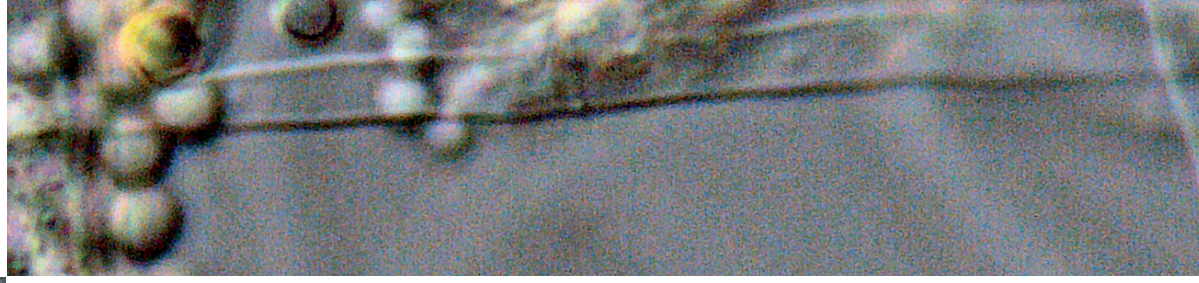
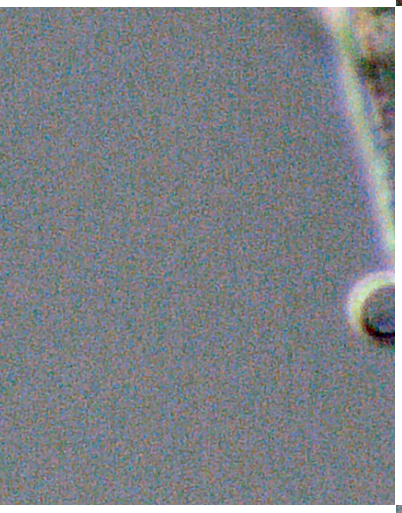
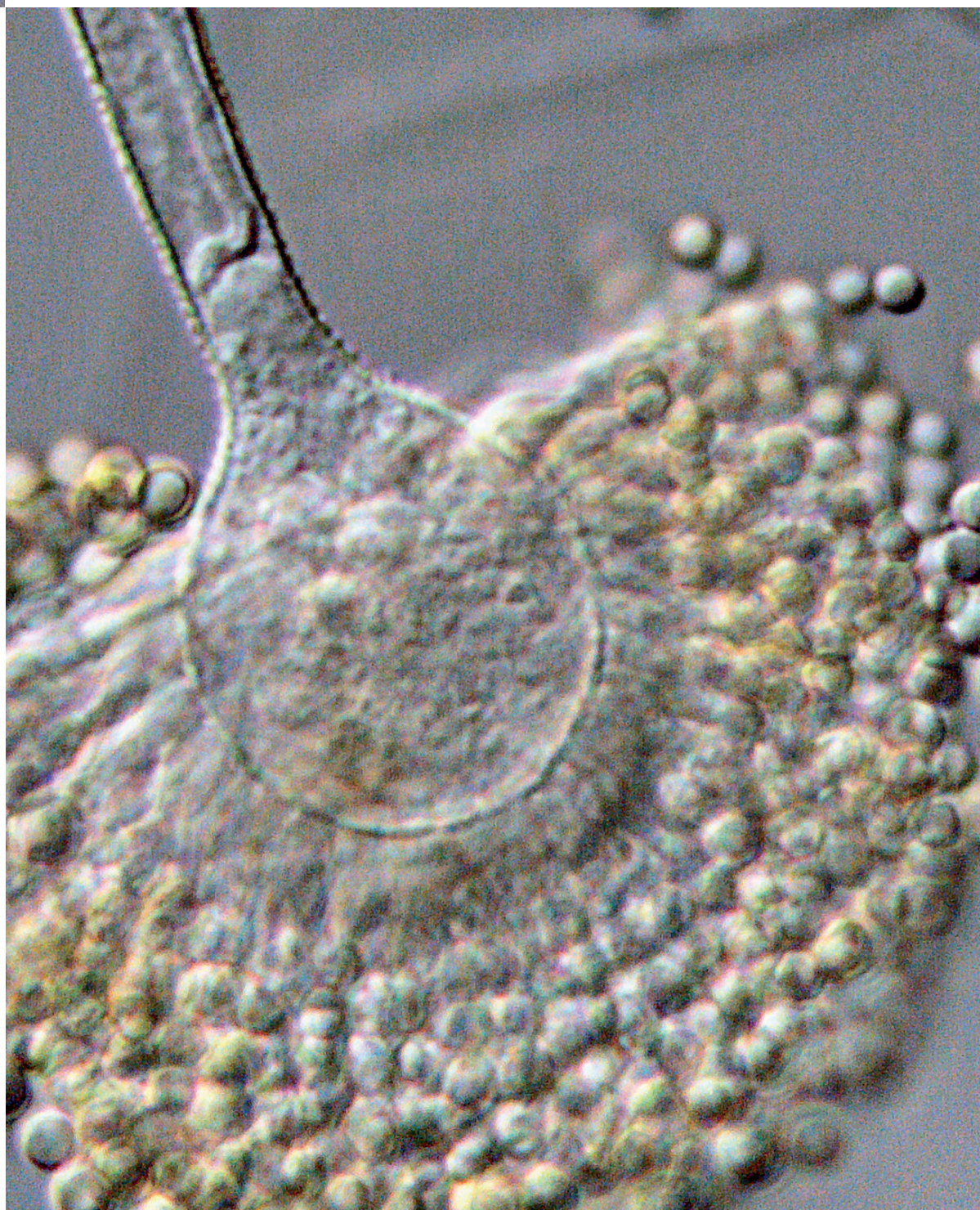




Mycotoxins and assessment of environmental risks in laboratory conditions in The Netherlands



CGM 2013-01

ONDERZOEKSRAPPORT

Mycotoxins and assessment of environmental risks in laboratory conditions in The Netherlands

COGEM Research Report CGM 2013-01

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Keywords

Mycotoxins, Risk Assessment, Occupational Health

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De uitvoering van het onderzoek is begeleid door een commissie samengesteld uit de volgende leden:

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Preface

Risk classification of the construction and handling of genetically modified organisms (gmo's) largely depends on the potential danger of the involved organisms for humans, animals, or plants. The production of toxins often results in classification of (micro-) organisms in pathogenicity class 2 or higher. Consequently, the construction and use of gmo's producing toxins are usually legally restricted to laboratory environment with a biosafetely II (ML-II) or higher.

A potential problem in biosafety classification of toxin producing organisms may arise when otherwise harmless organisms produce toxins. These toxins may cause direct tissue damage in humans, animals, or plants, but equally well may behave like chemical compounds that may only be toxic after prolonged or repeated exposure. Moreover, it is possible that the toxins are produced only under defined environmental conditions that may not exist in the standard laboratory environment. It seems irrational and undesired to apply strict biosafety regulations to the conditions where biological risks are minimal and chemical safety regulations seem more appropriate.

The scenario of tightly regulated production of toxins by otherwise seemingly harmless organisms applies particularly for fungal species. In a previous project supported by COGEM, classification of fungi was complicated because some mycotoxins (e.g. aflatoxins) may be present on food products and a major threat for human health, whereas others may be non-pathogenic or even be used in food production (e.g. *Penicillium roquefortii* in cheese production). This raises the question as to how the production of mycotoxins by distinct fungi should be considered as a risk factor in the classification of fungal species. To address this issue, the COGEM and GMO office have commissioned the writing of an independent report on mycotoxin occurrence, regulation, production and toxicity with the ultimate goal to have a more solid basis for a rational risk assessment of mycotoxin producing fungal strains.

The research project was assigned to dr. Cees Waalwijk of Plant Research International (PRI) and dr. Ir. W.C.M. de Nijs of the RIKILT Institute of Food safety. Based on their expertise and analysis of the literature, an overview has been compiled about the chemical, physical and toxic properties of mycotoxins as well as about the biosynthesis of mycotoxins under different environmental conditions. The report emphasizes that the identification of the used organism(s) with novel molecular techniques should be a first key step in any classification of genetically modified fungi and provides a flow chart of criteria that may aid the classification process.

Prof. dr. J.P.M. van Putten
Chairman advisory committee
Faculty of Veterinary Medicine, Utrecht University
COGEM subcommittee 'Medisch Veterinair'

Content

Abbreviations

AF	Aflatoxin
BSC	Biological species concept
DON	Deoxynivalenol
EFSA	European Food Safety Authority
EU	European Union
FFSC	*Fusarium fujikuroi* species complex
FUM	Fumonisin
GM	genetically modified
GRAS	Generally recognized as safe
IARC	International Agency for Research on Cancer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	Lethal dose at which 50% of the organisms die of the treatment
MSC	morphological species concept
MT	Mycotoxin
NRPS	Non-ribosomal protein synthetase
OTA	Ochratoxin A
PKS	Polyketide synthase
PSC	phylogenetic species concept
QPS	Qualified Presumption of Safety
SM	Secondary metabolite
SMB	Secondary metabolism
ST	Sterigmatocystin
TDI	Tolerable daily intake
TF	Transcription factor

Summary

Working with genetically modified organisms (GMOs) in the laboratory always requires a certain level of containment. Main issue is to prevent spreading of GMOs to the environment. In addition, the occupational health of the personnel working with GMOs in the laboratory must be secured. In case the GMO is a fungus evaluation with respect to (1) pathogenicity of the fungus towards humans, animals or plants is required as well as its (2) ability to produce mycotoxins.

The evaluation starts with the adequate identification of the fungus at strain level. This allows a reliable assessment of pathogenicity and mycotoxin producing capacities. Up to recent, identification of fungal strains was mainly based on morphological characteristics which, in many cases, has proven to be insufficient for identification at the species level, let alone at strain level. However, DNA based methods currently enables unambiguous identification of the fungus. Nevertheless, when assessing (older) literature for pathogenicity or mycotoxin production awareness of possible misidentification must be considered.

In addition, mycotoxin production of the fungal strain needs to be assessed. Over 300 mycotoxins are known but only a few have been evaluated for toxicity for humans and animals. The toxic effects of mycotoxins depend on animal species and on concentrations ingested. Since mycotoxins are often secreted into food and raw materials, EU regulations have been set for mycotoxins in food and feed commodities.

Mycotoxin production by a fungus depends on genetic make-up and growth conditions (both under field or laboratory conditions). The recent notion that many genes are involved in mycotoxin production occur in gene clusters opened the possibility of unambiguous identification of the ability and inability of a fungus to produce certain mycotoxin. Currently, these gene clusters have been recognized for most common mycotoxins.

Aspects to substantiate risk assessment on potentially mycotoxin producing fungi should include fungal taxonomy, toxin production, and relevant genetic make-up and growth conditions of these fungi in laboratory situations.

1. Introduction

Fungi can both benefit and pose risks to human health. Several fungi can produce beneficial products, such as antibiotics, and fungi are also involved in food fermentation for preservation (e.g. cheese) and degradation of certain macromolecules to allow digestion (e.g. tempeh). However, fungi may cause deterioration of food and feed materials or excrete metabolites into the food and feed. Some of these metabolites are toxic and can thus challenge the health of humans (and animals) that are consuming these products. However, toxin production is highly variable and depends on many factors such as the availability of substrates, water activity, light and temperature. This explains why potential toxigenic fungi that have a long history of use in multiple commodities, e.g. *Penicillium roqueforti* in the production of blue cheeses, lack noticeable adverse health effects. This apparent contradiction of a fungus in use in food processing for centuries and its capacity to produce mycotoxins, requires knowledge concerning the conditions that are conducive for mycotoxin production.

There is a need for guidelines to evaluate fungi for their environmental safety in laboratory conditions, particularly when these fungi are going to be genetically modified.

The environmental and occupational health aspects of working with genetically modified toxigenic fungi in a laboratory situation are discussed in this study.

2. Scope of this study

This survey will focus on factors relevant for risk assessment of fungi potentially producing a restricted set of mycotoxins. It should be noted that allergens will not be considered in this review.

A literature survey showed that 42% of ~ 23,000 bioactive microbial products, i.e., antifungal, antibacterial, antiviral, cytotoxic and immunosuppressive agents, can be produced by fungi (Lazzarini *et al.,* 2000). It is, however, estimated that fungi can produce more than 200,000 secondary metabolites, 300 of which may be regarded as mycotoxins (Cole and Cox, 1981). It is not the scope of this survey to evaluate all these 300 mycotoxins since solid toxicological evaluations exist for only a few of them. The list of mycotoxins considered in the study presented here, is based on current legislation and upcoming EFSA opinions (http://www.efsa.europa.eu/en/publications.htm). Literature was reviewed for fungi that are associated with the production of these mycotoxins. EFSA and JECFA documents were analysed for toxicological evaluations and QPS status of fungi.

The study presented here will not consider the application of genetically modified fungi under field conditions. Also, fungi capable of causing human or animal mycoses will not be considered, as mycoses are the result of the pathogenicity of fungi capable of causing infections with adverse health effects in humans and/or animals. Likewise, fungi can be opportunistically pathogenic when they cause adverse health effects in immuno-compromised patients (Boekhout, 2011).

Mycotoxins

3.1. Definitions

Fungi produce primary and secondary metabolites, some of which are toxic, while others may be potent allergens. The definitions of the different compounds that have been adopted in the present study are indicated below. It should be noted that according to these definitions mycotoxin toxicity is related to the consumption of contaminated food or feed, but that it is believed that professional workers may also be exposed to mycotoxins via inhalation and/or skin contact.

Mycotoxins (MTs) are secondary metabolites (SMs) produced by fungi that may have adverse effects on the health of humans and animals after consumption of contaminated food or feed.

Primary metabolites are a diverse group of molecules produced by all organisms that are essential for survival of the producing organism (i.e. being part of the central metabolism), e.g..

Secondary metabolites (SMs) are an extremely diverse group of molecules produced by most living organisms, including many filamentous fungi. These compounds are the result of the combined action of multiple enzymes, of which the encoding genes may or may not occur in [gene] clusters in the genome of the producing organism (Khaldi *et al.,* 2008). Secondary metabolism (SMB) may be regarded as a safe way to eliminate so-called waste products as they are most commonly produced at highest levels during the transition from active growth to stationary phase. This view is in agreement with the finding that the producing organism can survive (at least for some time) without SMB, suggesting that it is not essential (Firn and Jones, 2009). Nevertheless, SMB and SMs are integral parts of the organism's biology, which is supported by the fact that substrates/precursors (e.g. amino acids, acyl-CoA and carbohydrates) and the energy required for the synthesis of SMs are recruited from primary metabolism. In addition, SMs may add to the survival and/or proliferation of the producer (e.g. protection to UV radiation, recognition of mating partners, inhibition of the growth of competitors, repulsion of predators [Rohlfs *et al.,* 2007], virulence factors, etc). SM production is the result of enzyme activities, that mainly occur when growth and primary metabolism

cease and is primarily influenced by the genetic make-up of the organism, but also by environmental factors such as temperature, substrate, humidity and time. SMs may be excreted under conducive conditions into crops during infection of plants in the field by plant pathogenic fungi (Jestoi *et al.,* 2008; Palencia *et al*., 2010; Pirgozliev *et al*., 2002; von der Ohe *et al*., 2010). Alternatively, when products are not stored under optimal conditions, opportunistic fungi can invade commodities, proliferate and excrete SMs into the product. Some of the SMs excreted by the fungi can be toxic and are, therefore, mycotoxins.

Toxic primary metabolites. Toxic primary metabolites are products from primary metabolism that are toxic for humans and animals, e.g. **proteinaceous toxins** or [fungal] **allergens**.

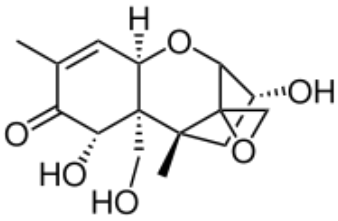
Allergens are products of primary or secondary metabolism, that are capable of causing an allergic response.

In this review, only mycotoxins as described in the definitions above will be discussed.

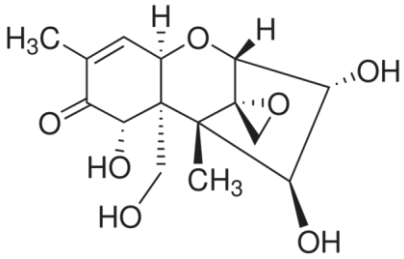
3.2. Classification and physical properties of mycotoxins

3.2.1. Chemical classification of mycotoxins

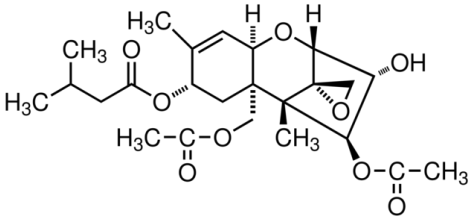
The total number of compounds which can be considered as mycotoxins is estimated to exceed 300 (Cole and Cox, 1981). Mycotoxins are grouped into different chemical classes based on the backbone of the molecule. However, the structure of the various mycotoxins is very divers. Nevertheless, five major classes can be identified: (i) trichothecenes, like deoxynivalenol (DON, see Figure 1), nivalenol (NIV), T-2 toxin and HT-2 toxin and derivatives thereof, belonging to the sesquiterpenes. (ii) MTs are also found in multiple classes of polyketides: different forms of aflatoxins (Figure 2) that belong to this class of compounds, which also encompass *Fusarium* MTs such as fumonisins (Figure 3) and zearalenone (Figure 4) and ochratoxin which is produced by several *Aspergillus* and *Penicillium* species (Figure 5). (iii) Non-ribosomal protein synthases are involved in the production of cyclic depsi-peptides like beauvericin and enniatins (Figure 6). (iv) Ergot alkaloids produced by *Claviceps purpurea* and (v) other MTs that cannot be easily grouped, such as moniliformin (Figure 7).



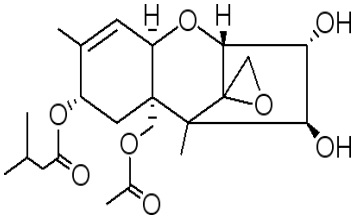
Deoxynivalenol



Nivalenol



T2-toxin



HT2-toxin

Figure 1. Chemical structure of important trichothecenes: Deoxynivalenol; CAS # 51481-10-8; molecular formula $C_{15}H_{20}O_6$; soluble in common polar organic solvents as acetonitrile, methanol and ethyl acetate, slightly soluble in water; Nivalenol; CAS # 23282-20-4; molecular formula $C_{15}H_{20}O_7$; soluble in acetonitrile; T2-toxin CAS # 21259-20-1; molecular formula $C_{24}H_{34}O_9$; soluble in chloroform. HT2- toxin; CAS # 26934-87-2; molecular formula $C_{22}H_{32}O_8$, soluble in chloroform.

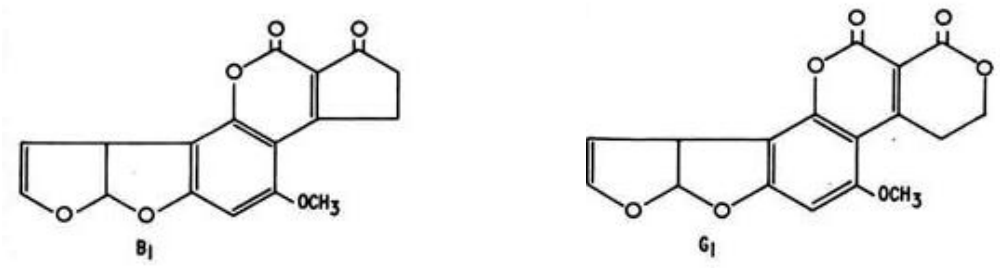


Figure 2. Chemical structure of Aflatoxin B_1; CAS # 1162-65-8; Molecular formula $C_{17}H_{12}O_6$; Soluble in water and polar organic solvents. Aflatoxin G_1 – CAS # 1165-39-5; Molecular formula $C_{17}H_{12}O_7$; Soluble in chloroform.

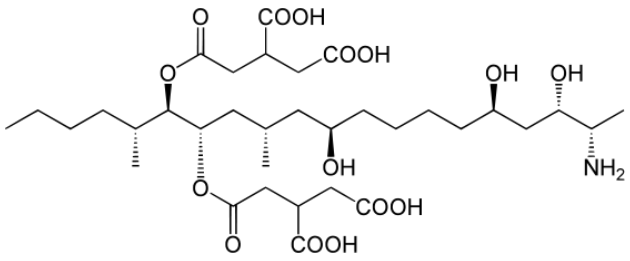


Figure 3. Fumonisin B_1; CAS # 116355-83-0; Molecular formula $C_{34}H_{59}NO_{15}$; soluble in methanol and acetonitrile.

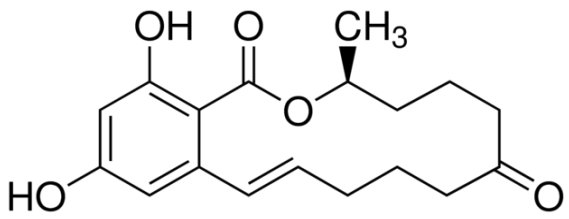


Figure 4. Zearalenone; CAS # 17924-92-4; Molecular formula $C_{18}H_{22}O_5$; soluble in methanol.

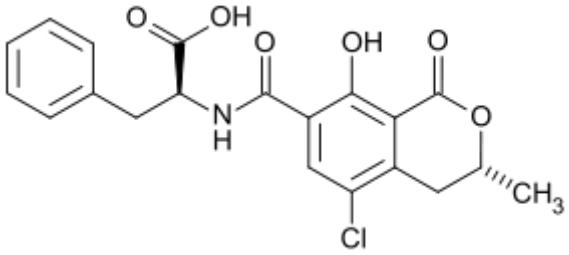
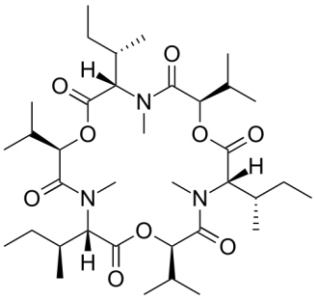
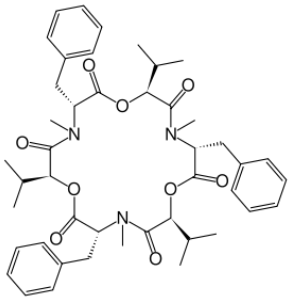


Figure 5. Ochratoxin A $C_{20}H_{18}ClNO_6$; CAS # 303-47-9; soluble in DMSO, Methanol, Ethanol.



Enniatin A1



Beauvericin

Figure 6. Enniatin A1; CAS # 4530-21-6; Molecular formula $C_{35}H_{61}N_3O_9$; soluble in ethanol, methanol, DMF or DMSO; and Beauvericin; CAS # 26048-05-5; molecular formula $C_{45}H_{57}N_3O_9$; soluble in acetonitrile and methanol.

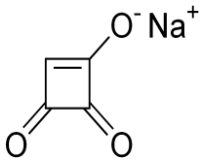


Figure 7. Moniliformin sodium salt; CAS # 71376-34-6; Molecular formula C_4HO_3Na; soluble in water and other polar solvents.

3.2.2. Physical properties of mycotoxins

Several physical properties are relevant for the toxicity assessment of mycotoxins, including solubility, processing stability, decontamination recalcitrance.

Solubility. Mycotoxins have very different molecular structures, as is shown in figures 1-7. The variations in polarity make them more better or less soluble in polar or organic solvent. Most MTs are rather heat stabile which complicates degradation during e.g. food processing. The majority of MTs is fairly polar and, therefore soluble in watery solutions. Special attention needs to be paid to ochratoxin A, since this mycotoxin is soluble in fat. This means that its half-life in the animal or human body is long, with a $t_{1/2}$ of about 35 days in humans (Duarte *et al.,* 2011).

Processing. Most MTs are rather heat stabile which complicates degradation during processing. *Fusarium* mycotoxins DON, NIV, T-2/HT-2 toxin and zearalenone are stable under food processing conditions applied for bakery products (Schwake-Anduschus *et al*., 2012; Scudamore *et al*., 2009; Voss and Snook, 2010). Cleaning of raw cereals may reduce the concentration of certain MTs, as was shown for ergot alkaloids (Franzman *et al*., 2011).

Decontamination. Several methods, based on application of chemicals or enzymes, are under investigation to reduce mycotoxins in raw materials. Ammoniation can be applied to corn, peanuts, cottonseed and flour to effectively reduce aflatoxin levels (Park, 2002). Alkaline treatment, or nixtamalisation, as traditionally used for tortilla preparation, reduces fumonisin levels (Hartinger and Moll, 2011). Enzyme treatment may also be promising to reduce MTs in feed. However, more research is needed to verify the safety issues of the products that are formed in during chemical and enzymatic processes. Treatments of food to reduce MT levels, however, are not allowed in the EU.

3.3. Toxicity

3.3.1. Routes of exposure

The main route of exposure of humans and animals to mycotoxins is through consumption of contaminated foods. It is believed that workers in the cereal industry may get exposed to high levels of mycotoxins by inhalation of contaminated dust. In the mid-1990s Snijders *et al.* (1996) noticed that planters replanting stored marram grass developed lesions of the skin and mucous membranes.

In a laboratory, humans may be exposed to fungal spores through inhalation when working in a laboratory without proper protection. This may lead to the development of allergic reactions and other symptoms such as skin rashes and mucosal irritation or even bleeding (Samson *et al.*, 2010). Spores can be contaminated with mycotoxins but exposure of humans to mycotoxins through spores is generally regarded as low (Kelman *et al.,* 2004; Hardin *et al.,* 2009). An exception is formed by the spores of the indoor mould *Stachybotrys chartarum* that may can contain high levels of mycotoxins (Pestka *et al*., 2008).

3.3.2. Mode-of-action

For few mycotoxins the mode-of-action is known. Modes-of-action vary greatly between MTs and effects may vary dramatically between animal species. Examples here are given for aflatoxin B_1 and fumonisin B_1. When aflatoxin B_1 reaches the liver, it is transformed to the active compound (Figure 8). The adverse reaction, in particular cancer, takes place in the liver, but can also happen in other organs (Gross-Steinmeyer and Eaton, 2012).

Fumonisin, on the other hand, is structurally related to sphingolipids and can bind to mammalian ceramide synthase thus disrupting sphingolipid metabolism (Figure 9). There seem to be large differences in sensitivity between the various animal species. The disruption leads to the hole-in-the-head syndrome in horses, lung oedema in pigs and renal toxicity in rabbits. From epidemiological studies in various parts of the world, fumonisins have been associated with oesophageal cancer in humans (Voss *et al.*, 2007). Moreover, fumonisin has also been implicated with the increased frequency of neural tube defects in new-borns (Gelineau-van Waes *et al.,* 2009).

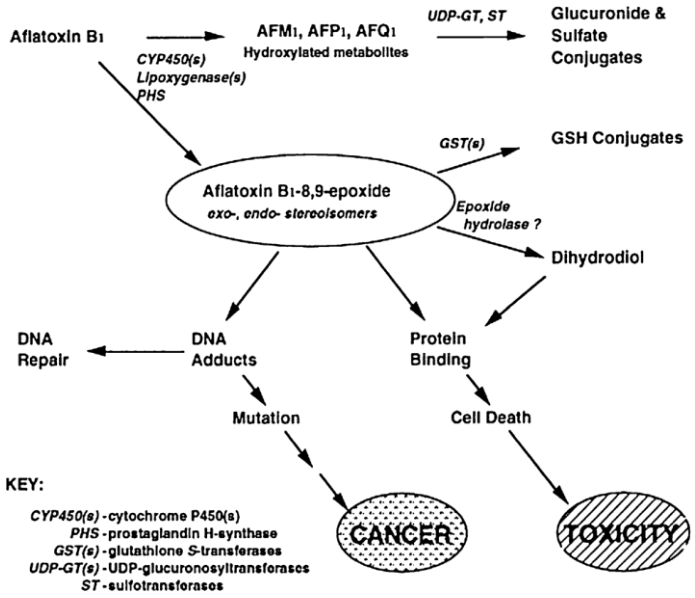


Figure 8: Role of biotransformation of aflatoxin in hepatocarcinogenesis (reproduced from Gross-Steinmeyer and Eaton, 2012).

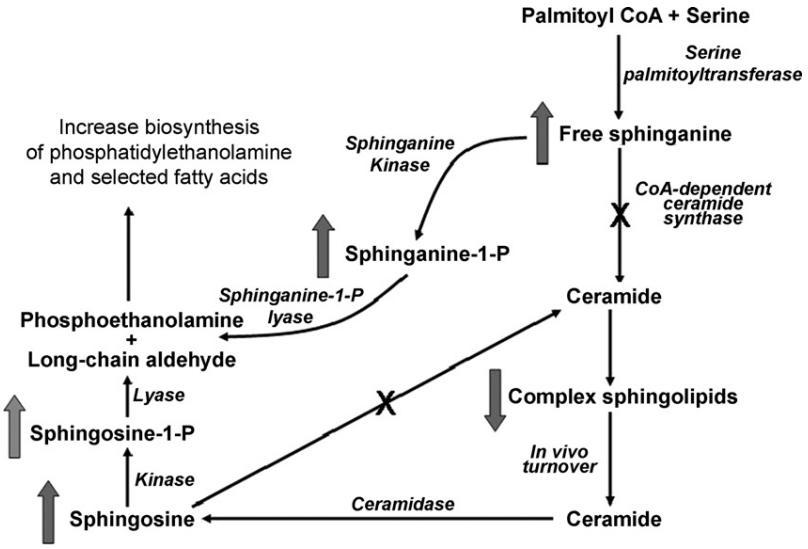


Figure 9 Schematic summary of sphingolipid metabolism showing the inhibition of ceramide synthase (✕) by fumonisins. Inhibition blocks the formation of ceramide from sphinganine and fatty acyl-CoA (not shown in figure) via de novo synthesis or from sphingosine produced by the ceramidase-mediated breakdown of ceramide. The consequences of ceramide synthase inhibition include increased tissue and serum concentrations (up arrows) of sphinganine (to a lesser extent), sphingosine, and their 1-phosphate metabolites as well as decreases (down arrow) in complex sphingolipids downstream of ceramide (reproduced from Voss *et al.*, 2007).

Exposure to low levels of mycotoxins may lead to illness only after a live-long chronic exposure. In contrast, acute toxicity is known for various MTs. Human diseases and fatalities have been reported, e.g. human fatalities in Kenya in 2004 due to the consumption of maize heavily contaminated with aflatoxins (Kensler *et al.,* 2011; Probst *et al*., 2007). The chronic exposure to aflatoxins is associated with the incidence of hepatocellular carcinoma in humans (Kensler *et al.,* 2011). Studies in The Gambia and Benin showed a correlation between maternal exposure to aflatoxin and stunted growth of their children (Kensler *et al.,* 2011; Wild, 2007). The mycotoxin OTA is related to the disease Endemic Balkan Nephropathy (Bennett and Klich, 2003). Rice, contaminated with citreoviridin, was associated with 32 fatal human beriberi cases in Brazil in 2006 (Rosa *et al*., 2010). Beriberi is generally caused by thiamine (vitamin B1) deficiency and administration of thiamine to patients improved their health condition considerably (Rosa *et al*., 2010). However, the authorities decided to withdraw the rice stock from the market and to replace it by rice from another region (Rosa *et al.,* 2010). Ergot alkaloids have been associated with human intoxication in Europe in the Middle Ages (Bennett and Klich, 2003). The vasoconstrictive properties of these mycotoxins led to the notorious Saint Anthony's fire depicted in the painting entitled the beggars by Pieter Breugel the Elder (Matossian, 1989). Interestingly, ergot alkaloids are also used in the treatment of Parkinsonism and are being tested as medicines to treat migraine (Bennett and Klich, 2003).

3.3.3. Toxicity evaluation

Toxicity is evaluated based on the results of animal studies published in peer reviewed journals, and if described, human intoxications. Evaluation of the toxicity is the first part of the risk assessment. EFSA and JECFA are currently the two authorities in the world that perform toxic evaluations. Most known are EFSA and JECFA that publish the results of the toxicity evaluations on the internet (http://www.efsa.europa.eu/en/publications.htm; http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/en/). If enough reliable toxicological data are available, a tolerable daily intake (TDI) can be determined. A TDI can only be set for unavoidable substances with threshold of toxicity and when chronic exposure is relevant, as for many mycotoxins such as deoxynivalenol. The TDI is the amount of an unintended substance in air, food or drinking water that can be consumed on a daily basis over a lifetime without appreciable health risk.

3.3.4. Toxicity of several of the most known mycotoxins

An overview of toxic effects of several of the regulated MTs and some other MTs of interest is shown in Appendix 5. The data are based on EFSA and JECFA risk assessment documents, that are available on the internet. The data presented in Table 1 show that most mycotoxins can have more than one adverse effect on the health of humans and animals.

In general, fungal secondary metabolites have been identified as mycotoxins after animals suffered from illnesses not being caused by infectious agents. Ingested feeds were subsequently analysed for the presence of fungi and fungal metabolites. It was often concluded that the detected metabolite was the cause of the disease. Furthermore, metabolites can be indicated as mycotoxins after adverse reactions have been identified in cytotoxicity assays or if the backbone of the molecule resembles a known mycotoxin (Cole and Cox, 1981). *In vitro* toxicity experiments require a small amount of pure metabolite but the predictive value of these results for animal or human health is often unclear.

Published studies on toxic evaluations of mycotoxins are scarce. Isolation of sufficient pure metabolite is costly, as are animal experiments. Animal experiments using fungal culture material spiked to feed lots, or naturally contaminated feed, instead of pure metabolite, may be easier to perform but may also be hard to interpret due to (synergistic or antagonistic) interactions between multiple SMs. In addition, expression of the SMs may be different in culture compared to natural substrata. Finally, interpretation of the data may be confused due the presence of masked MTs or other hitherto unrecognized MTs (Dall'Asta *et al.,* 2010)

4. Mycotoxin producers

4.1. Mycotoxin producing fungi

Mycotoxins are produced by diverse groups of fungi, however almost all belong to the phylum Ascomycota The majority of toxigenic species can be found in the genera: *Aspergillus*, *Fusarium* and *Penicillium*. In addition, several other genera have been implicated in the production of toxic SMs as indicated in Table 1.

Mycotoxin producing fungi from the genera *Aspergillus*, *Fusarium* and *Penicillium* are commonly found on food and feed commodities, e.g. small grain cereals, maize, nuts, coffee, grapes and fruits. Most prominent are *Fusarium* species in small grain cereals like wheat, barley, triticale, oats and rye (Nielsen, 2011), *Fusarium* and *Aspergillus* species in maize and *Aspergillus* and/or *Penicillium* species on nuts, grapes and coffee.

All of the before mentioned fungi infect crops in the field, either as infectious plant pathogen or as opportunistic pathogen. The fungi can form mycotoxins during growth and excrete mycotoxin in the plant under favourable field conditions. In fact, some MTs are required for an efficient infection of the plant (Kang and Buchenauer, 1999; Maier *et al.,* 2006). Since species of the genera *Aspergillus* and *Penicillium* are able to grow at low water activity, these fungi can continue to grow and synthetize MTs during storage of the crop.

4.2. Fungal identification

4.2.1. Morphology

Classical identification is based on morphological characters, such as (spore) morphology and colour of colonies on different media, microscopic characteristics and production of odours (Samson *et al*., 2010). The establishment of culture collections and the need to exchange strains for research and industrial purposes has led to the development of morphological identification keys. The re-evaluation of whole genera has resulted in more standardised identification but also in renaming of species. Among mycologists, agreement has been reached that older names have priority (Samson *et al*., 2010). Another point of attention is that many species have an asexual as well as a sexual state. Both states have their own identification system. The morphological species concept (MSC) is useful in many situations, but other methods have been developed to overcome

some of the issues associated with it. To avoid confusion several research communities working with specific fungal species have adopted the principle of one fungus, one name (www.aspergilluspenicillium.org; Geiser *et al.,* 2012; Wingfield *et al.,* 2012). Advantage of morphological identification is that the required equipment has limited financial impact. It requires agar based media, an incubator and a microscope. One can have a quick and dirty impression of the unknown isolate. However, to make reliable identifications, as for publication, highly skilled personnel is required.

4.2.2. Genetics

Next to the MSC, the biological species concept (BSC) has been introduced into mycology. Principle of BSC is that when two individual strains are able to cross and produce viable progeny, they are considered to belong to the same mating population (Taylor *et al.,* 2000). These genetic boundaries were used to delineate the species. Impediment of this system is the fact that many species are asexual, or rarely show crossing ability under laboratory conditions or in the field. These species are, therefore, considered to be either asexual or exhibit a form of cryptic sexuality. In addition so-called bridging isolates have been identified that are able to cross (with low efficiency) with members of two separate mating populations (Leslie *et al.,* 2007).

4.2.3. Molecular characterization

To avoid discussions on morphological identification and overcome the drawbacks of asexual species, the phylogenetic species concept (PSC) has been developed. The method is based on DNA sequencing of single loci. Large numbers of single loci of isolates are sequenced and the sequences are compiled in databases, like NCBI (http://www.ncbi.nlm.nih.gov/) and *Fusarium* ID (www.fusariumdb.org/*)*. These databases are available to be queried by researchers to establish the identity of the [unknown] sample. To understand the power of the various identification methods the species complex comprising a.o. *Fusarium* species producing fumonisins, the *Fusarium fujikuroi* species complex, FFSC, can be separated into three species by morphology. These can be further divided into 15 species based on the BSC, while identification using multiple sequence loci resulted in > 50 species according to the PSC (O'Donnell *et al.,* 1998; 2000)

5. Biochemistry of mycotoxins and genetics of the related genes

As indicated in Appendix 4 the biosynthetic pathways of a number of MTs has been elucidated and the genetic basis of the enzymes involved in the production of the most important MTs is known.

5.1. Biochemical pathways

SMs are small bioactive molecules with molecular weights less than 1000 daltons. Their biosynthesis requires the production of a C-backbone, usually involving either a polyketide synthase (PKS), a non-ribosomal peptide synthetase (NRPS), a (sesqui)terpene synthase or a dimethylallyl transferase (DMATS). In subsequent steps, these backbones are modified to contain acyl-, hydroxyl-, keto- groups, unsaturated bonds and alike. Finally, these intermediates are decorated by the addition of single or multiple side-groups, which can lead to a variety of compounds. Elucidation of these complex biochemical pathways required over a decade of research, including feed-and-chase experiments with labelled intermediates. Figure 10 illustrates the biosynthesis of the trichothecenes DON, NIV and T-2 toxin from the primary metabolite farnesyl pyrophosphate. The biosynthetic pathway leading from acetate to aflatoxins AFB_{1-2} and AFG_{1-2} is shown in figure 11.

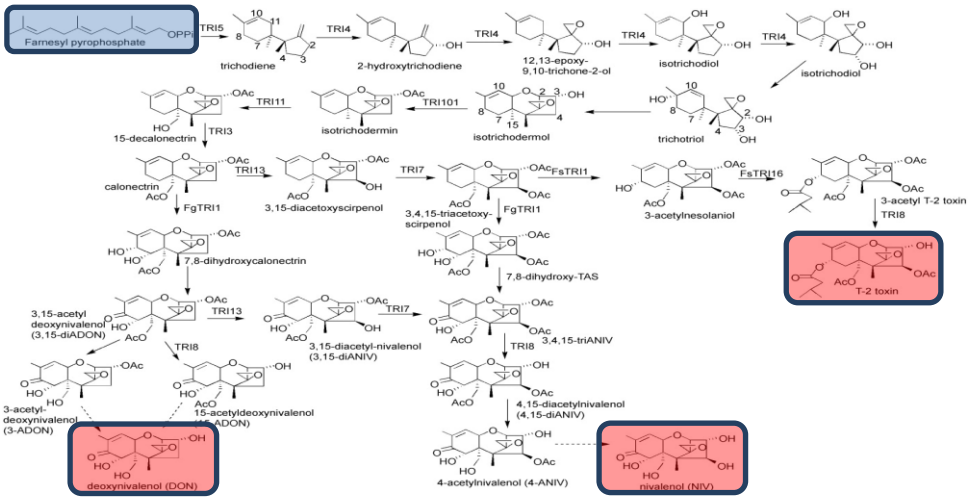


Figure 10. Biosynthesis of trichothecenes DON, NIV and T2-toxin (shown in red) from farnesyl PPi (adopted from Alexander *et al.,* 2009)

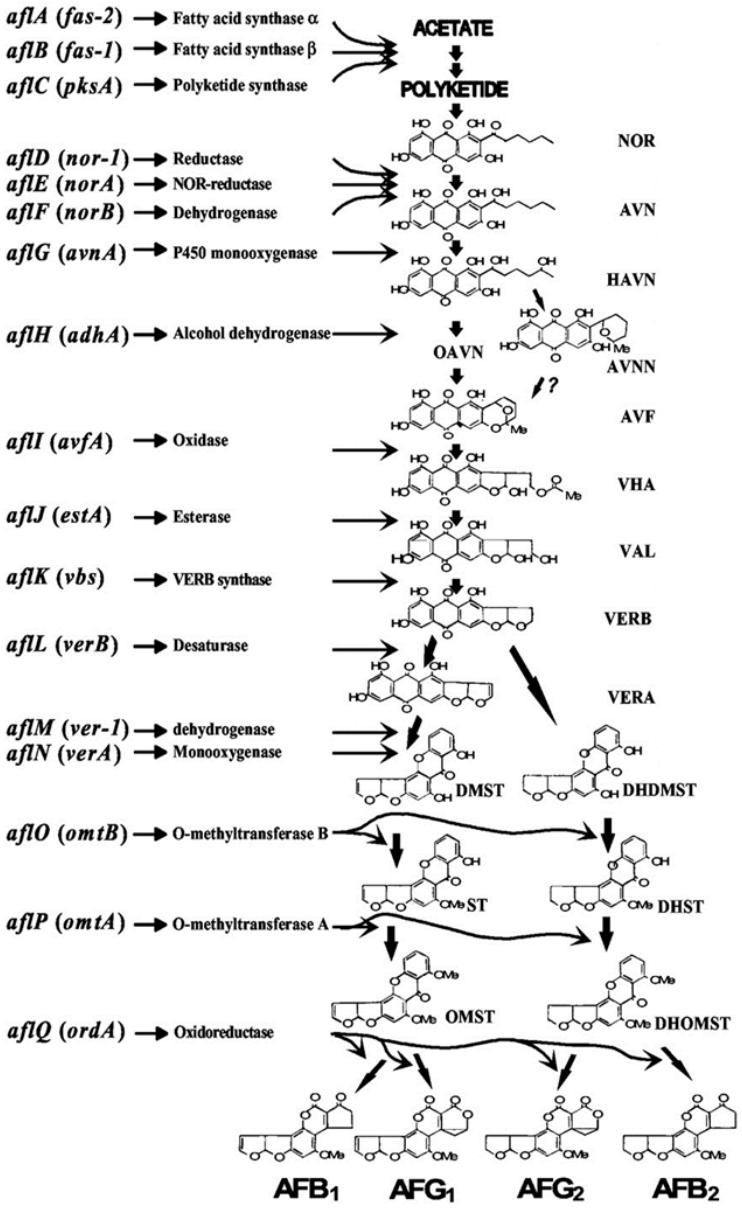


Figure 11. The generally accepted pathway for the biosynthesis of aflatoxins. The enzymes involved in each bioconversion step are shown. Arrows indicate the connections from the genes to the enzymes they encode, from the enzymes to the bioconversion steps they are involved in, and from the intermediates to the products in the aflatoxin bioconversion steps. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5'-hydroxyaverantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; AFG2, aflatoxin G2 (reproduced from Yu *et al.,* 2004a).

5.2 Genetic aspects of mycotoxins

The genes encoding the enzymes involved in the synthesis of any particular SM are usually clustered and act as a single genetic locus (Keller *et al.,* 2005; Yu and Keller, 2005, Hohn *et al.,* 1993; Ward *et al.,* 2002; Brown *et al.,* 2004). In addition, SM gene clusters are not randomly distributed in the genome, but are often located in subtelomeric regions (Perrin *et al.,* 2007; Palmer and Keller, 2010). Examples of such MT-gene clusters are depicted in figure 12.

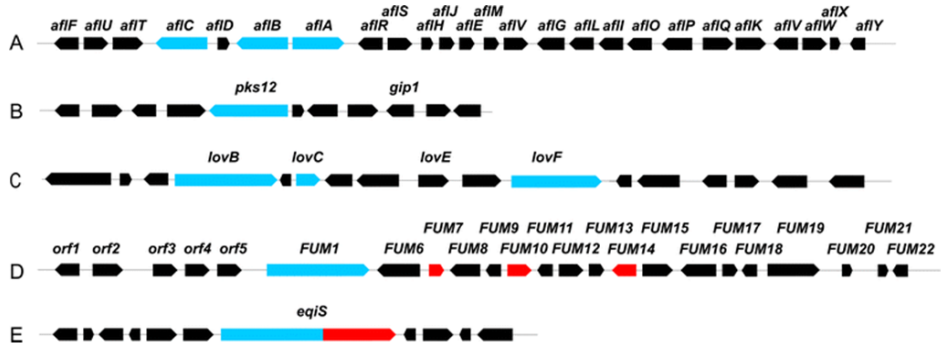


Figure 12 Selected biosynthetic gene clusters for fungal polyketides. The PKS genes are indicated in blue, while the equisetin hybrid gene is shown in blue (PKS domain) and red (NRPS domain). A, aflatoxin cluster; B, aueofusarin cluster; C, lovastatin cluster; D, fumonisin cluster (with in red FUM7, FUM10 and FUM14, to symbolize separate domains of a multimodular NRPS-like enzyme), E, equisetin cluster (reproduced from Hoffmeister and Keller, 2007).

5.3. Global regulation by broad domain transcription factors

As the synthesis of SMs is not constitutive, it is apparent that a molecular switch is needed to regulate the pathways leading to their production. The production of SM is an energetically costly process, and fungi are able to regulate secondary metabolism under certain environmental conditions. Carbon and nitrogen sources, temperature, light and pH are well known parameters that strongly influence the synthesis of SMs. CreA, AreA and PacC are Cys2His2 type zinc finger transcription factors that are involved in signalling of carbon, nitrogen and pH, respectively (Dowzer and Kelly, 1989 & 1991; Hynes, 1975; Tilburn *et al.*, 1995). These Cys2His2 zinc finger transcription factors can affect regulation of gene clusters in a positive as well as a negative fashion. PacC regulates gene expression based on ambient pH and penicillin production is positively regulated by PacC in contrast to sterigmatocystin (ST) which is negatively regulated (Espeso *et al.,* 1993; Keller *et al.,* 1997; Martin, 2000). In addition, PacC seems to

negatively regulate fumonisin production in *F. verticillioides* (Flaherty *et al.,* 2003) and ochratoxin A production in *A. ochraceus* (O'Callaghan *et al.,* 2006).

5.4. In-cluster transcription factors

Many SM gene clusters contain one or more pathway specific genes (e.g. Yu *et al.,* 1996; Yu *et al.,* 2004b; Flaherty and Woloshuk, 2004; Wiemann *et al.,* 2009). The AF/ST cluster specific transcription factor AflR, is model for this class of DNA binding proteins which contain a Zn(II)2Cys6 domain that is unique for fungi. AflR binds to a canonical DNA motif, 5'-TCG(N5)GCA-3' that has been found in most promotors of the AF/ST biosynthetic gene cluster. Disruption of AlfR abolishes the expression of most of the genes in the AF gene cluster. Conversely, overexpression of AlfR augments the production of AF/ST, whereby AlfR from *Aspergillus flavus* can complement ΔaflR in *Aspergillus nidulans* and vice versa (Flaherty and Payne, 1997).

The 28 kb gene cluster involved in gliotoxin biosynthesis contains a *gliZ*, a gene encoding a Zn(II)2Cys6 protein, GliZ (Gardiner and Howlett, 2005) and deletion of the *gliZ* gene completely abolishes gliotoxin production (Bok *et al.,* 2006). Similarly, in *F. verticillioides*, Zn(II)2Cys6 transcription factors ZFR1 and FUM21 have been found, that regulate FUM production. ZFR1 is an example of a TF that is located outside of the SM gene cluster it regulates (Bluhm *et al.*, 2008). The *FUM21* gene, on the other hand, is an integral part of the gene cluster and deletion of this gene completely abolishes FUM production (Brown *et al.,* 2007).

TRI6, the transcription factor responsible for the production of trichothecenes, like DON and T2 toxin, is member of the class of Cys2His2 zinc finger proteins. Its binding motif, YNAGGCC, is found in nine *tri* genes and deletion of the *tri6* gene leads to strongly reduced expression of *tri* genes and accumulation of the trichothecene precursor trichodiene (Hohn *et al.,* 1999, Seong *et al.,* 2009; Nasmith *et al.,* 2011).

5.5. Transcription complexes

The regulation of the *AflR* gene itself was elucidated upon a mutagenesis strategy, which led to the identification of LaeA (loss of AlfR expression-A) in *A. nidulans* (Butchko *et al.,* 1999). LaeA orthologs were soon identified in other aspergilli (Bok and Keller, 2004). Loss of LaeA results in the reduction of expression of genes in SM gene clusters such as

AF/ST, penicillin, gliotoxin etc. In *Aspergillus fumigatus*, animal virulence was greatly reduced by deletion of the *LaeA* gene (Bok *et al.,* 2005)

It was shown that LaeA controls ~9.5 % of all genes in *A. fumigatus*, whereas over half (13 out of 22) SM gene clusters are under the control of LaeA (Perrin *et al.*, 2007). Orthologs of LaeA have been identified in many other filamentous fungi, including *P. chrysogenum, P. citrinum, F. fujikuroi* (Kosalkova *et al.,* 2009; Xing *et al.,* 2010; Wiemann *et al.,* 2010). In the latter organism, the orthologous protein, FflaeA1, positively regulates gibberellic acid, FUM and fusarin C production, while bikaverin production is repressed (Wiemann *et al.,* 2010). It was shown that in *P. chrysogenum* LaeA also controls pigmentation and sporulation (Kosalkova *et al.,* 2009) and these findings have been confirmed in other fungi (Wiemann *et al.,* 2010).

LaeA was found to form a heterotrimeric complex with VeA, also known as *velvet*, that was long known to be associated with (a)sexual development, and VelB, whose function still needs to be elucidated (Bayram *et al.,* 2008). VeA appears to be essential for activation of AlfR expression (Calvo *et al.,* 2004) and redlight–induced conidiation (Mooney and Yager, 1990). Strains carrying a VeA deletion were unable to produce any sexual fruiting bodies, while overexpression of VeA led to constitutive fruiting body formation, irrespective of the light conditions. In light, VeA accumulates in the cytoplasm, while in the dark the protein is localized in the nucleus (Stinnett *et al.,* 2007). The overlapping phenotypes of LaeA and VeA mutations can be explained by their interaction in the Velvet complex (Bayram *et al.,* 2008). Under light conditions, VeA levels in the nucleus are low and the Velvet complex is not formed (asexual sporulation and no synthesis of SMs) while in the dark, VeA levels are raised and complexation with LaeA and VelB to form the Velvet complex occurs, which triggers both SM synthesis and sexual development (Bayram *et al.,* 2008). In all *Aspergillus, Penicillium* and *Fusarium* species examined so far, the Velvet complex regulates spore formation and SM synthesis in a concerted fashion (Bayram *et al.,* 2008; Kosalkova *et al.,* 2009: Wiemann *et al.,* 2010).

In yeast as well as in plants (e.g. *Arabidopsis thaliana*) and in animals (e.g. *Xenopus leavis*) Hap-like regulatory complexes are functional. These CCAAT-binding complexes have also been found in fungi, like *A. nidulans*, where it appears to be involved in response to iron stress through interaction with the iron-sensing bZIP protein HapX (Hortschansky *et al.,* 2007). In *A. fumigatus*, the HapX ortholog regulates siderophore production which is needed for full virulence (Schrettl *et al.,* 2010). bZIP proteins

regulate development and morphology, stress responses as well as several metabolic processes. Recently a bZIP-like protein, RsmA (remediation of secondary metabolism) was identified in *A. nidulans* that could overcome ΔLaeA/ΔVeA double mutations, suggesting that bZIP proteins, like Velvet, may be involved in global SM regulation (Shaaban *et al.,* 2010).

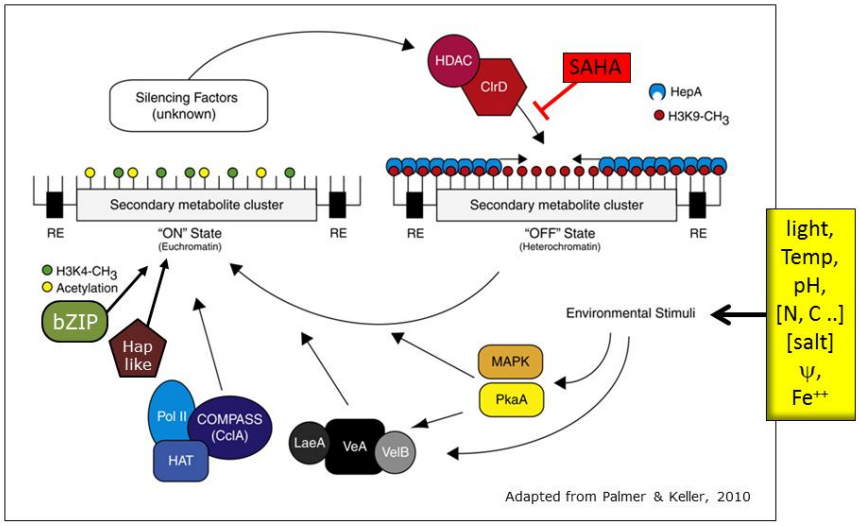


Figure 13. A proposed model for chromatin-mediated control of secondary metabolite gene clusters. Secondary metabolite gene clusters are often flanked by repetitive elements (REs) and located in subtelomeric regions of the genome. The epigenetic marks of H3K4 methylation (H3K4-CH3) and general histone acetylation have been shown to be associated with active gene transcription. Thus, histone acetyltransferases (HAT) and the H3K4 methylation protein complex (COMPASS) are involved in initiation of transcription through RNA polymerase II (Pol II). Environmental stimuli are translated by signal transduction cascades, including but not limited to MAPK and PkaA, to trigger production of secondary metabolites. These signals work independently and dependently through the LaeA containing velvet complex. On the other hand, in several eukaryotic systems heterochromatin protein 1 has been shown to bind H3K9 methylation (H3K9-CH3) and is associated with gene silencing. In *Aspergillus nidulans*, null mutants of the H3K9 methyltransferase (ClrD) and heterochromatin protein 1 (HepA) result in derepression of the ST gene cluster. Currently, the genetic components involved in initiation of heterochromatin at secondary metabolite gene clusters is unknown, RNAi-mediated heterochromatin formation could function this way as well as DNA binding repressors. (adapted after Palmer and Keller, 2010)

The impact of location in the genome on the expression of genes came from the finding that expression of one of the AF cluster genes was 500-fold reduced, when it was located outside the cluster (Liang *et al.,* 1997). As mentioned before, genes involved in SMB are typically located in gene clusters and co-regulated. They are frequently found near the ends of chromosomes (Perrin *et al.*, 2007), where chromatin structure influences transcription. Euchromatin consists of an "open" structure with acetylated and H3K4-methylated histones which supports active gene transcription. In contrast,

heterochromatin is "condensed" with deacetylated nucleosomes containing histone 3 proteins, methylated at the K9 position. These H3K9 methylated proteins are bound by hepA, with the overall consequence that genes in heterochomatic regions are silenced. Addition of an inhibitor of histone deacetylase, relieves the repression by increasing the acetylation status of the chromatin, as does addition of the DNA methyltransferase inhibitor 5 azacytidine (Williams *et al.,* 2008).

The notion that LaeA controls expression of subtelomeric SM gene clusters was highly substantiated by expression profiling, where *A. fumigatus* growing under normal laboratory conditions was compared with the fungus during infection of laboratory mice. Under laboratory conditions, no expression of genes involved in SMB was observed, but these genes were highly upregulated during infection (McDonagh *et al.,* 2008).

5.6. Discovery of novel compounds

As the genomic sequences of more and more fungi become available, new SM gene clusters are regularly being discovered. PKS and NRPS genes contain highly conserved domains, and bioinformatics tools [e.g. SMURF, Khaldi et al., 2010] have been developed that allowed the prediction of 25 PKSs, 18 NRPSs, 14 NRPS-like synthases and several hybrid enzymes in *A. flavus* (Amaike and Keller, 2011). In total, 55 SM clusters were predicted, but even under many different conditions (n=28) expression of only a few of these SM clusters was observed (Georgianna *et al*., 2010). Most SM clusters appear to be silent under standard laboratory conditions (Hertweck, 2009) and hence different approaches are required to identify their products. These include (i) identification of the SM based on prediction of physiochemical properties using bioinformatics; (ii) methods based on inactivation or removal of putative SMB genes, (iii) overexpression of a single SM gene cluster gene (iv) overexpression of a pathway specific transcription factor may be successful, as exemplified by the discovery of aspyridones A and B (Bergmann et al., 2007), (v) overexpression of pleiotropic regulators like LaeA and VeA, which has led to the identification of terrequinone A in *A. nidulans*, (vi) interference with chromatin modelling and (vii) through environmental manipulation e.g. co-cultivation with the soil bacterium *Streptomyces rapamycinicus* induced the production of multiple SM by *A. nidulans* (Schroeckh *et al.*, 2009; Brakhage and Schroeckh, 2011).

6. Legislation related to working with (GMO-) fungi and legislation on mycotoxins

6.1. Legislation related to working with (GMO-) fungi

6.1.1. Legislation on working with fungi

All laboratory work must be performed according to the guidelines of Directive 2000/54/EC when working with micro-organisms of class 2 and higher, as listed in Directive 2000/54/EC (pathogenic to humans). The responsible authorities, being the arbeidsinspectie of the Ministry of Social Affairs and Employment in the Netherlands, must be notified in advance. The authorities determine whether, and if so, which adaptations must be made to the work place. They also perform the periodic controls on the work place. Legislation also requires the registration of a 'list of exposed workers', from both microbiological and biological agent point of view. The latter includes potentially allergenic or toxic effects as a result of the work of the workers (EU 2000/54/EC article 3.3.d).

6.1.2. Legislation on working with GMO

When working with genetically modified organisms, the laboratory must follow additional regulations. The interpretation of the EU legislation for the Netherlands is the responsibility of the Bureau Genetisch Gemodificeerde Organismen (Bureau GGO) of the Ministry of Infrastructure and the Environment (Ministry of IenM). The Netherlands legislation concerning working with GMO's is published on the website of the Ministry of IenM (Bureau GGO). The GMO regulations, analogous to the occupational health regulations, require evaluation of the safety of the working place and registration of personnel working with the GMO's. A licence is required for before the actual activities start. Main aim of the measures for the working place is the containment of the GMOs to prevent accidental escapes leading to contamination of the environment.

The Netherlands Commission on Genetic Modification (COGEM) is an independent scientific advisory committee composed of scientists. The main functions of COGEM are to give statutory advice to the Dutch Minister of Environment on the risks to human health and the environment from experiments under contained conditions (laboratories, greenhouse, production facilities) with Genetically Modified Organisms (GMOs), and the

release and marketing of GMOs, and to inform the Dutch government on ethical and societal issues linked to genetic modification.

6.2. Legislation on mycotoxins

6.2.1. European legislation on mycotoxins in food and feed

European legal limits on mycotoxins in food and feed are laid down in Regulation (EC) 1881/2006 (food) (EU Regulation 1881/2006) and Directive (EC) 2002/32 (feed) (EU Directive 2002/32), respectively, and their amendments. In addition, guidelines for legal limits on several mycotoxins in feed are indicated in Recommendation 2006/576/EC (EU Recommendation 2006/576/EC). An overview of MTs in food and feed as regulated by the EU is given in Appendix 1.

New European legislation is based on risk assessment, performed in an independent way and based on scientific knowledge (EU Regulation (EC) No 178/2002). Risk assessment for European mycotoxin legislation is done by the European Food Safety Authority (EFSA) while the Joint FAO/WHO Committee on Food Additives (JECFA) is responsible for the risk assessment for FAO/WHO standards worldwide. Both institutions have access to published scientific data on toxicology. The risk assessments are communicated via internet publications (EFSA; JECFA, 1998) and risk managers subsequently decide on possible regulations, with the use of the data from the risk assessments and other considerations, such as social and economic consequences.

6.2.2. European legislation on the safety of consumption of fungi

EFSA makes use of the Qualified Presumption of Safety (QPS) methodology, to assess suitability of micro-organisms to be consumed by humans and / or livestock (EFSA, 2005). A body of knowledge must be installed that can provide adequate assurance that any potential to produce adverse effects in humans, livestock or the wider environment is understood and predictable (EFSA, 2005a). The list of QPS recommended biological agents was last updated in 2011 (EFSA, 2011a). No filamentous fungi appear on the QPS list due to frequent occurrence of inaccuracies and inconsistencies in fungal species identification, insufficient knowledge concerning the regulation mechanisms underlying the production of fungal metabolites, the poor knowledge concerning the toxic impact of fungal secondary metabolites and the increased activity in the discovery of new

mycotoxins, designed as emerging mycotoxins (EFSA, 2011a). This also accounts for fungi, such as *Penicillium camemberti*, that have a long history in cheese making, but where production of toxic quantities of metabolites in other matrices cannot be excluded (EFSA, 2007).

Relevant to this subject is the EFSA opinion on the risk assessment of genetically modified microorganisms and their products intended for food and feed (EFSA 2011d). This guidance mentions the relevance and importance of a proper identification of the recipient strain. It also provides relevant issues that must be addressed to before recipient strain can be considered for use. These issues, supplemented with the issues in regards to plants, must be included in the documents as mentioned in Appendix 2.

7. Environmental impact

7.1. Level of containment for working with mycotoxin producing fungi

The Netherlands Regulation on Genetically Modified Organisms (Regeling Genetische Gemodificeerde Microorganismen) must be met when applying for genetic modification of fungi. However, when there is a request for use of a fungus that is not listed in one of the appendices for containment and when this fungus is not pathogenic to humans, animals or plants, several issues must be considered.

Main issue is on how mycotoxin production must be evaluated with respect to containment for safety of laboratory personnel, for a fungus that is not pathogenic to humans, animals or plants. An overview of the steps to de addressed is presented in the flowchart in Appendix 2.

The decision making starts with step 1, judgement of the provided documents on identity of the recipient (or parental) strain to evaluate the need for specific analyses or potential concerns (much according to EFSA, 2011d). This requires comprehensive information on scientific name, taxonomy, other names, phenotypic markers, pathogenicity (human, animal and plant), ecological and physiological traits. Source and natural habitat must be provided. This allows the evaluation of specific containment requirements related to mycotoxin production. This is the case when a mycotoxin-producing strain is suggested to be used in an area where strain of the same species does occur naturally, generally meaning that ecological factor are identical, but does not produce mycotoxins. All other relevant information must be provided by the applicant. The information must also provide enough information for the committee to decide on pathogenicity in step 2.

After estimation of the identity and pathogenicity in steps 1 and 2, step 3 continues by evaluation of information on toxin production. The applicant must provide comprehensive information on how toxin production is evaluated. This could be based on genetic make-up or analysis of known mycotoxins after growing in liquid or solid media.

Step 4 evaluates the relevance of the mycotoxin production. The provided documentation must indicate which mycotoxins can be produced and how production can be prevented. The evaluation committee will decide to the level of risk in the risk classification.

When there is enough information to establish the risk, the fungus can be classified (step 5) and the level of containment and method of waste treatment can be determined (step 6).

Science based documents must be provided to substantiate all statements. Furthermore, according to Netherlands regulations the applicant is at all times responsible to provide newly emerged information, even after granting of the license.

Note that regardless of mycotoxin production, exposure of laboratory personnel and contamination of laboratory environment must be prevented to avoid possible allergic reactions.

7.2. Waste treatment

Laboratory waste materials always need to be treated with validated methods to inactivate the microorganisms. Growth media, both solid and liquid, only need to be treated as carcinogenic waste material when the fungus of concern has been identified as being able to produce carcinogenic mycotoxins. The material then needs to be properly incinerated.

8. Discussion and conclusions

Working with fungi in the laboratory always requires protection of personnel and the laboratory environment, irrespective of the pathogenicity of the fungus. Fungi can spread easily by means of spores which may be contaminated with mycotoxins, and thus may contaminate the laboratory and possibly other samples with both fungal parts and/or mycotoxins. In addition, laboratory personnel must always be protected from the harmful fungal allergens. Therefore, containment requirements for working with fungi should always follow national regulations on occupational health.

Additional laboratory containment may be required for genetic modification of fungi or heterologous gene expression. For pathogenic fungi or the cloning of fungal genes, the standard risk assessment and evaluation procedures apply. The key question and basis of this report is how to deal with non-pathogenic but potential mycotoxin producing organisms. On the basis of state-of-the-art presented in this report, it can be concluded that mycotoxins are fungal products that may be harmful after consumption via contaminated food or feed and, possibly, after inhalation and skin contact. Mycotoxin production is tightly regulated by environmental factors. Risk of exposure may vary dependent of culture conditions and the nature of the involved gene modifications. Particular attention is warranted with alterations in gene regulation resulting in constitutive or overproduction of mycotoxins, or with transfer of large genomic segments (>20 kb) that may contain the complete cluster of mycotoxin biosynthesis genes.

The first step in the assessment is to ensure adequate identification of the fungus at strain level. This step must be lucid and unambiguous, to allow a reliable assessment of pathogenicity and mycotoxin producing capacities (see also paragraph 4.2). Until recently the identification of fungi relied on morphological characteristics. This included growing under strict conditions, followed by colour and/or odour analysis and microscopy of the fungal structures. It includes the exploration of the anamorphic (a-sexual state) and teleomorph (sexual state) forms, with identification systematics for each form, thus resulting in different names for anamorph and teleomorph of the same fungus. However, interpretation of the morphological characteristics can be complicated and it can unintentionally lead to misidentification. Therefore, DNA based techniques are the methods of choice for unambiguous identification of the fungus. In fact, this has led to the resolution of ambiguities in the nomenclature of several important fungal genera, encompassing many toxigenic species, under the principle of one fungus, one name

(Geiser *et al.,* 2012; Wingfield *et al.,* 2012; www.aspergilluspenicillium.org). Nevertheless, when assessing literature for pathogenicity or mycotoxin production, awareness of possible misidentifications is required. Only correct identification allows correct assessment of pathogenicity towards humans, animals and plants and the ability of mycotoxin production.

When the fungal strain is considered non-pathogenic, the next step in the risk assessment is characterization of mycotoxin production. Evaluation of the ability of fungi to produce mycotoxins can be complicate, as mentioned in paragraphs 5.3-5.5. As for the field conditions, the actual production of secondary metabolites depends on genetic make-up of the fungus and the growth conditions in the field. Laboratory methods based on standardised growth conditions may give simple insight in the ability for mycotoxin production. However, even under laboratory conditions correct identification of fungi could have been ambiguous in the past, or strains may be renamed, which can result in deviations in ability for mycotoxin production by the various fungal species. The recent identification of clusters of genes responsible for/involved in mycotoxin production has opened the possibility of unambiguous identification of the ability or inability to produce a certain mycotoxin. However, these clusters are currently known for a limited range of the most common mycotoxins.

When there are indications (literature, genetic data) of mycotoxin production, the risk can be further classified as low, medium or high based on a set of possible criteria as indicated in Appendix 3. These include known toxicity (Table 1), although interferences with other constituents in the matrix, including synergism and/or antagonism between different MTs in a single commodity add to this complexity. Several opinions from both EFSA and JECFA are available (in food and feed) on toxicity and human exposure to mycotoxins. Other criteria that should be taken into consideration when evaluating toxicity include data on mycotoxin levels in spores, 'porte d'entrée', mode-of-action, nature of toxic effect, LD_{50} differences in susceptibility between different species and chronic or acute effects of mycotoxin exposure. When only minimal information about the mycotoxin is available, the risk of the mycotoxin for health and environment may be considered minimal considering the widespread availability of the compounds in the natural environment, but careful follow up of literature is required to underpin this policy.

It can be concluded that when fungi that can produce mycotoxins are proposed for use in genetic modification, the ability to produce mycotoxins may influence the level of containment advised for the work.

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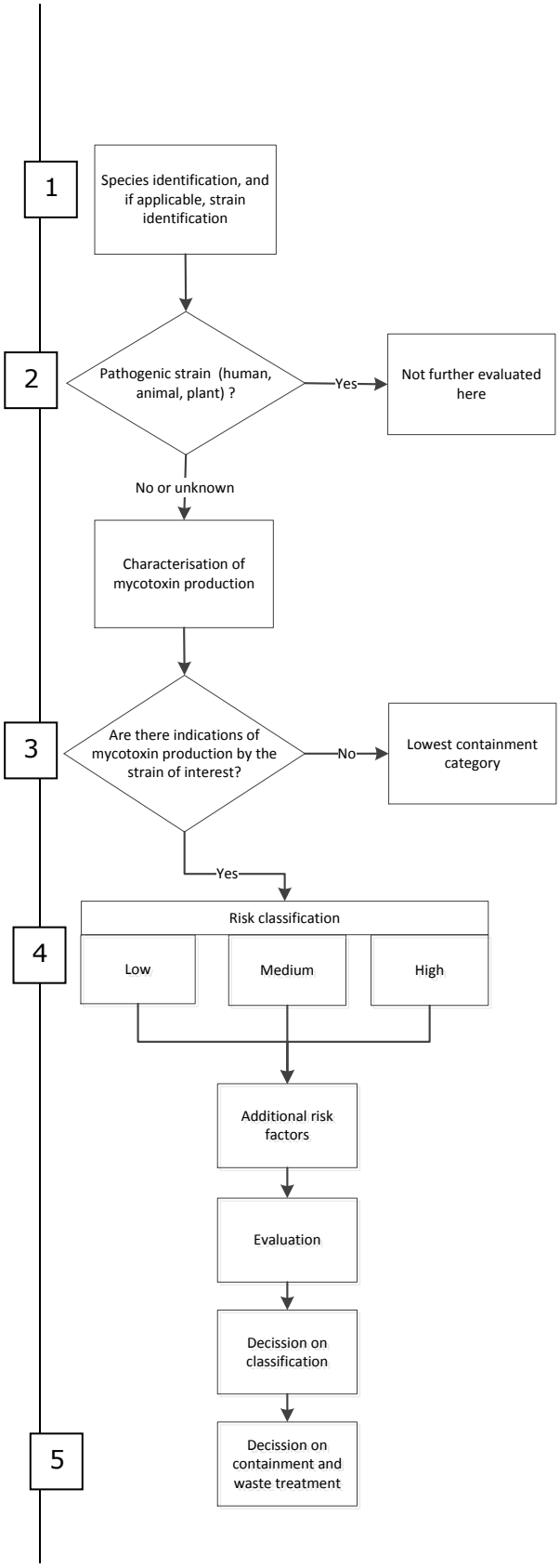
Appendix 1. Regulated moulds and mycotoxins in food and feed. EU regulation 1881/2006 (food); EU directive 2002/32 (feed); EU recommendation 2006/576/EC (feed).

	Food	Feed
Aflatoxin B_1	X	X
Aflatoxins B_1, B_2, G_1, G_2	X	
Aflatoxin M_1	X	
Ochratoxin A	X	X[1)]
Patulin	X	
Deoxynivalenol	X	X[1)]
Zearalenone	X	X[1)]
Fumonisins B_1, B_2	X	x[1)]
Claviceps purpurea sclerotia		X

[1)] Recommended as guidelines

Appendix 2. Flowchart for decision making on containment of mycotoxin producing fungi



Provide documentation on all mentioned issues, and include state-of-the-art reviews on all subjects

- Give details on origin of strain
- Give method of identification of strain (including name, historic identification, morphology, sporulation, sexual/asexual state)
- Genetic characterisation, e.g. sequence analysis
- Explain taxonomic tool
- Other relevant infomation

- Give evidence on how pathogenicity/ non-pathogenicity is determined
- Does the strain have a GRAS or QPS registration?
- Other relevant information

- How is (non-)producing estimated?
- E.g. Literature review
- Genetic information
- Bioinformatic analysis on mycotoxins by comparative genomics
- Analysis of mycotoxins
- Other
- Other relevant information

- Growth conditions for mycotoxin production
- What fungal parts hold the mycotoxin(-s) and up to what concentration
- Are there EU regulations on the produced mycotoxin(-s)
- Most sensitive animal species
- Porte d'entrée: ingestion, contact, inhalation
- Toxicity:
- * mechanism of action
- * severity of toxic effect
- * acute and chronic effects
- * Is there a known LD_{50}
- Other relevant information

Appendix 3. Major mycotoxins and their toxic effects

	Mycotoxin	Fungal genus related to the mycotoxin production[1)]	IARC[2)]	Carc.[#]	Hepa[#]	Imm[#]	Neph[#]	Neuro[#]	Terato[#]	Specific[#]	REF
Trichothecenes type A	Diacetoxy scirpenol	*Fusarium, Trichothecium*	-	NI[$]	NI	NI	NI	NI	NI	NI	
	HT-2 Toxin		-	NI	NI	NI	NI	NI	NI	NI	EFSA, 2011b
	T-2 Toxin		3	-	-	**X**	-	-	-	Haemato-toxic	EFSA, 2011b
Trichothecenes type B	Deoxynivalenol		3	-	-	**X**	-	**X**	-	-	EFSA, 2004
	DON-3-glucoside		-	NI	NI	NI	NI	NI	NI	NI	
	Σ (3&15-Acetyl-DON)		-	NI	NI	NI	NI	NI	NI	NI	
	Nivalenol		3	-	-	**X**	**X**	-	-	Haemato-toxic	Battilani *et al.* 2008
Zearalenone-group	Zearalenone	*Fusarium*	3	-	-	-	-	-	-	Estrogenic	EFSA, 2011c
	α-Zearalenol		-	NI	NI	NI	NI	NI	NI	NI	
	ß-Zearalenol		-	NI	NI	NI	NI	NI	NI	NI	
Fumonisins	Fumonisin B_1	*Aspergillus, Fusarium* **Fumonisin-like**: *Alternaria, Aspergillus, Cochliobolus, Paecilomyces*	2B	**X**	**X**	**X**	**X**	**X**	-	Lung lesions in pig	EFSA, 2005b
	Fumonisin B_2		2B								
	Fumonisin B_3		-								
	Moniliformin	*Fusarium*	-	-	**X**	-	**X**	-	-	Hearth	Battilani *et al.* 2008
	Beauvericin	*Beauveria, Fusarium, Isaria*	-	NI	NI	NI	NI	NI	NI	NI	

	Mycotoxin	Fungal genus related to the mycotoxin production[1)]	IARC[2)]	Carc.[#]	Hepa[#]	Imm[#]	Neph[#]	Neuro[#]	Terato[#]	Specific[#]	REF
Aflatoxins	Aflatoxin B_1	*Aspergillus*	1	**X**	**X**	**X**	**-**	**-**	**X**	Mutagenic	JECFA, 1998
	Aflatoxin B_2										
	Aflatoxin G_1										
	Aflatoxin G_2										
	Sterigmatocystin	*Aspergillus, Bipolaris, Chaetomium, Emericella, Humicola*	2B	**X**	**-**	**-**	**-**	**-**	**X**	Mutagenic	Battilani *et al.* 2008
	Agroclavine (ergot alkaloid)	*Aspergillus, Claviceps*[3)]	**-**	**-**	**-**	**-**	**-**	**-**	**X**	Gangrene	Battilani *et al.* 2008
	Citrinin	*Aspergillus, Blennoria, Clavariopsis, Monascus, Penicillium*	3	Concern carcino-genic	**-**	**-**	**X**	**-**	**X**	Concern for genotoxicity	EFSA, 2012
	Ochratoxin A	*Aspergillus, Neopetromyces, Penicillium, Petromyces,*	2B	**-**	**X**	**X**	**X**	**-**	**X**	**-**	EFSA, 2006
	Mycophenolic acid	*Byssochlamys, Penicillium, Septoria*	**-**	NI	NI	NI	NI	NI	NI	NI	
	Roquefortine C	*Penicillium*	**-**	NI	NI	NI	NI	NI	NI	NI	
Alternaria toxins	Alternariol	*Alternaria*	**-**	-	-	-	-	-	Feto-toxic[4)]	*In vitro* genotoxic[4)]	EFSA 2011d
	Alternariol-methylether										

	Mycotoxin	Fungal genus related to the mycotoxin production[1)]	IARC[2)]	Carc.[#]	Hepa[#]	Imm[#]	Neph[#]	Neuro[#]	Terato[#]	Specific[#]	REF
	Nitropropionic acid	*Arthrinium, Aspergillus*	NI	NI	NI	NI	NI	NI	NI	NI	
	Patulin	*Aspergillus, Byssochlamus, Penicillium*	3	-	-	X	X	Gastro	-	Genotoxic	JECFA, 1995

NI = No information available in EFSA opinions or JECFA reports.

1) Boekhout, T. (2011).

2) IARC groups and:
Group 1 Carcinogenic to humans
Group 2A Probably carcinogenic to humans
Group 2B Possibly carcinogenic to humans
Group 3 Not classifiable as to its carcinogenicity to humans
Group 4 Probably not carcinogenic to humans
- no information

3) Group of Ergot alkaloids

4) EFSA reports toxicity for the combined group of *Alternaria* toxins:

5) Battilani *et al*. (2008) is a scientific document. Information on toxicity of DAS was too limited for toxic evaluation.

Carc. =carcinogenic;

Hepa= hepatotoxic (liver);

Imm= immunotoxic;

Neph=nephrotoxic (kidney);

Neuro= neurotoxic;

Terato=teratogenic (offspring);

Specific= other toxic effects.

Appendix 4. Overview of mycotoxins, environmental stimuli, global and pathway specific transcription factors in most common fungal genera

Mycotoxin	Biochemical pathway[a)]	Genes clustered[b)]	Regulation	Global TFs[c)]	Pathway specific TFs	References
Trichothecenes (Diacetoxyscirpenol, T-2 and HT-2 toxin, Deoxynivalenol DON-3-glucoside Nivalenol	+	+	N, C, pH, stress, water activity, Mg++, temp.	CreA, AreA, PacC,	TRI6 (Cys2His2) TRI10	1, 2 3, 4 5, 6
Zearalenone and derivatives	(+)	+				7
Fumonisins	+	+	Amylopectin, water activity, temp.	CreA, PacC, LaeA	FUM21 (Zn2Cys6)	8, 9 10, 11
Moniliformin	-	-				
Beauvericin	-	-				
Aflatoxins and sterigmatocystin	+	+	C-, N-, pH, cell cycle, temp	CreA, AreA PacC,	AflR (Zn2Cys6) AflJ	12, 13 14, 15 16
Agroclavine	-	-	C-, PO_4	CreA		
Citrinin	(+)	+				

Mycotoxin	Biochemical pathway[a)]	Genes clustered[b)]	Regulation	Global TFs[c)]	Pathway specific TFs	References
Ochratoxin A	-	-	pH, salt, light	PacC		17
Mycophenolic acid	-	-				
Roquefortine C	-	-				
Alternariol	-	-				
AOL-methylether	-	-				
Nitropropionic acid	-	-				
Patulin	-	-	N-			18

a) biochemical pathway of the MT is +, known; (+), pathway is partially known or -, pathway is not known.
b) +, [part of the] genes involved in biosynthesis of the MT are clustered in the genome.
c) TF, transcription factor.

1) Merhej *et al.,* 2011; 2) Marin *et al.,* 2010; 3) Kulik *et al.,*2012; 4) Pinson-Gadias et al., 2008; 5) Schmidt-Heydt *et al.,* 2011a; 6) Ochiai *et al.,* 7) Lysøe *et al.,* 2009; 8) Bluhm and Woloshuk, 2005; 9) Flaherty *et al.,* 2003; 10) Jurado *et al.,* 2008; 11) Kim *et al.,* 2008; 12) O'Brian *et al.,* 2007; 13) Ehrlich *et al.,* 2011; 14) Schmidt-Heydt *et al.,* 2010; 15) Huang *et al.,* 2009; 16) Reverberi *et al.,* 2010; 17) Price *et al.*, 2005; 18) Puel *et al.,* 2010)

Appendix 5: proposal tekst dd. oktober 2011

'Mycotoxines en milieurisicoanalyse van ggo-werkzaamheden'

Indeling in pathogeniteitsklassen van organismen is een van de hoekstenen voor de milieurisicoanalyse en inschaling van ggo-werkzaamheden onder Ingeperkt gebruik. De COGEM heeft eerder een onderzoekproject laten uitvoeren naar de classificatie van schimmels in pathogeniteitsklassen. Eén van de complicerende factoren bij de classificatie van schimmels is het feit dat sommige schimmels toxines (mycotoxines) kunnen produceren. Mycotoxines kunnen een belangrijke bedreiging voor de volksgezondheid vormen, zoals de aanwezigheid van kankerverwekkende aflatoxines in landbouwproducten uit (sub)tropische regio's. Toxineproductie is een belangrijke aanwijzing om micro-organismen in pathogeniteitsklasse 2 of hoger te plaatsen. Dit betekent dat ggo-werkzaamheden op ML-II niveau of hoger moeten plaatsvinden. Echter naar gebleken is zijn sommige toxine producerende schimmels apathogeen en worden zelfs gebruikt bij voedselproductie e.d., bijvoorbeeld *Penicillium roquefortii* voor de productie van blauwschimmelkazen. Het lijkt in dergelijke gevallen onlogisch en onwenselijk om strenge veiligheidsmaatregelen in laboratoria op te leggen. Het is de vraag hoe met deze ogenschijnlijke discrepantie in de milieurisicoanalyse omgegaan moet worden.

Doel van het project:

Inzicht verkrijgen in de expressiecondities, werkingsmechanismen en toxiciteit van mycotoxines, en analyse hoe omgegaan moet worden in de ggo-milieurisicoanalyse met apathogene schimmels die mycotoxines produceren.

Resultaat:

Een omvattend overzicht over de eigenschappen van deze toxines, de condities waaronder expressie plaatsvindt en hoe met mycotoxines in de risicoanalyse moet worden omgegaan.