committee on Food, Opinion on Fusarium toxins, part 4: Nivalenol (NIV). Recommendation for a temporary tolerable daily intake (tTDI) for NIV. Date 19-10-2000, see http://europa.eu.int/comm/food/fs/sc/scf/out74_en.pdf
- European Commission, Scientific Committee on Food, Opinion on Fusarium toxins, part 5: T-2 toxin and HT-2 toxin. Recommendation for a temporary tolerable daily intake (tTDI) for T-2 toxin and HT-2 toxin, see http://europa.eu.int/comm/food/fs/sc/scf/out88_en.pdf
- European Commission, Scientific Committee on Food, Opinion on Fusarium toxins, part 5: T-2 toxin, HT-2 toxin, NIV and DON, see http://europa.eu.int/comm/food/fs/sc/scf/out123_en.pdf

5.6 Harmonisation of standards for mycotoxins in the Codex Alimentarius

D.G. Kloet

In the Codex Committee on Food Additives and Contaminants (CCFAC) mycotoxins are regularly being discussed. There are interesting recent developments in the risk analysis process regarding mycotoxins. The CCFAC is one of the horizontal Committees operating under the Codex Alimentarius Commission (CAC), and convenes once a year in March. The CAC is an international organisation supported by FAO and WHO, aiming at facilitating world trade and protecting the health of the consumer by developing international standards for foods and feeds. The CCFAC develops standards in a procedure which follows the principles of risk analysis as far as possible, according to rules and methods as they are laid down in the general Codex Procedural Manual and more specifically in the Codex General Standard for Contaminants and Toxins in Food. The procedure operates by requesting discussion papers and position papers about all relevant aspects of a food contaminant when there is reason to expect health concerns and trade problems, followed by developing proposals for maximum levels when all necessary requirements for standard setting are fulfilled. These requirements are that health concerns can be substantiated, preferably on the basis of a toxicological and exposure assessment by JECFA, and that sufficient reliable data about levels in foods are available (preferably world wide distributed) to develop a maximum level on the basis of the ALARA principle.

Regarding mycotoxins, progress within the CCFAC had been slow until recently because of the lack of clear advice about the toxicological evaluation of compounds and often also the lack of reliable information about levels in foods world wide. Zearalenone was evaluated by JECFA in 1999; the CCFAC decided however that development of a ML was not necessary for the time being. In February 2001 the JECFA (WHO and FAO Committee of experts, which acts as a scientific advisory committee to the CCFAC) performed toxicological evaluations (hazard assessments) of DON, T-2 and HT-2-toxin and fumonisin B_1, leading to the establishment of PMTDIs for these compounds. The JECFA also reevaluated Ochratoxin A (OTA) and retained the existing PTWI. Much attention of the JECFA was also devoted to exposure assessments for all these mycotoxins and a quantitative risk assessment was e.g. performed for ochratoxin A, in relation to a CCFAC debate about suitable maximum levels (MLs). It was shown that high level consumers may approach the PTWI for OTA from consumption of cereals alone, which are the main contributors to the intake of OTA. This has led to an enhancement of the risk management discussions about the mycotoxins in the CCFAC. With a view on the risk assessment by JECFA establishment of MLs in cereals has been prioritised especially for OTA and DON; a proposal for OTA (at the level of 5 μg/kg in wheat, barley and rye and

derived products) is now at step 8 (near to adoption), while for DON a discussion paper has been discussed in March 2002 and proposals for MLs can be expected in 2003.

The CCFAC has apart from its goal to develop standards (MLs) where necessary also decided to devote much attention to developing Codes of Practice in which principles and advice about practical measures to control mycotoxins during cultivation, storage and processing are assembled. A proposed draft Code of Practice for the prevention (reduction) of mycotoxin contamination in cereals, including Annexes on OTA, zearalenone, fumonisin and trichothecenes, has been developed and is in discussion; it is expected to be finalised in 2003. Reports of Committee meetings, agendas and papers for future meetings, JECFA reports etc. are generally available on the Codex web site http://www.codexalimentarius.net.

6. The Chain-wide approach: Final conclusions and needs for further research

O.E. Scholten, P. Ruckenbauer, A. Visconti, W.A. van Osenbruggen and A.P.M. den Nijs

6.1 Future breeding and research needs in the EU

Within Europe the range of resistance available in commercial varieties differs between countries. In general, varieties from the UK are more susceptible than those from France and Germany. Clearly there is overlap between countries and this analysis says nothing about which varieties are successful commercially, and indeed whether disease prone areas, such as the south of Germany, only grows the more resistant varieties. However, this snapshot does show that German and French breeders have achieved a better standard of resistance than their UK counterparts, probably because they have seen a greater need for resistance, over a number of years, in their regions than UK breeders

For the future there is an urgent need for better resistance to meet both farmer and end user needs. Three strategies can be envisaged; continued emphasis on conventional disease selection, deployment of molecular markers for resistance and the possibility of using transgenic resistance. Breeders are trying to combine resistances from within the current German and French material to 'stack' the resistance factors and to introduce the better resistance from lines such as Sumai#3. Effective selection in the presence of the disease at key stages in the breeding process is essential for success. The ability to identify the 'fingerprint' of specific genes can offer considerable advantages in breeding. This fingerprint' of key genes which describes the resistance in Suma#3 has been identified (Sixin *et al.,*, 2001). Use of these molecular markers gives advantages such as the ability to identify the plant the breeder needs without using the pathogen and the ability to test at any time of the year as the assay is not tissue specific. In addition high throughput technology would allow much more material to be handled.

6.2 Final recommendations for the chain-wide approach

The general conclusion of the concerted action Mycotochain is that more research is needed to minimise mycotoxin-contaminated food and feed products. Looking chain wide,

there is a need for the development of a tracing and tracking system for harvested lots of grain. Furthermore, it is concluded that special attention is required for the following new topics of research, which are listed below for the various parts of the chain.

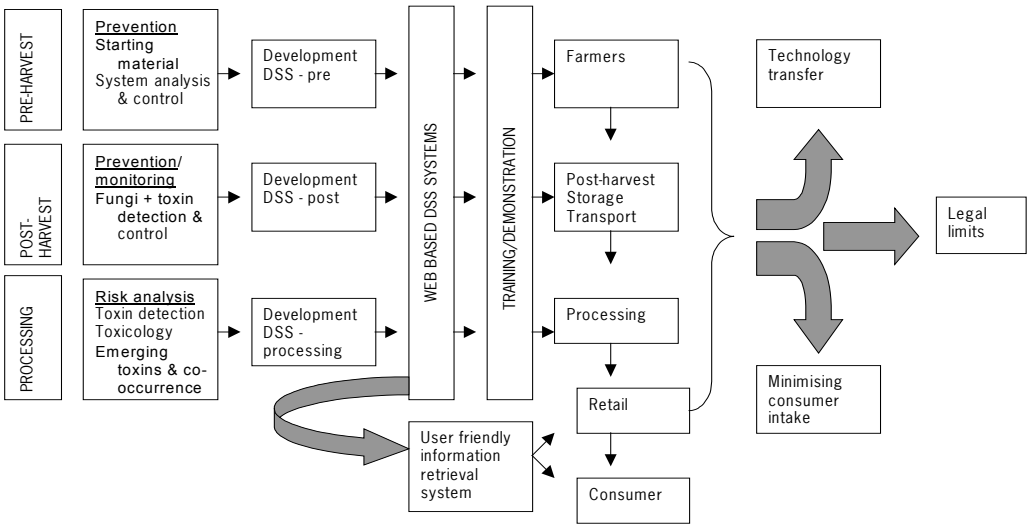
Under **crop rotation** it is recommended to concentrate further research on …
- Development of soil sanitation procedures
- Developments of bio-pesticides
- Designing multiple durable resistant varieties through application of genomics
- Designing GMO's with detoxifying genes
- Improving fungal detection in soil and plants
- Development of precision agriculture with GPS for real time monitoring crop and fungi

Under **storage and processing** it is recommended to concentrate further research on …
- Improving fungal detection in plants and harvested products
- Use of alternative toxicological assessments to reduce the use of animals (e.g. by using cell lines or artificial models)
- Interaction effect of toxins when they are combined in food or feed (see WHO Technical Report Series 906)
- Fate of mycotoxins during processing (including decontamination)
- Further development of decontamination and separation procedures (e.g. ASTER)

Regarding **analytical methods** it is recommended to concentrate further research on…
- Development of rapid and validated screening techniques for outside use, to obtain levels close to legal limits (e.g. NIT, biosensors)
- Further development of using sophisticated methods for confirmation of quick tests (e.g. LC-MS, MS-MS)
- Development and validation of horizontal methods of analysis (e.g. raw and processed materials)
- Sampling statistics

For Framework Programme 6 Expressions of Interests were requested to identify areas of research that need further research to be carried out. For analytical methods an Expression of Interest was prepared by partner H. Pettersson, together with R. Krska, IFA-Tulln, Austria, H.P. van Egmond, RIVM, The Netherlands and S. Mac Donald, CSL, United Kingdom and submitted to the European Commission. The title of this Expression of Interest is 'Methods of Analysis, sampling and quality assurance for effective control of mycotoxins in cereals'. The overall aim is to provide tools for an efficient control of the contamination of cereals with mycotoxins.

Discussions at Plant Research International regarding mycotoxins in cereals and other crops have led to the compilation of the following scheme that is based on the chain-wide approach:



(compiled by R. van den Bulk and P. Boonekamp, Plant Research International)

In April and May 2002, this scheme has been used in discussions among several participants of the Concerted Action Mycotochain together with other scientists from all over Europe. They have expressed their concern about the mycotoxin problem of food in general (so not only produced by *Fusarium* spp.). and have identified research questions. These discussions resulted in the preparation of an Expression of Interest entitled 'Food safety and quality: An integrated European management system for mycotoxins in food and feed' by N. Magan, Cranfield University, United Kingdom, and others, and has been submitted to the European Committee. The overall objective is to minimise consumer exposure to mycotoxins through development of innovative, qualitative and reasoned approaches to integrated management systems in the food chain. It addresses all research questions mentioned earlier by following the chain-wide approach. Submission of such Expressions of Interest again underline the need for further research in the area of mycotoxins.

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Appendix I. Partners of Mycotochain

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- G.H.J. (Gert) Kema
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Key words: Wheat, maize, resistance, inheritance, molecular markers, mycotoxin analysis (DON/NIV-HPLC), characterisation and detection of *Fusarium* species (quantitative pcr), ABC transporters, trichothecenes biosynthesis, epidemiology, crop rotation

Partner 2: Dutch Board for Arable Crop Production

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Other specialists
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Key words: Wheat , maize, breeding, resistance, inheritance, molecular markers, mycotoxin analysis (various mycotoxins and methods)

Partner 5: Agence Française de Sécurité Sanitaire des Aliments (AFSSA)

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Key words: Mycotoxin analysis, risk assessment

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Key words: Mycotoxin analysis, animal feed

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Key words: Mycotoxin analysis

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- M. (Michelangelo) Pascale
- A. (Alessandra) Ricelli
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Key words: Characterisation and detection of *Fusarium* species (molecular methods), fungal genetics, mycotoxin analysis (various mycotoxins and methods), standardization of analytical methods, epidemiology, risk assessment, food processing, detoxification, biomarkers, *Fusarium* phytotoxins.

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Key words: Mycotoxin analysis, characterisation and detection of Fusarium species (quantitative PCR), chain management, risk evaluation, detoxification

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Key words: Maize, breeding

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Key words: Wheat, research from crop production to consumer

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Key words: Wheat, storage

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Key words: Wheat, resistance, inheritance, molecular markers

Appendix II. European Research Projects related to Mycotoxins in cereals

- The Mycotoxin-Prevention Cluster: Prevention of mycotoxins entering the human and animal food chain
  Cluster co-ordinator: N. Magan, Cranfield University, United Kingdom
  http://www.mycotoxin-prevention.com

  1. Prevention of ochratoxin A in cereals
     (QLK1-CT-1999-00433; OTA PREV)
     Co-ordinator: M. Olsen, National Food Adminstration, Sweden
  2. Prevention of Fusarium mycotoxins entering the human and animal food chain
     (QLK1-CT-1999-00996; Control Mycotox Food)
     Co-ordinator: N. Magan
  3. Early detection of toxigenic Fusarium species and ochratoxigenic fungi in plant products
     (QLK1-CT-1999-001380; Detox-Fungi)
     Co-ordinator: G. Mulé, CNR, Bari, Italy

- The EMAN Network (European Mycotoxin Awareness Network) (QLK1-CT-2000-01248; EMAN)
  Co-ordinator: Richard Lawley. Leatherhead Food Research Association, United Kingdom
  http://www.mycotoxins.org/

- Sustainability, product safety and quality in cereals: development of novel quantitative models for risk assessment for *mycotoxigenic Fusarium*
  (QLK5-CT-1999-31517; RAMFIC)
  Co-ordinator: I. Hardie, Horticulture Research International, United Kingdom
  http://europa.eu.int/comm/research/quality-of-life/ka5/en/31517.html

- Risk assessments of biological control agents
  (QLRT-2000-01391, RAFBCA)
  Co-ordinator: T. Butt, Singleton Park, Swansea, United Kingdom
  http://www.swan.ac.uk/biosci/rafbca

- Novel tools for developing Fusarium resistant & toxin fee wheat (QLRT-2000-02044; FUCOMYR)
  Co-ordinator: P. Ruckenbauer, IFA-Tulln, Austria
  http://www.ifa-tulln.ac.at/Fucomyr/fucomyr.html

- Novel test kits for mycotoxins
  (CRAFT-199-70556; MycoSens)
  Co-ordinator: S. Holmes, ADGEN Agrifood Diagnostics, United Kingdom

- Genetic improvement of maize to introduce resistance to Fusarium moniliforme
  (ICA4-2000-3003; Safemaize)
  Co-ordinator: D. Berger, University of Pretoria, South Africa
  http://fabinet.up.ac.za/personals/dave2.html

- Development, validation and harmonisation of screening and confirmatory tests to distinguish zeranol abuse from fusarium toxin contamination in food animals
  (FAIR-CT97-3443; Natural zearaelone)
  Co-ordinator: G. Kennedy, Veterinary Sciences Division, Department of Agriculture and Rural Development, Belfasr, United Kingdom
  http://europa.eu.int/comm/research/agro/fair/en/uk3443.html

- Concerted Action QLK1 "Food Safety in Europe"
  Co-ordinator: B. Danse, ILSI EUROPE, Brussels, Belgium
  Food and Chemical Toxicology-Special Issue, vol. 40, n.2/3, 2002, p.237-427.

- IAEA-RCP on evaluation of methods of analysis of mycotoxins in foods end feeds
  Co-ordinator: B. Doko, IAEA, Vienna, Austria